

Monitoring Opioid Use for Pain Management Using a Fully Automated DBS-SPE-LC/MS/MS Approach

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Overview

Purpose

- To develop and validate an automated sample preparation and analysis procedure for dried blood spots (DBS) relevant for pain management monitoring.

Method

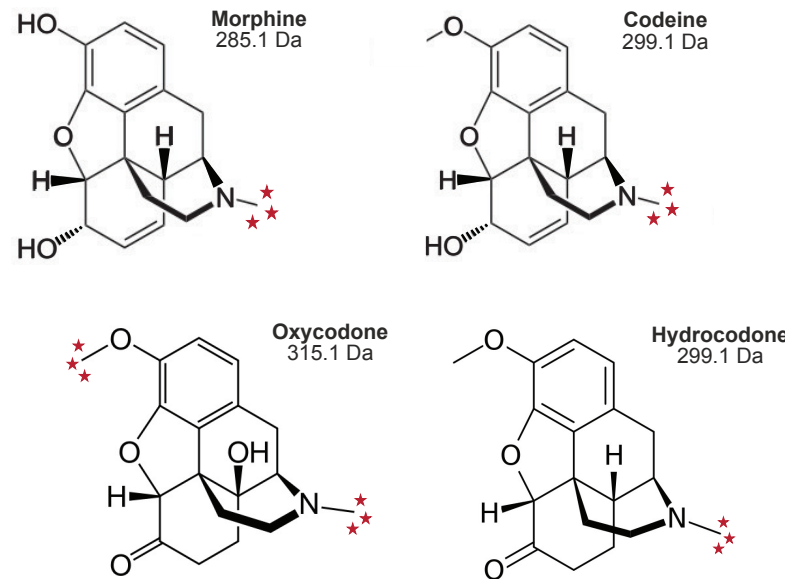
- Automated flow-through elution of DBS cards is followed by on-line solid phase extraction (SPE) and analysis by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS).

Results

- Selective, sensitive, accurate, and reproducible quantitation of opioids in blood using a fully automated DBS-SPE-LC/MS/MS approach.
- Our data support the potential of DBS sampling for monitoring opioids as well as other pharmaceuticals in both anti-doping and pain management regimens.

Introduction

- Opioids represent a highly effective class of drugs widely prescribed and used for pain management. They include natural (e.g., morphine, codeine) and semi-synthetic (e.g., oxycodone, hydrocodone) 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.
- Due to less-invasive sample collection, small sample volumes, and easy sample shipping and storage, DBS sampling provides advantages for doping control and pharmaceutical and clinical analyses [1, 2]. DBS sampling allows easy point-of-care as well as out-of-competition sample collection. Modern analytical instruments are capable of the sensitive measurements required for analyzing micro samples. As such, labor-intensive off-line sample preparation can be considered a bottleneck of DBS analyses.
- We present the development and validation of fully automated flow-through elution of DBS micro samples with on-line SPE and LC/MS/MS bioanalysis used for the detection of opioids.
- For this study, we selected four representative compounds covering the analytical challenges posed by the opioids (e.g., isomers).

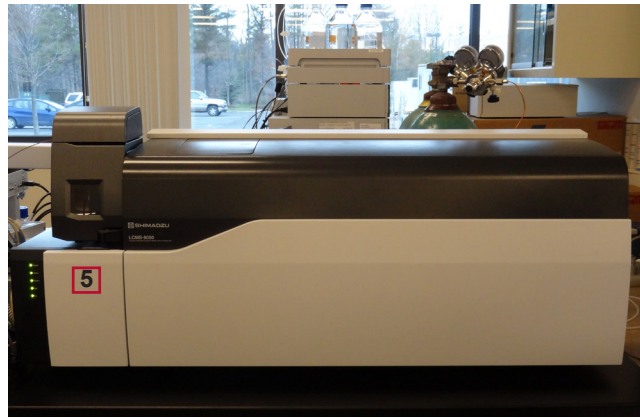
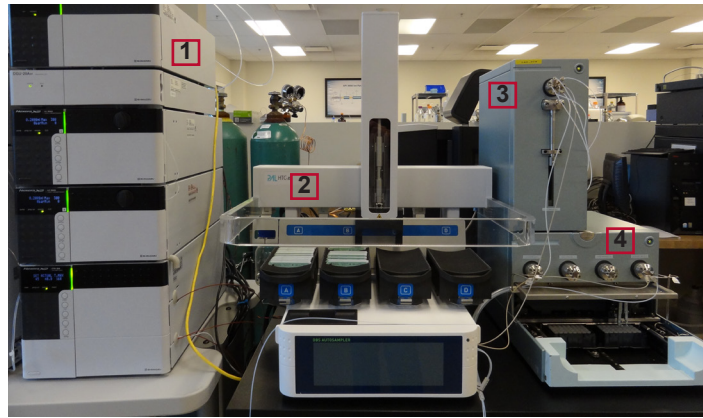


★ Indicates the location of the deuterium stable-isotope labels.

References

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- Cox, HD., et al. *Bioanalysis* **2014**, 6(19), 2651-2662.
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- Verplaetse, R., et al. *J Chrom B* **2010**, 878(22), 1987-1996.

Method



The numbers in the photos at left are keyed to the text below.

Automated flow-through DBS elution

- DBS system:**
- 2 DBS card autosampler (DBSA, Spark-Holland)
 - 3 High Pressure Dispenser pump (HPD, Spark-Holland)

- Sample:**
- 10 μ L of blood spotted on DBS card (Ahlstrom 226, Perkin Elmer) and dried for ≥ 3 hours

- Internal standard (IS):**
- Loop injection of 20 μ L of IS (1 ng/mL codeine- d_3 , hydrocodone- d_3 , morphine- d_3 , and oxycodone- d_6)

- DBS clamp diameter:**
- 2 mm

- DBS desorption:**
- 1 mL of 0.1% ammonium hydroxide at 2 mL/min

- DBS clamp wash:**
- 1 mL of 0.1% ammonium hydroxide at 5 mL/min
 - 1 mL of methanol at 5 mL/min
 - 1 mL of 20:40:30:10 water/methanol/acetonitrile/isopropanol at 5 mL/min
 - 1 mL of 0.1% formic acid at 5 mL/min

Automated SPE

- SPE system:**
- 4 Automated SPE Cartridge Exchanger module (ACE, Spark-Holland)

- SPE cartridge:**
- HySphere C18 HD, 7 μ m, 2 x 10 mm (Spark-Holland)

- SPE conditioning:**
- 1 mL of methanol at 5 mL/min

- SPE equilibration:**
- 1 mL of 0.1% ammonium hydroxide at 5 mL/min

- SPE load:**
- Same as DBS desorption solvent

- SPE wash:**
- 1 mL of 0.1% ammonium hydroxide at 5 mL/min

- SPE elution:**
- (A) 0.1% formic acid in 5 mM ammonium formate
 - (B) methanol
 - 5 to 100% B in 3 min at 0.4 mL/min, 45 $^{\circ}$ C

- SPE wash:**
- Same as DBS clamp wash solvents

LC/MS/MS conditions

- LC/MS/MS system:**
- 1 Nexera ultra-high performance liquid chromatography (UHPLC) system (Shimadzu)
 - 5 LCMS-8050 mass spectrometer (Shimadzu)

- LC column:**
- Raptor Biphenyl (2.7 μ m, 2.1 x 50 mm) with guard column (2.7 μ m, 2.1 x 5 mm) (Restek)

- LC gradient:**
- Same as SPE elution solvents

- MS source:**
- Electrospray ionization (ESI), 2 kV, using nebulizer (2 L/min), heating (5 L/min), and dry (5 L/min) gas (N_2)

- MS/MS scan mode:**
- Selected reaction monitoring (SRM) in positive mode

Table 1: MS/MS parameters

Analyte	pKa	logP	Q1 MH ⁺ (m/z)	Quantifier SRM fragment (m/z)	Qualifier SRM fragment (m/z)	Retention time (min)
Morphine	8.0, 9.9	-0.1	286.1	165.1	201.1	1.4
Codeine	8.0	0.6	300.1	165.1	215.1	2.0
Oxycodone	9.0	0.7	316.1	241.1	256.1	2.1
Hydrocodone	8.9	NA	300.1	199.1	171.1	2.2

NA: Not available

Figure 1: Schematic drawing of work flow (Colors in the schematic drawing below correspond to colors in the text at left.)

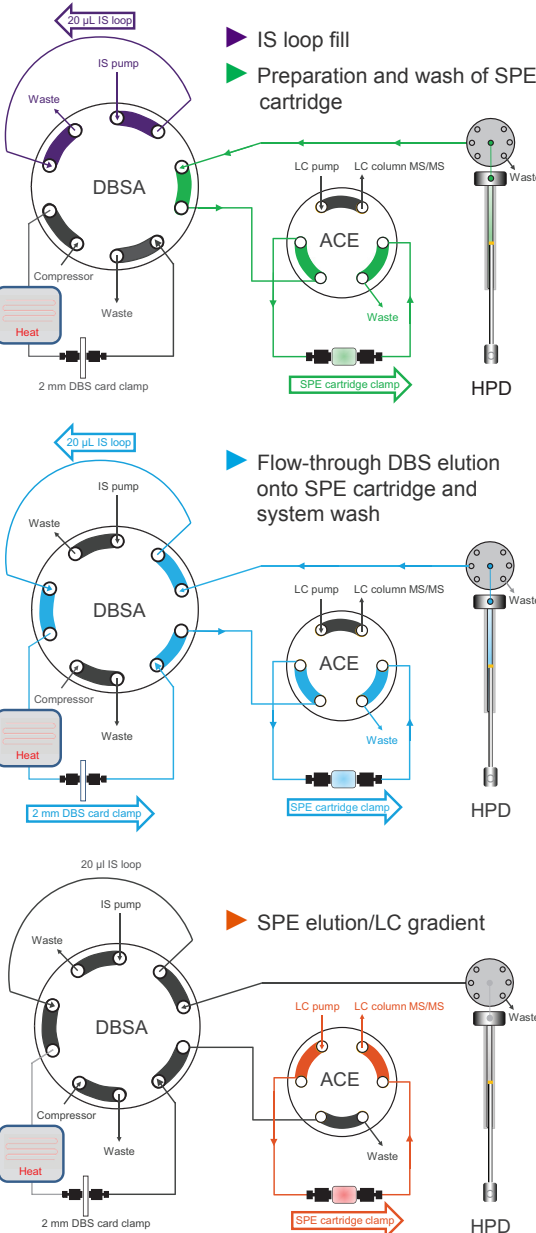
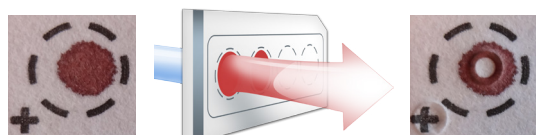


Figure 2: Flow-through DBS elution



A DBS card with four spots was placed in the online DBS autosampler. A 2-mm area was desorbed from a spot and loaded onto the SPE cartridge by flow-through elution with 1 mL of 0.1% ammonium hydroxide. A blank part of the card (left of the spot) was clamped for washing afterward to minimize carryover.

Method Optimization

- A variety of SPE cartridges are available to use on the ACE system [3]. Initially, mixed-mode SPE (cation exchange) was preferred as it provides orthogonality to reversed-phase LC. Moreover, elution of basic compounds from these SPE cartridges requires a high pH, in turn providing so called "wrong-way ionization" and more retention on the LC/MS/MS [4]. However, SPE elution was only achieved after pumping a high percentage of organic solvent, making chromatographic separation of the isomers impossible. Moreover, some band broadening was observed, caused by the 10- μ m SPE particles.
- C18 and C2 SPE cartridges (particle size 7 μ m) were tested. No organic solvent was used in the DBS desorption/SPE loading solvent as 5% already results in loss of SPE loading. The composition of the solvent, volume, flow rate, and temperature used during DBS desorption/SPE were optimized (see Figure 3).
- Several wash steps, flushing both the DBS clamp and SPE cartridge, were necessary to minimize carryover. Moreover, this allows for re-use of the SPE cartridges.
- Three different LC columns were evaluated to determine which provided the best peak shape, separation, and sensitivity for the four analytes (see Figure 4).

Figure 3: DBS desorption/SPE load optimization

Compared: C2 vs. C18 SPE cartridges;
0.1% vs. 1% ammonium hydroxide;
Room temperature (RT) vs. 45 $^{\circ}$ C vs. 80 $^{\circ}$ C

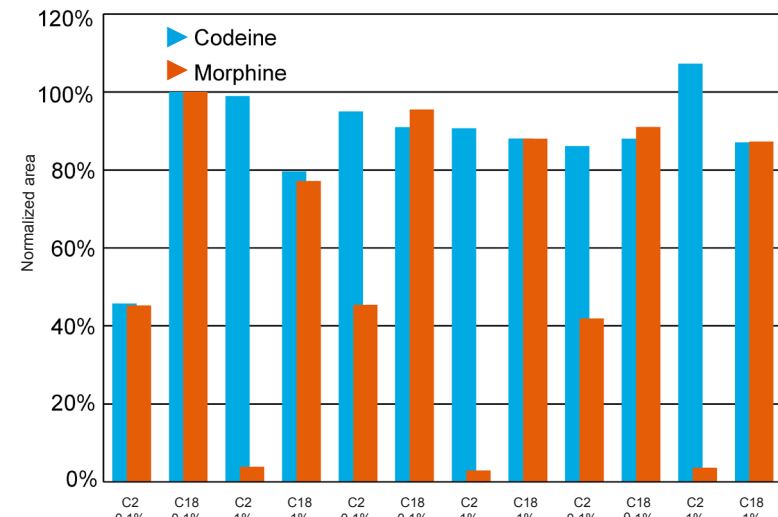
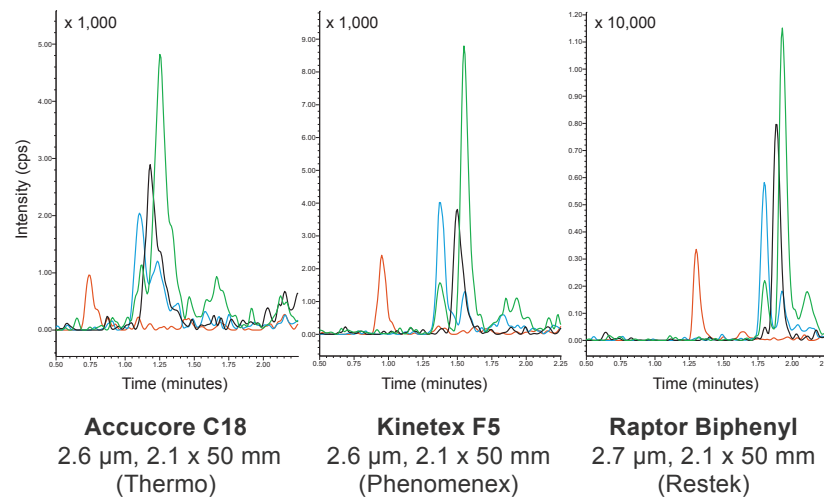


Figure 4: LC column optimization

Blood spiked at the lower limit of quantitation (LLOQ) was spotted onto a DBS card, extracted, and analyzed for the four analytes using three different LC columns. Note: No guard column was used.

Key: ► Morphine ► Codeine ► Oxycodone ► Hydrocodone



Method Validation

The sensitivity, selectivity, linearity, accuracy and precision, stability, and lack of carryover of the method were demonstrated for morphine, codeine, oxycodone, and hydrocodone (see Tables 2, 3, and 4). Representative chromatograms for the four analytes are shown below. Chromatogram key: ► Quantifier SRM ► Qualifier SRM

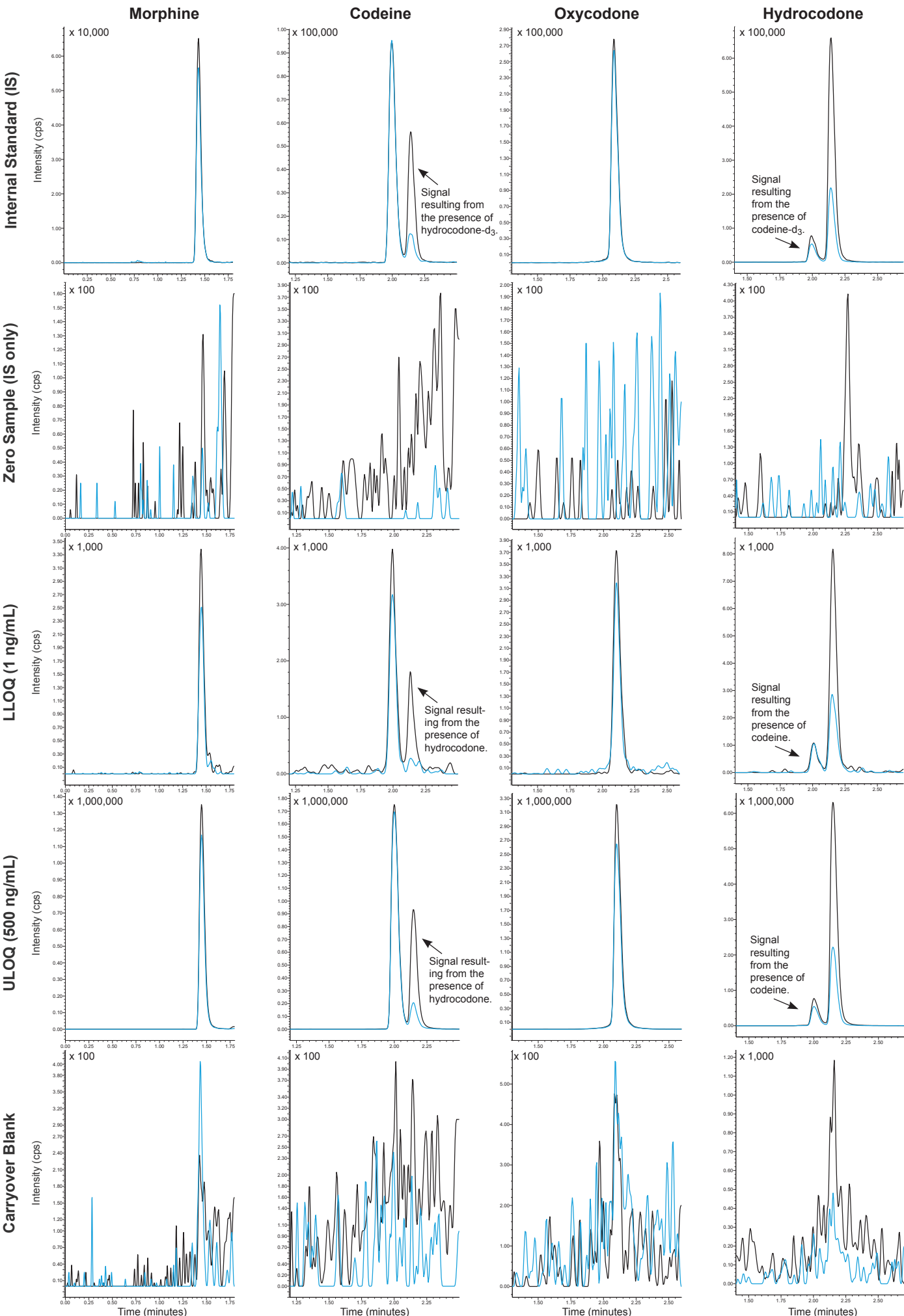


Table 2: Linearity

Analyte	Therapeutic range (ng/mL blood)	Toxic range (ng/mL blood)	Calibration range (ng/mL blood)*	R ² * (quadratic regression, 1/x ²)
Morphine	10 to 100	>100	1 (LLOQ) to 500 (ULOQ)	0.998
Codeine	10 to 200	>250	1 (LLOQ) to 500 (ULOQ)	0.998
Oxycodone	10 to 100	>200	1 (LLOQ) to 500 (ULOQ)	0.995
Hydrocodone	10 to 50	>100	1 (LLOQ) to 500 (ULOQ)	0.997

*The calibration curve was defined by six concentration levels (n=2 on each of three days).
LLOQ: Lower limit of quantitation
ULOQ: Upper limit of quantitation

Table 3: Matrix inter-lot accuracy and precision

Analyte	LLOQ		ULOQ	
	RE%	CV%	RE%	CV%
Morphine	-1.6	14.7	13.4	8.3
Codeine	15.4	12.9	14.4	9.9
Oxycodone	6.7	8.5	8.9	8.4
Hydrocodone	20.0	7.9	11.5	8.0

Matrix effects were evaluated by analyzing six different lots of whole blood.
LLOQ: Lower limit of quantitation
ULOQ: Upper limit of quantitation
RE% (relative error) = [(calculated mean – nominal value)/nominal value] x 100
CV% (coefficient of variation) = (standard deviation/mean) x 100

Table 4: Accuracy and precision

Analyte	LLOQ QC		Low QC		Medium QC		High QC	
	RE%	CV%	RE%	CV%	RE%	CV%	RE%	CV%
Morphine	-1.4	13.4	-3.3	11.4	-1.1	10.4	0.2	8.8
Codeine	0.1	10.7	1.5	10.9	3.6	11.2	0.4	9.0
Oxycodone	7.2	7.8	3.4	9.8	6.0	9.3	4.0	8.6
Hydrocodone	2.2	7.4	11.2	10.7	10.6	8.1	5.1	6.9

Accuracy and precision were evaluated at four concentration levels (n=6 on each of three days).
RE% (relative error) = [(calculated mean – nominal value)/nominal value] x 100
CV% (coefficient of variation) = (standard deviation/mean) x 100

Conclusions

- An automated DBS-SPE-LC/MS/MS method for the quantitation of four representative opioids in whole blood was developed and validated over the concentration range of 1 to 500 ng/mL blood, allowing detection of subtherapeutic, therapeutic, and toxic levels.
- The total cycle time per sample was 4.5 minutes. This included wash steps allowing re-use of the SPE cartridges. Overlap between cycles of different samples is possible.
- We demonstrated the feasibility of a fully automated approach that combines simple point-of-care sample collection and on-line sample preparation with sensitive and selective detection. Our data suggest considerable potential of the presented work flow for other bioanalytical applications.

Acknowledgements

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