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.

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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. Isotopically labeled aripiprazole, aripiprazole-D8, has been used as the internal standard (IS) for both analytes. Only 200 μ l of human plasma was needed for analyte extraction, using effective phospholipids-eliminating three-steps microelution-solid-phase extraction (SPE, Oasis PRiME HLB 96-well μ Elution Plate). An ACE C18-PFP column was applied for chromatographic separation at 25°C, protected by a 0.2- μ m 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 for 5 minutes-followed by a re-equilibration time of 3 minutes, to give a total run time of 8 minutes. 5 μ l 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 ARI and DARI, 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.

Keywords: aripiprazole; dehidro-aripiprazole; antidepressants and antipsychotics; liquid chromatography in tandem with mass spectrometry; solid phase extraction; pharmacokinetics.

49	Abbreviations used:
50	ARI: aripiprazole
51	AUC: Area under curve
52	CAL: Calibration standard
53	C _{min} : plasma trough levels
54	C _{max} : Maximum plasma concentration
55	CV: Coefficient of variation
56	DAD: diode array detection
57	DARI: dehydro-aripiprazole
58	EDTA: Ethylenediaminetetraacetic acid
59	EIC: Extraction ion chromatogram
60	EMA: European Medicines Agency
61	ESI: Electrospray ionization
62	FDA: Food and Drug Administration
63	h: hour
64	HLB: hydrophilic-lipophilic balance
65	HPLC: High-performance liquid chromatography
66	IL-IS: isotopically labeled internal standards
67	IS: Internal standard
68	LC-MS: Liquid chromatography-mass spectrometry
69	LC-MS/MS: Liquid chromatography-tandem mass spectrometry
70	LLE: Liquid-liquid extraction
71	LLOQ: Lower limit of quantification
72	min: minute

73	MRM: Multiple-reaction monitoring
74	PFP: pentafluorophenyl
75	PPT: Protein precipitation
76	QC: Quality control
77	R^2 : Correlation coefficient
78	tR: Retention time
79	SD: Standard deviation
80	SPE: Solid phase extraction
81	$T_{\max}$: Time of occurrence of $C_{\max}$
82	$T_{1/2}$: Half-life
83	TIC: Total ion chromatogram
84	UPLC-MS/MS: ultra liquid chromatography-tandem mass spectrometry
85	UV: Ultraviolet
86	
87	
88	

1. Introduction

Aripiprazole (ARI), a quinolinone derivative, is an atypical antipsychotic drug indicated in schizophrenia and schizoaffective disorder [1,2]. It displays a partial agonist/antagonist activity at dopamine D2 and D3 receptors and serotonin 5-HT1A receptors, whereas at serotonin 5-HT2A receptors acts as an antagonist [3]. The efficacy of ARI is reducing the positive and negative symptoms of schizophrenia due to the activity at dopamine and serotonin receptors, respectively [4].

ARI is mainly metabolized by the cytochrome P450 (CYP) 3A4 and CYP2D6 enzyme systems to dehydro-aripiprazole (DARI), its main active metabolite. Both enzyme systems are subjected to drug interactions and genetic polymorphism, therefore dose-adjustment is necessary when it is coadministered with drugs that inhibit CYP3A4 and CYP2D6 or induce CYP3A4 [5,6]. Additionally, allelic variants of ABCB1 and ABCG2 genes were found to influence the plasma concentrations of antipsychotics, including ARI. ABCB1 2677TT/3435TT genotype caused a significantly lower ARI plasma concentration/dose ratio if compared with patients carrying other ABCB1 genotypes [7]. Therefore, ARI plasma monitoring is suggested to improve efficacy, avoid drug-drug interactions or decrease adverse effects.

To date, numerous analytical methods have been used for ARI and DARI human plasma determination by high performance liquid chromatography (HPLC, also called 'LC') with coulombimetric detection, ultraviolet detection (UV) and diode array detection (DAD) [8–11]. However, the lower limit of quantification (LLOQ) is higher or the run time is longer than other LC-tandem mass spectrometry (LC-MS/MS) methods with electrospray ionization (ESI). Despite improved sensitivity and selectivity of the LC-MS/MS-based methods, one of the main problems of ESI sources is the ion suppression or enhancement caused by the sample matrix (also known as 'matrix effect') and interferences from metabolites [12]. Endogenous phospholipids cause ion suppression in both positive and negative ESI modes leading to matrix effect and as a consequence variability and irreproducibility in bioanalytical LC-MS/MS methods [13–15]. Lipids are the most variable components in human plasma. They can vary significantly among individuals according to diet and metabolic rate. However, glycerophosphatidylcholines, such as phosphatidylcholine, the main phospholipids circulating in the human plasma, constitute up to 70% of total plasma phospholipids [16]. Lysophospholipids, including lysophosphatidylcholine, compose up to 10% of total phospholipids [17]. Therefore, an optimization of an appropriated extraction method which is able to eliminate phospholipids combined with the use of isotopically labeled

internal standards (IL-IS) is extremely important to achieve reliable results and maintain the LC/MS-MS system clean [12,18].

Nonetheless, the majority of the methods of LC-MS/MS and ultra-LC-MS/MS (UPLC-MS/MS) with ESI have used protein precipitation (PPT) or liquid liquid extraction (LLE) in addition to non IL- IS instead of solid phase extraction (SPE) and stable IL- IS [19–21,21–31]. PPT is the fastest, but the least effective sample preparation technique, often resulting in significant matrix effects due mainly to the presence of endogenous phospholipids [17]. As follows, some HPLC-UV or LC-MS/MS methods choose SPE as extraction method. Reversed-phase SPE methods are known to provide cleaner extracts and reduce matrix effects compared to PPT [14,17,18]. In spite of the fact that SPE has been applied in many of published methods, it is characterized by longer performing times, need of evaporation and reconstitution steps and no usage of IL- IS [10,32,33]. Between all these methods, only Patel and co-workers were able to achieve the best results employing SPE [34]. Patel et al applied UPLC, which offers shorter analyte separation time due to a higher pressure in the UPLC system. However, none of the cited methods investigated phospholipids' elimination efficacy of the extraction method applied.

Consequently, the aim of the present study was to develop a simple LC-MS/MS method based on effective phospholipids removing three-steps microelution-SPE method compared to PPT for simultaneous plasma quantification of ARI and DARI as well as its further application to pharmacokinetic studies and clinical practice.

2. Materials and Methods

2.1. Chemicals and reagents

ARI, DARI and IL-IS [2H8]-ARI (ARI-D8) were provided by Toronto Research Chemicals (North York, Canada). Acetonitrile, methanol, ammonia solution (at 25%) and ammonia hydroxide 5N (HPLC Grade) were purchased from SYMTA (Madrid, Spain). Formic acid was supplied by Sigma-Aldrich (Madrid, Spain). All chemicals were analytical or LC-MS grade. The water for HPLC was obtained using a Milli-Q system (Millipore-Ibérica, Madrid, Spain). Blank human plasma samples of healthy patients were from the Transfusion Center of the Autonomous Community of Madrid (Madrid, Spain).

2.2. Stock solutions, calibration standards (CALs), and quality controls (QCs)

Stock solutions of CALs and QCs of ARI, DARI and ARI-D8 were prepared by dissolving an accurately weighed quantity in 0.5% formic acid solution in methanol to obtain a concentration of 1 mg/ml for all compounds. CALs were made from independent dilutions of each stock solution and spiked in the blank plasma to obtain 8 CALs with the concentrations of 0.18, 0.5, 1.0, 5.0, 25, 50, 100 and 120 ng/ml for ARI and 0.35, 0.5, 1.0, 2.5, 10, 50, 90 and 110 ng/ml for DARI. QC samples were prepared in the same fashion to obtain the four corresponding QC levels (LLOQ, low, medium, and high), as follows: 0.18, 0.5, 55, 110 ng/ml for ARI and 0.35, 0.7, 45, and 90 ng/ml for DARI. Secondary IS solution of 1000 ng/ml ARI-D8 was diluted 20 times to give a working solution of 50 ng/ml.

A drug-free blank plasma sample and a drug-free zero plasma sample (processed with IS) were included according to the recommendations for bioanalytical method validation of the US Food and Drug Administration (FDA) [35] and the European Medicines Agency (EMA) [36]. All CALs, QC, and IS solutions were stored at -80°C until use or analysis.

2.3. Chromatographic conditions

The HPLC system consisted of a 1200 Series separation module (Agilent Technologies, Madrid, Spain) controlled by Agilent MassHunter Workstation Data Acquisition software for programming samples and chromatographic conditions. Separations were carried out at 25°C in an ACE C18-PFP column (3- μ m, 4.6 x 100 mm; SYMTA, Madrid, Spain) at 0.6 ml/min. The mobile phase consists of ammonium formate (5 mM, solvent A)-acetonitrile (solvent B) (pH 4.0; 65:35, v/v). The chromatogram was run under gradient condition as follows: initial conditions: 65% (A) and 35% (B); 0-0.1 min, gradually increasing eluent B to 75% (B); 0.1-0.5 min, gradually increasing eluent B to 90%; 0.5-1.5 min, gradually increasing eluent B to 99% and maintain from 1.5 to 3.0 min; 3.0-3.2 min returning to the initial conditions (65% A and 35% B) and maintain from 3.2 to 5.0 min. The chromatogram was followed by a re-equilibration time of 3.0 min. The volume injected into the HPLC was 5 μ l.

2.4. Mass spectrometry

The mass spectrometry detection system consisted of an Agilent Technologies 6410, triple quadrupole with ESI in positive ion mode. The mass spectrometry was operated in multiple-reaction monitoring (MRM) mode. The desolvation gas (nitrogen) and flow is operated at 350°C and 12 L/min, respectively. The nebulizer pressure was 60 psi and the capillary voltage was 5 kV. The MS collision gas was highly pure N₂ (>99.9995).

The dwell time, fragmentor voltage and collision energy were optimized for all the compounds (Table 1). After separation by HPLC, the peak area corresponding to the mass-to-charge ratio (m/z) transition for the quantifier ion was measured as relative to the m/z transition for its IS. The m/z transition for the qualifier ion was also monitored to add specificity to the results. Additionally, for endogenous phospholipids monitoring, m/z 184.1 $\rightarrow$ 184.1 transition was used (Table 1). The integration peak area of the MRM transitions of each analyte was calculated using MassHunter Workstation Quantitative Analysis software (Agilent Technologies, Madrid, Spain).

2.5. Sample preparation

For sample clean-up two different types of extraction were tested in order to check phospholipids' elimination capacity. Therefore to choose the best sample preparation method, twelve different plasma samples were extracted using simple PPT or three steps microelution-SPE. For PPT, 200 μ l of plasma sample was spiked with 10 μ l of IS and 800 μ l of precipitating agent (ACN with 1% formic acid in a 4:1 proportion; v/v) and centrifuged at 14000 rpm and 4°C for 5 minutes. Then the supernatant was evaporated to dryness using a concentrator (5301, Eppendorf, Germany) at 45°C for 1 hour and 15 minutes. Consequently, the dry residue was reconstituted with 200 μ l of the acetonitrile/methanol/buffer (ammonium formate, 5 mM at pH 4.0) reconstitution solution (8:1:1, v/v/v) and 5 μ l of sample was injected into the LC-MS/MS system.

On the other hand, Oasis PRiME HLB (hydrophilic-lipophilic balance) 96-well μ Elution Plate, 3 mg Sorbent per Well (Waters, Madrid, Spain) was used for SPE of the analytes. Sample preparation was fast and simple. Microelution-SPE consisted of only three steps: sample loading, washing and elution. 200 μ l of plasma sample was spiked with 10 μ l of IS and 290 μ l of 0.2% formic acid, pH 2.0, and loaded (2 x 250 μ l) into the μ Elution Plate. Next, a washing step with 400 μ l (2 x 250 μ l) of 5% methanol solution in MilliQ water and addition of 2% ammonia was applied. Vacuum between 5-15 mmHg was applied in each step of the SPE process. Elution was performed with 200 μ l (2 x 100 μ l) of acetonitrile/methanol/buffer (ammonium formate, 5 mM at pH 4.0) solution (8:1:1, v/v/v) and collected in a 1 ml 96-well plate (Agilent Technologies, Santa Clara, USA). Then, 5 μ l of eluate was directly injected into the LC-MS/MS system without evaporation and reconstitution. Finally, SPE was chosen as sample preparation for method validation.

2.6. Assay validation procedures

The method was validated according to the recommendations published online by the FDA [35] and EMA [36].

2.6.1. Calibration curve, selectivity and LLOQ

Quantitative analysis of ARI and DARI in plasma was carried out using the method of IS. Eight CALs were used for validation to cover the range of clinically relevant concentrations expected in most healthy volunteers. A quadratic regression was used to calculate the equation relating the area ratio of each analyte versus IS to the concentration of the analytes in the CALs. Weighting factor of 1/x was used to obtain the best fit for all CALs. Six standard curves from different days were analyzed. To fulfill validation criteria, the error of accuracy and coefficient of variation (CV) should be less than 15% for all CALs, except for the LLOQ, which was less than 20%. To be considered LLOQ, the response of the analyte should be at least 5 times higher than the blank response.

The selectivity of the method was studied by analyzing 6 different batches from human blank plasma, with the addition of IS (zero plasma) or without the IS. The method is considered selective, when the response is less than 5 times of the LLOQ for ARI and DARI, and less than 20 times for the IS.

2.6.2. Precision and accuracy

The precision and accuracy of the method were evaluated by analyzing 6 replicates per 4 concentration levels (LLOQ and low, medium, and high QCs) in a single analytical run that covered the calibration curve range. The intra-day precision and accuracy were assessed by analyzing a single analytical run in a single day. The inter-day variation was evaluated over 3 analytical runs from 3 different days. Precision was measured as a coefficient of variation (%) and accuracy as the percentage difference between the theoretical and the measured value according to the following equation:

$$\text{Accuracy (\%)} = \frac{(\text{concentration found} - \text{theoretical concentration})}{(\text{theoretical concentration})} \times 100\%$$

To verify precision and accuracy, the error must be less than 15% for all samples except the LLOQ (<20%).

2.6.3. Extraction recovery and matrix effect

Recovery is measured as the ratio of the compound concentrations in plasma following SPE (pre-extracted analyte concentration) to the same concentration spiked directly in blank plasma sample that had undergone the process of SPE (post-extracted analyte concentration). The matrix effect of human plasma was evaluated by adding a known concentration of analyte to a blank plasma sample that had undergone the process of SPE (post-extracted analyte concentration). The response was compared with the addition of the same amount of analyte to the final elution solution [acetonitrile/methanol/buffer, pH 4.0 (8:1:1, v/v/v)], which had not undergone the extraction process (pure solution of the analyte).

In both tests six repetitions of the LLOQ, low QC and high QC were analyzed in at least 6 different batches of human plasma for all analytes. The recovery of QC samples had to be precise, reliable, and reproducible. To validate the matrix effect, the CV could not be larger than 15% for all the QCs and less than 20% for LLOQ. All recovery and matrix effect values were calculated as absolute and relative to its IS.

2.6.4. Stability

The stability of ARI and DARI at different conditions has been already evaluated in many articles, but we conducted the following stability assays with 4 replicates at low and high QC for ARI and DARI to check it in our laboratory conditions:

- after 24 hours at 19°C in the autosampler, at room temperature and at 4°C in the fridge

- after 3 cycles of freeze-thaw in the freezer at –80°C

Analyte stability must not be 100%, but it had to be between 85-115% and CV should be less than 15% for all the QCs studied.

2.6.5. Carry-over

Carry-over was evaluated by injecting blank samples after CAL at the highest concentration. The peak area of blank sample injected after CAL at the upper limit of quantification could not be greater than 20% of the LLOQ and 5% of the IS. To prevent carry-over the needle was washed 5 times between injections (vial and flash port) with water and acetonitrile solution (50:50, v/v).

2.6.6. Application of the method to pharmacokinetics of ARI and DARI in healthy volunteers

This developed method was successfully applied to pharmacokinetic analysis of 1133 samples from 103 healthy volunteers who received a single oral dose of ARI (10 mg) in several bioequivalence studies under fasting conditions. Blood samples were taken at the following time intervals after dosing: 0, 1, 2, 4, 6, 8, 10, 12, 24, 48 and 72 hours. Blood samples were collected in EDTA dipotassium dihydrate (EDTA K2) tubes (Vacuette) and centrifuged at 3000 rpm for 10 minutes at 24°C. The plasma was separated and stored at -80°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. The pharmacokinetic analysis was carried out by means of a model-independent method with WinNonLin Professional Edition, version 2.0 (Scientific Consulting, Inc., Cary, NC). Maximum plasma concentration (C_{max}) and time to 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. The half-life ($T_{1/2}$) was calculated as $\ln 2/k$.

3. Results and Discussion

3.1. Optimization of MS/MS conditions and chromatography

The full scan spectra in positive ESI mode indicated that all analytes were protonated molecules $[M+H]^+$ which were therefore selected to detect the most abundant products. Figure 1 shows the percentages of abundance of the precursor and product ions of the quantifier, qualifier, or IL-IS versus mass to charge (m/z) under MRM and the fragmentation pattern of quantifier transition. Table 1 summarized all the optimized mass spectrometer parameters, such as the transition in MRM mode, dwell time, fragmentor voltage and collision energy for each analyte (quantifier and qualifier ion) and their ISs. For each analyte, a second transition named qualifier ion was optimized to confirm the analysis.

The chromatographic separation was run under gradient conditions to reach better resolution of the three compounds. Figure 2 displays the typical extraction ion chromatogram (EIC) of a blank human plasma, pure solution and blank human plasma spiked with LLOQ before and after SPE of both, ARI and DARI. The retention times (t_R) were very consistent all through the recordings (Table 1). The t_R of ARI was 3.644 minutes, of DARI 3.451 minutes and of the ARI-D8 was 3.582 minutes. Although the t_R s were very similar with respect to their compounds (Table 1), they can be separated by the EIC analysis based on reconstructed ion currents using the Agilent MassHunter

Workstation software package (Santa Clara, USA). The recording time was 5.0 minutes followed by 3.0 minutes for re-equilibration owing to gradient elution.

Due to the similarities of ARI and its metabolite, the chromatographic seems to be complicated. However, when large sample amount for clinical pharmacokinetic studies is processed, the time of analysis of a single sample is extremely important. Thus, XBridge BEH C18 column (2.5- μ m, 2.1 x 75 mm) and ACE C18-pentafluorophenyl (PFP) column (3- μ m, 4.6 x 100 mm) were applied to separate both of the drugs under the same mobile phase condition. Based on our results, we have chosen C18 column with PFP group for analyte separation. It has a better resolution and improves analyte separation compared to ordinary C18 column due to π - π interactions with the aromatic ring and NH₂ groups. Our total chromatographic run lasted 8 minutes, similar to other authors [11,22]. Although shorter analytical runs ranging from 1.2 minutes to 7.5 minutes exists [21,25,26,34], there are mostly isocratic separations, which tend to cause problems in repeatability in retention time, peak area and peak height or linearity of the calibration curve [37].

Another important factor to consider is the injection volume. Taking into account the particle size of the column, we have injected 5 μ l of the sample into the LC-MS/MS system. On the contrary, several LC-MS/MS method have used larger injection volume between 20-100 μ l [20,21,25,26,32], which can cause carry-over effect and shorter the analytical column life-time.

3.2. Calibration curve, selectivity and LLOQ

The method was linear in the range of 0.18-120 ng/ml for ARI and 0.35-110 ng/ml for DARI. The correlation coefficient (R^2) values were 0.9979 ± 0.0026 for ARI and 0.9995 ± 0.0004 for DARI (n=6). We obtained satisfactory R^2 , similar to other methods [21–24]. Our calibration range was adjusted according to the real samples' concentration (55.72 ± 14.58 ng/ml for ARI and 7.01 ± 2.44 ng/ml for DARI), enabling us to obtain pharmacokinetic data for both ARI and its metabolite.

The present method was selective, as no significant interference was found in the detection of ARI and DARI in the absence or presence of their ISs in 6 different blank samples. Figure 2 shows the selectivity of the method. Figure 2A shows an EIC of blank sample, while figure 2B, 2C and 2D shows ARI and DARI at LLOQ concentration, sample. LLOQs peak area is 5 times more than the one of the blank sample and remained at similar concentrations in pure solution, in pre-extracted and post-extracted, corroborating the selectivity of the method.

The LLOQs of 0.18 ng/ml for ARI and 0.35 ng/ml for DARI presented an intra- and inter-day accuracy and CV values that are adequate (Table 2). The analyte area/blank area ratio was 18.03 ± 13.60 for ARI and 12.82 ± 8.19 for DARI, more than 5 times higher than that of any target plasma in all cases. The area of the zero plasma, with its IS, is 20 times larger than the area of the blank plasma (725.04 ± 212.14 for both, ARI and DARI). To date, the lowest LLOQs were obtained by Patel and co-workers [34] with the value of 0.05 ng/ml for ARI and by Song and colleagues [23] with the value of 0.01 ng/ml for DARI. While not reaching the before-mentioned values, we still outperformed other UPLC- and LC-MS/MS methods developed by different authors [20,24–32,32,33,38].

3.3. Precision and accuracy

Table 2 summarizes the results for precision and accuracy with standard deviation (SD), CV and accuracy calculated for each sample. The intra- and inter-day precision was optimal with CV less than 11% and accuracy lower than 10%. These results are similar to those obtained by other authors [23,24,26,38] and better comparing to Kubo [21] and Choong [33] and co-workers. Thus, the method is reproducible and accurate for ARI and DARI.

3.4. Extraction recovery, matrix effect and phospholipids removal

The extraction recovery and matrix effect were determined at three concentration levels: LLOQ, low and high QC. Table 3 summarizes the results for absolute and relative extraction recoveries and matrix effects in percentages (100% is referred as 100% of recovery/no matrix effect) with mean and CV values calculated for each sample. The absolute mean recoveries were found between 88% and 96% for both compounds, as well as for IS. However, the relative recovery values were much better comparing with absolute values, as they have been achieved between 96% and 106% for ARI and DARI. We have achieved good values of absolute mean matrix effect for all compounds (between 90% and 100%). The relative mean of matrix effect was in the range of 92% to 105%. Therefore, the extraction procedure was accurate and reproducible, no significant matrix effect was observed with CV values which were less than 9% in all cases.

Extraction procedure applied in the present work enables direct sample injection into the LC-MS/MS without evaporation and reconstitution process. Three steps microelution-SPE applied for sample preparation and the use of IL-IS enables the decrease of matrix effect and improves assay selectivity, which is similar to that

reported by other authors [10,34]. However, most of the methods employed PPT or LLE, which are not efficient in eliminating phospholipids, surfactants, salts [17,39] and may interfere with ionization process and consequently producing the loss of the analyte signal [40]. On account of this, our extraction recovery is better (between 96% and 106%) than other method applying PPT or LLE as sample preparation reported in the bibliography [9,11,19,25,26,32,33,38] and ranging from 46% to 123%. Moreover, in spite of large impact of matrix effects in LC-MS/MS analysis [41,42], it has not been taken into account in some of described methods [19–21]. Usually matrix effects' evaluation is based on post-column infusion method [24,28,31,38,43] or post-extraction addition method [30,41], similar to our assay. The advantage of post-extraction addition method over post-column infusion method is the quantitative and not qualitative estimation of ionization suppression/enhancement. Consequently, our results on matrix effects were among the best achieved in the bibliography, similar to those achieved by Patel and co-workers [34]. Patel et al used SPE as well, but with UPLC-MS/MS system, which is known of producing less matrix effects due to smaller injection volume and lower flow rates.

To contrast our own results on phospholipids removal with the literature, we have tested twelve human plasma samples with SPE and PPT extraction methods. Monitoring of phospholipids was useful in method development to avoid the matrix interferences with no negative influence on analyte quantification. Due to the amphiphilic characteristic of phospholipids, the application of hydrophilic-lipophilic SPE sorbent enabled us to remove efficiently 99.45% endogenous plasma phospholipids compared to PPT method ($100.00 \pm 11.16\%$ vs $0.55 \pm 0.47\%$). Figure 3 displays endogenous plasma phospholipids content in percentages using PPT or microelution-SPE, respectively. Additionally, we were able to chromatographically separate phospholipids ($t_R=1.477$ minutes) from analyte elution (ARI, $t_R=3.644$ minutes; DARI, $t_R=3.451$ minutes and ARI-D8, $t_R=3.582$ minutes). This is the first report monitoring simultaneously human plasma ARI, DARI and phospholipids in the same acquisition LC-MSMS method.

3.5. Stability

Table 4 summarizes the results of the stability assays at low and high QC for ARI (0.5 and 110 ng/ml) and DARI (0.7 and 90 ng/ml). The stability of both analytes after 24 hours at 19°C in the autosampler revealed a value in the range of 99% and 108% (CV no higher than 5%). The short-term stability after 24 hours at room temperature was from 87% to 93%, with CV values in the range of 0% to 4%. The stability test after 24

hours at 4°C in the fridge also showed a range of 91% and 99% (CV no higher than 7%). Finally, the stability tests after 3 freeze-thaw cycles in the freezer at -80°C showed no significant degradation, with stability in the range of 90% and 101% and CV in the range of 1 to 5%. The mean was close to 100% and the CV was less than 7% in all cases.

The EMA guide stresses that analyte stability cannot be proven by literature data [36], further outlining the importance of analyte stability testing. The reason is that if the analyte is unstable, its decomposition influences the precision and accuracy of the procedure and is, thus, accounted for by these two parameters [42]. Therefore, we guaranteed stability of the analytes during sample preparation, the analytical process, and the storage conditions, similar to other authors [33,34,38]. In spite of its importance, methods without evaluating stability tests are present in the bibliographic data [22,43].

3.6. Carry-over

The carry-over in the blank sample following after CAL at highest concentration was 14.31% ± 4.45% of the LLOQ for ARI and 13.71% ± 8.56% for DARI, less than 20%; for IS was 0.01%, less than 5%. Thus, in line with EMA regulations, no significant carry-over effect was observed.

Despite the fact, that evaluation of carry-over effect is not mandatory in all guidelines for bioanalytical method, such as in International conference on harmonisation (ICH) [44] or FDA guidelines [35], it should be assessed within the method development and validation. Determination of carry-over effect is a very simple way to detect a possible column contamination after injection of high concentrated CALs and can be included routinely in each analytical run. Unfortunately, the carry-over effect was only evaluated by few authors [29,30,33,34,43], showing excellent results.

3.7. Application of the method to patients' plasma samples

This procedure has been successfully applied to several pharmacokinetic studies. Figure 4A exhibits ARI and DARI plasma concentration (ng/ml) versus time (from 0 to 72 hours) acquired after a single oral administration of ARI (10 mg) in 103 healthy volunteers (1133 samples). The average of maximal plasma concentration (C_{max}) was 55.72 ± 14.58 ng/ml at 3.25 ± 2.41 hours (T_{max}) for ARI and 7.01 ± 2.44 ng/ml at 52.71 ± 16.57 hours for DARI. Fig. 4B shows mean pharmacokinetic parameters of ARI and DARI. These results are in line with previous reports on pharmacokinetics by Ravinder and co-workers as well as by Patel et al (C_{max} of ≈50 ng/ml), who used the same single

oral dosage. Other authors have reported different levels of C_{max} due to higher-30 mg [11] or lower administration dose of 5-6 mg [9,23]. Besides the different dose, the differences in the pharmacokinetic results are due to different populations (Caucasian, Chinese and Indian) as well as healthy volunteers versus patients. The high inter-subject variability showed by the error bars in Fig 4, is similar to those results obtained by other authors making them suitable for monitoring. It was not possible to calculate the $T_{1/2}$ of DARI because the last sample was taken at 72 hours as EMA guidelines requires for long half-life drugs [36].

4. Conclusion

In summary, the present method is fast and simple, easily applicable to large sample number. The method is linear and covers wide range of the concentration curve from 0.18 to 120 ng/ml for ARI and from 0.35 to 110 ng/ml for DARI. This LC-MS/MS assay is precise, accurate, sensitive, specific, stable and not showing significant carry-over effects according to FDA and EMA recommendations and is currently being applied in pharmacokinetic studies. The present method applies effective phospholipids' removal and therefore good recoveries as well as no significant matrix effect, thanks to three-steps microelution-SPE sample preparation method and usage of IL-IS.

Acknowledgements

The authors wish to thank Instituto-Fundación Teófilo Hernando for its continued financial support. This work was supported by FPU12/02220 from MECED to AW.

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Compound	Retention time (tR, min)	Quantification MRM	Confirmation MRM	Dwell time (ms)	Fragmentor voltage (V)	Collision energy (eV)
		(transition)	(transition)			
Aripiprazole	3.644	448.2>285.2	448.2>176.2	100	100	25
Dehydro-aripiprazole	3.451	446.2>285.2	446.2>188.0	100	100	35
Aripiprazole-D8	3.582	456.2>293.2		100	110	30
Phospholipids	1.477	184.1>184.1		100	100	5

Table 1. Retention time (tR) in minutes (min), ions and fragmentation conditions used for selected reaction monitoring (SRM) of aripiprazole, dehydro-aripiprazole, the stable isotope-labeled internal standard, aripiprazole-D8 and phospholipids.

Compound	Concentration (ng/mL)	Intra-day (n=5)			Inter-day (n=15)		
		Mean ± SD (ng/mL)	CV (%)	Accuracy (%)	Mean ± SD (ng/mL)	CV (%)	Accuracy (%)
Aripiprazole	0.18 (LLOQ)	0.19±0.00	2.34	4.49	0.18±0.01	6.07	1.22
	0.5	0.49±0.02	3.49	-2.37	0.50±0.02	4.15	0.80
	55	57.26±1.43	2.50	4.12	56.11±4.83	8.61	2.02
	110	119.81±31.88	1.57	8.92	120.51±12.02	9.97	9.56
Dehydro-aripiprazole	0.35 (LLOQ)	0.35±0.01	4.13	0.94	0.37±0.02	4.53	4.30
	0.7	0.67±0.01	1.43	-4.11	0.67±0.03	4.73	-4.28
	45	49.15±1.68	3.43	9.23	47.93±5.08	10.61	6.51
	90	95.23±5.36	5.63	5.82	96.35±10.10	10.48	7.05

Table 2. Intra- and inter-day precision and accuracy assays. Data were obtained from 4 quality controls (QCs) –the lower limit of quantification (LLOQ), low, medium and high – on the same day as intra-day and on 3 consecutive days as inter-day precision and accuracy assays. Precision data are expressed as the percentage of the coefficient of variation (CV) for repeated measurement and accuracy 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 is shown in parenthesis.

Compound	Concentration (ng/mL)	Recovery (n=6)				Matrix effect (n=6)			
		Mean ± SD (%)		CV (%)		Mean ± SD (%)		CV (%)	
		Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
Aripiprazole	0.18 (LLOQ)	91.12±6.70	98.75±6.81	7.35	6.89	95.34±7.49	105.25±8.66	7.85	8.23
	0.5	95.78±8.46	105.74±3.59	8.84	3.39	95.57±8.51	97.63±6.03	8.91	6.18
	110	95.40±4.77	106.41±3.05	5.00	2.87	100.00±8.98	99.99±0.60	8.98	0.60
Dehydro-aripiprazole	0.35 (LLOQ)	88.12±7.96	96.35±5.74	9.03	5.96	92.73±4.81	101.53±5.22	5.18	5.14
	0.7	89.90±1.07	100.66±4.10	1.19	4.07	90.00±18.05	92.10±6.61	8.47	7.18

Figure 1.

Figure 1: Precursor/product ion mass spectra of $[M+H]^+$ and chemical structures of aripiprazole (ARI, A), dehydro-aripiprazole (DARI, B) and the isotope-labeled internal standard (IL-IS), aripiprazole-D8 (ARI-D8, C), under multiple-reaction monitoring (MRM). The fragmentation patterns of all analytes are indicated by an arrow on the chemical structure of each analyte. The 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 chromatograms have been normalized to the largest peak.

Figure 2.

Figure 2: Representative extraction ion chromatograms (EIC) of aripiprazole (ARI) and dehydro-aripiprazole (DARI). Human blank plasma (A), lower limit of quantification (LLOQ) of aripiprazole (ARI) and dehydro-aripiprazole (DARI) in pure solution (B), human plasma spiked with LLOQ of ARI and DARI before (C) and after microelution-solid phase extraction (SPE, D) procedure. Retention times (tR) and concentration values calculated from calibration curves are given for both analytes. The results are presented as the percentage of counts versus acquisition time in minutes (min). All chromatograms have been normalized to the largest peak.

Figure 3.

Figure 3: Comparison of two extraction methods, protein precipitation (PPT) and microelution-solid phase extraction (SPE), in order to evaluate endogenous phospholipids removal from biological matrix. Representative extraction ion chromatogram (EIC) of phospholipids are shown for protein precipitation (PPT) or SPE, retention times (tR) in minutes (min) and area at the top of chromatograms. Data are expressed as mean percentages $\pm$ SEM of twelve human plasma samples. Paired t-test, two tailed was performed to compare phospholipids removal using PPT versus microelution-SPE, *** $p < 0.001$.

Figure 4.

B

Pharmacokinetic parameters of ARI and DARI (mean $\pm$ S.D.); (n=103)

Parameter	ARI	DARI
C_{max} (ng/ml)	55.72 $\pm$ 14.58	7.01 $\pm$ 2.44
T_{max} (h)	3.25 $\pm$ 2.41	52.71 $\pm$ 16.57
$T_{1/2}$ (h)	53.16 $\pm$ 22.54	no data
AUC_{0-t} (ng $\times$ h/ml)	1944.34 $\pm$ 531.00	396.29 $\pm$ 141.80