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A Fully Automated On-line Forensic Toxicology Solution for the Determination of Opioids in Blood by Smart SPE Clean-Up and GC-MS

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Introduction

Many forensic toxicology service labs are facing increasing casework, often times with reduced staff numbers. While there is significant pressure on the lab analysts to reduce case turnaround times (TATs), it is essential that the results are reproducible and reliable for consideration as evidence in a Court of Law.

Automation of the sample preparation workflow can not only offer faster TATs and excellent repeatability of results, but it can also free up the lab analysts for thinking tasks such as data analysis, method development and reporting.

This application note describes the fully automated on-line workflow for the determination of four forensically-relevant opiates in blood by gas chromatography-mass spectrometry (GC-MS), including morphine, 6-monoacetylmorphine (6-MAM), codeine and dihydrocodeine (DHC).

Opioids encompass natural opiate alkaloids and the semi-synthetics derived from them. Natural opiates are found in the opium poppy plant *Papaver Somniferum* and are used medicinally for the treatment of acute pain. Although opiates are effective for relieving moderate to severe chronic pain, they are highly addictive drugs and are abused globally.

Some manual workflows for the extraction of opiates from blood can involve solid phase extraction (SPE) to enrich the analyte concentration and reduce matrix interference prior to GC-MS or LC-MS analysis.

The automation of on-line SPE employs miniaturised Instrument Top Sample Prep (ITSP) cartridges packed with customised sorbent (smart SPE)¹ to match the material used for standard size SPE cartridges. The miniaturisation of the SPE cartridge consequently requires a scale down of the whole method.



Figure 1: Instrument Top Sample Prep (ITSP) miniaturised cartridges for smart SPE and 96 well plate format ITSP tray.

The most unique aspect of smart SPE using ITSP single use miniaturised cartridges is the precise flow control and the ability to achieve high levels of chromatographic performance. Hence, in contrast to manual larger volume SPE, smart SPE flow profiles follow the expected Van Deemter curves with clearly defined optima. As an outcome of the accurate flow control, absolute

recoveries of >99% can be achieved along with a significant reduction in background matrix.

In this study, spiked blood samples were fully prepared and extracted by the MPS using smart SPE and then injected directly on a GC-MS single quadrupole system for instrumental analysis.

Instrumentation

The fully automated workflow for the determination of opiates in blood by GC-MS was developed on a GERSTEL MultiPurpose Sampler (MPS) 2 XT Dual Rail (Figure 1) equipped with the following objects:

- Solvent Filling Station
- Solvent Reservoirs (3 positions, 100 mL each)
- Standard Large Wash Station (2 washes and 1 waste line)
- 1 x Tray VT-98
- Smart SPE ITSP kit
- 2 x Trays VT-40
- Agitator
- GERSTEL Multi-Position Vortexer (mVortex)
- GERSTEL Multi-Position Evaporation Station (mVAP)
- Anatune CF-200 Robotic Centrifuge

GC-MS analysis was performed using the Agilent 7890B Gas Chromatograph coupled to an Agilent 5975C MSD.



Figure 2: GERSTEL Dual Rail MPS with Agilent GC-MS for fully automated determination of opiates in blood

Methods

Optimised automated sample preparation and spiking:

An aliquot of defibrinated horse blood was transferred manually into 2 mL glass vials with magnetic screw cap.

Using the PrepSequence option in the GERSTEL Maestro software, phosphate buffer pH 6/water (2: 1 v/v) was then added to the samples to promote protonation of the basic drug targets.

Deuterated internal standard (containing morphine-d3, 6-MAM-d3, codeine-d3 and DHC-d6) was added to each blank and calibrator sample to achieve a final concentration of 100 µg/L.

Calibration standards were prepared by spiking the horse blood aliquots with defined volumes of methanolic drug standard to achieve a concentration range of 25 µg/L- 500 µg/L for DHC, codeine and morphine and 5 µg/L- 200 µg/L for 6-MAM. Negative blank samples with deuterated internal standard were also prepared.

After spiking, samples were vortex mixed using the multi-position Vortexer (mVortex). Once mixing had been completed, vials were moved by the robot to the CF-200 centrifuge whereby the samples were centrifuged to obtain a clear extract.

Optimised automated solid phase sample extraction and derivatisation:

Post-centrifugation, samples were extracted and cleaned up using smart SPE (cation exchange mode).

ITSP cartridges (UCT DAU, 10 mg and 30 mg) were conditioned with methanol, water and phosphate buffer pH 6, respectively. The sample supernatant was then loaded onto the ITSP cartridge and allowed to drip through by the positive pressure applied by the syringe.

After loading, the cartridges were washed with water, acetate buffer pH 4.5 and methanol respectively to remove whole blood matrix interferences. The basic opiate compounds were then eluted with dichloromethane/isopropanol/ammonium hydroxide 78:20:2 (v/v/v) into a clean high recovery vial.

The eluate was transferred to a capped vial and evaporated to dryness using the multi-position evaporation station (mVAP). Once dry, the sample was reconstituted with BSTFA + 1% TMCS and the mixture was incubated. After derivatisation, the sample was injected directly for GC-MS analysis.

GC/MS conditions:

GC:

- Column: HP-5MS Ultra inert+ Duraguard (10 m) 30 m x 0.25 mm x 0.25 µm
- Injection mode: Pulsed Splitless
- GC ramp: 150 °C held for 2 min, 10 °C/min to 300 °C, held for 5 min

MS:

- Inert EI source
- Scan/SIM mode

Results and Discussion

In this study, sample batches including blanks with internal standard and five calibrators were prepared over three separate days to test robustness and reproducibility of the system.

Two different smart SPE bed sizes (10 mg and 30 mg, respectively) were tested within each batch to investigate potential breakthrough.

A blank and five calibrator samples were prepared without being extracted via smart SPE (i.e. derivatisation only) to evaluate ITSP recovery of the target analytes.

Automated on-line solutions (e.g. an MPS autosampler mounted or linked to a dedicated instrumental technique such as GC-MS or LC-MS) allows the exploitation of the PrepAhead function within the GERSTEL Maestro software. This software feature includes the ability to complete sample preparation for each sample immediately preceding the GC-MS injection, whilst the previous sample is running.

Figure 3 shows the PrepAhead function in the timeline for the preparation of one sample batch (blank + five calibrators for 10mg and 30 mg sorbent bed sizes, for a total of 12 samples per batch).

The green and yellow multi-coloured bands represent the sample preparation and beige bands the GC run-time.

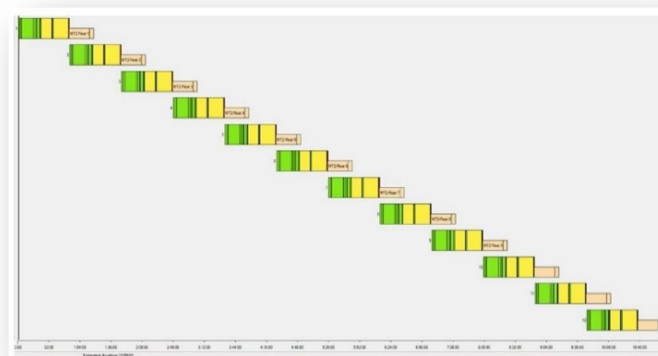


Figure 3: Sequence timeline preview for 12 samples using PrepAhead.

A notable advantage of automating this procedure was the significant reduction in solvent consumption, reflecting in significant savings on consumables costs and solvent disposal costs which can be high (in particular for the chlorinated waste).

Table 1 summarises the differences in solvent consumption between the manual and automated workflow for the preparation of a sample batch (12 samples).

Solvent Type	Volume [mL]	
	Manual	Automated
Phosphate Buffer pH 6	72	7.2
Methanol	72	2.4
Deionised water	72	2.4
Acetate buffer pH 4.5	24	1.2
DCM/IPA/NH4OH	30	3

Table 1: Volumes of solvents required for the preparation of 12 samples using the manual (left hand side column) and the automated (right hand side column) workflows, respectively.

The fully automated solution was demonstrated to give excellent linearity and reproducibility. Table 2 reports the average ($n=3$) slope, intercept and R^2 for each of the investigated analytes (10 mg bed size, 30 mg bed size and derivatisation only). Figure 4 shows as an example of the calibration plots obtained using 30 mg bed size for all four opiates.

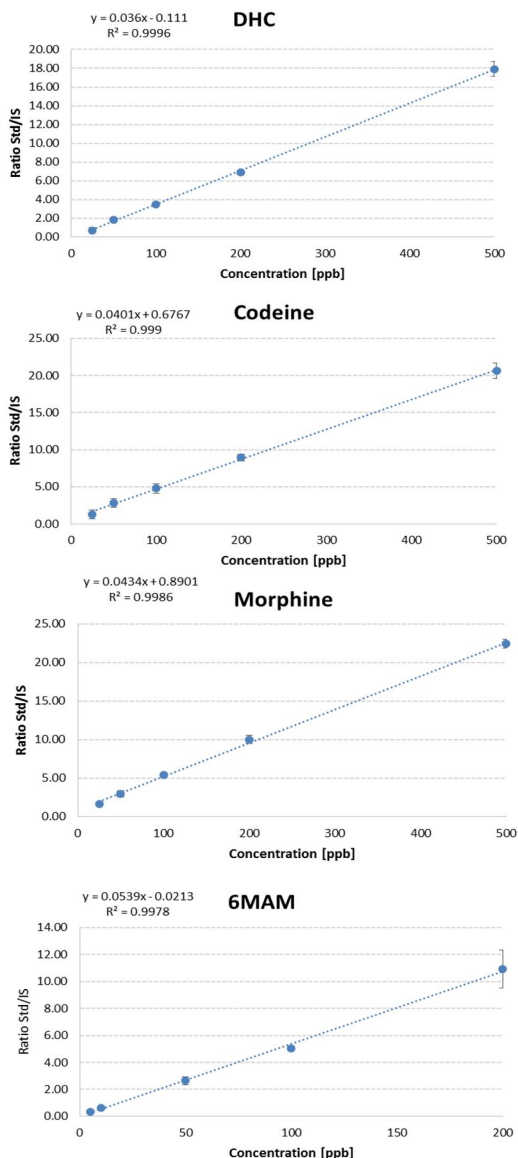


Figure 4: Calibration plots for the four opiates using 30 mg bed size for smart SPE.

As shown in Table 2, performances in terms of linearity for the two different bed sizes were comparable to the results obtained without the smart SPE extraction (derivatisation only).

	10mg			30mg			derivatisation only		
	Slope	Intercept	R ²	Slope	Intercept	R ²	Slope	Intercept	R ²
DHC	0.0338	0.3034	0.9996	0.0360	-0.111	0.9996	0.0374	0.1205	0.998
Codeine	0.0376	0.5345	0.9993	0.0401	0.6747	0.999	0.038	0.3607	0.9998
Morphine	0.0415	1.0438	0.9975	0.0434	0.8901	0.9986	0.0417	0.846	0.9985
6MAM	0.0509	-0.0268	0.9972	0.0539	-0.0213	0.9978	0.0531	-0.0153	0.9998

Table 2: Average ($n=3$) slope, intercept and R² for the four investigated opiates using 10 mg bed size, 30 mg bed size and no extraction (i.e. derivatisation only).

The % Relative Standard Deviations (% RSD) for the analytes of three calibrator replicates over three different days ranged between 2% and 10% for 10 mg bed size, 1% and 20% for 30 mg and 0.2% and 14% for derivatisation only.

Recoveries for the smart SPE were calculated using the average calibration curve slopes obtained for each analyte in the tested conditions (i.e. 10 mg and 30 mg) against the average calibration curve slopes obtained without performing the solid phase extraction (derivatisation only). Recovery results are reported in Table 3, with all % RSDs $\leq 10\%$.

Analyte	Recovery %	
	10 mg	30 mg
DHC	90 \pm 7	96 \pm 5
Codeine	99 \pm 2	106 \pm 6
Morphine	100 \pm 4	104 \pm 3
6MAM	98 \pm 6	100 \pm 10

Table 3: Smart SPE % recoveries using 10 mg and 30 mg bed size for the extraction of opiates from 200 μ L spiked blood calibrators

Recoveries using 30 mg bed size were not statistically different from the ones obtained using 10 mg bed size (t -test, $\alpha=0.05$).

Figure 5 shows the peak responses for the four opiates at the lowest calibration level (25 μ g/L for DHC, codeine and morphine as well as 5 μ g/L for 6-MAM).

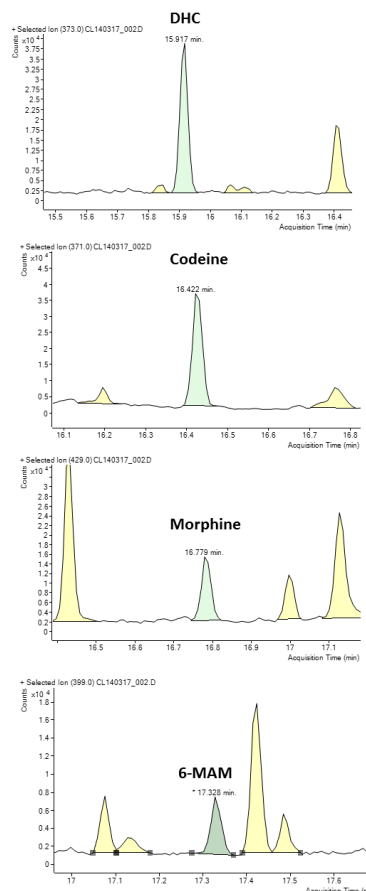


Figure 5: Extracted Ion Chromatograms for the four target opiates at the lowest calibration level using 10mg smart SPE



Table 4 lists the average ion ratios obtained for the quantifier ion/qualifier ion ratios for each analyte over the whole calibration range (5 calibrators) over the three separate days using the two bed sizes ($n=30$). As shown by the % RSDs, the ratios between the ions were generally less than 20% and consistent within calibration concentration range. The qualifier 2 ions for DHC and codeine were present at lower abundance hence the likely reason for the higher %RSD values, although still less than 30%.

	DHC		Codeine	
	Quant/Qual 1 (m/z 373/236)	Quant/Qual 2 (m/z 373/178)	Quant/Qual 1 (m/z 371/196)	Quant/Qual 2 (m/z 371/146)
Qualifier Ratio Average ($n=30$)	42	22	64	50
Qualifier Ratio SD	6	6	8	15
Qualifier Ratio RSD%	14	26	13	29

	Morphine		6MAM	
	Quant/Qual 1 (m/z 429/414)	Quant/Qual 2 (m/z 429/236)	Quant/Qual 1 (m/z 399/340)	Quant/Qual 2 (m/z 399/287)
Qualifier Ratio Average ($n=30$)	60	96	111	60
Qualifier Ratio SD	4	8	23	7
Qualifier Ratio RSD%	6	8	20	12

Table 4: Average, standard deviation and % RSD ($n=30$) of the quantifier/qualifier ratios for both selected ions (Quant 1 and Qual 2) for the investigated analytes.

Table 5 lists average signal-to-noise (S/N) ratios ($n=6$) obtained for the four analytes at the lowest calibration point. S/N was calculated using the peak-to-peak algorithm within the Mass Hunter Quantitative Analysis method selecting 0.5 min noise region on the side of the peak

Analyte	Average S/N
DHC	30
Codeine	18
Morphine	13
6MAM	8

Table 5: Average ($n=6$) signal-to-noise ratios for the four target opiates at the lowest calibration level

Signal-to-noise (S/N) was above 10 for all analytes with the exception of 6-MAM. According to The United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) guidelines, the LOQ can be derived by adding ten deviations to the true value of the blank but it is preferable to determine the LOQ experimentally as the lowest concentration for which an acceptable %CV can be achieved.² For 6-MAM the % RSD at the 5 $\mu\text{g/L}$ calibrator point was 2% despite having an average S/N of 8.

Limits of detection (LODs) were calculated for the investigated analytes using a S/N acceptance criteria of 3 on 3 different days. These were determined as 9 $\mu\text{g/L}$ for DHC, 5 $\mu\text{g/L}$ for codeine, 6 $\mu\text{g/L}$ for morphine and 3 $\mu\text{g/L}$ for 6-MAM, respectively

It is worth highlighting the fact that this study was carried out on a GC coupled with a single quadrupole mass analyser. As shown in Figure 5, the quantifier ion trace for the 6-MAM (m/z 399) was affected by background interference due to the low selectivity of the single quadrupole. This was also true for other 6-MAM acquired ion traces (qualifier 1 m/z 340 and qualifier 2 m/z 287).

Analysis of the same sample on a MS/MS system (e.g. GC triple quadrupole) would provide the increase in selectivity and sensitivity necessary to improve the S/N for the low concentrations of 6-MAM.

Conclusions

A robust and reproducible fully automated on-line solution was developed and tested for the GC-MS determination of four forensically relevant opiates in spiked horse blood using smart SPE.

Excellent linearity, reproducibility and absolute recoveries were observed for all four target analytes using both smart SPE bed sizes, with consistent ion quantifier/qualifier ratios (majority less than 20%) across the calibration range for each analyte.

The average S/N ratio of the LOQ (lowest calibrator point) were all greater than 10 with the exception of the 6-MAM. However the % RSD value for the lowest 6-MAM calibrator was 2% which would be acceptable according to UKIAFT method validation guidelines².

Initial results in spiked horse blood look promising however the next step will involve an assessment of this automated system for the analysis of whole blood post-mortem samples for opiates.

References

1. ITSP Solutions Inc. application note, Automated Online μ SPE-LC/MS/MS for the Measurement of Basic Drugs in Blood.
2. The United Kingdom and Ireland Association of Forensic Toxicologists Forensic Toxicology laboratory guidelines (2010). *Science and Justice* 50: (2010), 166-176.

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