Research article

Drug Testing
and Analysis

Received: 17 September 2015 Revised: 28 October 2015 Accepted: 29 October 2015 Published online in Wiley Online Library: 26 November 2015

(www.drugtestinganalysis.com) DOI 10.1002/dta.1927

Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS

Ruth Verplaetse and Jack Henion*

Opioids are well known, widely used painkillers. Increased stability of opioids in the dried blood spot (DBS) matrix compared to blood/plasma has been described. Other benefits provided by DBS techniques include point-of-care collection, less invasive micro sampling, more economical shipment, and convenient storage. Current methodology for analysis of micro whole blood samples for opioids is limited to the classical DBS workflow, including tedious manual punching of the DBS cards followed by extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS) bioanalysis. The goal of this study was to develop and validate a fully automated on-line sample preparation procedure for the analysis of DBS micro samples relevant to the detection of opioids in finger prick blood. To this end, automated flow-through elution of DBS cards was followed by on-line solid-phase extraction (SPE) and analysis by LC-MS/MS. Selective, sensitive, accurate, and reproducible quantitation of five representative opioids in human blood at sub-therapeutic, therapeutic, and toxic levels was achieved. The range of reliable response ($R^2 \geq 0.997$) was 1 to 500 ng/mL whole blood for morphine, codeine, oxycodone, hydrocodone; and 0.1 to 50 ng/mL for fentanyl. Inter-day, intra-day, and matrix inter-lot accuracy and precision was less than 15% (even at lower limits of quantitation (LLOQ) level). The method was successfully used to measure hydrocodone and its major metabolite norhydrocodone in incurred human samples. Our data support the enormous potential of DBS sampling and automated analysis for monitoring opioids as well as other pharmaceuticals in both anti-doping and pain management regimens. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: automation; dried blood spot; opioids; anti-doping; LC-MS/MS

Introduction

Opioids represent a highly effective class of drugs widely prescribed and used for pain management.[1] They include natural (e.g. morphine, codeine), semi-synthetic (e.g. hydrocodone, oxycodone), and synthetic (e.g. fentanyl) compounds. In addition to correct therapeutic use, these compounds are also abused by some pain patients and recreational drug users, as well as during athletic competition.[1,2] In 2013, the global number of opioid users was estimated to be 0.7% of the total adult population (i.e. 32.4 million users), with the highest prevalence in North America (3.8% of the adult population).[3]

Dried blood spot (DBS) refers to a micro blood sampling technique without the need for a phlebotomist where small volumes of blood (5 to 20 µL) are collected and spotted on an appropriate cellulose substrate paper, dried and sent to the laboratory via conventional mail services for bioanalysis. Due to less-invasive sample collection (e.g. from a finger prick), small sample volumes, increased analyte stability as a result of enzyme deactivation, and easy sample shipping/storage, DBS sampling is an appealing approach for bioanalysis. The use of DBS techniques has been extensively reviewed recently.[4–6] DBS sampling may allow patient-friendly point-of-care sampling and easy in-competition testing as well as out-of-competition sample collection/testing, providing advantages for anti-doping control, therapeutic drug monitoring, and clinical analyses/chemistry.[7–12] Modern analytical instruments are capable of the sensitive measurements required for analyzing micro samples (<50 µL). Labour-intensive off-line sample preparation includes punching a disk from the DBS card followed by transferring the punched spot to a tube or multi-well device, extracting the disk and sometimes additional sample treatment such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE). These steps are often considered the bottleneck of DBS analyses which can be circumvented by the approach described herein.

Several reports have described effective, but tedious off-line sample preparation of DBS-containing opioids.[13–23] Analysis of DBS samples from one up to six of the following compounds has been reported: buprenorphine, codeine, despropionylfentanyl, fentanyl, hydromorphone, methadone, 6-monoacetylmorphine (6-MAM), morphine and its glucuronides, methadone, norfentanyl, noroxycodone, oxycodone, and sufentanyl. These studies demonstrated the applicability of DBS for bioanalysis of opioids as well as a stabilizing effect of DBS for 6-MAM and morphine-glucuronides.[13–15] There is one more automated DBS extraction procedure for several model compounds including opioids.[17,18] However, punching disks out of the DBS cards is still needed and concentrations of morphine in the lower therapeutic range could not be detected, which limits the method's applicability.

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* *Correspondence to: Jack Henion, Q^2 Solutions, 19 Brown Rd, Ithaca, NY 14850, USA.*
E-mail: henionj@advion.com

Q^2 Solutions, 19 Brown Rd, Ithaca, NY, 14850, USA

Approaches enabling automated handling of DBS samples without any treatment or manual disk punching include flow-through elution of DBS and direct extraction from the surface of the card.[4–6] A limited number of feasibility studies using these approaches have been published.[24–32] To the best of our knowledge, fully automated on-line DBS analysis has not been evaluated for opioids.

This report presents the rigorous development and validation of fully automated flow-through elution of DBS micro samples with on-line SPE and liquid chromatography-tandem mass spectrometry (LC-MS/MS) bioanalysis used for the detection of opioids. For this study, five representative compounds covering the analytical challenges posed by the opioids were chosen. These included morphine, codeine, oxycodone, hydrocodone, and fentanyl. This selection includes isomers (codeine and hydrocodone) as well as compounds with varying polarities (logP values ranging from -0.1 for morphine, a hydrophilic drug up to 2.3 for fentanyl, a highly lipophilic drug). These five compounds are widely prescribed to pain patients and are on the Prohibited List as established by the World Anti-Doping Agency (WADA).[1,2] As the parent drugs of these opioids predominate in blood, metabolites were not included in the method development and validation (morphine and hydrocodone are metabolites of codeine, but they are parent compounds themselves and therefore included in our selection).[33]

Experimental

Chemicals, reagents, and materials

Morphine, morphine-d$_3$, codeine, codeine-d$_3$, oxycodone, oxycodone-d$_6$, hydrocodone, hydrocodone-d$_3$, fentanyl, fentanyl-d$_5$, norhydrocodone, and norhydrocodone-d$_3$ were purchased from Cerilliant (Round Rock, TX, USA). LC-MS-grade acetonitrile, LC-grade isopropanol, and LC-MS-grade methanol were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). MilliQ water from a Millipore system was used. Ammonium formate, ammonium hydroxide and formic acid were obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). Whole blood from adult healthy volunteers containing Na$_2$EDTA was used within 7 days of collection. Blood containing Na$_2$EDTA with corrected haematocrit values (30%, 45%, 60%) was purchased from BioreclamationIVT (Westbury, NY, USA). Ahlstrom 226 DBS cards were supplied by Perkin Elmer (Boston, MA, USA). Desiccant pellets and glassine envelopes used for storage of DBS cards were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Stock and working solutions were stored in 4 mL and 20 mL borosilicate glass vials from Kimble Chase (Vineland, NJ, USA). Blood samples were prepared in 1.5 mL Protein LoBind tubes from Eppendorf (Hamburg, Germany). Volumetric pipettes were Pipet-Lite XLS series from Rainin Instrument LLC (Oakland, CA, USA) and a Repeater plus multi-pipette from Eppendorf.

On-line DBS-SPE

Hardware instrumentation from Spark Holland (Emmen, the Netherlands) was used in this work.[24,25] It consists of a DBS card autosampler (DBSA) to perform flow-through elution of the DBS card, an automated SPE cartridge exchange module (ACE) for on-line SPE, and a high pressure dispenser pump (HPD) to deliver solvents. The DBS-SPE system was controlled via Spark Link software.

The DBS cards were manually placed into the card rack of the DBSA and then sequentially inserted into the autosampler by a robotic arm. An initial digital picture was taken in sequence of each DBS card and then the card was clamped such that the center of the detected spot was eluted within a rim diameter of 2.0 mm. The compounds were eluted or desorbed from the DBS card on-line onto the SPE cartridge by flow-through elution with 1 mL 0.1% NH$_4$OH at a flow rate of 2 mL/min (delivered by the HPD). Twenty µL of the internal standard (IS) solution (0.1 ng/mL fentanyl-d$_5$ and 1 ng/mL morphine-d$_3$, codeine-d$_3$, oxycodone-d$_6$, hydrocodone-d$_3$ in 20:80 methanol:water) was loaded onto the clamped card via the flow-through elution solvent. After desorption, a second photograph of the DBS card was automatically taken and saved within the data file for each analytical run.

The disposable SPE cartridge used was a HySphere C18HD, 7 µm, 2 x 10 mm cartridge (Spark Holland, Emmen, the Netherlands). It was conditioned using 1 mL methanol at 5 mL/min and equilibrated with 1 mL 0.1% NH$_4$OH at 5 mL/min before the compounds were eluted from the card to the cartridge. After loading of the SPE cartridge with the desorbed target opioids, washing of the SPE cartridge to remove interferences occurred using 1 mL 0.1% NH$_4$OH at 5 mL/min. The targeted analytes were then eluted from the SPE cartridge onto the LC column using the LC mobile phase under gradient conditions.

After each SPE elution, both the SPE cartridge and the DBS clamp (with a DBS card clamped in a blank region of the DBS paper) were washed sequentially with four solvents to minimize carry over: 1 mL 0.1% NH$_4$OH, 1 mL methanol, 1 mL 20:40:30:10 water:methanol:acetonitrile:isopropanol with 0.1% formic acid and finally 1 mL 0.1% formic acid at 5 mL/min.

LC-MS/MS

LC-MS/MS analysis was performed with a Nexera UHPLC system coupled to a LCMS-8050 mass spectrometer and LabSolutions software from Shimadzu (Marlborough, MA, USA).

The LC column employed was a Raptor Biphenyl, 2.7 µm, 2.1 x 50 mm protected by a guard column (2.7 µm, 2.1 x 5 mm) from Restek (Bellefonte, PA, USA). The mobile phase consisted of (A) 5 mM ammonium formate and 0.1% formic acid and (B) methanol. The gradient program used for SPE elution and LC separation was: start: 5% B, 2.5 min: 62.5% B, 3 min: 100% B kept for 0.4 min before returning to initial conditions. Flow rate was 0.4 mL/min at 45°C.

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode using the following conditions: interface voltage: 2 kV, interface temperature: 300°C, desolvation line temperature: 250°C, heat block temperature: 400°C, heating gas flow: 5 L/min N$_2$, drying gas flow: 5 L/min N$_2$, nebulizing gas flow: 5 L/min N$_2$, CID gas: 270 kPa argon. For each compound two selected reaction monitoring (SRM) transitions were monitored: morphine (*m/z* 286.1 to *m/z* 165.1 and *m/z* 201.1), morphine-d$_3$ (*m/z* 289.1 to *m/z* 165.1 and *m/z* 201.1), codeine (*m/z* 300.1 to *m/z* 165.1 and *m/z* 215.1), codeine-d$_3$ (*m/z* 303.1 to *m/z* 165.1 and *m/z* 215.1), oxycodone (*m/z* 316.1 to *m/z* 241.0 and *m/z* 256.1), oxycodone-d$_6$ (*m/z* 322.1 to *m/z* 247.1 and *m/z* 262.1), hydrocodone (*m/z* 300.1 to *m/z* 199.1 and *m/z* 171.0), hydrocodone-d$_3$ (*m/z* 303.1 to *m/z* 199.1 and *m/z* 171.0), fentanyl (*m/z* 337.1 to *m/z* 188.1 and *m/z* 105.1), fentanyl-d$_5$ (*m/z* 342.1 to *m/z* 188.1 and *m/z* 105.1), norhydrocodone (*m/z* 286.1 to *m/z* 199.1 and *m/z* 171.1) and norhydrocodone-d$_3$ (*m/z* 289.1 to *m/z* 202.1 and *m/z* 174.1). The listed precursor and product ions are in good agreement with MS/MS spectra observed by others.[34]

Preparation of calibrators and QC samples

Primary stock solutions of the target opioid compounds were purchased as methanolic solutions at a concentration of 1 mg/mL

(0.1 mg/mL for the deuterated analogues). These stock solutions were diluted with 50:50 methanol:water to obtain working standard solutions at the following concentrations: for morphine, codeine, oxycodone and hydrocodone: 0.05, 0.25, 1.25, 5, 12.5, 25 μg/mL; for fentanyl the corresponding concentrations were: 0.005, 0.025, 0.125, 0.5, 1.25, 2.5 μg/mL. The working standard solutions were used for the preparation of calibrators. Quality control (QC) working solutions were prepared in 50:50 methanol:water at the following concentrations: for morphine, codeine, oxycodone and hydrocodone: 0.05, 0.15, 7.5, 22.5 μg/mL; concentrations were 10 times lower for fentanyl. These working solutions were used for the preparation of QC samples. All solutions were stored at -20°C.

Calibrators and QC samples were prepared by diluting the appropriate working solution with human blood (volume of working solutions was 2% of the total volume). The final concentrations of the calibrators were: 1 (lower limit of quantification (LLOQ)), 5, 25, 100, 250 and 500 (upper limit of quantification (ULOQ)) ng/mL blood for morphine, codeine, oxycodone and hydrocodone; 0.1 (LLOQ), 0.5, 2.5, 10, 25 and 50 (ULOQ) ng/mL blood for fentanyl. QC samples contained 1 (LLOQ), 3 (LOW), 150 (MED) and 450 (HIGH) ng/mL morphine, codeine, oxycodone and hydrocodone in whole blood; 0.1 (LLOQ), 0.3 (LOW), 15 (MED) and 45 (HIGH) ng/mL fentanyl. Fortified blood samples were shaken for 15 min at 37°C and allowed to cool to room temperature prior to spotting. DBS cards were spotted with 10 μL whole blood and dried at room temperature for at least 3 h. After drying, they were stored at room temperature in glassine envelopes in sealed plastic bags with desiccant pellets until analysis.

Method validation

A method validation covering all aspects (selectivity, linearity, accuracy, precision, matrix interferences, recovery, carry over, and stability) required to establish the feasibility of a validated fully automated DBS-SPE-LC-MS/MS approach for analysis of opioids was performed according to Food and Drug Administration (FDA) regulatory bioanalysis guidelines. Control blanks (i.e. DBS cards spotted with blank blood analyzed without IS, n=2) and zero samples (i.e. DBS cards spotted with blank blood analyzed with IS, n=2) were evaluated during each run. Matrix inter-lot selectivity was assessed by analyzing six different lots of whole blood matrix (without IS, n=1 and with IS, n=1). Calibrators at six concentration levels (n=2 at each level) were analyzed on each of three days. The intra- and inter-day accuracy and precision of the method was evaluated using QC samples at four concentration levels (n=6) on each of three days. Matrix inter-lot accuracy and precision were assessed by analyzing six different lots of matrix at both LLOQ and ULOQ level (n=1). For determination of recovery, flow-through elution of a spot was repeated up to 5 times (LLOQ level, 6 different lots) or 10 times (ULOQ level, 6 different lots). The effect of haematocrit (HCT) was evaluated using QC samples at three concentration levels (n=6) prepared in blood at 30% (low HCT), 45% and 60% (high HCT). At each HCT value, flow-through elution of a spot was repeated up to 5 times (LLOQ level) or 10 times (QC HIGH) to determine the impact of HCT on recovery. Carry over was investigated by analyzing blank blood DBS cards after the analysis of the highest calibrator and QC sample. The stability was investigated by storing QC samples at four concentration levels (n=4 for each concentration level on one DBS card) at room temperature for 2 and 4 h before spotting. To establish the stability on the DBS card, QC samples at four concentration levels (n=4) were spotted onto DBS cards and stored for 3, 7, 15, 30, 55, and 95 days at three different storage temperatures (room temperature, 4°C and -20°C) before DBS-SPE-LC-MS/MS analysis. The analyte concentrations were determined using calibration curves generated with calibrators prepared and analyzed the day of analysis of the stability QCs.

Incurred samples

Hydrocodone (10 mg immediate release formulation) was administered orally to a healthy male volunteer. Non-volumetric DBS samples were collected from a finger prick at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, and 19 h after intake. The DBS cards were dried at room temperature for at least 3 h and stored in glassine envelopes in sealed plastic bags with desiccant pellets until analysis. In addition to the targeted opioids, norhydrocodone was also monitored in a semi-quantitative way (the quantitative determination of this compound was not included in the method validation).

Results and discussion

Method development

The automated DBS-SPE-LC-MS/MS workflow that was optimized and validated for analysis of whole blood micro samples for opioids included seven major steps: (1) conditioning and equilibration of the SPE cartridge (Figure 1a), (2) flow-through desorption of the target compounds of the DBS cards onto the SPE cartridge including loading of the IS via the DBS card onto the SPE cartridge (Figure 1b), (3) washing of interferences from the SPE cartridge (Figure 1a), (4) elution of the target compounds from the SPE cartridge onto the LC column (using LC gradient) (Figure 1c), (5) elution of the compounds from the LC column to the mass spectrometer, (6) detection of the target compounds by MS/MS, and (7) washing of the DBS-SPE system (Figure 1b). These steps are carried out in an automated manner using three different valve settings (Figure 1). Due to the on-line nature of all parts of the automated system, all the processes should be considered and evaluated simultaneously during method development as the different steps will influence each other. Optimized parameters were the DBS elution solvent (composition, flow rate, volume, and temperature), SPE cartridge, SPE solvents (composition, flow rate, and volume), LC column, LC mobile phase (composition, flow rate, temperature, and gradient) and MS/MS parameters. A brief summary showing the most important experiments performed during method development is shown.

A variety of SPE cartridges is available to use on the ACE system.[24] Initially mixed-mode SPE (cation exchange) was preferred as it provides a degree of selectivity to reversed-phase LC. Moreover, elution of basic compounds from these SPE cartridges requires a high pH, in turn providing so-called wrong-way round electrospray ionization and more retention on the LC-MS/MS.[35–37] Flow-through elution of the DBS card and loading onto the mixed-mode SPE cartridge was performed with 0.1% formic acid. Unfortunately, elution of the compounds from the SPE cartridge onto the LC column was only achieved after pumping a high percentage of organic solvent, making chromatographic separation of the isomers impractical.

Next, reversed-phase cartridges were tested. C2 and C18 SPE cartridges performed equally well, except for morphine, where significantly more retention was observed on a C18 SPE cartridge. No organic solvent was used in the DBS desorption/SPE loading solvent as even 5% organic content already decreases SPE loading.

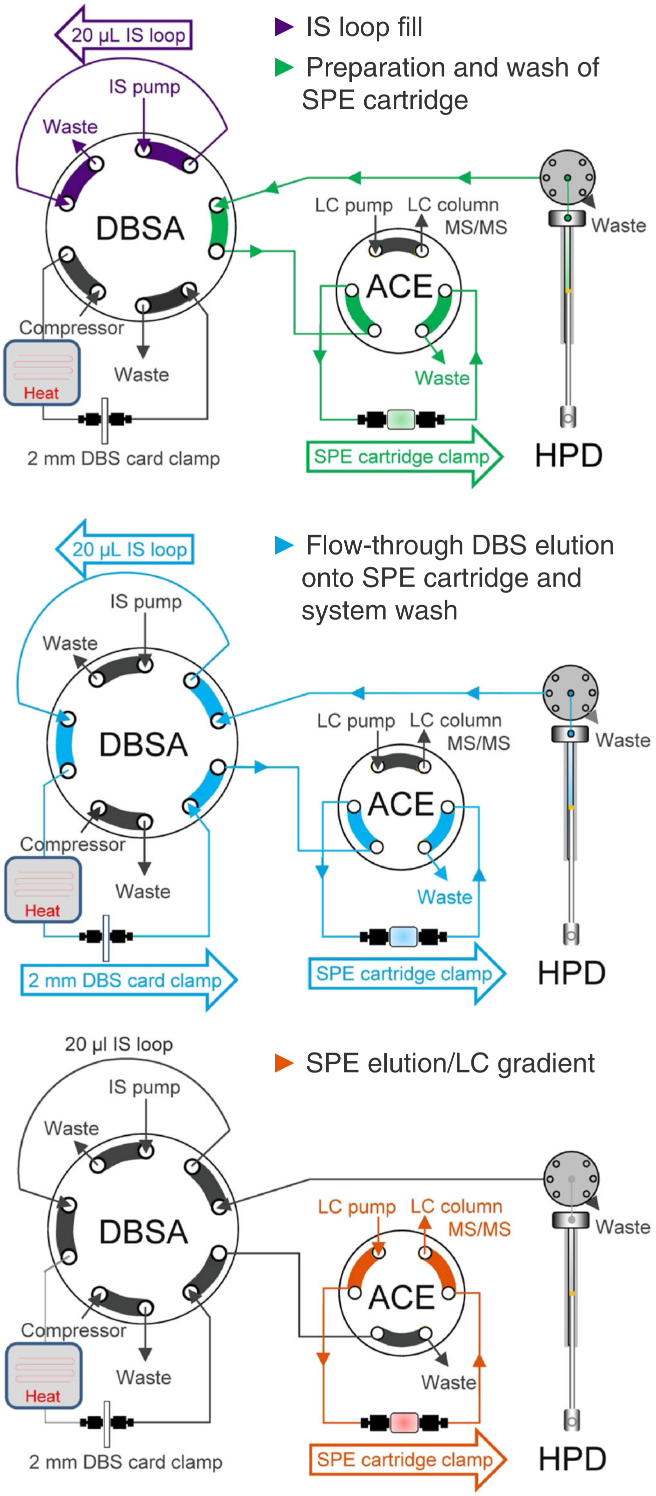
20 µL IS loop
IS pump
Waste
DBSA
Compressor
Heat
Waste
2 mm DBS card clamp
▶ IS loop fill
▶ Preparation and wash of SPE cartridge
LC pump
LC column
MS/MS
ACE
Waste
SPE cartridge clamp
Waste
HPD
20 µL IS loop
IS pump
Waste
DBSA
Compressor
Heat
Waste
2 mm DBS card clamp
▶ Flow-through DBS elution onto SPE cartridge and system wash
LC pump
LC column
MS/MS
ACE
Waste
SPE cartridge clamp
Waste
HPD
20 µl IS loop
IS pump
Waste
DBSA
Compressor
Heat
Waste
2 mm DBS card clamp
▶ SPE elution/LC gradient
LC pump
LC column
MS/MS
ACE
Waste
SPE cartridge clamp
Waste
HPD

Figure 1. Three sets of valve settings used in the automated on-line DBS-SPE-LC-MS/MS workflow: (a) IS loop fill, preparation and wash of SPE cartridge, (b) flow-through desorption of DBS card and system wash of both SPE cartridge and DBS clamp, (c) SPE elution with LC gradient.

For retention of basic compounds such as the opioids onto a reversed-phase SPE cartridge, a basic solvent is needed. 0.1% NH_4OH was preferred over 1% NH_4OH. Temperature had no significant effect on desorption from DBS and loading onto SPE cartridge (room temperature, 45°C and 80°C were compared for desorption with both 0.1% NH_4OH and 1% NH_4OH). Elution with 100% aqueous solvent left a blank spot on the card, indicating not only the compounds but also endogenous constituents of blood were eluted from the card onto the SPE cartridge (Figure 2). To reduce matrix effects during LC-MS/MS analysis, the SPE cartridge was washed with the desorption solvent in order to remove interferences before elution of the target compounds onto the LC column. Moreover, this step was absolutely required to prevent abnormal clogging of the LC column. Again, in this step no organic solvent was used as this resulted in loss of the analytes of interest from the SPE cartridge.

Chromatographic separation by core-shell particles was selected in order to cope with the current maximum pressure limit of the DBS-SPE system (300 bar) while still taking advantage of the gain in sensitivity caused by LC with smaller particles. Three reversed-phase LC columns were compared with respect to sensitivity, isomeric resolution and peak shape (Figure 3). A biphenyl column provided the best results and was selected for further use. The total inter DBS card cycle time of the optimized DBS-SPE-LC-MS/MS method was 4.5 min. This can be further reduced as the availability of two clamps for SPE cartridges allows overlap between different samples (e.g. SPE equilibration and conditioning on clamp 1 in parallel with loading, washing and elution on other clamp).[25] The possibility to couple UHPLC-MS/MS to the DBS-SPE system is another promising approach.

Method validation

Selectivity was evaluated by monitoring DBS blanks with and without IS (including different whole blood matrix lots). No interfering ion current signals were observed at the retention times of the analytes of interest or the IS.

Calibration curves were constructed by plotting analyte/IS peak area ratio versus nominal concentration in DBS micro samples. Calibration curves were linear over the analyzed range ($R^2 \geq 0.997$, back-calculated concentrations were within ±15.0% of nominal value) (Table 1). A weighted $1/x^2$ linear regression provided the most accurate and precise response over the concentration range of 1 to 500 ng/mL whole blood (0.1 to 50 ng/mL for fentanyl). This range encompasses the concentration levels that can be expected in real-life samples from opioid users and abusers (Table 1).[1,38,39]

DBS sample concentrations in the QC samples were determined from the calibration curves within each analytical run. Accuracy was reported as relative error (RE% = (calculated mean – nominal value)/nominal value x 100) and precision as coefficient of variation (CV% = standard deviation/mean). The results obtained for the intra-day, inter-day and matrix inter-lot accuracy and precision were within the acceptance criteria: RE% within ±15.0% (±20% at LLOQ level) and CV% ≤15% (≤20% at LLOQ level) (Table 2).

The described automated platform does not allow a conventional determination of recovery (i.e. comparison of a pre- and post-extraction spiked sample). Recovery determination in this report was performed by consecutively extracting the same DBS sample up to five times (at LLOQ level) or 10 times (at ULOQ level) without removing the DBS card from the clamp. The ratio of the analyte peak area of the first extraction and the sum of the analyte peak of all 5 or 10 extractions defined the recovery.[30] A comparable recovery (between 68.8 and 78.1%) was observed for all compounds and tested concentration levels with very good precision (CV% lower than 5%) (Table 1).

Unacceptable carry over (i.e. peak area ratio in a zero sample after analysis of a sample containing a high concentration was greater than 20% of the area ratio observed for the LLOQ samples) was seen

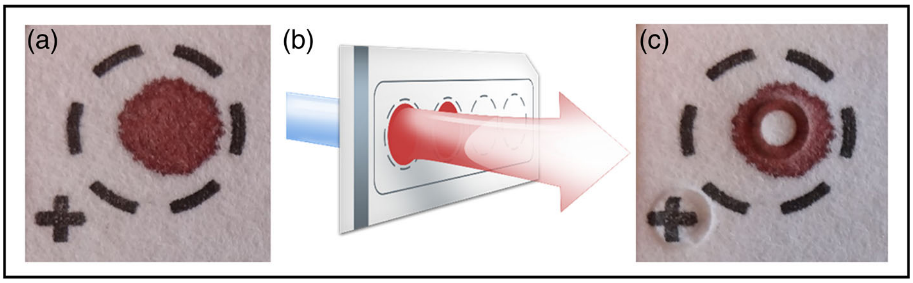

Figure 2. (a) A spot before elution. (b) A cartoon of flow-through elution of a DBS card. (c) A picture taken after a 2 mm area within the spot was clamped and eluted with 1 mL 0.1% NH_4OH at 2 mL/min. The clamped 2 mm area is now free of blood. A blank part of the card (left of the blood spot) was clamped for washing afterwards to minimize carry over.

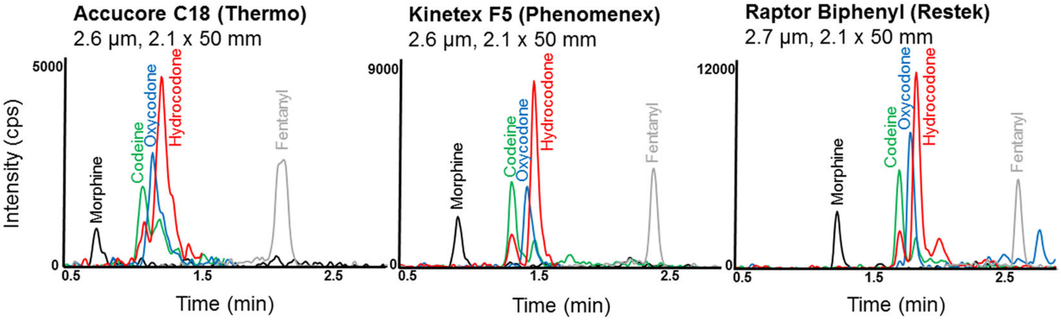

Figure 3. Blood spiked at the LLOQ level was spotted onto a DBS card, extracted and analyzed with three different LC columns (without guard columns). Unsmoothed quantifier SRM transitions for the five targeted opioids are shown. Best separation of the isomers codeine and hydrocodone was obtained on the F5 and biphenyl column. The biphenyl column provided the best sensitivity.

Table 1. Linearity and recovery

	Therapeutic range (ng/mL plasma)	Toxic range (ng/mL plasma)	Calibration range (ng/mL blood)	R^2	Recovery (± CV%)	
					LLOQ	ULOQ
Morphine	10 – 100	>100	1 (LLOQ) – 500 (ULOQ)	0.998	78.1 ± 4.7	77.3 ± 1.3
Codeine	10 – 250	>250	1 (LLOQ) – 500 (ULOQ)	0.998	68.8 ± 4.1	70.1 ± 2.2
Oxycodone	5 – 100	>200	1 (LLOQ) – 500 (ULOQ)	0.997	78.0 ± 3.6	72.9 ± 1.7
Hydrocodone	10 – 100	>100	1 (LLOQ) – 500 (ULOQ)	0.997	74.0 ± 3.6	71.7 ± 1.7
Fentanyl	1 – 3	>3	0.1 (LLOQ) – 50 (ULOQ)	0.998	73.5 ± 4.9	74.6 ± 1.8

Note: large inter-individual variations in opioid concentrations are described.[1] Only for three of the studied compounds blood-to-plasma concentration ratios were available: around 1.5 for oxycodone and around 1.0 for morphine and fentanyl.[20,52,53] As such, interpretation of detected concentrations in blood should be performed with care.[50,51]

in the absence of washing the DBS clamp and SPE cartridge. The carryover was dramatically reduced to acceptable levels by washing the entire DBS-SPE elution path sequentially with four different solvents. This did not result in a higher cycle time as these wash steps could be conducted simultaneously with the last stage of the LC gradient. As an additional benefit, repeated usage of a SPE cartridge up to more than 100 times without any loss in sensitivity or reduced peak shape quality was possible. The LC guard column was preventively replaced every 250–300 injections ensuring good LC column performance after more than 1000 injections. As such, the overall performance of the used system can be considered robust.

Carry over between DBS cards and/or envelopes used for storage was also tested. To this end, blank cards were stored for one month in an envelope that had contained a DBS card at the highest concentration level for another month before and in an envelope between two DBS cards at the highest concentration level. No carry over was seen when analyzing the blank cards after one month of storage at room temperature.

The compounds were stable in wet blood for at least 4 h at room temperature before spotting them onto DBS cards (RE% within ±15.0% (±20% at LLOQ level) and CV% $\leq$15% ($\leq$20% at LLOQ level)). Notwithstanding clear differences between liquid blood and drying blood (e.g. cell lysis) these data suggest that drying the DBS cards at room temperature does not influence the stability of the targeted opioids. While it is generally accepted that DBS as a matrix can have a stabilizing effect on analytes, existing literature contains limited data regarding the on-card stability of the studied opioids: morphine was stable up to 7 days at 4°C, -20°C, -80°C and up to 5 days at 40°C.[14,16] There was instability described for morphine and codeine after six months storage at 4°C, which was not seen when storing the DBS cards at -20°C.[19] For fentanyl, acceptable on-card stability was described up to 5 days at room temperature.[15] In this study, morphine and fentanyl were stable (i.e. RE% within ±15.0% (±20% at LLOQ level) and CV% $\leq$15% ($\leq$20% at LLOQ level)) for 30 days after spotting and storage at room temperature (tested at 3, 7, 15, 30, 55, and 95 days) (Supporting Information 1). When stored at 4°C, they

Table 2. Intra-day, inter-day and matrix inter-lot accuracy and precision

		Day1		Day 2		Day 3		Over 3 days		6 different lots	
		RE%	*CV%*	RE%	*CV%*	RE%	*CV%*	RE%	*CV%*	RE%	*CV%*
Morphine	LLOQ	13.1	*6.7*	6.4	*6.1*	-4.5	*9.6*	5.0	*10.0*	-9.3	*6.5*
	LOW QC	-0.4	*4.8*	0.6	*7.5*	-4.6	*8.7*	-1.5	*7.1*	-	-
	MED QC	-1.8	*2.0*	-3.0	*5.5*	-2.6	*5.1*	-2.5	*4.2*	-	-
	HIGH QC	2.4	*3.0*	-1.7	*4.8*	-3.4	*3.5*	-0.9	*4.4*	-	-
	ULOQ	-	-	-	-	-	-	-	-	-7.3	*6.9*
Codeine	LLOQ	14.2	*7.2*	7.0	*5.2*	-3.1	*6.9*	6.1	*9.2*	-1.1	*3.2*
	LOW QC	3.4	*5.3*	2.4	*3.8*	0.8	*5.2*	2.2	*4.6*	-	-
	MED QC	-1.3	*3.0*	-1.7	*5.4*	-2.1	*3.8*	-1.7	*3.9*	-	-
	HIGH QC	4.0	*2.7*	-1.7	*4.1*	-5.4	*6.0*	-1.0	*5.8*	-	-
	ULOQ	-	-	-	-	-	-	-	-	-5.1	*8.7*
Oxycodone	LLOQ	9.0	*4.9*	-0.7	*3.9*	-9.3	*7.2*	-0.3	*9.2*	3.0	*2.9*
	LOW QC	3.0	*5.9*	-0.2	*7.3*	-3.9	*8.0*	-0.3	*7.2*	-	-
	MED QC	-4.7	*4.4*	-6.0	*4.4*	-5.6	*2.6*	-5.4	*3.7*	-	-
	HIGH QC	3.3	*1.7*	-1.4	*6.4*	-5.3	*3.8*	-1.1	*5.5*	-	-
	ULOQ	-	-	-	-	-	-	-	-	-8.8	*6.4*
Hydrocodone	LLOQ	9.2	*7.6*	2.2	*4.8*	-3.3	*7.1*	2.7	*8.1*	0.1	*2.3*
	LOW QC	-0.2	*7.5*	-4.2	*4.9*	-5.1	*5.3*	-3.2	*6.1*	-	-
	MED QC	-1.3	*2.2*	-3.2	*3.6*	-1.5	*5.3*	-2.0	*3.8*	-	-
	HIGH QC	6.1	*2.5*	2.1	*5.2*	-2.3	*4.8*	2.0	*5.3*	-	-
	ULOQ	-	-	-	-	-	-	-	-	-5.7	*8.9*
Fentanyl	LLOQ	10.5	*3.5*	3.7	*4.0*	3.3	*4.8*	5.8	*5.0*	-4.4	*5.3*
	LOW QC	0.6	*7.3*	-1.3	*4.3*	-3.2	*4.4*	-1.3	*5.5*	-	-
	MED QC	-0.7	*3.3*	-2.2	*5.6*	-1.3	*3.9*	-1.4	*4.1*	-	-
	HIGH QC	5.7	*2.8*	1.1	*3.7*	-3.1	*5.3*	1.2	*5.2*	-	-
	ULOQ	-	-	-	-	-	-	-	-	-10.4	*13.5*

were stable for up to 55 days after spotting (tested at 3, 7, 15, 30, 55, and 95 days). When stored at -20°C, they were stable for up to 95 days after spotting (tested at 3, 7, 15, 30, 55, and 95 days). Codeine, oxycodone, and hydrocodone were stable for 15 days after spotting and storage at room temperature. When stored at 4°C or -20°C, they were stable for up to 95 days after spotting. Storage of DBS cards at -20°C is thus recommended. This is contradictory to the popular statement that DBS cards can be simply placed in an envelope and send by regular mail without any temperature control. However, even with cooling being a requirement, shipping and storage of DBS card is still preferable over liquid blood: DBS cards take up less space than leak-proof recipients required for blood. Moreover, DBS cards do not need extra precautions to deal with possible biohazardous risks.

It is well known that whole blood haematocrit (HCT = the fraction of whole blood that consists of red blood cells) can be a disturbing factor when performing partial spot analysis, since the HCT value affects the spreading of the blood on the filter paper.[4–6,40] It is also increasingly evident that HCT can have an impact on the extraction efficiency and thus the recovery of an analyte.[40–42] Our experiments with blood at different HCT levels show acceptable accuracy and precision (i.e. RE% within ±15.0% (±20% at LLOQ level) and CV% ≤15% (≤20% at LLOQ level)) when analyzing blood with low and normal HCT levels (30% and 45%) (Supporting Information 2). We observed systematic higher concentrations in QC samples prepared in blood at high HCT values (60%) versus QCs at low and normal HCT. This is most likely caused by the use of partial spot analysis (spotting blood with higher HCT levels results in a smaller spot size and thus more analyte present in the clamped and desorbed 2 mm area). In accordance with this trend, one would expect the lowest concentrations detected in QC samples prepared at the lowest HCT. This was only the case for fentanyl. For the four other opioids, levels determined at 30% HCT were higher than those observed in normal blood, demonstrating that variation in assay bias is not as simple as might be expected. The impact of HCT on recovery was negligible. A comparable recovery (between 72.7 and 87.7%) was observed for all compounds and tested concentration levels in blood at low, normal and high HCT with very good precision (CV% lower than 5.8%) (Table 3). Overall, the results for QCs prepared in blood at 45% are in good agreement with the method validation that was performed with whole blood from adult healthy volunteers (HCT values between 36 and 50), defining the HCT range in which the validated method delivers acceptable results.

Clearly, the impact of HCT on DBS analysis can be compensated by preparing calibration curves in blood with HCT values close to the range of the target population.[40] For example, for athletes, HCT values are generally lower than that of an untrained population and extreme HCT levels (especially low values) are rare. Exercise causes an increase in the number of red blood cells, but also an increase in plasma volume (higher than the increase in red blood cells), resulting in a net decrease of HCT.[43,44] In the clinical world, decreased HCT values are associated with patients with anemia and tuberculosis, patients receiving chemotherapy and immune compromised patients. However, abnormal HCT values can never be excluded, so when using partial spot analysis it is a good idea to measure the HCT value. This can be done by sampling blood in a capillary from which the HCT can be read out after centrifugation or by measuring the potassium concentration, which is correlated to the level of HCT.[40] The latter approach is also possible from a

Table 3. Recovery (± CV%) for different levels of HCT

	HCT 30%		HCT 45%		HCT 60%	
	LLOQ	HIGH QC	LLOQ	HIGH QC	LLOQ	HIGH QC
Morphine	83.8 ± 2.3	79.6 ± 0.9	87.7 ± 2.9	87.2 ± 4.5	82.5 ± 4.3	84.8 ± 1.9
Codeine	77.7 ± 4.7	72.7 ± 1.0	80.2 ± 2.1	81.8 ± 5.8	75.4 ± 1.8	80.2 ± 2.3
Oxycodone	82.3 ± 1.5	74.9 ± 1.0	87.0 ± 3.6	83.8 ± 5.3	82.0 ± 3.4	81.3 ± 2.0
Hydrocodone	77.9 ± 1.3	74.1 ± 1.1	83.3 ± 2.6	83.4 ± 5.5	77.6 ± 1.8	80.9 ± 2.3
Fentanyl	77.3 ± 4.3	76.7 ± 1.3	81.3 ± 3.4	82.4 ± 3.7	77.8 ± 1.3	81.1 ± 1.8

DBS sample. Information about an athlete's HCT does not require extra testing, as it is available by means of the Athlete Biological Passport (ABP). The ABP was introduced in 2009 as a tool to detect blood manipulation through longitudinal monitoring of selected hematological markers.[45] A long-term evaluation of these markers allows for discrimination of doping use from changes caused by exercise which impacts the plasma volume and red cell mass and/or hypoxia (e.g. induced by altitude) which increases red cell mass. One of the measured parameters is the athlete's HCT value. When the HCT of the blood is known, it can be checked whether it is within the accepted HCT range defined during method validation. In case a significant deviation in HCT value is found, it could be incorporated into an established algorithm that enables the correction of the analyte concentration.[40]

Instead of dealing with the HCT effect as described above, it can also be avoided by analysis of the entire blood spot instead of a partial spot coupled with a quantitative volume of applied whole blood.[40] The Spark Holland system can be configured with a 6 mm or 8 mm DBS desorption clamp, allowing automated analysis of an entire spot.[41] For whole spot analysis, volumetric application of spots is the most critical parameter. Moreover, in order to avoid/minimize possible effects of HCT (i.e. not only the effect on spot size but also on analyte recovery), the recovery of the whole spot assay must be high enough.[41,42] Several promising approaches are currently being developed/tested to deal with these challenges and will be explored by the authors in future research.[41,42,46,47]

Incurred samples

The above-described validated DBS-SPE-LC-MS/MS method was successfully applied to human DBS samples obtained from a healthy volunteer who received a single oral dose of 10 mg hydrocodone.

Previously a maximum hydrocodone plasma level of 37.3 ng/mL at 1.0 h (t_{max}) after intake was reported in a similar experimental setting.[48] Another study reports a maximum peak concentration of 23.6 ± 5.2 ng/mL serum achieved at 1.3 ± 0.3 h (t_{max}) after a 10 mg oral dose of hydrocodone administered to five adult male subjects. The elimination half-life ($t_{1/2}$) for hydrocodone following oral administration in humans is around 4 h for hydrocodone and 8 h for norhydrocodone.[38,39,49]

We observed a maximum peak concentration in blood of 44.9 ± 1.9 ng/mL hydrocodone and 7.7 ± 0.3 ng/mL norhydrocodone 1 h after intake (Figure 4). These concentrations were reduced by approximately 50% after another 3 h and 8 h for hydrocodone and norhydrocodone respectively (20.5 ± 1.0 ng/mL hydrocodone was measured 4 h after intake and 3.3 ± 0.4 ng/mL norhydrocodone 9 h after intake). The time points (t_{max}, $t_{1/2}$) reported in literature are in good agreement with our results. Interpretation of the concentration values is more difficult, as the blood-to-plasma ratio for hydrocodone is not known.[50,51] For morphine, it is approximately 1.0, for oxycodone 1.5.[20,52,53] Considering the structural similarities with hydrocodone, a similar value can be expected, suggesting good agreement between our results and the literature. This demonstrates that the presented automated workflow can be implemented in real-life applications such as doping control, therapeutic drug monitoring and clinical chemistry. However, in addition to the HCT issue described above, the lack of reference values measured in DBS and/or whole blood and the use of plasma and serum values to interpret a result is another concern that should be addressed when using DBS. Bridge experiments

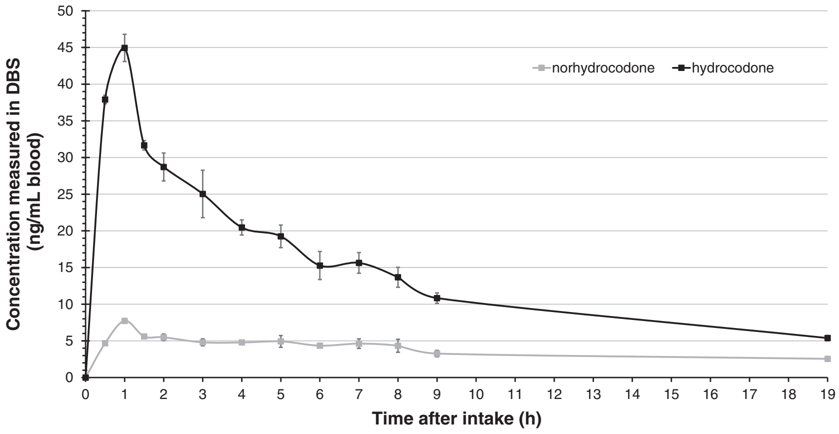

Figure 4. Concentrations of hydrocodone and its major metabolite norhydrocodone measured in DBS collected after oral intake of 10 mg hydrocodone by a healthy volunteer. The average concentration ± standard deviation measured in three analyzed spots was plotted for each time point.

that may be helpful to further understand the correlation between DBS and plasma/serum measurements can benefit from an automated workflow as described here.

Conclusions

A fully automated DBS-SPE-LC-MS/MS method for the quantitative determination of five representative opioids in whole blood has been developed and validated over the concentration range of 1 to 500 ng/mL morphine, codeine, hydrocodone, and oxycodone (0.1 to 50 ng/mL for fentanyl), allowing detection and bioanalytical confirmation of sub-therapeutic, therapeutic and toxic levels within 5 min analysis time after placing a DBS card into the card rack. The method was successfully applied to analyze incurred whole blood samples obtained from simple finger pricks.

These data demonstrate the feasibility of a fully automated approach that combines simple, point-of-care micro sample collection without the aid of a phlebotomist, on-line sample preparation and sensitive detection employing state-of-the-art LC-MS/MS. This automated workflow dramatically reduces the challenging traditional off-line workload for punching and DBS extraction which can minimize potential manual handling errors. The range of possible DBS applications that could benefit from the presented workflow is vast: therapeutic drug monitoring, clinical analysis, doping control and roadside testing of drivers among many other applications.

Future research will focus on the applicability of the automated DBS-SPE-LC-MS/MS system for other relevant compounds and the refinement of the current approach (e.g. decreasing analysis time, avoiding haematocrit issues by whole spot analysis combined with volumetric application of blood onto DBS cards).

Acknowledgements

This project was supported by generous funding from the Partnership for Clean Competition Research Collaborative. The content of this manuscript does not necessarily reflect the views or policies of Research Collaborative. This project was realized thanks to the generous consignments of the LC-MS/MS system by Shimadzu and the DBS autosampler and on-line SPE system by Spark Holland.

References

[1] G. S. Bodor. The laboratory's role in opioid pain medication monitoring. *EJIFCC*. **2012**, *23*, 1.
[2] The WADA 2015 Prohibited List. http://list.wada-ama.org [September 2015].
[3] United Nations World Drug Report 2014. http://www.unodc.org/wdr2014 [September 2015].
[4] J. Henion, R. V. Oliveira, D. H. Chace. Microsample analyses via DBS: challenges and opportunities. *Bioanalysis*. **2013**, *5*, 2547.
[5] R. J. Meesters, G. P. Hooff. State-of-the-art dried blood spot analysis: an overview of recent advances and future trends. *Bioanalysis*. **2013**, *5*, 2187.
[6] M. Wagner, D. Tonoli, E. Varesio, G. Hopfgartner. The use of mass spectrometry to analyze dried blood spots. *Mass Spectrom. Rev.* **2014**, *9999*, 1.
[7] M. Thevis, T. Kuuranne, H. Geyer, W. Schanzer. Annual banned-substance review: analytical approaches in human sports drug testing. *Drug Test. Anal.* **2015**, *7*, 1.
[8] A. J. Wilhelm, J. C. den Burger, E. L. Swart. Therapeutic drug monitoring by dried blood spot: progress to date and future directions. *Clin. Pharmacokinet.* **2014**, *53*, 961.
[9] H. D. Cox, J. Rampton, D. Eichner. Quantification of insulin-like growth factor-1 in dried blood spots for detection of growth hormone abuse in sport. *Anal. Bioanal. Chem.* **2013**, *405*, 1949.
[10] S. Hoppner, P. Delahaut, W. Schanzer, M. Thevis. Mass spectrometric studies on the in vivo metabolism and excretion of SIRT1 activating drugs in rat urine, dried blood spots, and plasma samples for doping control purposes. *J. Pharm. Biomed. Anal.* **2014**, *88*, 649.
[11] L. Tretzel, A. Thomas, H. Geyer, P. Delahaut, W. Schanzer, M. Thevis. Determination of Synacthen((R)) in dried blood spots for doping control analysis using liquid chromatography tandem mass spectrometry. *Anal. Bioanal. Chem.* **2015**, *407*, 4709.
[12] L. Tretzel, A. Thomas, H. Geyer, G. Gmeiner, G. Forsdahl, V. Pop, W. Schanzer, M. Thevis. Use of dried blood spots in doping control analysis of anabolic steroid esters. *J. Pharm. Biomed. Anal.* **2014**, *96*, 21.
[13] N. Sadones, S. Capiau, P. M. De Kesel, W. E. Lambert, C. P. Stove. Spot them in the spot: analysis of abused substances using dried blood spots. *Bioanal.* **2014**, *6*, 2211.
[14] R. Garcia Boy, J. Henseler, R. Mattern, G. Skopp. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. *Ther. Drug Monit.* **2008**, *30*, 733.
[15] C. F. Clavijo, J. J. Thomas, M. Cromie, B. Schniedewind, K. L. Hoffman, U. Christians, J. L. Galinkin. A low blood volume LC-MS/MS assay for the quantification of fentanyl and its major metabolites norfentanyl and despropionyl fentanyl in children. *J. Sep. Sci.* **2011**, *34*, 3568.
[16] C. F. Clavijo, K. L. Hoffman, J. J. Thomas, B. Carvalho, L. F. Chu, D. R. Drover, G. B. Hammer, U. Christians, J. L. Galinkin. A sensitive assay for the quantification of morphine and its active metabolites in human plasma and dried blood spots using high-performance liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* **2011**, *400*, 715.
[17] A. Thomas, J. Deglon, T. Steimer, P. Mangin, Y. Daali, C. Staub. On-line desorption of dried blood spots coupled to hydrophilic interaction/reversed-phase LC/MS/MS system for the simultaneous analysis of drugs and their polar metabolites. *J. Sep. Sci.* **2010**, *33*, 873.
[18] F. Versace, J. Déglon, E. Lauer, P. Mangin, C. Staub. Automated DBS Extraction Prior to Hilic/RP LC–MS/MS Target Screening of Drugs. *Chromatographia*. **2012**, *76*, 1281.
[19] E. Saussereau, C. Lacroix, J. M. Gaulier, J. P. Goulle. On-line liquid chromatography/tandem mass spectrometry simultaneous determination of opiates, cocainics and amphetamines in dried blood spots. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2012**, *885*, 1.
[20] R. Jantos. Comparison of the determination of drugs with influence on driving performance in serum, whole blood and dried blood spots. *Toxichem. Krimtech.* **2013**, *80*, 49.
[21] A. Antelo-Dominguez, J. A. Cocho, M. J. Tabernero, A. M. Bermejo, P. Bermejo-Barrera, A. Moreda-Pineiro. Simultaneous determination of cocaine and opiates in dried blood spots by electrospray ionization tandem mass spectrometry. *Talanta*. **2013**, *117*, 235.
[22] J. Mommers, Y. Mengerink, E. Ritzen, J. Weusten, J. van der Heijden, S. van der Wal. Quantitative analysis of morphine in dried blood spots by using morphine-d3 pre-impregnated dried blood spot cards. *Anal. Chim. Acta*. **2013**, *774*, 26.
[23] S. Odoardi, L. Anzillotti, S. Strano-Rossi. Simplifying sample pretreatment: application of dried blood spot (DBS) method to blood samples, including postmortem, for UHPLC-MS/MS analysis of drugs of abuse. *Forensic Sci. Int.* **2014**, *243*, 61.
[24] J. A. Ooms, L. Knegt, E. H. Koster. Exploration of a new concept for automated dried blood spot analysis using flow-through desorption and online SPE-MS/MS. *Bioanalysis*. **2011**, *3*, 2311.
[25] R. V. Oliveira, J. Henion, E. R. Wickremsinhe. Automated direct extraction and analysis of dried blood spots employing on-line SPE high-resolution accurate mass bioanalysis. *Bioanalysis*. **2014**, *6*, 2027.
[26] R. V. Oliveira, J. Henion, E. Wickremsinhe. Fully-automated approach for online dried blood spot extraction and bioanalysis by two-dimensional-liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry. *Anal. Chem.* **2014**, *86*, 1246.
[27] R. V. Oliveira, J. Henion, E. R. Wickremsinhe. Automated high-capacity on-line extraction and bioanalysis of dried blood spot samples using liquid chromatography/high-resolution accurate mass spectrometry. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 2415.
[28] Y. Li, J. Henion, R. Abbott, P. Wang. Semi-automated direct elution of dried blood spots for the quantitative determination of guanfacine in human blood. *Bioanalysis*. **2012**, *4*, 1445.
[29] R. Fingerhut, M. L. Silva Polanco, J. Silva Arevalo Gde, M. A. Swiderska. First experience with a fully automated extraction system for simultaneous on-line direct tandem mass spectrometric analysis of amino acids and (acyl-)carnitines in a newborn screening setting. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 965.

[30] N. Ganz, M. Singrasa, L. Nicolas, M. Gutierrez, J. Dingemanse, W. Dobelin, M. Glinski. Development and validation of a fully automated online human dried blood spot analysis of bosentan and its metabolites using the Sample Card And Prep DBS System. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2012**, *885-886*, 50.
[31] K. Heinig, T. Wirz, F. Bucheli, A. Gajate-Perez. Determination of oseltamivir (Tamiflu(R)) and oseltamivir carboxylate in dried blood spots using offline or online extraction. *Bioanalysis*. **2011**, *3*, 421.
[32] R. L. Griffiths, A. Dexter, A. J. Creese, H. J. Cooper. Liquid extraction surface analysis field asymmetric waveform ion mobility spectrometry mass spectrometry for the analysis of dried blood spots. *Analyst*. **2015**, *140*, 6879.
[33] A. Z. DePriest, B. L. Puet, A. C. Holt, A. Roberts, E. J. Cone. Metabolism and disposition of prescription opioids: a review. *Forensic Sci. Rev.* **2015**, *27*, 115.
[34] MS/MS library. Available from: www.chemicalsoft.de. [September 2015].
[35] A. Apffel, J. A. Chakel, S. Fischer, K. Lichtenwalter, W. S. Hancock. Analysis of Oligonucleotides by HPLC-Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **1997**, *69*, 1320.
[36] L. Peng, T. Farkas. Analysis of basic compounds by reversed-phase liquid chromatography-electrospray mass spectrometry in high-pH mobile phases. *J. Chromatogr. A*. **2008**, *1179*, 131.
[37] R. Verplaetse, J. Tytgat. Development and validation of a sensitive UPLC-MS/MS method for the analysis of narcotic analgesics in urine and whole blood in forensic context. *Forensic Sci. Int.* **2012**, *215*, 136.
[38] R. Regenthal, M. Krueger, C. Koeppel, R. Preiss. Drug levels: therapeutic and toxic serum/plasma concentrations of common drugs. *J. Clin. Monit. Comput.* **1999**, *15*, 529.
[39] M. Schulz, S. Iwersen-Bergmann, H. Andresen, A. Schmoldt. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. *Crit. Care*. **2012**, *16*, R136.
[40] P. M. De Kesel, N. Sadones, S. Capiau, W. E. Lambert, C. P. Stove. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis*. **2013**, *5*, 2023.
[41] C. M. Hempen, E. H. Maarten Koster, J. A. Ooms. Hematocrit-independent recovery of immunosuppressants from DBS using heated flow-through desorption. *Bioanalysis*. **2015**, *7*, 2019.
[42] P. Abu-Rabie, P. Denniff, N. Spooner, B. Z. Chowdhry, F. S. Pullen. Investigation of different approaches to incorporating internal standard in DBS quantitative bioanalytical workflows and their effect on nullifying hematocrit-based assay bias. *Anal. Chem.* **2015**, *87*, 4996.
[43] J. F. Brun, C. Bouchahda, D. Chaze, A. A. Benhaddad, J. P. Micallef, J. Mercier. The paradox of hematocrit in exercise physiology: which is the 'normal' range from an hemorheologist's viewpoint? *Clin. Hemorheol. Microcirc.* **2000**, *22*, 287.
[44] H. Mairbaurl. Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. *Front Physiol.* **2013**, *4*, 332.
[45] WADA Athletic Biological Passport Operating Guidelines. www.wada-ama.org/en/what-we-do/science-medical/athlete-biological-passport [September 2015].
[46] L. A. Leuthold, O. Heudi, J. Deglon, M. Raccuglia, M. Augsburger, F. Picard, O. Kretz, A. Thomas. New microfluidic-based sampling procedure for overcoming the hematocrit problem associated with dried blood spot analysis. *Anal. Chem.* **2015**, *87*, 2068.
[47] N. Spooner, P. Denniff, L. Michielsen, R. D. Vries, Q. C. Ji, M. E. Arnold, K. Woods, E. J. Woolf, Y. Xu, V. Boutet, P. Zane, S. Kushon, J. B. Rudge. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis*. **2015**, *7*, 653.
[48] M. Darwish, R. Yang, W. Tracewell, P. Robertson Jr., M. Bond. Single- and multiple-dose pharmacokinetics of a hydrocodone bitartrate extended-release tablet formulated with abuse-deterrence technology in healthy, naltrexone-blocked volunteers. *Clin. Ther.* **2015**, *37*, 390.
[49] D. M. Navani, B. C. Yoburn. In vivo activity of norhydrocodone: an active metabolite of hydrocodone. *J. Pharmacol. Exp. Ther.* **2013**, *347*, 497.
[50] G. Emmons, M. Rowland. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis*. **2010**, *2*, 1791.
[51] P. Hinderling. Red blood cells: a neglected compartment in pharmacokinetics and pharmacodynamics. *Pharmacol. Rev.* **1997**, *49*, 279.
[52] C. W. Hand, R. A. Moore, J. W. Sear. Comparison of whole blood and plasma morphine. *J. Anal. Toxicol.* **1988**, *12*, 234.
[53] G. Skopp, L. Potsch, B. Ganssmann , R. Aderjan, R. Mattern. A preliminary study on the distribution of morphine and its glucuronides in the subcompartments of blood. *J. Anal. Toxicol.* **1998**, *22*, 261.

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