Fully automated forensic routine dried blood spot screening for workplace testing

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Abstract

In this study we describe the transfer of a new and fully automated workflow for the cost effective drug screening of large populations based on the dried blood spot (DBS) technology. The method was installed at a routine poison control center and applied for DBS and dried urine spot (DUS) samples. A fast method focusing on the high interest drugs and an extended screening method were developed on the automated platform. The dried cards were integrated into the automated workflow, in which the cards were checked in a camera recognition system, spiked with deuterated standards via an in-built spraying module and directly extracted. The extract was transferred online to an analytical LC column and then to the electrospray ionization tandem mass spectrometry system. The target compounds were analyzed in positive multiple reaction monitoring mode. Before each sample batch or analysis day, calibration samples were measured to balance inter day variations and to avoid false negative samples. An internal standard was integrated prior the sample extraction to allow in process control. 28 target compounds were analyzed and directly extracted within 5 minutes per sample. This fast screening method was then extended to 20 minutes, enabling the usage of a Forensic Toxicology Database to screen over 1200 drugs. The method gives confident positive/negative results for all tested drugs at their individual cut-off concentration. Good precision (+/- 15 %, respectively +/- 20 % at LOQ) and correlation within the calibration range from 5 to 1000 ng/ml was obtained. The method was finally applied to real cases from the lab and cross checked with the existing methodologies.

Key words: Drugs of abuse, Drug screening, Dried blood spot, Dried urine spot, Automation

1. Introduction

The fast and cost effective detection of prescription and illicit drugs is of major interest in workplace drug testing programs, roadside testing, rehabilitation programs and post-mortem investigations [1]. Psychoactive drugs act on normal brain functions and may alter an individual's consciousness, mood or thinking processes. There are legal drugs used for medication such as benzodiazepines, antidepressants and sedatives and illicit drugs such as opiates, cannabis, hallucinogens and cocaine [2], [3]. The integration of the dried blood spot (DBS) technology in drug testing offers various advantages such as simplified blood collection, reduced shipping, storage costs, reduced analysis time and labor [4]–[7]. After sample collection, each sample is anonymized using a barcode, which is later connected to the analysis results in a database. The advantages of DBS technology are not restricted to blood samples but can also be applied to other matrices such as urine spots (DUS).

The DBS technology was introduced in 1963 by Guthrie and Susi for the detection of phenylketonuria in newborns [8], [9]. The technology evolved to be the method of choice in newborn screening (NBS) laboratories around the world [10]. One major drawback was the sensitivity, since very small sample volumes are applied on the DBS cards. However, with modern mass spectrometry instruments this issue is no longer a hurdle. The DBS technology emerged to further applications and markets such as therapeutic drug monitoring [11]–[13], pharmaco-toxicokinetic studies [14][15] and forensics. Although the DBS technology has been applied to individual drug tests, no fully automated system that includes a screening of a wide range of drugs is available [16]–[20]. When it comes to high throughput of tests or to drug testing where no professional laboratory environment or expert knowledge is available, automation is crucial.

In this study, two fully automated methods for DBS and DUS were established, including card recognition, sample preparation and extraction, and online analysis by ultra-high performance liquid chromatography-tandem mass spectrometry (DBS-LC-MS/MS). First, a fast 5 minute screening method for the 28 most abundant illicit drugs at the Regional Poison Control Center Dammam was developed and second, the DBS samples were analyzed using a Forensic Toxicology Database method [21]. Automation for this study was implemented by the CAMAG DBS-MS 500 equipment and a triple quadrupole coupled to a UHPLC System. The method has been applied to real cases from the laboratory to illustrate the process and to show the potential of this approach. The focus of this study was to develop an automated method that gives a confident positive/negative result at the cut-off concentration of the individual drug.

2. Experimental

a. Apparatus

Gradient grade water and methanol for liquid chromatography (rinsing solvents 2-propanol and acetonitrile), and formic acid were purchased from Carl Roth (Carl Roth, Germany). Ammonium formate (LCMS Grade) was purchased from Sigma Aldrich (Sigma Aldrich, USA).

All analytical standards were purchased from Lipomed (Lipomed, Switzerland); Codeine, 6-Acetylmorphine, 3,4-Methylenedioxymethamphetamine, Amphetamine, Methamphetamine, N-Methyldiethanolamine, 4,4'-Methylene dianiline, Heroin, Cocaine, Morphine, 7-Aminoclonazepam, Chlorphenamine, Clozapine, Bromazepam, Midazolam, Clonazepam, Clobazam, Oxazepam, Alprazolam, Chlorpromazine, Nordiazepam, Diazepam, 11-nor-delta9- tetrahydrocannabinol carboxylic acid, 4'-methyl-AM-2201, 5"-fluoro-JWH-122, THCA-A, Delta-9- tetrahydrocannabinol, and Cannabidiol. Also, the

1 deuterated internal standards Alprazolam-D5 and Cocaine-D3 were purchased from Lipomed (Lipomed,
2 Switzerland).

3
4 Dried blood spot cards (Ahlstrom TFN filter paper) were provided by CAMAG (Muttensz, Switzerland).
5 Fresh whole blood was obtained from the local blood donation center (Basel, Switzerland) and from the
6 regional poison control center (Dammam, Saudi Arabia). EDTA tubes and blood drawing equipment were
7 supplied by the university hospital Basel (Basel, Switzerland).

8 9 **b. Methods**

10
11 The DBS-MS 500 system (CAMAG, Switzerland) was attached as front end to a modular HPLC system
12 from Shimadzu (Kyoto, Japan); it contains a system controller (CBM-20A), a Nexera X2 pump and a
13 degasser (DGU-20ASR). Analysis was performed in positive multiple reaction monitoring (MRM) mode on
14 an electrospray ionization tandem mass spectrometry system 8040 and 8060 (Shimadzu, Kyoto). Data
15 interpretation was performed by Labsolution, all samples are referred to quality control samples.

16 17 **i. LC-MS/MS instrumentation and settings**

18
19 Chromatography was performed on Shim-pack GIST (2.1 x50 mm, 2 µm C18) (GL Science, Japan) and
20 Kinetex (2.1 x 100 mm, 2.6µm, XB-C18) (Phenomenex, USA) analytical columns. An inline filter
21 (KrudKatcher Ultra, Phenomenex, USA) was connected upstream to the analytical column. Mobile phase
22 A consisted of water with 10 mmol/L ammonium formate + 0.1% formic acid and mobile phase B
23 methanol with 10 mmol/L ammonium formate + 0.1% formic acid. For the 5 minute method on the
24 Shim-pack GIST column, the following stepwise gradient was applied: 45%B (0.0-2.0 min), 45-90%B (2.0-
25 2.5 min), 90%B (2.5-3.5 min), 45%B (3.51-5.0 min). The flow rate was set to 0.5 mL/min at 50°C. The
26 screening method on the Kinetex column was set according to the Shimadzu protocol [21]. The HPLC
27 liquid stream was connected via the DBS-MS 500 autosampler to the LCMS system. At least 5 MRM
28 transitions were recorded for each analytical compound. The most abundant mass transition was used
29 for quantitation. The signals of all acquired mass transitions were used for compound identification. The
30 detailed MS settings are listed in Table 1.
31

Table 1, m/z transitions of all compounds

Name	Dwell [ms]	Pre.	Quant.	Qual. 1	Qual. 2	Qual. 3	Qual. 4	CE [V]
Codeine	20	300.15	152.10	215.15	165.15	128.10	153.15	-15
6-Acetylmorphine (6-MAM)	5	328.15	165.10	211.15	152.10	193.10	181.15	-16
3,4-Methylene-dioxymethamphetamine (MDMA)	5	194.10	105.10	163.10	135.05	77.05	133.05	-23
Amphetamine	5	136.10	91.10	119.15	65.10	39.10	51.10	-16
Methamphetamine	5	150.15	91.10	119.15	65.10	39.10	51.10	-26
N-Methyldiethanolamine (MDEA)	5	208.15	163.10	105.10	135.05	133.05	77.05	-14
4,4'-Methylene dianiline (MDA)	5	180.00	77.10	105.10	135.00	133.10		-21
Heroin	20	370.15	165.05	268.10	328.10	211.05	152.10	-19
Cocaine-D3	5	307.15	85.05					-20
Cocaine	2.5	304.15	182.15	82.05	77.05	105.05	150.15	-20
Morphine	20	286.15	152.10	201.10	165.10	128.05	153.10	-14
7-Aminoclonazepam	5	286.05	121.10	222.10	250.05	195.10	77.00	-20
Chlorphenamine	5	275.15	230.10	167.10	166.15	139.10	201.10	-20
Clozapine	5	327.15	270.10	192.10	227.05	164.10	296.10	-20
Bromazepam	5	316.00	182.15	209.10	181.10	208.15	288.00	-21
Midazolam	5	326.10	291.10	223.10	244.00	249.05	209.05	-22
Clonazepam	5	316.05	270.05	214.05	241.15	151.10	207.10	-22
Clobazam	5	301.05	259.05	224.10	223.10	153.05	105.10	-21
Oxazepam	5	287.05	241.00	104.05	77.00	231.00	269.00	-19
Alprazolam-D5 (internal standard)	5	314.10	286.00					-30
Alprazolam	2.5	309.10	281.05	205.10	274.10	151.10	241.05	-21
Chlorpromazine	5	319.10	86.10	58.05	246.00	214.05	239.05	-22
Nordiazepam	5	271.05	140.05	208.10	165.05	243.05	104.10	-18
Diazepam	5	285.10	154.00	193.05	222.10	257.00	228.00	-19
11-nor-delta9- tetrahydrocannabinol carboxylic acid (11-nor-delta9-THC COOH)	20	345.10	327.05	299.20	118.90	193.00	229.15	-19
4'-methyl-AM-2201, 5''-fluoro-JWH-122 (MAM-2201)	2.5	374.10	168.95	141.10	115.00	232.05	143.85	-30

THCA-A	20	345.20	299.20	193.15	119.20	187.15	327.20	-17
Delta-9- tetrahydrocannabinol	20	315.25	193.15	259.15	123.15	135.15	235.20	-11
Cannabidiol	20	314.95	192.90	93.05	135.10	123.00	259.15	-26

The general settings of the mass spectrometer were: nebulizing gas 2 L/min (N₂), heating gas 10 L/min (N₂), drying gas 10 L/min, positive mode only, and source temperature 300°C. Labsolutions software (Shimadzu Kyoto, Japan) was used to operate the LC-MS/MS system. Dwell = duration of collecting each m/z ion signal; Pre. = precursor ion; Quant. = daughter ion for quantification; Qual. = qualifier ion; Qual. 2,3 = qualifier ion 2 and 3; CE = collision energy of quantifier ion

ii. DBS-MS 500 Instrumentation and Settings

The extraction solvent on the DBS-MS 500 (CAMAG, Switzerland) was a mixture of methanol and water (70:30 v/v) and was connected to the extraction port (Figure 1). The wash solution, consisting out of methanol, acetonitrile, 2-propanol and water with 0.1% formic acid (25:25:25:25, v/v/v/v), was connected to the rinsing bottle. The internal standards D5-alprazolam or D3-cocaine were connected to internal standard port 1. The calibration mix 1 (bases) and calibration mix 2 (acids) were connected to port 2 and 3 (explained in section 3.c. Quality control cards and internal standard) and Internal standard port 4 and the wash port were filled with methanol. The system was prepared by priming methanol through the internal standard port 4 (10 cycles) followed by 2 cycles through port 1. The extraction head was cleaned in an ultra sound bath at 40°C for 10 min prior a large set of analyses. The extraction solvent was primed for 5 cycles and the rinsing solvents were flushed for 1 minute (this process is an automated system prime method). The DBS cards were photographed with the built-in camera of the DBS-MS 500 before and after each run to check for the presence of a blood spot and to adjust the extraction head to the center of each spot. The Chronos for CAMAG software automatically recognized inadequate dried blood spots based on their roundness, diameter, and area. Inadequate DBS were excluded from analysis. 20 µl of internal standard was sprayed in a homogenous layer onto each spot. After a 20 second drying time the samples were extracted with a volume of 22 µl and 50 µl/min flow rate. To complete the automated DBS extraction cycle, the extraction outlet was rinsed for 20 seconds with the wash solution and for 20 seconds with the extraction solution followed by 5 seconds inlet rinsing with the extraction solution [15].

Figure 1, Fully automated workflow of the DBS-MS 500

c. Sample preparation

i. Working standards for LC-MS/MS tuning and QC

To balance inter day variations of this screening method, each day a high and low quality control sample is measured before starting the sample analysis. The sample amount of those QC's is known and the later results are referred to these values. The QC cards were prepared in the according laboratory by using the DBS-MS 500 spraying module. Here, two calibration mixes were prepared in methanol, first all alkali target compounds are mixed to prepare a 100 ng/mL standard solution (drug panel except for THCA-A and 11-nor-delta9-THC COOH) and second the same is done for the two acidic target compounds. Both solutions are connected to the DBS-MS 500 internal standard ports (2 and 3). DBS cards with negative blood were prepared by pipetting 20 µl whole blood into the dashed rings and allowed to dry for 2 hours. The dried cards were inserted the DBS-MS 500 instrument and the cards were coated with the two 100 ng/mL standard solutions by using the spray option only. Low controls are coated with each standard mix with 10 µl and high control with 40 µl (both in fast mode), the acidic and alkali standard solutions were sprayed on top of each other. Separate cards for low and high controls were used. The DBS-MS 500 sprays a quadratic area of 1 x 1 cm. If 10 µl of a 100 ng/mL solutions are sprayed on the blank DBS, this equals a concentration of 0.126 ng under the plunger area which is 4 mm in diameter. The stock solutions are stable for 1 month at -20 °C. All target analytes from the panel of the 5 minute method, except for heroin, are stable on the dried card for 2 days at room temperature.

ii. Internal standard integration

To get an in-process control, at least one deuterated standard must be integrated into the analysis workflow. Depending on which laboratory, either D5-alprazolam or D3-cocaine was used, those analytes were chosen since they elute in the middle of the chromatographic separation and they show good ionization properties. The internal standard is dissolved in methanol to generate a 0.1 µg/mL solution, which is connected to the spray port (1) of the DBS-MS 500. If the method will be used for absolute quantitative analysis, each target compound must be compared to the according deuterated analogue.

iii. Dried blood spot samples

According to documented cut-off levels in whole blood [22] and according to the reference values of the Regional Poison Control Center Dammam, the target concentration range for the fast screening method was chosen between 5-1000 ng/mL. 6 calibration points were chosen to simplify the sample preparation, since it was intended to acquire a five point calibration from 5 to 500 ng/mL for certain target compounds and also a 5 point calibration from 25-1000 ng/mL for the remaining analytes. Freshly collected human blood was obtained from the local blood donation center. EDTA was used as an anticoagulation agent (vacutainer tubes, BD, Allschwil, Switzerland). Volunteer blood of a healthy male donor was chosen as zero control. A stock solution of the standards was prepared in methanol and gently mixed with the donor blood in six different concentrations to prepare the levels. The maximum organic spike was 2.5 % to the donor blood. 20 µL aliquots were spotted onto CAMAG DBS cards (CAMAG, Muttenz, Switzerland) and dried at room temperature for at least 2h. The cards were subsequently stored at 4 °C in sealed plastic bags containing desiccants. Stock solutions were stable for two weeks.

d. Analysis workflow

To avoid false positive and false negative, the following analysis workflow was defined (Table 2):

Table 2, analysis workflow

	Step	Comment
1	Solvent	Blank DBS card extraction without internal standard (ISTD) spray
2	Negative matrix	Negative blood sample with ISTD spray, signals must be below LOD
3	2 x Low control	Quality control low sample with ISTD
4	2 x High control	Quality control high sample with ISTD
5	2 x Negative matrix	Negative must be clean, signals must be below LOD
6	Patient 1	Negative: Only ISTD signal is detected Positive: Drug signal above cut-off detected, measure sample again with alternative technology
7	Negative matrix	Negative must be clean, signals must be below LOD
8	Patient 2	Repeat for further patients
9	Negative matrix	
n	Low control	Low control after 15 patients and at the end of each analysis batch
n+1	Negative matrix	

Blank DBS cards, cards with negative blood and the QC cards are filled in separate racks. Negative blood is measured between patients to get physical prove that there is no carry-over. If the samples are drawn from different sites, an optional process step can be integrated to exclude contamination of the whole filter paper card. Here, spot 1 is left empty while sampling and negative blood is applied in the laboratory prior the analysis.

3. Results

DBS and DUS cards were successfully automated for the intended analysis workflow. The cards are checked with image recognition software, sprayed with internal standard and extracted in a fully automated process. Up to 500 such cards with 4 samples each can be handled without any human interaction. The total time-to-result is 5 minutes per sample using the method with 28 target analytes and 20 minutes for the database method screening for over 1200 target compounds. Correlation was measured from below the according cut-off concentration to the toxic blood level of each drug (**Table 3**). Each compound is detected by five MRM transitions to enhance total signal intensity of a compound and to acquire enough data to confirm the target drugs. To further improve the signal of certain drugs and to lower their limit of quantification (LOQ), the dwell time of the mass spectrometer was adjusted for certain analytes to either increase or decrease their signal intensity (Table 1). To speed up the overall cycle time of the MRM data acquisition, specific retention time windows were defined for each compound (supplementary data).

a. Correlation and precision

The calibration levels were measured 6-fold on two different days to determine the method robustness and validity. The relative standard deviation of the internal standard was below 15 % for all target compounds by comparing the data through all levels (5-1000 ng/mL). The correlation and inter-day variations of all target compounds are listed in Table 3. The target to internal standard ratio was used to compare the results and to develop the calibration line. Excellent correlation was obtained for all target compounds. All points of the calibration functions were sufficiently precise with relative standard deviations below 15 %, respectively below 20 % at LOQ with the exception of THCA-A, 6-MAM and Diazepam which have to be further investigated and validated. Table 3 also includes the cut-off and toxicological levels from literature for blood samples [7], [22]. The same cut-off concentrations were used for the urine samples in this phase, however this will be adjusted with growing data from routine analysis. The goal of the analysis method was to detect the cut-off concentration with confidence; everything above this concentration will be reported and retested with alternative technologies.

Table 3, results form 6 point calibration on two separate days

	Relative inter-day standard deviation				Correlation R ²		Cut-off [22] [ng/mL]	Tox. Level [ng/mL]
	5 ng/mL [%]	25 ng/mL [%]	250 ng/mL [%]	500 ng/mL [%]	5-500 [ng/mL]	25-1000 [ng/mL]		
Codeine	10.4	10.5	9.7	11.6	0.992	0.997	50	1100
6-MAM	22.3	9	13.4	15.6	0.992	n/a	10	n/a
MDMA	14.3	8.5	6.8	6.3	0.995	0.999	100	1000
Amphetamine	12.6	9.6	4.9	7.1	0.996	0.999	100	1000
Methamphetamine	13.2	11.3	8	9	0.995	0.998	100	n/a
MDEA	11.8	8.7	6.9	6.6	0.994	0.997	100	n/a
MDA	9.4	5.1	5.3	5.8	0.995	0.998	100	n/a
Heroin (n/a, not stable on card)	4.8	7.4	4.6	5.6	0.995	0.997	50	200
Cocaine	14.8	13.3	14.7	7.7	0.999	n/a	20	1000
Morphine	18.6	9	8.2	8.6	0.993	0.998	50	200
7-Aminoclonazepam	12.6	7.7	7.4	6.6	0.995	n/a	25	n/a
Chlorphenamine	11.6	3.4	1.9	7.1	0.999	n/a	25	n/a
Clozapine	11.2	5.1	6.5	3.9	0.994	n/a	25	n/a
Bromazepam	15.2	7.6	6.5	5.7	0.998	n/a	25	n/a
Midazolam	10	4.5	3.1	4	0.995	n/a	25	n/a
Clonazepam	14.7	6.9	5.7	8.2	0.995	n/a	25	80
Clobazam	13.3	6.6	5.8	8.1	0.997	n/a	25	n/a
Oxazepam	13.8	13.3	11.1	12.6	0.999	n/a	25	1400
Alprazolam	11.9	3.9	4.5	7.1	0.991	n/a	25	350
Chlorpromazine	16.6	13.9	13.1	5.6	0.999	n/a	25	n/a
Nordiazepam	13	11.1	6.9	7	0.999	n/a	25	n/a
Diazepam	19.4	29.9	24.8	31.8	0.995	n/a	25	3000
11-nor-delta9-THC-COOH	15.4	11.8	11.9	7.4	0.998	n/a	10	n/a
MAM-2201	16.8	11.1	10	5.6	0.996	n/a	10	n/a
THCA-A	13.6	11	15.4	8.7	0.998	n/a	10	200
Delta-9- tetrahydrocannabinol	13.2	12.3	6.8	8.7	0.995	n/a	10	n/a
Cannabidiol	10.5	8.4	12.1	9	0.996	n/a	10	n/a

The calibration line is shown for amphetamine (25 ng/mL) in Figure 2, since this compound was found in one of the toxicological routine samples from the laboratory after method implementation (section: *Real samples from a routine environment*). Also illustrated in Figure 2 is the butterfly spectrum from the MRM spectrum mode and the according molecule structure generated by the Shimadzu Forensic Toxicology Database. There was a 100 % hit for the amphetamine spike and the MS fragment spectra are overlapping nicely. The spike in the measurement below was Alparzolam-D5.

Figure 2, Database report of amphetamine sample showing the peak, the internal standard peak, the calibration curve, the MRM spectrum mode with database spectra and the structure

b. Carry-over

Carry over was monitored by measuring blank blood right after extracting a mix of all standards at 1000 ng/mL. The criteria of bio-analytical method validation guidelines were fulfilled [18-19]. No carry over was observed for the chosen analyte panel. Figure 3 shows the amphetamine peak for 1000 ng/mL and the subsequent blank sample. This was optimized by programming a wash sequence within the DBS-MS 500 workflow. Here, the outlet capillary from the DBS-MS 500 system was rinsed with methanol, acetonitrile, 2-propanol and water with 0.1% formic acid (25:25:25:25, v/v/v/v) for 20 seconds at 45 bar after which the extraction chamber inlet was flushed with extraction solvent for 10 seconds.

Figure 3, 1000 ng/mL amphetamine peak (above) and subsequent blank sample (below) showing no carry-over

c. Quality control cards and internal standard

Prior the analysis, high and low control samples were measured. The low control reflects a concentration close to the cut-off of the THC derivatives and the high control gives a value significantly higher to allow controlling of the analysis performance. The control cards were prepared by the laboratory using the DBS-MS 500 spraying module.

10 µL of the deuterated alprazolam or cocaine standard is sprayed as 0.1 µg/mL solution in methanol directly on the DBS prior extraction. The internal standard solution can be used for up to two weeks if stored in the fridge overnight. Alprazolam-D5 was used during method development phase and cocaine-d3 for the method implementation due to standard availability.

4. Method transfer to routine laboratory

d. DBS method transfer

The method described in section 3 was developed in a research laboratory using a Shimadzu 8060 LCMS system. In the next step, this method was transferred to a routine laboratory equipped with an 8040 LCMS. After transferring the method and adjusting the MRM voltages of the target compounds by the Labsolution optimization tool, a five-point calibration each with three repetitions was measured in the range of 10 to 500 ng/mL target drug in blood, since this was the region of interest for the routine laboratory. Pre-sprayed quality control samples were measured before and after the calibration samples according to the described workflow. Excellent correlation >0.995 and standard deviation below 15 % was obtained for all compounds except for the THC derivatives, where the correlation was above 0.985 and standard deviation below 25 % (except for LOD, shown in supplementary data). The sensitivity of the THC derivatives is at its limit on the 8040 LCMS system, however, when the cut-off is put at 25 ng/mL rather than at 10 ng/mL the result can be detected reliably.

e. Method adaption for urine samples

Since sometimes samples are also acquired as urine by the laboratory, the method was adapted to measure the same analytes also from a dried urine spot (DUS). Negative urine was spiked with the same standard mix used for the blood samples and a 3 point calibration was measured in the range of 10 to 400 ng/mL. Here, 10 µL of urine was spotted on a urine card. The urine cards are coated to make the colorless liquid visible (picture in supplementary data) and detectable by the DBS-MS 500 inbuilt camera. Excellent correlation >0.995 and standard deviation below 15 % (below 25 % at LOQ) was obtained for all compounds except for the THC derivatives, where the standard deviation was below 25 % (except for LOQ, shown in supplementary data). Also, morphine and MDA could not be detected due to interferences.

f. Real samples from a routine environment

To further investigate the method, a positive sample from the Dammam laboratory was measured with the newly installed DBS-LC-MS/MS method. Blood and urine from the same suspect was analyzed. The sample was positive for amphetamine and 11-nor- Δ^9 -tetrahydrocannabinol carboxylic acid in both, urine and blood, where the same cut-off concentration was considered for both matrices. Δ^9 -tetrahydrocannabinol and cannabidiol was positive in blood and THCA-A was positive in urine. This reflects the metabolism of THC, where the native THC is found in blood and the carboxylated THC is found in urine. All data was in correlation with the results from alternative analysis by the Dammam laboratory. This is a proof of concept for the function of the new dried matrix spot method.

5. Discussion

The method was successfully transferred from a research into a routine environment. The sensitivity of the 8040 LCMS instrument was sufficient for the described application, however the cut off level of the THC-derivates is at its limit of sensitivity on this instrument. On the other hand was the MRM signal saturated for e.g. Midazolam and Clozapine at the level 1000 ng/mL and therefore only the correlation up to 500 ng/mL is displayed in the results. Very small amount of drugs can be measured on these instruments allowing the trend towards micro sampling approaches. When reducing the volume to one droplet of blood with a realistic drug concentration, only a few picograms reach the analytical system. Nonetheless, this low amount is detectable and quantification is possible. Using the automated instrumentation, up to 500 sample cards can be processed without any human interaction enabling the fast and cost-effective screening of large populations.

When using tandem mass spectrometry, not all compounds have to be base line separated by the chromatography, since they can be distinguished by the MRM information. Attention must be paid if certain compound share the same MRM transitions and are not separated. Here, morphine and 7-aminoclonazepam both elute at 0.62 minutes and share a similar parent ion mass of 286 m/z. However, the fragmentation patterns are different and therefore both compounds can be identified.

To enhance the signal of certain drugs and to lower their LOQ, the dwell time was adjusted. Increasing the dwell time from 5 to 20 ms increases the signal to noise ratio (SNR) significantly. For example codeine has a SNR of 11.67 at 5 ms, 13.81 at 10 ms and 20.35 at 20 ms. The absolute signal area does not increase significantly, but the background noise is lowered and therefore increase the SNR, which then decreases the detection limit.

1 The internal standards are used to monitor and normalize the extraction efficiency, to ensure that the
2 system is working properly and to avoid false negatives [15],[25]. 20 µl of the internal standard solution
3 was sprayed on the DBS spot prior to extraction. This process of integrating the internal standard by
4 spraying is the method of choice for quantification. P. Abu-Rabie stated that the hematocrit level can
5 have an influence on the extraction efficiency, so that the internal standard should better be applied
6 prior to the extraction [25]. It is not possible to spike the internal standard to the blood sample prior
7 spotting, since the spots are usually drawn at remote areas.

8
9 Low and high quality control cards were introduced for the screening workflow. For the initial phase, the
10 quality control cards were prepared directly in the laboratory using the DBS-MS 500 spraying module.
11 This process step is evaluated to outsource into a certified reference laboratory to better control the
12 workflow at the routine site. The goal would be that the low control reflects the individual cut-off
13 concentration of each target drug and the high control should be around the toxic concentration. This
14 would allow a direct comparison of the routine samples to the low control to generate a either positive
15 or negative screening result with more confidence and to cover the complete analytical relevant range.
16 Also the analyte on card stability needs to be further investigated. The DBS-MS 500 reference cards are
17 stored at deep freeze and long term stability studies are running. Deep freeze and room temperature
18 stability studies are running investigation at the Dammam Poison control center, since the DBS-MS 500 is
19 not cooled and the cards remain at room temperature during sample queue. The cut-off and
20 toxicological range was defined according to literature [22] and internal reports of the Dammam Poison
21 control center.

22
23 The extraction setup of the DBS-MS 500 features a horizontal extraction, where a small volume of
24 solvent is flushed under increased pressure from the bottom through the sealed area on the DBS back to
25 the bottom of the card. No other HPLC grade solvents or consumables are required for the extraction
26 process, which makes this application more cost effective and therefore well suited for high throughput
27 applications such as the described method. Since the DBS card, which is the main consumable, is a
28 natural product from cellulose, the analysis workflow is eco-friendly compared to alternative sample
29 preparation processes.

30
31 The analysis method was transferred successfully to urine samples, except for MDA and morphine. Both
32 compounds are rapidly metabolized to sulfates and glucuronide before urine excretion [26]. Therefore,
33 the urine method should be adapted for their according metabolites. Those analytes have to be further
34 investigated.

35 Using the Shimadzu Forensic Toxicology Database method allows to detect next to the list of integrated
36 drugs also new drugs, since the search tool compares homologous fragment ions and does use the mass
37 of the precursor ion as a pre-filter of the candidates during the database search. With this new option,
38 for example new synthetic cannabinoids can be detected by the identical fragmentation pattern of the
39 molecule ground structure independent of their intact precursor mass. On the other hand, this can also
40 lead to more than just one database hits for certain structurally related drugs. For example a MDA
41 sample gives also a 100% database hit for MDMA and MDEA (Figure 4), since they share the same
42 fragmentation pattern. They need to be distinguished by the precursor mass in this case.

Figure 4, Insight report for MDA showing also a positive library hit for MDMA and MDEA

The whole analysis workflow was fully automated. Each process is monitored and documented without any human interaction required. Each sample preparation step is standardized through the automation. Using the 28 target drug method, each sample takes 5 minutes. This workflow was extended by using the Shimadzu Forensic Toxicology Database to open up the analysis possibility to identify and quantify more than 1200 drugs within 20 minute sample analysis time.

6. Conclusion

A new workflow for high throughput drug screening was developed and transferred into a routine environment. The method is already used on a daily basis to monitor workplace safety and for suspects from road side controls. Blood and urine samples are analyzed via the DBS-LC-MS/MS platform and depending on the context, either the drug panel or the data base method are acquired. The process from sampling to generating the report was linked with a barcode system. Quality control cards can be prepared by the laboratory using the spraying module of the DBS-MS 500. Each process step is well documented and all analysis steps follow Good Laboratory Practice (GLP) [23]. The method can be easily modified or extended and transferred to other routine laboratories.

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Competing interest

The authors have declared that no competing interests exist.

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Supplementary data

Attachment 1, Retention time, MRM window and dwell time of the DBS-LC-MS/MS method

Name	Ret. Time [min]	Start [min]	End [min]	Dwell [ms]	Event Time [ms]
Codeine	0.46	0	1.5	20	0.105
6-Acetylmorphine	0.47	0	1.5	5	0.030
3,4-Methylenedioxymethamphetamine	0.50	0	2.5	5	0.030
Amphetamine	0.51	0	1.5	5	0.030
Methamphetamine	0.51	0	1.5	5	0.030
N-Methyldiethanolamine	0.52	0	1.5	5	0.030
4,4'-Methylene dianiline	0.52	0	1.5	5	0.030
Heroin	0.52	0	1.5	20	0.105
Cocaine	0.58	0	1.5	2.5	0.018
Morphine	0.62	0	1.5	20	0.105
7-Aminoclonazepam	0.62	0	1.5	5	0.030
Chlorphenamine	0.99	0	1.5	5	0.030
Clozapine	1.45	1	2.5	5	0.030
Bromazepam	1.57	1	2.5	5	0.030
Midazolam	1.95	1.5	2.5	5	0.030
Clonazepam	2.08	1.5	2.5	5	0.030
Clobazam	2.66	2	3.5	5	0.030
Oxazepam	2.73	2	3.5	5	0.030
Alprazolam-D5 (internal standard)	2.79	2	4	5	0.006
Alprazolam	2.80	2	3.5	2.5	0.018
Chlorpromazine	2.91	2	3.5	5	0.030
Nordiazepam	2.95	2.5	4	5	0.030
Diazepam	3.00	2.5	4	5	0.030
11-nor-delta9- tetrahydrocannabinol carboxylic acid	3.22	2.5	4	20	0.105
4'-methyl-AM-2201, 5''-fluoro-JWH-122	3.27	2.5	4	2.5	0.018
THCA-A	3.28	2.5	4	20	0.105
Delta-9- tetrahydrocannabinol	3.33	2.5	4	20	0.105
Cannabidiol	3.33	2.5	4	20	0.105

Attachment 2, Calibration results from Dammam laboratory of DBS

	Relative standard deviation at each level			
	25 ng/mL [%]	50 ng/mL [%]	100 ng/mL [%]	correlation R ²
Morphine	5.8	12.4	5.5	0.999
Amphetamine	2.4	3.4	0.6	0.999
Diazepam	10.1	4.9	12.8	0.999
Alprazolam	5.8	7.8	2.3	0.999
Methamphetamine	2.3	6.7	3.9	0.998
Codeine	10.5	1.2	2.6	0.999
MDMA	1.1	1.9	1.1	0.999
Cocaine	0.5	5.3	1.4	0.998

Delta-9- tetrahydrocannabinol	29.5	3.6	0.5	0.999
Midazolam	2.7	6.0	2.9	0.999
Clozapine	2.6	5.2	1.4	0.999
Clonazepam	2.7	6.0	1.6	0.999
Bromazepam	5.4	1.2	1.4	0.999
Oxazepam	6.7	2.0	2.3	0.999
6-MAM	12.5	5.4	3.4	0.999
THCA-A	6.8	24.0	21.9	0.984
11-nor-delta9- tetrahydrocannabinol carboxylic acid	37.0	17.9	4.0	0.997
Nordiazepam	1.7	1.1	2.0	0.999
MDA	0.6	6.4	5.1	0.999
Cannabidiol	39.1	21.5	6.2	0.999
ISTD variation over all samples	5.4 %			

1

2 Attachment 3, Calibration results from Dammam laboratory of DUS

	Relative standard deviation at each level			
	25 ng/mL	50 ng/mL	100 ng/mL	correlation
	[%]	[%]	[%]	R ²
Morphine	141.4	26.4	27.3	0.999
Amphetamine	19.3	12.6	7.8	0.998
Diazepam	6.2	4.4	3.0	0.999
Alprazolam	4.2	2.4	2.5	0.999
Methamphetamine	9.5	14.5	21.8	0.999
Codeine	20.7	2.5	3.1	0.999
MDMA	3.6	1.3	4.3	0.999
Cocaine	4.6	2.6	0.8	0.999
Delta-9- tetrahydrocannabinol	25.1	13.1	9.8	0.999
Midazolam	6.8	6.9	2.2	0.999
Clozapine	9.7	5.3	6.2	0.999
Clonazepam	3.2	1.7	3.0	0.999
Bromazepam	9.5	3.6	15.0	0.999
Oxazepam	5.4	4.7	0.2	0.999
6-MAM	9.6	1.8	5.0	0.999
THCA-A	29.0	18.2	10.8	0.994
11-nor-delta9- tetrahydrocannabinol carboxylic acid	21.3	18.4	11.4	0.999
Nordiazepam	11.1	1.9	4.2	0.999
MDA	6.2	3.0	3.2	0.017
Cannabidiol	30.5	11.8	2.9	0.999
ISTD variation over all samples	2.5 %			

3

4

5

Attachment 4, Dried urine spot card

Attachment 5, Stability at room temperature for 2 days

Compound	Dev.*	Compound	Dev.*
	[rel. %]		[rel. %]
Codeine	-2.7	Midazolam	-6.5
6-Acetylmorphine	-10.6	Clonazepam	-1.1
3,4-Methylenedioxymethamphetamine	-4.3	Clobazam	-11.0
Amphetamine	-6.3	Oxazepam	-14.7
Methamphetamine	2.6	Alprazolam	-6.1
N-Methyldiethanolamine	0.7	Chlorpromazine	-2.5
4,4'-Methylene dianiline	-5.8	Nordiazepam	-9.5
Heroin	-176.1	Diazepam	-6.2
Cocaine	-9.9	11-nor-delta9- tetrahydrocannabinol carboxylic acid	2.7
Morphine	5.8	4'-methyl-AM-2201, 5''-fluoro-JWH-122	-14.8
7-Aminoclonazepam	4.8	THCA-A	1.3
Chlorphenamine	-5.7	Delta-9- tetrahydrocannabinol	3.8
Clozapine	-4.1	Cannabidiol	-2.5
Bromazepam	-2.3		

* Relative deviation from day 1 to day 2 at room temperature