Universidad Autónoma de Madrid
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Evaluation of genetic polymorphisms associated with the metabolic effects of aripiprazole and olanzapine

Doctoral Thesis

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Que Doña Dora Koller, ha realizado la presente Tesis Doctoral "Evaluation of genetic polymorphisms associated with the metabolic effects of aripiprazole and olanzapine" con objeto de obtener el Grado de Doctor.

Como director del trabajo hago constar que ha sido realizado con todas las garantías técnicas y metodológicas, y las conclusiones obtenidas son plenamente válidas, siendo considerado, por tanto, apto para ser presentado como Tesis Doctoral.

En Madrid, a 10 de junio de 2020

Fdo. Dr. Francisco Abad Santos

This thesis, submitted for the degree of Doctor of Philosophy (Ph.D.), has been elaborated in the Clinical Pharmacology Department, Instituto de Investigación Sanitaria of Hospital Universitario de La Princesa from June 2017 until June 2020 under the supervision of Dr. Francisco Abad Santos.

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Abbreviations

3’UTR: 3’ untranslated region

5-HT receptor: 5-hydroxytryptamine (serotonin) receptor

5’UTR: 5’ untranslated region

A: adenine

ABC: ATP-Binding Casette transporter

ACV: average constriction velocity

ADME: absorption, distribution, metabolism and excretion

ADRA2A: alpha-2A adrenergic receptor

ADV: average dilation velocity

ALP: alkaline phosphatase

APCI: atmospheric pressure chemical ionization

APOA5: apolipoprotein A5

APOC3: apolipoprotein C3

ARI-D8: aripiprazole-D8

ARI: aripiprazole

AUC: area under the curve

BDNF: brain-derived neurotrophic factor

BMI: body mass index

C: cytosine

C18: cyclo[18]carbon

CACNA1S: Calcium Voltage-Gated Channel Subunit Alpha1 S

CAF: caffeine

CFTR: Cystic fibrosis transmembrane conductance regulator

Cl: clearance

CLO-D3: clozapine-D3

CLO: clozapine

C_{max}: maximum concentration

C_{min}: minimum concentration

CNV: copy number variation

COMT: catechol-O-methyltransferase

CON: percentage of constriction

CPIC: Clinical Pharmacogenetic Implementation Consortium

CYP: cytochrome P450 enzyme

DARI: dehydro-aripiprazole

DISC1: disrupted in schizophrenia 1

DME: drug-metabolizing enzyme

DPWG: Dutch Pharmacogenetics Working Group

DPYD: dihydropyrimidine dehydrogenase

DRD: dopamine receptor

DTNBP1: dystrobrevin-binding protein 1

ECG: electrocardiogram

EM: extensive metabolizer

EMA: European Medicines Agency

ESI: electrospray ionization

FDA: U.S. Food and drug administration

FMO3: flavin-containing monooxygenase 3

Free T4: free thyroxine

G: guanine

G6PD: glucose-6-phosphate dehydrogenase

GC: gas chromatography

GGT: gamma-glutamyl transferase

GOT: glutamate-oxaloacetate transaminase

GPT: glutamate-pyruvate transaminase

GTT: glucose tolerance test

GWAS: genome-wide association study

HbA1c: hemoglobin A1c

HDL: high-density lipoprotein

HLA: human leukocyte antigen

HPLC: high-performance liquid chromatography

ICH: International Council on Harmonisation

IFNL3: interferon lambda 3

IM: intermediate metabolizer

IS: internal standard

LAT: latency

LC: liquid chromatography

LDL: low-density lipoprotein

LEP: leptin

LEPR: leptin receptor

LLE: liquid-liquid extraction

LLOQ: lower limit of quantification

LOD: lower limit of detection

m/z: mass-to-charge ratio

MALDI: matrix assisted laser desorption ionization

MAX: maximum pupil diameter

MCV: maximum constriction velocity

MDR: multidrug resistence

MEC: minimum effective concentration

MHC: Major Histocompatibility Complex

MIN: minimum pupil diameter

MRM: multiple reaction monitoring mode

MS: mass spectrometry

MTC: maximum tolerated concentration

NM: normal metabolizer

NRG1: neuregulin 1

NUDT15: nudix hydrolase 15

OLA-C1-D3: olanzapine C1-D3

OLA: olanzapine

OPRM1: opioid receptor mu 1

P-gp: P-glycoprotein

PAL-D4: paliperidone-D4

PAL: paliperidone

PGRN: Pharmacogenomics Research Network

PharmGKB: The Pharmacogenomics Knowledge Base

PM: poor metabolizer

PPT: protein precipitation

Q: quadropole

QTc: heart rate-corrected QT

QUE-C4: quetiapine-C4

QUE: quetiapine

RIS-D4: risperidone-D4

RIS: risperidone

Rs: resolution factor

RSD: relative standard deviation

RT, tR: retention time

RYR1: Ryanodine receptor 1

SIL-IS: stable isotope-labeled internal standard

SLC: solute carrier

SNP: single nucleotide polymorphism

SPE: solid phase extraction

STR: short tandem repeat; microsatellite

T: thymine

$T_{1/2}$: elimination half-life

T75: total time taken by the pupil to recover 75% of the initial resting pupil size

TDM: therapeutic drug monitoring

Tmax: time to maximum concentration

TOF: time-of-flight

TPMT: Thiopurine S-methyltransferase

TSH: thyroid stimulating hormone

UGT1: UDP Glucuronosyltransferase Family 1

UHPLC: ultra-high-performance liquid chromatography

UM: ultrarapid metabolizer

Vd: volume of distribution

VIP: very important pharmacogenes

VKORC1: Vitamin K epOxide Reductase Complex (VKORC) subunit 1

VNTR: variable number of tandem repeat

XIC: extracted ion chromatogram

µ-SPE: microelution-solid phase extraction

ABSTRACT

Introduction:

Aripiprazole and olanzapine are atypical antipsychotics. Both drugs can induce metabolic and cardiovascular changes and adverse drug reactions. Pupillography is a non-invasive and cost-effective method to determine autonomic nerve activity during antipsychotic treatment. The aims of the study were to optimize and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure drug concentrations; to evaluate the effects of aripiprazole and olanzapine on pupillary light reflex; prolactin levels, lipid and glucose metabolism; hepatic, hematological, thyroid and renal performance; adverse events and safety and to assess the relationship between these parameters and pharmacogenetics. Genetic variants in cytochrome P450 (*CYP*), dopamine receptor (*DRD2*, *DRD3*), serotonin receptor (*HTR2A*, *HTR2C*) and ATP-binding cassette subfamily B (*ABCB1*) genes, among others, may be associated with the pharmacokinetics and pharmacodynamics of antipsychotic drugs.

Methods:

A simple and sensitive liquid chromatography-tandem mass spectrometry method was developed and validated in human plasma for the simultaneous determination of aripiprazole and its metabolite dehydro-aripiprazole; olanzapine, risperidone, paliperidone, quetiapine, clozapine and caffeine. The above mentioned compounds and their isotope-labeled internal standards were extracted from 200 µL human plasma samples by effective phospholipids-eliminating three-step microelution-solid-phase extraction (µ-SPE) and protein precipitation (PPT) for comparison. All analytes were monitored by mass spectrometric detection operating in multiple reaction monitoring mode and the method was validated covering the corresponding therapeutic ranges. Twenty-four healthy volunteers received 5 daily oral doses of 10 mg aripiprazole and 5 mg olanzapine, separated by a 28-day washout period in a crossover randomized clinical trial and were genotyped for 51 polymorphisms in 17 genes by qPCR. Drug plasma concentrations were measured by the previously explained LC-MS/MS method. Pupil examination was performed by automated pupillometry. Biochemical and hematological analyses were performed by enzymatic methods. Blood pressure and 12-lead ECG were measured in supine position. For safety evaluation, the Ramsay sedation scale and the UKU side effect rating scale were used. In addition, spontaneously notified adverse events were also recorded.

Abstract

Results:

The analytical method was validated based on the recommendations of regulatory agencies through tests of precision, accuracy, extraction recovery, identity confirmation, trueness, matrix effects, process efficiency, stability, selectivity, linearity and carry-over effect fulfilling the guideline requirements. Our μ-SPE method eliminated more than 99% of early eluting and more than 92% of late-eluting phospholipids compared to PPT. Additionally, the method was successfully applied for the quantification of aripiprazole and olanzapine plasma concentrations in healthy volunteers. Aripiprazole pharmacokinetics was affected by CYP2D6 and CYP1A2 phenotypes and polymorphisms in the *ABCB1* gene. Dehydro-aripiprazole pharmacokinetics were variable based on CYP2D6, CYP3A and CYP1A2 phenotypes and *ABCB1* gene polymorphisms. Olanzapine pharmacokinetics were affected by CYP3A phenotype and polymorphisms in *ABCB1* and *UGT1A1* genes. Aripiprazole affected pupil contraction: it caused dilatation after the administration of the first dose, then caused constriction after each dosing. Olanzapine only altered minimum pupil size. Polymorphisms in *CYP3A*, *HTR2A*, UDP-glucuronosyltransferase 1A1 (*UGT1A1*), *DRD2* and *ABCB1* affected pupil size, the time of onset of constriction, pupil recovery and constriction velocity. Olanzapine induced hyperprolactinemia but not aripiprazole. *DRD3* rs6280 and *ABCB1* rs10280101, rs12720067 and rs11983225 polymorphisms and CYP3A phenotype had an impact on plasma prolactin levels. Only olanzapine caused weight gain, what was influenced by *HTR2C* alleles. C-peptide concentrations were higher after aripiprazole administration and were influenced by *COMT* rs4680 and rs13306278 polymorphisms. Olanzapine and the *UGT1A1* rs887829 polymorphism were associated with elevated glucose levels. CYP3A poor metabolizers had increased insulin levels. Triglyceride concentrations were decreased due to olanzapine and aripiprazole treatment and were variable based on CYP3A phenotypes and the *APOC3* rs4520 genotype. Cholesterol levels were also decreased and depended on *HTR2A* rs6314 polymorphism. All hepatic enzymes, platelet and albumin levels and prothrombin time were altered during both treatments. Additionally, olanzapine reduced the leucocyte count, aripiprazole increased free T4 and both decreased uric acid concentrations. Aripiprazole decreased diastolic blood pressure on the first day of treatment and decreased QTc on the third and fifth day of treatment. Olanzapine had a systolic and diastolic blood pressure, heart rate and QTc lowering effect on the first day of treatment. Polymorphisms in *ADRA2A*, *COMT*, *DRD3* and *HTR2A* genes

were significantly associated to these changes. More adverse drug reactions were associated to aripiprazole than to olanzapine, especially psychiatric and nervous system disorders based on the UKU side effect rating scale and those spontaneously notified. The most frequent adverse drug reactions to aripiprazole were somnolence, headache, insomnia, dizziness, restlessness, palpitations, akathisia and nausea while were somnolence, dizziness, asthenia, constipation, dry mouth, headache and nausea to olanzapine. Ramsay scores showed low level of sedation Additionally, *HTR2A*, *HTR2C*, *DRD2*, *DRD3*, *OPRM1*, *UGT1A1* and *CYP1A2* polymorphisms had a role in the development of adverse drug reactions to aripiprazole and olanzapine.

Conclusions:

Aripiprazole altered pupil contraction. Olanzapine caused significant prolactin and weight elevation. Glucose levels in glucose tolerance test were higher after olanzapine treatment. Moreover, olanzapine had more cardiovascular effects than aripiprazole. However, aripiprazole was associated to more psychiatric and nervous system adverse drug reactions. Many polymorphisms may influence pupillometric and metabolic parameters along with cardiovascular changes and adverse events. Moreover, several polymorphisms had an effect on aripiprazole, dehydro-aripiprazole and olanzapine pharmacokinetics. It seems that aripiprazole provokes less severe metabolic and cardiovascular changes, however, more adverse drug reactions were registered to it compared to olanzapine.

Abstract

Introducción:

El aripiprazol y la olanzapina son antipsicóticos atípicos. Ambos fármacos pueden inducir cambios metabólicos y cardiovasculares y reacciones adversas. La pupilografía es un método no invasivo y económico para determinar la actividad nerviosa autonómica durante el tratamiento antipsicótico. Los objetivos del estudio fueron optimizar y validar un método de cromatografía líquida y espectrometría de masas en tándem (LC-MS/MS) para medir las concentraciones de los fármacos; evaluar los efectos del aripiprazol y la olanzapina en el reflejo pupilar; los niveles de prolactina, el metabolismo de los lípidos y la glucosa y la función hepática, hematológica, tiroidea y renal; los efectos adversos y la seguridad, y evaluar la relación de la farmacogenética con todos estos parámetros. Las variantes genéticas de los genes del citocromo P450 (*CYP*), los receptores de la dopamina (*DRD2*, *DRD3*), los receptores de la serotonina (*HTR2A*, *HTR2C*) y la subfamilia B de transportador de unión a ATP (*ABCB1*), entre otros, se pueden asociar a la farmacocinética y la farmacodinamia de los antipsicóticos.

Métodos:

Se desarrolló y validó un método sencillo y sensible de cromatografía líquida y espectrometría de masas en tándem en plasma humano para la determinación simultánea de aripiprazol y su metabolito dehidro-aripiprazol, olanzapina, risperidona, paliperidona, quetiapina, clozapina y cafeína. Los compuestos mencionados y sus patrones internos marcados con isótopos se extrajeron de muestras de plasma humano de 200 µl por medio de extracción en fase sólida de microelución en tres pasos (µ-SPE) y precipitación de proteínas (PPT) para su comparación. Todos los analitos se evaluaron mediante detección por espectrometría de masas que funcionó en el modo de monitorización de reacción múltiple y el método se validó cubriendo los rangos terapéuticos correspondientes. Veinticuatro voluntarios sanos recibieron 5 dosis orales diarias de 10 mg de aripiprazol y 5 mg de olanzapina, separados por un periodo de lavado de 28 días, en un ensayo clínico aleatorio cruzado, y se genotiparon para 51 polimorfismos en 17 genes mediante qPCR. Las concentraciones plasmáticas del fármaco se midieron por el método LC-MS/MS explicado anteriormente. El examen de las pupilas se realizó mediante pupilometría automatizada. Los análisis bioquímicos y hematológicos se realizaron por métodos enzimáticos. La presión sanguínea y el ECG de 12 derivaciones se midieron en posición supina. Para evaluar la seguridad se utilizó

la escala de sedación de Ramsay y la escala de calificación de efectos secundarios de la UKU, y también se registraron los eventos adversos notificados espontáneamente.

Resultados:

El método analítico fue validado de acuerdo a las recomendaciones de los organismos reguladores mediante pruebas de precisión, exactitud, recuperación de la extracción, confirmación de la identidad, veracidad, efectos de la matriz, eficiencia del proceso, estabilidad, selectividad, linealidad y efecto de arrastre que cumplían los requisitos de las directrices. Nuestro método µ-SPE eliminó más del 99% de los fosfolípidos de elución temprana y más del 92% de los fosfolípidos de elución tardía en comparación con el PPT. Además, el método se aplicó con éxito para la cuantificación de las concentraciones plasmáticas de aripiprazol y olanzapina en voluntarios sanos. La farmacocinética del aripiprazol se vio afectada por los fenotipos CYP2D6 y CYP1A2 y polimorfismos del gen *ABCB1*. La farmacocinética del dehidro-aripiprazol fue variable en base a los fenotipos CYP2D6, CYP3A y CYP1A2 y los polimorfismos del gen *ABCB1*. La farmacocinética de la olanzapina se vio afectada por el fenotipo CYP3A y los polimorfismos de los genes *ABCB1* y *UGT1A1*. El aripiprazol afectó a la contracción de la pupila: causó dilatación después de la administración de la primera dosis, y luego causó constricción después de cada dosis. La olanzapina sólo alteró el tamaño mínimo de las pupilas. Los polimorfismos en *CYP3A*, *HTR2A*, UDP-glucuronosiltransferasa 1A1 (*UGT1A1*), *DRD2* y *ABCB1* afectaron el tamaño de la pupila, el tiempo de inicio de la constricción, la recuperación de la pupila y la velocidad de constricción. La olanzapina indujo hiperprolactinemia pero no el aripiprazol. Los polimorfismos *DRD3* rs6280 y *ABCB1* rs10280101, rs12720067 y rs11983225 y el fenotipo CYP3A tuvieron un impacto en los niveles de prolactina plasmática. Sólo la olanzapina causó aumento de peso, lo que fue influenciado por los alelos *HTR2C*. Las concentración de péptido C fueron más altas después de la administración de aripiprazol y fueron influenciadas por los polimorfismos *COMT* rs4680 y rs13306278. La olanzapina y el polimorfismo *UGT1A1* rs887829 se asociaron con niveles elevados de glucosa. Los metabolizadores lentos de la CYP3A tenían niveles de insulina elevados. Las concentraciones de triglicéridos disminuyeron debido al tratamiento con olanzapina y aripiprazol y fueron variables en base a los fenotipos de CYP3A y el genotipo *APOC3* rs4520. Los niveles de colesterol también se redujeron y dependieron del polimorfismo *HTR2A* rs6314. Todas

las enzimas hepáticas, los niveles de plaquetas y albúmina y el tiempo de protrombina se alteraron durante ambos tratamientos. Además, la olanzapina redujo el recuento de leucocitos, el aripiprazol aumentó la T4 libre y ambos disminuyeron las concentraciones de ácido úrico. El aripiprazol disminuyó la presión arterial diastólica en el primer día de tratamiento y disminuyó el QTc en el tercer y quinto día de tratamiento. La olanzapina tuvo un efecto de disminución de la presión arterial sistólica y diastólica, la frecuencia cardíaca y el QTc en el primer día de tratamiento. Los polimorfismos en los genes *ADRA2A*, *COMT*, *DRD3* y *HTR2A* se asociaron significativamente a estos cambios. Se relacionaron más reacciones adversas con el aripiprazol que con la olanzapina, especialmente los trastornos psiquiátricos y del sistema nervioso, según la UKU, la escala de calificación de efectos adversos y los notificados espontáneamente. Las reacciones adversas más frecuentes al aripiprazol fueron somnolencia, dolor de cabeza, insomnio, mareos, inquietud, palpitaciones, acatisia y náuseas, mientras que a la olanzapina se le atribuyeron somnolencia, mareos, astenia, estreñimiento, sequedad de boca, dolor de cabeza y náuseas. Las puntuaciones de Ramsay mostraron un bajo nivel de sedación. Además, los polimorfismos en los genes *HTR2A*, *HTR2C*, *DRD2*, *DRD3*, *OPRM1*, *UGT1A1* y *CYP1A2* desempeñaron un papel en el desarrollo de reacciones adversas al aripiprazol y la olanzapina.

Conclusiones:

La olanzapina causó una elevación significativa de la prolactina y el peso, pero el aripiprazol alteró la contracción de la pupila. El test de tolerancia oral a la glucosa fue más alto después del tratamiento con olanzapina que también produjo más efectos cardiovasculares que el aripiprazol. Sin embargo, el aripiprazol se asoció a más reacciones adversas psiquiátricas y neurológicas. Muchos polimorfismos pueden influir en los parámetros pupilométricos y metabólicos, así como en los cambios cardiovasculares y los acontecimientos adversos. Además, varios polimorfismos tuvieron un efecto en la farmacocinética del aripiprazol, el dehidro-aripiprazol y la olanzapina. Parece que el aripiprazol provoca cambios metabólicos y cardiovasculares menos graves; sin embargo, se registraron más reacciones adversas asociadas a él que a la olanzapina.

I. INTRODUCTION

1. Clinical trials

Before including humans, animal studies have to be performed to analyze drug safety in equivalent doses to human exposures, to explore pharmacodynamics (mechanisms of action and the connection between drug levels and clinical response) and pharmacokinetics (drug absorption, distribution, metabolism, excretion and drug–drug interactions) [1].

Following animal studies, it is necessary to test the drug in 4 human study phases (*Figure* 1). Phase I clinical trials are the first when the drug is tested in humans and are designed to test safety, maximum tolerated dose, human pharmacokinetics and pharmacodynamics and drug-drug interactions. Phase II clinical trials are performed in a few patients with the disease of interest to assess preliminarily efficacy. They are designed to test safety, pharmacokinetics, and pharmacodynamics, similarly to phase I trials, but in people with the illness. Additionally, the planning of phase III trials already starts in phase II trials with the investigation of optimal doses, dose frequencies, administration routes and endpoints. They offer preliminary evidence of drug efficacy by comparing the study drug with controls, by examining different dosing arms within the trial, or by randomizing some subjects to control arms. However, given the low number of participants, it is necessary to perform the phase III trials. Phase III trials are conducted in a large and often more diverse target population with the disease of interest in order to demonstrate efficacy and to identify and estimate the incidence of common adverse drug reactions.

Finally, phase IV trials are conducted after European Medicines Agency (EMA) approval to identify less-common adverse drug reactions and to evaluate cost and/or drug effectiveness in different diseases, populations or doses [1] (***Figure* 1**). Post-marketing studies are conducted after EMA has approved a product for marketing, therefore it is already in use following EMA indications. The reason to perform these trials is to gather additional information about safety, efficacy or optimal use of the drug [2].

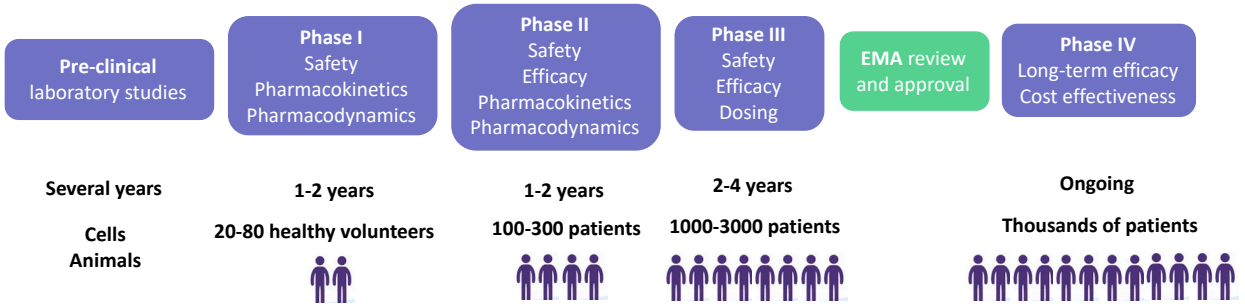

***Figure* 1.** The phases of clinical studies.

Schizophrenia treatment still remains a significant challenge due to the lack of efficacy and side effects of available drugs; and the stage of the illness. Moreover, the exact neuropathology, neurophysiology and neurochemistry of the disease are not clear to date, therefore, clinicians still rely on their subjective experience based on the symptoms to establish diagnoses [3].

Consequently, it is of importance to perform clinical trials to discover new information and replicate the existing findings. All clinical trials – unreplicated trials, uncontrolled systematic descriptions, case reports – offer valuable information. However, randomized clinical trials provide the most important evidence, although a single trial, even if it is large, cannot address the multiple questions that might be relevant in schizophrenia given its complexity. Nevertheless, although small trials address more factors, they may have insufficient statistical power to form conclusions. Therefore, meta-analyses are of particular interest as they combine results from all available trials [3].

Another important issue is that many of these clinical trials are performed for regulatory purposes and it is not required by regulatory agencies to inform clinicians about every detail prior to marketing. Moreover, many industry-sponsored studies are performed post-marketing and the results are frequently not published afterwards. However, studies funded by foundations and governmental agencies are commonly conducted for investigational purposes [3].

2. Liquid chromatography and mass spectrometry

2.1. Liquid chromatography

Liquid chromatography (LC) separates compounds in a mixture based on the differences in their structure; the sample is moving through the system with the mobile phase to the stationary phase, which is an analytical column. Analytes with strong interaction with the stationary phase move slowly in the column compared to those with weak interactions, thus they can be separated. High-performance liquid chromatography (HPLC) can alone separate, qualify and quantify analytes in a solution under high pressure (up to 400 bars) with a detector [4]. The ultra-HPLC (UHPLC) offers better analyte separation and better sensitivity due to using even higher pressure (up to 1000 bars) [5]. As compared to gas chromatography (GC), LC is not limited by sample volatility or thermal stability. Consequently, LC is ideal for the separation of almost any molecule of interest such as proteins, polysaccharides, synthetic polymers, nucleic acids, plant pigments, surfactants, amino acids, polar lipids, pharmaceuticals, dyes, explosives and plant and animal metabolites, therefore, almost any compound which can be dissolved in liquid can be analyzed [6].

2.1.1. Components and workflow of LC

In general, the components of an LC system consist of solvents, pump, sample injector, analytical column and detector. The reservoir holds the solvent (mobile phase). A high-pressure pump is used to generate a specified flow rate of the mobile phase (mL/min). A sample injector (autosampler) injects the sample into the continuously flowing mobile phase stream and finally arrive together to the HPLC column. The packing material of the column, i.e. the stationary phase is needed for chromatographic separation. Finally, a detector is needed to visualize the separated peaks (***Figure* 2**).

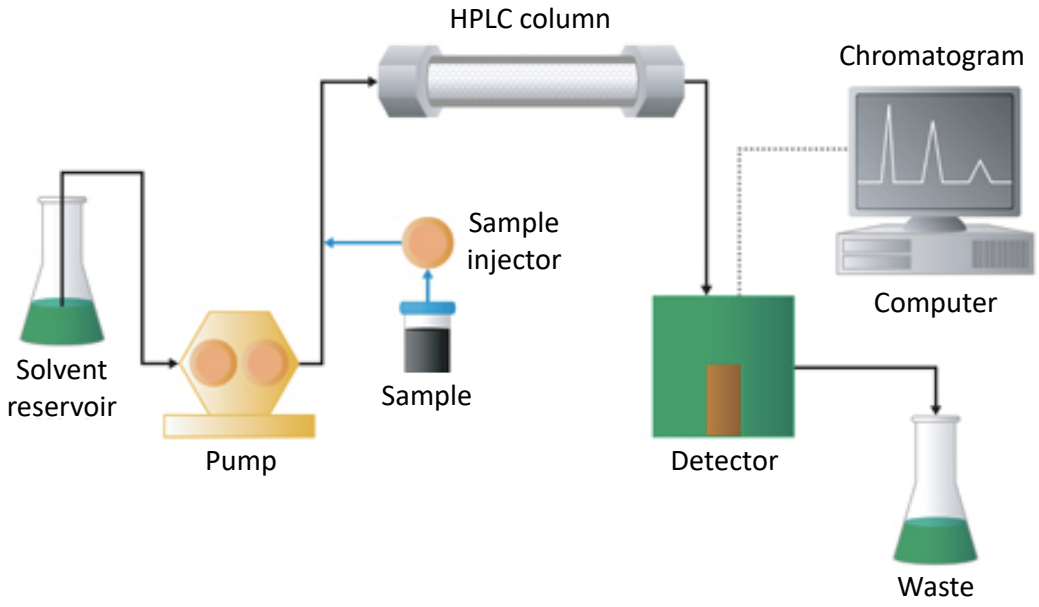

***Figure* 2**. The workflow of the HPLC system.

Source: www.waters.com, modified.

The exact time for a molecule in a solution to pass through a chromatography column (the stationary phase) is called retention time (RT). It is calculated from time of injection to detection. RT is specific for each analyte and provides qualitative information, while the area and the height of the peak are for quantitative analysis [5]. RT is often called acquisition time, which is the time that it takes for the Analog-to-Digital Converter to acquire and convert an analog signal to a digital value (***Figure* 3**).

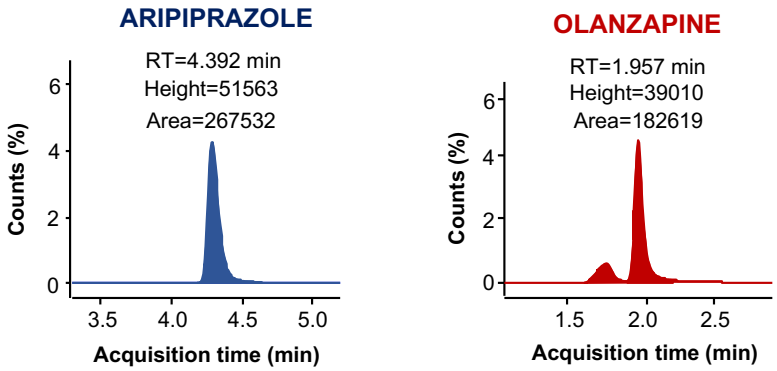

***Figure* 3**. Chromatograms of aripiprazole and olanzapine.

Source: Koller *et al.*, 2019 [7], modified.

2.1.2. Analytical column and mobile phase

High-performance columns should be designed for efficient separation. The packing particles (the stationary phase) should be small with similar geometry. In general, the size of the column should be appropriate for the number and properties of the analytes of interest [5]. There are two types of LC: normal and reversed phase. Normal-phase refers to using a column packed with unmodified silica with polar, i.e. hydrophilic properties and here, a non-polar solvent is used. Contrastively, reversed-phase refers to the use of non-polar, i.e. hydrophobic column packed with porous silica gel or polymer particles with specific chemical ligands on their surface. The most common package for a reversed-phase column is octadecyl carbon chain (C18)-bonded silica. In reversed-phase chromatography, a mixture of water and polar organic solvent is used for mobile phase. These different column packings are due to the distinct properties of the molecules. For instance, polar molecules are poorly retained on reverse phase columns and elute at the beginning of the chromatogram with a low organic content [8].

2.1.3. Chromatographic separation

When the composition of the mobile phase does not change during the whole separation process, it is called isocratic elution. Normally, the ratio of polar compounds to non-polar compounds is changed throughout the process to be able to elute various compounds in the sample in a short time, what is called gradient elution [9].

After completing the separation process with LC, a detector is needed for analysis. Several types of detectors can be used, such as electrochemical, ultraviolet and fluorescence detectors. However, mass spectrometry (MS) detectors are the most frequently used currently due to their high specificity, sensitivity and low detection limits (LOD) [8].

2.2. Mass spectrometry

Ideal detectors for LC are MS detectors. Although the first LC-MS hybrid method was developed in 1968 [10], its use was limited for many years due to the relative incompatibility of existing MS ion sources with a continuous liquid stream until the development of the electrospray ionization (ESI) method by Fenn [11]. Fenn was awarded the Nobel Prize in 2002 with Koichi Tanaka [12] who

developed matrix assisted laser desorption ionization (MALDI), another MS ionization technique for the analysis of biological molecules [13].

MS generates ions from molecules and then measures their specific mass-to-charge ratio (*m/z*). The "m" indicates the molecular mass number, while the "z" means the charge number of the ion [14]. This measurement allows to calculate the exact molecular weight of each molecule in the sample. MS can give qualitative results; it simply answers the question if the analyte of interest is in the sample. Besides, it can be quantitative; it calculates exactly how much of the analyte we have in the sample [14]. MS can be used for targeted or untargeted analysis. For targeted analysis, an acquisition method is designed to determine a list of known compounds. Untargeted analysis simultaneously measures as many compounds as possible from a biological sample [15]. Although many MS instruments exist, generally each of them consists of the following 5 components:

1. **Sample introduction site**

   Samples are usually separated previously by LC then arrive to the MS instrument.
2. **Ionization source**

   The uncharged analyte converts to positive or negative charged ions. The available ionization methods are ESI and MALDI and atmospheric pressure chemical ionization (APCI) [16].
3. **Mass analyzer**

   Ions get separated based on their m/z. The process is done under vacuum [14]. The types of mass analyzers are quadrupoles (Q), orbitraps, iontraps and time-of-flight (TOF).
4. **Ion detection system**

   The ions are detected with an abundance that is proportional to their original concentration. The product ions are passed to a transducer – an electron multiplier or Fourier transform – which converts their abundance into an electrical signal.
5. **Computer**

   Data output, acquisition and instrument control.

The generated mass spectrum of each molecule is a plot of ion abundance (%) versus *m/z*. The mass analyzer used in this study was MS/MS, also called as triple quadrupole (QQQ). It consists of 2 mass analyzers and a collision cell between them [17].

The components of an MS/MS system are the following (see in ***Figure* 4**):

1. **Ion source**

   ESI or APCI

2. **Mass Analyser (QQQ)**

   - **Q1:** first mass analyzer or quadrupole, the filter for precursor ions.
   - **Q2:** collision cell, the precursor ions get fragmentated to product ions.
   - **Q3:** second mass analyzer or quadrupole, the filter for product ions.

3. **Detector**

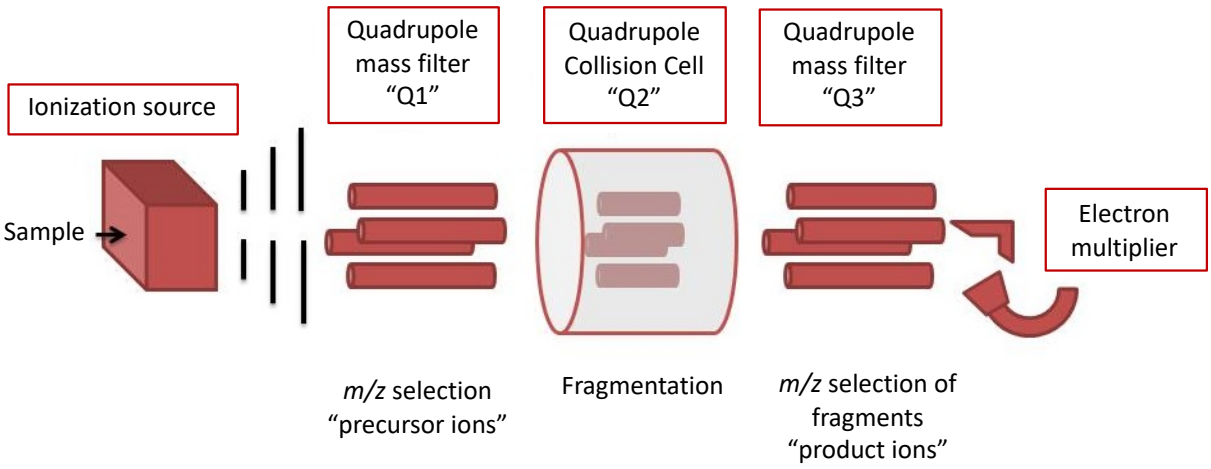

***Figure* 4**. Components of the MS/MS instrument.

***Figure* 5** shows the mass spectra of aripiprazole and olanzapine generated by a triple quadrupole mass analyzer.

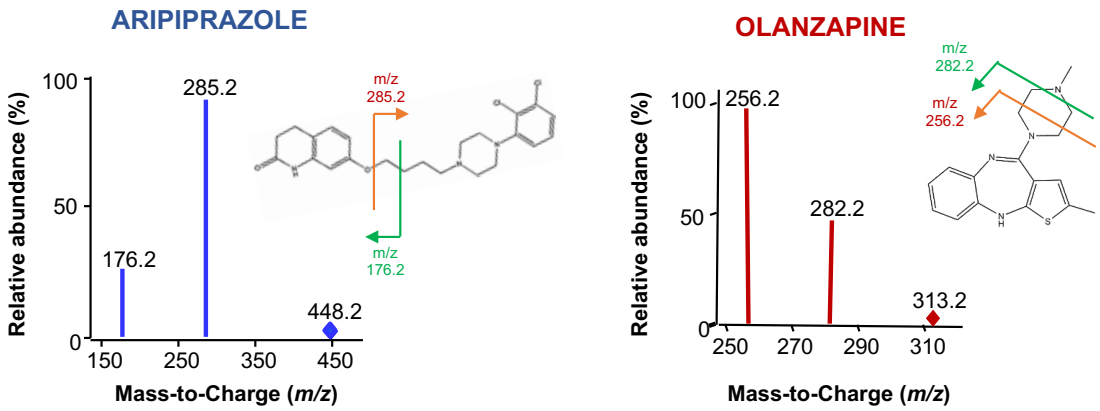

***Figure* 5.** Mass spectra of aripiprazole and olanzapine generated by a triple quadrupole mass analyzer.

Rhombus signs refer to precursor ions. The continuous lines show the product ions. The arrows display the site of fragmentation to generate the product ions in the second quadrupole.
Source: Koller *et al*., 2019, modified [7].

2.3. Sample preparation for LC-MS

Prior to injecting the sample to the LC system, the sample needs to be extracted appropriately, because tissue, blood, plasma, urine or cerebrospinal fluid cannot be injected directly. These matrices contain various endogenous and exogenous compounds that can interfere with the analysis. The most common methods for sample preparation in LC-MS are protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE).

PPT denatures the protein content of the sample while disrupting its drug binding ability. A strong acid or base, heat or organic solvents could be used for this process [18]. LLE is based on mixing an aqueous sample with an equal volume of organic solvent. Finally, the analyte will be extracted from the aqueous layer into the organic layer, however, it has to be soluble in the latter [19]. SPE separates compounds in the mixture based on their physical and chemical properties. It is used to concentrate and purify samples for analysis [20].

Choosing the appropriate sample extraction method for LC-MS analysis is of great importance as it has influence on the quality of the chromatogram and the ionization process [19]. Although PPT method eliminates big particles and proteins, lipids and salts still remain in the sample. Lipids are crucial LC-MS contaminants, especially when ESI is used as an ionization process [8]. SPE eliminates most of the interferences from the sample, thus reducing matrix effect (ion suppression or enhancement) [21].

2.4. Analytical method validation

Validation of an analytical method is necessary to demonstrate its reliability and reproducibility for quantitative analysis. Validation is specific for a particular matrix and has to be repeated in case of using another matrix. Guidelines of regulatory agencies – U.S. Food and Drug Administration (FDA), EMA and International Council on Harmonization (ICH) – explain the requirements for targeted method validation [22–24]. The following parameters should be evaluated during analytical method validation:

1. Selectivity or specificity
2. Linearity (calibration curve)
3. LOD and lower limit of quantification (LLOQ)
4. Accuracy and precision
5. Extraction recovery and matrix effects
6. Stability

2.4.1. Selectivity

Samples are usually a mixture of several compounds; thus the analytical method must be selective for the analyte of interest. When many interfering compounds are in the sample, the method is less selective. If its results are not affected by any other sample component, the method is completely specific. However, analytical methods are never 100% specific, but the analyst must be pursued to reach the highest specificity possible [25].

2.4.2. Linearity

Linearity is the method's ability to obtain directly proportional test results to the concentration of the analyte in the sample. Linearity refers to two sets of samples: calibrators and quality controls. Calibration curve with 6 to 8 calibration standards covering the expected calibration range should be performed for each analysis to quantify the analytes of interest. In addition, an LLOQ, a low, a medium and a high quality controls should be used; all of them need to be inside of the calibration range [22,23]. Three calibration methods can be used in quantitative analysis: external standard, internal standard and standard addition. In the present study, the IS method was used. An IS should be chemically similar to the analyte of interest and isotope-labeled. An equal amount of IS, that is not present in the sample, should be added to the samples, the calibrators and quality controls [26]. In addition, a blank and a zero sample should be also used during validation. The blank sample is a processed matrix sample without analyte and without IS, and the zero sample is a blank sample with IS [22].

2.4.3. LOD and LLOQ

LOD or detection limit is the smallest amount or concentration of analyte in the sample that can be reliably distinguished from zero. Contrastively, the LLOQ is the lowest concentration of the analyte that can be determined with an acceptable repeatability. The lower LOD and LLOQ are, more sensitive is the method [27].

2.4.4. Accuracy and precision

Accuracy indicates the closeness of a single measurement to the true value or an accepted reference value. Accuracy should be assessed on samples spiked with the quality controls that contain known amount of analytes. Precision describes the closeness of repeated individual measurements of the analyte within a single run and between different runs. Precision relates to the random error of a measurement system and it is a component of measurement uncertainty [22].

2.4.5. Extraction recovery and matrix effects

Recovery is calculated as the ratio of the peak area of the analyte spiked before and after the extraction process. It basically shows the efficiency of the extraction process. Matrix effect

compares the concentration of the analyte spiked after the extraction process to the neat solution of the analyte [22]. Matrix effects are often caused by the alteration of ionization efficiency of target analytes in the presence of coeluting compounds in the same matrix. Matrix effects can be observed either as a loss in response (ion suppression) or as an increase in response (ion enhancement). Both the ion suppression and enhancement dramatically affect the analytical performance of a method. The IS compensates for most of the matrix effects [28].

2.4.6. Stability

Stability is the chemical stability of a specific analyte in a matrix under specific conditions at different time points. Analyte stability must be ensured during sample collection, processing, storage, extraction and duration of the analysis. Thus, stability tests are usually among the most time-consuming tests in the validation procedure [22,23].

2.5. LC-MS in clinical pharmacokinetic studies

Clinical pharmacokinetics is described by a four-letter acronym, ADME: absorption, distribution, metabolism and excretion. It is used to describe mechanisms: crossing the gut wall (A); movement between compartments (D); mechanisms of metabolism (M); excretion or elimination (E); and transport (T) is usually added [29]. In general, the active moiety of the drug is followed through the body and during the whole time that the drug is present. ***Figure* 6** presents the pathway from the prescription of the drug until the patient's health. Although ADME is used traditionally, the acronym ABCD – administration, bioavailability, clearance and distribution – was developed for clinical pharmacology. Administration refers to dosing and adherence; bioavailability is the active moiety of the drug that arrives in the systemic circulation (A from ADME); clearance is the drug leaving the systemic circulation (ME from ADME) and distribution is the site or sites of action (D from ADME) [30].

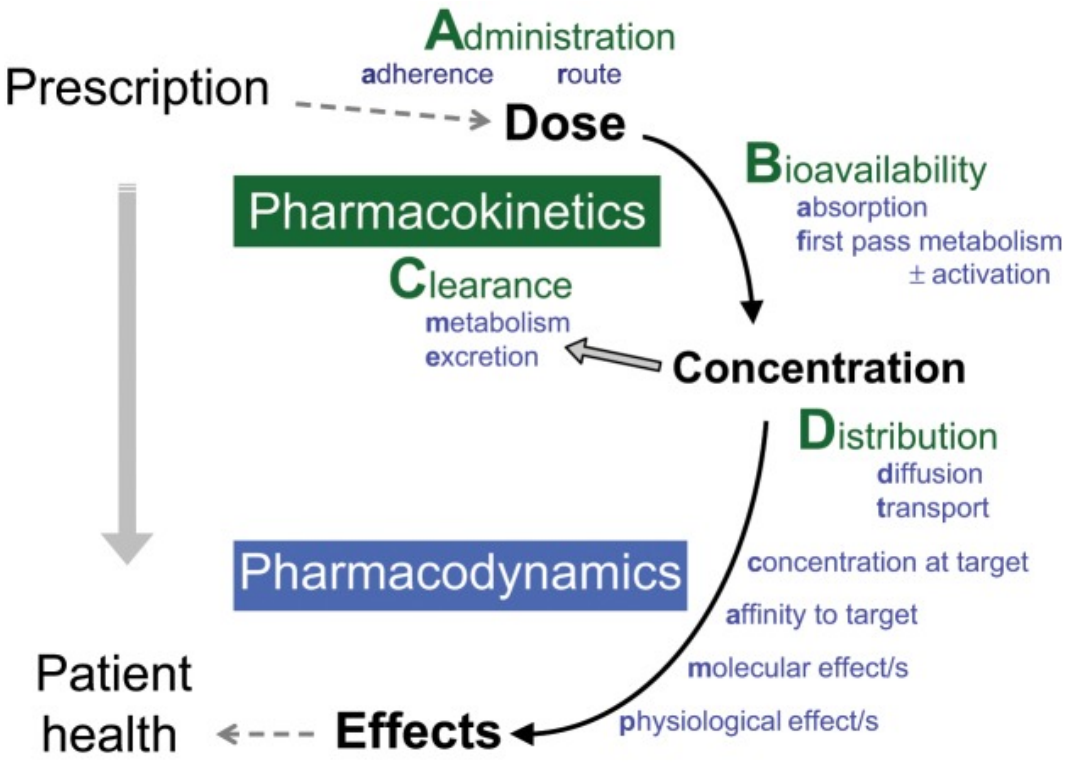

***Figure* 6**. The pathway from prescription to patient health.

Source: Doogue *et al*., 2013 [30].

The traditional factors for choosing the most appropriate drug for each patient are age, sex, weight, race, other diseases and other treatments [31–34]. Pharmacokinetic studies provide additional information for drug prescription. They are used to explore bioavailability, bioequivalence, drug discovery, safety, drug-drug interactions, dose adjustment, efficacy and toxicity [35]. Ideally, pharmacokinetic studies can be applied in personalized medicine [36]. Therapeutic drug monitoring (TDM) is a clinical laboratory measurement that can directly influence drug prescription. TDM is for the individualization of drug dosage by maintaining plasma or blood drug concentrations within a targeted therapeutic range or window. By combining knowledge of pharmaceutics, pharmacokinetics, and pharmacodynamics, TDM enables the assessment of the efficacy, safety and toxicity of a particular drug. The overall goal is to individualize treatment for each patient separately, thereby maximizing efficacy and minimizing toxicity [37].

The most important pharmacokinetic parameters are shown in ***Table* 1**.

***Table* 1**. Pharmacokinetic parameters.

Parameter	Description	Symbol	Unit
Maximum concentration	The peak plasma concentration of a drug after administration.	C_{max}	M, ng/mL
Time to maximum concentration	Time to reach C_{max}.	T_{max}	s, h
Minimum concentration	Through concentration; the lowest concentration that a drug reaches before the administration of the next dose	C_{min}	M, ng/mL
Volume of distribution	Volume of distribution; the apparent volume in which a drug is distributed.	Vd	m^3, L/kg
Clearance	The volume of plasma cleared of the drug per unit time.	Cl	m^3/s, mL/h/kg
Elimination half-life	The time required for the drug concentration to decrease to half of its original value for oral and other extravascular routes.	$T_{1/2}$	s
Area under the curve	The integral of the concentration-time curve.	AUC	M•s, ng•h/mL

The therapeutic range is the range of drug concentrations within the desired clinical response and low probability of toxicity. When drug concentrations reach to the maximum tolerated concentration (MTC), toxicity and side effects can appear. On the contrary, when drug concentrations do not reach the minimum effective concentration (MEC), the treatment can be ineffective [38] (***Figure* 7**).

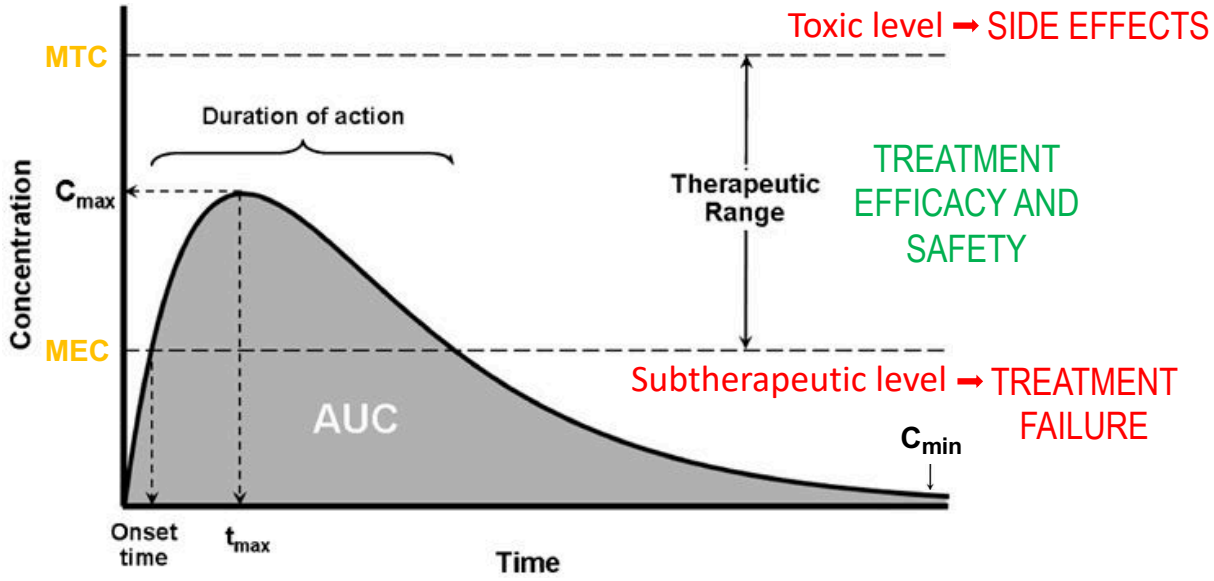

***Figure* 7**. Concept of the therapeutic range.

Source: Mehrotra *et al*., 2016 [38], modified.

Abbreviations: MEC: minimum effective concentration; MTC: maximum tolerated concentration; onset time: the time required for the drug to reach MEC after administration.

Definitions: duration of action: the time difference between the onset time and the time for the drug to decline back to MEC; therapeutic range: concentration between MEC and MTC; toxic level: level above MTC; subtherapeutic level: concentration below MEC.

Drugs undergo a series of chemical transformations by drug-metabolizing enzymes (DMEs) in order to facilitate their excretion from the body. This transformation is achieved through different metabolic pathways that are classified into two phases: phase I and phase II metabolism. Phase I reactions may occur by oxidation, reduction, hydrolysis, cyclization, decyclization and addition of oxygen or removal of hydrogen by cytochrome P450 (CYP) enzymes. CYPs metabolize 75% of drugs that are eliminated principally in the liver. In phase II reactions the drugs are conjugated with glutathione, sulfate, glycine or glucuronic acid by glutathione S-transferases, sulfotransferases, glycine N-acyltransferases and UDP-glucuronosyltransferases, respectively [39].

3. Pharmacogenetics and pharmacogenomics

3.1. Concept

As mentioned before, the goal of modern medicine is to individualize treatment for each patient. Apart from TDM, pharmacogenetics is also a highly important area worth to consider before prescribing a drug. Personalized medicine is also of interest to the pharmaceutical industry as it can help with drug development, drug testing and drug registration process while reducing the costs [40].

Pharmacogenetics investigates the variability in drug response due to heredity. Currently, the term pharmacogenomics is used more frequently that refers to all the genes in the genome that can determine drug response [41]. Pharmacogenetics originates from Pythagoras in Italy, where he found out that the ingestion of fava beans induced hemolytic anemia in some people, but not in all individuals [40]. Friedrich Vogel was the first who used the term pharmacogenetics in 1959 [42]. However, the field became of interest with the Human Genome Project [41].

The concept of pharmacogenetics is explained in ***Figure* 8**.

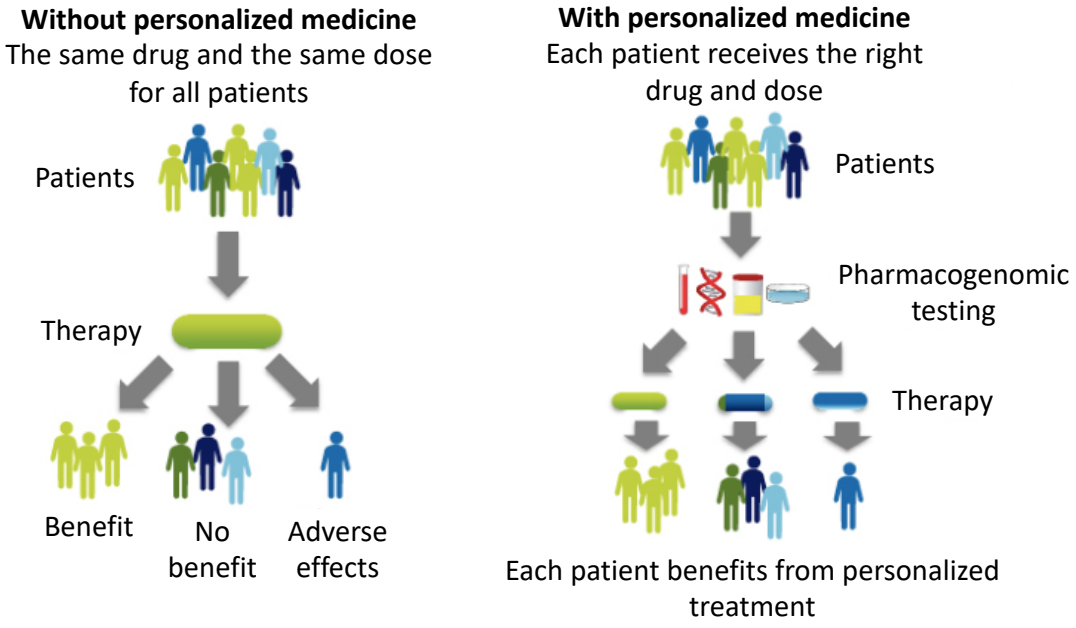

***Figure* 8**. The concept of personalized medicine based on pharmacogenetic testing.

Pharmacogenetic differences may cause reduced protein expression, increased resistance (usually a receptor mediated mechanism), altered response due to differences in enzyme induction and other disorders of unknown etiology. A difference does not mean disorder automatically, as low enzyme activity is not always associated with adverse reactions or ineffective treatment [41].

3.2. Genetic polymorphisms

The most important polymorphisms in pharmacogenetics are SNPs with the frequency of at least 1% in the population [43]. In addition, variable number of tandem repeats (VNTRs), short tandem repeats or microsatellites (STRs) and CNVs are also investigated regarding the variability in drug response [44]. CNVs are repeated sections of the genome and the number of repeats varies between individuals. They are structural variations: duplications or deletions that affect a considerable number of base pairs [45].

Each SNP represents a difference in a single nucleotide. For instance, a SNP may replace the nucleotide adenine (A) with the nucleotide guanine (G) or thymine (T) in a certain stretch of DNA (***Figure* 9**). SNPs, given their 1% frequency in the population, occur almost once in every 1000 nucleotides, therefore there are approximately 4 to 5 million SNPs in the genome. These variations may be rare or occur in many individuals.

An allele is a variant form of a gene, i.e. it is one of two or more versions of a mutation or SNP. Each individual inherits two alleles for each gene, one from the mother and one from the father. These alleles are in the same position on the homologous chromosomes (known as locus). On the autosomal chromosomes, the genotype for a SNP is the combination of the same two alleles at the same locus (one on each chromosome), known as homozygous. Subjects with two different alleles at one locus, one on each chromosome, are known as heterozygotes. For instance, the possible genotypes or allele pairs of a change from the C allele to the T allele are:

- C/C: the ancestral genotype, usually the most frequent and called as wild-type
- C/T: heterozygous genotype
- T/T: normally the least frequent genotype, called as mutant

Therefore, each individual can be classified as homozygous wild-type, heterozygous and homozygous mutant for each SNP.

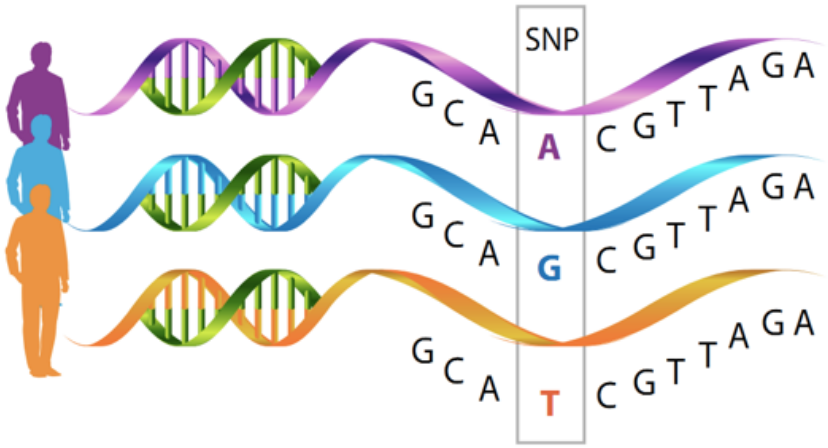

***Figure* 9**. Single nucleotide polymorphisms (SNPs).

SNPs can act as biological markers or play a direct role in disease development by affecting the gene's function. SNPs can be within coding sequences, non-coding regions or in the intergenic regions of genes. SNPs in the coding regions can be synonymous and nonsynonymous. Synonymous SNPs do not affect the protein sequence, while nonsynonymous SNPs change its amino acid sequence. The nonsynonymous SNPs can be missense or nonsense. Missense SNPs result in a codon (nucleotide triplets coding an amino acid) for a different amino acid. Nonsense SNPs cause a premature stop codon, therefore the truncation of the protein. SNPs that are outside of the protein-coding regions still can affect splicing, transcription factor and microRNA binding or messenger RNA degradation. These SNPs are usually in the promotor, upstream (5' untranslated region; 5'UTR) or downstream (3' untranslated region; 3'UTR) regions of the gene [46].

Since the completion of the Human Genome Project (2003), the entire sequence of the human genome – 3 billion base pairs – is known and freely accessible to everyone (e.g. https://www.ncbi.nlm.nih.gov/ or https://www.ensembl.org/index.html). However, all the details of this enormous amount of information are still unknown. For instance, the biological role of many mutations and polymorphisms and the exact function of transposons (genes that can change their position) is currently unknown. This can be due to the fact that individual variations have

little effect; many SNPs in many genes form the phenotype together with environmental factors [46]. Additionally, a consortium was founded between the pharmaceutical industry and charities to create a library of 300000 SNPs, however, finally it contains 1.42 million SNPs at an average density of one SNP every 1.9 kilobases. Theoretically, this could be used to create individual SNP profiles correlated with individual drug response [47].

The two main approaches for the analysis of the genetic predisposition related to diseases are candidate gene studies and genome-wide association studies (GWAS). Both approaches have several advantages and disadvantages. Candidate gene studies have higher statistical power, but cannot discover new genes of interest. GWAS can discover new genes or gene combinations, however, they have lower statistical power [48].

3.3. Pharmacogenes

SNPs present in metabolizing enzymes and transporters may affect the pharmacokinetics of drugs, while SNPs in receptors, ion channels or other molecules involved in the mechanism of action of drugs may affect their pharmacodynamics. Therefore, the response to drugs could be predicted by genetic polymorphisms that influence their pharmacokinetics and/or pharmacodynamics.

As mentioned before, SNPs in genes related to pharmacokinetics or pharmacodynamics are the main genetic factors accountable for individual drug response. Additionally, CNVs also take part in variable response. Several variants can be the cause of drug toxicity in particular patients and others are responsible for ineffectiveness of certain drugs. Pharmacogenes can be classified in different ways; the most suitable is functional characterization, which classify pharmacogenes on the basis of their protein function in three major classes: drug metabolizing enzymes, drug transporters and drug targets [49].

Currently 66 Very Important Pharmacogenes (VIP) are registered in the The Pharmacogenomics Knowledge Base (PharmGKB) database (https://www.pharmgkb.org/vips) based on recommendations from FDA and Clinical Pharmacogenetic Implementation Consortium (CPIC).

The listed VIP genes involve in the pharmacokinetic and pharmacogenetic variability of several drugs. Most of these genes (33) are involved in drug metabolism [49] (***Table* 2**).

***Table* 2**. List of Very Important Pharmacogenes (VIP).

Involve in	Biological function	VIP gene
Pharmacokinetics	Metabolizer	*ADH1A1, ADH1B, ADH1C, ALDH1A1, ALOX5, COMT, CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4F2, DPYD, G6PD, GSTP1, GSTT1, HMGCR, MTHFR, NAT2, NQO1, PTGIS, PTGS2, SULT1A1, TPMT, TYMS, UGT1A1, VKORC1*
	Transporter	*ABCB1, CFTR, SLC19A1, SLC22A1, SLCO1B1, ABCG2*
Pharmacodynamics	Receptor	*ADRB1, ADRB2, AHR, ALK, DRD2, EGFR, ERBB2, NR112, P2RY1, P2RY12, RYR1, HLA-B, VDR*
	Signaling	*ABL1, BRCA1, KIT, KRAS, NRAS, BRAF, BCR*
	Ion channel	*KCNH2, KCNJ11, SCN5A, CACNA15*
	Coagulation	*F5*
	Ribosomal RNA	*MT-RNR1*
	Regulator	*ACE*

Source: https://www.pharmgkb.org/vips.

3.3.1. Cytochrome P450 enzymes

The most studied drug metabolizer class is CYP450; the 57 existing CYP proteins metabolize thousands of endogenous compounds and xenobiotics. CYP450 enzymes are predominantly expressed in the liver, but they are present in the small intestine, lungs, placenta and kidneys as well [50]. To be a member in the CPY450 family, 40% of the amino acid sequence has to be identical and this is 55% when it is a member in the subfamily. Finally, the individual enzymes differ 3% in their amino acid sequence [51] (***Figure* 10**). From the 18 existing families, CYP1, CYP2 and CYP3 are the most relevant in the phase I drug metabolism [52]. Yet, 90% of the drugs are metabolized by CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 [50].

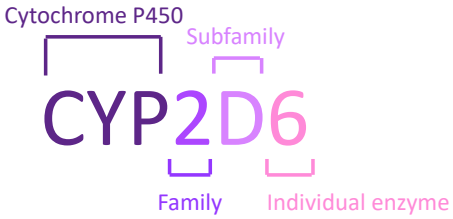

***Figure* 10**. Nomenclature of the Cytochrome P450 superfamily.

Each isoform varies in each population due to genetic, non-genetic and environmental factors, some of which are constant (genotype, sex), while others are dynamic (age, administered drugs, pathologies). These factors also do not influence different isoforms in the same manner. For instance, the activity of CYP2D6 is mainly influenced by genetic polymorphisms, while the activity of CYP3A4 is mainly influenced by sex and induction or inhibition by a wide range of drugs [53]. ***Figure* 11** shows the contribution of the most frequent drug metabolizing CYPs and factors that influence their variability.

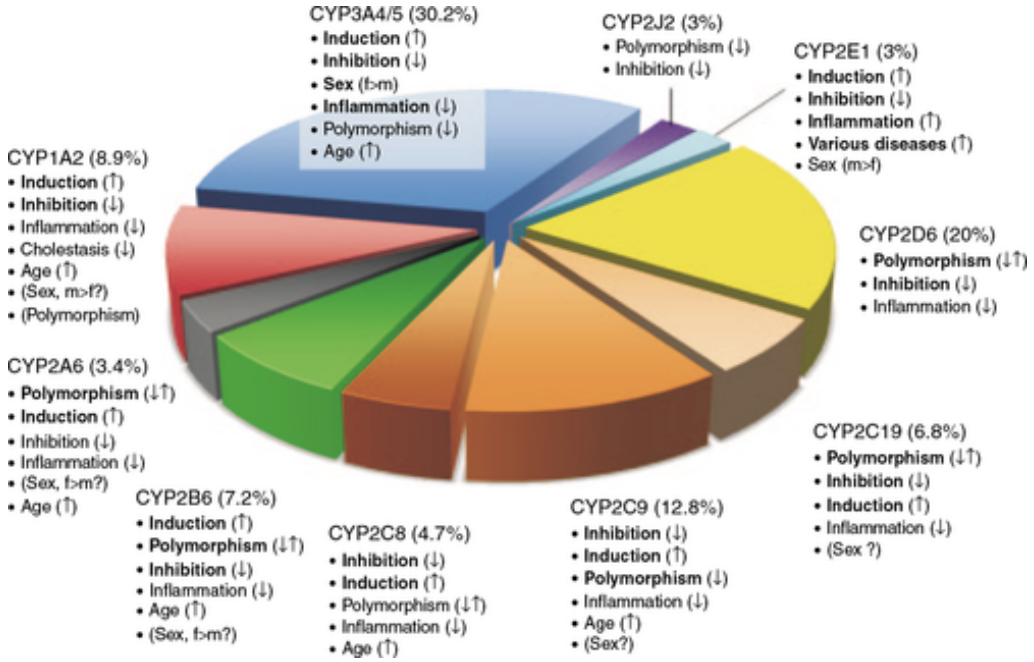

***Figure* 11**. Contribution (%) of individual cytochrome P450 isoforms to major drug metabolism pathways and factors influencing variability.

Variability factors are indicated by bold type with possible directions of influence indicated (↑: increased activity; ↓: decreased activity; ↑↓: increased and decreased activity).
Source: Zanger *et al*., 2014 [53].

3.3.2. Drug transporters

In pharmacogenetics, it is also of importance to analyze transporters, i.e. membrane proteins that control the access of endogenous and xenobiotic substances throughout the body. Unlike drug metabolizing enzymes, which are mainly expressed in the liver and intestine, drug transporters are found in all types of tissues. For this reason, their role is relevant in the absorption, distribution and elimination of drugs, thus influencing their pharmacokinetics and pharmacodynamics. The Human Genome Project identified more than 400 transporters belonging to the two main superfamilies: ATP-Binding Casette (ATP or ABC) transporters and the solute carrier (SLC) families [54].

The ABC carrier family plays a key role in the absorption of drugs in the gastrointestinal tract or in their transportation through the blood-brain barrier to the central nervous system. The most important protein in the ABC family is the P-glycoprotein (P-gp), a flow pump that transports xenobiotic compounds out of the tissue in which they are expressed [55]. P-gp was discovered by its ability to confer multidrug resistance (MDR) to cancer cells [56]. It is encoded by the *ABCB1* (*MDR1*) gene and it is expressed in specialized epithelial cells of secretory/excretory organs (liver, kidneys, small intestine) and in cells of the blood-brain barrier or the placenta [57]. Due to its particular location, P-gp may be involved in the ADME process of multiple drugs [58]. Therefore, P-gp expression level and its functional integrity may influence the pharmacokinetics of drugs that are its substrates [59].

3.4. Clinical Practice Guidelines

Clinical Practice Guidelines facilitate the access about recent findings and help to understand all the information to provide treatment recommendations. However, the fast development of pharmacogenetic knowledge does not always imply that the clinical evidence is enough to set guidelines for treatment or dosage. Therefore, an important barrier for the implementation of pharmacogenetics in clinical practice is the translation of the results of a genetic test into clinical application [60].

The first dosing recommendations were for antidepressants based on CYP2D6 and CYP2C19 genotypes related to pharmacokinetic data [61]. To date, the two most important groups that develop guidelines are the CPIC and the Dutch Pharmacogenetics Working Group (DPWG). The CPIC was established in 2009 as a joint project between the Pharmacogenomics Research Network (PGRN) and the PharmGKB. The objectives of CPIC are 1) to address the barriers of the implementation of pharmacogenetic tests into clinical practice, 2) to provide guidelines that enable the translation of genetic laboratory test results into prescribing decisions for drugs individually. To date, this database contains 40 gene-drug pairs with therapeutic recommendation and it is available for free in https://cpicpgx.org/guidelines/. The objectives of DPWG are 1) to develop pharmacogenetics-based therapeutic and dose recommendations, 2) to assist drug prescribers and pharmacists by integrating the recommendations into computerized systems for drug prescription and automated medication surveillance [62]. This database contains more than 50 gene-drug pairs with therapeutic recommendation and it is available for free in https://www.pharmgkb.org/page/dpwg.

These guidelines include several gene/drug pairs, such as CFTR/ivacaftor, CYP2B6/efavirenz, CYP2C19/clopidogrel, CYP2D6/tamoxifen, DPYD/fluoropyrimidines or HLA-B/abacavir, among others (***Table* 3**).

***Table* 3**. Gene-drug pair recommendations in Clinical Practice Guidelines.

Recommendation	**Drug**	**Gene**
CFTR and ivacaftor	Ivacaftor	*CFTR*
CYP2C19 and clopidogrel	Clopidogrel	*CYP2C19*
CYP2C19 and voriconazol	Voriconazol	*CYP2C19*
CYP2C9, *HLA-B* and phenytoin	Phenytoin	*HLA-B* *CYP2C9*
CYP2C9, *VKORC1*, *CYP4F2* and warfarin	Warfarin	*CYP2C9* *VKORC1* *CYP4F2*
CYP2D6 and atomoxetine	Atomoxetine	*CYP2D6*
CYP2D6 and codeine	Codeine	*CYP2D6*
CYP2D6 and ondansetron and tropisetron	Ondansetron, Tropisetron	*CYP2D6*

Recommendation	Drug	Gene
CYP2D6 and tamoxifen	Tamoxifen	*CYP2D6*
CYP2D6, *CYP2C19* and selective serotonin reuptake inhibitors	Citalopram Escitalopram Fluvoxamine Paroxetine Sertraline	*CYP2D6* *CYP2C19*
CYP2D6, *CYP2C19* and cyclic antidepressants	Amitriptyline Clomipramine Desipramine Doxepin Imipramine Nortiptyline Trimipramine	*CYP2D6* *CYP2C19*
CYP3A5 and tacrolimus	Tacrolimus	*CYP3A5*
DPYD and fluoropyrimidines	Capecitabine Fluoroacil Tegafur	*DPYD*
G6PD and rasburicase	Rasburicase	*G6PD*
HLA-A, *HLA-B* and carbamazepine and oxcarbamazepine	Carbamazepine Oxcarbamazepine	*HLA-A* *HLA-B*
HLA-B and abacavir	Abacavir	*HLA-B*
HLA-B and alopurinol	Alopurinol	*HLA-B*
IFNL3 and regimens based on pegylated interferon	Pegylated interferon alfa-2A Pegylated interferon alfa-2B	*IFNL3*
RYR1, *CACNA1S* and volatile anesthetic agents and succinylcholine	Desflurane Enflurane Halothane Methoxyflurane Isoflurane Sevoflurane Succinylcholine	*RYR1* *CACNA1S*
SLCO1B1 and simvastatin	Simvastatin	*SLCO1B1*
TPMT, *NUDT15* and thiopurines	Azathioprine Mercaptopurine Thioguanine	*TPMT* *NUDT15*
UGT1A1 and atazanavir	Atazanavir	*UGT1A1*

Source: https://cpicpgx.org/guidelines/.

4. Schizophrenia

Schizophrenia is a debilitating mental disorder that impairs mental and social functioning. The disease affects about 1% of the total population and it is equally prevalent in males and females. However, males habitually get diagnosed with the disease in their late teenage years or early twenties, while females usually start to show symptoms in their late twenties or early thirties. There are five types of schizophrenia: paranoid, disorganized, catatonic, undifferentiated and residual [63]. The disease is generally characterized by positive and negative symptoms. The most common positive symptoms are hallucinations, paranoid delusions and disorganized speech and behavior, while the most common negative symptoms are social withdrawal, difficulties with affection, speech impairment, apathy, anhedonia, loss of motivation and lack of social interest, among others. These changes hinder these patients from maintaining a normal life and also affect social relations, i.e. relationship with their families and friends. Additionally, there is a 10% lifetime risk of suicide [64]. Apart from this issue, substance abuse – especially consuming alcohol, nicotine, cocaine and cannabis – is very common. Normally, substance abuse is associated with substandard treatment outcomes, including increased psychotic symptoms and poorer treatment compliance [65].

Schizophrenia is considered as a polygenic disorder with the presence of several environmental factors. Possible risk factors to develop the disease are family history, season and birth location, socioeconomic status and maternal infections. However, to date, the evidence is still inconclusive [66,67]. The genetic risk of schizophrenia is highly sensitive to the patients' emotional status and family environment. Even if being genetically predisposed to the disease, several environmental factors could be protective against the manifestation of the symptoms [68]. Additionally, ethnicity also seems to be an important risk factor in the development of the disease. African Americans, Asians, and Hispanics are diagnosed more frequently with schizophrenia compared to Caucasians [69]. Likewise, the incidence of schizophrenia is higher among immigrant ethnic groups in Western Europe, what can be caused by the social stress due to their ethnic minority [70].

The most studied SNPs in the genetic background of schizophrenia are in the following candidate genes: disrupted in schizophrenia 1 (*DISC1*), dystrobrevin-binding protein 1 (*DTNBP1*),

neuregulin 1 (*NRG1*) and catechol-O-methyltransferase (*COMT*). DISC1 participates in the regulation of cell proliferation, differentiation, migration, neuronal axon and dendrite outgrowth, mitochondrial transport, fission and fusion and cell-to-cell adhesion [71]. DTNBP1 belongs to the dytrophin-associated protein complex of skeletal muscle cells [72]. NRG1 is a cell adhesion protein what belongs to the neuregulin family that act on the EGFR receptors [73]. COMT is one of several enzymes that degrade catecholamines [74].

Furthermore, several studies found associations between schizophrenia and genetic markers across the extended Major Histocompatibility Complex (MHC) locus on chromosome 6. Likewise, the role of rare and large CNVs, i.e. deletions on chromosome 1q21.1, 3q29, 15q13.3 and 22q11.2 and duplications on chromosome 16p11.2 and 16p13.11 seem to be related to the disease. Thus, genetics is definitely a strong risk factor for schizophrenia, however, its genetic architecture is very complex, heterogeneous and polygenic: the risk for the disease is constituted by numerous common genetic variants (e.g. SNPs) and by uncommon, but highly penetrant genetic variants with larger effect (e.g. CNVs) [75].

Furthermore, dopamine seems to play an important role. Drugs that cause psychoses similar to the positive symptoms of schizophrenia increase dopaminergic neurotransmission, and almost all antipsychotics decrease dopaminergic neurotransmission. Nevertheless, dopaminergic pathways cannot entirely explain the pathophysiology of schizophrenia and the role of other neurotransmitters, such as serotonin, should be considered [76].

5. Antipsychotic treatment

Antipsychotics are utilized for the treatment of several psychiatric conditions, including schizophrenia, bipolar disorder, reactive psychoses, depression with psychotic symptoms, psychoses due to substance abuse, mental retardation, delirium, borderline personality disorders, Huntington's disease, Tourette's syndrome, among others [77].

Effective pharmacologic treatment for schizophrenia has been available since the 1950s in Europe and the United States. After introducing the first antipsychotic to the market, the term

"neuroleptic" was introduced to distinguish their effects from other sedatives and antidepressants [78]. Since introducing clozapine, the first second-generation (atypical) antipsychotic, this term usually refers to first-generation (typical) antipsychotics. The term atypical antipsychotic refers to the less risk of extrapyramidal side effects compared to typical antipsychotics. ***Table* 4** presents currently available antipsychotic agents in Europe.

***Table* 4**. Currently available antipsychotic drugs.

Medication class	Drug	Year of approval by EMA	Usual effective dose
Dopamine D2 antagonists (high-potency)	Perphenazine	1957	16 mg twice daily
	Trifluoperazine	1959	6 mg twice daily
	Fluphenazine	1960	2.5 mg twice daily
	Haloperidol	1967	5 mg three times daily
	Thiothixene	1967	10 mg three times daily
	Fluphenazine decanoate	1972	25 mg intramuscular injection every three weeks
	Haloperidol decanoate	1986	100 mg intramuscular injection every four weeks
Dopamine D2 antagonists (mid-potency)	Molindone	1974	25 mg three times daily
	Loxapine	1975	50 mg twice daily
Dopamine D2 antagonists (low-potency)	Chlorpromazine	1957	100 mg three times daily
	Thioridazine	1962	100 mg three times daily
Atypical (mixed neuroreceptor antagonists: low-affinity dopamine D2 antagonists, high-affinity 5-HT_{2A} antagonists)	Clozapine	1989	125 mg twice daily
	Risperidone	1993	4 mg once daily
	Olanzapine	1996	10 mg once daily
	Quetiapine	1997	200 mg twice daily
	Ziprasidone	2001	40 mg twice daily
	Aripiprazole*	2002	20 mg once daily
	Paliperidone	2006	3 mg once daily
	Iloperidone	2009	12 mg twice daily
	Asenapine	2009	10 mg twice daily
	Lurasidone	2010	40 mg once daily

*Aripiprazole shows special atypicality compared to other atypical antipsychotics; it acts as a partial agonist at dopamine D2 receptors and it is a 5-HT_{2A} receptor antagonist. Source: Mauri *et al*., 2014 [79].

Nonadherence to medications, consequently relapse is a significant problem; in a previous study, 74% of patients discontinued their medication within 18 months after starting treatment [80]. Originally, atypical antipsychotics were thought to help with this issue due to the lower risk of neurological side effects. Nevertheless, drop-out rates and the risk of relapse did not seem to improve [81]. Additionally, it was found that many benefits of the atypical antipsychotics were dose-dependent; when administering low doses, the neurological side effects ceased [82]. Furthermore, delays in initiating antipsychotic therapy may result in lifetime psychotic episodes and difficulties with social adjustment [83].

Antipsychotic polypharmacy, i.e. the co-prescription of more than one antipsychotic drug to the patient, is frequently applied in the clinical environment. The general reason to do so is to gain a faster therapeutic response compared to antipsychotic monotherapy. The risk-benefit profile of polypharmacy is usually unfavorable due to the increased number of adverse effects and the difficulty to establish a maintenance dose [84]. Moreover, anticholinergic, antidepressant, anxiolytic and mood stabilizing drugs, among others, are also frequently prescribed in addition to antipsychotic drugs [85].

5.1. Adverse drug reactions

Medications can control the positive and negative symptoms; however, all antipsychotics have side effects. Typical antipsychotics usually cause several extrapyramidal side effects, such as dystonic reactions (fixed upper gaze, neck twisting, facial muscle spasms), parkinsonian symptoms (rigidity, bradykinesia, shuffling gait, tremor), akathisia (inability to sit still, restlessness, tapping of feet) and neuroleptic malignant syndrome (catatonia, stupor, fever, unstable blood pressure, myoglobinemia) [64]. Tardive dyskinesia is also considered as an extrapyramidal side effect. It is a movement disorder that is characterized by involuntary and repetitive body movements [86]. Normally, the Abnormal Involuntary Movement Scale is used to monitor the development of involuntary movements associated with typical antipsychotics [87].

Atypical antipsychotics are associated with fewer neurological side effects. However, the risk of metabolic side effects (diabetes, hypercholesterolemia, hyperprolactinemia, weight gain,

hyperinsulinemia, elevated triglyceride levels, elevated plasma uric acid levels) is higher compared to typical antipsychotics [88]. The incidence of metabolic side effects is considered certainly high, therefore their recurrent monitoring is recommended in patients (***Table* 5**). However, few data are available concerning the relative frequency of performing these tests [89].

***Table* 5**. Physical health monitoring recommendations for patients before and under antipsychotic treatment.

Condition	Recommendation	Monitoring Procedure
Weight gain	Monitoring body mass index (BMI)	BMI measurement
	The risk of weight gain is higher for patients with BMI ≥25	
	A weight gain of one BMI unit indicates a need for an intervention, except for underweight patients (BMI <18.5)	
Diabetes	Baseline plasma glucose level measurement before starting a new antipsychotic	Fasting plasma glucose level or hemoglobin A_{1c} value
	Patients with high risk for diabetes and weight gain should be frequently monitored	
	The symptoms of new-onset diabetes – weight loss, polyuria, polydipsia – should be diagnosed	Evaluation
Hyperlipidemia	Lipid levels should be monitored	Screening of total cholesterol, low- and high-density lipoprotein (LDL and HDL) cholesterol, and triglycerides
	Schizophrenic patients should be considered to be at high risk for coronary heart disease	
	National Cholesterol Education Program guidelines (http://www.nhlbi.nih.gov/about/ncep/) should be followed for screening and treating patients who are at high risk for cardiovascular disease	
	If the patient's LDL level is >130 mg/dl, the patient should be sent to a primary care provider or internist and the patient should change his/her diet to reduce fat intake. If the LDL level does not fall into the normal range, a cholesterol-lowering drug should be initiated	
QT interval prolongation	Thioridazine, mesoridazine, or pimozide should not be prescribed for patients with known heart disease, a personal history of syncope, a family history of sudden death at an early age or congenital prolonged QT syndrome	Electrocardiogram
	Patients with any of the risk factors described in the previous recommendation for whom ziprasidone is prescribed should be evaluated with an ECG	

Condition	Recommendation	Monitoring Procedure
Prolactin and sexual function	Female patients should be asked about changes in menstruation and libido and whether they have milk coming out of their breasts; male patients should be asked about libido and erectile and ejaculatory function	Asking patients about symptoms associated with prolactin elevation
	If a patient is receiving an antipsychotic known to be associated with prolactin elevation, the questions described in the previous recommendation should be asked more frequently	
	If having symptoms, the patient's prolactin level should be measured and, if possible, other medical causes of the symptoms should be ruled out; a medication change should be considered to a prolactin-sparing medication	Measuring prolactin level
Extrapyramidal side effects	Extrapyramidal side effects and tardive dyskinesia should be examined before the initiation of any antipsychotic medication to determine the possible preexistence of parkinsonian signs or abnormal involuntary movements	Examination for rigidity, tremor, akathisia
	After initiation of antipsychotic medication, patients should be evaluated frequently for extrapyramidal side effects until the dose has been stabilized	
	Tardive dyskinesia should be examined frequently; patients at high risk for tardive dyskinesia, including elderly patients, should be examined even more frequently	Examination for tardive dyskinesia
Cataracts	Change in vision, especially distance vision and blurry vision should be evaluated	Inquiry about change in vision
	Regular ocular evaluations should be performed	Evaluation
Myocarditis	Clozapine-treated patients should be monitored for myocarditis	No specific recommendations
	Myocarditis should be suspected in clozapine-treated patients with unexplained fatigue, dyspnea, tachypnea, fever, chest pain, palpitations, other signs or symptoms of heart failure, or ECG findings such as ST abnormalities and T wave inversions	
	If myocarditis is suspected, the white blood count and serum level of troponin should be measured	
	If myocarditis is identified, clozapine should be stopped and the patient should be urgently evaluated by a primary health care provider	

Source: Marder *et al*., 2004 [89].

5.2. Olanzapine

5.2.1. Pharmacokinetics

Olanzapine, a thienobenzodiazepine derivative, is an atypical antipsychotic. The FDA approved it in 1996. Doses from 5 to 20 mg once a day are effective and well tolerated by patients [90].

Olanzapine is well absorbed with 87% of absolute bioavailability and reaches T_{max} in approximately 6 hours following an oral dose. It is eliminated extensively by first pass metabolism, with approximately 40% of the dose metabolized before reaching the systemic circulation. Food does not affect the rate or extent of olanzapine absorption. Olanzapine displays linear kinetics over the clinical dosing range. Its $T_{1/2}$ ranges from 21 to 54 hours. When administered once daily, it reaches steady-state concentrations in approximately 1 week. Plasma concentrations, $T_{1/2}$ and Cl of olanzapine vary based on smoking status, gender, and age. Olanzapine is extensively distributed throughout the body, with a Vd of approximately 1000 L or 21.9 L/kg. 93% of the drug binds to plasma proteins, primarily to albumin and α1-acid glycoprotein.

Olanzapine is extensively metabolized by direct glucuronidation via the UDP-glucuronosyltransferase (UGT) enzyme family and oxidized by CYP1A2 and secondarily by CYP2D6 and CYP3A4. Direct glucuronidation and CYP mediated oxidation are the primary metabolic pathways for olanzapine. In vitro studies suggest that CYP1A2 and CYP2D6, and the flavin-containing monooxygenase 3 (FMO3) are involved in olanzapine oxidation [90,91]. CYP2D6 mediated oxidation appears to be a minor metabolic pathway in vivo as the clearance of olanzapine is not reduced in CYP2D6-deficient subjects [90]. After multiple dosing, the major circulating metabolites were the 10-glucuronide, present at steady state at 44% of the concentration of olanzapine, and 4'-desmethyl olanzapine, present at steady state at 31% of the concentration of olanzapine. Both metabolites lack pharmacological activity at the concentrations observed [90,92]. Olanzapine acts as an P-glycoprotein (P-gp) inhibitor [93]. The complex metabolic pathways of olanzapine are shown in ***Figure* 12**.

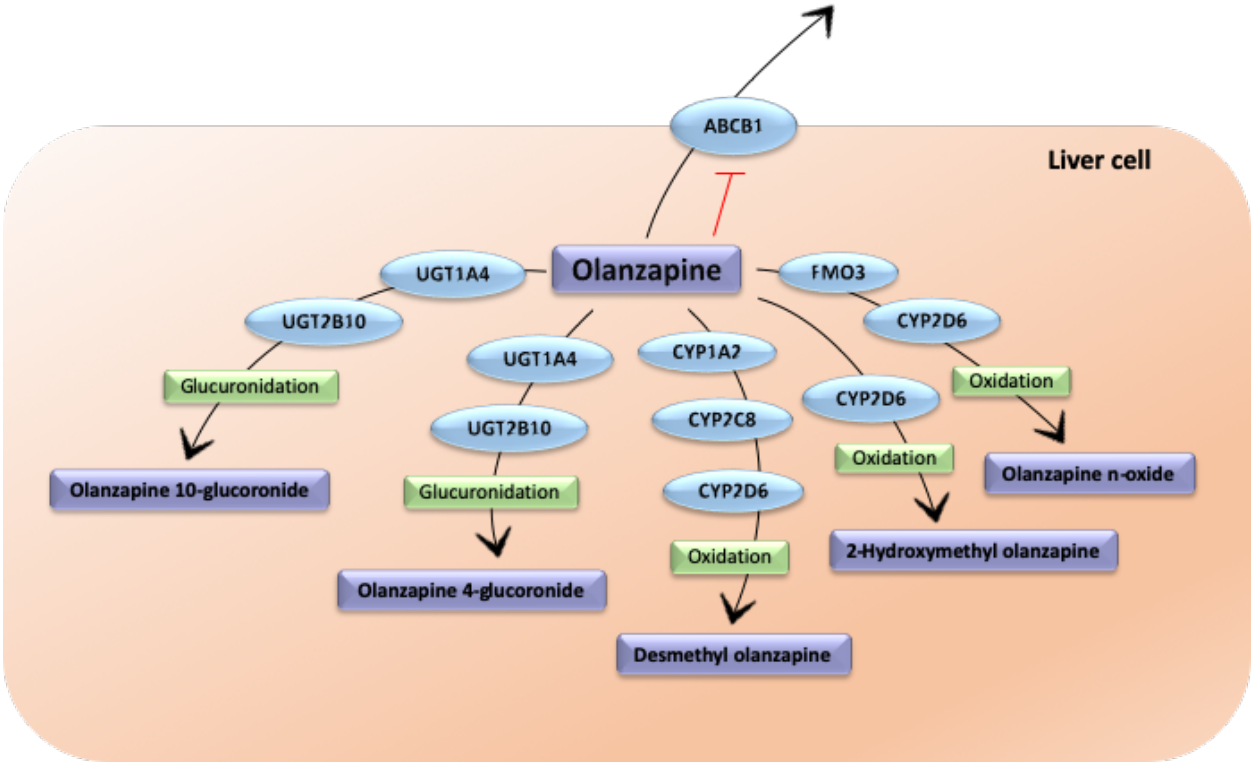

***Figure* 12**. The metabolic pathways of olanzapine.

Olanzapine is excreted mainly in the urine and partly in the feces. Following a single oral dose of ^{14}C labelled olanzapine, 7% of the dose was recovered in the urine as unchanged drug, indicating that it is highly metabolized. Approximately 57% and 30% of the dose was recovered in the urine and feces, respectively. Approximately half of the radiocarbon was excreted within 3 days, whereas > 70% of the dose was recovered within 7 days of dosing [94]. In plasma, olanzapine accounted for only 12% of the AUC indicating significant exposure to metabolites [95].

5.2.2. Mechanism of action

Olanzapine has significant *in vitro* inhibitory activity at dopamine D2, D3, D4, serotonin 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_6, histamine H1, α1-adrenergic and muscarinic M1-5 receptors. It has a higher 5-HT_{2A}/ dopamine D2 binding ratio compared to typical antipsychotics [95,96] (***Figure* 13**).

Olanzapine has high affinity for muscarinic M1-5 receptor subtypes, what could play an important

role in the suppression of extrapyramidal side effects through reducing dopamine receptor blockade [97]. Additionally, it has lower affinity for α1-adrenergic receptors than clozapine and risperidone what may increase its mesocorticolimbic selectivity. In fact, the ideal binding profile for an antipsychotic agent are 5-HT_{2A} antagonism, weak dopamine D2 antagonism and moderate α1-adrenergic antagonism [98]. Olanzapine has higher affinity for histaminergic H1 receptors than haloperidol and risperidone what may be partly responsible for side effects, such as sedation and weight gain [99]. Additionally, it has low affinity for ß-adrenergic receptors like clozapine and haloperidol, which suggests to be a factor in the development of olanzapine-induced weight gain [100]. Finally, it has lower activity for α2-adrenergic receptors than clozapine, which may reduce cardiovascular effects habitually produced by clozapine [101], however, it can lead to body weight changes [102].

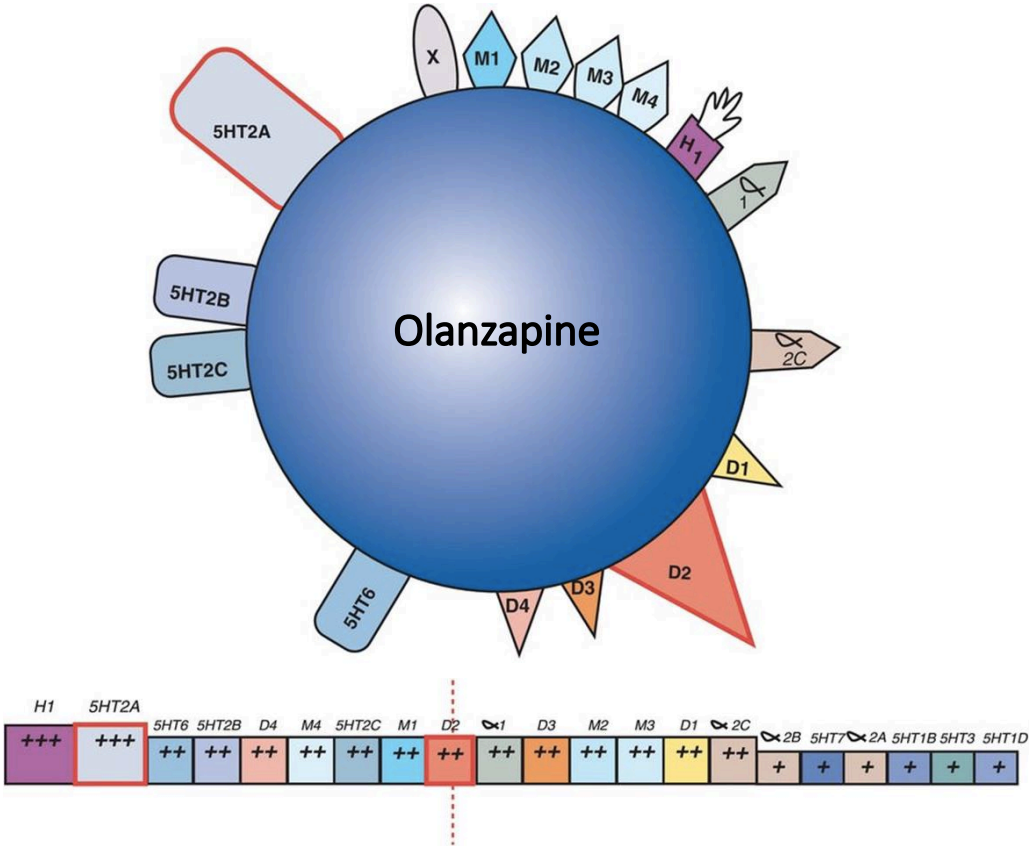

***Figure* 13**. The mechanism of action of olanzapine.

Boxes are placed in rank order of how potently olanzapine binds to the receptors, with the most potent and largest boxes to the far left and the weakest binding and smallest boxes to the far right. The vertical line indicates the weaker binding to dopamine D2 receptor compared to other typical antipsychotics.
Source: Riordan-Eva, Paul *et al.*, 2018 [103].

5.3. Aripiprazole

5.3.1. Pharmacokinetics

Aripiprazole, a quinolinone derivative, is an atypical antipsychotic approved in 2002 by FDA. Doses from 15 to 30 mg once a day are effective and well tolerated by patients [104]. Aripiprazole tablet is well absorbed with 87% absolute oral bioavailability. It reaches T_{max} within 3 to 5 hours with the C_{max} of 163 to 452 ng/mL with the dose of 10 to 30 mg/day at steady state (day 14) [105].

The single-dose pharmacokinetics of aripiprazole is linear and dose-proportional between the doses of 5 mg to 30 mg. Its steady-state Vd is high (404 L or 4.9 L/kg), indicating extensive extravascular distribution. At therapeutic concentrations, 99% of aripiprazole and its active metabolite, dehydro-aripiprazole bind to serum proteins, primarily to albumin. In healthy human volunteers 0.5 to 30 mg/day aripiprazole was administered for 14 days when dose-dependent D2 receptor occupancy was observed indicating brain penetration of aripiprazole in humans [106].

The metabolism and elimination of aripiprazole is mainly mediated through two CYP isoforms, CYP2D6 and CYP3A4. CYP3A4 is responsible for N-dealkylation and both CYP2D6 and CYP3A4 mediate dehydrogenation and hydroxylation of the drug leading to its main active metabolite, dehydro-aripiprazole [107,108]. It amounts to 40% of the parent compound in plasma and has similar affinity for the dopamine D2 receptor, therefore shows similar pharmacological activity [109]. The complex metabolic pathways of aripiprazole are shown in ***Figure* 14**.

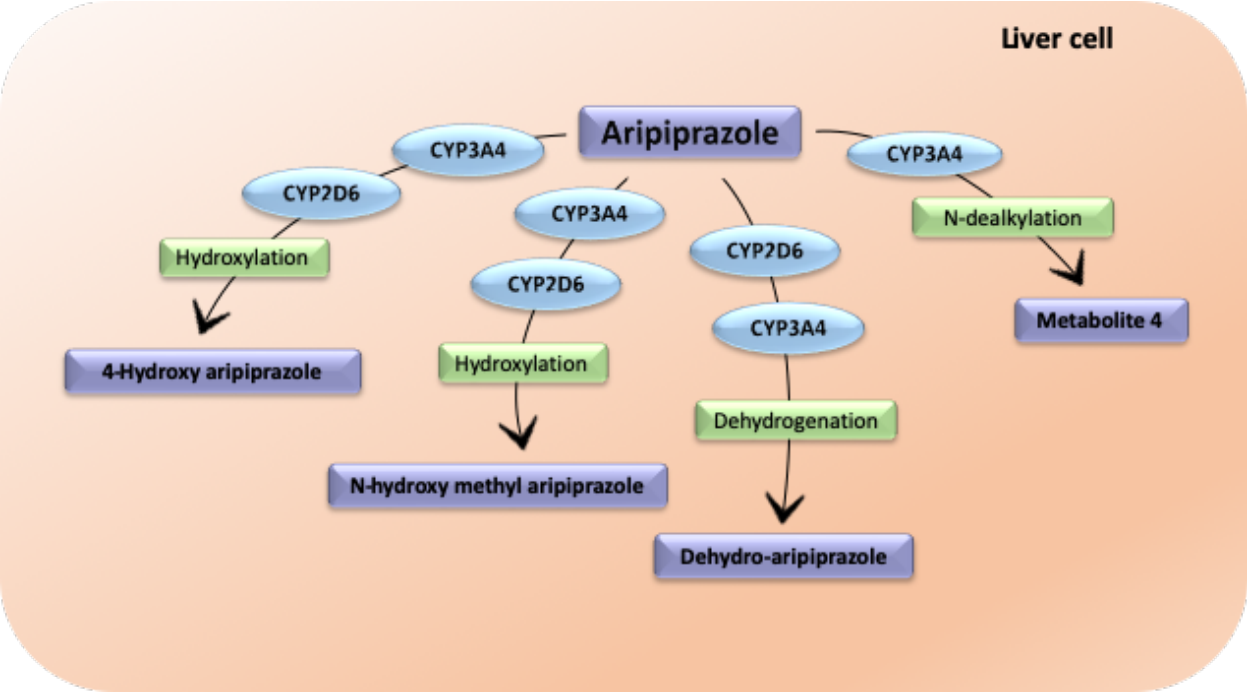

***Figure* 14**. The metabolic pathways of aripiprazole.

Following a single oral dose of ^{14}C-labeled aripiprazole, approximately 25% and 55% of the administered radioactivity was recovered in the urine and feces, respectively. Less than 1% of unchanged aripiprazole was excreted in the urine and approximately 18% of the oral dose was recovered unchanged in the feces [106]. Its apparent systemic clearance (Cl) is approximately 3.45 L/h [105]. The mean elimination half-life ($T_{1/2}$) is about 47-75 hours for aripiprazole [105], however, for individuals who do not have sufficient CYP2D6 activity (poor metabolizers; PMs), this can be about 146 hours [110]. $T_{1/2}$ for its metabolite, dehydro-aripiprazole is about 83 hours [109].

5.3.2. Mechanism of action

Aripiprazole acts as a partial agonist at dopamine D2, D3, D4 and serotonin 5-HT$_{1A}$, 5-HT$_{2C}$ and α1-adrenergic receptors and it is a 5-HT$_{2A}$ and 5-HT$_7$ receptor antagonist [111] (***Figure* 15**). Due to its unique pharmacological profile, it is frequently defined as a third-generation antipsychotic drug [112]. Being a dopamine D2 partial agonist, it can act either as a functional agonist or a functional antagonist, depending on the surrounding levels of dopamine, a full agonist at this receptor. In the absence of dopamine, aripiprazole shows functional agonist activity, i.e. produces a response while

binding to the receptor. In the presence of dopamine, aripiprazole shows functional antagonist activity: when binding to the receptor, it reduces the response from that seen with the full agonist. This mechanism of action seems to be the ideal treatment for schizophrenia, as aripiprazole should act as a functional antagonist in the mesolimbic dopamine pathway, where excessive dopamine activity causes positive symptoms. Contrastively, it shows functional agonist activity in the mesocortical pathway, where reduced dopamine activity is associated with negative symptoms and cognitive impairment. Consequently, aripiprazole reduces both positive and negative symptoms more effectively compared to first and second generation antipsychotics [113].

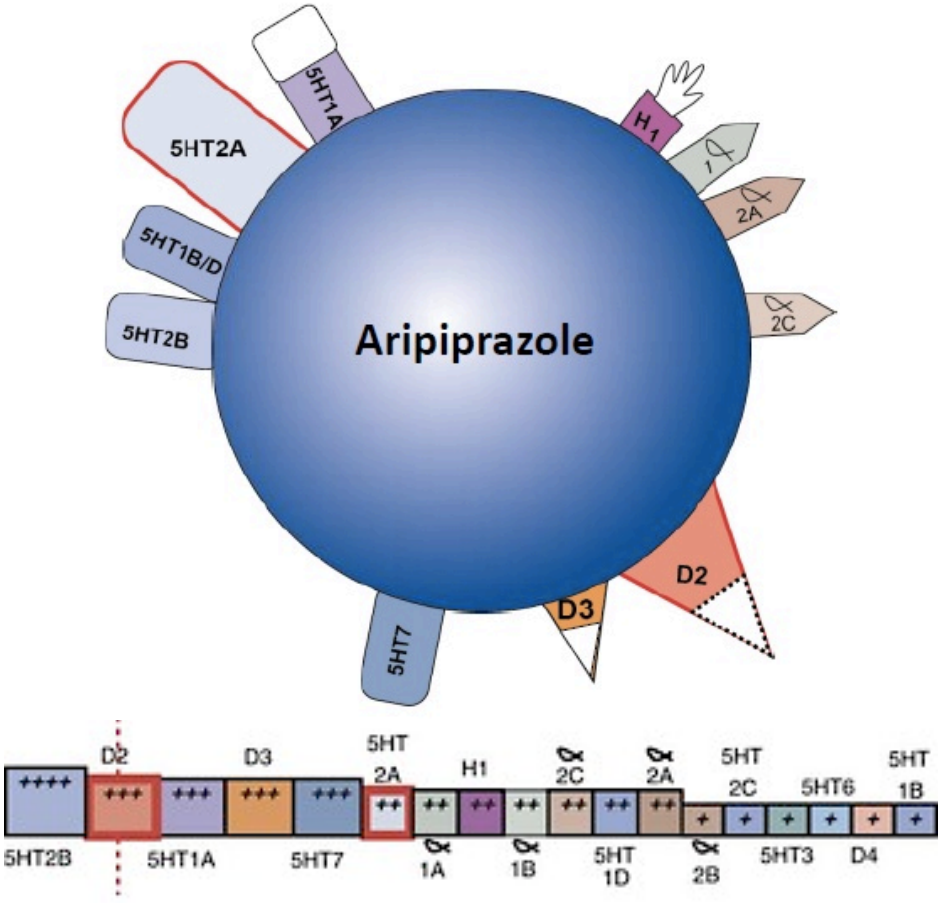

***Figure* 15**. The mechanism of action of aripiprazole.

Boxes are placed in rank order of how potently aripiprazole binds to the receptors, with the most potent and largest boxes to the far left and the weakest binding and smallest boxes to the far right. The vertical line indicates the strongest binding to dopamine D2 receptor among all receptors. Partial agonistic activity is shown with red squares. Source: Stahl, Stephen M., 2016 [114].

Regarding adverse effects, the high occupancy of D2 receptors occurs with less risk of extrapyramidal symptoms as its partial agonism produces a significantly lower level of functional antagonism of D2 receptor-mediated neurotransmission than other atypical antipsychotics. Additionally, patients who switched from other atypical antipsychotics, such as olanzapine and risperidone to aripiprazole showed improvement in their subjective well-being [112].

Dopamine is not the only factor why aripiprazole is effective and well tolerated in patients without producing many adverse effects. It can also interact with other neurotransmitter receptors, some of which may contribute to its therapeutic activity. The blockade of 5-HT_{2A} receptors may increase dopamine release at striatal level, thus reducing the incidence of motor side effects, and in the prefrontal cortex where, the decreased dopaminergic transmission contributes to cognitive dysfunction. Moreover, aripiprazole exhibits higher occupancy at D2 receptors (87%) than at 5-HT_{2A} receptors (52%), suggesting that, its atypical profile is not related to a high 5-HT_{2A}/D2 binding profile alike other atypical antipsychotics [115]. Moreover, aripiprazole shows affinity for muscarinic receptors, which combined with its partial agonist activity for D2 and 5-HT_{1A} receptors, could explain the improvements in neurocognitive functions [116].

The greatest factor for weight gain and sedation associated with antipsychotic therapy is thought to be the H1-histamine receptor antagonism, probably along with the antagonism for serotonin 5-HT_{2C} receptors. The moderate affinity of aripiprazole for H1-histamine receptors could explain its low risk to induce weight gain [117].

5.4. Adverse drug reactions to aripiprazole and olanzapine

The most common (≥ 5% prevalence and at least twice than placebo) adverse drug reactions to aripiprazole in schizophrenic patients are akathisia, extrapyramidal disorder, somnolence and tremor [106]. The following adverse reactions have been identified during post-approval clinical trials with aripiprazole: allergic reactions (anaphylactic reaction, angioedema, laryngospasm, pruritus and oropharyngeal spasm), pathological gambling, hiccups and blood glucose fluctuation [106]. On the contrary, the most common (≥5% and at least twice than placebo) adverse drug reactions to olanzapine in schizophrenic patients are constipation, weight gain, dizziness, personality disorder,

akathisia, postural hypotension, sedation, headache, increased appetite, fatigue, dry mouth and abdominal pain [90]. The adverse reactions reported since the introduction of olanzapine to the market were allergic reactions (anaphylactoid reaction, angioedema and pruritus), diabetic coma, diabetic ketoacidosis, diaphoresis, nausea, vomiting, jaundice, neutropenia, pancreatitis, priapism, rash, rhabdomyolysis, venous thromboembolic events (pulmonary embolism and deep venous thrombosis), hypercholesterolemia and hypertriglyceridemia [90].

Several atypical antipsychotics – including olanzapine – cause plasma prolactin level elevation [118]. Normally, while switching the therapy from olanzapine to aripiprazole mean prolactin levels decrease significantly even after one week of treatment and are maintained forth [119]. Nevertheless, aripiprazole can also cause mild prolactin elevation in less than 5% of patients [118]. Olanzapine is a D2 receptor antagonist and induce hyperprolactinaemia via inhibition of dopamine action at D2 receptors in the hypothalamus, where prolactin secretion is regulated [118]. On the contrary, serotonin stimulates prolactin secretion probably via stimulation of prolactin-releasing factors [120]. Aripiprazole acts as a functional antagonist under hyperdopaminergic conditions while it acts as a functional agonist under hypodopaminergic conditions at dopamine D2 receptors. D2 receptor stimulation provokes a suppression on prolactin secretion, therefore aripiprazole's high D2 receptor occupancy does not induce hyperprolactinemia in the majority of subjects [112].

Previous clinical trials with schizophrenic patients demonstrated that aripiprazole has more benign side effect profile – regarding weight gain, blood sugar level and lipid profile – as compared to olanzapine in short-term treatment. Weight gain was observed more frequently in olanzapine-treated patients when compared with aripiprazole. Mean serum triglyceride, blood glucose and cholesterol levels in patients treated with olanzapine were higher than in patients treated with aripiprazole [121,122]. Additionally, olanzapine was associated with significantly increased glucose levels compared to placebo and with a significantly greater change in glucose levels compared to other antipsychotics [123]. In addition, when comparing lean mice and others on high-fat diet, olanzapine induced hyperglycemia and therefore systemic insulin resistance [124].

Several typical and atypical antipsychotics increase the risk of heart rate-corrected QT (QTc)

prolongation and, as a consequence, *Torsades de Pointes* and sudden cardiac death [125]. In previous studies with schizophrenic patients, the mean QTc interval was decreased with aripiprazole and the QTc prolongation risk was lower with aripiprazole and olanzapine compared to placebo and other antipsychotics [125–128]. However, although aripiprazole usually does no produce QTc interval prolongation, some studies reported the contrary with patients and healthy volunteers [129–131].

Neuroleptic Malignant Syndrome, although it is rare, may occur with the administration of all antipsychotics. One of its symptoms is fluctuation in blood pressure [132]. Therefore, it is of emphasized importance to monitor the blood pressure during antipsychotic treatment. Aripiprazole and olanzapine may produce a decrease in blood pressure in both patients and healthy volunteers [129,133,134]. However, aripiprazole may also induce hypertension [135] and olanzapine can induce orthostatic hypotension [133].

Both aripiprazole and olanzapine can cause an increase in heart rate [129,136]. However, when changing the therapy from olanzapine to aripiprazole, a significant decrease was detected in heart rate [136]. Therefore, olanzapine had a stronger heart rate enhancing effect compared to aripiprazole and its effects were dose-dependent [136].

6. Pupillometry

The iris size, and therefore the pupillary diameter, is controlled by two muscles, the sphincter pupillae and the dilator pupillae, which are mainly under the regulation of the parasympathetic nervous system and the sympathetic nervous system, respectively. Contraction of the sphincter, accompanied by the relaxation of the dilator, produces pupil constriction (miosis), while contraction of the dilator, accompanied by the relaxation of the sphincter, produces pupil dilation (mydriasis) [137] (***Figure* 16**).

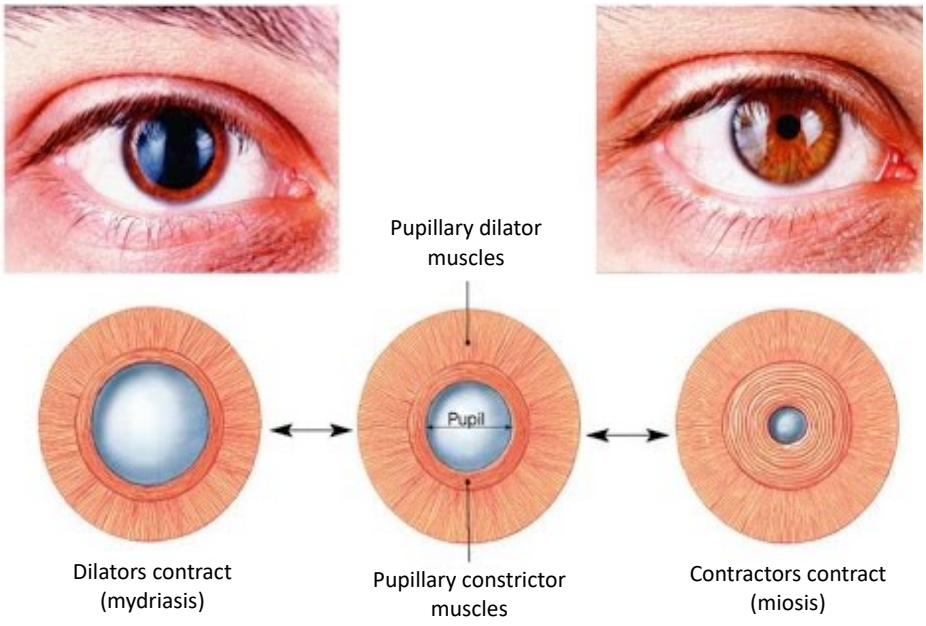

***Figure* 16**. The mechanism of pupil contraction.

Source: https://www.austincc.edu, modified.

Since pupillography was developed in 1958 [138], it is used for the detection of autonomic dysfunction associated with numerous diseases, such as diabetes [139], heart failure [140], Parkinson's disease [141], Alzheimer's disease [142] and hypertension [143]. Pupillometry is a non-invasive and cost-effective method to determine autonomic nerve activity [144]. The majority of studies analyzing the effect of drugs on the pupil diameter were performed with opioids finding that they cause pupil constriction (miosis) [145–148].

Several atypical antipsychotics caused pupil miosis in overdose patients. It can be due to inducing unopposed parasympathetic stimulation of the pupil with significant alpha-1 adrenergic receptor blockade [149]. On the other hand, these drugs could affect the pupil diameter due to their affinity for dopamine and serotonin receptors [150,151] as serotonin and dopamine are effectors on various types of muscles including the sphincter pupillae and the dilator pupillae [152]. Pupillography could be used for the determination of autonomic nerve activity during aripiprazole and olanzapine treatment [153].

7. The pharmacogenetics of aripiprazole and olanzapine

7.1. Genes related to pharmacokinetics

CYP2D6

CYP2D6 is highly polymorphic, with over 100 star (*) alleles described (***Table* 6**) (https://www.pharmvar.org/gene/CYP2D6). *CYP2D6**1 is the reference (or wild-type) allele encoding the enzyme with normal activity. *CYP2D6**2, *33, and *35 alleles are also considered to confer normal activity.

***Table* 6**. Most common *CYP2D6* alleles in Caucasians.

Allele	Effect	Enzyme activity	Allele frequency
***1**	No effect (wild-type)	Normal	0.2379
***2**	No effect	Normal	0.1852
***3**	Frameshift	Null	0.0158
***4**	Splicing defect	Null	0.1856
***5**	Complete gene deletion	No enzyme	0.0299
***6**	Frameshift	Null	0.0111
***7**	Missense	Null	0.0005
***9**	Deletion	Decreased	0.0275
***33**	No effect	Normal	0.0190
***35**	No effect	Normal	0.0464
***1xN**	Duplication	Increased	0.0083
***4xN**	Duplication	Null	0.0066

Allele frequencies are shown for Caucasians.
Source: https://www.pharmgkb.org/.

After genotyping the subject for *CYP2D6* alleles, a phenotype can be described for CYP2D6 metabolism through an activity score. A value is assigned to each allele based on its activity (e.g., alleles causing null enzymatic activity receive 0, while alleles causing increased activity receive 2) (***Table* 7**). The activity score of a genotype is the sum of the values assigned to each allele (e.g., *CYP2D6**1/*1 and *CYP2D6**2/*5 genotypes have activity score of 2 and 1, respectively) [154] (***Table* 8**).

***Table* 7**. Values assigned to *CYP2D6* alleles based on their activity.

Value assigned to allele	Alleles
0	*3, *4, *4xN, *5, *6, *7, *16, *36, *40, *42, *56B
0.5	*9, *10[#], *17, *29, *41, *45, *46
1	*1, *2, *35, *43, *45xN
2	*1xN, *2xN, *35xN

Source: https://www.pharmgkb.org/.

[#]Since 2020 January, the recommended value assigned to *10 is 0.25 [232]. However, the analysis was performed according to the previous method [154], which is shown in the *Table*.

***Table* 8**. CYP2D6 phenotypes based on the sum of the values assigned to both alleles of each subject.

Enzymatic activity	Phenotype
0	Poor metabolizer (PM)
0.5-1[#]	Intermediate metabolizer (IM)
1.5-2[#]	Normal metabolizer (NM)
>2[#]	Ultrarapid metabolizer (UM)

Source: https://www.pharmgkb.org/.

[#]Since 2020 January, IMs are defined as $0 < x < 1.25$, NMs as $1.25 \leq x \leq 2.25$ and UMs as $x > 2.25$ [232]. However, the analysis was performed according to the previous method [154], which is shown in the *Table*.

Subjects with more than two normal function copies of the *CYP2D6* gene are ultrarapid metabolizers (UMs), whereas subjects with two normal or one normal and one decreased function allele are classified as normal metabolizers (NMs). Subjects with one normal or one decreased and one no function allele or two decreased function alleles are categorized as intermediate metabolizers (IMs). Finally, subjects with two no function alleles are PMs.

The most common no function alleles are *CYP2D6**3, *4, *5, and *6, while the most common decreased function alleles include *CYP2D6**9, *10, *17, *29 and *41. The prevalence of CYP2D6 alleles is highly variable between different ethnicities. *CYP2D6**4 is the most common no function allele in Caucasians, but less abundant in Africans, and rare in Asians. Contrastively, the decreased function allele *CYP2D6*10* is the most common allele in Asians, and *CYP2D6**17 is almost

exclusively found in Africans. Consequently, the phenotype frequencies also vary among populations. Approximately 6-10% of Caucasians are PMs, mainly due to the prevalent no function *CYP2D6**4 and *5 alleles.

FDA recommends that the half of the usual dose of aripiprazole should be administered to CYP2D6 PMs. In addition, the quarter of the usual dose should be administered when they are taking CYP3A4 inhibitors as concomitant treatment, however, when it is withdrawn, the dosage should be adjusted to its original level [106]. Likewise, the DPWG recommends to reduce the maximum dose of aripiprazole to 10 mg/day in PMs [155]. On the contrary, CYP2D6 does not seem to have a dominating role in olanzapine biotransformation, it is apparently involved with a minor role [107].

CYP3A4

Unlike *CYP2D6*, genetic variation cannot explain *CYP3A4* variability. Although 26 allelic variants are currently described, the majority have not been shown to alter CYP3A4 activity (https://www.pharmvar.org/gene/CYP3A4). To date, the most studied no function *CYP3A4* alleles are *CYP3A4**6, *CYP3A4**20 and *CYP3A4**26. The *CYP3A4**20 allele has a frequency of about 0.2% in European Americans and 0.05% in African Americans, while it has a frequency of 1.2% in Spain; what frequency reached up to 3.8% in specific Spanish regions [156]. *CYP3A4**22, with the minor allele frequency of 5.0% in Caucasians, is a reduced function allele and was associated with tacrolimus dose requirements, however, its clinical utility is not confirmed to date [157]. Additionally, *CYP3A4**2 and *3 were identified as missense polymorphisms [158] decreasing the function of the enzyme with a frequency of 1.1 and 2.1% in Caucasians, respectively [159,160]. The exact role of these polymorphisms in aripiprazole and olanzapine pharmacokinetics is unknown.

CYP3A5

Due to the similar substrate specificity between CYP3A4 and CYP3A5 , some drugs metabolized by CYP3A4 are also metabolized by CYP3A5 [161]. To date, 26 allelic variants of *CYP3A5* are described. The most common no function variant is *CYP3A5**3 which results in a splicing defect and has high allele frequency (91% in the Spanish population). Subjects with the *3/*3 genotype do not express CYP3A5, therefore, higher aripiprazole concentrations are expected in them during aripiprazole treatment [162].

ABCB1

One of the most studied polymorphisms in *ABCB1* is C3435T, an SNP in exon 26 that seems to have a relevant role in the expression and function of the P-glycoprotein. Studies in knock-out mice for the *ABCB1* gene showed that both aripiprazole and its metabolite are substrates of P-glycoprotein [163]. However, the C3435T SNP does not appear to influence the pharmacokinetics of aripiprazole. Yet, it may have an important role in regulating the entry of aripiprazole and dehydro-aripiprazole into the central nervous system through the blood-brain barrier [163]. In addition, two other SNPs (C1236T and G2677T/A) may influence the response to antipsychotic treatment with clozapine, haloperidol and risperidone [164]. However, the pharmacokinetics of aripiprazole was not affected by the G2677T/A polymorphism [165]. Nevertheless, in a previous study by our laboratory, the G2677T/A and C1236T polymorphisms affected aripiprazole clearance [166]. Olanzapine has low to moderate inhibitor affinity for P-gp [107]. Two studies showed a correlation between the 2677T allele and higher serum levels of olanzapine [167,168]. Additionally, C3435T T/T subjects had significantly higher AUC [169]. Likewise, schizophrenic patients with the 1236T/2677T/3435T haplotype had higher serum and cerebrospinal fluid concentrations of olanzapine [167].

CYP1A2

CYP1A2 is a smoking-induced enzyme and smokers seem to have a higher clearance of olanzapine than nonsmokers. Smoking is highly important health concern for psychiatric patients. In fact, the rate of smoking among schizophrenic patients ranges from 70 to 90%, compared with only 28–40% in the general population [170]. Although 41 alleles have been reported (https://www.pharmvar.org/gene/CYP1A2) in the *CYP1A2* gene, only a few have been associated with variability in gene expression or inducibility. Five *CYP1A2* alleles (*1C, *1D, *1E, *1F and *1K) have been assessed for their potential influence on olanzapine exposure. The *CYP1A2**1F allele was shown to confer a higher inducibility as well as an elevated basal enzyme activity [171,172]. On the other hand, other studies were unable to find this correlation [173,174]. *CYP1A2**1D also influenced olanzapine plasma concentrations [175]. Currently, based on the few and contradictory information available about this enzyme, no recommendations exist for *CYP1A2* genotyping before prescribing olanzapine.

UGT1A4 and *UGT2B10*

The UGT1 family, especially UGT1A4, is the main enzyme responsible for addition of glucuronic acid to amino groups (N-glucuronidation) [176]. The two most studied variants in this enzyme are UGT1A4*2 and UGT1A4*3 [174]. Although the effects of these two variants on glucuronidation are substrate dependent *in vitro*, increased glucuronidation is usually associated with the *3 variant and reduced (or not changed) with the *2 variant [176]. Moreover, reduced systemic olanzapine exposure was reported for *UGT1A4**3 [177]. UGT2B10 from the UGT2 family catalyzes conjugation of nitrogen-containing heterocycles [178]. *UGT2B10**2 significantly altered olanzapine glucuronidation *in vitro* [177].

FMO3

Of the five active human FMO isoenzymes, FMO3 is the major hepatic form in adults. The three most studied *FMO3* polymorphisms, p.E158K, p.V257M and p.E308G, are associated with reduced enzyme activity *in vitro* in a substrate-dependent manner. Regarding olanzapine biotransformation, reduction was observed in its N-oxidation *in vitro* for the protein variant expressed by the K158–G308 allele [91]. The p.E158K variant also affected OLA N-oxidation: the minor allele carriers had its level 50% lower compared to wild-type subjects. However, the variants had no influence on the plasma levels of the parent compound [174].

7.2. Genes related to pharmacodynamics

Many pharmacokinetic studies were performed in order to understand how genetic variants in genes encoding drug targets can impact response and side effect profiles of antipsychotics. These targets include receptors involved in the mechanism of action of the drugs, synapse level transporters and components of the signaling pathways. Antipsychotics have different affinities for dopamine receptors (DRD1, DRD2, DRD3 and DRD4), serotonin receptors (e.g., 5-HT_{2A} and 5-HT_{2C}), noradrenergic, cholinergic and histaminergic receptors [179].

Dopamine receptor genes

Dopamine receptors are primary targets for atypical antipsychotics, among them aripiprazole and olanzapine. The most studied polymorphism is the Taq1A polymorphism (rs1800497) in the *DRD2*

gene. This variant is a nonsynonymous coding and its minor allele, T, correlates with low density of DRD2 receptors in the striatum [180]. Moreover, this allele was associated with higher prolactin concentrations and hyperprolactinemia associated adverse events after olanzapine treatment [181,182]. There is some evidence that the T allele may predict better treatment response to aripiprazole [183]. Contrastively, another study found no impact of the variant on response to olanzapine [184]. Moreover, C/C homozygotes for the rs6277 polymorphism of this gene were associated with poor aripiprazole response [185]. Additionally, minor allele carriers of rs6277 and rs1800497 had poorer cognitive performance in schizophrenic patients [186].

Variability of the *DRD3* gene seems to be associated with the response to olanzapine [96], especially rs6280, a non - synonymous coding variant (Ser9Gly). In addition, it was found that patients with rs6280 Gly/Gly genotype showed superior response to olanzapine compared with the other genotypes [187].

Serotonin receptor genes

Variations in the *HTR2A* gene (encoding the 5-HT_{2A} receptor) influence the binding affinities of aripiprazole and olanzapine [188]. Previous studies found that the His/His homozygotes of the His452Tyr (rs6314) and the C/C homozygotes of the T102C (rs6313) polymorphisms respond better to olanzapine [189]. The same association was found with aripiprazole: subjects with the GG/CC genotype of *HTR2A* A-1438G (rs6311)/T102C polymorphisms predict poor aripiprazole response, specifically for negative symptoms [190]. Additionally, patients carrying the HTR2A 1438A, 102T, and 452His haplotype had significantly higher C peptide levels compared with the 1438A, 102T, and 452Tyr haplotype during olanzapine treatment [191].

By contrast, it is well established that the 5-HT_{2C} receptor (encoded by the *HTR2C* gene) mediates several metabolic side effects of olanzapine [192]. Increased activity of the 5-HT_{2C} receptor due to genetic variation in HTR2C may increase leptin levels resulting in greater weight gain, and variants that decrease expression of the receptor may be protective against weight gain induced by olanzapine [192]. Moreover, the minor allele of -759C/T (rs3813929) of *HTR2C* was reported as protective for weight gain [193]. It should be noted that, in in-patient studies, in which the diet is

controlled and treatments are of shorter duration, are most likely to produce positive findings for an impact of the -759C/T polymorphism on weight gain [194]. Moreover, olanzapine-treated patients with the HTR2C -759C, -697C and 23Ser haplotype had higher BMI and C peptide levels compared to those with 759T, -697C and 23Cys haplotype [191].

II. OBJECTIVES

The overall goal of this work was to compare the metabolic effects and safety of two atypical antipsychotics (aripiprazole and olanzapine) and to find biomarkers than can help to predict which subject should have higher risk to develop these effects.

The specific aims of this thesis were:

1) To develop and validate a simple LC-MS/MS method for the simultaneous determination of atypical antipsychotics: aripiprazole, dehydro-aripiprazole, olanzapine, risperidone, paliperidone, quetiapine and clozapine in human plasma.

2) To evaluate the pharmacokinetics of aripiprazole and olanzapine after 5 days treatment in healthy subjects and how they are altered based on sex and genetic factors.

3) To compare the effects of aripiprazole and olanzapine on pupil contraction and its relationship with pharmacokinetics and pharmacogenetics.

4) To compare the metabolic effects of aripiprazole and olanzapine and its relationship with pharmacokinetics and pharmacogenetics.

5) To evaluate the adverse drug reactions induced by aripiprazole and olanzapine in healthy subjects and its relationship with pharmacokinetics and pharmacogenetics.

III. MATERIALS AND METHODS

1. Clinical trial

1.1. Study design

The clinical trial was a phase I trial with multiple oral dose design, open-label, randomized, crossover, two-periods, two-sequences, single-centre and comparative study. The aim of the study was to measure the effect of aripiprazole and olanzapine on different metabolic factors, such as oral glucose tolerance, insulin secretion, blood pressure, electrocardiogram, weight, lipid and prolactin levels; along with pupil contraction, evaluation of adverse drug reactions and bioavailability.

Ten mg/day aripiprazole tablets or 5 mg/day film-coated olanzapine tablets were administered during 5 consecutive days. Block randomization was used to assign the treatment to each volunteer on the first day. The drug was administered at 09:00 h each day under fasting conditions with 240 ml water. A mouth check was performed immediately after drug administration to ensure the swallowing of the drug. The subjects were hospitalized from 1 h before the first dose until 24 h after the last dose. In the second period, after a washout period of 28 days, each volunteer received the opposite drug they received in the first period. The random allocation sequence, the recruitment of participants and their assignment to interventions were performed by investigators of the Clinical Trials Unit.

The protocol was approved by the Research Ethics Committee (10 May 2018), fully authorized by the Spanish Drugs Agency (23 May 2018) and under the guidelines of Good Clinical Practice with the registration number of EUDRA-CT: 2018-000744-26.

1.2. Study population

A multiple-dose clinical trial including 24 healthy volunteers was performed in three groups at the Clinical Trials Unit of Hospital Universitario de La Princesa. The study started on 2 July 2018 and ended on 22 April 2019 (***Table* 9**). The trial complied with the international standards and with the

Declaration of Helsinki. The Principal Investigator of the trial was Francisco Abad Santos. All subjects were adequately informed about the study and, if agreed to participate, signed an informed consent form.

***Table* 9**. Dates of performing the study in groups.

Group	Date of performing the trial	Number of included subjects
Group 1	July-August 2018	11
Group 2	September-October 2018	10
Group 3	March-April 2019	3

The inclusion criteria were the following:

- Male and female volunteers between 18 and 65 years old.
- Free from any known organic or psychiatric conditions.
- Normal vital signs and electrocardiogram ECG.
- Normal medical records and physical examination.
- No clinically significant abnormalities in haematology, biochemistry, serology (HIV antibody, Hepatitis C antibody, Hepatitis B surface antigen) and urine tests.

The exclusion criteria were the following:

- Individuals who received pharmacological treatment in the last 15 days or any kind of medication in the last 48 hours prior to receiving the study medication, except of contraceptives.
- Individuals with BMI outside the 18.5-30.0 kg/m^2 range.
- Individuals with history of drug allergy.
- Individuals with galactose intolerance, Lapp lactase deficiency or glucose-galactose malabsorption.
- Suspected consumers of controlled substances.
- Smokers.
- Daily alcohol consumers and/or individuals who experienced acute alcohol poisoning the previous week.

- Individuals who donated blood the previous month.
- Pregnant or breastfeeding women.
- Investigational drug study participants in the previous 3 months.
- Subjects unable to follow instructions or collaborate during the study.

1.3. Procedures

1.3.1. Sample collection for pharmacokinetic analysis

Twenty-two blood samples were collected from each participant for pharmacokinetic assessments during each period, thus 44 samples in total: 7 samples on day 1 (predose and 1, 2, 3, 5, 8 and 12 h after dosing); 1 (predose) sample on days 2, 3 and 4; 7 samples on day 5 (predose and 1, 2, 3, 5, 8 and 12 h after dosing) and 1 sample on days 6, 7, 9, 11 and 15 (corresponding to 24, 48, 96, 144 and 240 h after the last dose, respectively) (***Table* 10**). Each blood sample was labelled with the protocol code, volunteer number, treatment period and day and extraction time without specifying the administered drug. Subsequently, the samples were centrifuged at 3500 rpm (1900 G) for 10 minutes and then the plasma was collected and stored at −80°C until the determination of drug concentrations.

***Table* 10**. Study schedule with all performed procedures during the study.

Day	Hour*	Procedure	Samples	Scales	Others
1	**8:00-9:00**	Admission	END, GTT, PK, PG, PRL, pregnancy, toxics	Ramsay	ECG, BP, HR, weight, pupillometry
	9:00 (0 h)	Dosing			
	10:00 (1 h)		PK		
	11:00 (2 h)	Breakfast	PK	Ramsay	ECG, BP
	12:00 (3 h)		PK		
	13:00 (4 h)				pupillometry
	14:00 (5 h)		PK, PRL	Ramsay	ECG, BP
	15:00 (6 h)	Lunch			
	17:00 (8 h)		PK		
	21:00 (12 h)		PK	Ramsay	ECG, BP

Day	Hour*	Procedure	Samples	Scales	Others
	22:00 (13 h)	Dinner			
2	**8:30**		PK	Ramsay, UKU	ECG, BP, HR, weight, pupillometry
	9:00 (0 h)	Dosing			
	11:00 (2 h)	Breakfast		Ramsay	ECG, BP
	13:00 (4 h)				pupillometry
	14:00 (5 h)			Ramsay	ECG, BP
	15:00 (6 h)	Lunch			
	21:00 (12 h)			Ramsay	ECG, BP
	22:00 (13 h)	Dinner			
3	**8:30**		H, C, BQ, PK, PRL	Ramsay	ECG, BP, HR, weight, pupillometry
	9:00 (0 h)	Dosing			
	11:00 (2 h)	Breakfast		Ramsay	ECG, BP
	13:00 (4 h)				pupillometry
	14:00 (5 h)			Ramsay	ECG, BP
	15:00 (6 H)	Lunch			
	21:00 (12 h)			Ramsay	ECG, BP
	22:00 (13 h)	Dinner			
4	**8:30**		PK	Ramsay, UKU	ECG, BP, HR, weight, pupillometry
	9:00 (0 h)	Dosing			
	11:00 (2 h)	Breakfast		Ramsay	ECG, BP
	13:00 (4 h)				Pupillometry
	14:00 (5 h)			Ramsay	ECG, BP
	15:00 (6 H)	Lunch			
	21:00 (12 h)			Ramsay	ECG, BP
	22:00 (13 h)	Dinner			
5	**8:30**		PK, PRL	Ramsay	ECG, BP, HR, weight, pupillometry
	9:00 (0 h)	Dosing			
	10:00 (1 h)		PK		
	11:00 (2 h)	Breakfast	PK	Ramsay	ECG, BP
	12:00 (3 h)		PK		
	13:00 (4 h)				pupillometry
	14:00 (5 h)		PK, PRL	Ramsay	ECG, BP

Day	Hour*	Procedure	Samples	Scales	Others
	15:00 (6 h)	Lunch			
	17:00 (8 h)		PK		
	21:00 (12 h)		PK	Ramsay	ECG, BP
	22:00 (13 h)	Dinner			
6	**9:00 (24 h)**		H, C, BQ, END, GTT, PK, PRL	Ramsay, UKU	ECG, BP
	10:00 (25 h)	Discharge			
7	**9:00 (48 h)**		PK	Ramsay	ECG, BP
9	**9:00 (96 h)**		PK	Ramsay, UKU	ECG, BP
11	**9:00 (144 h)**		PK	Ramsay	ECG, BP
15	**9:00 (240 h)**	Safety	PK, H, C, BQ, PRL, pregnancy, toxics	Ramsay, UKU	ECG, BP, HR, weight

The same procedure and schedule were followed in both periods of the clinical trial.

Abbreviations:
*The time after dosing is shown in parenthesis.
H: hematology: red blood cells, haemoglobin, haematocrit, mean corpuscular volume, platelets, leukocytes, leukocyte formula,
C: prothrombin activity,
BQ: biochemistry: glucose, GOT, GPT, LDH, alkaline phosphatase, GGT, total bilirubin, uric acid, creatinine, urea, albumin, total cholesterol, LDL-cholesterol, triglycerides,
END: peptide C, glycosylated hemoglobin, insulin, TSH, free T4,
GTT: glucose tolerance test
PK: pharmacokinetics,
PRL: prolactin,
PG: pharmacogenetics,
UKU: Udvalg for kliniske undersøgelser (UKU) Side Effect Rating Scale,
Ramsay: Ramsay sedation scale,
ECG: electrocardiogram,
BP: blood pressure,
HR: heart rate,
Serology: HIV antibody, Hepatitis C antibody, Hepatitis B surface antigen,
Pregnancy test in urine: for women,
Toxics: test in urine for cannabinoids, cocaine, opiates, amphetamines and cotinine.

1.3.2. Biochemical and haematological analyses

The biochemical and haematological analyses were carried out by Eurofins Megalab S.A. (Madrid, Spain). All subjects underwent the oral glucose tolerance test (GTT) on days 1 and 6 with 75 g of oral anhydrous glucose dissolved in 250 mL water; samples were collected before, and 1 and 2 h after glucose administration. Glucose, triglyceride, bilirubin, glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), gamma-glutamyl transferase (GGT), albumin, alkaline phosphatase (ALP), uric acid, urea and creatinine concentrations were measured spectrophotometrically and the samples were collected at screening and on days 3, 6 and 15. Total cholesterol, LDL and HDL levels were analysed by enzymatic colorimetric method at screening and on days 3, 6 and 15. Prolactin levels were analysed on days 1, 3 and 5 before and 5 h after dosing and on days 6 and 15. C-peptide, insulin, thyroid stimulating hormone (TSH) and free thyroxine (T4) concentrations were quantified on days 1 and 6 by Enzyme-Linked ImmunoSorbent Assay (ELISA). Hemoglobin A1c (HbA1c) was measured on days 1 and 6. Haematocrit, platelet, leucocyte, haemoglobin and red blood cell counts were measured by flow cytometry at screening and on days 3, 6 and 15. Finally, prothrombin time was determined by coagulometry at screening and on days 3, 6 and 15 (***Table* 10**). Tests to detect pregnancy and illicit drug use were performed from urine samples and were repeated each period before the first drug administration.

1.3.3. Analysis of blood pressure and electrocardiogram

The blood pressure was measured in supine position with an automatic monitor (Carescape V100, General Electric, Boston, MA, USA) at screening, before and 2 h, 5 h and 12 h after each dosing and on days 6, 7, 9 ,11 and 15. Likewise, the 12-lead electrocardiogram was obtained at the same time points with an Esaote P8000 instrument (Esaote S.P.A, Florence, Italy) (***Table* 10**). The corrected QT interval (QTc) and heart rate were automatically calculated by the electrocardiogram device. The Bazett correction formula was used to correct the QT interval [195]. According to the ICH E14 clinical guidance [196], a QTc interval greater than 450 milliseconds or a change from baseline greater than 30 milliseconds were considered as QTc interval prolongation.

1.3.4. Safety and tolerability assessments

The safety and tolerability of aripiprazole was assessed by clinical evaluation of adverse drug reactions and other parameters including vital signs, physical examinations and 12-lead electrocardiograms. During the development of the study, volunteers were asked if they had experienced any adverse event. Moreover, the Ramsay sedation scale [197] was evaluated before and 2 h, 5 h and 12 h after each dosing and on days 6, 7, 9 ,11 and 15. The UKU side effect rating scale [198] was evaluated on days 2 and 4 before dosing and on days 6, 9 and 15 (***Table* 10**). According to the algorithm of the Spanish pharmacovigilance system [199], the causality of adverse events were classified as definite, probable, possible, unlikely or unrelated. Only those adverse events that were definite, probable, or possible related to the treatment were considered as adverse drug reactions in the statistical analysis. Intensity (mild, moderate, and severe), time sequence and outcome of the adverse events were also registered.

The adverse drug reactions were classified using system organ class allocation as general (asthenia, fatigue, tiredness and gait alterations), cardiovascular (palpitations), gastrointestinal (constipation, nausea, vomiting, hyposalivation, hypersalivation, dry mouth and diarrhea), nervous system (akathisia, headache, difficulties with concentration, dizziness, paraesthesia, presyncope, syncope, tremor, somnolence and restless legs), psychiatric (restlessness, insomnia, anxiety, abnormal orgasms and nightmares), respiratory (epistaxis, hiccups, cough and sore throat), endocrine (galactorrhea), metabolic (lack of appetite, increased appetite and hyporexia), reproductive (dysmenorrhea, mastalgia and menstrual irregularity), skin (hair loss, pruritus, rash, facial rash and sweating), musculoskeletal (shoulder pain, knee pain, neck pain, upper limb weakness, lumbago, cramps, back pain and leg pain), infections (cold), eye (photophobia) and investigations (increased liver enzymes) [200].

1.3.5. Pupillometric measurements

Pupillometric measurements were performed right before and 4 h after drug administration on each day of hospitalization (***Table* 10**). The data was recorded with a PRL-200™ automated monocular infrared pupillometer (NeurOptics, Irvine, CA, United States). Each measurement was performed in a hospital room with artificial illumination. In order to adjust for differences in luminosity, light

intensity (*in lux*) was measured at the moment of the pupillometric determination with a lux meter (mobile application).

Before starting the measurement, the subject was instructed to focus on a small target object with the eye that was not being tested open (left eye). Stimuli were single light pulses with a fixed intensity of 180 µW during 154 milliseconds. Once the device was focused on the target pupil (right eye), a white light stimulus was flashed. The measurements were sampled at a frequency of 32-frames per second and lasted up to 5 seconds, allowing a full or partial recovery of the pupil size after light constriction.

Eight different pupillometric parameters were measured based on the user guide [201]. Maximum pupil diameter (MAX) and minimum pupil diameter (MIN) represent the pupil diameter before constriction and just at the peak of constriction, respectively. The percentage of constriction (CON) was calculated by (MAX—MIN)/MAX. Latency (LAT) is time of the onset of constriction. ACV and MCV are average and maximum constriction velocity, respectively. The negative sign differentiates constriction from dilation being the opposite movement. After reaching its constriction, the pupil tends to recover and dilate back to its initial resting size, which is measured as average dilation velocity (ADV). T75 is the total time taken by the pupil to recover 75% of the initial resting pupil size after it reached the peak of constriction.

2. Analytical method validation

2.1. Chemicals and Reagents

Aripiprazole, its internal standard [2H8]-aripiprazole (aripiprazole-D8) and dehydro-aripiprazole were provided by Toronto Research Chemicals (North York, Canada) and olanzapine, risperidone, paliperidone, quetiapine and clozapine and their internal standards [13C,2H3]-olanzapine (olanzapine-C1-D3), [2H4]-risperidone (risperidone-D4), [2H4]-paliperidone-D4 (paliperidone-D4), [13C4]-quetiapine (quetiapine-C4) and [13C,2H3]-clozapine (clozapine-C1-D3) were provided by AlsaChim (Illkirch Graffenstaden, France). Ultrapure water was acquired from a Milli-Q® Water Purification System (Millipore-Ibérica, Madrid, Spain). Acetonitrile, methanol

and ammonium hydroxide solutions in gradient HPLC grade were provided by SYMTA (Madrid, Spain). Formic acid was bought from Sigma-Aldrich (Madrid, Spain). All the used chemicals were of analytical grade. Plasma samples for the preparation of calibration and validation standards were supplied by the Transfusion Center of "Comunidad Autónoma de Madrid" (Madrid, Spain).

2.2. Preparation of calibration standards and quality controls

Stock solutions of aripiprazole, dehydro-aripiprazole and aripiprazole-D8 were dissolved in methanol containing 0.5% formic acid solution, while olanzapine and olanzapine -C1-D3 were supplied in acetonitrile solution with 0.1% formic acid. Risperidone and risperidone-D4 were dissolved in methanol -water 1:1 solution with 0.1% formic acid. Paliperidone and paliperidone-D4 were prepared in 100% acetonitrile solution while quetiapine, quetiapine-C4, clozapine and clozapine-C1-D3 were dissolved in 100% methanol solution. The concentration of all standard solutions was 1 mg/mL. Working solutions of each analyte were prepared in methanol and 0.1% formic acid by dilutions at the following concentrations from each stock solution: 0.1 mg/mL, 0.01 mg/mL and 0.001 mg/mL. All stock and working solutions were kept at -80ºC.

Calibration standards and quality controls were prepared by independent dilutions method of each stock solution, then they were spiked to blank plasma samples. Entirely 8 calibrators and 4 quality controls (LLOQ; low quality control; medium quality control and high quality control) were made. The used concentrations were calculated based on the therapeutic ranges of each drug described in the literature (summarized in ***Tables* 11**, **12 and 13**). A blank and a zero plasma sample processed without drugs were included to the analysis. Similarly, the stock and working solutions, calibrators, quality controls and internal standards were kept at -80ºC until utilization.

***Table* 11**. Concentrations of calibration standards (Cal) for each drug.

Drug/ metabolite	R^2	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
		(ng/mL)							
ARI	0.9991	0.18	0.25	0.50	2	10	50	100	120
DARI	0.9989	0.25	0.35	0.70	2	5	20	40	80
OLA	0.9944	1.00	1.10	1.50	5	10	20	50	100
RIS	0.9968	0.70	0.80	1.20	1.5	5	10	25	60
PAL	0.9815	0.20	0.30	0.50	1	2	5	18	30
QUE	0.9956	0.50	0.60	1.00	5	10	20	80	160
CLO	0.9981	0.50	0.80	2.00	20	100	500	750	1000
CAF	0.9363	1200	1450	1700	1950	2200	2700	3200	3700

R^2: correlation coefficient

Abbreviations: ARI: aripiprazole; DARI: dehydro-aripiprazole; OLA: olanzapine; RIS: risperidone; PAL: paliperidone; QUE: quetiapine; CLO: clozapine; CAF: caffeine. LLOQ: lower limit of quantification

***Table* 12.** Concentrations of quality controls (QC) for each drug.

Drug/ metabolite	LLOQ	QC 2	QC 3	QC 4
	ng/mL			
ARI	0.18	1.00	60	100
DARI	0.25	0.90	40	70
OLA	1.00	3.00	50	80
RIS	0.70	1.50	25	50
PAL	0.20	1.60	15	25
QUE	0.50	1.50	80	130
CLO	0.50	7.00	500	800
CAF	1200	2200	3200	3700

***Table* 13**. Therapeutic range of each drug.

Drug	Therapeutic range (ng/mL)
ARI	50-350 [202,203]
	150-300 [204]
	146–254 [205]
	32.1–188 [206]
OLA	10-100 [203,207]
	20–80 [208–210]
	1.2-208 [211]
RIS	10-100 [203,207]
	20–60 [212,213]
	0.6-25 [214]
PAL	10-100 [203,207]
	20-52 [212]
	20-60 [213]
	10-109 [214]
QUE	70-170 [203,207,215,216]
CLO	200-800 [203,207]
	350-600 [217,218]

2.3. Equipment

The chromatographic separation was performed with an HPLC system consisting a 1200 Series separation module (Agilent Technologies, Madrid, Spain) and was coupled to a triple quadrupole mass spectrometer (Agilent Technologies 6410B), with positive mode ESI. The instrument was controlled by the MassHunter Workstation Data Acquisition software (Agilent Technologies, Madrid, Spain). For separating the drugs, an ACE C18-PFP (pentafluorophenyl) column (3 µm, 4.6 × 100 mm; SYMTA, Madrid, Spain) maintained at 25 °C was used. The flow rate was 0.6 mL/min. The mobile phase was a mixture of formic acid (0.2%, solvent A) and acetonitrile (solvent D) (65:35, v/v). The final pH was 3.0 adjusted with 5 mol/L ammonium hydroxide. The chromatogram was run with the following gradient conditions: initial conditions: 60% of A and 40% of D from 0 to 1.0 min, 60% D maintained from 1.0 to 1.8 min, D increased to 65% from 1.8 to 2.5 min, 65% D kept from 2.5 to 2.7 min, D increased to 99% from 2.7 to 3.5 min, 99% D maintained from 3.5 to 4.0 min and finally, returned to the initial conditions (60% A and 40% D)

from 4.0 to 6.0 minutes. Afterwards, the chromatogram was followed by a re-equilibration time of 3.0 min.

Analytes were quantified in dynamic multiple reaction monitoring (MRM) mode. All results were based on the peak area ratio between the drug and the internal standard. The MS conditions were as follows: desolvation gas flow and temperature 12 L/min and 350 °C, respectively, nebulizer pressure 60 psi and capillary voltage 3 kV. Highly pure N_2 (>99.9995) was used as MS collision gas. For all the compounds, fragmentor voltage and collision energy were set using selected ion monitoring (SRM) for each transition at specific retention time. A confirmation transition was also monitored to acquire more specific results. Additionally, m/z 184>184 and 104>104 as common in-source collision-induced dissociation ion fragments produced by endogenous phospholipids (phosphatidylcholines and lysophosphatidylcholines) were measured [219]. Moreover, m/z 524.4>184.1, 524.4>104.1, 496.4>184.1 and 496.4>104.1 produced by late-eluting phospholipids (lysophosphatidylcholines 16:0 and 18:0) were also analyzed (***Table* 14**).

Furthermore, product ion qualifier ratio was defined along with retention time-, relative retention time-, and ion ratio identity confirmation according to SANTE/11813/2017 and 2002/657/EC COMMISSION DECISION guidelines [220,221]. The acceptance criteria were the following: retention time difference between extracted analyte and neat solution of the analyte should be lower than 0.1 min [220], relative retention time difference between extracted analyte and neat solution of the analyte should be lower than 2,5% [221], and ion ratio difference between calibrator and quality control samples should not differ more than 30% [220], respectively.

MassHunter Workstation Quantitative Analysis software (Agilent Technologies, Madrid, Spain) was used to quantify the concentrations based on MRM transitions of each analyte.

***Table* 14**. Relevant LC–MS/MS characteristics.

Compound	Retention time (tR, min)	Quantification SRM Transition (m/z)	Confirmation SRM Transition (m/z)	Qualifier ratio	Fragmentor voltage (V)	Collision energy (eV)
ARI	4.750	448.2 > 285.2	448.2 > 176.2	30.10	100	25
ARI-D8	4.790	456.2 > 293.0			110	30
DARI	4.439	446.2 > 285.0	446.2 > 188.0	0.16	125	22
OLA	1.941	313.2 > 256.2	313.2 > 282.2	10.51	90	25
OLA-C1-D3	1.940	317.3 > 256.2			110	25
RIS	3.373	411.3 > 191.2	411.3 > 110.1	6.47	50	30
RIS-D4	3.405	415.3 > 195.2			90	30
PAL	2.945	427.3 > 207.2	427.3 > 110.2	33.10	110	30
PAL-D4	2.944	431.3 > 211.2			100	30
QUE	3.945	384.3 > 253.2	384.3 > 221.2	45.70	110	35
QUE-C4	3.969	388.3 > 255.2			110	35
CLO	4.029	327.2 > 270.1	331.2 > 270.2	59.54	130	25
CLO-C1-D3	4.029	327.2 > 192.1			110	25
CAF	2.001	195.2 > 138.2	195.2 > 110.2		100	20
PC	1.485	184.1 > 184.1			100	5
LPC	1.537	104.1 > 104.1			100	10
LPC 16:0	8.348	496.4 > 184.1	496.4 > 104.1		135	30
LPC 18:0	8.186	524.4 > 184.1	524.4 > 104.1		135	30

Abbreviations: SRM: selected reaction monitoring; m/z: Mass-to-Charge; ARI-D8: [2H8]-aripiprazole; OLA-C1-D3: [13C,2H3]-olanzapine; RIS-D4: [2H4]-risperidone; PAL-D4: [2H4]-paliperidone-D4; QUE-C4: [13C4]-quetiapine; CLO-C1-D3: [13C,2H3]-clozapine; PC: phosphatidylcholine; LPC: lysophosphatidylcholine; LPC 16:0: lysophosphatidylcholine 16:0; LPC 18:0: lysophosphatidylcholine 18:0. Qualifier ratio: the peak area ratio quantifier/qualifier.

2.4. Sample preparation

Plasma samples were extracted by both microelution-solid phase extraction (µSPE) and protein precipitation (PPT).

For PPT, 200 µL plasma sample was spiked with 10 µL internal standard and 800 µL precipitating agent, acetonitrile with 0.1% formic acid (4:1, v/v) and was centrifuged at 14000 rpm at 4°C for 5 min. Afterwards, the supernatant was evaporated using a concentrator (5301, Eppendorf, Germany) at 45°C for 75 min. Consequently, the dry residue was reconstituted with 200 µL acetonitrile/methanol/buffer (formic acid, 0.2% at pH 3.0) reconstitution solution (8:1:1, v/v/v).

Three steps were applied in the µ-SPE: sample loading, washing and elution. Initially, 10 µL internal standard was added to 200 µL of plasma sample along with 290 µL of 0.2% formic acid

in water, pH 1.5, and loaded (2×250 µL) into the Oasis PRiME HLB (hydrophilic-lipophilic balance) 96-well µElution Plate (Waters, Madrid, Spain). It was followed by the washing step adding 400 µL (2×200 µL) 5% methanol solution with water and 2% ammonium hydroxide. After each step, a 5-15 mmHg vacuum was applied until the wells were dry. Then, the compounds were eluted with 200 µL (2×100 µL) acetonitrile/methanol/buffer (formic acid, 0.2% at pH 3.0) solution (8:1:1, v/v/v) and were collected in a 1 mL 96-well plate (Agilent Technologies, Santa Clara, USA).

Finally, 5 µL of eluate was injected directly to the LC-MS/MS system. SPE was chosen as sample preparation process for method validation due to its excellent phospholipid-elimination capacity.

2.5. Method validation

The validation of this method was based on the guidelines of FDA [222], EMA [223] and ICH [224]. Six validation series were assessed to determine linearity, precision and accuracy, 8 for selectivity, matrix effects, process efficiencies, while 4 validation series were used for different types of stabilities based on the peak area ratio of drug and internal standard.

2.5.1. Linearity

Calibrators were measured in duplicate at the concentrations shown in ***Table* 11** covering the plasma therapeutic ranges and expected patients' plasma concentrations. Six calibration curve replicates at the 8 concentration levels were analyzed from different days. To quantify the calibration data, a linear regression model was applied based on the analyte versus internal standard peak area ratio. In order to meet the validation criteria, the error of accuracy and relative standard deviation (RSD, %) should not exceed 15% for each calibrator corresponding to each drug. The final estimated linearity model was verified using the lack-of-fit test, to confirm that the selected regression and linearity are appropriate.

LLOQ was defined as the lowest point of the calibration curve (see ***Table* 12**) which can be quantified with acceptable precision and accuracy. Based on this practice, for LLOQ, the error of

accuracy and RSD should not exceed 20%. Therefore, the extraction ion chromatogram peak area ratio of each analyte should be at least 5 times higher than the blank.

2.5.2. Selectivity

Method selectivity was ascertained by analyzing 8 different blank plasma samples from human donors. These samples were extracted and injected at the beginning of the HPLC analysis to exclude any endogenous interference. In addition, 8 zero samples (blank sample with internal standard) were analyzed to verify the absence of analyte ions in the respective peaks of internal standard. The method is considered selective when the blank plasma matrix extraction ion chromatogram peak area ratio is less than 20% of the extraction ion chromatogram peak area of LLOQ in plasma matrix for each drug, and less than 5% for the internal standards.

2.5.3. Precision (repeatability and intermediate precision) and accuracy

Quality control samples 'LLOQ', 'Low quality control, 'Medium quality control' and 'High quality control' were prepared at concentrations described in ***Table* 12**. Six samples of each quality control were measured over a period of 4 following days. The determined concentrations were in the ranges of calibrators. Repeatability and intermediate precision were evaluated by analyzing a single analytical run in a single day and 3 analytical runs from 3 different days, respectively. The precision was defined as RSD, % and was determined at each concentration level. The acceptance limit was <15%, except for LLOQ (<20%). The accuracy describes the closeness of mean test results obtained by the method to the theoretical concentration of the analyte.

2.5.4. Extraction recovery, matrix effect and process efficiency

Extraction recovery was defined as the ratio of analyte response between plasma spiked before (Pre) and after (Post) the extraction process. Extraction recovery can be defined as relative: the ratio of the compound concentration; or absolute: the ratio of the extraction ion chromatogram peak area.

Matrix effect was established as quantitative approach comparing the extraction ion chromatogram peak area ratio (absolute) or the compound concentration ratio (relative) between Post-plasma and

neat solution, which was obtained by the addition of the same amount of analyte to the final elution solution [acetonitrile/methanol/buffer, pH 3.0 (8:1:1, v/v/v)] without undergoing the extraction process.

Process efficiency values were calculated as the ratio of analyte response between plasma spiked before (Pre) the extraction process and neat solution. Absolute and relative values were determined in the same fashion than for extraction recovery and matrix effect.

In each test, 8 repetitions of Low and High quality controls were analyzed in blank plasma samples from 8 different human donors for all analytes. To validate matrix effect, the RSD should not exceed 15% for all quality controls (except for LLOQ, when 20% is allowed).

The following equations were used to calculate the previously described values:
Extraction recovery: $RE\ (\%) = Pre * 100/Post$
Matrix effect: $ME\ (\%) = Post * 100/Neat\ solution$
Process efficiency: $PE\ (\%) = Pre * 100/Neat\ solution$

2.5.5. Trueness

'LLOQ', 'Low quality control', and 'High quality control' samples (Pre) were analyzed from 5 weeks in comparison with spiked samples as reference (Post). The results were expressed with Zeta-score which compares a test value to a reference value. Values less than 2 were considered satisfactory, while the values between 2 and 3 were questionable. Values higher than 3 were considered unsatisfactory.

2.5.6. Stability

Four replicates of low and high quality control aliquots were quantified after storing plasma samples at 20ºC (room temperature), at +4°C for 24 h and after 3 freeze-thaw cycles in the freezer at −80°C, as well as in extracted samples in the autosampler at 19°C. Additionally, long-term stability was determined by keeping one set of aliquots at −80°C for 1 month, 3 months and 6 months. The analyte stability should be between 85 and 115% and the RSD should not exceed

15% for the studied quality controls. The ratio of analyte concentration before and after storage conditions mentioned above was determined.

2.5.7. Carry-over

After the highest calibrator, a blank plasma sample was injected to determine any possible carry-over effect. The peak area of the blank samples should be lower than 20% of the LLOQ and 5% of the internal standard. Additionally, the needle was washed 5 times between injections with acetonitrile/water solution (50:50, v/v) to avoid carry-over.

2.5.8. Statistical analyses

Data were analyzed with Microsoft Excel (Microsoft® Office® 2010, Microsoft Corp., USA) and the results are presented as mean values and standard deviations. The lack-of-fit test results were compared to pure error variances at a 95% confidence level to evaluate the acceptability of the results and the adequacy of the regression models. For the evaluation of trueness, zeta-score test was applied.

3. Pharmacokinetic analyses

Pharmacokinetic parameters were calculated from drug concentrations measured with the previously described analytical method by noncompartmental analysis using Phoenix® WinNonlin® (version 8, Pharsight, Mountain View, CA, USA) as “single dose” (i.e. for the first day) and “multiple dose” (i.e. considering all time points). Peak plasma concentration (C_{max}) and time to reach maximum concentration (T_{max}) were obtained directly from the original data. The area under the plasma concentration-time curve from time zero to the last observed time point (AUC_{last}) was calculated using the trapezoidal rule. The AUC from time zero to infinity (AUC_{inf}) was determined as the sum of the AUC_{last} and the extrapolated area calculated as the last plasma concentration (C_{last}) divided by the terminal rate constant (k_e) that was determined by regression analysis of the log-linear part of the concentration-time curve. Elimination half-life was determined by $0.693/k_e$. The total apparent clearance adjusted for bioavailability (Cl/F) was calculated using the following formula: $Cl/F = dose/AUC_{inf}/weight$. For multiple-dose pharmacokinetics, the AUC for a dosing interval (AUC_{tau}), where tau was 24 h, was calculated.

The volume of distribution adjusted for bioavailability (Vd/F) was calculated as Cl/F divided by k_e. AUC and C_{max} were adjusted for dose/weight ratio (AUC/dW and C_{max}/dW, respectively) and were logarithmically transformed for statistical analysis.

4. Genotyping

4.1. DNA extraction

DNA was extracted from 1 mL of peripheral blood using a MagNA Pure LC DNA Isolation Kit in an automatic DNA extractor (MagNa Pure® System, Roche Applied Science, Indianapolis, Indiana, USA). Subsequently, it was quantified spectrophotometrically using a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) and the purity of the samples was determined by the by $A_{260/280}$ absorbance ratio.

4.2. Selected polymorphisms

Polymorphisms analysed in our study are listed in ***Table* 15**.

***Table* 15**. List of all analysed genes and polymorphisms.

Gene	Variant	Alleles	Type of variant	Amino acid change	Functional consequence
CYP2D6	*3 (rs35742686)	T/-	Frameshift	Arg>Gly	No ez activity
	*4 (rs3892097)	C/T	Splice acceptor	No	No ez activity
	*5		Full gene deletion	No	No ez activity
	*6 (rs5030655)	A/-	Frameshift	Trp>Gly	No ez activity
	*7 (rs5030867)	T/G	Missense	His>Pro	No ez activity
	*8 (rs5030865)	C/A	Stop gained	Early stop codon (TGA)	No ez activity
	*9 (rs5030656)	CTT/-	Inframe deletion	No	Decreased ez activity
	*10 (rs1065852)	C/T	Missense	Pro>Ser	Decreased ez activity
	*14 (rs5030865)	C/T	Stop gained	Early stop codon (TGA)	Decreased ez activity
	*17 (rs28371706)	G/A	Missense	Thr>Ile	Decreased ez activity
	*29 (rs16947)	G/A	Missense	Arg>Cys	Decreased ez activity

Gene	Variant	Alleles	Type of variant	Amino acid change	Functional consequence
	*41 (rs28371725)	C/T	Intron	No	Decreased ez activity
	*2xN (rs16947)	G/A	Duplication More than 1 copy of *2	Arg>Cys	Increased ez activity
CYP1A2	*1C (rs2069514)	G/A	5'UTR	No	Less ez activity
	*1F (rs762551)	A/C	Intron	No	Increased inducibility
	*1B (rs762551)	C/T	Synonymous	No	Increased inducibility
CYP3A4	*2 (rs55785340)	T/C	Missense	Ser>Pro	Decreased ez activity
	*6 (rs4646438)	-/A	Frameshift	Early stop codon (TGA)	No ez activity
	*20 (rs67666821)	-/T	Frameshift	Pro>Thr	No ez activity
	*22 (rs35599367)	C/T	Intron	No	Decreased ez activity
CYP3A5	*3 (rs776746)	T/C	Splice acceptor	No	No ez activity
	*6 (rs10264272)	C/T	Synonymous	No	No ez activity
ABCB1	C3435T (rs1045642)	G/A	Synonymous	No	Decreased activity
	C1236T (rs1128503)	G/A	Frameshift	No	Decreased activity
	G2677TA (rs2032582)	C/A/T	Frameshift	Ser>Ala/Thr	Decreased activity
	rs10248420	A/G	Intron	No	Unknown
	rs10276036	C/A	Intron	No	Unknown
	rs10280101	A/C	Intron	No	Unknown
	rs11983225	T/C	Intron	No	Unknown
	rs12720067	C/T	Intron	No	Unknown
	rs3842	T/C	3'UTR	No	Unknown
	rs4148737	T/C	Intron	No	Unknown
	rs4728709	G/A	Intron	No	Unknown
	rs7787082	G/A	Intron	No	Unknown
ADRA2A	rs1800544	G/C	5'UTR	No	Unknown
APOA5	rs662799	A/G	5'UTR	No	Unknown
APOC3	rs4520	C/T	Synonymous	No	Unknown
	rs5128	C/G	3'UTR	No	Unknown
BDNF	rs6265	C/T	Missense	Val>Met	Decreased activity
COMT	rs13306278	C/T	5'UTR	No	Unknown
	rs4680	G/A	Missense	Val>Met	Unknown
DRD2	rs6277	C/T	Synonymous	No	Unknown
	rs1799732	G/-	Deletion	No	Increased activity

Gene	Variant	Alleles	Type of variant	Amino acid change	Functional consequence
	rs1800497	C/T	Missense	Glu>Lys	Decreased activity
DRD3	rs6280	C/T	Missense	Gly>Ser	Unknown
HTR2A	rs6313	C/T	Synonymous	No	Unknown
	rs6314	C/T	Missense	His>Tyr	Unknown
	rs7997012	A/G	Intron	No	Unknown
HTR2C	rs1414334	C/G	Intron	No	Unknown
	rs518147	C/G	5'UTR	No	Unknown
	rs3813929	C/T	5'UTR	No	Altered DNA-nuclear factor interactions
LEP	rs7799039	A/G	5'UTR	No	Impaired signaling capacity of the leptin receptor
LEPR	rs1137101	A/G	Missense	Gln>Arg	Higher levels of Leptin binding activity
OPRM1	rs1799971	A/G	Missense	Asn>Asp	Decreased receptor levels
UGT1A1	rs887829	C/T	Intron	No	Decreased activity

Alleles are shown as wild type/mutant.
Abbreviations: A: adenine; Ala: alanine; Arg: arginine; Asn: asparagine; Asp: aspartic acid; C: cytosine; Cys: cysteine; ez: enzyme; G: guanine; Gln: glutamine; Glu: glutamic acid; Gly: glycine; His: histidine; Ile: isoleucine; Lys: lysine; Met: methionine; Pro: proline; Ser: serine; T: thymine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine; Val: valine.

4.3. Genotyping

Samples were genotyped with TaqMan® assays on an OpenArray® platform on a QuantStudio™ 12K Flex instrument. The assay included 120 SNPs, whereof 46 were analysed based on their importance in the metabolism and mechanism of action of aripiprazole and olanzapine (see ***Table* 15**).

During PCR, numerous copies are made of a DNA sequence of interest using primers and DNA-dependent DNA polymerase. The stages of PCR are denaturation, annealing, and elongation. During denaturation, the two strands of DNA are separated (hydrogen bonds are broken down),

requiring high temperatures. Upon annealing, the temperature is lowered so that primers can bind to the DNA strands. Finally, during elongation, the DNA-dependent DNA polymerase creates a new strand, starting from the primer. The copy is made from the original DNA strand.

Real-time PCR additionally uses allele-specific probes (oligonucleotides) which are fluorescently labelled, called TaqMan probes. They have a reporter dye at their 5'UTR ends and a quencher group at their 3'UTR ends. The quencher group is designed to prevent the reporter dye from emitting a fluorescent signal. When the PCR reaction starts, the DNA polymerase cleaves the probe with its 5'exonuclease activity, therefore the quencher group no longer inhibits the release of the reporter dye. Subsequently, a signal can be detected which is proportional to the amount of product. When SNP genotyping is performed, two labelled probes are used with different fluorescent molecules with different colours (FAM and VIC). These probes can bind to the different alleles of the SNP [225] (***Figure* 17**).

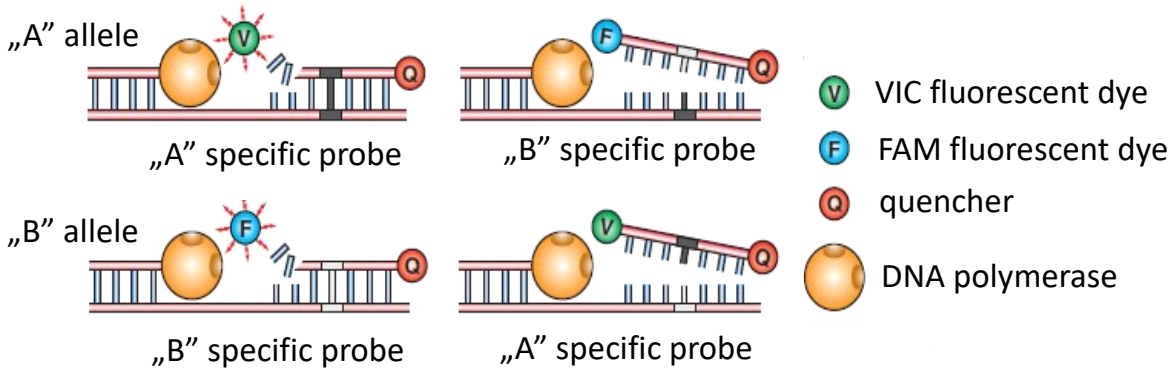

***Figure* 17**. Real-time PCR method with TaqMan® probes.

Real-time PCR method can be multiplexed - based on the TaqMan® OpenArray® Genotyping System (***Figure* 18**). We analysed 24 samples for 120 SNPs on two plates along with 2 negative controls/ plate. DNA samples were normalized to 50 ng/mL concentration. Six µL was used for analysis.

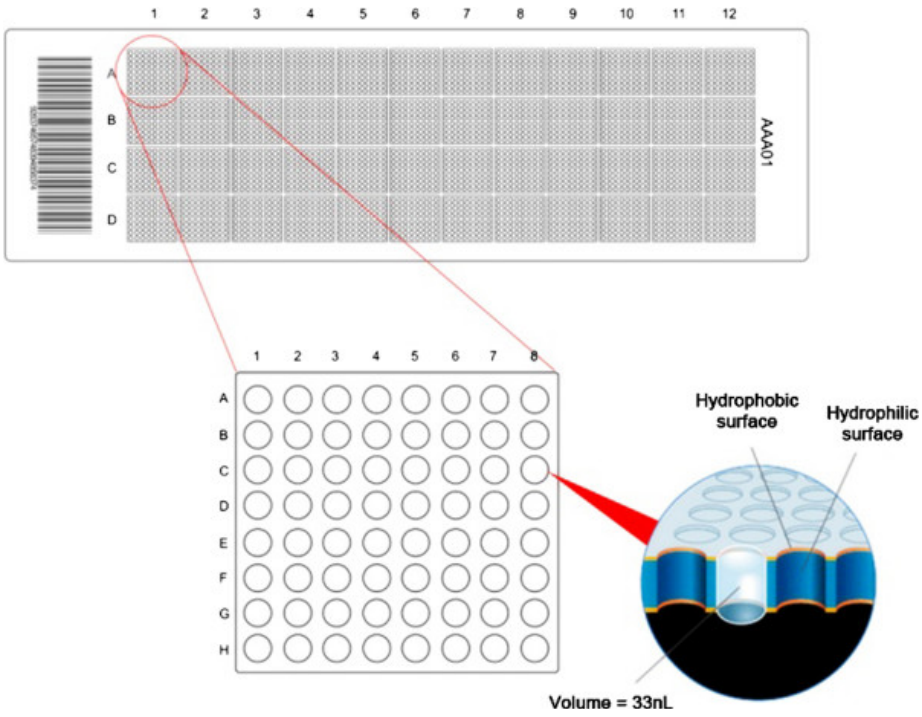

***Figure* 18**. The OpenArray® plate.

One array (circled area): can analyse 64 SNPs on one sample. The assay contains 48 arrays, therefore 24 samples can be analysed.
Source: Arrojo *et al*., 2013 [226].

Since the *CYP2D6* *29 (rs16947) polymorphism was not included in the array, it was genotyped with the same instrument using individual TaqMan® probes. CNVs and *5 deletion in the *CYP2D6* gene were determined with the TaqMan® Copy Number Assay (Assay ID: Hs00010001_cn; Thermo Fisher Scientific, Waltham, Massachusetts, USA) which detects a specific sequence in exon 9 [227]. Samples were run in the same instrument.

Results were analyzed with the QuantStudio™ 12K Flex and the TaqMan® Thermo Fisher Cloud software. The Thermo Fisher Cloud software automatically plots the samples on a graph with the color of each genotype group (***Figure* 19**). These clouds can be modified and the samples can be checked individually for each SNP in case of uncertainty.

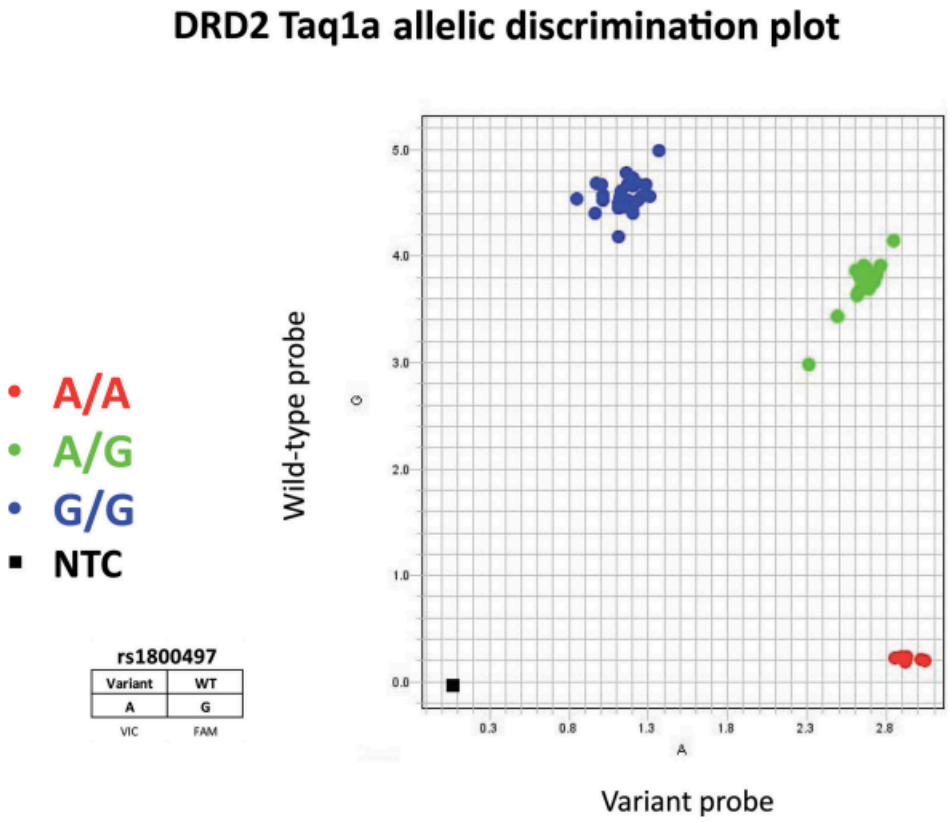

***Figure* 19**. An example for the allele discrimination plot obtained from OpenArray® genotyping data.

Source: Nerenz *et al.*, 2018 [228].

Additionally, the *CYP3A4**20 (rs67666821) polymorphism was genotyped by the KASPar SNP Genotyping System (LGC Genomics, Herts, UK) using 15 ng genomic DNA in the National Cancer Research Center (CNIO), Madrid, Spain. The assay included DNA control samples with known genotypes and negative controls. The ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for fluorescence detection and allele assignment [156].

5. Statistical analyses

Statistical analyses were performed with the SPSS 24.0 software (SPSS Inc., Chicago, Illinois). *P* values lower or equal than 0.05 were considered significant.

5.1. Genetic polymorphisms

Hardy-Weinberg equilibrium was estimated for all analysed variants. Deviations from the equilibrium were detected by comparing the observed and expected frequencies using a Fisher exact test based on the De Finetti program (available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The Hardy-Weinberg equilibrium states that allele and genotype frequencies in a population remain constant from generation to generation in the absence of evolutionary influences, such as mutation, natural selection and migration. A polymorphism meets the Hardy-Weinberg criteria if P>0.05 [229,230]. A significant deviation from Hardy-Weinberg equilibrium may indicate genotyping error, because the conditions of Hardy-Weinberg equilibrium are generally applicable to the control subjects in a well-designed study population. However, it can also be caused by the lack of mutated alleles or location of a gene on chromosome X [231].

CYP2D6 *3, *4, *5, *6, *7, *8, *9, *10, *14, *17, *29 and *41 alleles were classified in 4 phenotypes (poor metabolizer: PM; intermediate metabolizer: IM; normal metabolizer: NM and ultra-rapid metabolizer: UM), which is based on the functionality of alleles [232] and according to the standardizing pharmacogenetic terms consensus [233] (see Tables 7 and 8).

CYP3A4 *2, *20, *22 and *CYP3A5* *3 and *6 genotypes were merged into a CYP3A phenotype as follows: subjects with at least one CYP3A4 reduced activity allele (i.e. CYP3A4 *1/*22 or *22/*22) and no CYP3A5 activity (CYP3A5 *3/*3) were considered PM; subjects with normal CYP3A4 activity (CYP3A4 *1/*1) and no CYP3A5 activity (CYP3A5 *3/*3) were considered IM and subjects with normal CYP3A4 activity (CYP3A4 *1/*1) and at least one CYP3A5 functional allele (CYP3A5 *1/*1 or *1/*3) were categorized as extensive metabolizers (EM) [234].

Furthermore, a value was assigned to *CYP1A2* *1B, *1C and *1F alleles based on their functionality: 0.5 to *1C, 1 to *1, 1.5 to *1F and 1.25 to *1B. An activity score was calculated as the sum of the values assigned to each allele and finally was translated into phenotypes: normal/rapid metabolizers (NM) and ultrarapid metabolizers (UM) [235] (***Tables* **16** and **17**).

***Table* 16**. Values assigned to *CYP1A2* alleles based on their activity.

***CYP1A2* allele**	**Values assigned to the allele**
***1C**	0.5
***1**	1
***1F**	1.5
***1B**	1.25

***Table* 17**. CYP1A2 phenotypes based on the sum of the values assigned to both alleles of each subject.

Activity score	**Phenotype**
1-1.5	PM
1.75-2.5	NM/RM
2.75-3	UM

ABCB1 variants were merged into 3 groups: 0-8 mutant alleles were assigned to group 1, 9-12 mutant alleles were assigned to group 2 and 13-17 mutant alleles were assigned to group 3. Another *ABCB1* haplotype was assembled by only considering C3435T, G2677T/A and C1236T polymorphisms due to greater impact on the transporter's activity or expression levels [236]. Zero or one mutant allele carriers were assigned to group 1, carriers of 2 or 3 mutant alleles were assigned to group 2 and carriers of 4, 5 or 6 mutant alleles were assigned to group 3.

COMT rs13306278 and rs4680 polymorphisms were merged into a haplotype: carrying no mutant allele was assigned as wild-type, carrying one mutant allele was considered as heterozygous while carrying more than one mutant allele was considered as mutant.

The effect of sex on different genotypes was assessed by chi-square test.

5.2. Clinical parameters

ANOVA was used to compare mean pharmacokinetic values among different genotypes and sexes. Changes in pupillometric parameters (MAX, MIN, T75, MCV, CON, ACV, ADV, LAT) were

analysed by repeated measures ANOVA. The values were adjusted for differences in light intensity (*in lux*) before analysis. Pharmacokinetic parameters and polymorphisms were analysed as covariates. Estimate of effect size (partial eta squared, η_p^2), i.e. the proportion of the total variance that is attributed to an effect is reported for each ANOVA test to avoid type II errors. The Bonferroni post-hoc test for multiple comparisons was applied for each analysis to avoid type I errors. Multiple linear regression models were used to study factors related to all pupillometric and pharmacokinetic dependent variables.

Metabolic parameters were analysed by repeated measures ANOVA. Pharmacokinetic parameters and polymorphisms were analysed as covariates. MANOVA was used to study factors related to all metabolic data and pharmacokinetic variables. Estimate of effect size is reported for each ANOVA test. The Bonferroni post-hoc test for multiple comparisons was applied for each analysis.

Changes in blood pressure, heart rate and QTc were analysed by repeated measures ANOVA. Pharmacokinetic parameters and polymorphisms were analysed as covariates. Estimate of effect size is reported for each ANOVA test. The chi-square test was used to compare the incidence of adverse effects between different genotypes. The Bonferroni post-hoc test for multiple comparisons was applied for each analysis.

IV. RESULTS

1. Demographic characteristics

Twenty-four subjects were included in the study (12 males and 12 females). Ten subjects were Caucasian and 14 were Latin-American. Age was similar between males and females. Males had greater weight and height than females, however, BMI values did not differ significantly (***Table* 18**).

***Table* 18**. Demographic characteristics.

	N (%)	Age (y)	Weight (kg)	Height (m)	BMI (kg/m^2)
All	24 (100)	31.5 ± 11.6	71.4 ± 12.2	1.68 ± 0.11	25.3 ± 2.6
Males	12 (50)	28.5 ± 7.4	78.4 ± 12.2	1.76 ± 0.09	25.4 ± 2.8
Females	12 (50)	34.6 ± 14.3	64.3 ± 7.4	1.60 ± 0.07	25.1 ± 2.5
p		0.104	0.003	< 0.0001	0.798

Values are shown as mean ± SD unless otherwise indicated.

2. Analytical method validation

The main results of this chapter were published in:

Koller D, Zubiaur P, Saiz-Rodríguez M, Abad-Santos F, Wojnicz A. Simultaneous determination of six antipsychotics, two of their metabolites and caffeine in human plasma by LC-MS/MS using a phospholipid-removal microelution-solid phase extraction method for sample preparation. *Talanta*. 2019 Jun 1;198:159-168. doi: 10.1016/j.talanta.2019.01.112. Epub 2019 Feb 1. PubMed PMID: 30876545.

2.1. Experimental conditions in LC and MS/MS

pKa values were ranging from 7.06 to 8.76 for all drugs included in this method, except for caffeine with the pKa value of -0.92. Water solubility was ranging from 0.008 mg/mL to 0.297 mg/mL for all the compounds, except for caffeine with 11 mg/mL. To optimize chromatographic conditions, we used different analytical columns (XBridge BEH C18, Waters, Madrid, Spain and ACE C18-PFP, Agilent Technologies, Madrid, Spain), buffer compositions (ammonium formate, 0.1% and 0.2% formic acid), buffer pH (2, 3, 4 and 6), mobile phase flow rates (0.5 and 0.6 mL/min) and

variety of gradients. Additionally, the temperature of analytical separation was considered as well (25-30ºC). For the present method we applied the ACE C18-PFP column as it offers good resolution and improves analyte separation compared to ordinary C18 column due to π-π interactions with the aromatic ring and OH groups. Finally, 0.2% formic acid at pH 3.0 as aqueous buffer, flow rate of 0.6 mL/min and the temperature of 25ºC were chosen to improve the chromatographic performance of our method. After each injection, the injection needle and the analytical column were washed with a mixture of organic-aqueous solvent. A mixture of acetonitrile/water (50/50; v/v) and isopropanol/water (50/50; v/v) was tested to avoid carry-over phenomenon in subsequent injections. Finally, acetonitrile/water was found to be more effective in needle cleaning process. The total run time, including washing and re-equilibrating steps, was 9.0 min. Five µL of the sample was injected in the HPLC system. Human plasma was spiked with the LLOQ of each drug before µ-SPE. Retention times and concentration values were calculated from calibration curves given for all analytes. Concerning MS/MS conditions, ESI in positive mode was selected for scanning all analytes in dynamic MRM (dMRM) scan mode.

***Table* 14** displays relevant LC–MS/MS characteristics, ***Figure* 20** and **21** show XIC of the analytes.

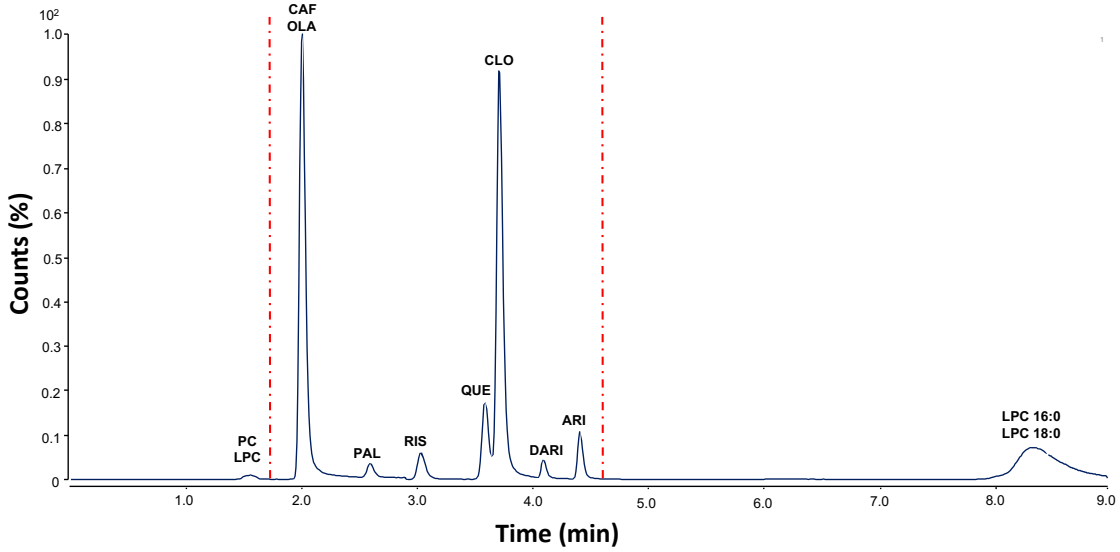

***Figure* 20**. Extraction ion chromatogram (XIC) of the compounds.

Results are presented as the percentage of counts versus time in minutes. All chromatograms were normalized to the largest peak. Dashed lines show the elution area of the antipsychotics and caffeine separated from early- and late-eluting (lysophosphatidylcholines, LPC) endogenous phospholipids.

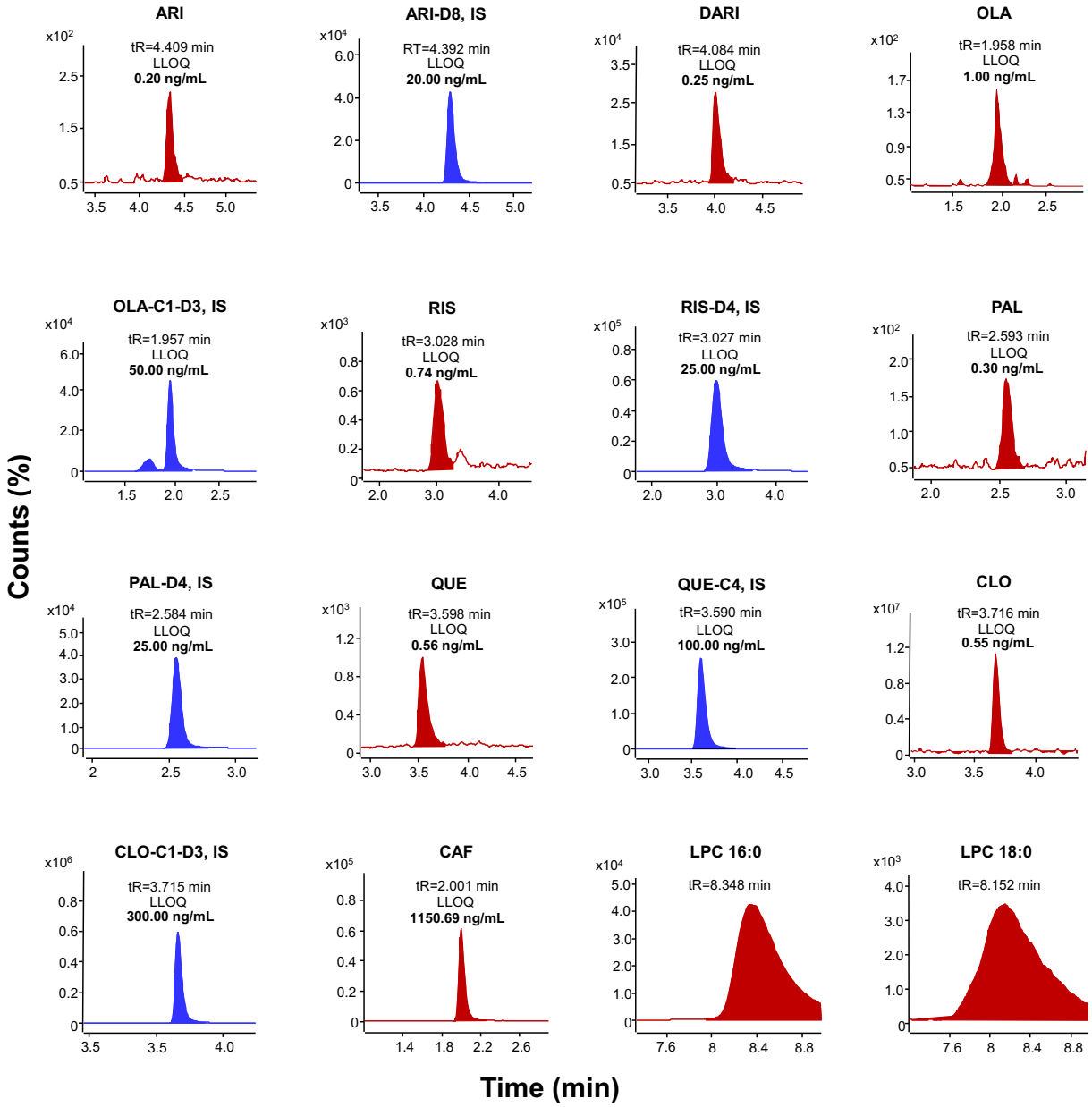

***Figure* 21**. Extraction ion chromatograms (XIC) of the compounds and their isotope-labeled internal standards.

Results are presented as the percentage of counts versus time in minutes.

***Figure* 22** depicts mass spectra obtained by collision-induced dissociation of the indicated precursor ions [M+H]+.

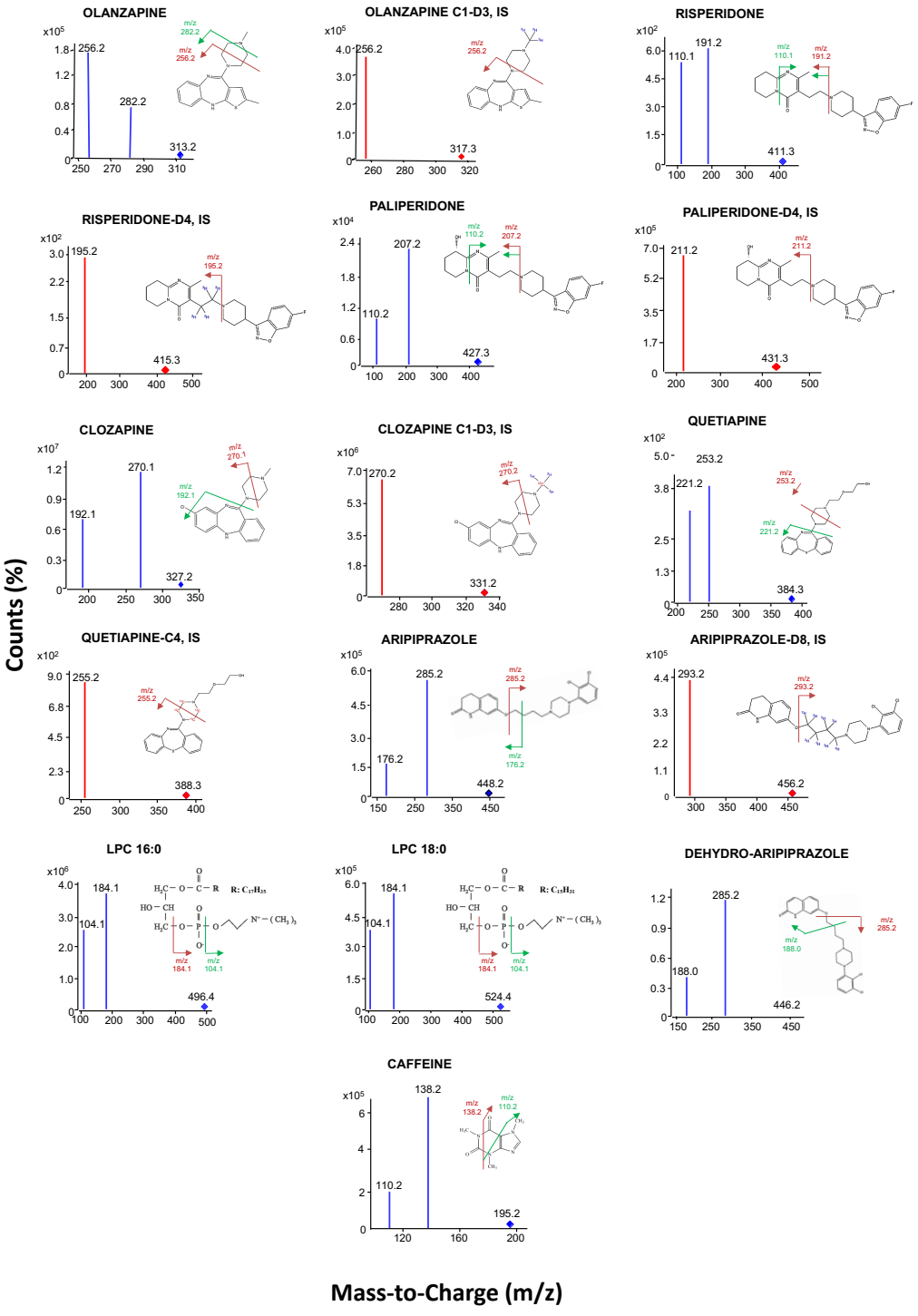

***Figure* 22**. Product ion spectra and chemical structures of the compounds and their stable isotope-labeled internal standards obtained by collision-induced dissociation (CID) of the indicated precursor ions $[M+H]^+$.

The resolution of elution, a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation, was also evaluated. It is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks:

$$Rs = 2[(tR)B - (tR)A)]WB + WA$$

Where rs = resolution factor, tR: retention time, B: compound B, A: compound A, W: width.

Resolutions between the analytes of interest are shown in ***Table* 19**.

***Table* 19**. Resolution factor (Rs) between the adjoining compounds.

Compounds	Rs
Caffeine-Olanzapine	1.28
Olanzapine-Paliperidone	12.63
Paliperidone-Risperidone	6.24
Risperidone-Quetiapine	7.35
Quetiapine-Clozapine	1.85
Clozapine-Dehydro-aripiprazole	7.81
Dehydro-aripiprazole-Aripiprazole	8.49

Apart from isotope-labeled internal standards, ion ratio (qualifier ratio) based identity confirmation was used to ensure the reliability of analyte detection. The ion ratio difference between calibrators and quality controls did not differ more than 30%. Additionally, we analyzed retention time and relative retention time difference between extracted analyte and neat solution of the analyte. The obtained value was lower than 0.1 min in all cases when analyzing retention time difference and lower than 2.5% when analyzing relative retention time – identity confirmation.

2.2. Sample preparation: phospholipid elimination, extraction recovery, matrix effects and process efficiency

We tested phospholipid cleaning ability of SPE compared to PPT in 36 different human blank plasma samples. With the SPE method we eliminated 99.56% and 99.46% of early eluting endogenous plasma phosphatidilcholines and lysophosphatidilcholines using hydrophilic-lipophilic SPE sorbent compared to PPT method (100.00 ± 33.49% vs 0.44 ± 0.73%, $p < 0.001$ for phosphatidilcholines and 100.00 ± 31.57% vs 0.54 ± 0.17%, $p < 0.001$ for lysophosphatidilcholines, respectively). Similarly, we evaluated the elimination capability of late-eluting phospholipids. SPE method was able to remove 92.34% and 97.68% of late-eluting endogenous plasma lysophosphatidilcholine 18:0-l and lysophosphatidilcholine 16:0-l compared to PPT method (100.00 ± 32.23% vs 7.66 ± 3.16%, $p < 0.001$ for lysophosphatidilcholine 18:0-l and 100.00 ± 26.16% vs 2.32 ± 0.74%, $p < 0.001$ for lysophosphatidilcholine 16:0-l, respectively). The results are shown in ***Figure* 23**. Early eluting phospholipids were eluted with at 1.485 min and at 1.537 min, while late-eluting phospholipids were eluted at 8.186 min and at 8.348 min. Analyte elution was between 1.940 min and 4.790 min.

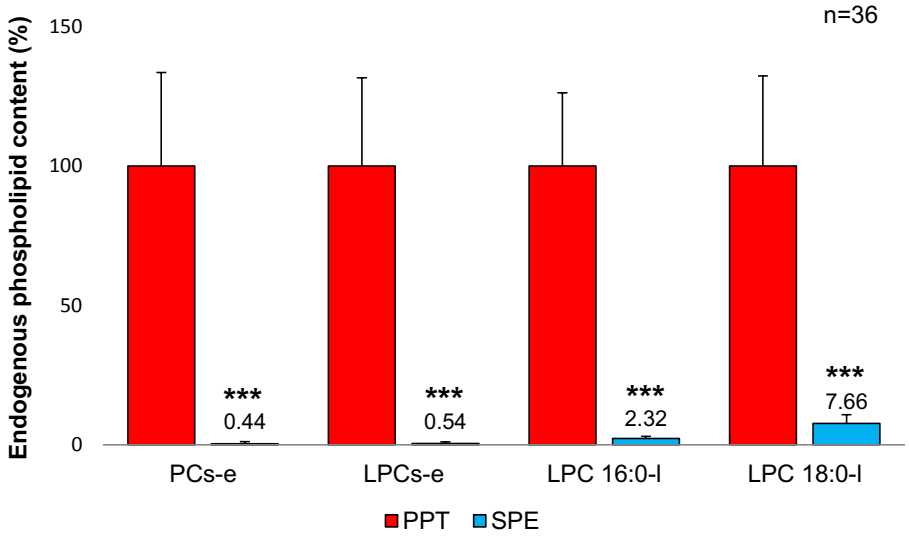

***Figure* 23**. Endogenous plasma phospholipid elimination efficiency in plasma after undergoing SPE and PPT processes including 36 experiments (RSD, %) at low and high concentrations.

Abbreviations: PCs-e: early eluting phosphatidilcholines, LPCs-e: early eluting lysophosphatidilcholines, LPC 16:0-l: late-eluting lysophosphatidylcholine 16:0, LPC 18:0-l: late-eluting lysophosphatidylcholine 18:0).

Low- and high-quality controls were applied for the determination of relative and absolute extraction recovery, matrix effects and process efficiency. These results are shown in ***Figures* 24** and **25**, respectively.

Relative mean extraction recovery when applying SPE was within 98.05-108.91% for all compounds, except for CAF with the value of 127.19% with an RSD lower than 10%, while absolute mean extraction recovery ranged between 81.31 and 119.02% with an RSD of 14%. Relative extraction recovery values for PPT ranged from 96.04 to 119.09% (except for CAF with the value of 171.54%) with an RSD not higher than 11.50%, while absolute extraction recovery had values between 57.42 and 75.91% with an RSD within 24.67%.

Regarding matrix effects, relative values were found between 99.05 and 105.14% with an RSD of 3.97% (except for caffeine with 77.52 and 118.85% and RSD of 27.04%), greatly better than absolute matrix effects ranging from 83.24 to 119.37% with an RSD of 15%. Only olanzapine represented higher ion suppression with a value of 75.80%. The results obtained with PPT as extraction method were as follows: relative matrix effects ranged from 94.09 to 106.57% with an RSD of 8.22%, absolute matrix effects for PPT was comprised between 63.92 and 91.11% with an RSD of 12.02%. Regarding extraction recovery and matrix effects, more preferable values were obtained when stable isotope-labeled internal standards were applied (relative) compared to absolute values. Absolute results obtained by SPE and PPT differed considerably. Absolute extraction recovery obtained with PPT compared to SPE was lower and highly variable (57.42 to 75.91% with an RSD of 24.67%), while ion suppression was also significant (mean matrix effects of 77.51%). Therefore, SPE was chosen as extraction procedure for method validation.

Regarding process efficiency during SPE, relative values were comprised between 95.28 and 113.15% with an RSD of 7.11%, while absolute process efficiency was extent from 95.75 to 119.59% with an RSD less than 13.10%, except for caffeine having values between 77.52 and 152.41% with an RSD of 13.50%. Relative process efficiency during PPT was found between 88.43 and 114.55% with an RSD of 10.45% (except for caffeine with a value of 156.34% and RSD

of 10.75%); absolute process efficiency was relatively lower ranging from 38.00 to 61.53% with an RSD of 24.72%.

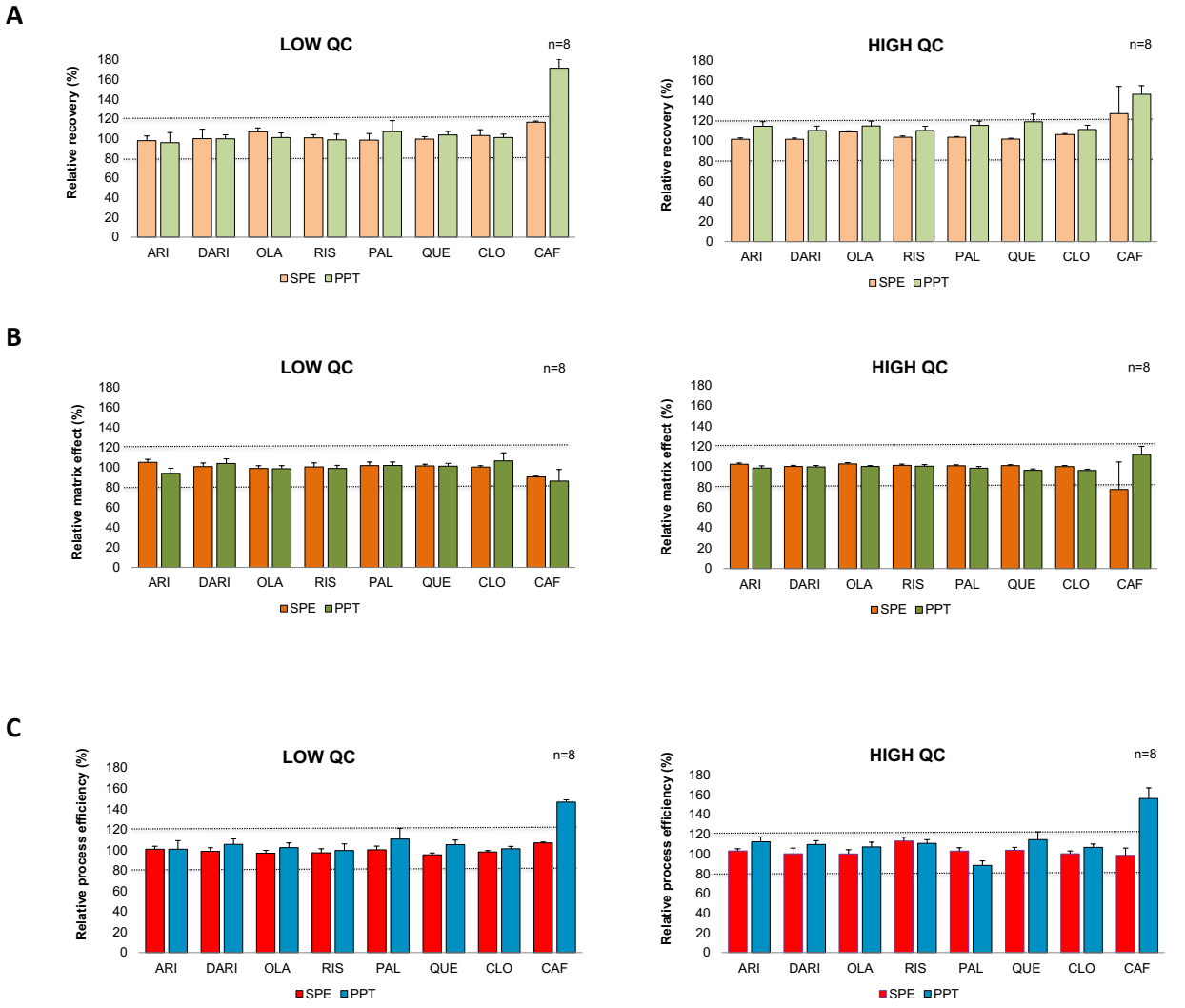

***Figure* 24**. Relative values of extraction recovery, matrix effects and process efficiency tests calculated for each analyte after SPE and PPT processes in low (Low QC) and high (High QC) concentrations.

Panel A shows extraction recovery, Panel B shows matrix effects while Panel C shows process efficiency values with the relative standard deviation (RSD, %) of 8 experiments.

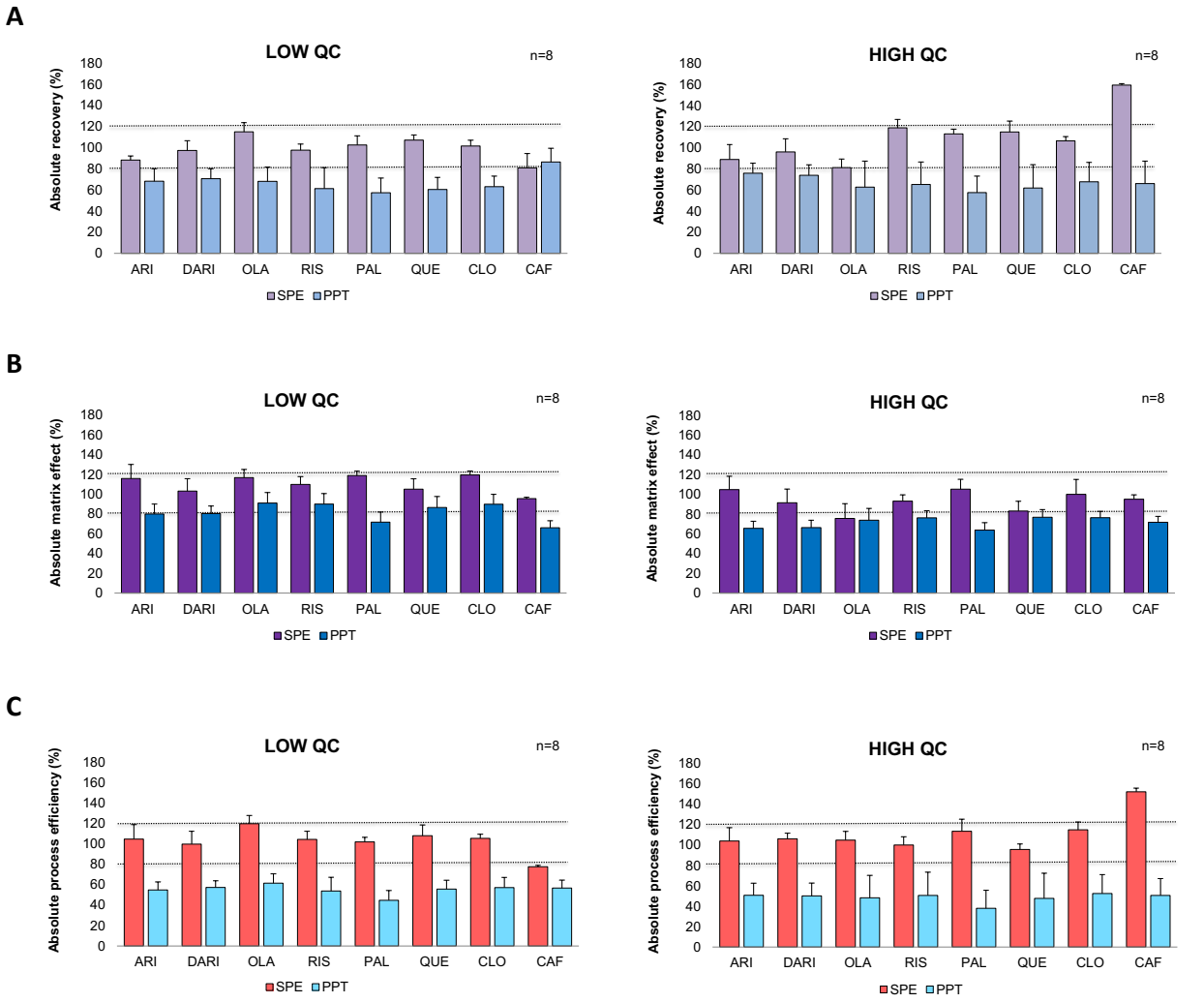

***Figure* 25**. Absolute values of extraction recovery, matrix effects and process efficiency tests calculated for each analyte after SPE and PPT processes in low (Low QC) and high (High QC) concentrations.

Panel A shows extraction recovery, Panel B shows matrix effects while Panel C shows process efficiency values with the relative standard deviation (RSD, %) of 8 experiments.

2.3. Selectivity, LLOQ and linearity

Blank plasma XIC peak area ratio was less than 10.6% compared to XIC peak area of the LLOQ of all drugs, except for caffeine (13.49%), and less than 0.07% for XIC peak area for all internal standards, except for caffeine (0.27%). LLOQs for the analytes in human plasma were the

following: aripiprazole: 0.18, dehydro-aripiprazole: 0.25, olanzapine: 1.00, risperidone: 0.70, paliperidone: 0.20, quetiapine: 0.50, clozapine: 0.50 ng/mL and caffeine: 1200 ng/mL (***Table* 11**).

After performing lack-of-fit test to evaluate linearity of the calibration curve, the F calculated value (0.381-1.687) was lower than the F tabulated value (2.685) for dehydro-aripiprazole, olanzapine, clozapine and caffeine. Thus, for these analytes, a linear regression model was performed. For the rest of the compounds the F calculated value was higher than F tabulated value, therefore non-linear calibration curve adjustment was applied. The most applicable weighting factors were 1/x for aripiprazole, dehydro-aripiprazole, olanzapine, quetiapine and caffeine, $1/x^2$ for paliperidone and 1/y for clozapine and risperidone. In all cases, we obtained correlation coefficients (R^2) higher than 0.99, except for caffeine (***Table* 20**).

***Table* 20**. Linearity of the compounds.

Compound	R^2	Regression model	Weighting factor	F calculated value
Aripiprazole	0.999	Quadratic	1/x	8.754
Dehydro-aripiprazole	0.999	Linear	1/x	0.875
Caffeine	0.939	Linear	1/x	1.687
Clozapine	0.998	Linear	1/y	1.239
Olanzapine	0.996	Linear	1/x	0.381
Paliperidone	0.985	Quadratic	$1/x^2$	18.230
Risperidone	0.998	Quadratic	1/y	14.566
Quetiapine	0.999	Quadratic	1/x	21.345

2.4. Precision and accuracy

As shown in ***Table* 21**, precision and accuracy were within the acceptance criteria for the evaluated assay range. The overall RSDs, for all quality controls except for LLOQ, ranging from 1.53 to 13.16% and from 1.78 to 14.84%, were achieved for repeatability and intermediate precision tests,

respectively. Global RSDs for accuracy assessment averaged -7.56 and 12.45%, -14.91 and 14.58%, respectively. LLOQs showed higher RSD values, however, they were consistently below the acceptance limits (20%).

***Table* 21**. Repeatability and intermediate precision and accuracy values.

Compound	Concentration (ng/mL)	Repeatability n=6 Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)	Intermediate precision n=24 Mean ± SD (ng/mL)	Pooled RSD (%)	Accuracy (%)
Aripiprazole	0.18	0.17 ± 0.01	0.07	-3.27	0.19 ± 0.02	7.69	2.56
	1.00	1.12 ± 0.08	0.07	12.33	1.08 ± 0.06	5.18	8.17
	60	58.84 ± 1.34	2.29	-1.93	59.59 ± 2.00	2.83	-0.69
	100	108.00 ± 2.60	2.41	8.00	112. 86 ± 9.24	6.91	12.86
Dehydro-aripiprazole	0.25	0.25 ± 0.01	3.72	0.75	0.25 ± 0.02	6.89	0.64
	0.90	0.85 ± 0.04	4.11	-5.23	0.86 ± 0.05	6.02	-4.87
	40	42.03 ± 0.72	1.72	5.07	41.73 ± 1.97	4.71	4.32
	70	68.85 ± 1.44	2.10	-1.64	66.28 ± 3.18	4.80	-5.31
Olanzapine	1.00	1.05 ± 0.03	3.32	5.09	1.08 ± 0.18	9.43	7.54
	3.00	3.01 ± 0.40	13.16	0.33	3.27 ± 0.40	9.42	9.00
	50	47.28 ± 0.99	2.08	-5.43	51.04 ± 4.59	2.90	2.09
	80	79.14 ± 1.98	2.50	-1.08	75.94 ± 5.15	3.15	-5.07
Risperidone	0.70	0.73 ± 0.02	3.20	4.89	0.61 ± 0.10	8.08	-12.33
	1.50	1.68 ± 0.07	3.98	12.01	1.71 ± 0.10	5.73	13.70
	25	25.02 ± 0.56	2.22	0.07	27.79 ± 0.91	2.51	3.14
	50	48.79 ± 1.25	2.56	-2.41	46.06 ± 3.07	2.37	-7.88
Paliperidone	0.20	0.24 ± 0.03	13.04	19.15	0.24 ± 0.04	16.51	17.58
	1.60	1.67 ± 0.05	3.19	4.39	1.70 ± 0.08	3.68	6.27
	15	14.77 ± 0.98	6.63	-1.55	14.71 ± 0.64	3.71	-1.92
	25	23.11 ± 0.55	2.36	-7.56	22.71 ± 1.09	2.14	-9.16
Quetiapine	0.50	0.48 ± 0.04	7.58	-3.65	0.45 ± 0.05	8.11	-10.69
	1.50	1.67 ± 0.10	5.93	11.16	1.69 ± 0.10	5.37	12.74
	80	89.96 ± 1.84	2.04	12.45	91.21 ± 6.16	6.95	14.00
	130	123.47 ± 2.20	1.78	-5.03	133.26 ± 11.30	3.00	2.50
Clozapine	0.50	0.53 ± 0.02	3.97	5.12	0.46 ± 0.05	7.35	-8.57
	7.00	7.85 ± 0.40	5.03	12.11	7.46 ± 0.51	4.12	6.56
	500	499.64 ± 13.29	2.66	-0.07	510.27 ± 21.25	2.51	2.05
	800	766.07 ± 19.99	2.61	-4.24	745.22 ± 50.99	2.79	-6.85
Caffeine	1200	974.48 ± 82.39	8.45	-18.79	1286.27 ± 322.33	19.36	7.19
	2200	2364.41 ± 126.05	5.33	7.47	2366.92 ± 137.84	6.06	7.59
	3200	3151.70 ± 48.25	1.53	-1.51	3080.38 ± 392.77	9.78	-3.74
	3700	3647.80 ± 143.63	3.94	-1.41	3290.99 ± 313.51	6.86	-11.05

Precision values are expressed as percentage of RSD (%) for repeated measurement from one day and as pooled RSD (%) from 4 consecutive days. Accuracy is shown as the percentage of the closeness of theoretical concentration to the measured value present in the matrix. The corresponding concentrations are displayed as the mean ± standard deviation (SD) of the number of total experiments (n).

2.5. Trueness

Analyzing trueness interpreted with Zeta-score resulted satisfactory (values less than 2) in case of all drugs and concentrations (between -0.10 and 1.20 for LLOQ, -1.07 and 1.16 for Low quality control and 0.56 and 1.70 for High quality control).

2.6. Stability

We performed stability tests in unextracted sample (room temperature at 20ºC, fridge at 4ºC and freezer at -80ºC) and after sample extraction in the autosampler. Results of the stability assays at low and high concentrations are summarized in ***Figure* 26**. Stability tests in unextracted plasma after 24h at 20ºC (room temperature) showed global RSDs between 1.16 and 10.32% and mean stabilities of 89.03 and 105.35% (except for caffeine with the value of 111.81%); after 24 h at 4°C in the fridge demonstrated overall RSDs between 0.55 and 8.66% and mean stabilities of 92.93 and 103.49%; after three freeze–thaw cycles (24 h each cycle) in the freezer at –80°C they exhibited global RSDs between 0.17 and 6.02% and mean stabilities of 91.55 and 101.67%; after 1 month in the freezer at –80°C they showed overall RSDs between 0.67 and 6.55% and mean stabilities of 87.28 and 109.04%; for all compounds included in the present method. Stability tests in extracted samples after 24 h at 20ºC in the autosampler presented RSDs of minimum 0.76 and maximum 7.88% and mean stabilities ranging from 92.63 to 107.57%. Overall, the RSD value was less than 10.32% and the mean stabilities were close to 100% for all tested compounds.

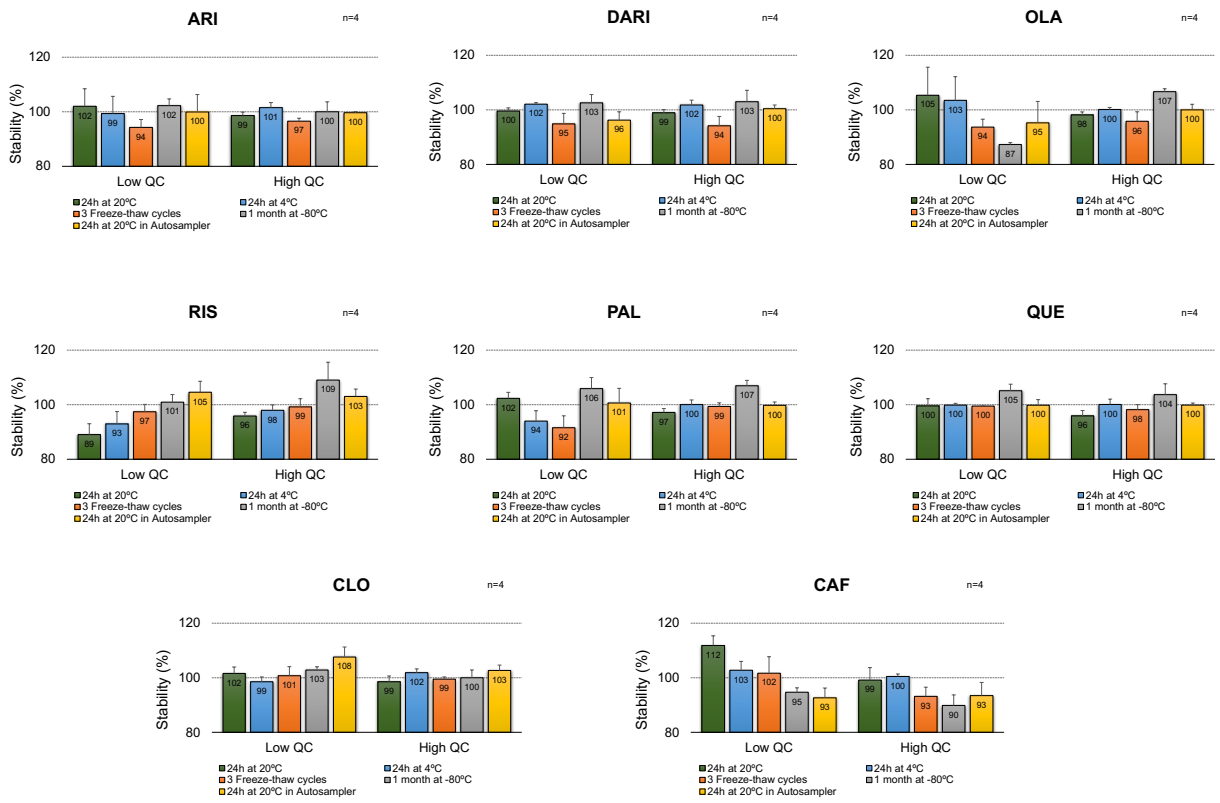

***Figure* 26**. Stability tests.

Storage stability of at low and high concentrations (Low QC and High QC) in human plasma samples during 24 h at 20ºC (room temperature), 24 h at 4°C in the fridge, after 3 freeze-thaw cycle (-80°C), stored in the freezer at -80°C for one month and in extracted samples after 24h at 20°C in the autosampler.
Results are given as mean percentages ± RSD. Low- and high quality-control (Low QC and High QC) samples in quadruplicates were used for each stability test.

2.7. Carry-over

We applied a mixture of acetonitrile and water (1/1, v/v) for needle wash as well as for blank sample injection after measuring samples with high concentration, in order to reduce carry-over effect. The carry-over effect was lower than 15% for antipsychotic drugs and lower than 16.5% for caffeine (***Table* 22**).

***Table* 22**. Carry-over effect.

Compound	Carry over (%) ± SD
Aripiprazole	7.41 ± 3.23
Dehydro-aripiprazole	4.92 ± 3.22
Caffeine	16.13 ± 19.23
Clozapine	14.39 ± 8.76
Olanzapine	14.68 ± 10.65
Paliperidone	7.28 ± 2.93
Risperidone	1.05 ± 0.76
Quetiapine	7.68 ± 3.65

3. Genotype frequencies

Genotype and phenotype frequencies of the analysed variants are shown in ***Table* 23**. *HTR2C* rs3813929 and rs518147, *ABCB1* rs4728709, *COMT* rs13306278, *CYP2D6* *14 (rs5030865), *17 (rs28371706), *3 (rs35742686), *6 (rs5030655), *7 (rs5030867) and *8 (rs5030865) and *CYP3A4* *2 (rs55785340) and *6 (rs4646438) were not in Hardy-Weinberg equilibrium ($p \leq 0.05$), what can be explained by the low number of subjects. The rest of the polymorphisms were in Hardy-Weinberg equilibrium ($p \geq 0.05$).

Genotype frequencies of *ABCB1* rs1128503, rs2032582, 10276036 and rs4148737 and *HTR2C* rs518147 polymorphisms were significantly different between males and females (***Table* 23**).

***Table* 23**. Genotype frequencies of the analysed polymorphisms.

Gene / variant	Genotype / Haplotype	Total	Males	Females
CYP2D6 phenotype	**NM**	16 (66.7)	8 (66.7)	8 (66.7)
	IM	6 (25.0)	3 (25.0)	3 (25.0)
	UM	2 (8.3)	1 (8.3)	1 (8.3)

Gene / variant	Genotype / Haplotype	Total	Males	Females
CYP1A2 phenotype	**NM/RM**	17 (70.8)	10 (83.3)	7 (58.3)
	UM	7 (29.2)	2 (16.7)	5 (41.7)
CYP3A4 phenotype	**EM**	4 (66.7)	3 (25.0)	1 (8.3)
	IM	17 (25.0)	7 (58.3)	10 (83.3)
	PM	3 (8.3)	2 (16.7)	1 (8.3)
ABCB1 phenotype*	**1**	7 (29.2)	6 (50.0)	1 (8.3)
	2	9 (37.5)	3 (25.0)	6 (50.0)
	3	8 (33.3)	3 (25.0)	5 (41.7)
ABCB1 phenotype#*	**1**	8 (33.3)	7 (58.4)	1 (8.3)
	2	10 (41.6)	3 (25.0)	7 (58.4)
	3	6 (25.0)	2 (16.7)	4 (33.3)
***ABCB1* C3435T**	**C/C**	7 (29.2)	6 (50.0)	1 (8.3)
	C/T	12 (50.0)	4 (33.3)	8 (66.7)
	T/T	5 (20.8)	2 (16.7)	3 (25.0)
ABCB1* G2677T/A	**C/C**	9 (37.5)	7 (58.3)	2 (16.7)
	C/A	12 (50.0)	5 (41.7)	7 (41.7)
	A/T	3 (12.5)	0 (0.0)	3 (41.7)
ABCB1* C1236T	**C/C**	8 (33.3)	6 (50.0)	2 (16.7)
	C/T	11 (45.8)	6 (50.0)	5 (41.7)
	T/T	5 (20.8)	0 (0.0)	5 (41.7)
***ABCB1* rs10248420**	**G/G**	1 (4.2)	0 (0.0)	1 (8.3)
	A/G	11 (45.8)	6 (50.0)	5 (41.7)
	A/A	12 (50.0)	6 (50.0)	6 (50.0)
ABCB1* rs10276036	**T/T**	8 (33.3)	6 (50.0)	2 (16.7)
	C/T	11 (45.8)	6 (50.0)	5 (41.7)
	C/C	5 (20.8)	0 (0.0)	5 (41.7)
***ABCB1* rs10280101**	**A/A**	16 (66.7)	7 (58.3)	9 (75.0)
	A/C	8 (33.3)	5 (41.7)	3 (25.0)
***ABCB1* rs11983225**	**T/T**	16 (66.7)	7 (58.3)	9 (75.0)
	C/T	8 (33.3)	5 (41.7)	3 (25.0)
***ABCB1* rs12720067**	**C/C**	16 (66.7)	7 (58.3)	9 (75.0)

Gene / variant	Genotype / Haplotype	Total	Males	Females
	C/T	8 (33.3)	5 (41.7)	3 (25.0)
***ABCB1* rs3842**	**T/T**	19 (79.2)	9 (75.0)	10 (83.3)
	C/T	4 (16.7)	3 (25.0)	1 (8.3)
	C/C	1 (4.2)	0 (0.0)	1 (8.3)
ABCB1* rs4148737	**T/T**	9 (37.5)	1 (8.3)	8 (66.7)
	C/T	12 (50.0)	9 (75.0)	3 (25.0)
	C/C	3 (12.5)	2 (16.7)	1 (8.3)
***ABCB1* rs4728709**	**A/A**	1 (4.2)	1 (8.3)	0 (0.0)
	A/G	2 (8.3)	1 (8.3)	1 (8.3)
	G/G	21 (87.5)	10 (83.3)	11 (91.7)
***ABCB1* rs7787082**	**A/A**	1 (4.2)	0 (0.0)	1 (8.3)
	A/G	12 (50.0)	7 (58.3)	5 (41.7)
	G/G	11 (45.8)	5 (41.7)	6 (50.0)
***ADRA2A* rs1800544**	**C/C**	16 (66.7)	8 (66.7)	8 (66.7)
	C/G	8 (33.3)	4 (33.3)	4 (33.3)
***APOA5* rs662799**	**A/A**	15 (62.5)	7 (58.3)	8 (66.7)
	A/G	9 (37.5)	5 (41.7)	4 (33.3)
***APOC3* rs4520**	**C/C**	4 (16.7)	2 (16.7)	2 (16.7)
	C/T	16 (66.7)	6 (50.0)	10 (83.3)
	T/T	4 (16.7)	4 (33.3)	0 (0.0)
***APOC3* rs5128**	**C/C**	12 (50.0)	7 (58.3)	5 (41.7)
	C/G	11 (45.8)	4 (33.3)	7 (58.3)
	G/G	1 (4.2)	1 (8.3)	0 (0.0)
***BDNF* rs6265**	**C/C**	16 (66.7)	6 (50.0)	10 (83.3)
	C/T	6 (25.0)	5 (41.7)	1 (8.3)
	T/T	2 (8.3)	1 (8.3)	1 (8.3)
COMT phenotype	**WT**	9 (37.5)	5 (41.7)	4 (33.3)
	HZ	9 (37.5)	3 (25.0)	6 (50.0)
	MUT	6 (25.0)	4 (33.3)	2 (16.7)
***COMT* rs13306278**	**C/C**	21 (87.5)	10 (83.3)	11 (91.7)

Gene / variant	Genotype / Haplotype	Total	Males	Females
	C/T	2 (8.3)	2 (16.7)	0 (0.0)
	T/T	1 (4.2)	0 (0.0)	1 (8.3)
***COMT* rs4680**	**G/G**	5 (20.8)	3 (25.0)	2 (16.7)
	A/G	10 (41.7)	4 (33.3)	6 (50.0)
	A/A	9 (37.5)	5 (41.7)	4 (33.3)
***DRD2* rs6277**	**A/A**	9 (37.5)	5 (41.7)	4 (33.3)
	A/G	13 (54.2)	7 (58.3)	6 (50.0)
	G/G	2 (8.3)	0 (0.0)	2 (16.7)
***DRD2* rs1799732**	**G/G**	18 (75.0)	7 (58.3)	11 (91.7)
	G/-	6 (25)	5 (41.7)	1 (8.3)
***DRD2* rs1800497**	**A1/A1**	14 (58.3)	7 (58.3)	7 (58.3)
	A1/A2	7 (29.2)	4 (33.3)	3 (25.0)
	A2/A2	3 (12.5)	1 (8.3)	2 (16.7)
***DRD3* rs6280**	**Ser/Ser**	6 (25.0)	3 (25.0)	3 (25.0)
	Ser/Gly	14 (58.3)	8 (66.7)	6 (50.0)
	Gly/Gly	4 (16.7)	1 (8.3)	3 (25.0)
***HTR2A* rs6313**	**C/C**	9 (37.5)	3 (25.0)	6 (50.0)
	C/T	9 (37.5)	6 (50.0)	3 (25.0)
	T/T	6 (25.0)	3 (25.0)	3 (25.0)
***HTR2A* rs6314**	**C/C**	22 (91.7)	11 (91.7)	11 (91.7)
	C/T	2 (8.3)	1 (8.3)	1 (8.3)
***HTR2A* rs7997012**	**G/G**	11 (45.8)	6 (50.0)	5 (41.7)
	A/G	12 (50.0)	6 (50.0)	6 (50.0)
	A/A	1 (4.2)	0 (0.0)	1 (8.3)
***HTR2C* rs1414334**	**C/C**	20 (83.3)	11 (91.7)	9 (75.0)
	C/G	3 (12.5)	0 (0.0)	3 (25.0)
	G/G	1 (4.2)	1 (8.3)	0 (0.0)
HTR2C* rs518147	**C/C**	5 (20.8)	4 (33.3)	1 (8.3)
	C/G	6 (25.0)	0 (0.0)	6 (50.0)
	G/G	13 (54.2)	8 (66.7)	5 (41.7)

Gene / variant	Genotype / Haplotype	Total	Males	Females
***HTR2C* rs3813929**	**C/C**	18 (75.0)	10 (83.3)	8 (66.7)
	C/T	3 (12.5)	0 (0.0)	3 (25.0)
	T/T	3 (12.5)	2 (16.7)	1 (8.3)
***LEP* rs7799039**	**A/A**	5 (20.8)	2 (16.7)	3 (25.0)
	A/G	12 (50.0)	5 (41.7)	7 (58.3)
	G/G	7 (29.2)	5 (41.7)	2 (16.7)
***LEPR* rs1137101**	**A/A**	9 (37.5)	5 (41.7)	4 (33.3)
	A/G	13 (54.2)	5 (41.7)	8 (66.7)
	G/G	2 (8.3)	2 (16.7)	0 (0.0)
***OPRM1* rs1799971**	**A/A**	19 (79.2)	10 (83.3)	9 (75.0)
	A/G	4 (16.7)	2 (16.7)	2 (16.7)
	G/G	1 (4.2)	0 (0.0)	1 (8.3)
***UGT1A1* rs887829**	**C/C**	8 (33.3)	2 (16.7)	6 (50.0)
	C/T	11 (45.8)	7 (58.3)	4 (33.3)
	T/T	5 (20.8)	3 (25.0)	2 (16.7)

*$P < 0.05$ females versus males.
[#]ABCB1 phenotype including only *ABCB1* C3435T, G2677T/A and C1236T variants.
Values are shown as n (%).

4. Pharmacokinetics and its relationship with polymorphisms

Mean and standard deviation (SD) of the pharmacokinetic parameters of aripiprazole, dehydro-aripiprazole and olanzapine are shown in ***Table* 24**. Females had higher $T_{1/2}$ ($p = 0.044$) and Vd/F ($p = 0.001$) of aripiprazole and higher Vd/F ($p = 0.048$) and Cl/F ($p = 0.015$) of dehydro-aripiprazole after multiple dose administration. Moreover, males had higher AUC_{24h} of dehydro-aripiprazole ($p = 0.035$) after single dose administration. No differences were found in the pharmacokinetic parameters of olanzapine between males and females.

***Table* 24**. Pharmacokinetic parameters of aripiprazole, dehydro-aripiprazole and olanzapine after administration of a single dose and 5 multiple doses.

	SINGLE DOSE			MULTIPLE DOSE		
	All	**Males**	**Females**	**All**	**Males**	**Females**
ARIPIPRAZOLE						
AUC (ng·h/mL)[#]	724.9 ± 236.5	647.0 ± 197.2	802.7 ± 254.6	11102.4 ± 8234.0	7790.0 ± 4071.5	14415.7 ± 10061.4
C_{max} (ng/mL)	50.6 ± 15.5	47.7 ± 14.4	53.5 ± 16.6	138.0 ± 45.9	129.6 ± 47.4	146.3 ± 44.9
T_{max} (h)	5.2 ± 2.4	4.8 ± 2.8	5.6 ± 2.0	3.2 ± 1.4	3.3 ± 1.4	3.2 ± 1.5
$T_{½}$ (h)	NA	NA	NA	66.1 ± 24.6	56.1 ± 19.9	76.1 ± 25.5*
Vd/F (L/kg)	NA	NA	NA	6.0 ± 1.6	5.1 ± 1.0	7.0 ± 1.4*
Cl/F (mL/h/kg)	NA	NA	NA	68.1 ± 21.4	69.1 ± 25.6	67.1 ± 17.5
DEHYDRO-ARIPIPRAZOLE						
AUC (ng·h/mL)	77.4 ± 43.9	90.3 ± 56.9	64.5 ± 20.6*	5149.8 ± 1628.6	4721.3 ± 1670.3	5578.3 ± 1534.8
C_{max} (ng/mL)	5.4 ± 8.5	6.5 ± 5.3	4.3 ± 1.2	34.9 ± 8.5	35.6 ± 9.6	34.1 ± 7.4
T_{max} (h)	21.9 ± 4.5	20.9 ± 5.4	22.9 ± 3.4	6.1 ± 4.4	7.1 ± 3.9	5.1 ± 4.8
$T_{½}$ (h)	NA	NA	NA	107.3 ± 62.5	89.4 ± 45.4	126.9 ± 74.2
Vd/F (L/kg)	NA	NA	NA	40.0 ± 44.0	22.4 ± 10.3	57.6 ± 57.1*
Cl/F (mL/h/kg)	NA	NA	NA	203.5 ± 51.0	181.3 ± 47.3	230.7 ± 44.2*
ARIPIPRAZOLE + DEHYDRO-ARIPIPRAZOLE						
AUC (ng·h/mL)	NA	NA	NA	14596.1 ± 6639.1	11883.5 ± 4788.9	17308.6 ± 7292.2
C_{max} (ng/mL)	NA	NA	NA	172.82 ± 48.74	165.27 ± 53.98	180.38 ± 43.93
AUC ratio	NA	NA	NA	0.64 ± 0.25	0.74 ± 0.27	0.54 ± 0.20*
C_{max} ratio	NA	NA	NA	0.27 ± 0.08	0.29 ± 0.07	0.25 ± 0.09

	SINGLE DOSE			MULTIPLE DOSE		
	All	**Males**	**Females**	**All**	**Males**	**Females**
OLANZAPINE						
AUC (ng·h/mL)#	127.6 ± 33.1	127.8 ± 38.6	127.4 ± 28.4	1289.5 ± 370.1	1142.7 ± 291.2	1436.2 ± 393.1
C_{max} (ng/mL)	7.9 ± 2.2	7.5 ± 2.0	8.2 ± 2.5	19.1 ± 4.8	18.4 ± 4.0	19.9 ± 5.5
T_{max} (h)	5.3 ± 2.3	5.4 ± 2.7	5.3 ± 1.9	4.4 ± 1.7	4.6 ± 1.6	4.3 ± 1.9
$T_{½}$ (h)	NA	NA	NA	77.1 ± 28.2	79.5 ± 33.4	74.8 ± 23.1
Vd/F (L/kg)	NA	NA	NA	26.6 ± 15.9	26.1 ± 17.2	27.0 ± 15.3
Cl/F (mL/h/kg)	NA	NA	NA	229.7 ± 54.7	218.9 ± 51.9	240.6 ± 57.5

*$P \leq 0.05$ vs. males after adjusting for weight. NA: not available.
#for single dose administration the 24 h area under the concentration-time curve (AUC_{24h}), while for multiple dose administration area under the curve from zero to infinity (AUC_{inf}) are shown.
Abbreviations: AUC ratio: dehydro-aripiprazole/aripiprazole AUC ratio; C_{max} ratio: dehydro-aripiprazole/ aripiprazole C_{max} ratio.

Univariate and multivariate analyses revealed associations between pharmacokinetic parameters of aripiprazole, dehydro-aripiprazole and olanzapine and several polymorphisms (***Tables* 25**, **26** and **27**). Additionally, results of the multivariate analysis are shown in ***Table* 28**. The effect of each polymorphism included in the study on pharmacokinetics was analysed, however, due to the large number of variables, only significant results are shown in the tables.

Aripiprazole

Cl/F was notably higher in CYP2D6 UMs than in NMs and IMs (p = 0.016 and p = 0.016, respectively). Moreover, although it did not reach the significant level, AUC, C_{max} and $T_{1/2}$ were lower in UMs compared to NMs and IMs. Additionally, AUC_{inf} and C_{max} on days 1 and 5 were significantly higher in CYP1A2 UMs than in NMs and RMs (p = 0.034, p = 0.040 and p = 0.012, respectively). Surprisingly, Cl/F was lower in CYP1A2 UMs compared to the other phenotypes (p = 0.033). Additionally, T_{max} was higher in *ABCB1* rs1045642 TT compared to CC subjects (p = 0.033). *ABCB1* rs4148737 C/C subjects had 2-times higher $T_{1/2}$ and T_{max} than T carriers (p = 0.024 and p = 0.004, respectively). Results are shown in ***Table* 25**. Several of these associations were

confirmed in the multivariate analysis: the effects of CYP2D6 phenotype on AUC, $T_{1/2}$, Cl/F and Vd/F; CYP1A2 phenotype on AUC, C_{max}, $T_{1/2}$ and Cl/F; *ABCB1* rs1045642 on T_{max} and *ABCB1* rs4148737 on AUC, T_{max} and $T_{1/2}$ (***Table* 28**).

***Table* 25**. Influence of genetic polymorphisms on pharmacokinetic parameters of aripiprazole.

Variable	AUC (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	Cl/F (L/h·kg)	Vd/F (L/kg)
CYP2D6 phenotype						
IM (n = 6)	89464 ± 34240	1025 ± 244	3.33 ± 1.37	72.4 ± 20.2	57.2 ± 11.4	5.89 ± 1.80
NM (n = 16)	79899 ± 59385	1006 ± 409	3.19 ± 1.52	67.3 ± 25.8	67.1 ± 18.5	6.11 ± 1.61
UM (n = 2)	24219 ± 15580	638 ± 52	3.00 ± 0.00	37.8 ± 8.2	108.5 ± 28.4*	5.74 ± 0.27
CYP1A2 phenotype						
NM/RM (n = 17)	63090 ± 35434	864 ± 219	3.12 ± 1.37	60.5 ± 18.5	74.0 ± 22.1	6.07 ± 1.51
UM (n = 7)	113009 ± 74372*	1261 ± 503*	3.43 ± 1.51	79.7 ± 33.2	53.8 ± 11.0*	5.93 ± 1.77
***ABCB1* rs1045642**						
C/C (n = 7)	63819 ± 44267	970 ± 296	3.14 ± 1.35	56.5 ± 23.2	67.9 ± 22.1	5.11 ± 1.52
C/T (n = 12)	85227 ± 64768	1020 ± 463	3.00 ± 1.54	73.2 ± 27.0	67.6 ± 16.9	6.75 ± 1.57
T/T (n = 5)	78827 ± 37731	897 ± 177	3.82 ± 1.12*	62.6 ± 18.6	69.5 ± 33.5	5.59 ± 0.57
***ABCB1* rs4148737**						
T/T (n = 9)	75711 ± 25536	861 ± 150	3.68 ± 1.33	69.0 ± 13.2	69.1 ± 16.5	6.74 ± 1.57
C/T (n = 12)	66727 ± 38774	1008 ± 437	2.67 ± 1.23	59.6 ± 20.7	70.8 ± 24.9	5.60 ± 1.71
C/C (n = 3)	127160 ± 132397	1226 ± 481	4.00 ± 1.73#	83.5 ± 56.3*	54.3 ± 21.4	6.03 ± 1.55

Values are presented as mean ± SD.
#$P \leq 0.05$ compared to the wild-type homozygote genotype.
*$P \leq 0.05$ compared to the other genotypes.

Dehydro-aripiprazole

C_{max} was significantly lower in CYP2D6 IM subjects than in NMs and UMs ($p = 0.006$). CYP3A4 PMs had higher AUC and C_{max} than IMs and EMs ($p = 0.001$ and $p = 0.001$, respectively). Additionally, Vd/F was higher in CYP1A2 UMs than in NMs and RMs ($p = 0.046$). Additionally, T_{max} was higher in *ABCB1* rs1045642 T/T and lower in *ABCB1* rs4148737 C/C subjects compared to the other genotypes ($p = 0.019$ and $p = 0.045$, respectively). Results are shown in ***Table* 26**. Several of these associations were confirmed in the multivariate analysis: the effects of CYP2D6 phenotype on C_{max}; CYP3A phenotype on AUC, C_{max} and T_{max}; CYP1A2 phenotype on C_{max} and Vd/F; *ABCB1* rs1045642 on T_{max} and *ABCB1* rs4148737 on T_{max} and Vd/F (***Table* 28**).

***Table* 26**. Influence of genetic polymorphisms on pharmacokinetic parameters of dehydro-aripiprazole.

Variable	AUC (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	Cl/F (L/h·kg)	Vd/F (L/kg)
CYP2D6 phenotype						
IM (n = 6)	32841 ± 10533	222.3 ± 56.1*	9.17 ± 4.49	120.6 ± 46.6	220.7 ± 50.5	66.8 ± 72.0
NM (n = 16)	39410 ± 13915	258.5 ± 83.9	5.03 ± 3.54	110.2 ± 68.2	202.9 ± 54.8	33.3 ± 28.5
UM (n = 2)	26862 ± 11426	248.5 ± 35.4	5.71 ± 8.90	51.1 ± 4.3	186.5 ± 28.2	13.7 ± 0.93
CYP3A phenotype						
EM (n = 4)	35366 ± 13389	239.0 ± 32.4	8.50 ± 4.04*	107.3 ± 58.6	195.0 ± 18.4	29.5 ± 14.2
IM (n = 17)	36871 ± 12796	235.1 ± 61.1	5.52 ± 4.42	111.0 ± 67.1	215.6 ± 52.3	35.5 ± 28.2
PM (n = 3)	37689 ± 20275*	337.7 ± 138.2*	6.33 ± 5.13	76.4 ± 37.4	166.0 ± 67.7	79.7 ± 113.2
CYP1A2 phenotype						
NM/RM (n = 17)	40899 ± 17468	268.2 ± 149.0	8.24 ± 0.63	116.4 ± 48.1	235.4 ± 116.4	35.7 ± 8.4
UM (n = 7)	55487 ± 17242	371.3 ± 181.6	10.48 ± 2.00	245.4 ± 72.5	249.0 ± 138.5	137.2 ± 16.8*
***ABCB1* rs1045642**						
C/C (n = 7)	35269 ± 13074	276.5 ± 66.3	6.01 ± 3.16	88.2 ± 49.7	181.6 ± 50.9	23.0 ± 13.4
C/T (n = 12)	36962 ± 12673	247.2 ± 84.1	4.32 ± 4.31	117.9 ± 77.1	215.1 ± 49.7	52.3 ± 59.1
T/T (n = 5)	38181 ± 17115	212.9 ± 55.3	10.60 ± 3.13*	110.9 ± 43.9	218.3 ± 55.1	34.5 ± 15.9
***ABCB1* rs4148737**						
T/T (n = 9)	35175 ± 13374	215.0 ± 45.8	6.20 ± 5.74	109.2 ± 33.7	226.8 ± 47.1	54.8 ± 59.5
C/T (n = 12)	38045 ± 14819	275.2 ± 88.9	6.51 ± 3.87	91.8 ± 42.7	189.9 ± 50.8	24.4 ± 10.8
C/C (n = 3)	36072 ± 7064	242.8 ± 53.7	4.33 ± 1.15*	164.4 ± 150.2	208.1 ± 61.5	58.1 ± 66.9

Values are presented as mean ± SD.
#$P \leq 0.05$ compared to the wild-type homozygote genotype.
*$P \leq 0.05$ compared to the other genotypes.

Olanzapine

No relationship was found with CYP2D6 or CYP1A2 phenotypes and olanzapine. $T_{1/2}$ was 2 times higher in CYP3A EM subjects compared to IMs and PMs (p = 0.025). Additionally, *ABCB1* rs10280101 A/A, rs12720067 C/C and rs11983225 T/T subjects had significantly higher $T_{1/2}$ compared to the other genotypes (p = 0.046, p = 0.046 and p = 0.046 respectively). Finally, *UGT1A1* rs887829 T/T homozygotes had higher T_{max} than C/T heterozygotes and C/C homozygotes (p = 0.016). The results are shown in ***Table* 27**. Several of these associations were confirmed in the multivariate analysis: the effects of CYP3A phenotype, *ABCB1* rs10280101, rs12720067 and rs11983225 on $T_{1/2}$ and *UGT1A1* rs887829 on T_{max} (***Table* 28**).

***Table* 27**. Influence of genetic polymorphisms on pharmacokinetic parameters of olanzapine.

Variable	AUC (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	Cl/F (L/h·kg)	Vd/F (L/kg)
CYP2D6 phenotype						
IM (n = 6)	17583 ± 2541	13.5 ± 2.0	4.56 ± 1.24	73.8 ± 16.0	223.5 ± 27.0	23.5 ± 4.1
NM (n = 16)	18741 ± 7099	14.0 ± 3.4	4.31 ± 1.66	74.5 ± 24.4	229.1 ± 59.5	25.4 ± 14.1
UM (n = 2)	17073 ± 5134	16.7 ± 11.4	5.00 ± 4.24	108.6 ± 76.9	253.6 ± 104.0	45.5 ± 44.4
CYP1A2 phenotype						
NM/RM (n = 17)	16924 ± 4990	13.9 ± 4.1	4.37 ± 1.63	77.2 ± 32.6	237.4 ± 57.8	27.9 ± 18.4
UM (n = 7)	21684 ± 7224	14.6 ± 3.4	4.58 ± 2.06	76.9 ± 14.9	211.2 ± 44.8	23.4 ± 7.0
CYP3A phenotype						
EM (n = 4)	15877 ± 3836	10.6 ± 1.4	5.00 ± 2.45	109.7 ± 41.2	265.0 ± 59.7	42.7 ± 23.0
IM (n = 17)	19186 ± 6586	14.8 ± 4.0	4.55 ± 1.51	72.3 ± 22.0	222.0 ± 55.7	24.0 ± 13.5
PM (n = 3)	16613 ± 4492	15.0 ± 3.1	3.01 ± 1.72	60.9 ± 3.3*	226.8 ± 35.0	19.8 ± 2.0
***ABCB1* rs10280101**						
A/A (n = 16)	18614 ± 5930	13.3 ± 3.2	4.83 ± 1.72	85.2 ± 31.4	231.5 ± 56.3	29.9 ± 18.4
C+ (n = 8)	17711 ± 6459	15.6 ± 4.9	3.63 ± 1.50	61.1 ± 7.4*	226.1 ± 55.1	20.0 ± 5.7
***ABCB1* rs12720067**						
C/C (n = 16)	18614 ± 5930	13.3 ± 3.2	4.83 ± 1.72	85.2 ± 31.4	231.5 ± 56.3	29.9 ± 18.4
T+ (n = 8)	17711 ± 6459	15.6 ± 4.9	3.63 ± 1.50	61.1 ± 7.4*	226.1 ± 55.1	20.0 ± 5.7
***ABCB1* rs11983225**						
T/T (n = 16)	18614 ± 5930	13.3 ± 3.2	4.83 ± 1.72	85.2 ± 31.4	231.5 ± 56.3	29.9 ± 18.4
T+ (n = 8)	17711 ± 6459	15.6 ± 4.9	3.63 ± 1.50	61.1 ± 7.4*	226.1 ± 55.1	20.0 ± 5.7
***UGT1A1* rs887829**						
C/C (n = 8)	17978 ± 7920	15.5 ± 3.5	3.54 ± 1.64	73.1 ± 27.7	232.1 ± 63.1	25.9 ± 18.4
C/T (n = 11)	18401 ± 5658	13.9 ± 4.5	4.28 ± 1.27	68.3 ± 19.4	221.2 ± 52.6	21.8 ± 7.5
T/T (n = 5)	18655 ± 3967	12.3 ± 2.7	6.20 ± 1.64*	103.0 ± 34.8	244.7 ± 53.3	38.1 ± 22.2

Values are presented as mean ± SD.

#$P \leq 0.05$ compared to the wild-type homozygote genotype.

*$P \leq 0.05$ compared to the other genotypes.

***Table* 28**. Influence of genetic polymorphisms on pharmacokinetic parameters of aripiprazole, dehydro-aripiprazole and olanzapine in the multivariate analysis.

Variable	AUC (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	Cl/F (L/h·kg)	Vd/F (L/kg)
Aripiprazole						
CYP2D6 phenotype	**β=-1063.9; *p*=0.049**	---	---	**β=16.2; *p*=0.005**	β=15.1; *p*=0.055	**β=1.9; *p*=0.001**
CYP1A2 phenotype	**β=64555; *p*=0.035**	**β=421; *p*=0.044**	---	**β=29.3; *p*=0.041**	β=-22.1; *p*=0.054	---
***ABCB1* rs1045642**	---	---	β=0.183; *p*=0.738	---	---	---
***ABCB1* rs4148737**	**β=5894; *p*=0.026**	---	β=0.183; *p*=0.545	**β=-17.5; *p*=0.027**	---	---
Dehydro-aripiprazole						
CYP2D6 phenotype	---	**β=17.3; *p*=0.043**	---	---	---	---
CYP3A4 phenotype	β=4257; *p*=0.273	**β=55.2; *p*=0.005**	**β=3.14; *p*=0.015**	---	---	---
CYP1A2 phenotype	---	**β=70.5; *p*=0.039**	---	---	---	β=10.2; *p*=0.387
***ABCB1* rs1045642**	---	---	**β=2.93; *p*=0.021**	---	---	---
***ABCB1* rs4148737**	---	---	β=-1.99; *p*=0.240	---	---	**β=17.1; *p*=0.041**
Olanzapine						
CYP3A4 phenotype	---	---	---	**β=-17; *p*=0.037**	---	---
***ABCB1* rs10280101**	---	---	---	β=-26.9; *p*=0.145	---	---
***ABCB1* rs12720067**	---	---	---	β=-26.9; *p*=0.145	---	---
***ABCB1* rs11983225**	---	---	---	β=-26.9; *p*=0.145	---	---
***UGT1A1* rs887829**	---	---	**β=1.58; *p*=0.006**	---	---	---

Results with $p \leq 0.05$ are highlighted in bold.

5. Pupillometry

The main results of this chapter were published in:

Koller D, Saiz-Rodríguez M, Zubiaur P, Ochoa D, Almenara S, Román M, Romero-Palacián D, de Miguel-Cáceres A, Martín S, Navares-Gómez M, Mejía G, Wojnicz A, Abad-Santos F.

The effects of aripiprazole and olanzapine on pupillary light reflex and its relationship with pharmacogenetics in a randomized multiple-dose trial.

Br J Clin Pharmacol. 2020 Apr 6. doi: 10.1111/bcp.14300. PubMed PMID: 32250470.

5.1. The effects of aripiprazole and olanzapine on pupillary light reflex

Following the first oral administration of aripiprazole, the pupil was significantly dilated. Subsequently, on the next four drug administration days it caused minor constriction. All pupillometric parameters changed significantly (MAX: $p = 0.008$; MIN: $p = 0.009$; CON: $p = 0.013$; LAT: $p = 0.009$; ACV: $p = 0.012$; MCV: $p = 0.006$; ADV: $p = 0.024$; T75: $p = 0.015$) (***Figure* 27**). Olanzapine showed the same tendency, but only MIN reached the statistically significant level ($p = 0.046$) (***Figure* 27**). No differences were found between males and females in any pupillometric parameters.

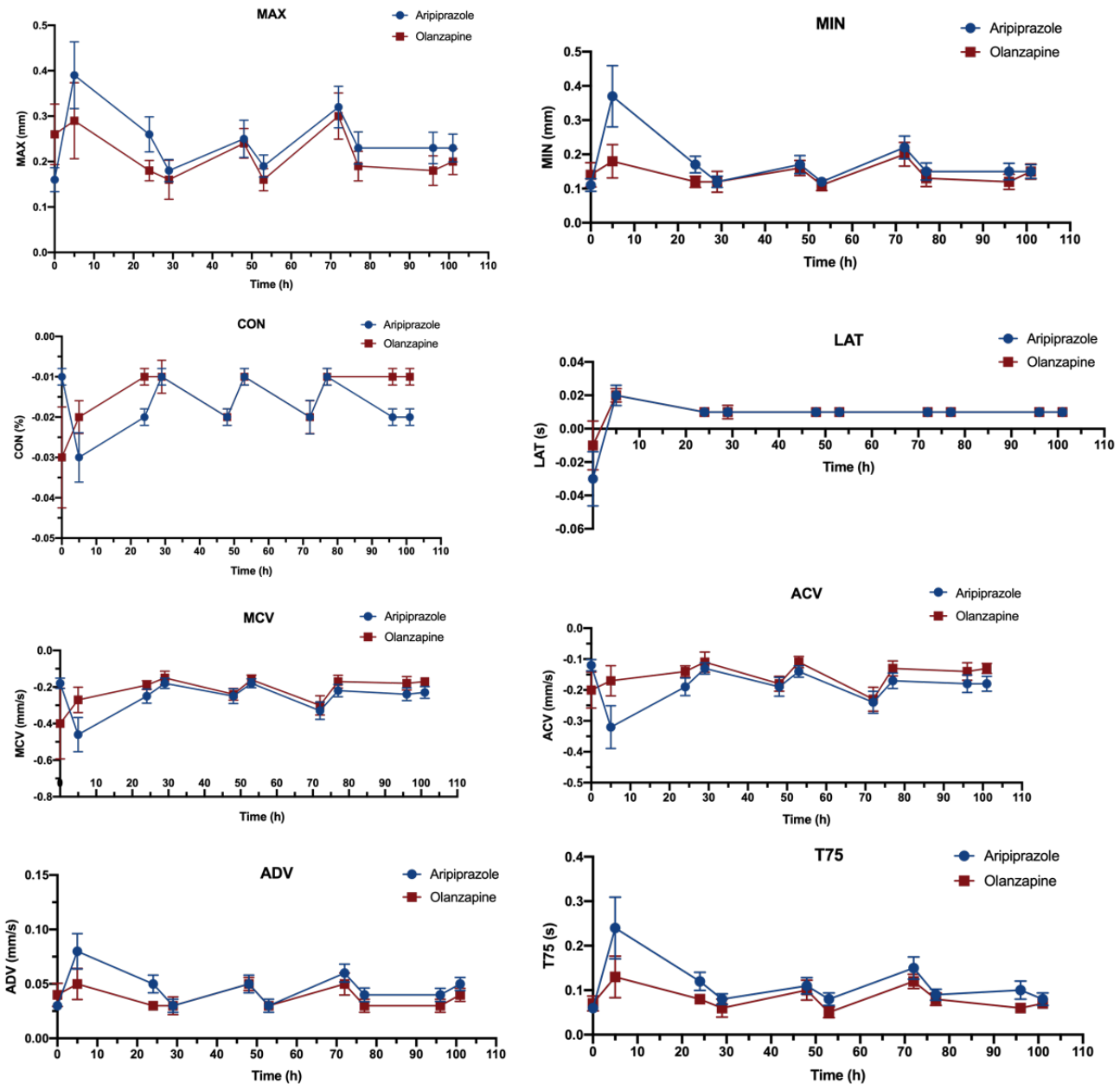

***Figure* 27.** Changes in pupillometric parameters after aripiprazole and olanzapine administration.

The figure shows original mean values ± SE corrected with light intensity in lux.
Abbreviations: MAX: maximum pupil diameter; MIN: minimum pupil diameter; CON: percent of constriction; LAT: latency; ACV: average constriction velocity; MCV: maximum constriction velocity; ADV: average dilation velocity; T75: total time taken by the pupil to recover 75% of the initial resting pupil size.

5.2. The influence of pharmacokinetics on pupillary light reflex

5.2.1. Single dose administration

AUC_{last} and T_{max} of aripiprazole had an influence on CON ($p = 0.029$ and $p = 0.043$, respectively). Moreover, AUC_{last} had an impact on MCV and ADV ($p = 0.004$ and $p = 0.034$, respectively) (***Table* 29**).

Furthermore, AUC_{24h} of dehydro-aripiprazole had an impact on MAX ($p = 0.042$), MIN ($p = 0.050$), CON ($p = 0.047$), ACV ($p = 0.049$) and MCV ($p = 0.046$) (***Table* 29**).

These associations were not confirmed in the multivariate analysis ($p > 0.05$). Additionally, olanzapine pharmacokinetics did not have an effect on any of the pupillometric parameters.

5.2.2. Multiple dose administration

C_{max} of dehydro-aripiprazole had an impact on several pupillometric parameters: MAX ($p = 0.029$), MIN ($p = 0.049$), CON ($p = 0.015$), ACV ($p = 0.041$), MCV ($p = 0.027$), ADV ($p = 0.033$) and T75 ($p = 0.045$) (***Table* 29**). These associations were not confirmed in the multivariate analysis ($p > 0.05$).

Aripiprazole and olanzapine pharmacokinetics did not have any association with any of the pupillometric parameters.

***Table* 29**. Influence of the pharmacokinetic parameters of aripiprazole and dehydro-aripiprazole on pupillometric parameters.

	Aripiprazole		Dehydro-aripiprazole	
	Single dose		Single dose	Multiple dose
Variable	**AUC_{last} (ng·h/mL)**	**T_{max} (h)**	**AUC_{24h} (ng·h/mL)**	**C_{max} (ng/mL)**
MAX	---	---	$p = 0.042$ ↓↓	$p = 0.029$ ↓↓
MIN	---	---	$p = 0.050$ ↓↓	$p = 0.049$ ↓↓
CON	$p = 0.029$ ↓↑	$p = 0.043$ ↓↓	$p = 0.047$ ↓↓	$p = 0.015$ ↓↓
ACV	---	---	$p = 0.049$ ↓↓	$p = 0.041$ ↓↓
MCV	$p = 0.004$ ↓↑	---	$p = 0.046$ ↓↑	$p = 0.027$ ↓↑
ADV	$p = 0.034$ ↓↓	---	---	$p = 0.033$ ↓↓
T75	---	---	---	$p = 0.045$ ↓↓

The arrows show the relationship between pharmacokinetic and pupillometric parameters. The first arrow refers to the pharmacokinetic parameter, while the second arrow refers to the pupillometric parameter. ↓↑ is indirectly proportional, while ↓↓ is directly proportional with the changes.

5.3. The influence of polymorphisms on pupillary light reflex

Aripiprazole

Subjects with the CYP3A IM phenotype had significantly higher MAX levels than PMs (p = 0.019). Moreover, *HTR2A* rs6314 T carriers and *UGT1A1* rs8877829 T/T homozygotes had higher MIN levels than C/C subjects (p = 0.025 and p = 0.039, respectively). Additionally, subjects with CYP3A PM phenotype and *DRD2* rs1800487 A2 carriers had higher CON values than with IM phenotype and A1 carriers, respectively, however, only CYP3A reached the significant level (p = 0.008 and p = 0.058, respectively).

Likewise, CYP3A IM and EM subjects, *DRD2* rs1800487 A2 carriers, *ABCB1* rs10280101 A/A, rs12720067 C/C and rs11983225 T/T subjects had higher LAT values than CYP3A PM subjects, *DRD2* rs1800487 A1 carriers and *ABCB1* 10280101 C, *ABCB1* rs12720067 T and *ABCB1* rs19983225 C carriers, respectively (p = 0.020, p = 0.039 and p = 0.034, respectively). Moreover, CYP3A PM subjects had lower ACV and MCV values than IM subjects (p = 0.028 and p = 0.022, respectively). Finally, *HTR2A* rs6314 T allele carriers had higher T75 levels than C/C homozygotes, although it did not reach the statistically significant level (p = 0.058).

After performing the multivariate tests, the influence of *HTR2A* rs6314 on MIN and T75 remained significant ($p = 0.001$ and $p = 0.020$, respectively) (***Figure* 28**).

Olanzapine

DRD2 rs1800497 A2 allele carriers had higher MAX, ACV and MCV values than A1/A1 homozygotes ($p = 0.025$, $p = 0.043$ and $p = 0.038$).

After performing the multivariate tests, the influence of *DRD2* rs1800497 on MAX remained significant ($p = 0.039$) (***Figure* 28**).

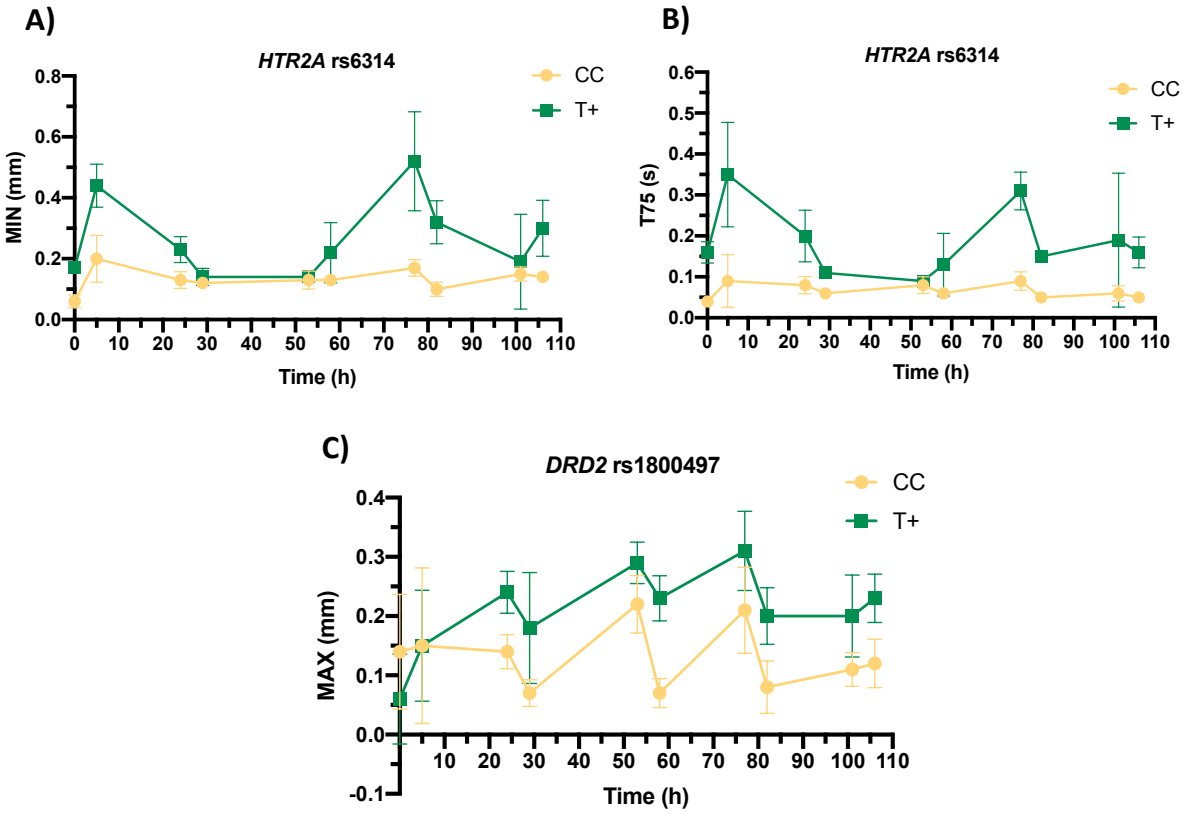

***Figure* 28**. The influence of *HTR2A* rs6314 and *DRD2* rs1800497 polymorphisms on pupillometric parameters.

A: The influence of *HTR2A* rs6314 on MIN during aripiprazole treatment. **B**: The influence of *HTR2A* rs6314 on T75 during aripiprazole treatment. **C**: The influence of *DRD2* rs1800497 on MAX during olanzapine treatment.
The figure shows original mean values ± SE corrected with light intensity *in lux*.

6. Metabolism

In this section, due to the high number of analysed variables, results are shown from the multivariate analysis.

6.1. Prolactin

6.1.1. The effects of aripiprazole and olanzapine on prolactin concentrations

Olanzapine caused a significant elevation in prolactin levels ($p < 0.001$, $\eta_p^2 = 0.474$) (***Figure* 29**). Males had lower prolactin levels than females, however, the extent of the increment did not differ between them. Additionally, a significant interaction was found between C_{max} of olanzapine and prolactin levels ($p = 0.006$, $\eta_p^2 = 0.168$). Compared to olanzapine, aripiprazole did not elevate prolactin levels. On the contrary, a tendency of decrease was observed but it did not reach the significant level ($p = 0.052$) (***Figure* 29**).

Prolactin levels were significantly higher during olanzapine treatment compared to aripiprazole ($p < 0.0001$, $\eta_p^2 = 0.356$). Prolactin levels were outside of the recommended range (males: 2-18 ng/mL; females: 3-30 ng/mL) during olanzapine treatment in 9 (75%) males and 9 (75%) females [237]. None of the volunteers showed prolactin levels outside of the normal range during aripiprazole treatment.

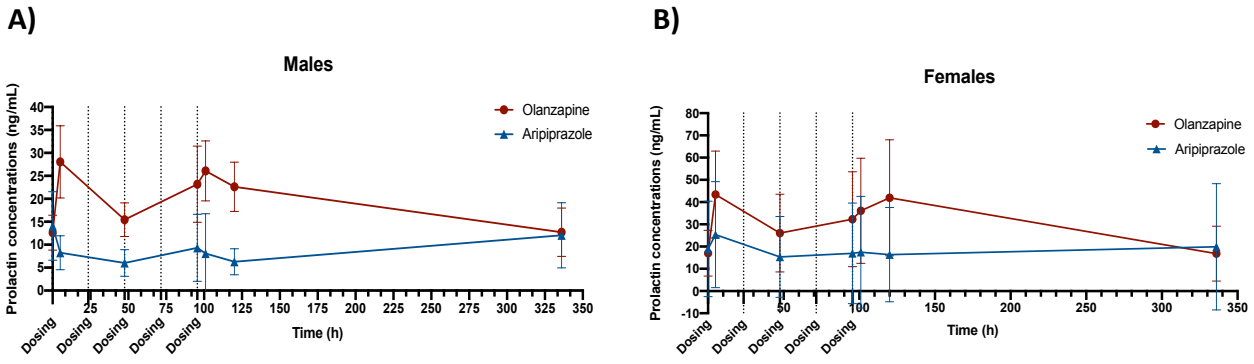

***Figure* 29**. Prolactin concentrations during the administration of 5 daily doses of aripiprazole 10 mg and olanzapine 5 mg tablets in males (A) and females (B).

Results are shown as mean ± SD.

6.1.2. The influence of polymorphisms on prolactin concentrations

Volunteers carrying the *DRD3* Gly carriers had significantly higher prolactin concentrations during olanzapine treatment than volunteers with Ser/Ser genotype (p = 0.036, η_p^2 = 0.121) (***Figure* 30**).

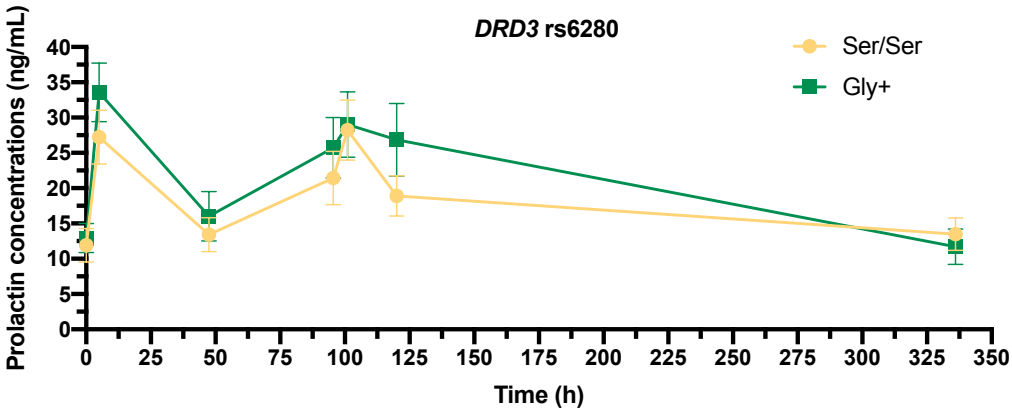

***Figure* 30**. The influence of *DRD3* rs6280 polymorphism on prolactin concentrations.

The figure shows original mean values ± SE.

CYP3A PMs had higher prolactin concentrations during aripiprazole treatment compared to IMs and EMs (p = 0.001, η_p^2 = 0.226). *ABCB1* rs10280101 A/A, rs12720067 C/C and rs11983225 T/T subjects had significantly higher prolactin concentrations compared to C, T and C allele carriers (p = 0.037, η_p^2 = 0.123) (***Figure* 31**). However, when analysing *ABCB1* haplotypes, this association could not be detected.

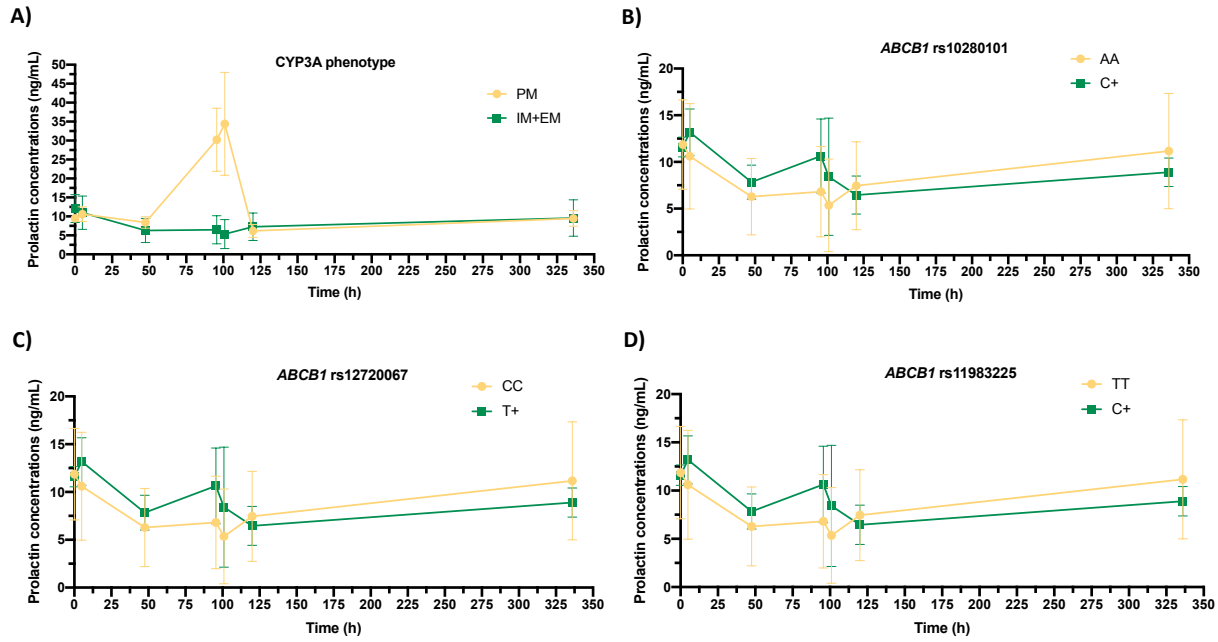

***Figure* 31**. The influence of CYP3A phenotype and ABCB1 rs10280101, rs12720067 and rs11983225 polymorphisms on prolactin concentrations during aripiprazole treatment.

The figure shows original mean values ± SE corrected with light intensity *in lux*.
A: The influence of CYP3A phenotype on prolactin concentrations. **B**: The influence of *ABCB1* rs10280101 polymorphism on prolactin concentrations. **C**: The influence of *ABCB1* rs12720067 polymorphism on prolactin concentrations. **D**: The influence of *ABCB1* rs11983225 polymorphism on prolactin concentrations.

The same genetic associations were found in males and females.

6.2. Glucose metabolism and its relationship with pharmacogenetics

6.2.1. The effects of aripiprazole and olanzapine on glucose metabolism

C-peptide concentrations were significantly higher after completing aripiprazole treatment compared to its initial levels ($p = 0.030$, $\eta_p^2 = 0.205$) (***Table* 25**). Likewise, AUC_{last} of dehydro-aripiprazole was indirectly proportional with the changes in insulin levels ($p = 0.045$, $\eta_p^2 = 0.228$).

After completing olanzapine treatment, the 1 h and 2 h glucose levels after performing GTT were higher compared to the measurements on the first day ($p = 0.007$, $\eta p2 = 0.213$) (***Table* 30**). Moreover, olanzapine administration increased the C-peptide/insulin ratio ($p = 0.044$, $\eta_p^2 = 0.196$).

On first day's GTT test, glucose levels were higher in olanzapine-treated subjects compared to aripiprazole (p = 0.011, ηp2 = 0.131), however, ARI showed the same tendency. However, the increment in glucose levels after GTT and the increment of C-peptide levels did not differ between aripiprazole and olanzapine. No changes were detected in HbA1c (***Table* 30**).

No differences were found between males and females nor in C-peptide, insulin, glucose and GTT levels neither in the genetic associations. No levels were outside of the normal range.

***Table* 30**. C-peptide, insulin, haemoglobin A1c and glucose levels during multiple dose treatment with aripiprazole and olanzapine.

ARIPIPRAZOLE						
	Day 1			**Day 6**		
C-peptide (ng/mL)*	1.60 ± 0.35			3.50 ± 3.85		
Insulin (mcU/mL)	7.84 ± 2.37			9.61 ± 4.37		
C-peptide/insulin ratio	0.22 ± 0.06			0.24 ± 0.09		
HbA1c (%)	5.30 ± 0.22			5.28 ± 0.21		
	Screening		**Day 3**	**Day 6**		**Day 15**
Glucose (mg/dL)	80.33 ± 6.64		79.71 ± 6.49	80.87 ± 9.01		79.17 ± 5.84
	Day 1			**Day 6**		
	0 h	**60 min**	**120 min**	**0 h**	**60 min**	**120 min**
GTT (mg/dL)	81.79 ± 7.79	103.38 ± 35.63	76.00 ± 13.40	84.54 ± 8.82	122.04 ± 31.80	97.38 ± 21.56
OLANZAPINE						
	Day 1			**Day 6**		
C-peptide (ng/mL)	1.69 ± 0.51			2.60 ± 2.29		
Insulin (mcU/mL)	8.19 ± 2.31			10.40 ± 12.76		
C-peptide/insulin ratio*	0.21 ± 0.03			0.25 ± 0.08		
HbA1c (%)	5.36 ± 0.23			5.07 ± 1.09		
	Screening		**Day 3**	**Day 6**		**Day 15**
Glucose (mg/dL)	79.96 ± 7.52		79.38 ± 6.50	79.14 ± 8.05		80.71 ± 6.22
	Day 1			**Day 6**		
	0 h	**60 min**	**120 min**	**0 h**	**60 min**	**120 min**
GTT (mg/dL)*	81.88 ± 6.73	104.08 ± 33.81	89.38 ± 20.98#	79.71 ± 8.65	124.21 ± 38.07	101.96 ± 28.51

*$P \leq 0.05$. #$p \leq 0.05$ compared to aripiprazole. Values are shown as mean ± SD.

6.2.2. The influence of polymorphisms on glucose metabolism

The increase in C-peptide levels was greater in *COMT* rs4680 G/G subjects and rs13306278 T carriers compared to A carriers and C/C homozygotes, respectively during aripiprazole treatment ($p = 0.010$, $\eta_p^2 = 0.289$; $p < 0.001$, $\eta_p^2 = 0.535$, respectively). This association could not be detected when analysing COMT phenotype. Moreover, although insulin levels only tended to increase after aripiprazole administration ($p = 0.073$), *BDNF* rs6265 C/C subjects had greater increment compared to other genotypes ($p = 0.040$, $\eta_p^2 = 0.237$) (***Table* 31**).

***Table* 31**. The influence of genetic polymorphisms on C-peptide and insulin levels during aripiprazole treatment.

ARIPIPRAZOLE		
	Day 1	**Day 6**
	C-peptide (ng/mL)	
***COMT* rs4680**		
G/G	2.05 ± 0.32	6.32 ± 6.27
T+	1.39 ± 0.32	1.93 ± 0.90
***COMT* rs13306278**		
C/C	1.50 ± 0.33	2.10 ± 1.25
T+	2.09 ± 0.53	14.56 ± 7.99
	Insulin (mcU/mL)	
***BDNF* rs6265**		
C/C	7.28 ± 2.42	9.66 ± 4.36
T+	9.13 ± 1.78	9.51 ± 4.76

Values are shown as mean ± SD.

UGT1A1 rs887829 C/C homozygote subjects had significantly higher glucose concentrations in GTT both after 1 h and 2 h than the T allele carriers after olanzapine treatment ($p = 0.014$, $\eta_p^2 = 0.186$). Moreover, this polymorphism was also related to higher increase in glucose levels in C/C subjects compared to the T allele carriers ($p = 0.013$, $\eta_p^2 = 0.258$). Additionally, insulin levels of CYP3A PMs increased more, compared to EMs and IMs during olanzapine administration ($p = 0.029$, $\eta_p^2 = 0.217$) (***Table* 32**).

***Table* 32**. The influence of genetic polymorphisms on insulin and glucose levels during olanzapine treatment.

OLANZAPINE						
Insulin (mcU/mL)						
CYP3A phenotype	**Day 1**			**Day 6**		
PM	7.77 ± 0.81			10.95 ± 4.17		
EM	8.53 ± 3.44			11.10 ± 5.53		
IM	7.69 ± 2.37			8.95 ± 4.25		
Glucose (mg/dL)						
***UGT1A1* rs887829**	**Screening**	**Day 3**		**Day 6**	**Day 15**	
C/C	80.63 ± 7.13	79.88 ± 4.05		85.60 ± 9.10	82.00 ± 5.48	
C/T	79.00 ± 9.00	77.55 ± 6.30		76.50 ± 5.54	78.91 ± 6.30	
T/T	81.00 ± 5.39	82.60 ± 9.61		73.67 ± 2.89	82.60 ± 7.33	
GTT (mg/dL)						
	Day 1			**Day 6**		
***UGT1A1* rs887829**	**0 h**	**60 min**	**120 min**	**0 h**	**60 min**	**120 min**
C/C	82.00 ± 4.87	94.38 ± 30.57	91.13 ± 21.00	83.13 ± 10.92	143.00 ± 31.21	108.25 ± 23.69
C/T	81.27 ± 7.11	109.18 ± 35.89	89.09 ± 21.53	77.91 ± 7.34	117.55 ± 37.75	99.36 ± 27.19
T/T	83.00 ± 9.46	108.40 ± 37.86	87.20 ± 24.25	78.20 ± 7.19	108.80 ± 44.04	97.60 ± 41.49

Values are shown as mean ± SD.

6.3. Weight and lipid metabolism

6.3.1. The effects of aripiprazole and olanzapine on weight and lipid metabolism

During aripiprazole treatment, volunteers' weight decreased significantly ($p < 0.0001$, $\eta_p^2 = 0.301$). On the contrary, a tendency to gain weight was observed during olanzapine treatment, but it did not reach the significant level ($p = 0.120$) (***Figure* 32**). Additionally, a significant difference was found when comparing weight changes between aripiprazole and olanzapine treatment ($p < 0.001$, $\eta_p^2 = 0.301$).

Triglyceride levels linearly decreased due to aripiprazole and olanzapine administration ($p = 0.009$, $\eta_p^2 = 0.177$; $p = 0.047$, $\eta_p^2 = 0.125$, respectively) (***Figure* 32**). No significant difference was found in the extent of this decrease between aripiprazole and olanzapine ($p = 0.593$). Additionally, C_{max} of aripiprazole, C_{max} of dehydro-aripiprazole and C_{max} of their sum were inversely proportional to triglyceride levels ($p = 0.003$, $\eta_p^2 = 0.203$; $p < 0.001$, $\eta_p^2 = 0.327$; $p < 0.001$, $\eta_p^2 = 0.258$, respectively).

Likewise, total cholesterol levels diminished significantly during aripiprazole and olanzapine treatment ($p = 0.002$, $\eta_p^2 = 0.250$; $p = 0.004$, $\eta_p^2 = 0.209$, respectively) (***Figure* 32**). No significant difference was found between aripiprazole and olanzapine in the extent of this reduction ($p = 0.241$).

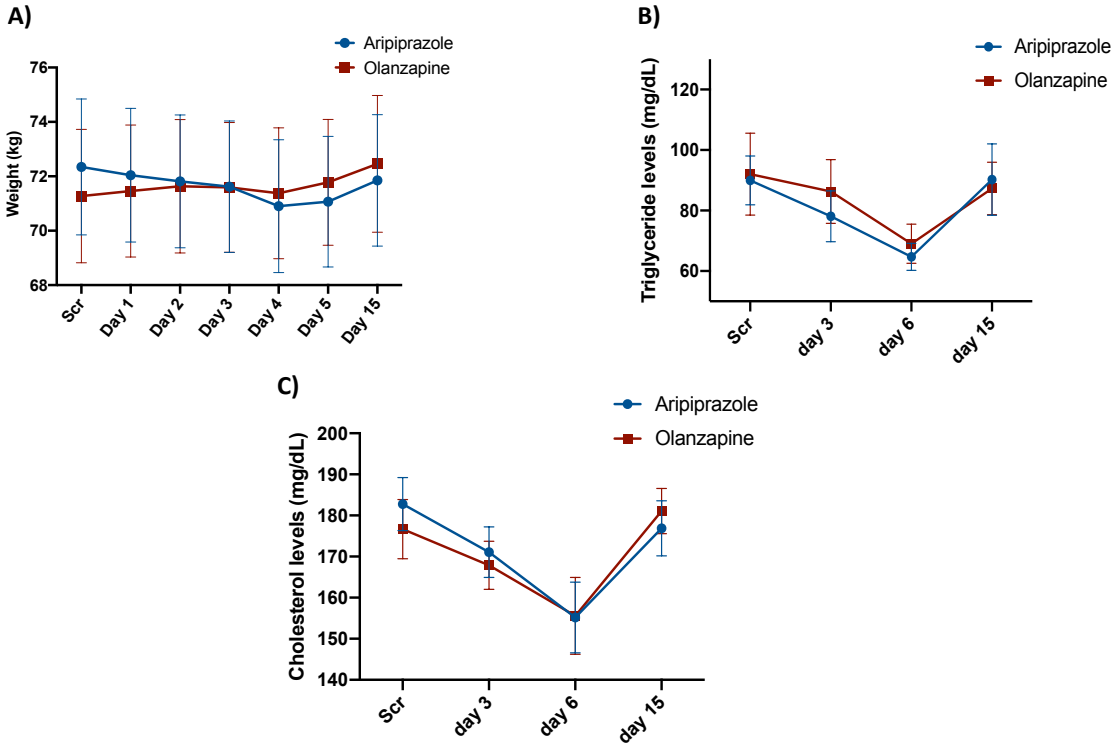

***Figure* 32**. Weight (A), triglyceride (B) and cholesterol (C) concentrations during aripiprazole and olanzapine treatment.

Values are shown as mean ± SD.

No differences were found between males and females weight changes and triglyceride and cholesterol levels. No levels were outside of the normal range.

6.3.2. The influence of polymorphisms on weight and lipid metabolism

CYP3A PMs had significantly greater decrease in triglyceride levels during aripiprazole treatment compared to the other phenotypes ($p < 0.001$, $\eta_p^2 = 0.296$). Moreover, *HTR2A* rs6314 T allele carriers had lower cholesterol concentrations during aripiprazole treatment compared to C/C subjects ($p = 0.037$, $\eta_p^2 = 0.141$) (***Figure* 33**).

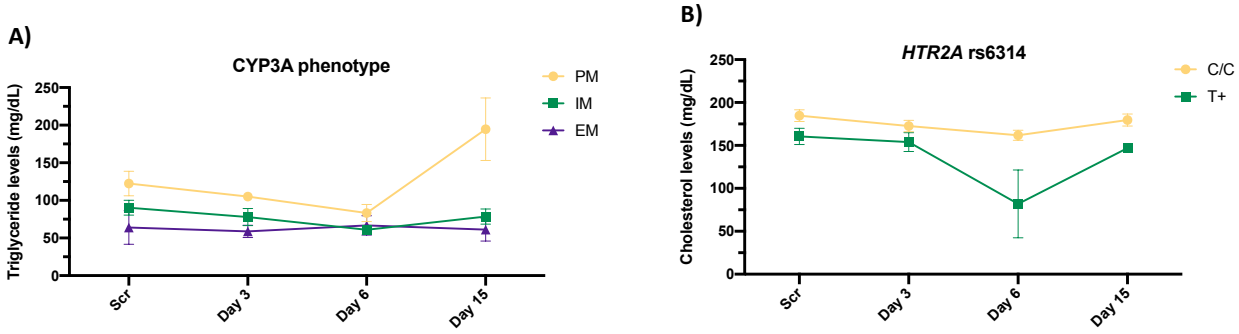

***Figure* 33.** The influence of CYP3A phenotype (A) and HTR2C rs6314 polymorphism (B) on triglyceride and cholesterol levels during aripiprazole treatment.

The figure shows original mean values ± SE.

HTR2C rs1414334 C/C subjects gained significantly more weight compared to T allele carriers during olanzapine treatment ($p = 0.002$; $\eta_p^2 = 0.196$). Furthermore, *APOC3* rs4520 C/C homozygotes had lesser decrease in triglyceride concentrations after olanzapine administration than T allele carriers ($p = 0.018$, $\eta_p^2 = 0.162$) (***Figure* 34**).

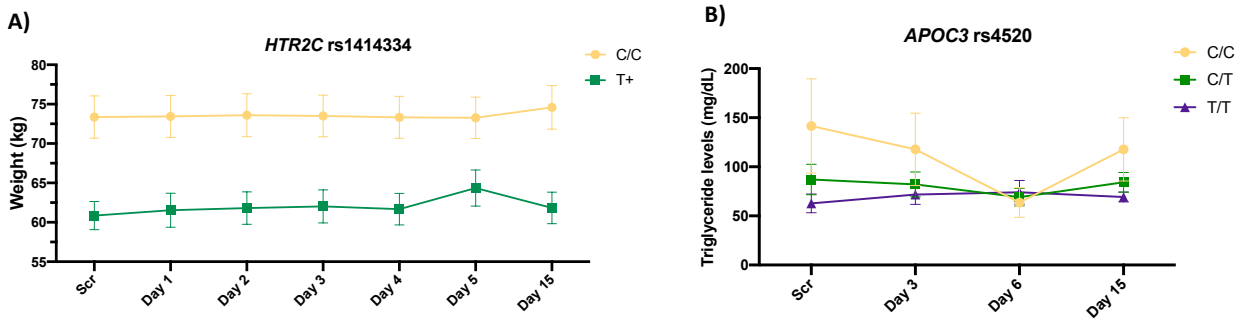

***Figure* 34**. The influence of *HTR2C* rs1414334 on weight (A) and *APOC3* rs4520 polymorphisms on triglyceride levels (B) during olanzapine treatment.

The figure shows original mean values ± SE.

6.4. Hepatic performance

GOT, GPT, GGT, ALP and albumin levels significantly decreased during aripiprazole treatment ($p = 0.001$, $\eta_p^2 = 0.249$; $p = 0.004$, $\eta_p^2 = 0.209$; $p = 0.001$, $\eta_p^2 = 0.224$; $p < 0.001$, $\eta_p^2 = 0.312$; $p < 0.001$, $\eta_p^2 = 0.307$, respectively) (***Table* 33**). Additionally, GGT levels were inversely proportional to C_{max} of dehydro-aripiprazole and sum of aripiprazole and dehydro-aripiprazole ($p = 0.050$, $\eta_p^2 = 0.116$; $p = 0.043$, $\eta_p^2 = 0.121$). Likewise, ALP levels were dependent on C_{max} of aripiprazole and sum of aripiprazole and dehydro-aripiprazole ($p = 0.042$, $\eta_p^2 = 0.121$; $p = 0.048$, $\eta_p^2 = 0.117$).

Olanzapine treatment produced a decline in GGT, bilirubin, ALP and albumin levels ($p < 0.001$, $\eta_p^2 = 0.281$; $p = 0.045$, $\eta_p^2 = 0.123$; $p = 0.007$, $\eta_p^2 = 0.215$; $p = 0.004$, $\eta_p^2 = 0.285$, respectively) (***Table* 33**).

All GOT, GPT, GGT, bilirubin and ALP levels normalized after discontinuing aripiprazole or olanzapine treatment (***Table* 33**).

***Table* 33**. Glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, gamma-glutamyl transferase, bilirubin, alkaline phosphatase and albumin levels during aripiprazole and olanzapine treatment.

ARIPIPRAZOLE				
	Screening	**Day 3**	**Day 6**	**Day 15**
GOT (U/L)*	18.38 ± 4.03	15.58 ± 3.65	16.25 ± 3.34	20.25 ± 7.22
GPT (U/L)*	18.50 ± 7.97	14.02 ± 4.66	15.40 ± 4.98	19.23 ± 9.16
GGT (U/L)*	19.71 ± 7.30	17.92 ± 6.82	16.75 ± 6.17	18.38 ± 6.09
Total bilirubin (mg/dL)	0.56 ± 0.32	0.66 ± 0.36	0.65 ± 0.43	0.68 ± 0.44
ALP (U/L)*	71.16 ± 15.21	65.23 ± 14.90	63.72 ± 12.53	67.21 ± 16.15
Albumin (g/dL)*	4.42 ± 0.32	4.24 ± 0.31	4.28 ± 0.32	4.48 ± 0.24
OLANZAPINE				
	Screening	**Day 3**	**Day 6**	**Day 15**
GOT (U/L)	18.58 ± 5.48	17.67 ± 4.83	18.25 ± 11.56	21.04 ± 7.46
GPT (U/L)	17.33 ± 6.57	18.02 ± 9.96	19.10 ± 12.17	21.56 ± 9.63
GGT (U/L)*	18.88 ± 5.83	18.00 ± 6.78	15.83 ± 6.94	20.29 ± 8.21
Total bilirubin (mg/dL)*	0.69 ± 0.41	0.55 ± 0.34	0.54 ± 0.41	0.58 ± 0.33
ALP (U/L)*	69.26 ± 16.56	64.81 ± 15.21	60.03 ± 19.32	70.86 ± 14.07
Albumin (g/dL)*#	4.38 ± 0.22	4.00 ± 0.26	3.81 ± 0.91	4.38 ± 0.21

*$P \leq 0.05$.
#$P \leq 0.05$ compared to aripiprazole.
Values are shown as mean ± SD.

No differences were found between males and females in GOT, GPT, GGT, ALP, bilirubin and albumin levels. Additionally, the changes in albumin levels differed between aripiprazole and olanzapine treatment (p = 0.009, η_p^2 = 0.183). Changes in the rest of the parameters were not dependent on the treatment (aripiprazole versus olanzapine). No levels were outside of the normal range.

6.5. Haematological performance

The platelet count significantly decreased during aripiprazole treatment ($p < 0.001$, $\eta_p^2 = 0.361$). Additionally, the prothrombin time increased and the prothrombin index decreased over time ($p < 0.001$, $\eta_p^2 = 0.360$; $p < 0.001$, $\eta_p^2 = 0.410$, respectively) (***Table* 34**).

On the contrary, the leucocyte and platelet count decreased during olanzapine treatment ($p = 0.004$, $\eta_p^2 = 0.217$; $p = 0.007$, $\eta_p^2 = 0.199$, respectively). Similarly to aripiprazole, the prothrombin index decreased over time ($p = 0.006$, $\eta_p^2 = 0.237$) (***Table* 34**).

***Table* 34**. Leucocyte, platelet, haemoglobin, red blood cell and haematocrit count and prothrombin time and index during aripiprazole and olanzapine treatment.

ARIPIPRAZOLE				
	Screening	**Day 3**	**Day 6**	**Day 15**
Leucocytes (/µL (10^3))	6.56 ± 2.04	6.35 ± 1.88	6.02 ± 2.31	6.77 ± 1.97
Platelets (/µL (10^3))*	263.08 ± 56.77	251.46 ± 53.48	244.04 ± 73.93	285.38 ± 54.91
Hemoglobin (mg/dL)	13.88 ± 1.65	13.80 ± 1.86	13.02 ± 3.29	13.44 ± 1.83
Red blood cells (/µL (10^6))	4.69 ± 0.56	4.66 ± 0.60	4.42 ± 1.10	4.52 ± 0.57
Hematocrit (vol%)	40.88 ± 5.20	40.97 ± 5.33	38.43 ± 9.74	39.76 ± 5.12
Prothrombin time (sec)*	11.40 ± 0.74	12.00 ± 0.74	12.15 ± 0.98	11.47 ± 0.85
Prothrombin index (%)*	109.04 ± 10.72	99.18 ± 10.60	96.76 ± 11.70	106.86 ± 12.35
OLANZAPINE				
	Screening	**Day 3**	**Day 6**	**Day 15**
Leucocytes (/µL (10^3))*	6.67 ± 1.90	5.85 ± 1.63	5.54 ± 1.79	6.62 ± 1.69
Platelets (/µL (10^3))*	267.42 ± 54.31	240.50 ± 53.80	236.67 ± 64.62	278.96 ± 56.17
Hemoglobin (mg/dL)	13.68 ± 1.56	13.43 ± 1.88	12.85 ± 3.36	13.44 ± 1.80
Red blood cells (/µL (10^6))	4.60 ± 0.51	4.52 ± 0.62	4.31 ± 1.11	4.55 ± 0.59
Hematocrit (vol%)	40.69 ± 4.11	39.75 ± 5.44	38.10 ± 9.84	39.75 ± 5.12
Prothrombin time (sec)	11.50 ± 0.82	11.97 ± 0.86	11.53 ± 2.61	11.37 ± 0.76
Prothrombin index (%)*	106.96 ± 11.21	100.48 ± 10.16	94.00 ± 22.47	108.78 ± 11.12

*$P \leq 0.05$. Values are shown as mean ± SD.

No differences were found between males and females in leucocyte, platelet, haemoglobin, red blood cell and haematocrit count and prothrombin time and index. No levels were outside of the normal range.

6.6. Thyroid performance

Free T4 levels significantly increased after aripiprazole treatment ($p = 0.035$, $\eta_p^2 = 0.180$). On the contrary, after olanzapine treatment, decreased levels were observed. However, this association did not reach the significant level ($p = 0.230$). Neither aripiprazole, nor olanzapine had a significant effect on TSH levels (***Figure* 35**).

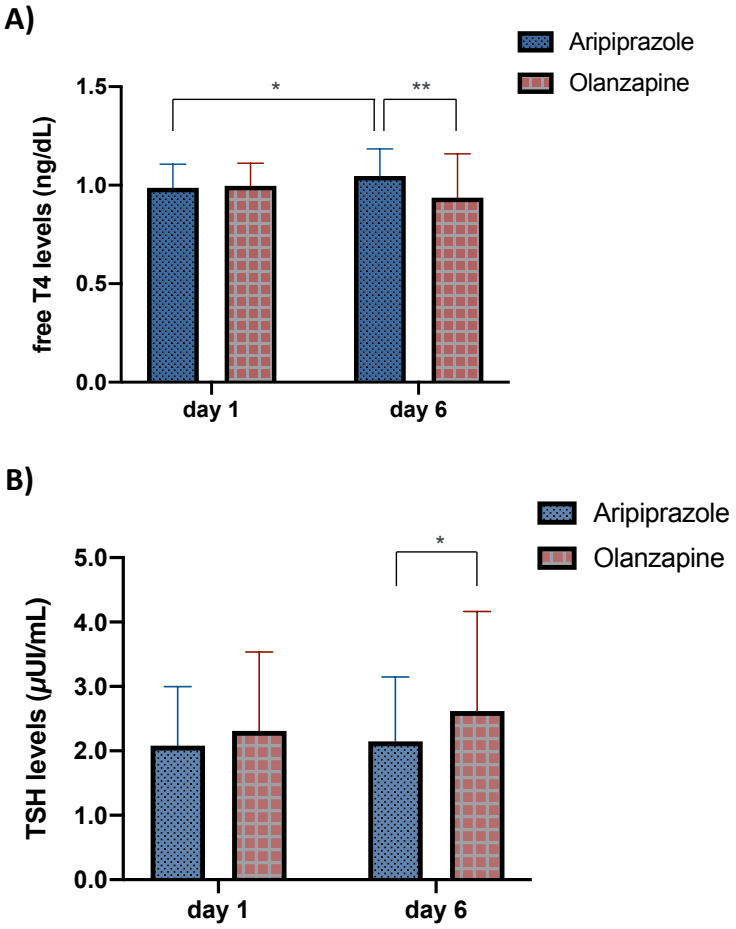

***Figure* 35**. Free T4 (A) and TSH concentrations (B) after multiple dose administration of aripiprazole and olanzapine.

Values are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$.

Nonetheless, a significant difference was found between aripiprazole and olanzapine treatment in both free T4 and TSH levels ($p = 0.010$, $\eta_p^2 = 0.267$; $p = 0.022$, $\eta_p^2 = 0.216$, respectively). No

differences were found between males and females in free T4 and TSH levels. No levels were outside of the normal range.

6.7. Renal performance

Uric acid levels significantly decreased during aripiprazole and olanzapine treatment ($p < 0.001$, $\eta_p^2 = 0.324$; $p = 0.045$, $\eta_p^2 = 0.116$) (***Table* 35**).

***Table* 35**. Urea, creatinine and uric acid levels during aripiprazole and olanzapine multiple dose treatment.

ARIPIPRAZOLE				
	Screening	**Day 3**	**Day 6**	**Day 15**
Urea (mg/dL)	32.58 ± 8.24	29.75 ± 5.41	31.42 ± 7.28	29.54 ± 10.18
Creatinine (mg/dL)	0.78 ± 0.13	0.76 ± 0.15	0.76 ± 0.15	0.80 ± 0.15
Uric acid (mg/dL)*	4.82 ± 1.43	4.20 ± 1.23	4.24 ± 1.20	4.34 ± 1.20
OLANZAPINE				
	Screening	**Day 3**	**Day 6**	**Day 15**
Urea (mg/dL)	30.08 ± 6.98	31.42 ± 5.22	32.13 ± 8.83	30.04 ± 9.32
Creatinine (mg/dL)	0.82 ± 0.17	0.76 ± 0.16	0.74 ± 0.22	0.78 ± 0.15
Uric acid (mg/dL)*	4.78 ± 1.61	4.53 ± 1.31	4.31 ± 1.63	4.68 ± 1.50

*$P \leq 0.05$. Values are shown as mean ± SD.

No differences were found between males and females in urea, creatinine and uric acid levels. Additionally, the changes in their levels were not dependent on the treatment (aripiprazole versus olanzapine). Uric acid levels were not outside of the normal range.

6.8. Summary of all metabolic effects

The metabolic effects of aripiprazole and olanzapine are summarized in ***Table* 36**. Overall, aripiprazole seems to have less harmful metabolic effects than olanzapine.

***Table* 36**. The effects of aripiprazole and olanzapine on all metabolic parameters.

	Aripiprazole	Olanzapine
Prolactin (ng/mL)*		⇧
C-peptide (ng/mL)	⇧	⇧ ns
Insulin (mcU/mL)	⇧ ns	⇧ ns
C-peptide/insulin ratio		⇧
HbA1c (%)		⇧
Glucose (ng/mL)		
GTT (mg/dL)*	⇧ ns	⇧
Weight (kg)*	⇩ ns	⇧ ns
Triglyceride (mg/dL)	⇩	⇩
Cholesterol (mg/dL)	⇩	⇩
GOT (U/L)	⇩	
GPT (U/L)	⇩	
GGT (U/L)	⇩	⇩
Total bilirubin (mg/dL)		⇩
ALP (U/L)	⇩	⇩
Leucocytes (/µL (10^3))	⇩ ns	⇩
Platelets (/µL (10^3))	⇩	⇩
Hemoglobin (mg/dL)	⇩ ns	⇩ ns
Red blood cells (/µL (10^6))	⇩ ns	⇩ ns
Hematocrit (vol%)	⇩ ns	⇩ ns
Prothrombin time (sec)	⇧	
Prothrombin index (%)	⇩	⇩
Albumin (g/dL)*	⇩	⇩
Free T4 (ng/dL)*	⇧	
TSH (µUI/mL)*		⇧
Urea (mg/dL)		
Creatinine (mg/dL)		
Uric acid (mg/dL)	⇩	⇩

*$P \leq 0.05$ aripiprazole compared to olanzapine.

7. Electrocardiogram and blood pressure

7.1. The effects of aripiprazole and olanzapine on ECG and blood pressure

Aripiprazole had a diastolic blood pressure lowering effect on the first day of treatment (5 mmHg, $p = 0.004$, $\eta_p^2 = 0.309$) and a QTc lowering effect on days 3 and 5 (20 ms, $p < 0.001$, $\eta_p^2 = 0.512$; 15 ms, $p = 0.028$, $\eta_p^2 = 0.193$, respectively) (***Table* 37**). None of the volunteers had a QTc value higher than 450 ms or showed more than a 30 ms change. HR incremented from predose to 5 h after drug administration on the 4th day in males, while it did not change in females ($p = 0.034$, $\eta_p^2 = 0.197$).

***Table* 37**. Effects of aripiprazole on blood pressure, heart rate and corrected QT interval.

ARIPIPRAZOLE		SBP (mmHg)		DBP (mmHg)		HR (bpm)		QTc (ms)	
		predose	5 h postdose	predose	5 h postdose	predose	5 h postdose	predose	5 h postdose
Dose 1	Males	125.0 ± 11.8	124.8 ± 11.9	67.9 ± 8.8	63.1 ± 7.0	71.4 ± 12.2	69.2 ± 8.8	391.7 ± 18.7	389.9 ± 18.8
	Females	114.7 ± 15.2	107.7 ± 15.8	65.8 ± 10.4	60.5 ± 7.9*	71.7 ± 8.4	74.6 ± 12.7	412.3 ± 17.4	418.6 ± 18.3
	All	119.8 ± 14.3	116.2 ± 16.2	66.8 ± 9.5	61.8 ± 7.4#	71.5 ± 10.2	71.9 ± 11.0	402.0 ± 20.6	404.3 ± 23.3
Dose 2	Males	121.8 ± 12.8	123.2 ± 12.6	67.7 ± 11.3	66.4 ± 10.9	69.6 ± 12.2	69.7 ± 10.9	388.4 ± 18.5	384.0 ± 19.4
	Females	113.7 ± 19.2	109.7 ± 12.8	64.8 ± 9.9	60.3 ± 8.8	73.5 ± 11.8	74.8 ± 9.6	412.5 ± 12.4	410.8 ± 19.1
	All	117.7 ± 16.5	116.4 ± 14.2	66.2 ± 10.5	63.4 ± 10.2	71.5 ± 11.9	72.3 ± 10.4	400.5 ± 19.7	397.4 ± 23.3
Dose 3	Males	124.0 ± 10.7	122.3 ± 13.3	70.5 ± 8.0	71.5 ± 10.9	72.8 ± 10.6	71.0 ± 13.1	394.2 ± 16.8	372.9 ± 19.0
	Females	114.9 ± 15.3	109.0 ± 12.5	65.3 ± 8.3	63.2 ± 7.2	76.3 ± 11.1	73.8 ± 8.1	414.9 ± 9.7	403.6 ± 15.8
	All	119.5 ± 13.7	115.6 ± 14.3	67.9 ± 8.4	67.3 ± 10.0	74.5 ± 10.8	72.4 ± 10.7	404.5 ± 17.1	388.3 ± 23.2†
Dose 4	Males	126.0 ± 15.7	122.7 ± 14.4	71.3 ± 12.5	70.1 ± 9.9	72.9 ± 11.9	79.3 ± 11.3	387.0 ± 19.9	388.8 ± 17.9
	Females	112.8 ± 11.8	111.5 ± 15.2	66.6 ± 9.3	66.6 ± 8.5	74.9 ± 12.3	73.7 ± 10.7~	414.4 ± 9.8	406.8 ± 14.3
	All	119.7 (15.2)	117.3 (15.5)	69.0 (11.1)	68.4 (9.4)	73.9 (11.9)	76.7 (11.2)	400.1 (20.9)	397.4 (18.7)

ARIPIPRAZOLE		SBP (mmHg)		DBP (mmHg)		HR (bpm)		QTc (ms)	
		predose	5 h postdose	predose	5 h postdose	predose	5 h postdose	predose	5 h postdose
Dose 5	Males	125.1 ± 22.9	122.8 ± 11.9	70.7 ± 9.9	68.8 ± 9.0	73.2 ± 9.2	74.6 ± 8.0	389.3 ± 22.9	380.3 ± 17.9
	Females	113.9 ± 9.1	112.9 ± 15.7	66.8 ± 6.6	66.4 ± 10.2	77.3 ± 9.1	78.8 ± 10.2	419.0 ± 14.9	411.7 ± 18.0
	All	119.5 ± 18.0	117.9 ± 14.5	68.7 ± 8.5	67.6 ± 9.5	75.3 ± 9.2	76.7 ± 9.3	404.1 ± 24.2	396.0 ± 23.8*

*$P < 0.05$, #$p < 0.01$, †$p < 0.001$ compared to predose. ~$P < 0.05$ change from predose compared to males. Abbreviations: SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate, QTc: corrected QT interval. Values are shown as mean ± SD.

Olanzapine had a systolic blood pressure, diastolic blood pressure, heart rate and QTc lowering effect on the first day 5 h after drug administration (16 mmHg, $p < 0.001$, $\eta_p^2 = 0.678$; 8 mmHg, $p < 0.001$, $\eta_p^2 = 0.537$; 10 bpm, $p < 0.001$, $\eta_p^2 = 0.129$; 10 ms, $p = 0.002$, $\eta_p^2 = 0.359$, respectively) **(*Table* 38).** None of the volunteers had a QTc value higher than 450 ms or showed more than a 30 ms change. HR increased from predose to 5 h after drug administration on the 4th day in males, while it did not change in females ($p = 0.002$, $\eta_p^2 = 0.350$). On the 4th day of drug administration, QTc values increased in males and the opposite effect was detected in females ($p = 0.018$, $\eta_p^2 = 0.227$).

***Table* 38**. Effects of olanzapine on blood pressure, heart rate and corrected QT interval.

OLANZAPINE		SBP (mmHg)		DBP (mmHg)		HR (bpm)		QTc (ms)	
		predose	5 h postdose	predose	5 h postdose	predose	5 h postdose	predose	5 h postdose
Dose 1	Males	128.8 ± 9.0	113.2 ± 8.5	73.3 ± 10.1	60.5 ± 5.4	74.1 ± 10.7	57.8 ± 8.0	396.6 ± 21.7	375.6 ± 21.2#
	Females	113.2 ± 11.3	99.3 ± 10.7	64.7 ± 7.9	57.2 ± 7.4	73.3 ± 8.6	62.8 ± 5.5	416.3 ± 16.3	409.5 ± 16.3
	All	121.0 ± 12.8	106.3 ± 11.8†	69.0 ± 9.9	58.8 ± 6.6†	73.7 ± 9.5	60.3 ± 7.2†	406.4 ± 21.3	392.5 ± 25.4#
Dose 2	Males	117.4 ± 7.6	115.0 ± 12.4	67.6 ± 9.5	63.7 ± 7.8	58.3 ± 7.2	62.9 ± 8.3	385.0 ± 21.0	384.7 ± 14.1
	Females	106.2 ± 12.2	100.6 ± 11.0	61.1 ± 8.3	57.8 ± 5.7	67.8 ± 9.0	69.1 ± 9.4	415.8 ± 14.5	412.6 ± 20.6
	All	111.8 ± 11.5	107.8 ± 13.6	64.3 ± 9.3	60.8 ± 7.3	63.1 ± 9.3	66.0 ± 9.2	400.6 ± 23.5	398.6 ± 22.4

OLANZAPINE		SBP (mmHg)		DBP (mmHg)		HR (bpm)		QTc (ms)	
		predose	5 h postdose	predose	5 h postdose	predose	5 h postdose	predose	5 h postdose
Dose 3	Males	120.1 ± 8.9	115.8 ± 13.3	66.9 ± 7.5	61.9 ± 6.0	60.6 ± 6.9	60.8 ± 8.1	378.6 ± 23.3	368.2 ± 19.9#
	Females	109.3 ± 11.5	107.5 ± 16.3	63.4 ± 9.6	61.5 ± 9.0	71.0 ± 8.8	72.3 ± 10.0	416.7 ± 14.0	418.2 ± 15.1
	All	114.7 ± 11.5	111.6 ± 15.1	65.2 ± 8.6	61.7 ± 7.5	65.8 ± 9.4	66.5 ± 10.6	397.6 ± 27.1	393.2 ± 30.8
Dose 4	Males	117.5 ± 10.4	116.7 ± 10.5	65.6 ± 8.9	65.3 ± 7.8	60.8 ± 10.3	67.8 ± 9.0#	373.7 ± 17.9	378.1 ± 16.4#
	Females	111.6 ± 15.8	104.5 ± 12.7*	64.5 ± 7.8	60.4 ± 9.3*	74.5 ± 8.3	72.9 ± 6.5~	422.8 ± 20.1	414.4 ± 23.1~
	All	114.5 ± 13.4	110.6 ± 13.0	65.0 ± 8.2	62.8 ± 8.7	67.6 ± 11.6	70.3 ± 8.1	398.2 ± 31.2	396.3 ± 27.0
Dose 5	Males	120.7 ± 8.4	118.2 ± 9.4	66.8 ± 8.6	63.8 ± 6.6	62.6 ± 9.1	68.0 ± 10.8*	378.5 ± 21.4	378.3 ± 20.8
	Females	113.6 ± 13.5	107.6 ± 14.7	63.5 ± 7.8	61.8 ± 7.5	75.7 ± 8.1	76.5 ± 9.3	431.8 ± 19.2	425.2 ± 26.1
	All	117.1 ± 11.6	112.9 ± 13.2	65.1 ± 8.2	62.8 ± 7.0	69.1 ± 10.8	72.3 ± 10.8	405.1 ± 33.7	401.8 ± 33.2

*$P < 0.05$, #$p < 0.01$, †$p < 0.001$ compared to predose. ~$p < 0.05$ change from predose compared to males. Abbreviations: SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate, QTc: corrected QT interval. Values are shown as mean ± SD.

The effects of aripiprazole and olanzapine on blood pressure, heart rate and QTc are compared in ***Figure* 36**. The changes in systolic blood pressure, diastolic blood pressure and QTc were not statistically significant between aripiprazole and olanzapine ($p = 0.110$, $p = 0.145$ and $p = 0.236$, respectively). However, although it did not reach the statistically significant level, olanzapine lowered both systolic blood pressure and diastolic blood pressure to a greater extent compared to aripiprazole. After the 4th day, tolerance was developed for the hypotensive effect of olanzapine. Additionally, HR was significantly lower during olanzapine treatment compared to aripiprazole ($p < 0.0001$, $\eta_p^2 = 0.236$).

No relationship was found between drug concentrations and blood pressure, heart rate and QTc.

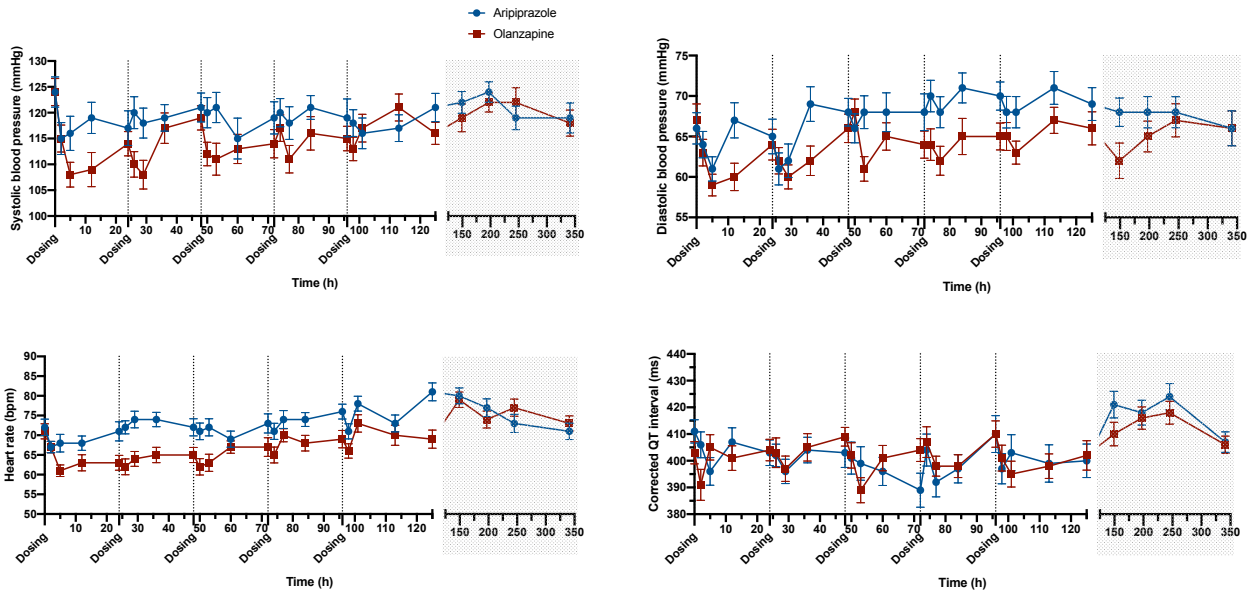

***Figure* 36.** Effects of aripiprazole and olanzapine on blood pressure, heart rate and corrected QT interval.

The shaded section shows the values after the end of treatment. Results are shown as mean ± SD.

7.2. The influence of polymorphisms on ECG and blood pressure

Diastolic blood pressure on the first day of aripiprazole treatment decreased more in *HTR2A* rs6313 C allele carriers and in *ADRA2A* rs1800544 C/C subjects compared to T/T and C/G subjects (7 vs +3 mmHg, $p = 0.006$, $\eta_p^2 = 0.296$; 7 vs 0 mmHg, $p = 0.020$, $\eta_p^2 = 0.224$, respectively).

Systolic blood pressure on the first day of olanzapine treatment decreased more in *DRD3* rs6280 Ser/Ser and Ser/Gly and in *ADRA2A* rs1800544 C/C subjects compared to volunteers with Gly/Gly and C/G genotypes (21 and 14 vs 6 mmHg, $p = 0.025$, $\eta_p^2 = 0.209$; 18 vs 8 mmHg, $p = 0.048$, $\eta_p^2 = 0.167$, respectively). Moreover, diastolic blood pressure on the first day diminished more in *COMT* wild-type subjects compared to those with heterozygous and mutant phenotype (16 vs 8 and 5 mmHg, $p = 0.022$, $\eta_p^2 = 0.218$). Additionally, heart rate on the first day decreased more in *DRD3* rs6280 Ser/Ser and Ser/Gly subjects compared to those with Gly/Gly genotype (16 and 15 vs 4 mmHg, respectively, $p = 0.013$, $\eta_p^2 = 0.249$).

8. Adverse events

During the study, no serious or life-threatening adverse events were documented. All volunteers experienced at least one adverse drug reaction.

8.1. Adverse drug reactions to aripiprazole and olanzapine

The most frequent adverse drug reactions to aripiprazole based on the UKU side effect rating scale and those spontaneously notified were somnolence (79%), headache (54%), insomnia (33%), dizziness (21%), restlessness (21%), palpitations (21%), akathisia (17%) and nausea (17%) (***Table* 39**). The number of adverse drug reactions was similar between males and females (3.9 ± 2.3 and 4.1 ± 2.0 adverse drug reaction/ subject, respectively). Palpitations were only registered in females (5 volunteers, $p = 0.012$ compared to males).

The most frequent adverse drug reactions to olanzapine based on the UKU side effect rating scale and those spontaneously notified were somnolence (100%), dizziness (29%), asthenia (13%), constipation (13%), dry mouth (13%), headache (13%) and nausea (13%) (***Table* 39**). The number of adverse drug reactions was similar between males and females (1.7 ± 1.8 and 1.7 ± 1.1 ADR/ subject, respectively).

Ramsay scores showed low level of sedation with the average level of activity of 2 (cooperative, orientated and calm) in case of both drugs. No relationship was found between drug concentrations and adverse drug reactions.

***Table* 39**. Adverse drug reactions to aripiprazole and olanzapine.

Adverse event	**Aripiprazole N (%)**	**Olanzapine N (%)**
Nervous system disorders		
Akathisia	4 (17)	-
Dizziness	5 (21)	7 (29)
Headache	13 (54)	3 (13)
Restless legs	1 (4)	-
Somnolence	19 (79)	24 (100)
Pre-syncope	-	1 (4)

Adverse event	Aripiprazole N (%)	Olanzapine N (%)
Syncope	1 (4)	1 (4)
Left hand tremor	-	1 (4)
Psychiatric disorders		
Anxiety	2 (8)	-
Early awakening	1 (4)	-
Restlessness	5 (21)	1 (4)
Insomnia	8 (33)	2 (8)
General disorders		
Asthenia	2 (8)	3 (13)
Fatigue	2 (8)	-
Gait alterations	1 (4)	-
Tiredness	1 (4)	-
Weakness	1 (4)	1 (4)
Gastrointestinal disorders		
Constipation	3 (13)	3 (13)
Dry mouth	-	3 (13)
Gastric discomfort	1 (4)	-
Hypersalivation	-	1 (4)
Hyposalivation	1 (4)	-
Nausea	4 (17)	3 (13)
Vomiting	2 (8)	-
Skin and subcutaneous tissue disorders		
Facial rash	1 (4)	1 (4)
Hair loss	1 (4)	-
Rash	-	1 (4)
Sweating	3 (13)	-
Pruritus	-	1 (4)
Respiratory, thoracic and mediastinal disorders		
Hiccups	3 (13)	-
Metabolism and nutrition disorders		
Hyporexia	1 (4)	-
Increased appetite	-	1 (4)
Investigations		
Increased liver enzymes	2 (8)	1 (4)
Cardiac disorders		
Palpitations	5 (21)	2 (8)
Musculoskeletal and connective tissue disorders		
Upper limb weakness	1 (4)	-
Left shoulder pain	-	1 (4)
Eye disorders		
Photophobia	-	1 (4)

Adverse drug reactions to aripiprazole and olanzapine classified by system organ class allocation are shown in ***Figure* 37**. The number of registered adverse drug reactions was significantly higher after aripiprazole administration (91 vs 60, $p < 0.035$). Likewise, more psychiatric and cardiac adverse drug reactions were detected during aripiprazole treatment compared to olanzapine (16 vs 3 and 7 vs 2, respectively, $p < 0.001$).

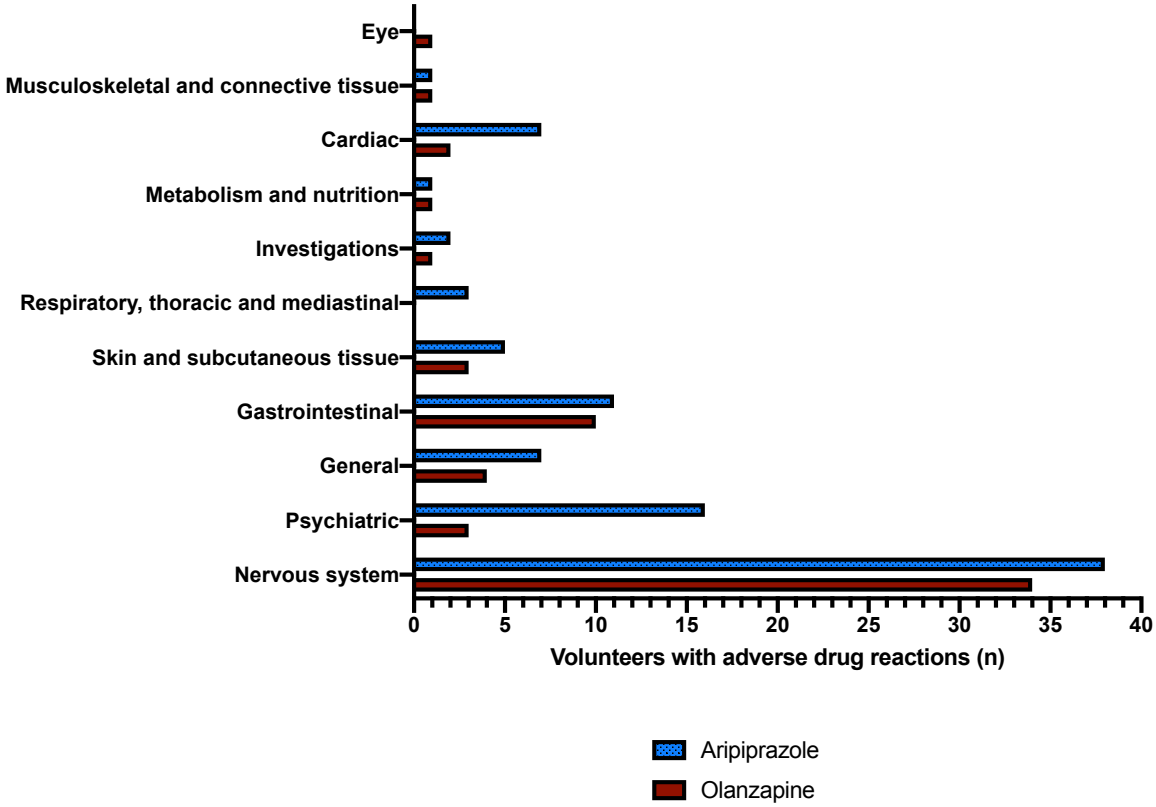

***Figure* 37.** Adverse drug reactions to aripiprazole and olanzapine classified in groups.

8.2. The incidence of adverse drug reactions among different genotypes

The incidence of akathisia was higher in *DRD3* rs6280 Ser/Ser homozygotes compared to Gly allele carriers (22.2% vs 0%, respectively, $p = 0.003$). Moreover, only *DRD2* rs1799732 G/- subjects experienced asthenia compared to G/G homozygotes (33.3% vs 0%, respectively, $p = 0.011$). The incidence of headache was significantly higher in *HTR2C* rs3813929 T carriers than in C/C homozygotes (66.7% vs 28.6%, respectively, $p = 0.030$). Additionally, an association was found between CYP1A2 NM/RM phenotype and the incidence of insomnia (41.2% vs 0% in UM,

$p = 0.044$). Finally, somnolence was detected more frequently in *HTR2A* rs6314 C/C and *OPRM1* rs1799971 A/A subjects compared to C/T subjects and G carriers, respectively (86.4% vs 0%, $p = 0.004$ and 89.5% vs 40%, $p = 0.029$, respectively).

Constipation was detected more frequently in *HTR2A* rs6314 T allele carriers, *HTR2A* rs7997012 A allele carriers and *UGT1A1* rs887829 T/T subjects compared to C/C homozygotes, G/G homozygotes and C allele carriers (100.0% vs 4.5%, $p < 0.001$; 15.4% vs 9.0%, $p = 0.026$ and 60.0% vs 0%, $p = 0.001$, respectively). Moreover, only *DRD3* rs6280 Ser/Ser subjects experienced dry mouth compared to Gly allele carriers (50.0% vs 0%, respectively, $p = 0.006$). The incidence of insomnia was higher in *HTR2C* rs1414334 G/G homozygotes compared to C allele carriers (100.0% vs 4.2%, respectively, $p = 0.003$). In addition, nausea was only detected in *HTR2C* rs518147 C/T heterozygotes and not in C/C and T/T homozygotes (33.3% vs 0%, respectively, $p = 0.038$). Finally, palpitations were only reported in CYP1A2 UM and *HTR2A* rs7997012 A allele carriers and not in NM/RMs and G/G homozygotes (28.6% vs 0%, $p = 0.021$ and 15.4% vs 0%, $p = 0.002$, respectively).

V. DISCUSSION

1. Analytical method validation

The method was developed and validated including 6 antipsychotic drugs and 2 of their metabolites. For the present study, we only needed to detect aripiprazole, dehydro-aripiprazole and olanzapine concentrations. Nevertheless, the reason of including other antipsychotics was to use this method for further studies, mostly in clinical environment for therapeutic drug monitoring. Additionally, we included caffeine to the method. The metabolism of clozapine and olanzapine is mainly dependent on CYP1A2 [107], similar to caffeine, which is a central nervous system stimulant [238]. It was shown that caffeine increases plasma levels of these drugs due to CYP1A2 competitive inhibition [238]. Therefore, caffeine was added to the method to be able to monitor its effect on their metabolism. To date, there is no documented record on the simultaneous determination of these antipsychotics along with caffeine in a single-run process. The method was developed based on the recommendations from EMA, FDA and ICH regulatory agencies including the following tests: precision, accuracy, sensitivity, matrix effects, extraction recovery, process efficiency, trueness, linearity, stability and carry-over effect [22–24].

1.1. Experimental conditions in LC and MS/MS

Strongest basic pKa values are ranging from 7.06 to 8.76 [239] for all drugs included in this method. Due to this matter, it was possible to optimize extraction method and validate an LC-MS/MS method for simultaneous quantification of aripiprazole, dehydro-aripiprazole, olanzapine, risperidone, paliperidone, quetiapine and clozapine. However, caffeine has a pKa value of -0.92, thus the extraction process was less efficient for this compound. Drugs' water solubility ranging from 0.008 mg/mL to 0.297 mg/mL, except for CAF with 11 mg/mL [239] enabled us to perform an excellent chromatographic separation. Additionally, the column we used, ACE C18-PFP, is characterized by a good resolution and improves analyte separation compared to ordinary C18 column due to π-π interactions with the aromatic ring and OH groups. After each injection, the injection needle and the analytical column were washed with a mixture of acetonitrile and water (50/50; v/v) which was chosen due to its better efficacy compared to the mixture of isopropanol and water (50/50; v/v). The total run time, including washing and re-equilibrating steps, was 9.0 min. Although our run time – 9 minutes – was longer compared to the methods described in the

literature (5.5-6 minutes) [240,241], it was considered essential to elute endogenous phospholipids outside of the elution profile of the analytes. Additionally, our method was performed with HPLC which requires longer run time compared to UPLC methods.

LC-MS/MS with ESI and small particle sizes of the analytical column (up to 3.5 µm) required small injection volume in order to avoid overloading, matrix effects and instrument contamination [242]. Thus, we injected 5 µL of the sample, which was applicable for the column particle size and the HPLC system.

Concerning MS/MS conditions, ESI in positive mode was selected for scanning all analytes. As their polarity was positive, dynamic MRM (dMRM) scan mode was applied to significantly improve analytes' peak shape and selectivity. Ion ratio (qualifier ratio) identity confirmation was used in addition to stable isotope-labeled internal standards to ensure the reliability of analyte detection as aripiprazole and dehydro-aripiprazole have the same product ions and can undergo crosstalk phenomenon in the collision cell.

1.2. Sample preparation: phospholipid elimination, extraction recovery, matrix effects and process efficiency

We tested phospholipid cleaning ability of SPE compared to PPT in 36 different human blank plasma samples. As PPT extraction methods were associated with the highest degree of matrix effects compared to SPE [243], we used this method as a positive control (100% of phospholipid content). According to our knowledge, there is no method which is able to remove 100% of phospholipids from the matrix. However, we were able to eliminate efficiently more than 92% of early- and late-eluting endogenous plasma phosphatidylcholines and lysophosphatidylcholines using hydrophilic-lipophilic SPE sorbent compared to PPT method. It was impossible to eliminate 100% of endogenous phospholipids, yet we were able to separate them chromatographically from the target compounds. Among LC-MS/MS and UHPLC-MS/MS methods for antipsychotic drug quantification [241,244], only our previous publication about the simultaneous determination of aripiprazole and dehydro-aripiprazole in human plasma considered endogenous phospholipid elimination efficiency during method development [245].

Caffeine had less efficient extraction recovery values due to the lack of its stable isotope-labeled internal standard and the extraction method, which was adjusted for antipsychotic drugs. When comparing the extraction recovery achieved in the present extraction method to the literature, we found that other authors [246,247] accomplished similar values for relative extraction recovery ranging from 71-123% using SPE as sample preparation method. Our results are slightly better, ranging from 98-109%. The extraction recovery of PPT method was between 50 and 111% (except for olanzapine, 185%) for LC-MS/MS methods, while improved when UHPLC-MS/MS was applied, ranging from 65 to 114% [202,241,248]. These differences can be due to the lower injection volume and less important matrix effects in UHPLC-MS/MS analysis.

Regarding matrix effects, more preferable values were obtained when stable isotope-labeled internal standards were applied (relative) compared to absolute values. Moreover, extraction recovery and matrix effects of SPE were slightly better than PPT values. These results were expected, because stable isotope-labeled internal standards compensate for analyte loss during extraction process. Absolute extraction recovery obtained with PPT compared to SPE was lower and highly variable, while ion suppression was also significant. Therefore, SPE was chosen as extraction procedure for method validation.

Matrix effects are commonly evaluated using qualitative methods, applying post-column infusion [249]. Nevertheless, manipulating the chromatographic separation to avoid matrix effects due to co-elution is not always applicable [250]. Matrix effects for SPE in the literature range from 91 to 133% [246], while for PPT it varies between 89 and 119% [249,251]. Although these results are analogous, the differences lie in the RSD values. RSD is greatly higher for PPT sample preparation method and cause method irreproducibility and as a consequence compromised precision and accuracy [22].

Regarding process efficiency, the results obtained with PPT were certainly worse compared to SPE. There are only a few methods evaluating relative process efficiency as a test included in method validation [247,248], ranging from 42 to 147%. The best process efficiency was achieved by Patel *et al*. [247], with values between 92.3 and 98.9% for only aripiprazole included in the analytical method. Our global relative process efficiency was found between 95 and 113%. Relative process efficiency of aripiprazole ranged from 101 to 103%, being closer to 100% than Patel *et al*.' results.

Based on our experience, process efficiency and phospholipid elimination ability should be included to method validation, along with extraction recovery and matrix effects. Process efficiency and phospholipid removal efficiency offer a more complete overview regarding the effect of possible analyte loss during sample preparation and ionization suppression/ enhancement in the ion source.

1.3. Selectivity, LLOQ and linearity

The present approach was selective and able to differentiate and quantify the analytes from endogenous matrix components, as no significant interferences were found in analyte detection. Moreover, the mobile phase and gradient modifications improved the response of dehydro-aripiprazole in the present method compared to our previously reported method [245].

LLOQ for caffeine was considerably higher, 1200 ng/mL, due to its high plasma levels after oral coffee consumption described in the literature [252]. Although more sensitive LC-MS/MS approaches were reported, with LLOQs lower than ours, some of these methods used larger plasma volumes for extraction between 250 and 500 µL [248,253], than we have (200 µL), except for Vecchione *et al*. [241], who used only 100 µL of plasma. Our LLOQs were better than those reported by Choong *et al.* [246] and Fisher *et al*. [254] (***Table* 40**). Of note, our LLOQ for paliperidone was the best compared to bibliographic values. It can be due to the applied sample preparation method. Fisher *et al.* applied liquid-liquid extraction with methyl-tertbutyl ether as sample extraction method, which is known to produce lower extraction recovery and higher LLOQ values.

***Table* 40**. LLOQ values of the included antipsychotics compared to bibliographic data.

Compound	Our LLOQ value (ng/mL)	LLOQ value by Choong *et al.* [246] (ng/mL)	LLOQ value by Fisher *et al.* [254] (ng/mL)
Aripiprazole	0.18	5.00	1-5 (not specified for each drug)
Dehydro-aripiprazole	0.25	5.00	
Clozapine	0.50	2.00	
Olanzapine	1.00	2.00	
Paliperidone	0.20	-	
Risperidone	0.70	-	
Quetiapine	0.50	-	

1.4. Precision and accuracy

Precision and accuracy were within the acceptance criteria for the evaluated assay range (RSD < 15%, except for LLOQ (< 20%)). These results indicate that it is possible to generate accurate data with the present method for the plasma determination of antipsychotics and caffeine.

1.5. Trueness

Due to our low Zeta-score values, which shows the level of systematic error, our LC-MS/MS method was verified [22–24].

1.6. Stability

Overall, the RSD value was less than 10.32% and mean stabilities were close to 100% for all tested compounds. Therefore, the concentration of the analytes remained the same in time. Degradation usually lowers the analyte content. If the analyte is unstable, its decomposition influences trueness and precision of the procedure. Furthermore, besides the analyte in the samples, analyte in the standards can also decompose. If both occur at the same rate then decomposition only affects precision [22]. Olanzapine was shown to be unstable in whole blood and oral fluid under most of the

conditions studied by Fisher *et al.* [255], although the addition of ascorbic acid to blood or serum showed protective properties against olanzapine degradation. Likewise, we added formic acid to all plasma samples resulting in the preservation of olanzapine in all stability tests.

1.7. Carry-over

Carry-over effect, if detected, should be prevented to ensure the precision and accuracy of the method. Application of the optimal washing procedures of the injection needle and the analytical column should be optimized. Similarly to others [246,256], we managed to avoid a significant carry-over effect with the mixture of acetonitrile and water (50/50; v/v). The carry-over phenomenon detected by other authors was higher [246] than ours (1.05-16.13%), except for Patel *et al.* [247], who were able to reduce the carry-over effect to less than 0.05%, however, only aripiprazole was included in the method. Most methods do not test carry-over effect. We included this test to ascertain that no significant contamination was present in the instrument.

2. The influence of sex on pharmacokinetics and pharmacogenetics

In the current study, pharmacokinetics of aripiprazole and dehydro-aripiprazole were affected by sex. Females had higher $T_{1/2}$ and Vd/F of aripiprazole than males, which is consistent with our previous single-dose studies [153,257]. However, in the present study females had higher Vd/F and Cl/F and lower AUC_{last} of dehydro-aripiprazole. Based on previous studies, Cl/F should be lower in males compared to females [90,258]. However, it can still be higher in females due to differences in metabolism or pharmacogenetics. Additionally, differences in Vd/F can be due to higher fat content in females [259]. Nonetheless, no dosage adjustment is recommended for aripiprazole or olanzapine based on sex differences, because they are predominantly explained by the differences in body weight [90,106].

The differences observed in the prevalence of *HTR2C* rs3813929, rs518147, *ABCB1* rs1128503, rs2032582, rs10276036 and rs4148737 polymorphisms regarding sex may be explained by the reduced sample size. Regarding *HTR2C*, it is explained by the location of the gene on chromosome X. Some polymorphisms were not in Hardy Weinberg equilibrium. The disequilibrium for *HTR2C* rs3813929, rs518147, *ABCB1* rs4728709 and *COMT* rs13306278 could be explained by the small

sample size. Regarding the *CYP2D6* and *CYP3A4* polymorphisms it is explained by the low frequency of mutated alleles.

3. The influence of polymorphisms on pharmacokinetics

Our study confirms the impact of CYP2D6 phenotypes on the pharmacokinetics of aripiprazole and dehydro-aripiprazole [153,165,260]. AUC was 4 times less, C_{max} and $T_{1/2}$ were about half and Cl/F was two times higher in UMs compared to the other phenotypes, similarly to our previous single-dose study [166].

CYP3A had an impact on the pharmacokinetics of dehydro-aripiprazole and olanzapine confirming its involvement in aripiprazole and olanzapine metabolism [261]. The lack of association with the parent drug, aripiprazole, may be due to the low sample size, because CYP2D6 and CYP3A4 contribute about equally to the metabolism of aripiprazole [261]. Moreover, CYP3A activity varies predominantly by sex and inhibition or induction of a wide range of substrates, rather than by polymorphisms [262].

Based on the literature, aripiprazole is not a substrate of CYP1A2 [106]. Unexpectedly, in our study, the CYP1A2 phenotype influenced the pharmacokinetics of aripiprazole and dehydro-aripiprazole as UMs showed a lower disposition compared to the other phenotypes. To the best of our knowledge, this is the first study to report a similar result, therefore, it can be a false positive. Based on our findings, more studies should be performed to confirm the role of CYP1A2 in the pharmacokinetics of aripiprazole.

Based on previous studies, CYP1A2, CYP3A4 and CYP2D6 are involved in the metabolism of olanzapine [107]. However, we did not find any association, what can be due to the lack of effect or small number of subjects in some phenotype groups.

To date, there are no consistent findings about the role of polymorphisms in *ABCB1*. In our previous studies the C1236T (rs1128503) polymorphism had an influence on the pharmacokinetics of aripiprazole [153,166]. In the current study, the C3435T (rs1045642) and rs4148737 polymorphisms

were related to $T_{1/2}$ and C_{max} of aripiprazole and dehydro-aripiprazole and the rs10280101, rs12720067 and rs11983225 polymorphisms had an influence on $T_{1/2}$ of olanzapine. Presumably *ABCB1* has an effect on the pharmacokinetics of these antipsychotics being substrates of P-gp [263]. According to our knowledge, no other study analysed polymorphisms in *ABCB1* other than C3435T (rs1045642), G2677TA (rs2032582), C1236T (rs1128503). More studies are needed including more polymorphisms in *ABCB1* to provide a wider insight of its role in the pharmacokinetics of these drugs. Additionally, as stated previously, the lack of consensus on P-gp pharmacogenetics is partially explained by the lack of studies and the guidelines describing phenotype interference from variants.

Finally, the *UGT1A1* rs887829 polymorphism affected the pharmacokinetics of olanzapine. In a previous study, this polymorphism was related to some adverse effects but not to pharmacokinetics [264]. Olanzapine is metabolized predominantly by direct glucuronidation via the UGT enzyme family [261], but clear evidence was found only for UGT1A4 [265]. Additionally, *UGT1A4* and *UGT2B10* polymorphisms significantly contributed to the interindividual variability in its metabolism in previous studies [177,266]. We did not include polymorphisms in these genes in our study, as the array we used did not contain them. Our study is the first reporting an association between an *UGT1A1* polymorphism and the pharmacokinetics of olanzapine.

4. Pupillometry

The mechanism of action of aripiprazole and olanzapine is still not perfectly understood [267]. Aripiprazole and dehydro-aripiprazole achieve their pharmacological effect possibly by partial agonistic activity at dopamine D2 and 5-HT1A receptors and antagonistic activity at 5-HT2A receptors. Pharmacodynamic effects on receptors other than dopamine D2, 5-HT1A and 5-HT2A may explain other clinical effects: changes in pupillary light reflex could be caused by partial agonistic activity at adrenergic alpha1 receptors [106]. Pupil dilatation is primarily an α1-adrenergic receptor mediated effect [268], while it is mediated to a lesser extent by dopamine and serotonin receptors [269,270].

The dilatation observed after the administration of the first dose of aripiprazole could be explained by its partial agonism at these receptors. On the contrary, olanzapine is an antagonist at these receptors [95], therefore, it could explain the lack of pupil dilatation. Both aripiprazole and dehydro-aripiprazole have higher affinity for dopamine D2 and 5-HT1A than for alpha1 adrenergic receptors [112]. When dehydro-aripiprazole was present, neither aripiprazole nor dehydro-aripiprazole bound to α1-adrenergic receptors due to competitive inhibition caused by the higher affinity for dopamine D2 and 5-HT1A receptors. Hence, a constriction was observed after drug administration. Our results could confirm the fact that pupillary changes may rather be caused by the metabolite than the parent compound [268]. This could be the reason why C_{max} and AUC_{last} of dehydro-aripiprazole had an influence on several pupillometric parameters, while the pharmacokinetic parameters of aripiprazole influenced only a few of them.

In conclusion, aripiprazole and dehydro-aripiprazole caused changes in pupillary light reflex due to their unique pharmacological profile. Measuring dynamic pupillary light reflex is already a valid test for the pharmacodynamic effects of opioid- and some noradrenergic drugs [271,272]. Both drugs caused pupil constriction in two previous studies [153,273], however, in another study, neither aripiprazole nor olanzapine affected pupil contraction [274]. Hence, more studies are needed to alleviate the ambiguity and they should be repeated in patients. Afterwards, pupillometry could be introduced in the practice to assess autonomic nerve activity.

4.1. The influence of polymorphisms on pupillary light reflex

In previous studies with opioids, CYP2D6 UMs experienced increased and PMs experienced decreased pupil size compared to EMs [271,272]. We could not replicate these findings with aripiprazole and olanzapine, which may be due to their different mechanism of action. Additionally, we did not find any PM and only 2 UMs were present in our population. Our results confirm those in our previous single-dose study with healthy volunteers that no associations can be found between CYP2D6 phenotypes and pupillometric parameters [153].

CYP3A phenotypes are unrelated to opioid pharmacokinetics [275]. Notwithstanding, in the present study, CYP3A IM pupil size was increased compared to that of PMs after aripiprazole

administration. This was expected as aripiprazole caused pupil constriction after multiple dose administration; the pupil was under prolonged aripiprazole exposure in PM subjects.

The effects of dopamine and serotonin on pupillary light reflex are well known. High serotonin levels cause pupil dilatation [270] and dopamine may cause pupil dilatation or constriction through sympathetic and parasympathetic nerves, respectively [269]. Based on our results, pupil constriction could be due to the antagonist activity of aripiprazole at 5-HT_{2A} and 5-HT_7 receptors, while its dilatation could be explained by its partial agonism at dopamine D_2, D_3, D_4 and serotonin 5-HT_{1A}, 5-HT_{2C} receptors [112]. This theory was confirmed by our study: both *HTR2A* rs6314 and *DRD2* rs1800487 had an influence on the pupil size, the proportion of its change, the time of onset of constriction and pupil recovery. Additionally, *DRD2* rs1800487 also affected the pupil size and its constriction velocity after olanzapine treatment. In our previous single-dose study some *HTR2A*, *HTR2C*, *DRD2* and *DRD3* polymorphisms were also related to pupillometric parameters [153]. The lack of associations with *HTR2C* and *DRD3* polymorphisms in the present study could be due to the low sample size.

Both aripiprazole and olanzapine are transported by P-gp [263]. However, to our knowledge, no previous study could associate the genetic differences in *ABCB1* to pupil contraction. We previously analysed 3 polymorphisms: rs1045642 (C3435T), rs1128503 (C1236T) and rs2032582 (G2677T/A), but no associations were found [153]. In the current study we analysed 12 *ABCB1* polymorphisms. No association was found with the 3 previously mentioned polymorphisms, however, 3 others had an influence on pupil recovery after aripiprazole treatment. It may suggest that though the 3 most common polymorphisms do not affect the pupillary light reflex, others could have an influence. Further research including other less studied *ABCB1* polymorphisms should be performed.

UGT1A1 rs8877829 subjects with the mutated T/T genotype had greater pupil size after aripiprazole treatment. Based on in vitro studies, aripiprazole does not undergo direct glucuronidation by UDP-glucuronosyltransferase (UGT) enzymes [106], however, no sufficient

evidence is available thus far. Considering our results, *UGT1A1* may be involved in aripiprazole metabolism. Nevertheless, it can be a false positive result due to the analysis of many variables.

5. Metabolic effects

5.1. Prolactin concentrations

Olanzapine caused prolactin elevation instantly after administering the first dose. Hyperprolactinemia is a common side effect of olanzapine treatment along with other atypical antipsychotics [276], what is produced by DRD2 blockage. Therefore, it causes loss of the dopaminergic prolactin inhibitory factor in the lactotroph cells in the anterior pituitary. Hence, antipsychotics with a greater D2 occupation index produce significant prolactin elevation [277]. Our previous study revealed that prolactin levels significantly increase after administering a single dose of olanzapine [278]. Additionally, prolactin concentration was higher in all time points in subjects with higher C_{max}. Our current study confirms that the administration of 5 multiple doses of olanzapine also causes prolactin elevation. Nevertheless, this increment was much less than that produced with single-dose risperidone treatment in our previous study [279].

Prolactin levels decreased after changing the therapy from other atypical antipsychotics - including olanzapine - to aripiprazole [280]. Our previous single-dose study showed that a single dose of aripiprazole mildly increases prolactin levels compared to the controls [281]. However, compared to olanzapine, no change was observed in prolactin levels after administration in the present study. Our study is the first to report a comparison between prolactin elevation induced by aripiprazole and olanzapine in the same subjects. Therefore, the results can be considered reliable as the intraindividual variability is discarded. Schizophrenic patients usually receive several antipsychotic agents before aripiprazole, therefore they are almost never drug-naïve [280]. Our healthy subjects had not received any antipsychotic medication previously, hence no prior drug treatment could cause prolactin elevation. The current clinical practice recommends switching to aripiprazole monotherapy in case of having high prolactin levels and if it does not appear to be normalized after 4 weeks of treatment, it should be discontinued [282].

Sex has a clear effect on prolactin concentrations, what was confirmed in several studies [283,284]. Its levels tend to be higher in females than in males [284]. Therefore, the effects of aripiprazole and olanzapine on prolactin secretion were analysed both jointly and separately.

HTR2C rs17326429 and rs3813929, *COMT* rs4680, *DRD2* rs1800497, *DRD3* rs6280 and *ABCB1* rs1045642, rs1128503, rs2032582 and rs2235048 polymorphisms and CYP2D6 phenotypes were previously associated to prolactin levels after risperidone, quetiapine, clozapine, aripiprazole or olanzapine treatment [279,281,285,286]. We expected similar results as aripiprazole is a partial agonist at DRD2 and at 5HT1A receptors and an antagonist at 5HT2A receptors while olanzapine is an antagonist at DRD2 and at 5HT2A and 2C receptors [116,287]. In addition, aripiprazole is metabolized by CYP2D6 and CYP3A4. However, aripiprazole usually does not induce hyperprolactinemia [118], therefore a clear difference in prolactin levels would not be expected among phenotype groups. However, CYP3A PM subjects had significantly higher prolactin concentrations compared to the other phenotypes during aripiprazole treatment. These subjects were under prolonged aripiprazole exposure what could cause mild prolactin increase. Regarding *ABCB1*, subjects with rs10280101, rs12720067 and rs11983225 A-C-T haplotype had higher prolactin concentrations compared to those carrying the mutated alleles. This confirms the hypothesis that *ABCB1* polymorphisms and haplotypes might affect P-glycoprotein activity, therefore aripiprazole brain availability and prolactin levels [286].

Regarding olanzapine, *DRD3* rs6280 Ser/Ser subjects had lower prolactin levels compared to those carrying the Gly allele. Consequently, they may show higher DRD3 occupancy, thus dopamine can inhibit prolactin release [118]. Previous findings imply that *DRD3* does not play a major role in olanzapine-induced prolactin secretion [182]. Notwithstanding, we did not expect these results considering that none of the *DRD2* polymorphisms affected prolactin levels. More studies are needed to resolve the ambiguity.

5.2. Glucose metabolism

5.2.1. C-peptide levels

C-peptide levels were significantly higher after aripiprazole treatment. However, these levels were not significantly different between aripiprazole and olanzapine. Clozapine, olanzapine, risperidone and sulpiride were associated with an increase in C-peptide levels in schizophrenic patients [288]. In our study, olanzapine tended to increase its levels without reaching statistical significance; however, the C-peptide/ insulin ratio was higher after the 5 days treatment. This ratio is an indirect index of hepatic insulin clearance [289]. The observed increase in the ratio may be due to the increase in hepatic insulin clearance therefore decreased insulin secretion [290]. Teff *et al*. with similar study design – 3 days of olanzapine treatment in healthy volunteers – found the contrary: a decrease was observed in the ratio what may imply an increase in insulin secretion [291]. In the latter study, opposite to us, aripiprazole did not cause elevation in C-peptide levels [291]. High C-peptide levels can imply insulin resistance and finally can lead to type 2 diabetes, atherosclerosis and metabolic syndrome. Thus, it may serve as a biomarker to identify the risk to develop these diseases [292].

COMT rs4680 G/G and rs1330678 T carriers had higher increase in C-peptide levels after ARI treatment. *COMT* polymorphisms were previously associated with glycaemic function and type 2 diabetes, what can alter catecholamine production [293]. *COMT* rs4680 A carriers achieved a significantly lower change in C-peptide levels compared to subjects with G/G genotype what is consistent with a previous study [294]. Thus, A may be the protective allele against changes in glucose metabolism. Aripiprazole and olanzapine seem to have an effect on C-peptide levels, however, more studies are needed both in patients and healthy volunteers to confirm these findings.

5.2.2. Glucose levels

Olanzapine is associated with glucoregulatory abnormalities. The 5-HT_1 antagonism may decrease the responsiveness of the pancreatic beta cells, thus reducing the secretion of insulin and causing hyperglycemia [295]. In our study, basal glucose levels did not change during acute treatment, however, the GTT performed after treatment showed higher 1 h and 2 h glucose levels compared to the first day. These levels were significantly higher than during ARI treatment. Previous findings show the same association in patients undergoing chronic treatment and healthy

volunteers with acute treatment [296,297]. *UGT1A1* rs887829 C/C homozygotes had higher basal glucose levels and also higher glucose levels in GTT after 1 h and 2 h on day 6 compared to the first day. OLA is metabolized predominantly by the UGT enzyme family, but clear evidence was found only for UGT1A4 [265]. Based on our results, T allele carriers may be under prolonged OLA exposure and therefore show higher glucose concentrations. This result is confirmed by our previous study as this polymorphism affected OLA pharmacokinetics [298].

5.2.3. Insulin levels

Aripiprazole and olanzapine tended to increase insulin levels. In addition, *BDNF* rs6265 C/C subjects showed higher insulin levels compared to the other genotypes after aripiprazole administration and in CYP3A PMs compared to the other phenotypes after olanzapine administration. In a previous study the *BDNF* rs6265 polymorphism did not affect insulin levels during chronic risperidone and olanzapine treatment [299]. Based on our knowledge, our study is the first to report this relationship with aripiprazole. C/C subjects may have more predisposition to develop high insulin levels and finally insulin resistance during aripiprazole treatment.

5.3. Weight gain and lipid metabolism

5.3.1. Weight gain

It is not completely understood how antipsychotics cause weight gain, but 5-HT_{2C} and 5-HT_{1A} receptors, histamine H_1 receptor and DRD_2 presumably play a role [300]. However, olanzapine pharmacology is not the only factor to affect weight gain; the diet and activity level may also play a role. Weight increases rapidly within the first 6 weeks of olanzapine treatment and patients continue to gain weight [301]. Based on our knowledge, our study is the first to report olanzapine-related weight gain during only 5 days of treatment. Aripiprazole did not induce weight gain in the same volunteers, what strengthens our results as we can discard the effect of the diet. *HTR2C* polymorphisms are clearly linked to susceptibility to gain weight with antipsychotics [194]. The *HTR2C* rs1414334 polymorphism was widely analysed and the C allele was associated to olanzapine, clozapine and risperidone-induced weight gain and metabolic syndrome [302], what we confirm in our study.

5.3.2. Lipid metabolism

Based on current knowledge, olanzapine, but not aripiprazole, increments triglyceride and cholesterol levels in chronic treatment [303]. Moreover, in a previous study, after administering 3 daily doses of olanzapine to healthy volunteers, cholesterol and triglyceride levels were higher [297]. These results suggest that olanzapine may have acute adverse effects on lipid profiles as well. However, in our study, both triglyceride and cholesterol levels decreased during aripiprazole and olanzapine treatment. The observed decrease could be due to the low carbohydrate diet during their stay [304]. It could explain why triglyceride and cholesterol levels recovered by the safety visit (10 days after discontinuing the treatment).

CYP3A PMs showed a greater decrease in triglyceride levels during aripiprazole treatment compared to the other phenotypes. PMs could have a prolonged aripiprazole exposure, therefore higher effect on triglyceride levels as aripiprazole is metabolized by CYP3A [92]. Furthermore, *APOC3* rs4520 C/C homozygotes had higher triglyceride concentrations after OLA administration than T allele carriers, what is consistent with a previous study [305]. Polymorphisms in this gene influence serum or plasma triglyceride levels as the APOC3 protein raises plasma triglyceride levels by the inhibition of lipoprotein lipase, stimulates low-density lipoprotein secretion and intestinal triglyceride trafficking modulation [306]. *HTR2A* rs6314 C/C homozygotes had greater cholesterol levels during aripiprazole therapy. In a previous study the contrary was found: T carriers had higher cholesterol levels. However, Korean population when compared to Iberians, have a lower frequency of *HTR2A* rs6314 C allele (approximately 0.515 versus 0.893, respectively), therefore the frequency variation among different ethnic groups could cause this difference [307]. Our study is the first to report differences between *HTR2A* rs6314 alleles in cholesterol level changes during antipsychotic therapy.

5.4. Hepatic performance

In a previous study, aripiprazole elevated mildly, while olanzapine elevated greatly the transaminase levels (eg. GOT and GPT) [308]. The effects of aripiprazole on GGT and ALP levels were not reported to date. However, olanzapine was reported to increase GGT, ALP and bilirubin levels [308]. Based on our knowledge, our study is the first to report changes in hepatic enzyme and

bilirubin levels during short time antipsychotic treatment in healthy volunteers. In our study, GOT, GPT, GGT and ALP levels significantly decreased during aripiprazole treatment while GGT, bilirubin and ALP levels significantly decreased during olanzapine treatment. The observed decrease could be explained by the low carbohydrate diet during their stay [304]. Additionally, the albumin levels also decreased during aripiprazole and olanzapine treatment alike risperidone and clozapine, what suggest that these drugs have a negative impact on serum antioxidant protection [309]. In addition, none of these levels were outside of the reference range.

5.5. Haematological performance

Olanzapine may cause leukopenia [310], thrombocytopenia [311] and thromboembolism [312]. Aripiprazole only causes these conditions when co-administered with other CYP2D6 substrates [313]. Our study confirms that both antipsychotics cause significant decrease in platelet count and olanzapine additionally induces a decrease in leucocyte count. Additionally, hemoglobin levels were also decreased during treatment, what can be due to the several blood extractions throughout the study. The current study is the first to report that these alterations are detected immediately after starting treatment, although none of these levels were outside of the reference range.

5.6. Thyroid performance

The free T4 levels significantly increased after aripiprazole treatment. Aripiprazole drug label states that it can induce both hypo,- and hyperthyroidism. Nevertheless, the underlying mechanism is currently unknown [106]. In studies with quetiapine, only free T4 changes were detected, and not TSH [314], similar to our results. Additionally, compared to the aripiprazole group, the olanzapine group had higher TSH and lower free T4 levels after treatment. Olanzapine was associated with lower free T4 and higher TSH levels in patients compared to healthy controls in a previous study [315]. Our study is the first to report increase in free T4 levels after aripiprazole treatment in healthy volunteers.

5.7. Renal performance

Atypical antipsychotics can increase the risk to develop chronic kidney diseases through the elevation of urea and creatinine levels [316,317]. After the acute treatment with aripiprazole and olanzapine we could not see this effect. Uric acid levels decreased during haloperidol [318], but not risperidone or clozapine treatment [309]. In the current study, we observed that both aripiprazole and olanzapine reduced its levels during treatment, but the levels were normalized after discontinuing the drugs. This can be due to the controlled diet what subjects received throughout the study. Uric acid is one of the principal antioxidants in the human plasma, therefore its low levels may cause oxidative stress [309]. Based on the authors' knowledge, this is the first study to analyse uric acid alterations in acute aripiprazole and olanzapine treatment.

6. Effects on blood pressure, heart rate and corrected QT interval

Aripiprazole caused both hyper- and hypotension in previous studies. Hypertension was reported in case reports, and the elevated blood pressure dropped immediately after aripiprazole withdrawal [135]. In contrast, in other studies, dose-related hypotension was also reported: when aripiprazole was reduced to 5 mg/day [134] or discontinued [319], the blood pressure returned to normal range. On the contrary, olanzapine has little effects on the cardiovascular system, if any [320]. Nevertheless, it can cause orthostatic hypotension [321]. Bradycardia was also reported previously during olanzapine treatment [321,322].

In our study, aripiprazole decreased systolic (without reaching significance) and diastolic blood pressure on the first day of treatment, but this effect was not repeated on the rest of the days. The blood pressure lowering mechanism may be due to blocking the α1-adrenergic receptors. Moreover, its 5-HT_{2A} antagonism could induce vasodilation and its 5-HT_{1A} agonism could produce hypotension and bradycardia [323]. Regarding olanzapine, systolic and diastolic blood pressure and heart rate significantly decreased after the first dose. These changes could be explained by its α1-adrenergic antagonism [324]. These effects were only significant on the first day of treatment and progressively diminished on the following days (*Figure* 2) as tolerance was developed. It was reported previously that only the first dose of olanzapine caused hypotension and bradycardia [325]. Our study is the first to report this association with aripiprazole.

The mean QTc interval normally decreases with aripiprazole and the QTc prolongation risk is lower compared to other atypical antipsychotics [127]. The QTc interval was overall decreased in case reports and clinical trials including schizophrenic patients and healthy volunteers, however, QTc prolongation events were also discovered [127]. Our study confirms that aripiprazole induces QTc decrease what started on the second day of drug administration and was maintained forth until the last day. Therefore, based on our results, aripiprazole does not seem to cause QTc prolongation and consequently *Torsades de Pointes* and sudden cardiac death. Olanzapine does not induce QTc prolongation [126]. Based on the authors knowledge, the current study is the first to report QTc decrease. However, it was produced only on the first day of drug administration alike blood pressure and heart rate. To date, the clinical significance of its QTc-shortening is uncertain. Nevertheless, it may induce proarrhythmia [326].

6.1. The influence of polymorphisms on blood pressure and heart rate

Systolic and diastolic blood pressure decreased more in *ADRA2A* rs1800544 C/C subjects compared to C/G subjects during olanzapine and aripiprazole treatment, respectively. α_{2A}-adrenergic receptors have important roles in sympathetic cardiovascular regulation. Mice that do not express *ADRA2A* had increased blood pressure and heart rate [327]. Consequently, rs1800544 mutant allele carriers may have increased blood pressure compared to wild-type homozygotes.

Diastolic blood pressure decreased more in *HTR2A* rs6313 C allele carriers compared to T/T subjects during aripiprazole treatment. Carriers of the wild-type allele of *HTR2A* rs6313 could have induced vasodilatation, and therefore a decrease in blood pressure [323].

During olanzapine treatment, systolic blood pressure and heart rate decreased more in *DRD3* rs6280 Ser/Ser and Ser/Gly subjects compared to those with Gly/Gly genotype. Dopamine causes cardiac stimulation and therefore vasoconstriction and increase in blood pressure. Olanzapine blocks dopamine receptors, therefore less dopamine binds to them what can result in decreased blood pressure and heart rate [328]. D_3 dopamine receptor blockage depends on the genotype, what explains that carriers of the mutant genotype may have a less efficient interaction between the drug

and the receptor, causing smaller variations in blood pressure and heart rate.

In previous studies, *COMT* rs4680 A (Val, wild-type) allele carriers had lower systolic and diastolic blood pressure throughout the study [329,330]. Our study confirmed these findings: subjects with the COMT wild-type phenotype (including COMT rs4680) had significantly higher diastolic blood pressure decrease after olanzapine administration. However, another study found the opposite: the wild-type allele was associated with systolic blood pressure elevation [331]. Thus, there is no clear consensus about the role of *COMT* polymorphisms in its blood pressure-lowering capacity. Nevertheless, this association could be due to its role in modulating dopamine function [332].

Habitually, females show higher heart rate and QTc than males [333,334]. In our previous single-dose study both were higher in females after a single dose of aripiprazole [129]. In our current study we found the contrary with aripiprazole and olanzapine, however, as this difference could only be seen on the 4th day of drug administration, it may be considered an artefact.

7. Adverse drug reactions

7.1. Most common adverse drug reactions to aripiprazole

According to the drug label, the most common (≥5% and at least twice than placebo) ADRs to aripiprazole in schizophrenic patients are akathisia, extrapyramidal symptoms, somnolence and tremor [106]. Somnolence and akathisia were among the most frequent ADRs that we observed. The most common ADRs in clinical trials in healthy volunteers and psychotic patients were somnolence, nausea, vomiting, constipation, headache, dizziness, akathisia, anxiety, insomnia, and restlessness [106]. All these ADRs were detected in our clinical trial in 79, 17, 8, 13, 54, 21, 17, 8, 33 and 21% of the volunteers, respectively. Somnolence was detected in 79% of the subjects. There is no evidence on sex differences in the prevalence of ADRs [335]. Our study confirms this hypothesis. In general, the most common ADRs to aripiprazole were nervous system and psychiatric conditions.

7.2. Most common adverse drug reactions to olanzapine

According to the drug label, the most common (≥5% and at least twice than placebo) ADRs to olanzapine in schizophrenic patients are constipation, weight gain, dizziness, personality disorder, akathisia, postural hypotension, sedation, headache, increased appetite, fatigue, dry mouth and abdominal pain [90]. Constipation, dizziness, headache and dry mouth were among the most frequent ADRs that we observed in 13, 29, 13 and 13% of the volunteers, respectively. Somnolence was detected in all subjects, however, it was mostly present at the first 2 days of treatment and they developed tolerance to the drug on the following days. ADRs that we could not detect in our clinical trial were personality disorder, fatigue, sedation and abdominal pain. The absence of these findings could be due to the short treatment; the majority of these ADRs usually appear after at least 6 weeks of treatment [90]. There are possible sex differences in the prevalence of ADRs to olanzapine [336]. However, we did not see a difference between the two sexes in our study. The lack of association could be due to the short-time treatment and the low sample size.

7.3. Cardiac alterations

Palpitations are considered infrequent ADRs to aripiprazole [106]. However, 5 female volunteers and no males experienced it in our study. Palpitations may also occur during olanzapine treatment, however, they are not considered as frequent ADRs [337]. Our study confirms this finding; only 2 volunteers were registered with palpitations. Interestingly, both were females, similar to aripiprazole. We are the first to report these findings. Palpitations can be associated with arrhythmias. The proarrhythmic effect, drug-induced *Torsade de Pointes* occurs more frequently in females than in males [338] and was observed with aripiprazole [127], but not with olanzapine [339]. Therefore, as in the current study, palpitations, a proarrhythmic sign, may occur more frequently in females.

In addition, during olanzapine treatment palpitations were only reported in CYP1A2 UMs and *HTR2A* rs7997012 A allele carriers. Olanzapine is metabolized by CYP1A2 [92]. UMs may reach high concentrations of olanzapine metabolites rapidly, which could be related to the development of palpitations. Additionally, palpitations were only reported in *HTR2A* rs7997012 mutant (A)

allele carriers. Serotonin can induce the development of palpitations [340]. Therefore, A allele carriers may have reduced 5-HTR2A blockage and consequently higher serotonin levels.

7.4. Nervous system alterations

Akathisia is commonly associated with first generation antipsychotics. It would be expected that aripiprazole had a low incidence of akathisia being an antagonist at 5-HT2A receptors [341]. However, aripiprazole seems to increase the risk of akathisia. Therefore, its pathophysiology seems complex, involving several neurotransmitters including dopamine, acetylcholine, □-aminobutyric acid, norepinephrine, serotonin and neuropeptides [342]. Consequently, *DRD3* mutant (Gly) allele carriers could be more protected from developing akathisia. The fact that *DRD2* rs1799732 G/- subjects experienced asthenia but not G/G homozygotes strengthens the dopamine theory: polymorphisms in its receptors seem to have a role in developing nervous system ADRs. On the contrary, olanzapine does not cause akathisia [341] what was confirmed in our study. It appears that its sedating properties could be responsible for attenuating the effects of akathisia [341].

5-HT receptors are related to the development of somnolence and headache during antipsychotic treatment, but the molecular background is unknown to date [343,344]. Aripiprazole relates to a lower risk of somnolence and headache compared to other atypical antipsychotics [343], however, they are still among the most common ADRs [106]. 100% and 79% of the volunteers experienced somnolence at least once during the study with aripiprazole and olanzapine, respectively. Somnolence was detected more frequently in *HTR2A* rs6314 wild-type subjects while headache was observed more in mutant allele carriers of *HTR2C* rs3813929. Our study is the first to report these findings. It strengthens the hypothesis that 5-HT receptor variability can lead to the development of these ADRs.

In our previous study, *OPRM1* rs1799971 mutant (G) allele carriers were associated with the increased likelihood of somnolence to fentanyl in healthy volunteers [345]. In the current study we observed the contrary: wild-type (A/A) homozygous subjects developed somnolence more frequently during aripiprazole treatment. The G variant is reported as a protective allele against ADRs [346]. However, its role in developing ADRs to aripiprazole is currently unknown. Opioid

receptor activation inhibits GABAergic interneurons in order to increase dopamine release [347]. Therefore, higher dopamine concentration in *OPRM1* rs1799971 wild-type homozygotes may increase the risk of somnolence.

In addition, CYP1A2 NM/RM subjects showed a higher prevalence of insomnia during ARI treatment than UM subjects. Based on current knowledge, aripiprazole is not metabolized by CYP1A2 [106]. However, in our population, CYP1A2 UMs showed lower ARI and DARI disposition compared to the other phenotypes [298]. Therefore, NM/RM subjects were under prolonged aripiprazole exposure what could cause the development of insomnia. Regarding olanzapine, insomnia is not among the most common ADRs to olanzapine [90]. However, 2 volunteers experienced it during our study, both carrying *HTR2C* rs1414334 G/G genotype. The lack of serotonin can cause insomnia [348]. *HTR2C* rs1414334 mutant (G/G) homozygotes may have less 5-HTR2C receptor blocking effect and consequently higher risk to experience insomnia.

7.5. Gastrointestinal alterations

Serotonin and acetylcholine activate the colonic smooth muscles inducing their contraction. Olanzapine, being a 5-HT antagonist, inhibits their contraction and consequently causes constipation [349]. Carriers of *HTR2A* rs6314 and rs7997012 mutant alleles (T and A, respectively) displayed a higher incidence of constipation, therefore, in these subjects, OLA possibly has higher binding affinity to 5-HT2A receptors. In our previous study, the prevalence of fatigue in *UGT1A1* rs887829 T/T subjects was significantly higher compared to wild-type (C) allele carriers [173]. In the current study, we found the same association, but with constipation. This enzyme may be responsible for the phase II metabolism of olanzapine [298]. These associations were not found with aripiprazole what we expected as it is not metabolized by the UGT enzyme family. However, similar to olanzapine, it is a 5-HT2A antagonist. Nevertheless, this mechanism should be more complex; dopamine or other neurotransmitters could have a role in the development of constipation.

Normally, a balance is maintained between acetylcholine and dopamine. When this balance is disturbed, acetylcholine levels increase, while dopamine levels decrease [350]. Dry mouth is an

anticholinergic side effect of olanzapine, but not to aripiprazole given its special mechanism of action: olanzapine blocks muscarinic receptors but not aripiprazole [95,96]. *DRD3* rs6280 wild-type (Ser/Ser) subjects may have higher dopamine level due to the low amount of acetylcholine, therefore experiencing dry mouth after olanzapine.

Nausea during olanzapine treatment was only detected in *HTR2C* rs518147 C/T heterozygotes and not in C/C and T/T homozygotes what can be due to the low sample size. This association was not found with aripiprazole.

8. Study limitations

Only 24 subjects were included in the study, what we consider its main limitation. Therefore, it is important to interpret these results with caution: studies including more subjects are necessary to increase the statistical reliability of the results. Moreover, the present study should be repeated in schizophrenic patients, whose brain structure and genetics may differ from healthy volunteers, to demonstrate the clinical utility of these results.

Moreover, neither aripiprazole, nor olanzapine reached steady state during 5 days of treatment. Both could have had a greater effect on autonomic nerve activity and metabolism if they had reached steady-state. Likewise, several ADRs could appear later than 5 days. However, the Research Ethics Committee would not have authorized a treatment longer than 5 days with antipsychotics in healthy volunteers. Additionally, Cl/F and Vd/F values were calculated without knowing bioavailability, which can yield questionable results, especially for dehydro-aripiprazole.

Nevertheless, despite of applying the Bonferroni post hoc test to each analysis, some of our results could be false positives due to the high number of analysed variables. Nevertheless, we have very well controlled conditions what can reduce the influence of other factors, such as comorbidity, smoking and nutrition. In addition, we administered both drugs to each volunteer which make them perfectly comparable.

VI. CONCLUSIONS

Analytical method and pharmacokinetics:

1) Our LC-MS/MS method was validated based on the recommendations from regulatory guidelines and it is adequate for precise and accurate simultaneous measurement of antipsychotic drugs and caffeine in human plasma.
2) Simple sample preparation and three step μ-SPE offer high phospholipid removal efficiency compared to PPT.
3) Pharmacokinetics of aripiprazole and dehydro-aripiprazole were affected by sex. Females had higher $T_{1/2}$ and Vd/F of aripiprazole; higher Vd/F and Cl/F and lower AUC_{last} of dehydro-aripiprazole compared to males.
4) Pharmacokinetics were significantly affected by polymorphisms in *CYP2D6*, *CYP3A*, *CYP1A2*, *ABCB1* and *UGT1A1* genes.

Pupillometry:

5) Aripiprazole, but not olanzapine produced pupil dilatation after the first dosing, which was followed by constriction after each day of treatment.
6) The effects of aripiprazole on the pupil were associated with polymorphisms in *CYP3A*, *HTR2A*, *UGT1A1*, *DRD2* and *ABCB1* genes.
7) Pupillography could be a non-invasive tool to assess autonomic nervous system activity during antipsychotic drug treatment.

Metabolic effects:

8) Olanzapine caused significant prolactin elevation, but not aripiprazole, which was influenced by *CYP3A*, *ABCB1* and *DRD3* polymorphisms.
9) Aripiprazole caused C-peptide elevation what was dependent on *COMT* genotypes, while the C-peptide/ insulin ratio was higher after olanzapine treatment.
10) Glucose levels in GTT were higher after olanzapine treatment and were influenced by *UGT1A1* genotypes.
11) Insulin levels did not change with any treatment, but were dependent on *BDNF* genotypes and CYP3A phenotypes.

12) Olanzapine, but not aripiprazole caused weight gain, which was influenced by *HTR2C* alleles.
13) Uric acid, triglyceride and cholesterol levels decreased during aripiprazole and olanzapine treatment.
14) Although both antipsychotics had significant metabolic effects in acute treatment, we can confirm that aripiprazole has a more benign metabolic profile compared to olanzapine.

Safety:

15) Olanzapine had more cardiovascular effects than aripiprazole. However, blood pressure, heart rate and QTc decreased significantly only on the first day of drug administration. Therefore, it seems that a rapid tolerance was developed to the drug.
16) *HTR2A*, *ADRA2A*, *DRD3* and *COMT* polymorphisms establish the interindividual variability of the cardiovascular effects of aripiprazole and olanzapine.
17) More adverse drug reactions were related to aripiprazole than to olanzapine, especially psychiatric and nervous system disorders.
18) Some polymorphisms in *HTR2A*, *HTR2C*, *DRD2*, *DRD3*, *OPRM1*, *UGT1A1* and *CYP1A2* genes may explain the difference in the incidence of adverse drug reactions among subjects.

Método analítico y farmacocinética:

1) Nuestro método LC-MS/MS fue validado de acuerdo a las recomendaciones regulatorias y es adecuado para la medición simultánea precisa y exacta de fármacos antipsicóticos y cafeína en el plasma humano.
2) La simple preparación de la muestra y los tres pasos de μ-SPE ofrecen una alta eficiencia de eliminación de fosfolípidos en comparación con el PPT.
3) La farmacocinética del aripiprazol y del dehidro-aripiprazol se vio afectada por el sexo. Las mujeres tenían una mayor $T_{1/2}$ y Vd/F de aripiprazol; una mayor Vd/F y Cl/F y una menor AUC_{last} de dehidro-aripiprazol en comparación con los hombres.
4) La farmacocinética se vio afectada significativamente por los polimorfismos en los genes *CYP2D6*, *CYP3A*, *CYP1A2*, *ABCB1* y *UGT1A1*.

Pupilometría:

5) El aripiprazol, pero no la olanzapina, produjo dilatación de la pupila después de la primera dosis, a la que siguió una constricción después de cada día de tratamiento.
6) Los efectos del aripiprazol en la pupila se asociaron con polimorfismos en los genes *CYP3A*, *HTR2A*, *UGT1A1*, *DRD2* y *ABCB1*.
7) La pupilografía podría ser una herramienta no invasiva para evaluar la actividad del sistema nervioso autónomo durante el tratamiento con fármacos antipsicóticos.

Efectos metabólicos:

8) La olanzapina causó una importante elevación de la prolactina, pero no el aripiprazol, que fue influenciado por los polimorfismos en *CYP3A*, *ABCB1* y *DRD3*.
9) El aripiprazol causó una elevación del péptido C, lo que dependía de los genotipos de la *COMT*, mientras que la relación péptido C/ insulina fue mayor después del tratamiento con olanzapina.
10) Los niveles de glucosa en el GTT fueron más altos después del tratamiento con olanzapina y se vieron influidos por los genotipos del *UGT1A1*.
11) Los niveles de insulina no cambiaron con ningún tratamiento, pero dependieron de los genotipos del *BDNF* y los fenotipos del *CYP3A*.

12) La olanzapina, pero no el aripiprazol, causó un aumento de peso, que fue influenciado por los alelos *HTR2C*.
13) Los niveles de ácido úrico, triglicéridos y colesterol disminuyeron durante el tratamiento con aripiprazol y olanzapina.
14) Aunque ambos antipsicóticos tuvieron efectos metabólicos significativos, podemos confirmar que el aripiprazol tiene un perfil metabólico más benigno en comparación con la olanzapina.

Seguridad:

15) La olanzapina tuvo más efectos cardiovasculares que el aripiprazol. Sin embargo, la presión arterial, la frecuencia cardíaca y el QTc disminuyeron significativamente sólo en el primer día de administración del medicamento. Por lo tanto, parece que se desarrolló una rápida tolerancia al fármaco.
16) Los polimorfismos en *HTR2A*, *ADRA2A*, *DRD3* y *COMT* establecen la variabilidad interindividual de los efectos cardiovasculares del aripiprazol y la olanzapina.
17) Más reacciones adversas se relacionaron con el aripiprazol que con la olanzapina, especialmente trastornos psiquiátricos y del sistema nervioso.
18) Algunos polimorfismos en los genes *HTR2A*, *HTR2C*, *DRD2*, *DRD3*, *OPRM1*, *UGT1A1* y *CYP1A2* pueden explicar la diferencia en la incidencia de las reacciones adversas entre los sujetos.

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ANNEXES

Publications related to the Doctoral Thesis

Article 1: Simultaneous determination of six antipsychotics, two of their metabolites and caffeine in human plasma by LC-MS/MS using a phospholipid-removal microelution-solid phase extraction method for sample preparation.

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Simultaneous determination of six antipsychotics, two of their metabolites and caffeine in human plasma by LC-MS/MS using a phospholipid-removal microelution-solid phase extraction method for sample preparation

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ABSTRACT

A simple and sensitive liquid chromatography-tandem mass spectrometry method was developed and validated in human plasma for the simultaneous determination of aripiprazole (ARI) and its metabolite dehydro-aripiprazole (DARI); olanzapine (OLA), risperidone (RIS), paliperidone (PAL), quetiapine (QUE), clozapine (CLO) and caffeine (CAF). CAF is included to the method because it can have an influence on drug metabolism due to competitive inhibition. The above mentioned compounds and their isotope-labeled internal standards were extracted from 200 µL human plasma samples by both, effective phospholipids-eliminating three-step microelution-solid-phase extraction (µ-SPE) and protein precipitation (PPT) for comparison. A combination of formic acid (0.2%)-acetonitrile (pH 3.0; 65:35, v/v) was used as mobile phase and the chromatogram was run under gradient conditions at a flow rate of 0.6 mL/min. Run time lasted 6 min, followed by a re-equilibration time of 3 min. All analytes were monitored by mass spectrometric detection operating in multiple reaction monitoring mode and the method was validated covering the corresponding therapeutic ranges: 0.18–120 ng/mL for ARI, 0.25–80 ng/mL for DARI, 1.00–100 ng/mL for OLA, 0.70–60 ng/mL for RIS, 0.20–30 ng/mL for PAL, 0.50–160 ng/mL for QUE, 0.50–1000 ng/mL for CLO, and finally 1200–3700 ng/mL for CAF. The method was validated based on the recommendations of regulatory agencies through tests of precision, accuracy, extraction recovery, identity confirmation, trueness, matrix effect, process efficiency, stability, selectivity, linearity and carry-over effect fulfilling the guideline requirements. Our µ-SPE method results in the elimination of more than 99% of early eluting and more than 92% of late-eluting phospholipids compared to PPT. Additionally, the method was successfully applied for quantifying ARI and OLA plasma concentrations from healthy volunteers.

1. Introduction

Aripiprazole (ARI), olanzapine (OLA), risperidone (RIS), paliperidone (PAL; 9-hydroxyrisperidone), quetiapine (QUE) and clozapine (CLO) are commonly used atypical antipsychotics with demonstrated efficacy in schizophrenia and bipolar disorder [1]. PAL is not only a parent compound, but also a metabolite of RIS [2]. These denominated second generation antipsychotics are effective in the treatment of

Abbreviations: CAN, acetonitrile; ARI, aripiprazole; AUC_{0-t}, area under the concentration-time curve from time 0 until the last measurable concentration; CAF, caffeine; CAL, calibration standard; CID, collision-induced dissociation; CLO, clozapine; C_{max}, maximum plasma concentration; CV, coefficient of variation; CYP, cytochrome P450; DAD, diode array detection; DARI, dehydro-aripiprazole; EDTA, ethylenediaminetetraacetic acid; EMA, European Medicines Agency; ESI, electrospray ionization; FDA, US Food and Drug Administration; HLB, hydrophilic-lipophilic balance; HPLC, high-performance liquid chromatography; ICH, International Conference on Harmonization; IS, internal standard; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLE, liquid-liquid extraction; LLOQ, lower limit of quantification; LPC, lysophophatidylcholine; ME, matrix effects; MeOH, methanol; MRM, multiple-reaction monitoring; MS, mass spectrometry; OLA, olanzapine; PAL, paliperidone, 9-hydroxyrisperidone; PC, phosphatidylcholine; PE, Process efficiency; PFP, pentafluorophenyl; PPT, protein precipitation; QC, quality control; QUE, quetiapine; R^2, correlation coefficient; RE, Extraction recovery; RIS, risperidone; SD, standard deviation; SEM, standard error of mean; SIL-IS, stable isotopically labeled internal standards; SLE, supported liquid extraction; SPE, solid phase extraction; SRM, selected ion monitoring; TIC, total ion chromatogram; T_{max}, time of occurrence of C_{max}; $T_{1/2}$, half-life; tR, retention time; UHPLC-MS/MS, ultra-high performance liquid chromatography-tandem mass spectrometry; UV, ultraviolet; XIC, extraction ion chromatogram

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schizophrenia - including both positive and negative symptoms - and are less likely to produce extrapyramidal symptoms and tardive dyskinesia when compared to first generation antipsychotics [1]. These six drugs were included in the analysis based on their importance in the worldwide market. The metabolite of ARI, dehydro-aripiprazole (DARI) was also selected, because it shows pharmacological activity similar to ARI.

The metabolism of CLO and OLA is mainly dependent on cytochrome P450 isoenzyme 1A2 (CYP1A2) [3], similar to CAF, which is a central nervous system stimulant [4]. It was shown that CAF increases the plasma levels of these drugs due to CYP1A2 competitive inhibition [4]. Therefore, CAF was added to the method to be able to monitor its effect on their metabolism. To date, there is no documented record on the simultaneous determination of these antipsychotics along with CAF in a single-run process.

In a clinical environment, the analysis of antipsychotics in blood is necessary in order to monitor patient compliance and to maintain drug concentrations within the recommended therapeutic range. Therapeutic drug monitoring (TDM) of several antipsychotics is already introduced into the clinical practice. Based on controlled clinical trials and clinical data, it is 1) strongly recommended for OLA and CLO, 2) recommended for ARI, RIS and PAL, and 3) useful for QUE [5].

Several methods are available in the bibliography about the quantification of a single compound with its related metabolite [6,7]. Simultaneous quantification of various psychotropic drugs were reported as well [8,9]. Numerous methods are applied to determine these drugs in human plasma using high performance liquid chromatography (HPLC, hereafter LC) coupled to ultraviolet (UV) detector (LC-UV) [10] or tandem mass spectrometry (LC-MS/MS) [6]. However, LC-MS/MS allows to achieve shorter run times and better lower limits of quantification (LLOQs).

Despite improved sensitivity and selectivity in LC-MS/MS, matrix components may cause ion suppression or enhancement of the analyte, especially when electrospray is used as the ionization source (electrospray ionization, ESI) [11]. The evaluation of matrix effects (ME) in quantitative drug analysis in plasma is an important aspect of assay validation. ME should be investigated in each analysis, despite of using stable isotope-labeled internal standards (SIL-ISs), as they might not compensate all ME. Endogenous phospholipids often cause ion suppression, therefore less efficient extraction recovery (RE), increased variability and irreproducibility of the analytical method. Blood contains several subtypes of phospholipids from which phosphatidylcholine (PC), sphingomyelin and lysophosphatidylcholine (LPC) account for 70% [12], 20% [12] and 8% [13], respectively. Phospholipids impair not only the ionization process but the chromatographic separation as well. Highly abundant PCs and LPCs with the polar head groups of strong ionic characteristics are eluted later in a reverse-phase C18 column when high percentage of organic solvent is applied. They are known to cause broad analyte peaks with tailing, by the late elution of their apolar hydrocarbon chains. On the contrary, the early eluted polar PCs and LPCs influence the early eluted analytes [14]. Therefore, they should be eliminated from plasma during the extraction process in order to obtain reliable results and to preserve the instrument clean.

Process efficiency (PE) represents the combination of ME and RE of the analyte from the matrix by the sample extraction process [15]. Its low value can be pernicious to the accuracy and the LLOQ of a particular method [16].

Despite the advances in separation and quantitation techniques, several sample preparation procedures are based on traditional technologies such as protein precipitation (PPT) and liquid-liquid extraction (LLE) [17]. PPT is the simplest and fastest method for sample preparation. However, it does not lead to a completely clean extract. PPT often causes ion suppression in ESI, since this method does not sufficiently remove endogenous compounds such as lipids, phospholipids and fatty acids. Moreover, LLE procedure is longer requiring multiple extraction steps to increase analyte recovery and to obtain cleaner extracts [11]. Compared to the sample preparation methods mentioned previously, solid phase extraction (SPE) offers reduced processing time, lower solvent consumption and it can be considered more environment friendly. Furthermore, SPE enables to enrich the concentration of target compounds and allows utilizing a smaller quantity of sample [17].

Our aim was to develop a simple LC–MS/MS method based on effective phospholipids' removal three-step microelution-SPE (μ-SPE) sample preparation process that can determine ARI, DARI, OLA, RIS, PAL, QUE, CLO and CAF using a small sample quantity −200 μL– while comparing it to PPT. According to our knowledge, apart from our previous study measuring ARI and DARI levels [6], none of the analytical methods designed for the monitorization of antipsychotic drugs compared the phospholipids' elimination efficacy of different extraction methods nor included CAF.

2. Materials and methods

2.1. Chemicals and reagents

ARI, DARI and [2H8]-aripiprazole (ARI-D8) were provided by Toronto Research Chemicals (North York, Canada) and OLA, RIS, PAL, QUE and CLO and their internal standards [13C,2H3]-olanzapine (OLA-C1-D3), [2H4]-risperidone (RIS-D4), [2H4]-paliperidone-D4 (PAL-D4), [13C4]-quetiapine (QUE-C4) and [13C,2H3]-clozapine (CLO-C1-D3) were provided by AlsaChim (Illkirch Graffenstaden, France). Ultrapure water was acquired from a Milli-Q® Water Purification System (Millipore-Ibérica, Madrid, Spain). Acetonitrile (ACN), methanol (MeOH), ammonia and ammonia hydroxide 5 N solutions in gradient HPLC grade were provided by SYMTA (Madrid, Spain). Formic acid was bought from Sigma-Aldrich (Madrid, Spain). All the used chemicals were of analytical grade. Plasma samples for the preparation of calibration and validation standards were supplied by the Transfusion Center of "Comunidad Autónoma de Madrid" (Madrid, Spain).

2.2. Preparation of calibration standards and quality controls

Stock solutions of ARI, DARI and ARI-D8 were dissolved in MeOH containing 0.5% formic acid solution, while OLA and OLA-C1-D3 were supplied in ACN solution with 0.1% formic acid. RIS and RIS-D4 were dissolved in MeOH-water 1:1 solution with 0.1% formic acid. PAL and PAL-D4 were prepared in 100% ACN solution while QUE, QUE-C4, CLO and CLO-C1-D3 were dissolved in 100% MeOH solution. The concentration of all standard solutions was 1 mg/mL. Working solutions of each analyte were prepared in MeOH and 0.1% formic acid by dilutions at the following concentrations from each stock solution: 0.1 mg/mL, 0.01 mg/mL and 0.001 mg/mL. All stock and working solutions were kept at −80 °C.

The calibration standards (CALs) and the quality controls (QCs) were prepared by independent dilutions method of each stock solution and spiked to blank plasma samples. Entirely 8 CALs and 4 QCs (LLOQ; low, Low QC; medium, Medium QC and high, High QC) were made. The used concentrations were calculated based on the therapeutic ranges of each drug described in the literature (summarized in Supplementary Tables 1 and 2). A blank and a zero (IS added) plasma sample processed without drugs were included to the analysis according to the recommendations for bioanalytical method validation of the US Food and Drug Administration (FDA) [18], the European Medicines Agency (EMA) [19] and the International Conference on Harmonization (ICH) [20]. Similarly to stock and working solutions, CALs, QCs, and ISs were kept in −80 °C until utilization.

2.3. Equipment

The chromatographic separation was performed with an HPLC system consisting a 1200 Series separation module (Agilent Technologies, Madrid, Spain) and was coupled to a triple quadrupole

mass spectrometer (Agilent Technologies 6410B), with positive mode ESI. The instrument was controlled by the Agilent MassHunter Workstation Data Acquisition software. For separating the drugs an ACE C18-PFP (pentafluorophenyl) column (3 µm, 4.6 × 100 mm; SYMTA, Madrid, Spain) maintained at 25 °C was used. The flow rate was 0.6 mL/min. The mobile phase was a mixture of formic acid (0.2%, solvent A) and ACN (solvent D) (65:35, v/v). The final pH was 3.0 adjusted with 5 mol/L ammonium hydroxide. The chromatogram was run with the following gradient conditions: initial conditions: 60% of A and 40% of D from 0 to 1.0 min, 60% D maintained from 1.0 to 1.8 min, D increased to 65% from 1.8 to 2.5 min, 65% D kept from 2.5 to 2.7 min, D increased to 99% from 2.7 to 3.5 min, 99% D maintained from 3.5 to 4.0 min and finally, returned to the initial conditions (60% A and 40% D) from 4.0 to 6.0 min. Afterwards, the chromatogram was followed by a re-equilibration time of 3.0 min. Five µL volume was injected in the HPLC system.

Analytes were quantified in dynamic multiple reaction monitoring (MRM) mode. All results were based on the peak area ratio between the drug and the IS. The MS conditions were as follows: desolvation gas flow and temperature 12 L/min and 350 °C, respectively, nebulizer pressure 60 psi, capillary voltage 3 kV. Highly pure N_2 (> 99.9995) was used as MS collision gas. For all the compounds, fragmentor voltage and collision energy were set using selected ion monitoring (SRM) for each transition at specific retention time (tR) (Table 1). A confirmation transition was also monitored to acquire more specific results. Additionally, *m/z* 184 > 184 and 104 > 104 as common in-source collision-induced dissociation (CID) ion fragments produced by endogenous phospholipids were measured [21]. Moreover, *m/z* 524.4 > 184.1, 524.4 > 104.1, 496.4 > 184.1 and 496.4 > 104.1 produced by late-eluting phospholipids were analyzed. Furthermore, product ion qualifier ratio was defined along with tR-, relative tR-, and ion ratio identity confirmation according to SANTE/11813/2017 and 2002/657/EC COMMISSION DECISION guidelines [22,23]. The acceptance criteria were the following: retention time difference between extracted analyte and neat solution of the analyte should be lower than 0.1 min [22], relative tR difference between extracted analyte and neat solution of the analyte should be lower than 2,5% [23], and ion ratio difference between CALs and QCs (samples) should not differ more than 30% [22], respectively. MassHunter Workstation Quantitative Analysis software (Agilent Technologies, Madrid, Spain) was used to quantify the concentrations based on MRM transitions of each analyte.

2.4. Sample preparation

2.4.1. Plasma samples were extracted by both SPE and PPT

For PPT, 200 µL plasma sample was spiked with 10 µL IS and 800 µL precipitating agent, ACN with 0.1% formic acid (4:1, v/v) and centrifuged at 14,000 rpm at 4 °C for 5 min. Afterwards, the supernatant was evaporated using a concentrator (5301, Eppendorf, Germany) at 45 °C for 75 min. Consequently, the dry residue was reconstituted with 200 µL ACN/MeOH/buffer (formic acid, 0.2% at pH 3.0) reconstitution solution (8:1:1, v/v/v).

Three steps were applied in the µ-SPE: sample loading, washing and elution. Initially, 10 µL IS was added to 200 µL of plasma sample along with 290 µL of 0.2% formic acid in water, pH 1.5, and loaded (2 × 250 µL) into the Oasis PRiME HLB (hydrophilic-lipophilic balance) 96-well µElution Plate (Waters, Madrid, Spain). It was followed by the washing step adding 400 µL (2 × 200 µL) 5% MeOH solution with water and 2% ammonia. After each step, a 5–15 mmHg vacuum was applied until the wells were dry. Then, the compounds were eluted with 200 µL (2 × 100 µL) ACN/MeOH/buffer (formic acid, 0.2% at pH 3.0) solution (8:1:1, v/v/v) and collected in a 1 mL 96-well plate (Agilent Technologies, Santa Clara, USA).

Finally, 5 µL of eluate was injected directly to the LC-MS/MS system. SPE was chosen as sample preparation process for method validation.

2.5. Method validation

The validation of this method was based on the guidelines of FDA [18], EMA [19] and ICH [20]. Six validation series were assessed to determine linearity, precision and accuracy, eight for selectivity, matrix effects, process efficiencies, while 4 validation series were used for different types of stabilities based on the peak area ratio of drug and IS.

2.6. Linearity

CALs were measured in duplicate at the concentrations shown in Supplementary Table 1 covering the plasma therapeutic ranges and expected patients' plasma concentrations (see Supplementary Table 2).

Table 1
Relevant LC–MS/MS characteristics.

Compound	Retention time (tR, min)	Quantification SRM Transition (*m/z*)	Confirmation SRM Transition (*m/z*)	Qualifier ratio	Fragmentor voltage (V)	Collision energy (eV)
ARI	4.750	448.2 > 285.2	448.2 > 176.2	30.10	100	25
ARI-D8	4.790	456.2 > 293.0			110	30
DARI	4.439	446.2 > 285.0	446.2 > 188.0	0.16	125	22
OLA	1.941	313.2 > 256.2	313.2 > 282.2	10.51	90	25
OLA-C1-D3	1.940	317.3 > 256.2			110	25
RIS	3.373	411.3 > 191.2	411.3 > 110.1	6.47	50	30
RIS-D4	3.405	415.3 > 195.2			90	30
PAL	2.945	427.3 > 207.2	427.3 > 110.2	33.10	110	30
PAL-D4	2.944	431.3 > 211.2			100	30
QUE	3.945	384.3 > 253.2	384.3 > 221.2	45.70	110	35
QUE-C4	3.969	388.3 > 255.2			110	35
CLO	4.029	327.2 > 270.1	331.2 > 270.2	59.54	130	25
CLO-C1-D3	4.029	327.2 > 192.1			110	25
CAF	2.001	195.2 > 138.2	195.2 > 110.2		100	20
PC	1.485	184.1 > 184.1			100	5
LPC	1.537	104.1 > 104.1			100	10
LPC 16:0	8.348	496.4 > 184.1	496.4 > 104.1		135	30
LPC 18:0	8.186	524.4 > 184.1	524.4 > 104.1		135	30

Abbreviations: SRM: selected reaction monitoring; *m/z*: Mass-to-Charge; ARI: aripiprazole; ARI-D8: [2H8]-aripiprazole; DARI: dehydro-aripiprazole; OLA: olanzapine; OLA-C1-D3: [13C,2H3]-olanzapine; RIS: risperidone; RIS-D4: [2H4]-risperidone; PAL: paliperidone; PAL-D4: [2H4]-paliperidone-D4; QUE: quetiapine; QUE-C4: [13C4]-quetiapine; CLO: clozapine; CLO-C1-D3: [13C,2H3]-clozapine; CAF: caffeine; PC: phosphatidylcholine; LPC: lysophosphatidylcholine; LPC 16:0: lysophosphatidylcholine 16:0; LPC 18:0: lysophosphatidylcholine 18:0. Qualifier ratio: the peak area ratio quantifier/qualifier.

Six calibration curve replicates at the 8 concentration levels were analyzed from different days. To quantify the CAL data, a linear regression model was applied based on the analyte versus IS peak area ratio. In order to meet the validation criteria, the error of accuracy and relative standard deviation (RSD, %) should not exceed 15% for each CALs corresponding to each drug. The final estimated linearity model was verified using the lack-of-fit test, to confirm that the selected regression and linearity are appropriate.

LLOQ was defined as the lowest point of the calibration curve (see Supplementary Table 1) which can be quantified with acceptable precision and accuracy. Based on this practice, for LLOQ, the error of accuracy and RSD should not exceed 20%. Therefore, the extraction ion chromatogram (XIC) peak area ratio of each analyte should be at least 5 times higher than the blank.

2.7. Selectivity

Method selectivity was ascertained by analyzing 8 different blank plasma samples from human donors. These samples were extracted and injected at the beginning of the HPLC analysis to exclude any endogenous interference. In addition, 8 zero samples (blank sample with IS) were analyzed to verify the absence of analyte ions in the respective peaks of IS. The method is considered selective when the blank plasma matrix XIC peak area ratio is less than 20% of the XIC peak area of LLOQ in plasma matrix for each drug, and less than 5% for the ISs.

2.8. Precision (repeatability and intermediate precision) and accuracy

QC samples 'LLOQ', 'Low QC, 'Medium QC' and 'High QC' were prepared at concentrations described in Table 2. Six samples of each QC concentration were measured over a period of 4 following days. The determined concentrations were in the ranges of CALs. Repeatability and intermediate precision were evaluated by analyzing a single analytical run in a single day and 3 analytical runs from 3 different days, respectively. The precision was defined as the relative standard deviation (RSD, %) and determined at each concentration level. The acceptance limit was < 15%, except for LLOQ (< 20%). The accuracy describes the closeness of mean test results obtained by the method to the theoretical concentration of the analyte.

2.9. Extraction recovery, matrix effect and process efficiency

RE was defined as the ratio of analyte response between plasma spiked before (Pre) and after (Post) the extraction process. RE can be defined as relative: the ratio of the compound concentration; or absolute: the ratio of the XIC peak area.

ME was established as quantitative approach comparing the XIC peak area ratio (absolute) or the compound concentration ratio (relative) between Post-plasma and neat solution, which was obtained by the addition of the same amount of analyte to the final elution solution [ACN/MeOH/buffer, pH 3.0 (8:1:1, v/v/v)] without undergoing the extraction process.

PE values were calculated as the ratio of analyte response between plasma spiked before (Pre) the SPE process and neat solution. Absolute and relative values were determined in the same fashion than for RE and ME.

In each test, 8 repetitions of Low QC and High QC were analyzed in blank plasma samples from 8 different human donors for all analytes.

Table 2
Repeatability and intermediate precision and accuracy values. Precision values are expressed as the percentage of the relative standard deviation (RSD, %) for repeated measurement from one day and as pooled RSD (%) from 4 consecutive days. Accuracy is shown as the percentage of the closeness of theoretical concentration to the measured value present in the matrix. The corresponding concentrations are displayed as the mean ± standard deviation (SD) of the number of total experiments (n).

Compound	Concentration (ng/mL)	Repeatability n = 6 Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)	Intermediate precision n = 24 Mean ± SD (ng/mL)	Pooled RSD (%)	Accuracy (%)
ARI	0.18	0.17 ± 0.01	0.07	− 3.27	0.19 ± 0.02	7.69	2.56
	1.00	1.12 ± 0.08	0.07	12.33	1.08 ± 0.06	5.18	8.17
	60	58.84 ± 1.34	2.29	− 1.93	59.59 ± 2.00	2.83	− 0.69
	100	108.00 ± 2.60	2.41	8.00	112. 86 ± 9.24	6.91	12.86
DARI	0.25	0.25 ± 0.01	3.72	0.75	0.25 ± 0.02	6.89	0.64
	0.90	0.85 ± 0.04	4.11	− 5.23	0.86 ± 0.05	6.02	− 4.87
	40	42.03 ± 0.72	1.72	5.07	41.73 ± 1.97	4.71	4.32
	70	68.85 ± 1.44	2.10	− 1.64	66.28 ± 3.18	4.80	− 5.31
OLA	1.00	1.05 ± 0.03	3.32	5.09	1.08 ± 0.18	9.43	7.54
	3.00	3.01 ± 0.40	13.16	0.33	3.27 ± 0.40	9.42	9.00
	50	47.28 ± 0.99	2.08	− 5.43	51.04 ± 4.59	2.90	2.09
	80	79.14 ± 1.98	2.50	− 1.08	75.94 ± 5.15	3.15	− 5.07
RIS	0.70	0.73 ± 0.02	3.20	4.89	0.61 ± 0.10	8.08	− 12.33
	1.50	1.68 ± 0.07	3.98	12.01	1.71 ± 0.10	5.73	13.70
	25	25.02 ± 0.56	2.22	0.07	27.79 ± 0.91	2.51	3.14
	50	48.79 ± 1.25	2.56	− 2.41	46.06 ± 3.07	2.37	− 7.88
PAL	0.20	0.24 ± 0.03	13.04	19.15	0.24 ± 0.04	16.51	17.58
	1.60	1.67 ± 0.05	3.19	4.39	1.70 ± 0.08	3.68	6.27
	15	14.77 ± 0.98	6.63	− 1.55	14.71 ± 0.64	3.71	− 1.92
	25	23.11 ± 0.55	2.36	− 7.56	22.71 ± 1.09	2.14	− 9.16
QUE	0.50	0.48 ± 0.04	7.58	− 3.65	0.45 ± 0.05	8.11	− 10.69
	1.50	1.67 ± 0.10	5.93	11.16	1.69 ± 0.10	5.37	12.74
	80	89.96 ± 1.84	2.04	12.45	91.21 ± 6.16	6.95	14.00
	130	123.47 ± 2.20	1.78	− 5.03	133.26 ± 11.30	3.00	2.50
CLO	0.50	0.53 ± 0.02	3.97	5.12	0.46 ± 0.05	7.35	− 8.57
	7.00	7.85 ± 0.40	5.03	12.11	7.46 ± 0.51	4.12	6.56
	500	499.64 ± 13.29	2.66	− 0.07	510.27 ± 21.25	2.51	2.05
	800	766.07 ± 19.99	2.61	− 4.24	745.22 ± 50.99	2.79	− 6.85
CAF	1200	974.48 ± 82.39	8.45	− 18.79	1286.27 ± 322.33	19.36	7.19
	2200	2364.41 ± 126.05	5.33	7.47	2366.92 ± 137.84	6.06	7.59
	3200	3151.70 ± 48.25	1.53	− 1.51	3080.38 ± 392.77	9.78	− 3.74
	3700	3647.80 ± 143.63	3.94	− 1.41	3290.99 ± 313.51	6.86	− 11.05

Abbreviations: ARI: aripiprazole; DARI: dehydro-aripiprazole; OLA: olanzapine; RIS: risperidone; PAL: paliperidone; QUE: quetiapine; CLO: clozapine; CAF: caffeine.

To validate the ME, the RSD should not exceed 15% for all QCs (except of LLOQ, when 20% is allowed).

The following equations were used to calculate the previously described values [19,24,25]:

$$RE\,(\%) = Pre*100/Post$$

$$ME\,(\%) = Post*100/Neat\ solution$$

$$PE\,(\%) = Pre*100/Neat\ solution$$

2.10. Trueness

'LLOQ', 'Low QC', and 'High QC' samples (Pre) were analyzed from 5 weeks in comparison with spiking samples as reference (Post). The results were expressed with Zeta-score which compares a test value to a reference value. Values less than 2 were considered satisfactory, while the values between 2 and 3 were questionable. Values higher than 3 were considered unsatisfactory.

2.11. Stability

Four replicates of low and high QC aliquots were quantified after storing plasma samples at 20 °C (room temperature), at +4 °C for 24 h and after 3 cycles of freeze-thaw in the freezer at −80 °C, and in extracted samples in the autosampler at 19 °C. Additionally, long-term stability was determined by keeping one set of aliquots at −80 °C for 1 month. The analyte stability should be between 85% and 115% and the RSD should not exceed 15% for the studied QCs. The ratio of analyte concentration before and after storage conditions mentioned above was determined.

2.12. Carry-over

After the highest CAL, a blank plasma sample was injected to determine any possible carry-over effect. The peak area of the blank samples should be lower than 20% of the LLOQ and 5% of the IS. Additionally, the needle was washed 5 times between injections with ACN/water solution (50:50, v/v) to avoid carry-over.

2.13. Statistical analyses

Data were analyzed with Microsoft Excel (Microsoft® Office® 2010, Microsoft Corp., USA) and the results are presented as mean values and standard deviations. The lack-of-fit test results were compared to pure error variances at a 95% confidence level to evaluate the acceptability of the results and the adequacy of the regression models. For the evaluation of trueness, zeta-score test was applied.

2.14. Pharmacokinetic applications of the method

Our method was successfully applied to pharmacokinetic analysis of 66 samples from 3 healthy volunteers who received 5 oral doses of ARI (10 mg) or OLA (5 mg) in fasting conditions. Blood samples were collected at 22 times during 14 days in EDTA dipotassium dihydrate (EDTA K2) tubes (Vacuette, Madrid, Spain) and centrifuged at 1900 × g for 10 min at 4 °C. The plasma was separated and stored at −20 °C. The study was approved by the local Ethics Committee (Clinical Research Ethics Committee of "Hospital Universitario de La Princesa", Madrid, Spain), and informed consent was obtained from the healthy volunteers. Pharmacokinetic parameters were estimated by noncompartmental analysis using PKSolver, a menu-driven add-in program for Microsoft Excel [26]. Maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were determined directly from plasma concentration data. The area under the plasma concentration–time curve from time 0 to the time of the last measurable concentration (AUC_{0-t}) was calculated using the trapezoidal method. Half-life was calculated by dividing 0.693 by k_e.

3. Results and discussion

3.1. Experimental conditions in LC and MS/MS

Due to the similar pKa values ranging from 7.06 to 8.76 [27], for all drugs included in this method, it was possible to optimize extraction method and validate an LC-MS/MS method for simultaneous quantification of ARI, DARI, OLA, RIS, PAL, QUE, and CLO. Only CAF has different pKa value of −0.92, thus the extraction process was less efficient for this compound. Differences in water solubility ranging from 0.008 mg/mL to 0.297 mg/mL, except for CAF, which has very high water solubility of 11 mg/mL [27] enabled us to perform an excellent chromatographic separation. To optimize chromatographic conditions, we used different analytical columns (XBridge BEH C18, Waters, Madrid, Spain and ACE C18-PFP, Agilent Technologies, Madrid, Spain), buffer compositions (ammonium formate, 0.1% and 0.2% formic acid), buffer pH (2, 3, 4 and 6), mobile phase flow rates (0.5 and 0.6 mL/min) and variety of gradients. Additionally, the temperature of analytical separation was considered as well (25–30 °C). For the present method we applied the ACE C18-PFP column. This column is characterized by a good resolution and improves analyte separation compared to ordinary C18 column due to π-π interactions with the aromatic ring and OH groups. It enables us to obtain good resolution factor for all antipsychotic drugs and their metabolites included in the present method (see Supplementary Table 3).

Finally 0.2% formic acid at pH 3.0 as aqueous buffer, flow rate of 0.6 mL/min and the temperature of 25 °C were chosen to improve chromatographic performance of our method. After each injection, the injection needle and the analytical column were washed with a mixture of organic-aqueous solvent. A mixture of ACN/water (50/50; v/v) and isopropanol/water (50/50; v/v) was tested to avoid carry-over phenomenon in subsequent injections, while ACN/water was found to be more effective in needle cleaning process. The total run time, including washing and re-equilibrating steps, was 9.0 min. Although our run time is longer compared to the methods described in the literature [9,28], it was considered essential to elute endogenous phospholipids outside of the elution profile of the analytes (Fig. 1).

LC-MS/MS with ESI and small particle sizes of the analytical column (up to 3.5 μm) required small injection volume in order to avoid overloading, ME and instrument contamination [29]. Thus, we injected 5 μL of the sample, which was applicable for the column particle size and the HPLC system. Table 1 displays relevant LC–MS/MS characteristics, Supplementary Fig. 1 depicts mass spectra obtained by collision-induced dissociation (CID) of the indicated precursor ions [M + H] + and Fig. 1 shows XIC chromatograms of ARI, DARI, OLA, RIS, PAL, QUE, CLO and CAF. Human plasma was spiked with the LLOQ of each drug before μ-SPE procedure. Retention times (tR) and concentration values were calculated from calibration curves given for all analytes. Concerning MS/MS conditions, ESI in positive mode was selected for scanning all analytes. As their polarity was positive, dynamic MRM (dMRM) scan mode was applied to significantly improve analytes' peak shape and selectivity.

As long as some compounds has the same product ions (ARI and DARI) and may undergo crosstalk phenomenon in the collision cell, we used, apart from SIL-ISs, ion ratio (qualifier ratio) based identity confirmation to ensure the reliability of the analyte detection. The ion ratio difference between CALs and QCs did not differ more than 30%. Additionally, we have analyzed tR - and relative tR - difference between extracted analyte and neat solution of the analyte and the obtained value was lower than 0.1 min in all cases when analyzing tR - difference and lower than 2.5% when analyzing relative tR - identity confirmation.

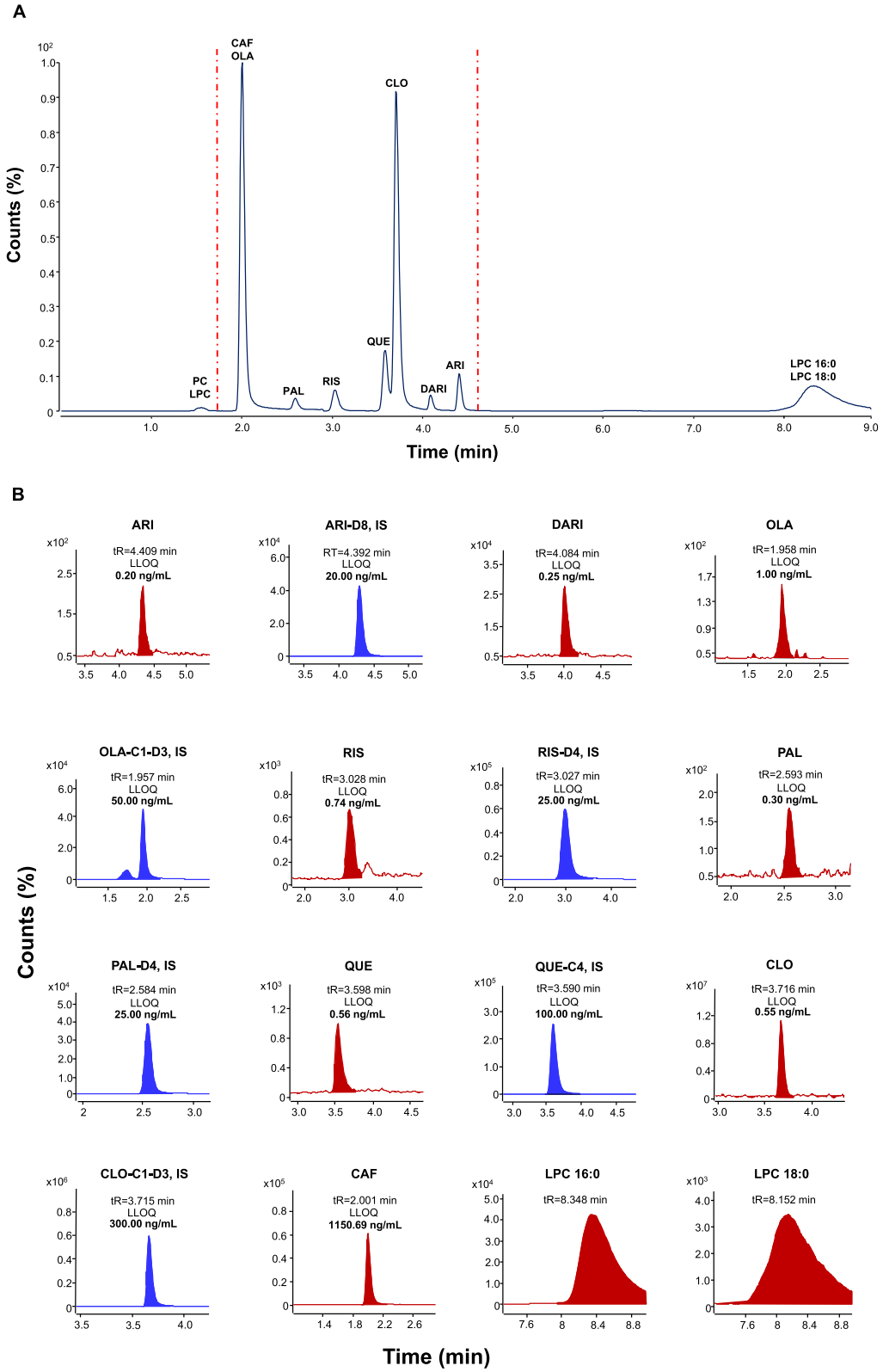

Fig. 1. Extraction ion chromatograms (XIC) of aripiprazole (ARI), dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS), paliperidone (PAL), quetiapine (QUE), clozapine (CLO), and caffeine (CAF) stable isotope-labeled internal standards (SIL-ISs): aripiprazole-D8 (ARI-D8), olanzapine-C1-D3 (OLA-C1-D3), risperidone-D4 (RIS-D4), paliperidone-D4 (PAL-D4), quetiapine-C4 (QUE-C4) and clozapine C1-D3 (CLO-D3). The results are presented as the percentage of counts versus time in minutes. All chromatograms have been normalized to the largest peak. The dashed lines show the elution area of the antipsychotics and CAF separated from early- and late-eluting endogenous phospholipids.

3.2. Sample preparation: phospholipid elimination, extraction recovery, matrix effect and process efficiency

We tested phospholipid cleaning ability of SPE compared to PPT in 36 different human blank plasma samples. As PPT extraction methods were associated with the highest degree of ME in comparison with SPE [30], we used this method as a positive control (100% of phospholipids) compared to SPE.

According to our knowledge, there is no method which is able to remove 100% of phospholipids from the matrix. However, we were able to eliminate efficiently 99.56% and 99.46% of early eluting endogenous plasma PCs-e (in source CID ion fragment 184.1 > 184.1) and LPCs-e (in source CID ion fragment 104.1 > 104.1) using hydrophilic-lipophilic SPE sorbent compared to PPT method (100.00 ± 33.49% vs 0.44 ± 0.73%, $p < 0.001$ for PCs-e and 100.00 ± 31.57% vs 0.54 ± 0.17%, $p < 0.001$ for LPCs-e, respectively). Similarly, we evaluated the elimination capability of late-eluting phospholipids. SPE method was able to remove 92.34% and 97.68% of late-eluting endogenous plasma LPC 18:0-l and LPC 16:0-l compared to PPT method (100.00 ± 32.23% vs 7.66 ± 3.16%, $p < 0.001$ for LPC 18:0-l and 100.00 ± 26.16% vs 2.32 ± 0.74%, $p < 0.001$ for LPC 16:0-l, respectively). The results are shown in the Supplementary Fig. 2D.

As it was impossible to eliminate 100% of endogenous phospholipids, we were able to separate them chromatographically from the target compounds. Early eluting phospholipids were eluted with the tR = 1.485 min and tR = 1.537, late-eluting phospholipid species with the tR = 8.186 min and tR = 8.348, while analyte elution was found between tR = 1.940 min and tR = 4.790 min, outside of the phospholipid elution times. Among LC-MS/MS and UHPLC-MS/MS methods for antipsychotic drug quantification available in the bibliography [28,31], only our previous publication about the simultaneous determination of ARI and DARI in human plasma [6] considered endogenous phospholipid elimination efficiency during method development.

Two QC levels, Low QC and High QC, were applied for the determination of relative and absolute RE, ME and PE. The RE, ME and PE results for SPE and PPT sample preparation are exhibited in Fig. 2 and Supplementary Fig. 2 as mean percentages of relative (Fig. 2A, B, C) and absolute (Supplementary Fig. 2A, B, C) RE, ME and PE, while RSD values are shown as error bars.

The relative mean RE applying SPE was achieved within 98.05–108.91% for all compounds, except for CAF with the value of 127.19% with RSD lower than 10%, while absolute RE ranges between 81.31% and 119.02% and RSD within 14%. CAF is characterized by less efficient extraction recovery values due to the lack of its SIL-IS and extraction method itself, which was adjusted for the antipsychotic drugs and not for CAF being a polar compound. Relative RE values for PPT ranged from 96.04% to 119.09% (except for CAF with the value of 171.54%) and RSD not higher than 11.50%, while absolute RE represents values achieved from 57.42% to 75.91%, and RSD within 24.67%.

When comparing the RE achieved in the present extraction method to the literature, we found that other authors [32,33] accomplished similar values for relative RE ranging from 71% to 123% using SPE as sample preparation method. Our results are slightly better, ranging from 98% to 109%. Among the bibliography, PPT RE results oscillated between 50% and 111% (except for OLA, 185%) for LC-MS/MS methods, while improved when UHPLC-MS/MS was applied, ranging from 65% to 114%. These differences can be due to the lower injection volume and less important ME in UHPLC-MS/MS analysis.

Regarding ME, relative values were found between 99.05% and 105.14% and RSD within 3.97% (except for CAF being found between 77.52% and 118.85% and RSD of 27.04%), greatly better than absolute ME ranging from 83.24% to 119.37% and RSD of 15%. Only OLA represented higher ion suppression, therefore ME value of 75.80%. The results obtained with PPT as extraction method were as follows: relative ME ranged from 94.09% to 106.57% and RSD within 8.22%, absolute ME for PPT was comprised between 63.92% and 91.11% and RSD of 12.02%. According to RE and ME results, more preferable values were obtained when SIL-ISs was applied (relative) in comparison to absolute values. However, SPE RE and ME were slightly better than PPT values. These results were expected, because SIL-ISs compensate for ME analyte loss during extraction process. Nevertheless, the absolute results obtained by SPE and PPT differed considerably. Absolute RE obtained with PPT compared to SPE was lower and highly variable (57.42–75.91%, and CV within 24.67%), while ion suppression was also significant (mean ME of 77.51%). Therefore, SPE was chosen as extraction procedure for method validation.

ME is commonly evaluated using qualitative methods, applying post-column infusion [34]. Nevertheless, manipulating the chromatographic separation to avoid ME due to co-elution is not always applicable [14]. ME calculated for SPE in the literature ranges from 91% to 133%, while for PPT it varies between 89% and 119%. Although these results are analogous, the differences lie in the RSD values. RSD is greatly higher for PPT sample preparation method and cause method irreproducibility and as a consequence compromised precision and accuracy.

Regarding SPE-PE, relative values were comprised between 95.28% and 113.15% and RSD within 7.11%, while absolute PE was extent from 95.75% to 119.59% and RSD less than 13.10%, except for CAF having values between 77.52% and 152.41% and CV of 13.50%. On the contrary, the results obtained with PPT were certainly worse compared to SPE. Relative PPT-PE were found between 88.43% and 114.55% and RSD within 10.45% (with the exception of CAF with a value of 156.34% and RSD of 10.75%); absolute PE were relatively lower ranging from 38.00% to 61.53%, and RSD of 24.72%. There are only a few methods evaluating relative PE as a test included in method validation [33,35], ranging from 42% to 147%. The best PE results were achieved by Patel et al. [33], with values between 92.3% and 98.9% for only ARI included in the analytical method. Our global relative PE was found between 95% and 113%, in regard to ARI, its relative PE ranged from 101% to 103%, being closer to 100% than Patel et al.' results.

Based on our study, PE and phospholipid elimination ability should be included, along with RE and ME, in the tests performed during method development and validation. PE and phospholipid removal efficiency offer a more complete overview regarding the effect of possible analyte loss during sample preparation and ionization suppression/enhancement in the ion source.

3.3. Selectivity, LLOQ and linearity

The present approach was selective and able to differentiate and quantify the analytes from endogenous matrix components, as no significant interferences were found in analyte detection. Blank plasma XIC peak area ratio was less than 10.6% compared to XIC peak area of the LLOQ for all the drugs, except for CAF (13.49%), and less than 0.07% for XIC peak area for all ISs, except for CAF (0.27%). Moreover, the mobile phase and gradient modifications improved DARI response in the present method compared to our previously reported method [6].

LLOQs achieved for antipsychotic drugs, their two metabolites and CAF in human plasma were as follows: ARI: 0.18, DARI: 0.25, OLA: 1.00, RIS: 0.70, PAL: 0.20, QUE: 0.50 and CLO: 0.50 ng/mL. For CAF the LLOQ value was considerably higher, 1200 ng/mL, due to high CAF plasma levels after oral coffee consumption described in the literature [36]. Although more sensitive LC-MS/MS approaches were reported, with LLOQs lower than ours, some of these methods used larger plasma volumes for extraction between 250 and 500 µL [35,37], than we have (200 µL), except for Vecchione et al. [28], who used only 100 µL of plasma. Our LLOQs were better than those reported by Choong et al. [32] and Fisher et al. [38]. Of note, LLOQ for PAL achieved by our method was the best compared to bibliographic values. It can be due to the applied sample preparation method. Fisher et al. applied LLE with methyl-tertbutyl ether as sample extraction method, which is known to

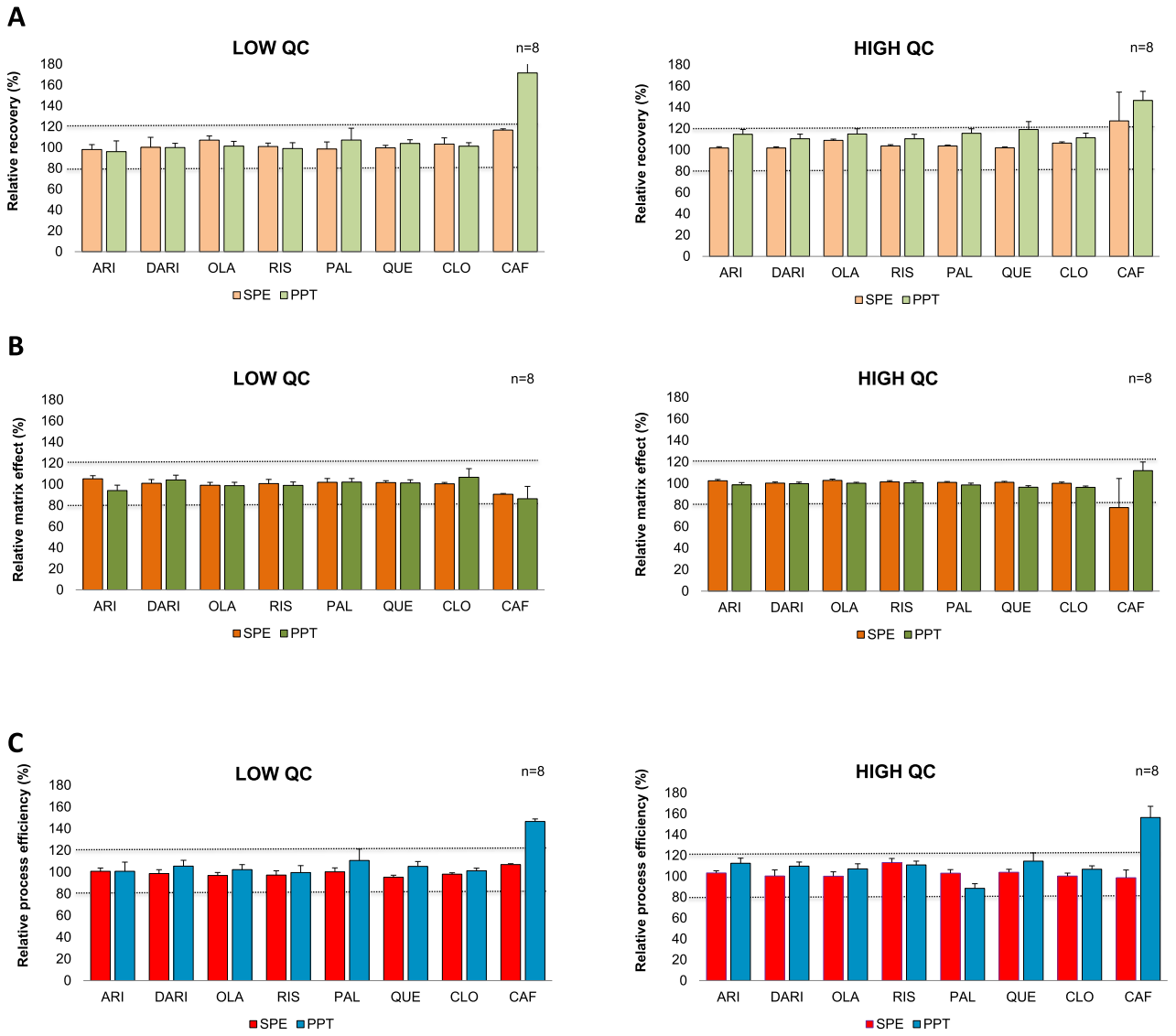

Fig. 2. Relative values of extraction recovery, matrix effect, and process efficiency tests calculated for aripiprazole (ARI), dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS), paliperidone (PAL), quetiapine (QUE), clozapine (CLO), and caffeine (CAF) in plasma for each compound after undergoing solid phase extraction (SPE) and protein precipitation (PPT) processes in low and high concentrations (Low QC and High QC). *Panel A* shows extraction recovery, *Panel B* displays matrix effect while *Panel C* presents process efficiency values showing the relative standard deviation (RSD, %) of 8 experiments.

produce lower RE and higher LLOQ values.

After performing lack-of-fit test to evaluate the linearity of the calibration curve, the F calculated value (0.381–1.687) was lower than the F tabulated value (2.685) for DARI, OLA, PAL, CLO and CAF. Thus, for these analytes, a linear regression model was performed. For ARI, RIS and QUE the F calculated value was higher than F tabulated value, therefore non-linear calibration curve adjustment was applied. The most applicable weighting factors were 1/x for ARI, DARI, OLA and QUE, 1/x2 for PAL and 1/y for CLO and RIS. In all cases, we obtained correlation coefficients (R^2) higher than 0.99 except for CAF (R^2 = 0.94).

3.4. Precision and accuracy

As shown in Table 2, precision and accuracy were within the acceptance criteria for the evaluated assay range. The overall RSDs, for all QCs except for LLOQ, ranging from 1.53% to 13.16% and from 1.78% to 14.84%, were achieved for repeatability and intermediate precision tests, respectively. Global RSDs for accuracy assessment averaged −7.56% and 12.45%, −14.91% and 14.58%, respectively. LLOQs showed higher RSD values, however, they were consistently below the acceptance limits. These results indicate that it is possible to generate accurate data with the present method for antipsychotics and CAF plasma determination.

3.5. Trueness

Analyzing trueness interpreted with Zeta-score resulted satisfactory (values less than 2) in case of all drugs and concentrations (between −0.10 and 1.20 for LLOQ, −1.07 and 1.16 for Low QC and 0.56 and 1.70 for High QC). Therefore, due to the low Zeta-score values, which shows the level of systematic error, our LC-MS/MS method was verified.

3.6. Stability

We performed stability tests in unextracted sample (room temperature at 20 °C, fridge at 4 °C and freezer at −80 °C) and after sample extraction in autosampler. The results of the stability assays at low and high concentrations are summarized in Supplementary Fig. 3. Stability tests in unextracted plasma after 24 h at 20 °C (room temperature) showed global RSDs between 1.16% and 10.32% and mean stabilities of 89.03% and 105.35% (except for CAF: 111.81%); after 24 h at 4 °C in the fridge demonstrated overall RSDs between 0.55% and 8.66% and mean stabilities of 92.93% and 103.49%; after three freeze–thaw cycles (24 h each cycle) in the freezer at −80 °C they exhibited global RSDs between 0.17% and 6.02% and mean stabilities of 91.55% and 101.67%; after 1 month in the freezer at −80 °C they showed overall RSDs between 0.67% and 6.55% and mean stabilities of 87.28% and 109.04%; for all compounds included in the present method. Stability tests in extracted samples after 24 h at 20 °C in the autosampler presented RSDs of minimum 0.76 and maximum 7.88% and mean stabilities ranging from 92.63% to 107.57%. Overall, the RSD value was less than 10.32% and the mean stabilities were close to 100% for all tested compounds. OLA was shown to be unstable in whole blood and oral fluid under most of the conditions studied by Fisher et al. [39], although the addition of ascorbic acid to the blood or serum showed protective properties against OLA degradation. Likewise, we added formic acid to all plasma samples resulting in the preservation of OLA in all stability tests.

3.7. Carry-over

Carry-over effect, if detected, should be prevented to ensure the precision and accuracy of the method. Application of the optimal washing procedures of the injection needle and the analytical column should be optimized. We applied a mixture of ACN and water (1/1, v/v) for needle wash as well as for blank sample injection after measuring high concentration samples, in order to reduce carry-over effect. Similarly to others [32,40], we managed to avoid a significant carry-over effect, which was lower than 15% for the antipsychotic drugs and lower than 16.5% for CAF. The carry-over phenomenon detected by other authors was even higher [34] than ours, except for Patel et al. [33], who were able to reduce the carry-over effect to less than 0.05%.

3.8. Pharmacokinetic applications of the method

The method was successfully applied to the multiple-dose pharmacokinetic study. Supplementary Fig. 4 exhibits ARI, DARI (*Panel A*) and OLA (*Panel B*) plasma concentrations (ng/mL) versus time acquired after 5 multiple doses of ARI (10 mg) and OLA (5 mg) oral administration in 3 healthy volunteers (1 for ARI and DARI; 2 and 3 for OLA). Supplementary Fig. 4C shows mean pharmacokinetic parameters of ARI, DARI and OLA. C_{max} data were collected only for day 1 and 5. The obtained C_{max} in day 1 was consistent with the results by Mallikaarjun et al., however, as their study design was different, C_{max} and AUC in day 5 were not available. In the same study, T_{max} was lower than ours, while $T_{1/2}$ was comparable [41]. To date there is no other study analyzing the pharmacokinetics of ARI and DARI in a multiple dose design during 5 days performed in healthy volunteers. OLA samples were checked for CAF interference. No CAF plasmatic levels were observed in volunteers taking OLA and therefore we obtained expected OLA concentrations.

4. Conclusion

Our LC-MS/MS method is adequate for precise and accurate simultaneous measurement of antipsychotic drugs and CAF in human plasma. Simple sample preparation and three step μ-SPE offer high phospholipid removal efficiency compared to PPT, as required by our clinical studies. Validation data have assessed its precision, accuracy, sensitivity, and immunity from matrix effects, good extraction recoveries and process efficiency, trueness, as well as competitive LLOQs and calibration range, good analyte stabilities and with no significant carry-over effect. The present method can be successfully implemented in the clinical laboratory and applied for routine TDM.

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Declaration of conflicting interests

F. Abad-Santos has been consultant or investigator in clinical trials sponsored by the following pharmaceutical companies: Abbott, Alter, Chemo, Cinfa, FAES, Farmalíder, Ferrer, GlaxoSmith*Kl*ine, Galenicum, Gilead, Janssen-Cilag, Kern, Normon, Novartis, Servier, Silverpharma, Teva, and Zambon. The remaining authors declare no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.01.112.

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Supplementary Table 1. Concentrations of the calibration standards (CALs) for each drug.

Drug/ metabolite	R^2	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
		(ng/mL)							
ARI	0.9991	0.18	0.25	0.50	2	10	50	100	120
DARI	0.9989	0.25	0.35	0.70	2	5	20	40	80
OLA	0.9944	1.00	1.10	1.50	5	10	20	50	100
RIS	0.9968	0.70	0.80	1.20	1.5	5	10	25	60
PAL	0.9815	0.20	0.30	0.50	1	2	5	18	30
QUE	0.9956	0.50	0.60	1.00	5	10	20	80	160
CLO	0.9981	0.50	0.80	2.00	20	100	500	750	1000
CAF	0.9363	1200	1450	1700	1950	2200	2700	3200	3700

Abbreviations: ARI: aripiprazole; DARI: dehydro-aripiprazole; OLA: olanzapine; RIS: risperidone; R^2: correlation coefficient; PAL: paliperidone; QUE: quetiapine; CLO: clozapine; CAF: caffeine.

Supplementary Table 2. Therapeutic drug ranges for each drug.

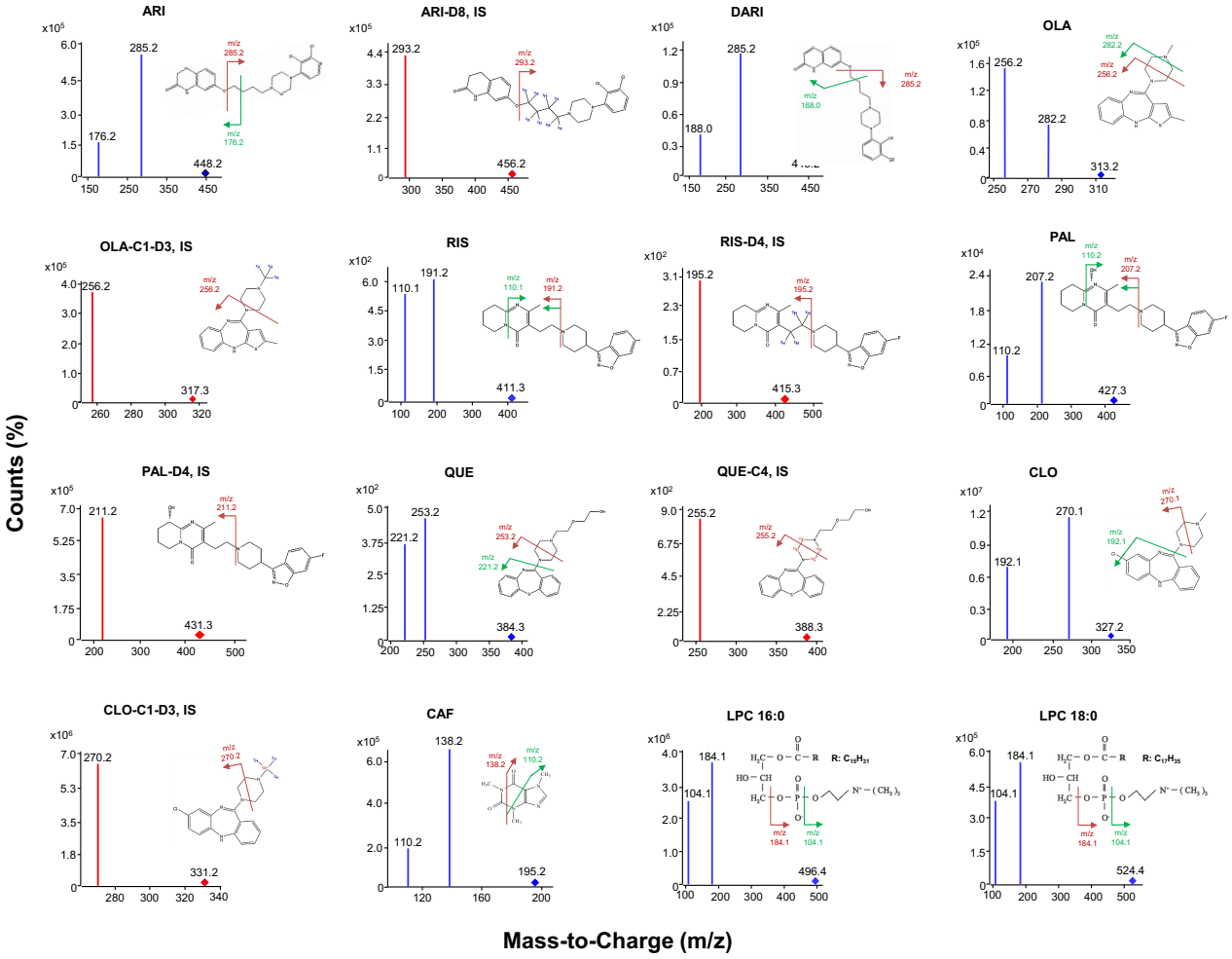
Drug	Therapeutic range (ng/mL)	References
ARI	50-350	[1,2]
	150-300	[3]
	146–254	[4]
	32.1–188	[5]
OLA	10-100	[2,6]
	20–80	[7–9]
	1.2-208	[10]
RIS	10-100	[2,6]
	20–60	[11,12]
	0.6-25	[13]
PAL	10-100	[2,6]
	20-52	[11]
	20-60	[12]
	10-109	[13]
QUE	70-170	[2,6,14,15]
CLO	200-800	[2,6]
	350-600	[16,17]

Abbreviations: ARI: aripiprazole; DARI: dehydro-aripiprazole; OLA: olanzapine; RIS: risperidone; R^2: correlation coefficient; PAL: paliperidone; QUE: quetiapine; CLO: clozapine; CAF: caffeine.

***Supplementary Table 3*.** The resolution factor between the adjoining compounds.

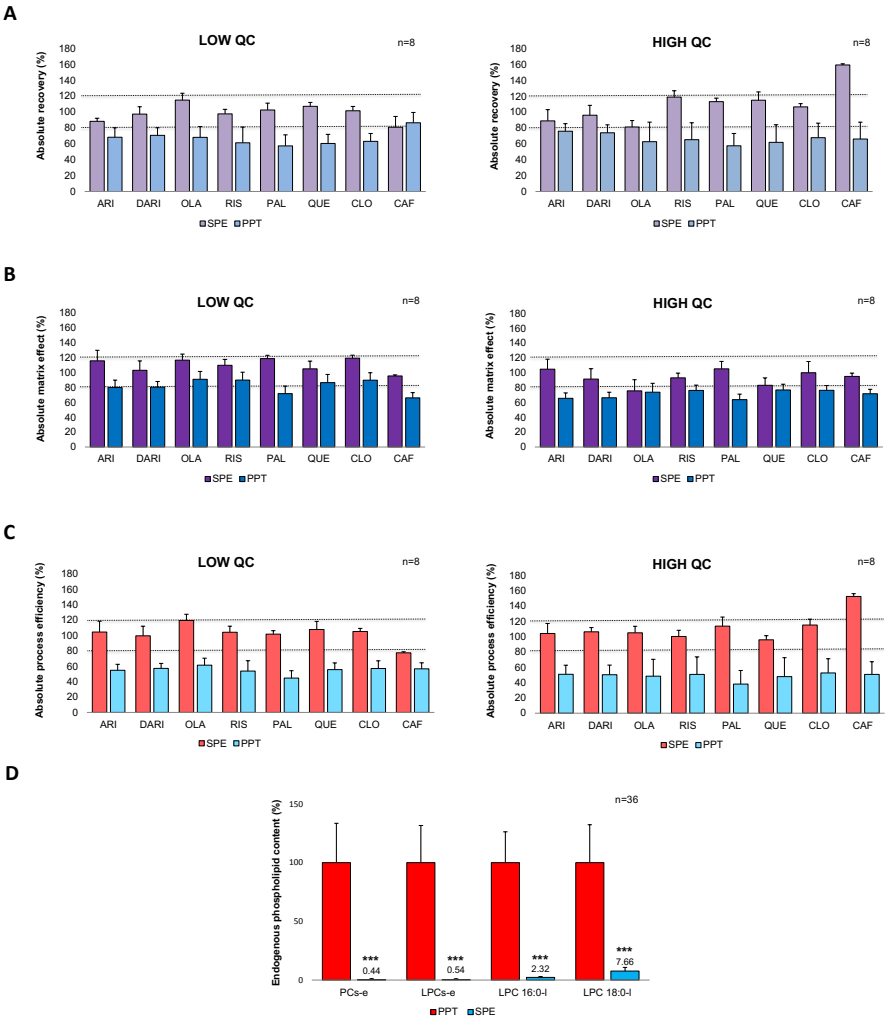
Compounds	Rs
CAF-OLA	1.28
OLA-PAL	12.63
PAL-RIS	6.24
RIS-QUE	7.35
QUE-CLO	1.85
CLO-DARI	7.81
DARI-ARI	8.49

Abbreviations: ARI: aripiprazole; DARI: dehydro-aripiprazole; OLA: olanzapine; RIS: risperidone; PAL: paliperidone; QUE: quetiapine; CLO: clozapine; CAF: caffeine; Rs: resolution factor.



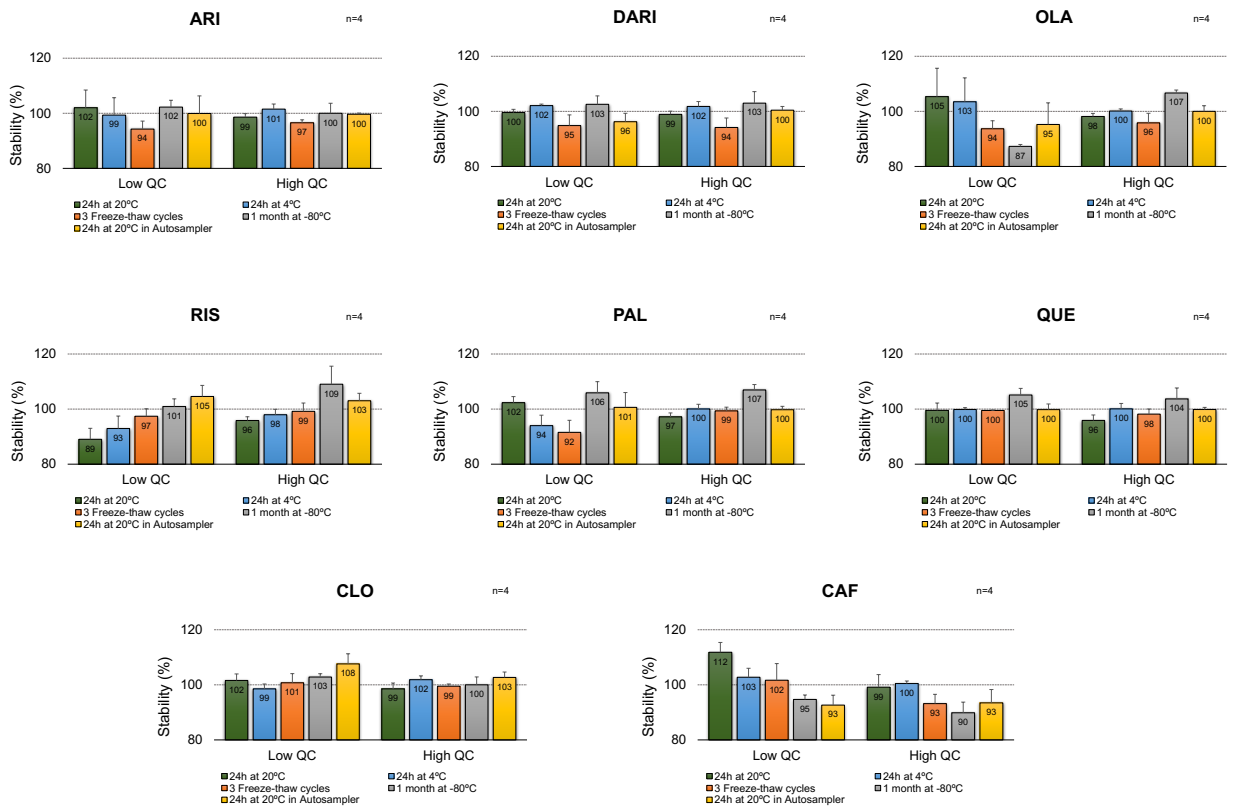
Supplementary Figure 1. Product ion spectra and chemical structures of aripiprazole (ARI), dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS), paliperidone (PAL), quetiapine (QUE), clozapine (CLO), and caffeine (CAF) and stable isotope-labeled internal standards (SIL-ISs): aripiprazole-D8 (ARI-D8), olanzapine-C1-D3 (OLA-C1-D3), risperidone-D4 (RIS-D4), paliperidone-D4 (PAL-D4), quetiapine-C4 (QUE-C4) and clozapine C1-D3 (CLO-D3). obtained by collision-induced dissociation (CID) of the indicated precursor ions $[M+H]^+$.

The fragmentation patterns of all analytes are indicated by an arrow on their chemical structure of each analyte. The residual precursor and the product ions, quantifier and qualifier, are shown in the figure. The results are presented as the percentage of counts versus Mass-to-Charge (m/z). All mass peaks have been normalized to the most abundant.



***Supplementary Figure* 2**. Absolute values of extraction recovery, matrix effect, process efficiency tests obtained for aripiprazole (ARI), dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS), paliperidone (PAL), quetiapine (QUE), clozapine (CLO), and caffeine (CAF) as well as endogenous plasma phospholipid elimination efficiency in plasma after undergoing solid phase extraction (SPE) and protein precipitation (PPT) processes at low and high concentrations (Low QC and High QC).

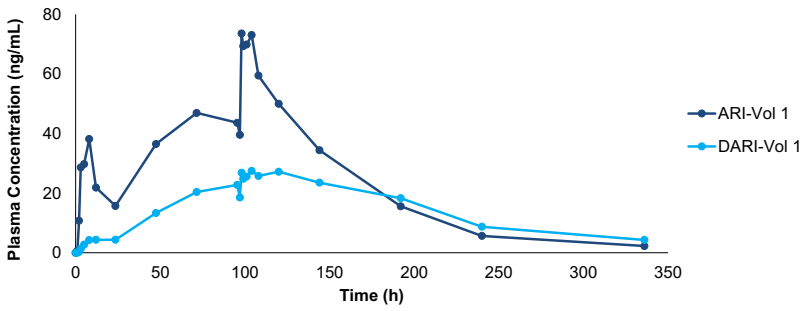
Panel A shows extraction recovery, *Panel B* displays matrix effect while *Panel C* presents process efficiency in percentages along with the relative standard deviation (RSD, %) of 8 experiments. *Panel D* depicts endogenous plasma phospholipid content (PCs-e: early eluting phosphatidilcholines, LPCs-e: early eluting lysophosphatidilcholines, LPC 16:0-l: late-eluting lysophosphatidylcholine 16:0, LPC 18:0-l: late-eluting lysophosphatidylcholine 18:0) in percentages, including the RSD (%) of 36 experiments, after PPT or SPE used as a sample preparation method.



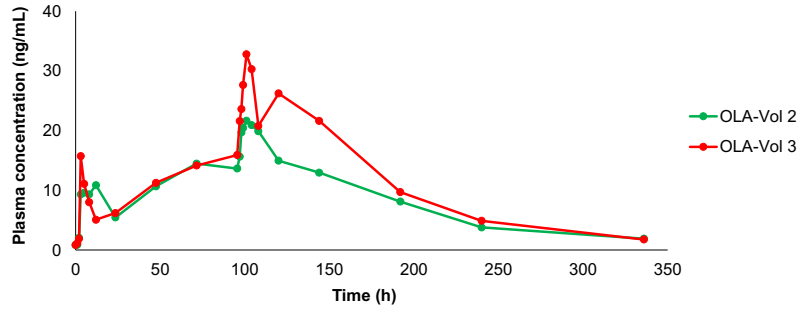
Supplementary Figure 3. Stability tests. Storage stability of aripiprazole (ARI), dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS), paliperdione (PAL), quetiapine (QUE), clozapine (CLO) and caffeine (CAF) at low and high concentrations (Low QC and High QC) in human plasma samples being 24 h at 20ºC (room temperature), 24 h at 4°C in the fridge, the 3rd freeze-thaw cycle (-80°C), stored in the freezer at -80°C for one month, and in the human plasma samples extract after 24 h at 20°C in the autosampler.

Results are given as mean percentages ± relative standard deviation (RSD). Low and high quality control (Low QC and High QC) samples in quatruplicates were used for each single stability test.

A



B



C

Pharmacokinetic parameters of ARI, DARI and OLA

Parameter	ARI-Vol 1	DARI-Vol 1	OLA-Vol 2	OLA-Vol 3
C_{max} Day 1 (ng/mL)	38.15	22.79	10.87	15.71
C_{max} Day 5 (ng/mL)	73.05	27.49	21.65	32.77
T_{max} Day 1 (h)	8.00	12.00	12.00	3.00
T_{max} Day 5 (h)	8.00	8.00	5.00	5.00
$T_{1/2}$ Day 14 (h)	45.10	77.90	65.37	59.44
AUC_{0-t} Day 14 (ng x h/mL)	7670.43	4741.17	2876.97	3643.81

Supplementary Figure 4. Plasma concentrations versus time and obtained pharmacokinetic parameters of aripiprazole (ARI), dehydro-aripiprazole (DARI) and olanzapine (OLA) in a pharmacokinetic study with healthy volunteers under fasting conditions. *Panel A* shows ARI and DARI plasma concentrations after 5 multiple doses oral administration of 10 mg ARI in one volunteer (Vol 1) at 22 time points. *Panel B* exhibits OLA plasma concentrations after 5 multiple oral doses of 5 mg OLA in two volunteers (Vol 2 and 3) at 22 time points. *Panel C* displays pharmacokinetic parameters - maximum plasma concentration (C_{max}), time of occurence of C_{max} (T_{max}), half-life ($t_{1/2}$) and area under the concentration-time curve from time 0 until the last measurable concentration (AUC_{0-t}) - of ARI, DARI and OLA. All pharmacokinetic parameters are expressed as mean percentage. Data were analysed with PKSolver, a menu-driven add-in program for Microsoft Excel.

TP-17

Simultaneous determination of aripiprazole, dehydro-aripiprazole, olanzapine, risperidone, paliperidone, quetiapine and clozapine in human plasma by LC-MS/MS

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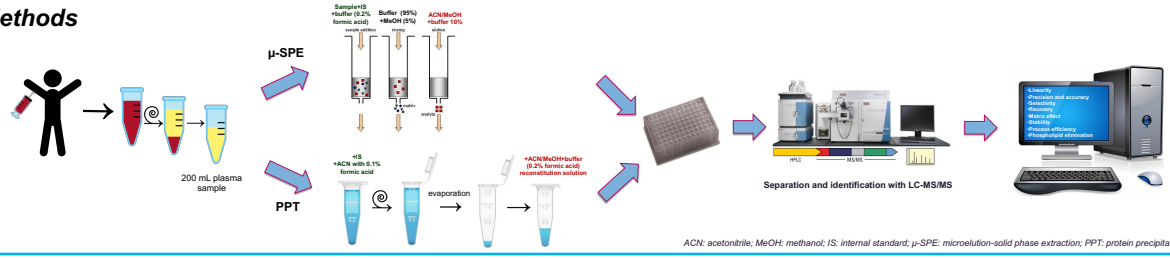
Introduction

A simple and sensitive liquid chromatography-tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous quantification of aripiprazole (ARI) and its active metabolite dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS) and its active metabolite paliperidone (PAL), quetiapine (QUE) and clozapine (CLO) along with caffeine (CAF) in human plasma. These drugs have been widely used in the treatment of schizophrenia and schizoaffective disorders.

Methods



ACN: acetonitrile; MeOH: methanol; IS: internal standard; μ-SPE: microelution-solid phase extraction; PPT: protein precipitation

Results

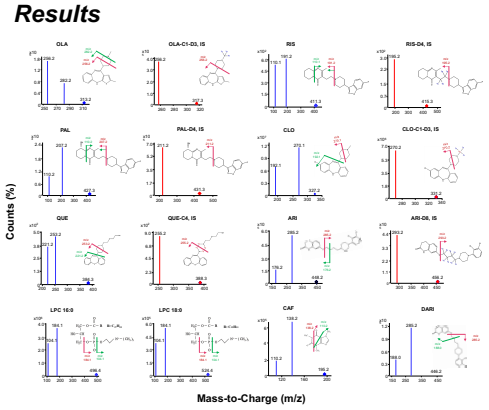


Figure 1. Product ion spectra and chemical structures of olanzapine (OLA), risperidone (RIS), paliperidone (PAL), clozapine (CLO), quetiapine (QUE), aripiprazole (ARI), dehydro-aripiprazole (DARI) and caffeine (CAF) and stable isotope-labeled internal standards (SIL-ISs): olanzapine-C1-D3, risperidone-D4, paliperidone-D4, clozapine C1-D3, quetiapine-C4 and aripiprazole-D8, obtained by collision-induced dissociation (CID) of the indicated precursor ions [M+H]+ along with lysophosphatidylcholine 16:0 (LPC 16:0) and lysophosphatidylcholine 18:0 (LPC 18:0).

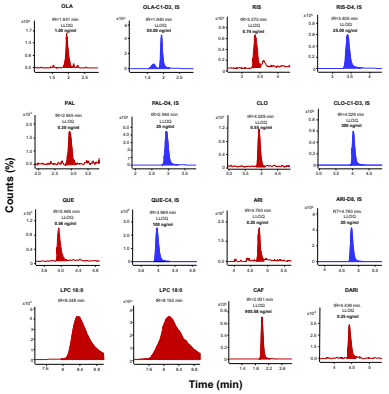


Figure 2. Extraction ion chromatograms (XIC) of olanzapine (OLA), risperidone (RIS), paliperidone (PAL), clozapine (CLO), quetiapine (QUE), aripiprazole (ARI), dehydro-aripiprazole (DARI) and caffeine (CAF) and stable isotope-labelled internal standards (SIL-ISs): olanzapine-C1-D3, risperidone-D4, paliperidone-D4, clozapine C1-D3, quetiapine-C4 and aripiprazole-D8. Human plasma was spiked with lower limit of quantification (LLOQ) of each drug before microelution-solid phase extraction (SPE) procedure. Retention times (R) and concentration values calculated from calibration curves are given for all analytes. The results are presented as the percentage of counts versus time in minutes (min). All chromatograms have been normalized to the largest peak.

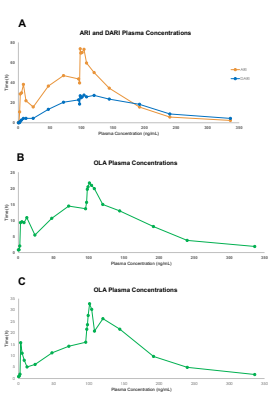


Figure 3. Aripiprazole (ARI), dehydro-aripiprazole (DARI) and olanzapine (OLA) plasma concentration versus time obtained after a 5 multiple dose oral administration of ARI (10 mg) and OLA (5 mg) under fasting conditions to 3 healthy volunteers. Panel A shows ARI and DARI plasma concentrations obtained from one volunteer. Panel B and C display OLA plasma concentrations obtained from two different volunteers.

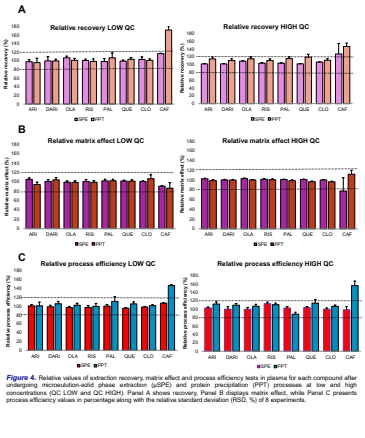


Figure 4. Relative values of extraction recovery, matrix effect and process efficiency tests in plasma for each compound after undergoing microelution-solid phase extraction (μSPE) and protein precipitation (PPT) processes at low and high concentrations (QC LOW and QC HIGH). Panel A shows recovery, Panel B displays matrix effect, while Panel C presents process efficiency values in percentage along with the relative standard deviation (RSD, %) of 8 experiments.

Table 1. **Stability tests.** Storage stability of aripiprazole (ARI), dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS), paliperidone (PAL), quetiapine (QUE), clozapine (CLO) and caffeine (CAF) at low and high quality controls' concentration in the human plasma sample after 24 h at room temperature, 24 h at 4 °C in the fridge, the 3rd freeze-thaw cycle (–80 °C), stored in the freezer at –80 °C for one month, and in the human plasma samples extract after 24 h at 23 °C in the autosampler. Results are given as mean percent difference from theoretical concentration ± standard deviation (SD). The total number of experiments is shown in parenthesis.

Compound	Concentration (ng/mL); n=4	Room temperature (24h)		Fridge (24h)		Freeze-thaw (3rd cycle)		Freezer -80 °C (1 month)		Autosampler (24h)	
		Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)
ARI	1.00	102.00 ± 6.83	6.69	99.33 ± 6.27	6.32	94.24 ± 2.76	2.93	109.80 ± 1.55	1.41	99.90 ± 6.41	6.42
	100	98.57 ± 1.27	1.29	101.49 ± 1.89	1.86	96.55 ± 1.02	1.06	100.00 ± 3.61	3.61	99.72 ± 10.35	10.39
DARI	0.90	100.27 ± 1.01	1.01	102.06 ± 0.56	0.55	94.81 ± 3.67	3.88	102.53 ± 3.16	3.09	96.23 ± 2.95	3.07
	70	98.88 ± 1.15	1.16	101.78 ± 1.79	1.76	94.13 ± 3.24	3.44	117.68± 4.92	4.18	100.41 ± 1.36	1.35
OLA	3.00	105.35 ± 10.87	10.32	103.49 ± 8.96	8.66	93.66 ± 2.73	2.92	87.28 ± 0.58	0.67	93.66 ± 2.73	2.92
	80	98.12 ± 1.12	1.14	100.11 ± 0.77	0.77	95.78 ± 3.35	3.50	118.90 ± 1.27	1.07	95.78 ± 3.35	3.50
RIS	1.50	89.03 ± 3.57	4.01	78.59 ± 6.76	8.60	97.39 ± 2.64	2.71	90.71 ± 12.81	14.12	104.57 ± 4.21	4.03
	50	95.84 ± 1.33	1.39	97.95 ± 1.95	1.99	99.21 ± 2.98	3.00	102.59 ± 11.71	11.41	103.04 ± 2.79	2.71
PALI	1.60	102.38 ± 5.43	5.31	96.65 ± 6.05	6.26	91.55 ± 4.00	4.37	105.88± 4.32	4.08	100.64± 5.42	5.38
	25	97. 16 ± 2.47	2.54	99.08 ± 1.27	1.27	99.33 ± 1.37	1.38	106.92 ± 2.13	1.99	99.77 ± 1.24	1.24
QUE	1.50	99.55 ± 2.66	2.67	99.86 ± 0.64	0.64	99.51 ± 0.17	0.17	105.10± 2.54	2.42	99.81 ± 2.03	2.03
	130	95.89 ± 1.91	1.99	100.06 ± 1.99	1.99	98.18 ± 1.82	1.86	113.40 ± 4.53	4.00	99.86 ± 0.76	0.76
CLO	7.00	101.58 ± 2.38	2.34	98.53 ± 1.73	1.75	100.72 ± 3.35	3.33	102.81 ± 1.22	1.18	107.57 ± 3.96	3.68
	800	98.51 ± 2.11	2.14	100.64 ± 1.44	1.42	99.50 ± 0.81	0.81	96.98 ± 2.87	2.97	102.66 ± 2.02	1.97
CAF	2200	120.29 ± 7.26	6.03	102.75 ± 3.35	3.26	101.67 ± 6.12	6.02	102.35 ± 15.43	15.07	92.63 ± 3.34	3.61
	3700	99.14 ± 4.52	4.56	100.46 ± 0.93	0.93	93.18 ± 3.14	3.37	93.88 ± 7.39	7.87	93.42 ± 4.54	4.86

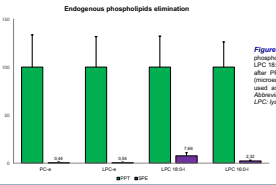


Figure 5. Endogenous plasma phospholipid content (PCS-a, LPCs-a, LPC 18:0-I and LPC 16:0-I) in percentages after PPT (protein precipitation) or SPE (microelution-solid phase extraction) used as a sample preparation method. *Abbreviations: PC: phosphatidylcholine; LPC: lysophosphatidylcholine.*

Table 2. **Repeatability and intermediate precision and accuracy values**. Data were obtained from the lower limit of quantification (LLOQ), low, medium and high quality controls (QCs) on one day as intra-day and on 3 consecutives days as inter-day precision and accuracy assays. Precision values are expressed as the percentage of the relative standard deviation (RSD, %) for repeated measurement from one day and as pooled RSD (%) from 4 consecutive days. Accuracy is shown as the percentage of the closeness of theoretical concentration to the measured value present in the matrix. The corresponding concentrations are displayed as the mean ± standard deviation (SD) of the number of total experiments (n).

Compound	Concentration (ng/mL)	Repeatability n=8 Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)	Intermediate precision n=24 Mean ± SD (ng/mL)	Pooled RSD (%)	Accuracy (%)
ARI	0.18	0.17 ± 0.01	0.07	-3.27	0.19 ± 0.02	7.69	2.56
	1.00	1.12 ± 0.08	0.07	12.33	1.08 ± 0.06	5.18	8.17
	60	58.84 ± 1.34	2.29	-1.93	59.59 ± 2.00	2.83	-0.69
	100	108.00 ± 2.60	2.41	8.00	112. 86 ± 9.24	6.91	12.86
DARI	0.25	0.25 ± 0.01	3.72	0.75	0.25 ± 0.02	6.89	0.64
	0.90	0.85 ± 0.04	4.11	-5.23	0.86 ± 0.05	6.02	-4.87
	40	42.03 ± 0.72	1.72	5.07	41.73 ± 1.97	4.71	4.32
	70	68.85 ± 1.44	2.10	-1.64	68.28 ± 3.18	4.80	-5.31
OLA	1.00	1.05 ± 0.03	3.32	5.09	1.08 ± 0.18	9.43	7.54
	3.00	3.01 ± 0.40	13.16	0.33	3.27 ± 0.40	9.42	9.00
	50	47.38 ± 0.99	2.08	-5.43	51.04 ± 4.59	2.96	2.09
	80	79.14 ± 1.98	2.50	-1.08	75.94 ± 5.15	3.15	-5.07
RIS	0.70	0.73 ± 0.02	3.20	4.89	0.61 ± 0.10	8.08	-12.33
	1.50	1.68 ± 0.07	3.98	12.01	1.71 ± 0.10	5.73	13.70
	25	25.02 ± 0.56	2.22	0.07	27.79 ± 0.91	2.51	3.14
	50	48.79 ± 1.25	2.56	-2.41	46.06 ± 3.07	2.37	-7.88
PAL	0.20	0.24 ± 0.03	13.04	19.15	0.24 ± 0.04	16.51	17.58
	1.60	1.67 ± 0.05	3.19	4.39	1.70 ± 0.08	3.68	6.27
	15	14.77 ± 0.98	6.63	-1.55	14.71 ± 0.54	3.71	-1.92
	25	23.11 ± 0.55	2.36	-7.56	22.71 ± 1.09	2.14	-9.16
QUE	0.50	0.46 ± 0.04	7.58	-3.65	0.45 ± 0.05	8.11	-10.68
	1.50	1.67 ± 0.10	5.93	11.16	1.69 ± 0.10	5.37	12.74
	90	89.96 ± 1.84	2.04	12.45	91.21 ± 6.16	6.95	14.00
CLO	130	123.47 ± 2.26	1.78	-5.03	133.26 ± 11.30	3.00	2.50
	0.50	0.53 ± 0.02	3.97	5.12	0.46 ± 0.05	7.35	-8.57
	7.00	7.85 ± 0.40	5.03	12.11	7.46 ± 0.51	4.12	6.56
	500	499.64 ± 13.29	2.66	-0.07	510.27 ± 21.25	2.51	2.05
CAF	600	766.07 ± 19.99	2.61	-4.24	745.22 ± 50.99	2.79	-6.85
	1200	974.46 ± 82.39	8.45	-18.79	1286.27 ± 322.33	19.36	7.19
	2200	2364.41 ± 126.05	5.33	7.47	2368.92 ± 137.84	6.06	7.59
	3200	3151.70 ± 48.25	1.53	-1.51	3080.38 ± 392.77	9.78	-3.74
	3700	3647.80 ± 143.63	3.94	-1.41	3290.99 ± 313.51	6.86	-11.05

Conclusions and future perspectives

We have developed a sensitive LC-MS/MS method to determine the levels of 5 antipsychotics and 2 active metabolites with the usage of stable isotopically labeled internal standards in human plasma. The method is currently being utilized in pharmacokinetic studies. Its application could help to optimize the effectiveness of treatment and manage side effects or toxicity leading to individualized therapeutic decisions for each patient.

Novel Aspect

The present method applies effective phospholipids' removal during SPE and therefore results in exceptional recoveries and low matrix effect.

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Article 2: The effects of aripiprazole and olanzapine on pupillary light reflex and its relationship with pharmacogenetics in a randomized multiple-dose trial.

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ORIGINAL ARTICLE

The effects of aripiprazole and olanzapine on pupillary light reflex and its relationship with pharmacogenetics in a randomized multiple-dose trial

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Aims: Pupillography is a noninvasive and cost-effective method to determine autonomic nerve activity. Genetic variants in cytochrome P450 (*CYP*), dopamine receptor (*DRD2*, *DRD3*), serotonin receptor (*HTR2A*, *HTR2C*) and ATP-binding cassette subfamily B (*ABCB1*) genes, among others, were previously associated with the pharmacokinetics and pharmacodynamics of antipsychotic drugs. Our aim was to evaluate the effects of **aripiprazole** and **olanzapine** on pupillary light reflex related to pharmacogenetics.

Methods: Twenty-four healthy volunteers receiving 5 oral doses of 10 mg aripiprazole and 5 mg olanzapine tablets were genotyped for 46 polymorphisms by quantitative polymerase chain reaction. Pupil examination was performed by automated pupillometry. Aripiprazole, dehydro-aripiprazole and olanzapine plasma concentrations were measured by high-performance liquid chromatography–tandem mass spectrometry.

Results: Aripiprazole affected pupil contraction: it caused dilatation after the administration of the first dose, then caused constriction after each dosing. It induced changes in all pupillometric parameters ($P < .05$). Olanzapine only altered minimum pupil size ($P = .046$). Polymorphisms in *CYP3A*, *HTR2A*, *UGT1A1*, *DRD2* and *ABCB1* affected pupil size, the time of onset of constriction, pupil recovery and constriction velocity. Aripiprazole, dehydro-aripiprazole and olanzapine pharmacokinetics were significantly affected by polymorphisms in *CYP2D6*, *CYP3A*, *CYP1A2*, *ABCB1* and *UGT1A1* genes.

Conclusions: In conclusion, aripiprazole and its main metabolite, dehydro-aripiprazole altered pupil contraction, but olanzapine did not have such an effect. Many polymorphisms may influence pupillometric parameters and several polymorphisms had an effect on aripiprazole, dehydro-aripiprazole and olanzapine pharmacokinetics.

The authors confirm that the Principal Investigator for this paper is Francisco Abad-Santos and that he had direct clinical responsibility for patients.

Pupillography could be a useful tool for the determination of autonomic nerve activity during antipsychotic treatment.

KEYWORDS
antipsychotics, genetics and pharmacogenetics, pharmacodynamics, pharmacokinetics, schizophrenia

1 | INTRODUCTION

Aripiprazole (ARI) and olanzapine (OLA) are atypical (second generation) antipsychotics commonly prescribed for patients with schizophrenia or schizoaffective disorders.[1] ARI has partial agonistic activity at **dopamine** D2, D3, D4 and **serotonin** 5-HT1A, 5-HT2C as well as α1-adrenergic receptors and also exhibits 5-HT2A and 5-HT7 receptor antagonism.[2] OLA has higher antagonistic affinity for 5-HT2A serotonin receptors than for D2 dopamine receptors. Additionally, it has antagonistic activity at dopamine D3 and D4, serotonin 5-HT3 and 5-HT6, histamine H1, α1-adrenergic, and muscarinic M1–5 receptors.[3,4]

ARI is extensively metabolized by cytochrome P450 (CYP) enzymes CYP3A4 and CYP2D6. Dehydro-aripiprazole (DARI), its main active metabolite, accounts for 40% of the parent compound in plasma. Moreover, the pharmacological activity of DARI is similar to ARI.[5] Olanzapine is predominantly metabolized by direct glucuronidation via the UDP-glucuronosyltransferase (UGT) enzyme family, principally by UGT1A4,[6] CYP1A2 and to a lesser extent by CYP2D6 and CYP3A4.[7]

Pupillography is a noninvasive and cost-effective method to determine autonomic nerve activity,[8] which was developed in 1958.[9] It was thoroughly described that opioid drugs cause pupil constriction (miosis).[10–13]

Several atypical antipsychotics caused pupil miosis in overdose patients. It can be due to inducing unopposed parasympathetic stimulation of the pupil with significant α1-adrenergic receptor blockade.[14] By contrast, these drugs could affect the pupil diameter due to their affinity for dopamine and serotonin receptors[15,16] as serotonin and dopamine are effectors on various types of muscles including the sphincter pupillae and the dilator pupillae.[17] Accordingly, genetic polymorphisms present in these genes can affect pupil response.[18]

The aim of the current study was to evaluate if ARI and OLA affect pupillometric parameters in healthy subjects after multiple dose administration. Furthermore, their relationship with pharmacokinetics and pharmacogenetics was also evaluated.

What is already known about this subject

- Atypical antipsychotics can provoke pupil contraction due to blocking α1-adrenergic receptors. However, these drugs could affect pupil diameter due to their affinity for dopamine and serotonin receptors. Accordingly, polymorphisms present in these genes can affect pupil response.

What this study adds

- This study is the first to reveal that aripiprazole has a significant influence on pupillary light reflex compared to olanzapine. Furthermore, several genetic polymorphisms affect these changes. Therefore, pupillography could be an important and useful tool to assess autonomic nervous system activity during antipsychotic drug treatment.

2 | MATERIALS AND METHODS

2.1 | Study population

A multiple-dose clinical trial including 24 healthy volunteers (12 males and 12 females) was performed at the Clinical Trials Unit of Hospital Universitario de La Princesa (Madrid, Spain). The protocol was approved by the Research Ethics Committee duly authorized by the Spanish Drugs Agency and under the guidelines of Good Clinical Practice (EUDRA-CT: 2018–000744-26). All subjects were adequately informed about the study and, if agreeing to participate, signed an informed consent form. The trial complied with the international standards and with the Declaration of Helsinki.

The inclusion criteria were the following: male and female volunteers between 18 and 65 years old; free from any known organic or psychiatric conditions; normal vital signs and electrocardiogram (ECG); normal medical records and physical examination; no clinically significant abnormalities in haematology, biochemistry, serology and urine tests.

2.2 | Study design

The clinical trial was phase I with multiple oral dose design, open-label, randomized, crossover, 2-periods, 2-sequences, single-centre and comparative study performed between June 2018–April 2019. ARI 10 mg/day tablets or OLA 5 mg/day film-coated tablets were administered during 5 consecutive days. Block randomization was used to

assign the treatment to each volunteer on the first day.[19] The drug was administered at 09:00 each day under fasting conditions. The subjects were hospitalized from 1 hour before the first dose until 24 hours after the last dose. In the second period, after a washout period of 28 days, each volunteer received the opposite drug they received in the first period. The random allocation sequence, the recruitment of participants and their assignment to interventions were performed by investigators of the Clinical Trials Unit.

Twenty-two blood samples were collected from each participant for pharmacokinetic assessments during each period, thus 44 samples in total: 7 samples on day 1 (predose and 1, 2, 3, 5, 8 and 12 hours after dosing); 1 (predose) sample on days 2, 3 and 4; 7 samples on day 5 (predose and 1, 2, 3, 5, 8 and 12 hours after dosing) and 1 sample on days 6, 7, 9, 11 and 15 (corresponding to 24, 48, 96, 144 and 240 hours after the last dose, respectively). Each blood sample was labelled with the protocol code, volunteer number, treatment period and day and extraction time without specifying the administered drug. Subsequently, the samples were centrifuged at 1900 *g* for 10 minutes and then the plasma was collected and stored at −20°C until the determination of drug concentrations.

2.3 | Pharmacokinetic analysis

Plasma concentrations of ARI, DARI and OLA were quantified by a high-performance liquid chromatography–tandem mass spectrometry method developed in our laboratory.[20]

The pharmacokinetic parameters were calculated by noncompartmental analysis by Phoenix WinNonlin (version 8, Pharsight, Mountain View, CA, USA) as *single dose* (i.e. for the first day) and *multiple dose* (i.e. considering all time points). Peak plasma concentration (C_{max}) and time to reach maximum concentration (T_{max}) were obtained directly from the original data. The area under the plasma concentration–time curve from time zero to the last observed time point (AUC_{last}) was calculated using the trapezoidal rule. The AUC from time zero to infinity (AUC_{inf}) was determined as the sum of the AUC_{last} and the extrapolated area calculated as the last plasma concentration (C_{last}) divided by the terminal rate constant (k_e) that was determined by regression analysis of the log-linear part of the concentration–time curve. Elimination half-life ($T_{1/2}$) was determined by $0.693/k_e$. The total apparent clearance adjusted for bioavailability (Cl/F) was calculated using the formula: $Cl/F = dose/AUC_{inf}$. The volume of distribution adjusted for bioavailability (Vd/F) was calculated as Cl/F divided by k_e. AUC and C_{max} were adjusted for dose/weight ratio (AUC/dW and C_{max}/dW, respectively) and were logarithmically transformed for statistical analysis.

2.4 | Pupillary light reflex measurements

Pupillometric measurements were performed right before and 4 hours after drug administration on each day of hospitalization. The data were recorded with a PRL-200 automated monocular infrared pupillometer (NeurOptics, Irvine, CA, USA). Each measurement was performed in a hospital room with artificial illumination. In order to adjust for differences in luminosity, light intensity (in lux) was measured at the moment of the pupillometric determination.

Before starting the measurement, the subject was instructed to focus on a small target object with the eye that was not being tested open (left eye). Stimuli were single light pulses with a fixed intensity of 180 μW during 154 ms. Once the device was focused on the target pupil (right eye), a white light stimulus was flashed. The measurements were sampled at a frequency of 32-frames/s and lasted up to 5 seconds, allowing a full or partial recovery of the pupil size after light constriction.

Eight different pupillometric parameters were measured based on the user guide.[21] Maximum pupil diameter (MAX) and minimum pupil diameter (MIN) represent the pupil diameter before constriction and just at the peak of constriction, respectively. The percentage of constriction (CON) was calculated by (MAX − MIN)/MAX. Latency (LAT) is time of the onset of constriction. ACV and MCV are average and maximum constriction velocity, respectively. The negative sign differentiates constriction from dilation being the opposite movement. After reaching its constriction, the pupil tends to recover and dilate back to its initial resting size, which is measured as average dilation velocity (ADV). T75 is the total time taken by the pupil to recover 75% of the initial resting pupil size after it reached the peak of constriction.

2.5 | Genotyping

DNA was extracted from 1 mL of peripheral blood using a MagNA Pure LC DNA Isolation Kit in an automatic DNA extractor (MagNa Pure System, Roche Applied Science, Indianapolis, Indiana, USA). Subsequently, it was quantified spectrophotometrically using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) and the purity of the samples was determined by the $A_{260/280}$ absorbance ratio.

Samples were genotyped with TaqMan assays on an OpenArray platform on a QuantStudio 12 K Flex instrument. Results were analysed with the QuantStudio 12 K Flex and the TaqMan Genotyper softwares (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The assay included 120 single nucleotide polymorphisms, of which the following 46 were analysed based on their importance in the metabolism and mechanism of action of ARI and OLA: *CYP1A2* *1C (rs2069514); *1F (rs762551); *1B 5347 T > C (rs2470890); *CYP2D6* *3 (rs35742686); *4 (rs3892097); *6 (rs5030655); *7 (rs5030867); *8 (rs5030865); *9 (rs5030656); *10 (rs1065852); *14 (rs5030865); *17 (rs28371706); *41 (rs28371725); *CYP3A4* *22 (rs35599367); *2 (rs55785340); *6 (rs4646438); *CYP3A5* *3 (rs776746); *6 (rs10264272); *ABCB1* C3435T (rs1045642); G2677T/A (rs2032582); C1236T (rs1128503); rs3842; 1000-44G > T (rs10276036); 2895 + 3559C > T (rs7787082); 330-3208C > T (rs4728709); 2481 + 788 T > C (rs10248420);

2686-3393 T > G (rs10280101); 2320-695G > A (rs12720067); 2482-707A > G (rs11983225); 2212-372A > G (rs4148737); *ADRA2A* rs1800544; *BDNF* Val66Met (rs6265); *COMT* rs4680; rs13306278; *DRD2* TaqIA (rs1800497); 957C > T (rs6277); −141 Ins/Del (rs1799732); *DRD3* Ser9Gly (rs6280); *HTR2A* T102C (rs6313); C1354T (rs6314); rs7997012; *HTR2C* −759C/T (rs3813929); −697G/C (rs518147); rs1414334; *OPRM1* rs1799971; and *UGT1A1* rs887829.

Copy number variations in the *CYP2D6* gene were determined with the TaqMan Copy Number Assay (Assay ID: Hs00010001_cn; Thermo Fisher Scientific, Waltham, MA, USA) which detects a specific sequence in exon 9.[22] Samples were run in the same instrument.

Since the *CYP2D6* *29 (rs16947) polymorphism was not included in the array, it was genotyped with the same instrument using individual TaqMan probes. Additionally, the *CYP3A4* *20 (rs67666821) polymorphism was genotyped by the KASPar SNP Genotyping System (LGC Genomics, Herts, UK). The ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific) was used for fluorescence detection and allele assignment.[23]

2.6 | Statistical analysis

Statistical analyses were performed with the SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). *P* values ≤.05 were considered significant. Hardy–Weinberg equilibrium was estimated for all analysed variants. Deviations from the equilibrium were detected by comparing the observed and expected frequencies using a Fisher exact test based on the De Finetti program (available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

ANOVA was used to compare mean pharmacokinetic values according to different categories, e.g. genotype, sex and race. Changes in pupillometric parameters (MAX, MIN, T75, MCV, CON, ACV, ADV, LAT) were analysed by repeated measures ANOVA. The values were adjusted for differences in light intensity (in lux) before analysis. Repeated measures ANOVA was used to associate pupillometric parameters to pharmacokinetic parameters and polymorphisms. A Bonferroni correction was applied for each analysis. Multiple linear regression models were used to study factors related to all pupillometric and pharmacokinetic dependent variables.

CYP2D6 genotypes were classified in 4 phenotypes (poor metabolizer: PM; intermediate metabolizer: IM; normal/rapid metabolizer: NM and ultra-rapid metabolizer: UM), which is based on the functionality of alleles[24] and according to the standardizing pharmacogenetic terms consensus.[25] *CYP3A4* *2, *20, *22 and *CYP3A5* *3 and *6 genotypes were merged into a CYP3A phenotype as follows: subjects with at least 1 CYP3A4 reduced activity allele (i.e. CYP3A4 *1/*22 or *22/*22) and no CYP3A5 activity (CYP3A5 *3/*3) were considered PM; subjects with normal CYP3A4 activity (CYP3A4 *1/*1) and no CYP3A5 activity (CYP3A5 *3/*3) were considered IM and subjects with normal CYP3A4 activity (CYP3A4 *1/*1) and at least 1 CYP3A5 functional allele (CYP3A5 *1/*1 or *1/*3) were categorized as extensive metabolizers (EM).[26] Furthermore, a value was assigned to *CYP1A2* *1B, *1C and *1F alleles based on their functionality: 0.5 to *1C, 1 to *1, 1.5 to *1F and 1.25 to *1B. An activity score was calculated as the sum of the values assigned to each allele and finally was translated into phenotypes: NMs and UMs.[27]

3 | RESULTS

3.1 | Demographic and genotypic characteristics

Ten subjects were Caucasian and 14 were Latin American. The average age was similar between males and females. Males had greater weight and height than females; however, the body mass index values did not differ significantly (Table 1).

Genotype and phenotype frequencies of the analysed variants are shown in Table S1. *HTR2C* rs3813929, rs518147, *ABCB1* rs4728709, *COMT* rs13306278, *CYP2D6* *14 (rs5030865), *17 (rs28371706), *3 (rs35742686), *6 (rs5030655), *7 (rs5030867), *8 (rs5030865), *CYP3A4* *2 (rs55785340) and *6 (rs4646438) were not in Hardy–Weinberg equilibrium ($P \leq .05$). The rest of the polymorphisms were in Hardy–Weinberg equilibrium ($P \geq .05$).

Genotype frequencies of *ABCB1* rs1128503, rs2032582, 10276036 and rs4148737 and *HTR2C* rs518147 polymorphisms were significantly different between males and females (Table S1).

3.2 | Pharmacokinetic analysis

Mean and standard deviation of ARI, DARI and OLA pharmacokinetic parameters are shown in Table 2. Females had higher ARI $T_{1/2}$

TABLE 1 Demographic characteristics

	n (%)	Age (y)	Weight (kg)	Height (m)	BMI (kg/m^2)
All	24 (100)	31.5 ± 11.6	71.4 ± 12.2	1.68 ± 0.11	25.3 ± 2.6
Males	12 (50)	28.5 ± 7.4	78.4 ± 12.2	1.76 ± 0.09	25.4 ± 2.8
Females	12 (50)	34.6 ± 14.3	64.3 ± 7.4	1.60 ± 0.07	25.1 ± 2.5
P		.104	.003	<.0001	.798

Values are shown as mean ± standard deviation unless otherwise indicated.
BMI, body mass index

TABLE 2 Pharmacokinetic parameters of aripiprazole, dehydro-aripiprazole and olanzapine after administration of a single dose and 5 multiple doses

Aripiprazole	All SINGLE DOSE	Males	Females	All MULTIPLE DOSE	Males	Females
AUC (ng·h/mL)[b]	724.9 ± 236.5	647.0 ± 197.2	802.7 ± 254.6	11 102.4 ± 8234.0	7790.0 ± 4071.5	14 415.7 ± 10061.4
C_{max} (ng/mL)	50.6 ± 15.5	47.7 ± 14.4	53.5 ± 16.6	138.0 ± 45.9	129.6 ± 47.4	146.3 ± 44.9
T_{max} (h)	5.2 ± 2.4	4.8 ± 2.8	5.6 ± 2.0	3.2 ± 1.4	3.3 ± 1.4	3.2 ± 1.5
$T_{½}$ (h)	NA	NA	NA	66.1 ± 24.6	56.1 ± 19.9	76.1 ± 25.5[a]
Vd/F (L/kg)	NA	NA	NA	6.0 ± 1.6	5.1 ± 1.0	7.0 ± 1.4[a]
Cl/F (mL/h/kg)	NA	NA	NA	68.1 ± 21.4	69.1 ± 25.6	67.1 ± 17.5
DEHYDRO-aripiprazole	**All SINGLE DOSE**	**Males**	**Females**	**All MULTIPLE DOSE**	**Males**	**Females**
AUC_{24h} (ng·h/mL)	77.4 ± 43.9	90.3 ± 56.9	64.5 ± 20.6[a]	5149.8 ± 1628.6	4721.3 ± 1670.3	5578.3 ± 1534.8
C_{max} (ng/mL)	5.4 ± 8.5	6.5 ± 5.3	4.3 ± 1.2	34.9 ± 8.5	35.6 ± 9.6	34.1 ± 7.4
T_{max} (h)	21.9 ± 4.5	20.9 ± 5.4	22.9 ± 3.4	6.1 ± 4.4	7.1 ± 3.9	5.1 ± 4.8
$T_{½}$ (h)	NA	NA	NA	107.3 ± 62.5	89.4 ± 45.4	126.9 ± 74.2
Vd/F (L/kg)	NA	NA	NA	40.0 ± 44.0	22.4 ± 10.3	57.6 ± 57.1[a]
Cl/F (mL/h/kg)	NA	NA	NA	203.5 ± 51.0	181.3 ± 47.3	230.7 ± 44.2[a]
OLANZAPINE	**All SINGLE DOSE**	**Males**	**Females**	**All MULTIPLE DOSE**	**Males**	**Females**
AUC (ng·h/mL)[b]	127.6 ± 33.1	127.8 ± 38.6	127.4 ± 28.4	1289.5 ± 370.1	1142.7 ± 291.2	1436.2 ± 393.1
C_{max} (ng/mL)	7.9 ± 2.2	7.5 ± 2.0	8.2 ± 2.5	19.1 ± 4.8	18.4 ± 4.0	19.9 ± 5.5
T_{max} (h)	5.3 ± 2.3	5.4 ± 2.7	5.3 ± 1.9	4.4 ± 1.7	4.6 ± 1.6	4.3 ± 1.9
$T_{½}$ (h)	NA	NA	NA	77.1 ± 28.2	79.5 ± 33.4	74.8 ± 23.1
Vd/F (L/kg)	NA	NA	NA	26.6 ± 15.9	26.1 ± 17.2	27.0 ± 15.3
Cl/F (mL/h/kg)	NA	NA	NA	229.7 ± 54.7	218.9 ± 51.9	240.6 ± 57.5

[a]$P \leq 0.05$ vs. males after adjusting for weight. NA: not available.
[b]for single dose administration the 24 h area under the concentration–time curve (AUC_{24h}), while for multiple dose administration area under the curve from zero to infinity (AUC_{inf}) are shown.
Abbreviations: C_{max}: maximum plasma concentration; T_{max}: time to reach the maximum plasma concentration; AUC: area under the curve; $T_{1/2}$: half-life; Cl/F: total drug clearance adjusted for bioavailability; Vd/F: volume of distribution adjusted for bioavailability.

(*P* = .044) and Vd/F (*P* = .001) and DARI Vd/F (*P* = .048) and Cl/F (*P* = .015) after multiple dose administration. Moreover, males had higher DARI AUC_{24h} (*P* = .035) after single dose administration. No differences were found in OLA pharmacokinetic parameters between males and females.

3.3 | The effects of aripiprazole and olanzapine on pupillary light reflex

Following the first oral administration of ARI, the pupil was significantly dilated. Subsequently, on the next 4 drug administration days ARI caused minor constriction. All pupillometric parameters changed significantly (MAX: *P* = .008; MIN: *P* = .009; CON: *P* = .013; LAT: *P* = .009; ACV: *P* = .012; MCV: *P* = .006; ADV: *P* = .024; T75: *P* = .015; Figure 1). OLA showed the same tendency, but only MIN reached the statistically significant level (*P* = .046; Figure 1). No differences were found between males and females regarding any pupillometric parameters.

3.3.1 | Single dose administration

ARI AUC_{last} and T_{max} had an influence on CON (*P* = .029 and *P* = .043, respectively). Moreover, AUC_{last} had an impact on MCV and ADV (*P* = .004 and *P* = .034, respectively; Table 3).

Furthermore, DARI AUC_{24h} had an impact on MAX (*P* = .042), MIN (*P* = .050), CON (*P* = .047), ACV (*P* = .049) and MCV (*P* = .046; Table 3).

These associations were not confirmed in the multivariate analysis (*P* > .05). Nonetheless, OLA pharmacokinetics did not have an effect on any of the pupillometric parameters.

3.3.2 | Multiple dose administration

DARI C_{max} had an impact on several pupillometric parameters: MAX (*P* = .029), MIN (*P* = .049), CON (*P* = .015), ACV (*P* = .041), MCV (*P* = .027), ADV (*P* = .033) and T75 (*P* = .045; Table 3). These associations were not confirmed in the multivariate analysis (*P* > .05).

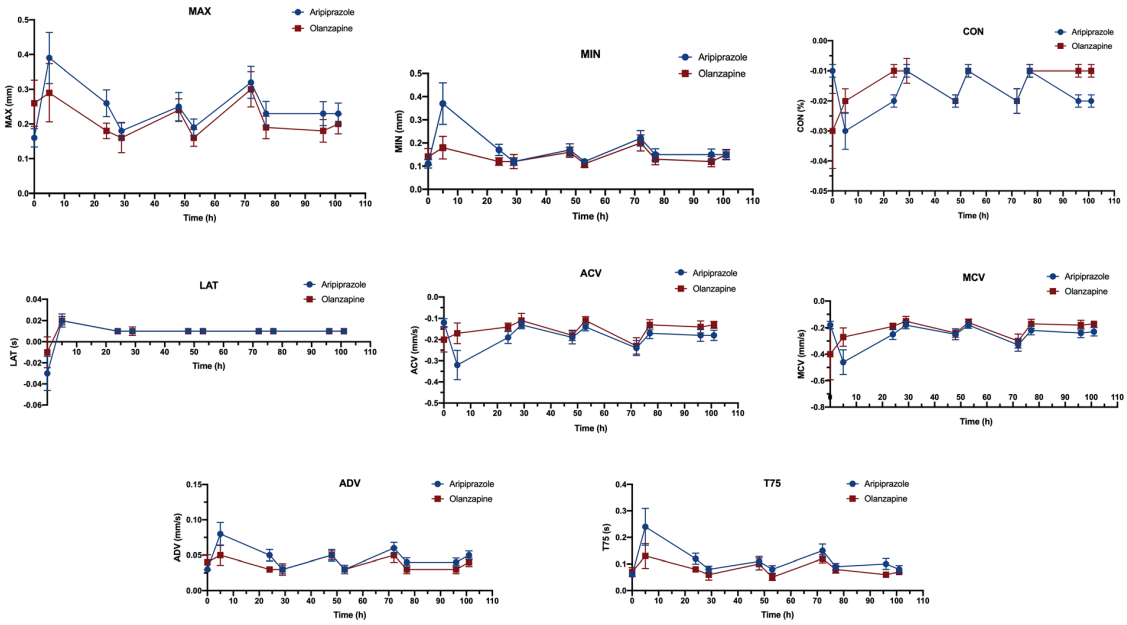

FIGURE 1 Changes in pupillometric parameters after aripiprazole and olanzapine administration. MAX, maximum pupil diameter, MIN, minimum pupil diameter; CON, percentage of constriction; LAT, latency; ACV, average constriction velocity; MCV, maximum constriction velocity; ADV, average dilation velocity; T75, total time taken by the pupil to recover 75% of the initial resting pupil size

TABLE 3 Influence of the pharmacokinetic parameters of aripiprazole and dehydro-aripiprazole on pupillometric parameters

	Aripiprazole		Dehydro-aripiprazole	
	Single dose		Single dose	Multiple dose
Variable	AUC_{last} (ng·h/mL)	T_{max} (h)	AUC_{24h} (ng·h/mL)	C_{max} (ng/mL)
MAX	---	---	P = .042 ↓↓	P = .029 ↓↓
MIN	---	---	P = .050 ↓↓	P = .049 ↓↓
CON	P = .029 ↓↑	P = .043 ↓↓	P = .047 ↓↓	P = .015 ↓↓
ACV	---	---	P = .049 ↓↓	P = .041 ↓↓
MCV	P = .004 ↓↑	---	P = .046 ↓↑	P = .027 ↓↑
ADV	P = .034 ↓↓	---	---	P = .033 ↓↓
T75	---	---	---	P = .045 ↓↓

The arrows show the relationship between pharmacokinetic and pupillometric parameters. The first arrow refers to the pharmacokinetic parameter, while the second arrow refers to the pupillometric parameter. ↓↑ is indirectly proportional, while ↓↓ is directly proportional with the changes.
Abbreviations: MAX: maximum pupil diameter; MIN: minimum pupil diameter; CON: percent of constriction; LAT: latency; ACV: average constriction velocity; MCV: maximum constriction velocity; ADV: average dilation velocity; T75: total time taken by the pupil to recover 75% of the initial resting pupil size. C_{max}: maximum plasma concentration; T_{max}: time to reach the maximum plasma concentration; AUC_{24h}: 24 h area under the concentration–time curve; AUC_{last}: area under the curve from time zero to the last observed time point.

Nonetheless, ARI and OLA pharmacokinetics did not have any association with any of the pupillometric parameters.

3.4 | The influence of polymorphisms on pupillometry

3.4.1 | Aripiprazole

Subjects with the CYP3A IM phenotype had significantly higher MAX levels than PMs (P = .019). Moreover, *HTR2A* rs6314 T carriers and *UGT1A1* rs8877829 T/T homozygotes had higher MIN levels than C/C subjects (P = .025 and .039, respectively). Additionally, subjects with the CYP3A PM phenotype and *DRD2* rs1800487 A2 carriers had higher CON values than with IM phenotype and A1 carriers, respectively; however, only CYP3A reached the significant level (P = .008 and .058, respectively).

Likewise, CYP3A IM and EM subjects, *DRD2* rs1800487 A2 carriers, *ABCB1* rs10280101 A/A, rs12720067 C/C and rs11983225 T/T subjects had higher LAT values than CYP3A PM subjects, *DRD2* rs1800487 A1 carriers and *ABCB1* 10280101 C, *ABCB1* rs12720067 T and *ABCB1* rs19983225 C carriers, respectively (P = .020, .039

and .034, respectively). Moreover, CYP3A PM subjects had lower ACV and MCV values than IM subjects (P = .028 and .022, respectively). Finally, *HTR2A* rs6314 T allele carriers had higher T75 levels than C/C homozygotes, although it did not reach the statistically significant level (P = .058).

After performing the multivariate tests, the influence of *HTR2A* rs6314 on MIN and T75 remained significant (P = .001 and .020, respectively; Figure 2).

3.4.2 | Olanzapine

DRD2 rs1800497 A2 allele carriers had higher MAX, ACV and MCV values than A1/A1 homozygotes (P = 0.025, .043 and .038).

After performing the multivariate tests, the influence of *DRD2* rs1800497 on MAX remained significant (P = .039; Figure 2).

3.5 | The influence of polymorphisms on pharmacokinetics

The univariate and multivariate analyses revealed associations between ARI, DARI and OLA pharmacokinetic parameters and several polymorphisms (Tables S2, S3 and S4). Additionally, the results of the multivariate analysis are shown in Table 4.

3.5.1 | Aripiprazole

Vd/F and Cl/F were notably higher in CYP2D6 UMs than in NMs and IMs (Vd/F: p = 0.001 and P = .016; Cl/F: P = .016 and .016, respectively). Additionally, AUC_{inf} and C_{max} on days 1 and 5 were significantly higher in CYP1A2 UMs than in NMs and RMs (P = .034, .040 and .012, respectively). Moreover, Cl/F was lower in CYP1A2 UMs compared to the other phenotypes (P = .033). Additionally, T_{max} was higher in *ABCB1* rs1045642 TT compared to CC subjects (P = .033). *ABCB1* rs4148737 C/C subjects had 2 times higher $T_{1/2}$ and T_{max} than T carriers (P = .024 and .004). The results are shown in Table S2. Several of these associations were confirmed in the multivariate analysis (Table 4).

3.5.2 | Dehydro-aripiprazole

C_{max} was significantly higher in CYP2D6 IM subjects than in NMs and UMs (P = .006). CYP3A4 PMs had higher AUC_{last} and C_{max} than IMs and EMs (P = .001 and .001, respectively). Additionally, Vd/F was higher in CYP1A2 UMs than in NMs and RMs (P = .046). Additionally, T_{max} was higher in *ABCB1* rs1045642 T/T and lower in *ABCB1* rs4148737 C/C subjects compared to the other genotypes (P = .019 and P = .045, respectively). The results are shown in Table S3. Several of these associations were confirmed in the multivariate analysis (Table 4).

3.5.3 | Olanzapine

$T_{1/2}$ was 2 times higher in CYP3A EM subjects compared to IMs and PMs (P = .025). Additionally, *ABCB1* rs10280101 A/A, rs12720067 C/C and rs11983225 T/T subjects had significantly higher $T_{1/2}$ compared to the other genotypes (P = .046, .046 and .046, respectively). Finally, *UGT1A1* rs887829 T/T homozygotes had higher T_{max} than

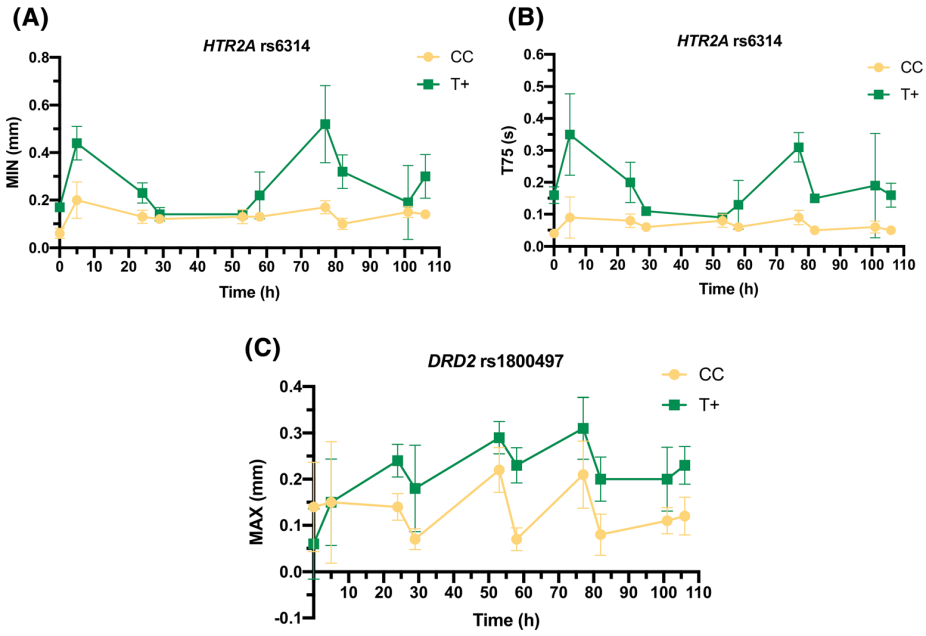

FIGURE 2 The influence of HTR2A rs6314 and DRD2 rs1800497 polymorphisms on pupillometric parameters. (A) The influence of HTR2A rs6314 on minimum pupil diameter (MIN) during aripiprazole treatment. (B) The influence of HTR2A rs6314 on total time taken by the pupil to recover 75% of the initial resting pupil size (T75) during aripiprazole treatment. (C) The influence of DRD2 rs1800497 on maximum pupil diameter (MAX) during olanzapine treatment

TABLE 4 Influence of genetic polymorphisms on aripiprazole, dehydro-aripiprazole and olanzapine pharmacokinetic parameters in the multivariate analysis. Results with $P \leq .05$ are highlighted in bold

Aripiprazole						
Variable	**AUC (ng·h/mL)**	**C_{max} (ng/mL)**	**T_{max} (h)**	**$T_{1/2}$ (h)**	**Cl/F (L/h·kg)**	**Vd/F (L/kg)**
CYP2D6 phenotype	**β = −1063.9; *P* = .049**	---	---	**β = 16.2; *P* = .005**	β = 15.1; *P* = .055	**β = 1.9; *P* = .001**
CYP1A2 phenotype	**β = 64555; *P* = .035**	**β = 421; *P* = .044**	---	**β = 29.3; *P* = .041**	β = −22.1; *P* = .054	---
ABCB1 rs1045642	---	---	β = 0.183; *P* = .738	---	---	---
ABCB1 rs4148737	**β = 58941; *P* = .026**	---	β = .183; *P* = .545	**β = −17.5; *P* = .027**	---	---
Dehydro-aripiprazole						
Variable	**AUC (ng·h/mL)**	**C_{max} (ng/mL)**	**T_{max} (h)**	**$T_{1/2}$ (h)**	**Cl/F (L/h·kg)**	**Vd/F (L/kg)**
CYP2D6 phenotype	---	**β = 17.3; *P* = .043**	---	---	---	---
CYP3A4 phenotype	β = 4257; *P* = .273	**β = 55.2; *P* = .005**	**β = 3.14; *P* = .015**	---	---	---
CYP1A2 phenotype	---	**β = 70.5; *P* = .039**	---	---	---	β = 10.2; *P* = .387
ABCB1 rs1045642	---	---	**β = 2.93; *P* = .021**	---	---	---
ABCB1 rs4148737	---	---	β = −1.99; *P* = .240	---	---	**β = 17.1; *P* = .041**
Olanzapine						
Variable	**AUC (ng·h/mL)**	**C_{max} (ng/mL)**	**T_{max} (h)**	**$T_{1/2}$ (h)**	**Cl/F (L/h·kg)**	**Vd/F (L/kg)**
CYP3A4 phenotype	---	---	---	**β = −17; *P* = .037**	---	---
ABCB1 rs10280101	---	---	---	β = −26.9; *P* = .145	---	---
ABCB1 rs12720067	---	---	---	β = −26.9; *P* = .145	---	---
ABCB1 rs11983225	---	---	---	β = −26.9; *P* = .145	---	---
UGT1A1 rs887829	---	---	**β = 1.58; *P* = .006**	---	---	---

Abbreviations: CYP: cytochrome p450 oxidase; *ABCB1*: ATP binding cassette subfamily B member 1; *UGT1A1*: UDP glucuronosyltransferase family 1 member A1; C_{max}: maximum plasma concentration; T_{max}: time to reach the maximum plasma concentration; AUC: area under the curve; $T_{1/2}$: half-life; Cl/F: total drug clearance adjusted for bioavailability; Vd/F: volume of distribution adjusted for bioavailability.

C/T heterozygotes and C/C homozygotes ($P = .016$). The results are shown in Table S4. Several of these associations were confirmed in the multivariate analysis (Table 4).

4 | DISCUSSION

4.1 | The influence of sex on pharmacokinetics, pharmacogenetics and pupillometry

In the current study, ARI and DARI pharmacokinetics were affected by sex. Females had higher ARI $T_{1/2}$ and Vd/F than males, which is consistent with our previous studies.[18,28] However, in the present study females had higher DARI Vd/F and Cl/F and lower AUC_{last}, which may be explained by the low sample size. Based on previous studies, Cl/F should be lower in males compared to females.[29,30] Nonetheless, no dosage adjustment is recommended for ARI or OLA despite sex differences because they are predominantly explained by the differences in body weight.[30,31]

The differences observed in the prevalence of *ABCB1* rs1128503, rs2032582, rs10276036 and rs4148737 polymorphisms regarding sex may be explained by the reduced sample size. Regarding *HTR2C*, it is explained by the location of the gene on chromosome X.

Some polymorphisms were not in Hardy–Weinberg equilibrium. Regarding *HTR2C*, it is due to the location of the gene on chromosome X. The disequilibrium for *ABCB1* rs4728709 and *COMT* rs13306278 could be explained by the small sample size. Regarding the *CYP2D6* and *CYP3A4* polymorphisms it is explained by the low frequency of mutated alleles.

No differences were found between males and females in any of the pupillometric parameters, which is consistent with previous studies.[18,32]

4.2 | Effects of aripiprazole, dehydro-aripiprazole and olanzapine on pupillometry

The mechanism of action of ARI and OLA is still not perfectly understood.[33] ARI and DARI achieve their pharmacological effect possibly by partial agonistic activity at dopamine D2 and 5-HT1A receptors and antagonistic activity at 5-HT2A receptors. Pharmacodynamic effects on receptors other than dopamine D2, 5-HT1A and 5-HT2A may explain other clinical effects: changes in pupillary light reflex could be caused by partial agonistic activity at α1-adrenergic receptors.[31] Pupil dilatation is primarily an α1-adrenergic receptor-mediated effect,[34] while it is mediated to a lesser extent by dopamine and serotonin receptors.[35,36]

The dilatation observed after the first ARI administration could be explained by its partial agonism at these receptors. On the contrary, OLA is an antagonist at these receptors,[3] therefore, it could explain the lack of pupil dilatation. Both ARI and DARI have higher affinity for dopamine D2 and 5-HT1A than for α1-adrenergic receptors.[37] When DARI was present, neither ARI nor DARI bound to α1-adrenergic receptors due to competitive inhibition caused by the higher affinity for dopamine D2 and 5-HT1A receptors. Hence, a constriction was observed after drug administration. Our results could confirm the fact that pupillary changes may rather be caused by the metabolite than the parent compound.[34] This could be the reason why DARI C_{max} and AUC_{last} had an influence on several pupillometric parameters, while the pharmacokinetic parameters of ARI influenced only a few of them.

In conclusion, ARI and DARI caused changes in pupillary light reflex due to their unique pharmacological profile. Measuring dynamic pupillary light reflex is already a valid test for the pharmacodynamic effects of opioid- and some noradrenergic drugs.[38,39] Both drugs caused pupil constriction in 2 previous studies[18,40]; however, in another study, neither ARI nor OLA affected pupil contraction.[41] Hence, more studies are needed to alleviate the ambiguity and they should be repeated in patients. Afterwards, pupillometry could be introduced in the practice to assess autonomic nerve activity.

4.3 | Polymorphisms and pupillometry

In previous studies with opioids, CYP2D6 UMs experienced increased and PMs experienced decreased pupil size compared to EMs.[38,39] We could not replicate these findings with ARI and OLA, which may be due to their different mechanism of action. Additionally, we did not find any PM and only 2 UMs were present in our population. Our results confirm those in our previous study with healthy volunteers that no associations can be found between CYP2D6 phenotypes and pupillometric parameters.[18]

CYP3A phenotypes are unrelated to opioid pharmacokinetics.[42] Notwithstanding, in the present study, CYP3A IM pupil size was increased compared to that of PMs after ARI administration. This was expected as ARI caused pupil constriction after multiple dose administration; the pupil was under prolonged ARI exposure in PM subjects.

The effects of dopamine and serotonin on the pupillary light reflex are well known. High serotonin levels cause pupil dilatation[36] and dopamine may cause pupil dilatation or constriction through sympathetic and parasympathetic nerves, respectively.[35] Based on our results, pupil constriction could be due to the antagonist activity of ARI at 5-HT2A and 5-HT7 receptors, while its dilatation could be explained by its partial agonism at dopamine D2, D3, D4 and serotonin 5-HT1A, 5-HT2C receptors.[37] This theory was confirmed by our study: both *HTR2A* rs6314 and *DRD2* rs1800487 had an influence on the pupil size, the proportion of its change, the time of onset of constriction and pupil recovery. Additionally, *DRD2* rs1800487 also affected the pupil size and its constriction velocity after OLA treatment. In our previous study some *HTR2A*, *HTR2C*, *DRD2* and *DRD3* polymorphisms were also related to pupillometric parameters.[18] The lack of associations with *HTR2C* and *DRD3* polymorphisms in the present study could be due to the low sample size.

Both ARI and OLA are transported by the P-glycoprotein (P-gp, ABCB1, MDR1).[43] However, to our knowledge, no previous study could associate the genetic differences in *ABCB1* to pupil contraction. We previously analysed 3 polymorphisms: rs1045642 (C3435T), rs1128503 (C1236T) and rs2032582 (G2677T/A), but no associations were found.[18] In the current study we analysed 12 *ABCB1* polymorphisms. No association was found with the 3 previously mentioned polymorphisms; however, 3 others had an influence on pupil recovery after ARI treatment. It may suggest that, even though the 3 most common polymorphisms do not affect the pupillary light reflex, others could have an influence. Further research including other less studied *ABCB1* polymorphisms should be performed.

UGT1A1 rs8877829 subjects with the mutated T/T genotype had greater pupil size after ARI treatment. Based on in vitro studies, ARI does not undergo direct glucuronidation by UGT enzymes[31]; however, no sufficient evidence is available thus far. Considering our results, *UGT1A1* may be involved in ARI metabolism.

4.4 | Pharmacokinetics and polymorphisms

Our study confirms the impact of CYP2D6 phenotypes on ARI and DARI pharmacokinetics.[18,44,45] All pharmacokinetic parameters, except for T_{max}, were different in UMs compared to NMs and IMs.

CYP3A only had an impact on DARI and OLA pharmacokinetics, confirming its involvement in ARI and OLA metabolism.[7] The lack of association with the parent drug, ARI, may be due to the low sample size, because CYP2D6 and CYP3A4 contribute about equally to the metabolism of aripiprazole.[7] Moreover, CYP3A activity varies predominantly by sex and inhibition or induction of a wide range of substrates, rather than by polymorphisms.[46]

Based on the literature, ARI is not a substrate of CYP1A2.[31] Unexpectedly, in our study, the CYP1A2 phenotype influenced ARI and DARI pharmacokinetics as UMs showed a lower disposition compared to the other phenotypes. To the best of our knowledge, this is the first study to report a similar result. Based on our findings, more studies should be performed to confirm the role of CYP1A2 in ARI pharmacokinetics.

To date, there are no consistent findings about the role of polymorphisms in ABCB1. In our previous studies the C1236T (rs1128503) polymorphism had an influence on ARI pharmacokinetics.[18,47] In the current study, the C3435T (rs1045642) and rs4148737 polymorphisms were related to ARI and DARI $T_{1/2}$ and C_{max}—and the rs10280101, rs12720067 and rs11983225 polymorphisms had an influence on OLA $T_{1/2}$ levels. Presumably *ABCB1* has an effect on the pharmacokinetics of these antipsychotics being substrates of P-gp.[43] According to our knowledge, no other study analysed polymorphisms in *ABCB1* other than C3435T (rs1045642), G2677TA (rs2032582), C1236T (rs1128503). More studies are needed including more polymorphisms in *ABCB1* to provide a wider insight of its role in ARI and OLA pharmacokinetics. Additionally, as stated previously, the lack of consensus on P-gp pharmacogenetics

is partially explained by the lack of studies and the guidelines describing phenotype interference from variants.

Finally, the *UGT1A1* rs887829 polymorphism affected OLA pharmacokinetics. In a previous study, this polymorphism was related to some adverse effects but not pharmacokinetics.[48] OLA is metabolized predominantly by direct glucuronidation via the UGT enzyme family,[7] but clear evidence was found only for UGT1A4.[6] Additionally, *UGT1A4* and *UGT2B10* polymorphisms significantly contributed to the interindividual variability in OLA metabolism.[49,50] Our study is the first reporting an association between an *UGT1A1* polymorphism and OLA pharmacokinetics.

4.5 | Study limitations

Only 24 subjects were included in the study, which we consider its main limitation. Therefore, it is important to interpret these results with caution: studies including more subjects are necessary to increase the statistical reliability of the results. Moreover, the present study should be repeated in schizophrenic patients, whose brain structure and genetics may differ from healthy volunteers. Moreover, neither ARI, nor OLA reached steady state during 5 days of treatment. Both could have had a greater effect on autonomic nerve activity if they had reached steady state. However, the Ethics Committee does not authorize a treatment longer than 5 days with antipsychotics in healthy volunteers. Accordingly, we cannot apply pupillometry to assess autonomic disfunction in the clinical practice yet. Additionally, the Cl/F and Vd/F values were calculated without knowing the bioavailability, which can yield questionable results, especially for DARI.

5 | CONCLUSIONS

ARI administration produced pupil contraction: it affected all pupillometric parameters. After the first dosing it caused dilatation, which was followed by constriction after each day of treatment. OLA did not cause any changes in any of the pupillometric parameters. Additionally, the effects of ARI on the pupil size, the time of onset of constriction, pupil recovery and constriction velocity were associated with polymorphisms in *CYP3A*, *HTR2A*, *UGT1A1*, *DRD2* and *ABCB1* genes. ARI, DARI and OLA pharmacokinetics were significantly affected by polymorphisms in *CYP2D6*, *CYP3A*, *CYP1A2*, *ABCB1* and *UGT1A1* genes. In conclusion, pupillography could be a noninvasive tool to assess autonomic nervous system activity during antipsychotic drug treatment.

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COMPETING INTERESTS

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CONTRIBUTORS

Wrote manuscript: Dora Koller; designed research: Dora Koller, Francisco Abad-Santos, Miriam Saiz-Rodríguez, Dolores Ochoa; performed clinical trial: Manuel Román, Gina Mejía, Francisco Abad-Santos, Daniel Romero-Palacián, Alejandro de Miguel-Cáceres, Samuel Martín, Dolores Ochoa; analysed data: Dora Koller, Susana Almenara, Francisco Abad-Santos; determination of drug concentrations: Dora Koller, Pablo Zubiaur, Aneta Wojnicz; pharmacogenetics: Dora Koller, Pablo Zubiaur, Marcos Navares, Miriam Saiz-Rodríguez.

DATA AVAILABILITY STATEMENT

Clinical Trial registry name, URL and registration number: TREATMENT-HV, EUDRA-CT: 2018–000744-26, https://eudract.ema.europa.eu/.

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***Table* S1. Genotype frequencies.** Values are expressed as number of subjects (percentage of the total).

Gene / variant	Genotype	Total	Males	Females
CYP2D6 phenotype	**NM**	16 (66.7)	8 (66.7)	8 (66.7)
	IM	6 (25.0)	3 (25.0)	3 (25.0)
	UM	2 (8.3)	1 (8.3)	1 (8.3)
CYP1A2 phenotype	**NM/RM**	17 (70.8)	10 (83.3)	7 (58.3)
	UM	7 (29.2)	2 (16.7)	5 (41.7)
CYP3A4 phenotype	**EM**	4 (66.7)	3 (25.0)	1 (8.3)
	IM	17 (25.0)	7 (58.3)	10 (83.3)
	PM	3 (8.3)	2 (16.7)	1 (8.3)
ABCB1* rs1045642	**C/C**	7 (29.2)	6 (50.0)	1 (8.3)
	C/T	12 (50.0)	4 (33.3)	8 (66.7)
	T/T	5 (20.8)	2 (16.7)	3 (25.0)
ABCB1* rs1128503	**C/C**	8 (33.3)	6 (50.0)	2 (16.7)
	C/T	11 (45.8)	6 (50.0)	5 (41.7)
	T/T	5 (20.8)	0 (0.0)	5 (41.7)
ABCB1* rs2032582	**C/C**	9 (37.5)	7 (58.3)	2 (16.7)
	C/A	12 (50.0)	5 (41.7)	7 (41.7)
	A/T	3 (12.5)	0 (0.0)	3 (41.7)
***ABCB1* rs10248420**	**G/G**	1 (4.2)	0 (0.0)	1 (8.3)
	A/G	11 (45.8)	6 (50.0)	5 (41.7)
	A/A	12 (50.0)	6 (50.0)	6 (50.0)
ABCB1* rs10276036	**T/T**	8 (33.3)	6 (50.0)	2 (16.7)
	C/T	11 (45.8)	6 (50.0)	5 (41.7)
	C/C	5 (20.8)	0 (0.0)	5 (41.7)
***ABCB1* rs10280101**	**A/A**	16 (66.7)	7 (58.3)	9 (75.0)
	A/C	8 (33.3)	5 (41.7)	3 (25.0)
***ABCB1* rs11983225**	**T/T**	16 (66.7)	7 (58.3)	9 (75.0)
	C/T	8 (33.3)	5 (41.7)	3 (25.0)
***ABCB1* rs12720067**	**C/C**	16 (66.7)	7 (58.3)	9 (75.0)
	C/T	8 (33.3)	5 (41.7)	3 (25.0)
***ABCB1* rs3842**	**T/T**	19 (79.2)	9 (75.0)	10 (83.3)
	C/T	4 (16.7)	3 (25.0)	1 (8.3)
	C/C	1 (4.2)	0 (0.0)	1 (8.3)

Gene / variant	Genotype	Total	Males	Females
ABCB1* rs4148737	**T/T**	9 (37.5)	1 (8.3)	8 (66.7)
	C/T	12 (50.0)	9 (75.0)	3 (25.0)
	C/C	3 (12.5)	2 (16.7)	1 (8.3)
***ABCB1* rs4728709**	**A/A**	1 (4.2)	1 (8.3)	0 (0.0)
	A/G	2 (8.3)	1 (8.3)	1 (8.3)
	G/G	21 (87.5)	10 (83.3)	11 (91.7)
***ABCB1* rs7787082**	**A/A**	1 (4.2)	0 (0.0)	1 (8.3)
	A/G	12 (50.0)	7 (58.3)	5 (41.7)
	G/G	11 (45.8)	5 (41.7)	6 (50.0)
***ADRA2A* rs1800544**	**C/C**	16 (66.7)	8 (66.7)	8 (66.7)
	C/G	8 (33.3)	4 (33.3)	4 (33.3)
***BDNF* rs6265**	**C/C**	16 (66.7)	6 (50.0)	10 (83.3)
	C/T	6 (25.0)	5 (41.7)	1 (8.3)
	T/T	2 (8.3)	1 (8.3)	1 (8.3)
***COMT* rs13306278**	**C/C**	21 (87.5)	10 (83.3)	11 (91.7)
	C/T	2 (8.3)	2 (16.7)	0 (0.0)
	T/T	1 (4.2)	0 (0.0)	1 (8.3)
***COMT* rs4680**	**G/G**	5 (20.8)	3 (25.0)	2 (16.7)
	A/G	10 (41.7)	4 (33.3)	6 (50.0)
	A/A	9 (37.5)	5 (41.7)	4 (33.3)
***DRD2* rs6277**	**A/A**	9 (37.5)	5 (41.7)	4 (33.3)
	A/G	13 (54.2)	7 (58.3)	6 (50.0)
	G/G	2 (8.3)	0 (0.0)	2 (16.7)
***DRD2* rs1799732**	**G/G**	18 (75.0)	7 (58.3)	11 (91.7)
	G/-	6 (25)	5 (41.7)	1 (8.3)
***DRD2* rs1800497**	**A1/A1**	14 (58.3)	7 (58.3)	7 (58.3)
	A1/A2	7 (29.2)	4 (33.3)	3 (25.0)
	A2/A2	3 (12.5)	1 (8.3)	2 (16.7)
***DRD3* rs6280**	**Ser/Ser**	6 (25.0)	3 (25.0)	3 (25.0)
	Ser/Gly	14 (58.3)	8 (66.7)	6 (50.0)
	Gly/Gly	4 (16.7)	1 (8.3)	3 (25.0)
***HTR2A* rs6313**	**C/C**	9 (37.5)	3 (25.0)	6 (50.0)
	C/T	9 (37.5)	6 (50.0)	3 (25.0)

Gene / variant	Genotype	Total	Males	Females
	T/T	6 (25.0)	3 (25.0)	3 (25.0)
***HTR2A* rs6314**	**C/C**	22 (91.7)	11 (91.7)	11 (91.7)
	C/T	2 (8.3)	1 (8.3)	1 (8.3)
***HTR2A* rs7997012**	**G/G**	11 (45.8)	6 (50.0)	5 (41.7)
	A/G	12 (50.0)	6 (50.0)	6 (50.0)
	A/A	1 (4.2)	0 (0.0)	1 (8.3)
***HTR2C* rs1414334**	**C/C**	20 (83.3)	11 (91.7)	9 (75.0)
	C/G	3 (12.5)	0 (0.0)	3 (25.0)
	G/G	1 (4.2)	1 (8.3)	0 (0.0)
HTR2C* rs518147	**C/C**	5 (20.8)	4 (33.3)	1 (8.3)
	C/G	6 (25.0)	0 (0.0)	6 (50.0)
	G/G	13 (54.2)	8 (66.7)	5 (41.7)
***HTR2C* rs3813929**	**C/C**	18 (75.0)	10 (83.3)	8 (66.7)
	C/T	3 (12.5)	0 (0.0)	3 (25.0)
	T/T	3 (12.5)	2 (16.7)	1 (8.3)
***OPRM1* rs1799971**	**A/A**	19 (79.2)	10 (83.3)	9 (75.0)
	A/G	4 (16.7)	2 (16.7)	2 (16.7)
	G/G	1 (4.2)	0 (0.0)	1 (8.3)
***UGT1A1* rs887829**	**C/C**	8 (33.3)	2 (16.7)	6 (50.0)
	C/T	11 (45.8)	7 (58.3)	4 (33.3)
	T/T	5 (20.8)	3 (25.0)	2 (16.7)

*$p < 0.05$ females versus males.

Abbreviations: CYP: cytochrome p450 oxidase; ABCB1: ATP binding cassette subfamily B member 1; ADRA2A: alpha-2A adrenergic receptor; BDNF: brain-derived neurotrophic factor; COMT: catechol-O-methyltransferase; DRD2: dopamine D2 receptor; DRD3: dopamine D3 receptor; HTR2A: serotonin receptor 2A; HTR2C: serotonin receptor 2C; OPRM1: μ1-opioid receptor 1; UGT1A1: UDP glucuronosyltransferase family 1 member A1; EM: extensive metabolizer IM: intermediate metabolizer;. NM/RM: normal metabolizer/ rapid metabolizer; NM: normal metabolizer; PM: poor metabolizer; UM: ultrarapid metabolizer.

***Table* S2. Influence of genetic polymorphisms on aripiprazole pharmacokinetic parameters.** Values are presented as mean ± SD.

	AUC (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	Cl/F (L/h·kg)	Vd/F (L/kg)
CYP2D6 phenotype						
NM (n = 16)	79899 ± 59385	1006 ± 409	3.19 ± 1.52	67.3 ± 25.8	67.1 ± 18.5	6.11 ± 1.61
IM (n = 6)	89464 ± 34240	1025 ± 244	3.33 ± 1.37	72.4 ± 20.2	57.2 ± 11.4	5.89 ± 1.80
UM (n = 2)	24219 ± 15580	638 ± 52	3.00 ± 0.00	37.8 ± 8.2	108.5 ± 28.4*	5.74 ± 0.27*
CYP1A2 phenotype						
NM/RM (n = 17)	63090 ± 35434	864 ± 219	3.12 ± 1.37	60.5 ± 18.5	74.0 ± 22.1	6.07 ± 1.51
UM (n = 7)	113009 ± 74372*	1261 ± 503*	3.43 ± 1.51	79.7 ± 33.2	53.8 ± 11.0*	5.93 ± 1.77
***ABCB1* rs1045642**						
C/C (n = 7)	63819 ± 44267	970 ± 296	3.14 ± 1.35	56.5 ± 23.2	67.9 ± 22.1	5.11 ± 1.52
C/T (n = 12)	85227 ± 64768	1020 ± 463	3.00 ± 1.54	73.2 ± 27.0	67.6 ± 16.9	6.75 ± 1.57
T/T (n = 5)	78827 ± 37731	897 ± 177	3.82 ± 1.12*	62.6 ± 18.6	69.5 ± 33.5	5.59 ± 0.57
***ABCB1* rs4148737**						
T/T (n = 9)	75711 ± 25536	861 ± 150	3.68 ± 1.33	69.0 ± 13.2	69.1 ± 16.5	6.74 ± 1.57
C/T (n = 12)	66727 ± 38774	1008 ± 437	2.67 ± 1.23	59.6 ± 20.7	70.8 ± 24.9	5.60 ± 1.71
C/C (n = 3)	127160 ± 132397	1226 ± 481	4.00 ± 1.73#	83.5 ± 56.3*	54.3 ± 21.4	6.03 ± 1.55

#: compared to the wild-type homozygote genotype.
*: compared to the other genotypes.

Abbreviations: CYP: cytochrome p450 oxidase; *ABCB1*: ATP binding cassette subfamily B member 1; C_{max}: maximum plasma concentration; T_{max}: time to reach the maximum plasma concentration; AUC: area under the curve; $T_{1/2}$: half-life; Cl/F: total drug clearance adjusted for bioavailability; Vd/F: volume of distribution adjusted for bioavailability.

***Table* S3. Influence of genetic polymorphisms on dehydro-aripiprazole pharmacokinetic parameters.** Values are presented as mean ± SD.

	AUC (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	Cl/F (L/h·kg)	Vd/F (L/kg)
CYP2D6 phenotype						
NM (n = 16)	39410 ± 13915	258.5 ± 83.9	5.03 ± 3.54	110.2 ± 68.2	202.9 ± 54.8	33.3 ± 28.5
IM (n = 6)	32841 ± 10533	222.3 ± 56.1	9.17 ± 4.49	120.6 ± 46.6	220.7 ± 50.5	66.8 ± 72.0
UM (n = 2)	26862 ± 11426	248.5 ± 35.4	5.71 ± 8.90	51.1 ± 4.3	186.5 ± 28.2	13.7 ± 0.93
CYP3A phenotype						
EM (n = 4)	35366 ± 13389	239.0 ± 32.4	8.50 ± 4.04*	107.3 ± 58.6	195.0 ± 18.4	29.5 ± 14.2
IM (n = 17)	36871 ± 12796	235.1 ± 61.1	5.52 ± 4.42	111.0 ± 67.1	215.6 ± 52.3	35.5 ± 28.2
PM (n = 3)	37689 ± 20275	337.7 ± 138.2	6.33 ± 5.13	76.4 ± 37.4	166.0 ± 67.7	79.7 ± 113.2
CYP1A2 phenotype						
NM/RM (n = 17)	40899 ± 17468	268.2 ± 149.0	8.24 ± 0.63	116.4 ± 48.1	235.4 ± 116.4	35.7 ± 8.4
UM (n = 7)	55487 ± 17242	371.3 ± 181.6	10.48 ± 2.00	245.4 ± 72.5	249.0 ± 138.5	137.2 ± 16.8*
***ABCB1* rs1045642**						
C/C (n = 7)	35269 ± 13074	276.5 ± 66.3	6.01 ± 3.16	88.2 ± 49.7	181.6 ± 50.9	23.0 ± 13.4
C/T (n = 12)	36962 ± 12673	247.2 ± 84.1	4.32 ± 4.31	117.9 ± 77.1	215.1 ± 49.7	52.3 ± 59.1
T/T (n = 5)	38181 ± 17115	212.9 ± 55.3	10.60 ± 3.13*	110.9 ± 43.9	218.3 ± 55.1	34.5 ± 15.9
***ABCB1* rs4148737**						
T/T (n = 9)	35175 ± 13374	215.0 ± 45.8	6.20 ± 5.74	109.2 ± 33.7	226.8 ± 47.1	54.8 ± 59.5
C/T (n = 12)	38045 ± 14819	275.2 ± 88.9	6.51 ± 3.87	91.8 ± 42.7	189.9 ± 50.8	24.4 ± 10.8
C/C (n = 3)	36072 ± 7064	242.8 ± 53.7	4.33 ± 1.15*	164.4 ± 150.2	208.1 ± 61.5	58.1 ± 66.9

#: compared to the wild-type homozygote genotype.
*: compared to the other genotypes.

Abbreviations: CYP: cytochrome p450 oxidase; *ABCB1*: ATP binding cassette subfamily B member 1; C_{max}: maximum plasma concentration; T_{max}: time to reach the maximum plasma concentration; AUC: area under the curve; $T_{1/2}$: half-life; Cl/F: total drug clearance adjusted for bioavailability; Vd/F: volume of distribution adjusted for bioavailability.

***Table* S4. Influence of genetic polymorphisms on olanzapine pharmacokinetic parameters.** Values are presented as mean ± SD.

	AUC (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	Cl/F (L/h·kg)	Vd/F (L/kg)
CYP3A phenotype						
EM (n = 4)	15877 ± 3836	10.6 ± 1.4	5.00 ± 2.45	109.7 ± 41.2	265.0 ± 59.7	42.7 ± 23.0
IM (n = 17)	19186 ± 6586	14.8 ± 4.0	4.55 ± 1.51	72.3 ± 22.0	222.0 ± 55.7	24.0 ± 13.5
PM (n = 3)	16613 ± 4492	15.0 ± 3.1	3.01 ± 1.72	60.9 ± 3.3*	226.8 ± 35.0	19.8 ± 2.0
***ABCB1* rs10280101**						
A/A (n = 16)	18614 ± 5930	13.3 ± 3.2	4.83 ± 1.72	85.2 ± 31.4	231.5 ± 56.3	29.9 ± 18.4
C+ (n = 8)	17711 ± 6459	15.6 ± 4.9	3.63 ± 1.50	61.1 ± 7.4*	226.1 ± 55.1	20.0 ± 5.7
***ABCB1* rs12720067**						
C/C (n = 16)	18614 ± 5930	13.3 ± 3.2	4.83 ± 1.72	85.2 ± 31.4	231.5 ± 56.3	29.9 ± 18.4
T+ (n = 8)	17711 ± 6459	15.6 ± 4.9	3.63 ± 1.50	61.1 ± 7.4*	226.1 ± 55.1	20.0 ± 5.7
***ABCB1* rs11983225**						
T/T (n = 16)	18614 ± 5930	13.3 ± 3.2	4.83 ± 1.72	85.2 ± 31.4	231.5 ± 56.3	29.9 ± 18.4
T+ (n = 8)	17711 ± 6459	15.6 ± 4.9	3.63 ± 1.50	61.1 ± 7.4*	226.1 ± 55.1	20.0 ± 5.7
***UGT1A1* rs887829**						
C/C (n = 8)	17978 ± 7920	15.5 ± 3.5	3.54 ± 1.64	73.1 ± 27.7	232.1 ± 63.1	25.9 ± 18.4
C/T (n = 11)	18401 ± 5658	13.9 ± 4.5	4.28 ± 1.27	68.3 ± 19.4	221.2 ± 52.6	2l.8 ± 7.5
T/T (n = 5)	18655 ± 3967	12.3 ± 2.7	6.20 ± 1.64*	103.0 ± 34.8	244.7 ± 53.3	38.1 ± 22.2

#: compared to the wild-type homozygote genotype.
*: compared to the other genotypes.

Abbreviations: CYP: cytochrome p450 oxidase; *ABCB1*: ATP binding cassette subfamily B member 1; *UGT1A1*: UDP glucuronosyltransferase family 1 member A1; C_{max}: maximum plasma concentration; T_{max}: time to reach the maximum plasma concentration; AUC: area under the curve; $T_{1/2}$: half-life; Cl/F: total drug clearance adjusted for bioavailability; Vd/F: volume of distribution adjusted for bioavailability.

Article 3: Metabolic effects of aripiprazole and olanzapine multiple-dose treatment in healthy volunteers. Association with pharmacogenetics.

Koller D, Almenara S, Mejía G, Saiz-Rodríguez M, Zubiaur P, Román M, Ochoa D, Navares-Gómez M, Santos E, Pintos E, Abad-Santos F.

Psychopharmacology. Under review.

Impact factor: 3.424.

ABSTRACT

Rationale: Aripiprazole and olanzapine are atypical antipsychotics. Both drugs can induce metabolic changes, however, the metabolic side effects produced by aripiprazole are more benign.

Objectives: To evaluate if aripiprazole and olanzapine alter prolactin levels, lipid and glucose metabolism and hepatic, hematological, thyroid and renal function.

Methods: Twenty-four healthy volunteers received 5 daily oral doses of 10 mg aripiprazole and 5 mg olanzapine in a crossover randomized clinical trial and were genotyped for 51 polymorphisms in 17 genes by qPCR. Drug plasma concentrations were measured by LC-MS. The biochemical and hematological analyses were performed by enzymatic methods.

Results and Conclusions: Olanzapine induced hyperprolactinemia but not aripiprazole. *DRD3* Ser/Gly and *ABCB1* rs10280101, rs12720067 and rs11983225 polymorphisms and CYP3A phenotype had an impact on plasma prolactin levels. C-peptide concentrations were higher after aripiprazole administration and were influenced by *COMT* rs4680 and rs13306278 polymorphisms. Olanzapine and the *UGT1A1* rs887829 polymorphism were associated with elevated glucose levels. CYP3A poor metabolizers had increased insulin levels. Triglyceride concentrations were decreased due to olanzapine and aripiprazole treatment and were variable based on CYP3A phenotypes and the *APOC3* rs4520 genotype. Cholesterol levels were also decreased and depended on *HTR2A* rs6314 polymorphism. All hepatic enzymes, platelet and albumin levels and prothrombin time were altered during both treatments. Additionally, olanzapine reduced the leucocyte count, aripiprazole increased free T4 and both decreased uric acid concentrations. Short term treatment with aripiprazole and olanzapine had a significant influence on the metabolic parameters. However, it seems that aripiprazole provokes less severe metabolic changes.

Article 4: Safety and cardiovascular effects of multiple-dose administration of aripiprazole and olanzapine in a randomised clinical trial.

Koller D, Almenara S, Mejía G, Saiz-Rodríguez M, Zubiaur P, Román M, Ochoa D, Wojnicz A, Martín E, Romero-Palacián D, Navares-Gómez M, Abad-Santos F.

Human Psychopharmacology: Clinical and Experimental. Under review.

Impact factor: 2.265.

ABSTRACT

Objective: To assess adverse events and safety of aripiprazole and olanzapine treatment.
Methods: Twenty-four healthy volunteers receiving 5 daily oral doses of 10 mg aripiprazole and 5 mg olanzapine in a crossover clinical trial were genotyped for 46 polymorphisms in 14 genes by qPCR. Drug plasma concentrations were measured by HPLC-MS/MS. Blood pressure and 12-lead ECG were measured in supine position. Adverse events were also recorded.
Results: Aripiprazole decreased diastolic blood pressure on the first day and decreased QTc on the third and fifth day. Olanzapine had a systolic and diastolic blood pressure, heart rate and QTc lowering effect on the first day. Polymorphisms in *ADRA2A*, *COMT*, *DRD3* and *HTR2A* genes were significantly associated to these changes. The most frequent adverse drug reactions to aripiprazole were somnolence, headache, insomnia, dizziness, restlessness, palpitations, akathisia and nausea while were somnolence, dizziness, asthenia, constipation, dry mouth, headache and nausea to olanzapine. Additionally, *HTR2A*, *HTR2C*, *DRD2*, *DRD3*, *OPRM1*, *UGT1A1* and *CYP1A2* polymorphisms had a role in the development of adverse drug reactions.
Conclusions: Olanzapine induced more cardiovascular changes; however, more adverse drug reactions were registered to aripiprazole. In addition, some polymorphisms may explain the difference in the incidence of these effects among subjects.

Other relevant publications:

Article 5: Effective quantification of 11 tyrosine kinase inhibitors and caffeine in human plasma by validated LC-MS/MS method with potent phospholipids clean-up procedure. Application to therapeutic drug monitoring.

Koller D, Vaitsekhovich V, Mba C, Steegmann JL, Zubiaur P, Abad-Santos F, Wojnicz A.

Talanta. 2020 Feb 1;208:120450. doi: 10.1016/j.talanta.2019.120450.

Impact factor: 4.916

ABSTRACT

Therapeutic drug monitoring (TDM) help to improve treatment efficacy and safety. Therefore, a simple and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous monitoring of 11 tyrosine kinase inhibitors (TKIs) in human plasma. TKIs included in the assay are used in the treatment of chronic myeloid leukemia (CML: imatinib, dasatinib, nilotinib, bosutinib, ponatinib), polycythemia vera (ruxolitinib), chronic lymphocytic leukemia (ibrutinib) and rheumatoid arthritis (filgotinib, tofacitinib, baricitinib, peficitinib). Caffeine was also included in the method. Caffeine increases the acidity of the stomach and decreases its pH as well as is a competitive inhibitor of cytochrome P450 isoenzymes. Thus, it may influence absorption and metabolism of some TKIs, by modifying their plasma levels. The analytes of interest and their stable isotope-labeled internal standards were extracted from 200 μL of human plasma. Microelution-solid phase extraction (μ-SPE) was optimized for method validation and compared to simple protein precipitation (PPT). A gradient elution on a Poroshell 120 EC-C18 column at 60ºC and a flow rate of 0.5 mL/min was applied for analyte separation. The analytical run lasted 8 minutes and it was followed by a re-equilibration time of 4 minutes. Dynamic multiple reaction monitoring scan in the positive ionization mode was applied to improve method sensitivity. Endogenous plasma phospholipids can strongly affect MS analysis. Hence, the monitoring of endogenous phospholipids was included in the assay. Full validation of the method was achieved, including tests of precision, accuracy, trueness, linearity, extraction recovery, matrix effect, process efficiency, stability, sensitivity (with excellent LLOQs), selectivity, identity confirmation and carry-over effect. Regarding sample cleanup, more than 91% of early eluting and more than 96% of late eluting endogenous phospholipids were eliminated by μ-SPE when compared to PPT. This method enables the simultaneous plasma monitoring of 11 TKIs and caffeine and ensures high effectiveness in phospholipids elimination. The present approach is currently used in our clinical practice, being applied to TDM of dasatinib, imatinib, nilotinib and ponatinib. TKIs plasma monitoring helps to individualize dose adjustment and manage adverse effects in CML patients.

Article 6: Effects of aripiprazole on pupillometric parameters related to pharmacokinetics and pharmacogenetics after single oral administration to healthy subjects.

Koller D, Belmonte C, Lubomirov R, Saiz-Rodríguez M, Zubiaur P, Román M, Ochoa D, Carcas A, Wojnicz A, Abad-Santos F.

J Psychopharmacol. 2018 Nov;32(11):1212-1222. doi: 10.1177/0269881118798605.

Impact factor: 4.738.

ABSTRACT

Background: Pupillometry is used for the detection of autonomic dysfunction related to numerous diseases and drug administration. Genetic variants in cytochrome P450 (*CYP2D6*, *CYP3A4*), dopamine receptor (*DRD2*, *DRD3*), serotonin receptor (*HTR2A*, *HTR2C*) and ATP-binding cassette subfamily B (*ABCB1*) genes were previously associated with aripiprazole response.

Aims: Our aim was to evaluate if aripiprazole affects pupil contraction and its relationship with pharmacokinetics and pharmacogenetics.

Methods: Thirty-two healthy volunteers receiving a 10 mg single oral dose of aripiprazole were genotyped for 15 polymorphisms in *ABCB1*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *HTR2C* genes by reverse transcription polymerase chain reaction. Aripiprazole and dehydro-aripiprazole plasma concentrations were measured by high-performance liquid chromatography tandem mass spectrometry. Pupil examination was performed by automated pupillometry.

Results and Conclusions: Aripiprazole caused pupil constriction and reached the peak value at C_{max}. *HTR2A* rs6313 T allele carriers and *HTR2C* rs3813929 C/T subjects showed higher maximum constriction velocity and maximum pupil diameter. Besides, Gly/Gly homozygotes for *DRD3* rs6280 showed significantly lower maximum constriction velocity values. A/G heterozygotes for *DRD2* rs6277 showed higher total time taken by the pupil to recover 75% of the initial resting size values. CYP2D6 intermediate metabolizers showed higher area under the curve, C_{max} and $T_{1/2}$ than extensive metabolizers. *ABCB1* G2677T/A A/A homozygotes had greater $T_{1/2}$ in comparison with C/C homozygotes. *ABCB1* C3435T T allele carriers and C1236T C/T subjects showed greater area under the curve than C/C homozygotes. Aripiprazole affects pupil contraction, which could be a secondary effect through dopamine and serotonin receptors. Pupillometry could be a useful tool to assess autonomic nervous system activity during antipsychotic treatment.

Article 7: Effects of aripiprazole on circadian prolactin secretion related to pharmacogenetics in healthy volunteers.

Koller D, Belmonte C, Saiz-Rodríguez M, Zubiaur P, Román M, Ochoa D, Abad-Santos F.

Basic Clin Pharmacol Toxicol. 2020 Mar;126(3):236-246. doi: 10.1111/bcpt.13323.

Impact factor: 2.452

ABSTRACT

Aripiprazole treatment in schizophrenic patients was previously associated with lower or normalized prolactin levels. Genetic variants in cytochrome P450 (CYP) (*CYP2D6*), dopamine receptor (*DRD2*, *DRD3*) and serotonin receptor (*HTR2A*, *HTR2C*) genes were previously associated with antipsychotic-induced hyperprolactinaemia. Our aim was to evaluate whether aripiprazole affects prolactin secretion and its relationship with pharmacogenetics. Thirty-one healthy volunteers receiving a 10-mg single oral dose of aripiprazole were genotyped for 12 polymorphisms in *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *HTR2C* genes by qPCR. Aripiprazole and dehydro-aripiprazole plasma concentrations were measured by HPLC-MS/MS. Prolactin concentrations of the 31 volunteers taking aripiprazole and 12 volunteers receiving ibuprofen were determined by ELISA. Prolactin concentrations after ibuprofen intake were considered as control, since it is known to cause no effect. Prolactin concentrations were slightly higher in the aripiprazole group compared to the ibuprofen group. All prolactin pharmacokinetic parameters were higher in females than in males. CYP2D6 poor and intermediate metabolizers had notably higher prolactin C_{max} and $AUC_{0\text{-}12}$ than normal and ultrarapid metabolizers. The *DRD3* rs6280 polymorphism affected prolactin levels: volunteers carrying Ser/Ser genotype had significantly lower prolactin levels than volunteers carrying the Gly allele. Furthermore, *HTR2C* rs3813929 C/C homozygotes had significantly lower prolactin levels than T allele carriers. Nevertheless, aripiprazole did increase prolactin levels compared to ibuprofen.

Article 8: The pharmacogenetics of aripiprazole-induced hyperprolactinemia: what do we know?

Koller D, Abad-Santos F.

Pharmacogenomics. 2020 Mar;126(3):236-246. doi: 10.1111/bcpt.13323. PMID: 31520576.

Impact factor: 2.265

Editorial article. No abstract available.

Article 9: Effect of the Most Relevant *CYP3A4* and *CYP3A5* Polymorphisms on the Pharmacokinetic Parameters of 10 CYP3A Substrates.

Saiz-Rodríguez M, Almenara S, Navares-Gómez M, Ochoa D, Román M, Zubiaur P, **Koller D**, Santos M, Mejía G, Borobia AM, Rodríguez-Antona C, Abad-Santos F.

Biomedicines. 2020 Apr 22;8(4):E94. doi: 10.3390/biomedicines8040094.

Impact factor: 3.60.

ABSTRACT

Several cytochrome P450 (CYP) *CYP3A* polymorphisms were associated with reduced enzyme function. We aimed to evaluate the influence of these alleles on the pharmacokinetic parameters (PK) of several CYP3A substrates. We included 251 healthy volunteers who received a single dose of ambrisentan, atorvastatin, imatinib, aripiprazole, fentanyl, amlodipine, donepezil, olanzapine, fesoterodine, or quetiapine. The volunteers were genotyped for *CYP3A4* and *CYP3A5* polymorphisms by qPCR. To compare the PK across studies, measurements were corrected by the mean of each parameter for every drug and were logarithmically transformed. Neither CYP3A phenotype nor individual *CYP3A4* or *CYP3A5* polymorphisms were significantly associated with differences in PK. However, regarding the substrates that are exclusively metabolized by CYP3A, we observed a higher normalized AUC ($p = 0.099$) and a tendency of lower normalized Cl ($p = 0.069$) in CYP3A4 mutated allele carriers what was associated with diminished drug metabolism capacity. *CYP3A4* polymorphisms did not show a pronounced influence on PK of the analysed drugs. If so, their impact could be detectable in a very small percentage of subjects. Although there are few subjects carrying CYP3A4 double mutations, the effect in those might be relevant, especially due to the majority of subjects lacking the CYP3A5 enzyme. In heterozygous subjects, the consequence might be less noticeable due to the high inducible potential of the CYP3A4 enzyme.

Article 10: Utility of Therapeutic Drug Monitoring of Imatinib, Nilotinib, and Dasatinib in Chronic Myeloid Leukemia: A Systematic Review and Meta-analysis.

García-Ferrer M, Wojnicz A, Mejía G, **Koller D**, Zubiaur P, Abad-Santos F.

Clin Ther. 2019 Dec;41(12):2558-2570.e7. doi: 10.1016/j.clinthera.2019.10.009.

Impact factor: 2.935.

ABSTRACT

Purpose: This study examined the utility of therapeutic drug monitoring (TDM) of imatinib, nilotinib, and dasatinib in adult patients with chronic-phase chronic myeloid leukemia (CML). TDM in CML entails the measurement of plasma tyrosine kinase inhibitor (TKI) concentration to predict efficacy and tolerability outcomes and to aid in clinical decision making. TDM was to be deemed useful if it could be used for predicting the effectiveness of a drug and/or the occurrence of adverse reactions. It was expected that the findings from the present study would allow for the definition of a therapeutic range of each TKI.

Methods: A systematic review of studies reporting trough TKI levels (C_{min}) and clinical outcomes was performed. We included randomized clinical trials, nonrandomized controlled studies, interrupted time series studies, and case series studies that provided information about plasma levels of imatinib, nilotinib, or dasatinib and relevant clinical end points in adult patients with chronic-phase CML treated with the corresponding TKI as the single antiproliferative therapy. Meta-analyses, Student t tests, and receiver operating characteristic analyses were performed to detect mean differences between groups of patients with or without: (1) the achievement of major molecular response and (2) adverse reactions.

Findings: A total of 38 studies (28 for imatinib, 7 for nilotinib, and 3 for dasatinib) were included in the systematic review. TDM was found useful in predicting the efficacy of imatinib, with a Cmin cutoff value of 1000 ng/mL, consistent with guideline recommendations. We suggest a therapeutic range of imatinib at a C_{min} of 1000-1500 ng/mL because higher concentrations did not increase efficacy. The findings from the rest of the comparisons were inconclusive.

Implications: TDM is useful in predicting the efficacy of imatinib in CML. Further research is needed to determine its validity with nilotinib and dasatinib.

Article 11: Effect of Polymorphisms in CYP2C9 and CYP2C19 on the Disposition, Safety and Metabolism of Progesterone Administrated Orally or Vaginally.

Zubiaur P, Ochoa D, Gálvez MÁ, Saiz-Rodriguez M, Román M, Aguilar M, de Pablo I, **Koller D**, Abad-Santos F.

Adv Ther. 2019 Oct;36(10):2744-2755. doi: 10.1007/s12325-019-01075-5.

Impact factor: 3.26

ABSTRACT

Introduction: Exogenous progesterone is prescribed for a variety of conditions with endogenous progesterone deficiency, e.g. menstrual alterations, primary or secondary infertility or premenopause. To the best of our knowledge, no pharmacogenetic studies have been published in relation to exogenous progesterone pharmacokinetic safety or progesterone metabolites so far.

Methods: Candidate-gene study where we evaluated whether five single-nucleotide polymorphisms (*CYP2C9**2, *3, *CYP2C19**2, *3 and *17) were related to the pharmacokinetics, safety and metabolism of progesterone in 24 healthy volunteers who received a 200-mg progesterone formulation either orally or vaginally.

Results: The vaginal formulation had an average AUCt value approximately 18 times greater than the oral formulation. CYP2C19 intermediate metabolizers (IM) consistently showed higher adjusted AUC_t and adjusted C_{max} than extensive metabolizers (EM) ($P < 0.05$); CYP2C9 EM incongruently exhibited higher adjusted C_{max} and longer half-life than IM ($p < 0.05$).

Conclusion: This is the first study that reports variability in progesterone disposition according to the CYP2C19 and CYP2C9 phenotype. We suggest that CYP2C19 may condition progesterone disposition and that it may be more relevant than CYP2C9. This study lays the foundations for further in-depth research to evaluate the pharmacogenetics of progesterone.

Article 12: Influence of CYP450 Enzymes, CES1, PON1, ABCB1, and P2RY12 Polymorphisms on Clopidogrel Response in Patients Subjected to a Percutaneous Neurointervention.

Saiz-Rodríguez M, Belmonte C, Caniego JL, **Koller D**, Zubiaur P, Bárcena E, Romero-Palacián D, Eugene AR, Ochoa D, Abad-Santos F.

Clin Ther. 2019 Jun;41(6):1199-1212.e2. doi: 10.1016/j.clinthera.2019.04.037.

Impact factor: 2.935

ABSTRACT

Purpose: Clopidogrel is a thienopyridine prodrug that inhibits platelet aggregation. It is prescribed to prevent atherothrombotic and thromboembolic events in patients receiving a stent implant in carotid, vertebral, or cranial arteries. The influence of cytochrome P-450 (CYP) 2C19 on the response to clopidogrel has been widely studied; however, the effect of other genes involved in clopidogrel absorption and metabolism has not been established in this cohort of patients.

Methods: This observational retrospective study assessed the antiplatelet response and the prevalence of hemorrhagic or ischemic events after percutaneous neurointervention in clopidogrel-treated patients, related to 35 polymorphisms in the genes encoding the clopidogrel-metabolizing enzymes (*CYP2C19*, *CYP1A2*, *CYP2B6*, *CYP2C9*, *CYP3A4*, *CYP3A5*, carboxylesterase-1 [*CES1*], and paraoxonase-1 [*PON1*]), P-glycoprotein transporter (*ABCB1*), and platelet receptor *P2Y12*. Polymorphisms were analyzed by quantitative real-time polymerase chain reaction and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Antiplatelet response was documented with the VerifyNow system (Accriva, San Diego, California).

Findings: We confirmed that CYP2C19 is the most important enzyme involved in clopidogrel response. The carriage of the *CYP2C19**2 allele was strongly associated with hyporesponse to clopidogrel, while the *CYP2C19**17 allele was a protective factor for the development of ischemic events (odds ratio = 0.149; P = 0.002) but a risk factor for bleeding (odds ratio = 3.60; P = 0.038). Patients carrying *ABCB1* mutated alleles showed lower aggregation values, suggesting that clopidogrel absorption is influenced by P-glycoprotein. In fact, the percentage of responders was significantly higher in the group carrying the mutated haplotype compared to the wild type (80.8% vs 43.3%; P = 0.009). Patients with the *CES1* G143E C/T genotype showed a considerably lower, aggregation value versus wild-type patients, although the

difference was not significant likely due to the small sample size (59.0 [21.2] vs 165.2 [86.0] PRU; P = 0.084), which suggests an increased active metabolite formation. No relationship was found between polymorphisms in other *CYP* genes, *PON1*, or *P2RY12* and response to clopidogrel in patients subjected to neurointervention procedures.

Implications: Therapeutic guidelines recommend that CYP2C19 intermediate and poor metabolizers with acute coronary syndromes undergoing percutaneous coronary intervention receive an alternative antiplatelet therapy; however, genotype-guided therapy is not a standard recommendation for neurovascular conditions. This is the first study to carry out a joint analysis of *CYP2C19* and other genes involved in clopidogrel treatment in patients receiving percutaneous neurointervention. Our findings support routine genotyping in clopidogrel-treated patients. Moreover, we encourage considering an alternative antiplatelet therapy in CYP2C19 intermediate, poor and ultrarapid metabolizers. Additionally, ABCB1 polymorphisms could be considered for a better pharmacogenetic approach.

Article 13: Polymorphisms in *CYP1A2*, *CYP2C9* and *ABCB1* affect agomelatine pharmacokinetics.

Saiz-Rodríguez M, Ochoa D, Belmonte C, Román M, Vieira de Lara D, Zubiaur P, **Koller D**, Mejía G, Abad-Santos F.

J Psychopharmacol. 2019 Apr;33(4):522-531. doi: 10.1177/0269881119827959.

Impact factor: 4.738.

ABSTRACT

Background: Agomelatine is an agonist of the melatoninergic receptors used for the treatment of depression. Our aim was to evaluate the effect of genetic polymorphisms in metabolising enzymes and the P-glycoprotein transporter on agomelatine pharmacokinetics and pharmacodynamics.

Methods: Twenty-eight healthy volunteers receiving a single 25 mg oral dose of agomelatine, were genotyped for nine polymorphisms in cytochrome P450 enzymes (*CYP1A2*, *CYP2C9* and *CYP2C19*) and adenosine triphosphate-binding cassette subfamily B member 1 (*ABCB1*), by real-time polymerase chain reaction . Agomelatine concentrations were measured by high performance liquid chromatography coupled to a tandem mass spectrometry detector.

Results and Conclusions: We calculated a CYP1A2 activity score that was directly correlated with agomelatine pharmacokinetics. Individuals with a decreased enzyme activity (*1C carriers) had a lower clearance and accumulated higher concentrations of agomelatine. In contrast, individuals with a high CYP1A2 inducibility (*1F or *1B carriers) showed an extensive clearance and lower agomelatine concentrations. The apparently marked differences between races were due to the different *CYP1A2* genotype distribution. CYP2C9 intermediate/poor metabolizers showed a higher area under the concentration-time curve and maximum concentration. *ABCB1* G2677T/A polymorphism affected the time to reach maximum concentration, as subjects carrying A/A+A/T genotypes showed higher values. No association was found for CYP2C19 phenotype. Agomelatine did not produce any change in blood pressure, heart rate or QT interval. CYP1A2 polymorphisms affect agomelatine pharmacokinetics. CYP1A2 phenotype inferred from the genotyping of *CYP1A2**1C, *1F and *1B alleles might be a potential predictor of agomelatine exposure. *ABCB1* G2677T/A could affect agomelatine absorption, as subjects with A/A+A/T genotypes had lower agomelatine concentration and they take more time to reach the maximum concentration.

Article 14: Polymorphisms associated with fentanyl pharmacokinetics, pharmacodynamics and adverse effects.

Saiz-Rodríguez M, Ochoa D, Herrador C, Belmonte C, Román M, Alday E, **Koller D**, Zubiaur P, Mejía G, Hernández-Martínez M, Abad-Santos F.

Basic Clin Pharmacol Toxicol. 2019 Mar;124(3):321-329. doi: 10.1111/bcpt.13141.

Impact factor: 2.452.

ABSTRACT

Fentanyl is an agonist of the μ-opioid receptor commonly used in the treatment of moderate-severe pain. In order to study whether pharmacogenetics explains some of the variability in the response to fentanyl, several genes related to fentanyl receptors, transporters and metabolic enzymes have been analysed. Thirty-five healthy volunteers (19 men and 16 women) receiving a single 300 μg oral dose of fentanyl were genotyped for 9 polymorphisms in cytochrome P450 (CYP) enzymes (*CYP3A4* and *CYP3A5*), ATP-binding cassette subfamily B member 1 (*ABCB1*), opioid receptor mu 1 (*OPRM1*), catechol-O-methyltransferase (*COMT*) and adrenoceptor beta 2 (*ADRB2*) by real-time PCR. Fentanyl concentrations were measured by ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS). Fentanyl pharmacokinetics is affected by sex. Carriers of the *CYP3A4**22 allele, which is known to reduce the mRNA expression, showed higher area under the concentration-time curve (AUC) and lower clearance (Cl) values. Although this finding might be of importance, its validity needs to be confirmed in other similar settings. Furthermore, carriers of the *ABCB1* C1236T T/T genotype presented a lower AUC and higher Cl, as well as lower half-life ($T_{1/2}$). As volunteers were blocked with naltrexone, the effect of fentanyl on pharmacodynamics might be biased; however, we could observe that fentanyl had a hypotensive effect. Moreover, *ADRB2* C523A A allele carriers showed a tendency towards reducing systolic blood pressure. Likewise, *OPRM1* and *COMT* minor allele variants were risk factors for the development of somnolence. *CYP3A5**3, *ABCB1* C3435T and *ABCB1* G2677T/A were not associated with fentanyl's pharmacokinetics, pharmacodynamics and safety profile.

Article 15: How to make P-glycoprotein (ABCB1, MDR1) harbor mutations and measure its expression and activity in cell cultures?

Zubiaur P, Saiz-Rodríguez M, Koller D, Ovejero-Benito MC, Wojnicz A, Abad-Santos F.

Pharmacogenomics. Review. 2018 Nov;19(16):1285-1297. doi: 10.2217/pgs-2018-0101.

Impact factor: 2.265.

ABSTRACT

Several polymorphisms have been identified in *ABCB1*, the gene encoding for the P-glycoprotein. This transporter alters the pharmacokinetics or effectiveness of drugs by excreting them from cells where it is expressed (e.g., blood-brain barrier, intestine or tumors). No consensus has been reached regarding the functional consequences of these polymorphisms in the transporter's function. The aim of this review was to describe a methodology that allows the assessment of P-gp function when harboring polymorphisms. We describe how to obtain cell lines with high expression levels of the transporter with polymorphisms and several tactics to measure its expression and activity. This methodology may help elucidate the contribution of polymorphisms in *ABCB1* to drug pharmacokinetics, effectiveness and safety or to cancer chemotherapy failure.

Article 16: Effect of ABCB1 C3435T Polymorphism on Pharmacokinetics of Antipsychotics and Antidepressants.

Saiz-Rodríguez M, Belmonte C, Román M, Ochoa D, Jiang-Zheng C, Koller D, Mejía G, Zubiaur P, Wojnicz A, Abad-Santos F.

Basic Clin Pharmacol Toxicol. 2018 Oct;123(4):474-485. doi: 10.1111/bcpt.13031.

Impact factor: 2.452.

ABSTRACT

P-glycoprotein, encoded by *ABCB1*, is an ATP-dependent drug efflux pump which exports substances outside the cell. Some studies described connections between C3435T polymorphism T allele and lower P-glycoprotein expression; therefore, homozygous T/T could show higher plasma levels. Our aim was to evaluate the effect of C3435T on pharmacokinetics of 4 antipsychotics (olanzapine, quetiapine, risperidone and aripiprazole) and 4 antidepressants (trazodone, sertraline, agomelatine and citalopram). The study included 473 healthy volunteers receiving a single oral dose of one of these drugs, genotyped by real-time PCR. Multivariate analysis was performed to adjust the effect of sex and genotype of the main cytochrome P450 enzymes. C3435T polymorphism had an effect on olanzapine pharmacokinetics, as T/T individuals showed lower clearance and volume of distribution. T/T individuals showed lower $T_{1/2}$ of 9-OH-risperidone, but this difference disappeared after multivariate correction. T/T homozygous individuals showed lower dehydro-aripiprazole and trazodone area under the concentration-time curve, along with lower half-life and higher clearance of trazodone. C/T genotype was associated to higher citalopram maximum concentration. C3435T had no effect on quetiapine, sertraline or agomelatine pharmacokinetics. C3435T can affect the elimination of some drugs in different ways. Regarding risperidone, trazodone and dehydro-aripiprazole, we observed enhanced elimination while it was reduced in olanzapine and citalopram. However, in quetiapine, aripiprazole, sertraline and agomelatine, no changes were detected. These results suggest that P-glycoprotein polymorphisms could affect CNS drugs disposition, but the genetic factor that alters its activity is still unknown. This fact leads to consider the analysis of ABCB1 haplotypes instead of individual variants.

Article 17: Influence of CYP2C19 Phenotype on the Effect of Clopidogrel in Patients Undergoing a Percutaneous Neurointervention Procedure.

Saiz-Rodríguez M, Romero-Palacián D, Villalobos-Vilda C, Caniego JL, Belmonte C, **Koller D**, Bárcena E, Talegón M, Abad-Santos F.

Clin Pharmacol Ther. 2019 Mar;105(3):661-671. doi: 10.1002/cpt.1067.

Impact factor: 6.336.

ABSTRACT

This observational retrospective study assessed the antiplatelet response and clinical events after clopidogrel treatment in patients who underwent percutaneous neurointervention, related to CYP2C19 metabolizer status (normal (NM), intermediate/poor (IM-PM), and ultrarapid (UM); inferred from *2, *3, and *17 allele determination). From 123 patients, IM-PM had a higher aggregation value (201.1 vs. 137.6 NM, 149.4 UM, $P < 0.05$) and lower response rate (37.5% vs. 69.8% NM, 61.1% UM), along with higher treatment change rate (25% vs. 5.7% NM, 10.5% UM). The highest ischemic events incidence occurred in NM (11.3% vs. 6.3% IM, 10.5% UM) and hemorrhagic events in UM (13.2% vs. 0% IM and 3.8% NM). No differences were found regarding ischemic event onset time, while hemorrhagic event frequency in UM was higher with shorter onset time ($P = 0.047$). CYP2C19 no-function and increased function alleles defined the clopidogrel response. UM patients had increased bleeding risk. Therapeutic recommendations should include dose reduction or treatment change in UM.

Article 18: Effective phospholipids removing microelution-solid phase extraction LC-MS/MS method for simultaneous plasma quantification of aripiprazole and dehydro-aripiprazole: Application to human pharmacokinetic studies.

Wojnicz A, Belmonte C, **Koller D**, Ruiz-Nuño A, Román M, Ochoa D, Abad-Santos F.

J Pharm Biomed Anal. 2018 Mar 20;151:116-125. doi: 10.1016/j.jpba.2017.12.049.

Impact factor: 2.983.

ABSTRACT

A simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for simultaneous quantification of aripiprazole and its active metabolite, dehydro-aripiprazole, in human plasma. Stable isotopically labeled aripiprazole, aripiprazole-D8, has been used as the internal standard (IS) for both analytes. Only 200 μl of human plasma was needed for analyte extraction, using effective phospholipids-eliminating three-step microelution-solid-phase extraction (SPE, Oasis PRiME HLB 96-well μElution Plate). An ACE C18-PFP column was applied for chromatographic separation at 25 °C, protected by a 0.2-μm on-line filter. A combination of ammonium formate (5 mM)-acetonitrile (pH 4.0; 65:35, v/v) was used as mobile phase and the chromatogram was run under gradient conditions at a flow rate of 0.6 ml/min. Run time lasted 5 min, followed by a re-equilibration time of 3 min, to give a total run time of 8 min. Five μl of the sample was injected into the chromatographic system. Aripiprazole, dehydro-aripiprazole and IS were detected using the mode multiple reaction monitoring in the positive ionization mode. The method was linear in the concentration range of 0.18-110 ng/ml and 0.35-100 ng/ml for aripiprazole and dehydro-aripiprazole, respectively. Our method has been validated according to the recommendations of regulatory agencies through tests of precision, accuracy, recovery, matrix effect, stability, sensitivity, selectivity and carry-over. Our microelution-SPE method removes more than 99% of main plasma phospholipids compared to protein precipitation and was successfully applied to several bioequivalence studies.

Article 19: Effect of Polymorphisms on the Pharmacokinetics, Pharmacodynamics and Safety of Sertraline in Healthy Volunteers.

Saiz-Rodríguez M, Belmonte C, Román M, Ochoa D, **Koller D**, Talegón M, Ovejero-Benito MC, López-Rodríguez R, Cabaleiro T, Abad-Santos F.

Basic Clin Pharmacol Toxicol. 2018 May;122(5):501-511. doi: 10.1111/bcpt.12938.

Impact factor: 2.452.

ABSTRACT

Sertraline is a selective serotonin reuptake inhibitor widely metabolized in the liver by cytochrome P450 (CYP) enzymes. Besides, it is a P-glycoprotein substrate. Moreover, serotonin transporters and serotonin receptors are involved in its efficacy and safety. The aim of this study was to evaluate the role of polymorphisms of metabolizing enzymes, transporters and receptors on the pharmacokinetics, pharmacodynamics and tolerability of sertraline in healthy volunteers. Forty-six healthy volunteers (24 men and 22 women) receiving a 100-mg single oral dose of sertraline were genotyped for 17 genetic variants of CYP enzymes (*CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP2D6*), ATP-binding cassette subfamily B member 1 (*ABCB1*), solute carrier family 6 member 4 (*SLC6A4*), 5-hydroxytryptamine receptor 2A (*HTR2A*) and 5-hydroxytryptamine receptor 2C (*HTR2C*) genes. Pharmacokinetic and pharmacodynamic parameters were similar in men and women. Polymorphisms in *CYP2C19* and *CYP2B6* genes influenced sertraline pharmacokinetics, with a greater effect of *CYP2C19*. Individuals carrying defective alleles for *CYP2C19* and *CYP2B6* showed higher area under the curve (AUC) and half-life ($T_{1/2}$). Moreover, *CYP2C19**17 was related to a decreased AUC and $T_{1/2}$. No significant effect was found for polymorphisms in *CYP2C9*, *CYP2D6* and *ABCB1* on sertraline pharmacokinetics. Sertraline had a small heart rate-lowering effect, directly related to maximum concentration (C_{max}) and the presence of *ABCB1* minor alleles. Sertraline had no significant effect on blood pressure and QTc. There was a tendency to present more adverse drug reactions in women and individuals with higher AUC of sertraline, such as CYP2C19 intermediate metabolizers and *CYP2B6* G516T T/T individuals.