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RESEARCH ARTICLE

Fully automated drug screening of dried blood spots using online LC-MS/MS analysis

Stefan Gaugler[1,*], Jana Rykl[2], Matthias Grill[3], Vicente Luis Cebolla[4]

[1]*CAMAG, Muttenz, Switzerland.*
[2]*Shimadzu Schweiz GmbH, Reinach, Switzerland.*
[3]*Lipomed, Arlesheim, Switzerland.*
[4]*CSIC, Instituto de Carboquímica, Zaragoza, Spain.*

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A new and fully automated workflow for the cost effective drug screening of large populations based on the dried blood spot (DBS) technology was introduced in this study. DBS were prepared by spotting 15 µL of whole blood, previously spiked with alprazolam, amphetamine, cocaine, codeine, diazepam, fentanyl, lysergic acid diethylamide (LSD), 3,4-methylenedioxymethamphet-amine (MDMA), methadone, methamphetamine, morphine and oxycodone onto filter paper cards. The dried spots were scanned, spiked with deuterated standards and directly extracted. The extract was transferred online to an analytical LC column and then to the electrospray ionization tandem mass spectrometry system. All drugs were quantified at their cut-off level and good precision and correlation within the calibration range was obtained. The method was finally applied to DBS samples from two patients with back pain and codeine and oxycodone could be identified and quantified accurately below the level of misuse of 89.6 ng/mL and 39.6 ng/mL respectively.

Keywords: drugs of abuse, dried blood spot, DBS, automation, TDM.

Introduction

The dried blood spot (DBS) technology was introduced in 1963 by Guthrie and Susi for the detection of phenylketonuria in newborns [1]. The technology evolved to be the method of choice in newborn screening laboratories around the world [2]. 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 [3–5], pharmaco-toxicokinetic studies [6] and forensics [7–13]. Advantages of using DBS are simplified blood collection, reduced shipping, and storage costs and reduced analysis time and labor costs due to full automation [4,14]. Automation of the DBS workflow was achieved by automated punching equipment [15], where a manual transfer step of transporting the discs remains, and by direct elution technologies [16,17].

Forensic applications are of major interest since the DBS technology allows screening of a large population with minimum equipment in a cost-effective way. Samples can be drawn easily and shipped to a centralized lab for analysis. The samples are non-hazardous after drying and

***Correspondence:**
CAMAG, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland. Phone: +41 614673435; Fax: +41 614610702; Email: stefan.gaugler@camag.com

can be shipped by standard mail to the fully automated laboratory. Each sample is anonymized after drawing using a barcode, which is later connected to the analysis results in a database. In this study, a panel of different psychoactive drugs was chosen for introducing the automated drug screening concept. 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 [18,19]. The detection of these drugs is of major interest in workplace drug testing programs, roadside testing, therapeutic drug monitoring, rehabilitation programs and post-mortem investigations.

We here present the development of an automated DBS process, including card recognition, sample preparation and extraction, and online analysis by ultra-high performance liquid chromatography-tandem mass spectrometry (DBS-LC-MS/MS) for the simultaneous determination of a panel of psychoactive drugs in dried blood spots. Automation for this study was implemented by the CAMAG DBS-MS 500 equipment and a Shimadzu LC-MS/MS-8060 triple quadrupole coupled to a Nexera X2 UHPLC System. The method has been applied to two real cases to illustrate the process and to show the potential of this approach. The acquired MRM data from MS was used for quantitation and additionally for compound verification by screening against a forensic toxicology spectral library from Shimadzu.

Materials and methods

Chemicals and consumables

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): alprazolam, amphetamine, cocaine, codeine, diazepam, fentanyl, lysergic acid diethylamide (LSD), 3,4-methylenedioxymethamphetamine (MDMA), methadone, methamphetamine, morphine and oxycodone, and their deuterated standards: alprazolam-D5, amphetamine-D3, cocaine-D3, codeine-D3, diazepam-D3, fentanyl-D5, lysergic acid diethylamide-D3, 3,4-methylenedioxymethamphetamine-D5, methadone-D3, methamphetamine-D5, morphine-D3 and oxycodone-D3. Dried blood spot cards (Ahlstrom TFN filter paper) were provided by CAMAG (Muttenz, Switzerland). Fresh whole blood was obtained from the local blood donation center (Basel, Switzerland). For the DBS drawing BD Microtainer contact-activated lancets (Becton, USA) and soft-zellin alcohol prep-pads (Paul Hartmann AG, Germany) were used.

Analytical material and methods

LC-MS/MS instrumentation and settings

Chromatography was performed on a modular HPLC system from Shimadzu (Kyoto, Japan), which contained a system controller (CBM-20A), two Nexera X2 pumps, a degasser (DGU-20ASR), and a column oven (CTO-20AC). Automated extractions were carried out with a DBS-MS 500 (CAMAG, Muttenz, Switzerland). Analytes were separated on a Shim-pack GIST (2.3 x 50 mm, 5 µm C18, PN 227-30017-3) analytical column (GL Science, Japan). An inline filter (KrudKatcher Ultra, Phenomenex, USA) was connected upstream to the analytical column. Mobile phase A consisted of water with 10 mM ammonia formate, and mobile phase B of methanol with 10 mM ammonia formate. The following stepwise gradient was applied: 5%-95% (0.0-6.0 min), 95% (6.0-8.0 min), 5% (8.1-10.0 min). The flow rate was set to 1.0 mL/min at 40°C. The HPLC liquid stream was connected to a 8060 tandem mass spectrometer (Shimadzu Kyoto, Japan). At least 5 MRM transitions were recorded for each analytical compound. The most abundant three mass transitions were used for quantitation. The detailed MS settings are listed in **Table 1**.

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. 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 standard mix was connected to internal standard port 2. Internal standard port 4 and the wash port were also filled with methanol. The system was prepared by priming methanol through the internal standard port 4 (10 cycles) followed by 2 cycles through port 2. 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,

Table 1. m/z transitions of all compounds.

Name	Pre.	Quant.	Qual.	Qual 2.	Qual 3.	Q1	Q2	Q3
	m/z	*m/z*	*m/z*	*m/z*	*m/z*	V	V	V
Alprazolam	309.1	281.05	205.1	274.1	151.1	-21	-25	-29
Alprazolam-D5	314.1	269.35	286.1	105.05	-	-30	-15	-28
Amphetamine	136.1	91.1	119.15	65.1	39.1	-16	-22	-16
Amphetamine-D3	139.0	92.1	122.15	93.1	-	-15	-18	-16
Cocaine	304.15	182.15	82.05	77.05	105.05	-20	-19	-21
Cocaine-D3	307.15	185.3	85.1	105.1	-	-22	-18	-30
Codeine	300.15	152.1	215.15	165.15	128.1	-15	-63	-28
Codeine-D3	303.05	215.1	226.05	199.1	-	-15	-27	-22
Diazepam	285.1	193.05	154.0	222.1	257.0	-19	-31	-20
Diazepam-D3	288.0	157.05	225.1	260.1	-	-30	-28	-30
Fentanyl	337.25	188.15	105.1	132.1	77.05	-20	-23	-22
Fentanyl-D5	342.25	105.25	188.15	137.2	-	-25	-31	-19
LSD	324.2	223.15	207.15	208.15	281.15	-22	-24	-23
LSD-D3	327.2	226.3	208.1	211.1	-	-24	-30	-23
MDMA	194.1	163.1	105.1	135.05	77.05	-23	-13	-28
MDMA-D3	197.0	163.25	105.1	135.1	-	-22	-14	-29
Methadone	310.2	265.15	105.05	77.05	223.15	-21	-15	-23
Methadone-D3	313.2	268.35	105.0	60.1	-	-23	-13	-29
Methamphetamine	150.15	91.1	119.15	65.1	39.1	-26	-22	-21
Methamphetamine-D5	155.15	92.2	121.1	-	-	-30	-20	-28
Morphine	286.15	152.1	201.1	165.1	128.05	-14	-59	-15
Morphine-D3	289.15	152.1	201.15	-	-	-14	-59	-26
Oxycodone	316.15	241.15	256.15	212.1	187.1	-16	-29	-25
Oxycodone-D3	319.15	301.1	259.1	-	-	-23	-19	-21

The general settings of the mass spectrometer were: nebulizing gas 2 L/min (N_2), heating gas 9.7 L/min (N_2), drying gas 10 L/min, positive and negative mode, and source temperature 300°C. Labsolutions software (Shimadzu Kyoto, Japan) was used to operate the LC-MS/MS system. Pre. = precursor ion; Quant. = daughter ion for quantification; Qual. = qualifier ion; Qual. 2,3 = qualifier ion 2 and 3; Q1 = first quadrupole massfilter ; Q2 = quadrupole collision cell ; Q3 = second quadrupole massfilter

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 20 µL and a 200 µL/min flow rate. To complete the automated DBS extraction cycle, the system was rinsed for 20 seconds [14].

Sample preparation

Working standards for LC-MS/MS tuning

The preset MRM transition coming from the forensic and toxicology LC-MS/MS method package of Shimadzu were confirmed using standard solutions (1 µg/mL) and were used afterwards for the LC- MS/MS method development. The deuterated drugs were dissolved in methanol to prepare 100 µg/mL standard solutions. Then, a mix was prepared by two dilution steps to generate a final concentration of 100 ng/mL for all deuterated standards. This solution was used as internal standard mix on the DBS-MS 500, each sample was sprayed with 20 µL in "fast" mode. The internal standard module of the DBS-MS 500 was purged with methanol prior mounting and priming the internal standard.

Dried blood spot samples

According to documented cut-off levels in whole blood

Table 2. Analyte concentration and levels [20].

Name	Cutt-off level	Toxic level	L1	L2	L3	L4
	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL
Alprazolam	100	> 350	10	100	500	1000
Amphetamine	100	> 1000	10	100	500	1000
Cocaine	150	> 1000	15	150	750	1500
Codeine	300	> 1100	30	300	1500	3000
Diazepam	100	> 3000	10	100	500	1000
Fentanyl	5	> 34	0.5	5	25	50
LSD	0.5	> 2	0.05	0.5	2.5	5
MDMA	5	> 1000	0.5	5	25	50
Methadone	200	> 1000	20	200	1000	2000
Methamphetamine	100	> 1000	10	100	500	1000
Morphine	300	> 200	30	300	1500	3000
Oxycodone	100	> 200	10	100	500	1000

[20], a calibration was set-up with four levels: level 1 was 10-fold below the cut-off, level 2 was the cut-off concentration, level 3 was 5-fold the cut- off concentration and level 4 10-fold the cut-off concentration (**Table 2**). Except for diazepam and MDMA, level 4 is above the toxic concentration and therefore covers the whole range. Freshly collected human blood was obtained from the local blood donation centre (Basel, Switzerland). EDTA was used as an anticoagulation agent (vacutainer tubes, BD, Allschwil, Switzerland). Endogenous 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 four different concentrations to prepare levels 1-4. 15 µL aliquots were spotted onto CAMAG DBS cards (CAMAG, Muttenz, Switzerland) and dried at room temperature for at least 2 h. The cards were subsequently stored at 4°C in sealed plastic bags containing desiccants. Stock solutions were stable for two weeks.

Results

Correlation and precision

The calibration levels were measured 7-fold to determine the method robustness and validity. The relative standard deviations of the internal standards, which were applied by spraying, are below 10% for all target compounds by comparing the data through all four levels **(Table 3)**.

The correlation and intra-day variations of all target compounds are listed in **Table 4**. The target to internal standard ratio was used to compare the results and to develop the calibration line. The calibration line is shown for codeine and oxycodone in **Figure 1**, since those compounds were measured in the patient samples using the developed method (section: Patient samples).

The intra-day variations as well as the coefficients of determination (R^2) of the four calibration standards are summarized in **Table 4**. Fentanyl, LSD, morphine and MDMA can be quantified at their cut-off level, however level 1 represents the limit of detection with a signal to noise ratio of 10.3 for fentanyl, 8.0 for LSD (shown as an example in **Figure 2**), 9.0 for morphine and 3.3 for MDMA, which are therefore not represented in **Table 4.** Excellent correlation was obtained for all target compounds in the panel (**Table 4**). All points of the calibration functions were sufficiently precise with relative standard deviations below 15%.

Table 3. Internal standard imprecision.

Name	Relative standard deviation
	%
Alprazolam-D5	6.0
Amphetamine-D3	6.7
Cocaine-D3	5.9
Codeine-D3	6.1
Diazepam-D3	9.6
Fentanyl-D5	6.0
LSD-D3	6.1
MDMA-D3	7.0
Methadone-D3	3.8
Methamphetamine-D5	9.4
Morphine-D3	5.4
Oxycodone-D3	5.9

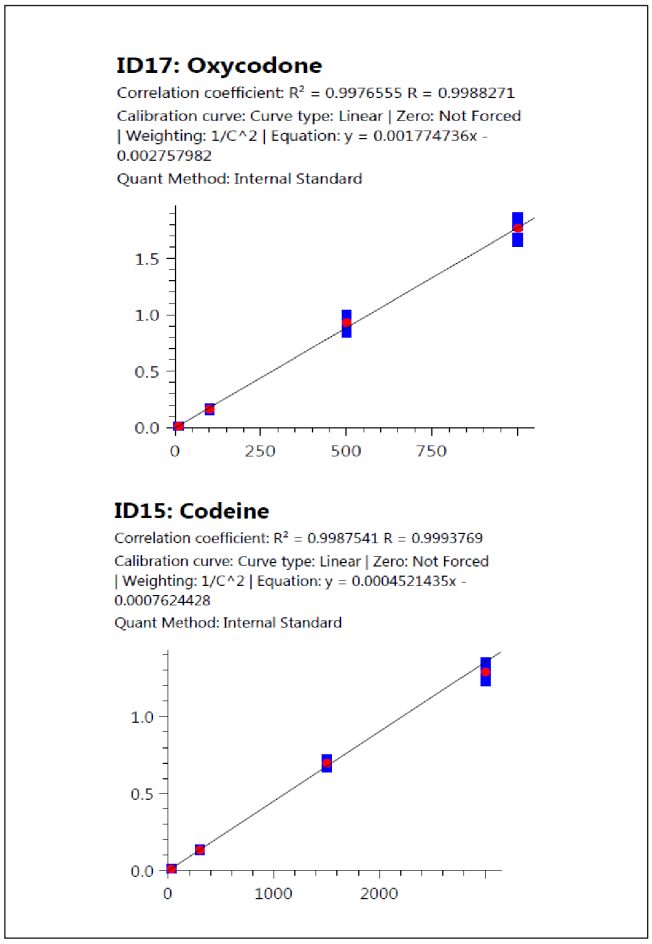

Figure 1. Calibration of top: oxycodone (n=7 each level) and bottom: codeine (n=7 each level).

Patient samples

To check the feasibility of the method and the applicability in a real setting, two anonymized DBS samples were acquired from healthy donors using medication for back pain. The samples were drawn by the patients themselves according to an introduction sheet. They were provided with a DBS card, a 1.8 mm lancet, an alcohol prep pad and packaging material with desiccant. The returned samples were analyzed using the newly developed DBS-LC-MS/MS method. 89.6 ng/mL codeine were detected in sample 1 and 39.6 ng/mL oxycodone in sample 2. The MRM data quality was sufficient enough to be used for screening against a spectral library search option to confirm the identity of the quantified compounds. The software displays the chromatographic peak, the calculated concentration according to the calibration function and the results from the library search (**Figure 3**).

Automation

The flow scheme of the fully automated card extraction system and the coupled LC-MS/MS is shown in **Figure 4**. The DBS cards are moved to the extraction unit, where a plunger seals a 4 mm circular hole in the card. The extraction solvent is pumped through the card and loaded into a loop (**Figure 4**: red arrows). By switching the 10-port valve, the loop volume is connected to the LC-MS/MS flow path (**Figure 4**: green arrows), guided to the column and to the MS/MS. Meanwhile, the extraction head is cleaned by a rinsing cycle to avoid carry over [21].

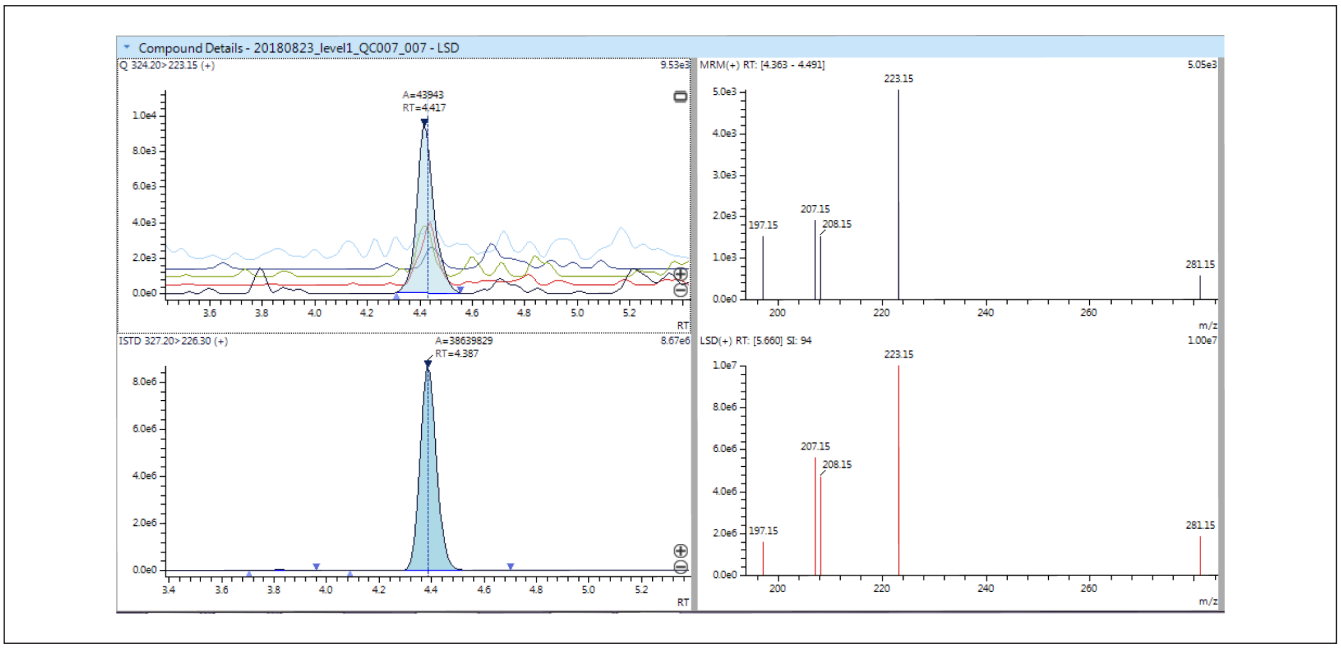

Figure 2. LSD peak at level 1 (0.05 ng/mL) (top) and its deuterated standard to represent the LOD (bottom).

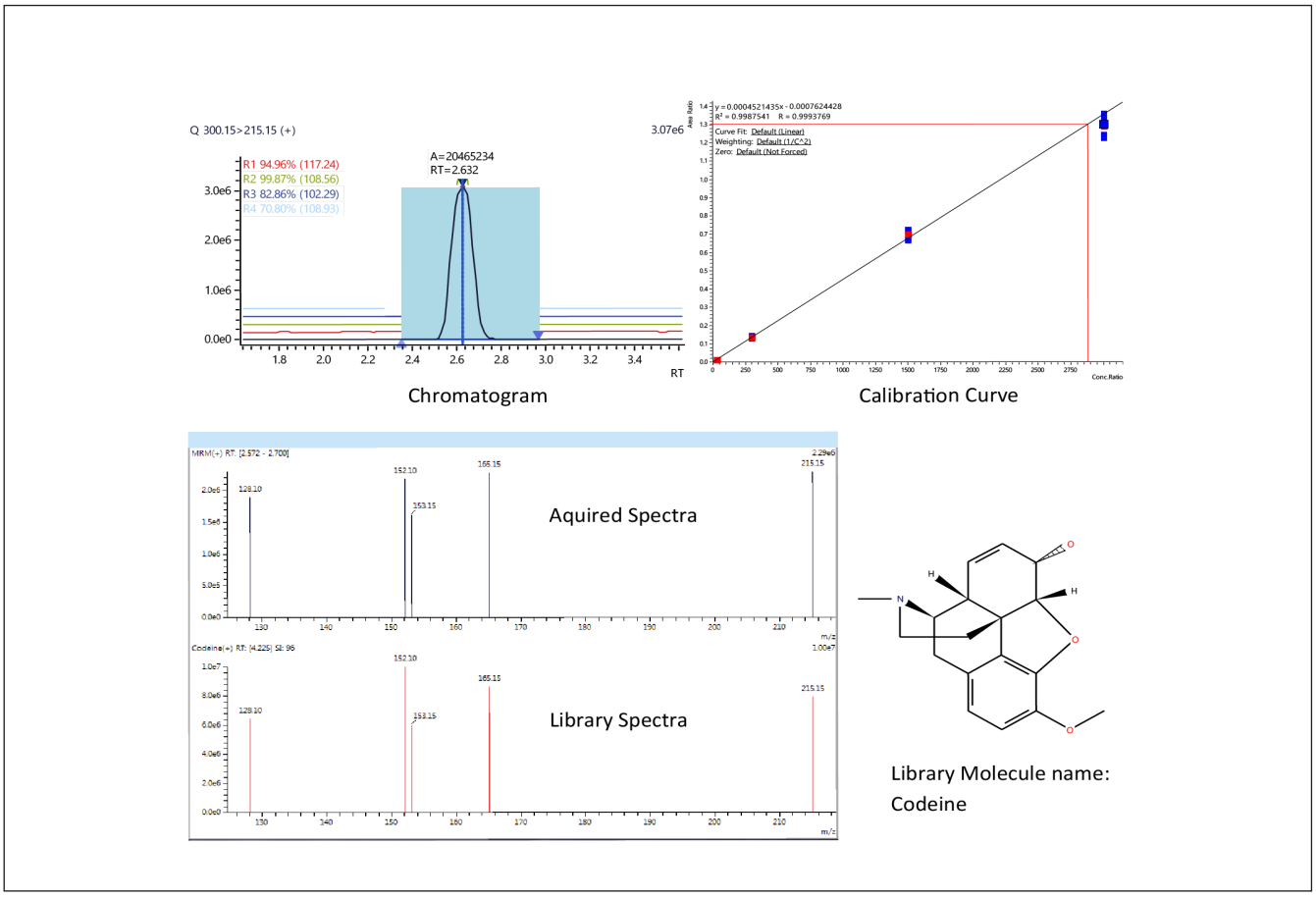

Figure 3. Sample report from the Insight® software with chromatographic peak, overlay of acquired and library spectra, calibration function and molecular structure.

The DBS card contains a barcode which is linked to a data administration sheet. The patient information is sent separately to an administration office and the DBS card containing the blood sample is sent to the centralized laboratory. The system reports the barcode, a picture of the card before and after extraction, blood spot details such as roundness, area, diameter and the location on the card, all fluidic pressures and the applied method. This report is then matched with the patient information. The advantage of this workflow is a completely anonymous and standardized sample handling process, which is suitable for anti-doping or police laboratories.

Carry-over

Carry over was monitored by measuring blank blood after injecting a mix of all standards at level 4 (**Table 2**). The criteria of bio-analytical method validation guidelines were fulfilled [22,23]. No carry over was observed for the chosen analyte panel. **Figure 5** shows the codeine peak for level 4 and the subsequent blank sample. This was optimized by programming a wash sequence by the DBS-MS 500. Here, the outlet capillary (between extraction and 10-port valve, **Figure 4**) 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.

The investigation of the feasibility and routine applicability of the method using real patient samples was successful. By spotting micro volumes, such as DBS, a very good sensitivity is obtained. The compounds fentanyl and LSD are among the most active drugs in the chosen panel and appear in very low blood concentrations. One blood droplet (15 µL) of the fentanyl sample at level 1 contains only 7.5 pg of standard. The droplet spreads with an average hematocrit to an area of approximately 40 mm^2. An extraction circle of 4 mm diameter equals 30 percent of the droplet, which means that with a theoretical extraction efficiency of 100%, only 2.5 pg of standard reaches the LC-MS/MS system. Nonetheless, this low amount is detectable and at slightly higher concentrations quantification is possible. The 8060 MS/MS system

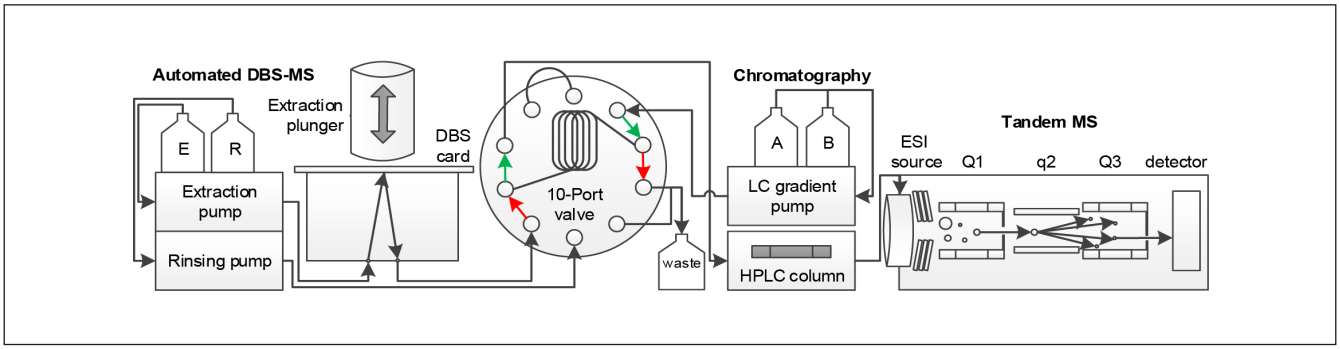

Figure 4. Flow scheme of the automated DBS-LC-MS/MS approach.

is a high-end mass spectrometer and those instruments are gaining in sensitivity with each new generation. This DBS-LC-MS/MS workflow allows screening of hundreds of drugs in parallel.

When analyzing smallest drug amounts in a complex matrix such as blood, it is useful to include a short separation on an analytical column prior the MS detection. The ion source of the MS can be protected from suppressing ions when the first portion from the column is guided directly to waste. Ions for example are not retained on the C18 column and can be eliminated by washing before opening the path into the ion source. In this method, a C18 column was used to isolate the target compounds from the blood extract. Also, 20 µL injection volume is a relatively high amount for the LC system. The extraction solvent should be as similar as possible to the mobile phase (at the time point of extraction) for a good LC separation. Here, it was found that the extraction volume does not corrupt the analytical separation, although the organic contend at the start conditions differs from the extraction solvent.

Both patient samples contained a drug concentration below the cut-off level of abuse (89.6 ng/mL codeine, cut-off 300 ng/mL and 39.6 ng/mL oxycodone, cut-off 100 ng/mL), which shows in both cases a good therapeutic dosage. Additionally a morphine signal was detectable in the patient taking codeine, since typically approximately 10 % of the codeine is metabolized via CYP2D6 to morphine [24].

The internal standards are used to monitor the extraction efficiency and to ensure that the system is working properly [14,25]. 20 µL of the internal standard mix was sprayed on the DBS spot prior extraction. This process of integrating the internal standard by spraying is the method of choice for quantification. Abu-Rabie et al. stated that the hematocrit level can have an influence on the extraction efficiency, so that the internal standard should better be applied prior to the extraction [25]. All deuterated standards were detected easily and could be used in much lower concentrations to decrease costs. Also, for a routine setup, internal standards can be used for substance classes to further reduce the analysis cost.

The extraction setup of the DBS-MS 500 features a horizontal extraction, where the solvent passes from the bottom through the sealed area on the DBS back to the bottom of the card. The extraction is performed under increased pressure to fully dissolve the target molecules producing 20 µL of highly concentrated extract (volume can be adjusted), which is online coupled to the analytical system. The full automation of the analysis workflow is important to exclude error sources. Each process is monitored and documented automatically and large batches of samples can be measured over night without any human interaction. Each dried blood spot card is prepared and handled the same way in a standardized process. Analytes are less stable in solution and this method minimizes this time period where those remain in solution.

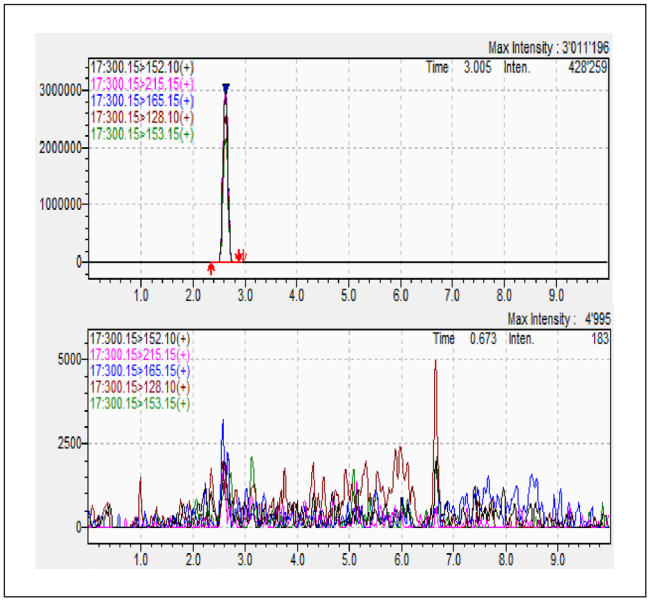

Figure 5. Codeine peak at level 4 (3000 ng/mL) (top) and the following blank blood injection (bottom).

With the presented method, the analysis of one sample takes 10 minutes. The analysis time could be decreased by using denser packed columns and a higher LC flow rate. The method needs to be validated as a next step prior to application for routine testing. Also the transportation and storage conditions have to be further examined to develop analysis guidelines.

Conclusion

A new workflow for drug screening in large populations was introduced for a panel of 12 drugs of abuse from a wide variety of structural categories. The analysis process is fully automated by using an online DBS-LC-MS/MS analysis system. The feasibility for such an approach was shown and the method has been successfully applied to real cases showing the potential. The panel was chosen with a variety of different illicit and legal drugs from different substance classes to cover a broad field of substances. Highly active drugs, such as LSD and fentanyl, which appear in very small blood concentrations, were included in the panel and quantified at their cut-off concentration. The process from sampling to generating the report was linked with a barcode system to enable forensic applications. Each process step is well documented and all analysis steps follow Good Laboratory Practice (GLP) [22]. The method can be easily extended and validated according to individual laboratory guidelines.

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