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Analysis of opiates in urine using microextraction by packed sorbent and gas Chromatography- Tandem mass spectrometry

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ABSTRACT

Opiates recreational consumption has always been a concern in society, public health, and in clinical toxicology analysis. The aim of this study was to develop and fully validate an analytical method, which was simple and rapid for the determination of tramadol, codeine, morphine, 6- acetylcodeine, 6-monoacetylmorphine and fentanyl using gas chromatography coupled to tandem mass spectrometry. The procedure includes the use of microextraction by packed sorbent for sample clean-up. A mixed mode sorbent was used, allowing the minimal use of solvents. The method was validated in urine samples, with the ability to detect and quantify all analytes with satisfactory linearity (in the range of 1 – 1000 ng/mL for all analytes, except for fentanyl (10–1000 ng/mL)). Extraction efficiency varied from 17 to 107%, which did not impair sensitivity, taking into account the low LLOQs obtained (1 ng/ mL for all analytes; and 10 ng/mL for fentanyl). The developed procedure proved to be fast, selective, and accurate for use in routine analysis, with a low volume of sample (250 μL).

1. Introduction

The consumption of opiates (natural and synthetic) has increased over the last years, accounting for a high number of deaths worldwide. In Europe, the drug market has been adapting to new drugs, namely synthetic opioids, which include 8 new substances reported for the first time in 2019 [1]. The addictive problems associated with their consumption are still a social and public health concern [2].

Urine is a widely used biological specimen in different clinical and toxicological contexts, namely on screening tests and workplace drug testing. The ease in acquiring such samples and the non-invasiveness to the patient, account as advantages. Moreover, the ability to collect great quantities, allows to find higher concentrations of substances and metabolites, also allowing for a more rapid analysis than blood [3]. However, a few drawbacks may be associated to urinalysis, namely the possibility of being tampered with (e.g. dilution with water) [4], or the fact that single "spot" concentrations must be interpreted with caution due to variable fluid intakes. One way to overcome the latter is to take into account the concentration of creatinine in the sample, normalizing analyte concentrations [5].

Several authors have published methods for the determination of opiates in urine, and several techniques have been used for sample clean-up, for instance liquid–liquid extraction (LLE) [6,7], solid-phase extraction (SPE) [8–11], dispersive liquid–liquid microextraction (DLLME) [12,13], enzymatic hydrolysis [14], liquid–liquid microextraction [15] and an extraction combining the use of dried urine spots and volumetric absorptive microsamples [16]. All techniques showed overall good results and applicability to authentic samples and usability on routine laboratorial analysis. However, some of these are not cost effective, using high volumes of solvents (LLE and SPE) and are not environmentally friendly. Therefore, miniaturized techniques have been recently proposed for the replacement of those approaches.

Microextraction by packed sorbent (MEPS) is adapted from the traditional solid-phase extraction (SPE) into a miniaturized procedure, where it is possible to work with lower solvent amounts, that is, it is possible to work in the range of microlitres. This clean-up procedure is

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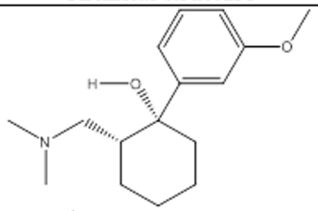
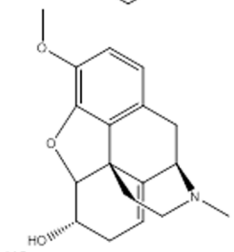
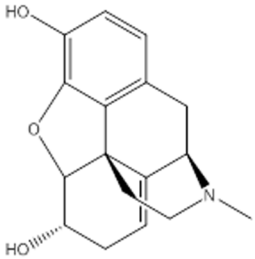
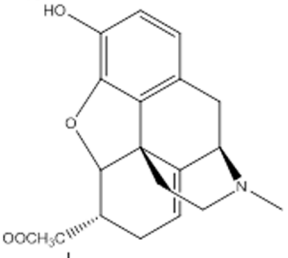
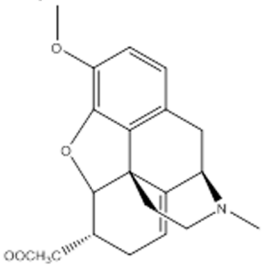
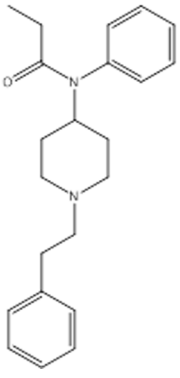
Analyte	Chemical structure	IUPAC name
Tramadol		(1*R*,2*R*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexan-1-ol
Codeine		(4*R*,4*aR*,7*S*,7*aR*,12*bS*)-9-methoxy-3-methyl-2,4,4*a*,7,7*a*,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-ol
Morphine		(4*R*,4*aR*,7*S*,7*aR*,12*bS*)-3-methyl-2,4,4*a*,7,7*a*,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol
6-monoacetylmorphine		(9-hydroxy-3-methyl-2,4,4*a*,7,7*a*,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl) acetate
6-acetylcodeine		[(4*R*,4*aR*,7*S*,7*aR*,12*bS*)-9-methoxy-3-methyl-2,4,4*a*,7,7*a*,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl] acetate
Fentanyl		*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide

Fig. 1. Chemical structures and chemical names (IUPAC) of the analytes under study.

an environment- and user-friendly technique [17,18]. Altogether, the ability of automation, the ease, the use of the same sorbents used in SPE, as well as the inexpensive costs, have made possible the application of this technique to a variety of different specimens [19–26]. Therefore, it presents several advantages, allowing high sensitivity, precision and accuracy and it was shown to be successful in clinical, forensic and toxicology analysis. MEPS has been used to extract other substances from urine samples [27–29]. Despite its known assets, online-MEPS (eVol MEPS syringe) has only been used once to determine oxycodone, morphine and codeine in urine, as described by Candish *et al.* [30].

Table 1
Retention time and GC–MS/MS parameters of opiates in urine sample.

Analyte	Retention time (minutes)	Quantifying transition (*m/z*)	Qualifying transition (*m/z*)	Collision energy (eV)	Dwell time (μs)
TRM	10.68	334.0 – 84.1	334.0 – 210.1	5	50
COD	12.99	371.0 – 234.0	371.0 – 343.0	10	50
COD-d3*	12.99	374.0 – 374.0	–	5	50
MOR	13.20	429.1 – 236.1	429.1 – 287.2	10 (20)	50
MOR-d3*	13.20	432.0 – 432.0	–	5	50
6-AC	13.32	341.0 – 282.2	341.0 – 229.0	10	50
6-MAM	13.54	399.0 – 287.3	399.0 – 340.3	15	50
6-MAM-d3*	13.54	402.4 – 402.4	–	5	50
FNT	14.39	244.0 – 146.1	244.0 – 189.2	15 (10)	50

* Internal standard.

Recently, Da Cunha *et al.* [31] developed a method that determine fentanyl in urine by MEPS and LC/MS-MS. However, no published methods are described for the determination of tramadol and 6-monoacetylmorphine (heroin metabolite). The determination of this latter metabolite is important, since heroin is still the most consumed opiate worldwide [2,32].

This work describes a method for the determination of tramadol (TRM), codeine (COD), morphine (MOR), 6-monoacetylmorphine (6-MAM), 6-acetylcodeine (6-AC) and fentanyl (FNT) in urine employing MEPS for sample clean-up and GC–MS/MS. The method was fully validated and as far as we know, this is the first time that MEPS coupled to gas chromatography/tandem mass spectrometry (GC–MS/MS) is used as sample clean-up for the determination of several opiates in urine samples.

2. Materials and methods

2.1. Reagents and standards

Standard solutions of TRM, COD, 6-AC, MOR, 6-MAM and FNT (Fig. 1), as well as of internal standards (IS) [codeine-d3 (COD-d3) (98.09 % and 99.38%, isotopic and chemical purity, respectively), morphine-d3 (MOR-d3) (96.11 % and 99.60% isotopic and chemical purity, respectively) and 6-acetylmorphine-d3 (6-MAM-d3) (87.65 % and 94.91 % isotopic and chemical purity respectively] were supplied by Sigma-Aldrich (Lisbon, Portugal). Methanol (Merck Co, Darmstadt, Germany), and acetonitrile (Prolabo, Lisbon, Portugal) were of analytical grade. Deionized (DI) water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA). Formic acid (Panreac Química SA, Barcelona, Spain) and ammonium hydroxide (J.T. Baker, Deventer, Holland) were pro-analysis grade. Hydrochloric acid (37% vol.) from Enzymatic (Santo Antão do Tojal, Portugal). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and trimethyl chlorosilane (TMS) were acquired from Macherey-Nagel (Düren, Germany), and the microwave employed in the derivatization step was purchased from Samsung (Lisbon, Portugal).

As for the MEPS technique, the used instruments consisted of a syringe of 250 μL and M1 cartridges needle with a sorbent of 4 mg packing; with a mixture of 80% (weight) C_8 and 20% (weight) SCX.

2.2. Preparation of working solutions

Working solutions were prepared by diluting stock solutions in methanol, except for 6-AC which was prepared in acetonitrile. The final concentrations for all analytes were 2.5 and 0.25 μg/mL. An IS working solution was prepared in methanol at a concentration of 0.5 μg/mL. All stock and working solutions were stored protected from light at 4 °C until use.

2.3. Urine samples

Urine samples (not containing opiates) used for all experiments were provided by laboratory colleagues from CICS-UBI, Covilhã, Portugal. Authentic urine samples were obtained from opioid addicts under surveillance at the Centro de Atendimento ao Toxicodependente—Casas de Santiago (Belmonte, Portugal), and were sent to the Laboratório de Fármaco-Toxicologia from UBImedical (Covilhã, Portugal). The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Casas de Santiago (protocol code CSB-2018–001).

2.4. Gas chromatographic and mass spectrometric conditions

Samples were analysed on an Agilent HP 7890A gas chromatography system coupled with an Agilent 7000B triple quadrupole mass spectrometer operated with a filament current of 35 μA and electron energy of 70 eV in the positive electron ionization mode (Agilent Technologies, Waldbronn, Germany), coupled to a MPS2 autosampler and a PTV-injector (Gerstel, Mülheim an der Ruhr, Germany). The capillary column (30 m × 0.25-mm I.D., 0.25-μm film thickness) with 5% (weight) phenylmethylsiloxane (HP-5 MS) was supplied by J & W Scientific (Folsom, CA, USA).

The chromatographic conditions were the same used by Rosado *et al.,* [33]. Helium was used as a carrier gas at a constant flow of 0.8 mL/min. The chromatographic conditions were set as follows: the oven temperature was held at 90 °C for 2 min, then increased to 300 °C at 20 °C/min (held for 3 min), giving a total run time of 15.5 min. The injection port was set in splitless mode at 240 °C and the transfer line was heated at 280 °C. The flow rate of the collision gas (nitrogen) was set at 2.5 mL/min. The mass detector was operated in the multiple reaction mode (MRM). The MassHunter WorkStation Acquisition Software Rev. B.02.01 (Agilent Technologies) was used for data acquisition. Mass spectrometric conditions were optimized previously, namely in what concerns collision energies and dwell times. Transitions were chosen based on selectivity and abundance to maximize signal-to-noise ratios (Table 1). To maximise sensitivity, the mass spectrometric method was constructed taking into account different windows of detection according to the compounds and their retention times.

2.5. Sample preparation

Urine samples were centrifuged at 2602 × g for 15 min. An acidic hydrolysis to release the conjugates [34] was performed prior to extraction. Hence, 125 μL of 37% (vol.) hydrochloric acid was added to 250 μL of urine. After vortex-mixing, the mixture was heated for 38 min at 115 °C. Then samples were cooled to room temperature and 50 μL of ISs solution was added prior to extraction.

2.6. Microextraction by packed sorbent

The sample clean-up technique was previously optimized resorting to the design of experiments (DOE) statistical tool, and the final conditions were as follows: the M1 MEPS cartridge was sequentially conditioned with 3 cycles of 250 μL of methanol (each) followed by 3 cycles of

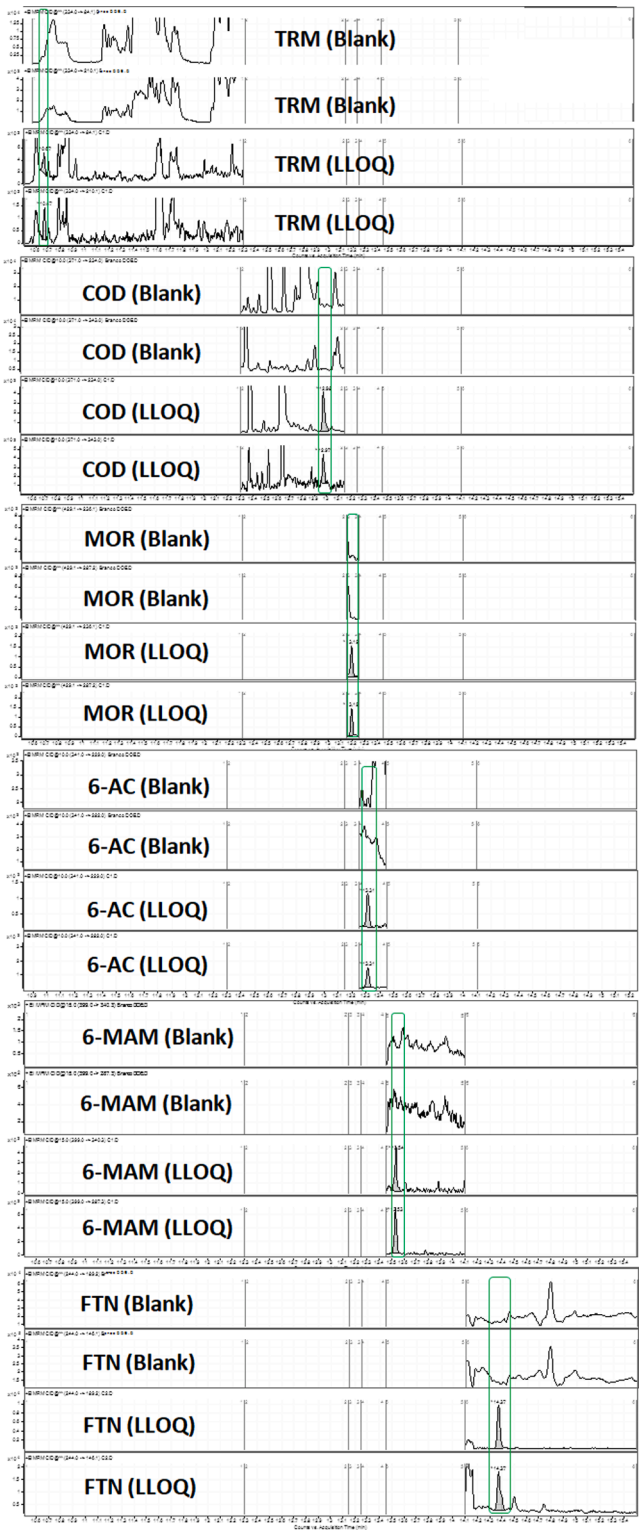


Fig. 2. Extracted ion chromatograms obtained from human urine (without spiked target drugs and with a spiked concentration of 1 ng/mL for all compounds except for fentanyl (10 ng/mL)) by the proposed method.

250 μL of 2 % (vol.) formic acid in water (each). Loading of the sample was performed with 5 cycles of 150 μL. The cartridge was washed with 50 μL of 3.36 % (vol.) formic acid. Subsequently, analytes were eluted using a solution containing 2.36% (vol.) ammonium hydroxide in methanol (4 cycles of 100 μL). Lastly, the extraction sorbent was reconstituted using methanol, followed by water (3 cycles of 250 μL each), so that the cartridges could be re-used.

Table 2
Linear range, calibration curve, correlation coefficients and LLOQ for each opiate in urine samples (n = 5).

Analyte	Weight	Linear range (ng/mL)	Linearity*		R^2*	LLOQ (ng/mL)
			Slope	Intercept		
TRM	1/x	1–1000	0.0004 ± 0.0001	0.0016 ± 0.0012	0.996 ± 0.004	1
COD	1/x	1–1000	0.0010 ± 0.0002	0.0385 ± 0.0320	0.998 ± 0.002	1
MOR	$1/x^2$	1–1000	0.0028 ± 0.0017	0.0078 ± 0.0101	0.992 ± 0.002	1
6-AC	1/x	1–1000	0.0032 ± 0.0005	0.0026 ± 0.0023	0.995 ± 0.003	1
6-MAM	1/x	1–1000	0.0020 ± 0.0002	0.0009 ± 0.0012	0.998 ± 0.002	1
FNT	1/x	10–1000	0.0149 ± 0.0016	− 0.0839 ± 0.0100	0.998 ± 0.001	10

*Mean values ± standard deviation.

Following the extraction procedure, dry extracts were then derivatized with 50 μL of MSTFA with 5% (vol.) TMS, which was microwave-assisted at 800 W for 2 min. Following derivatization, 2 μL of the resulting solution was directly injected into the GC–MS/MS system.

2.7. Method validation

The developed analytical method was fully validated according to the guiding principles of the ANSI/ASB Standard 036 [35], taking into consideration the following parameters: selectivity, linearity and limits of quantification, precision and accuracy, recovery and stability (autosampler, room temperature and freeze–thaw), which were evaluated following a 5-day validation protocol.

3. Results and discussion

3.1. Extraction optimization

In order to increase clean-up yield, it is highly important to evaluate the proper solvents and sorbent suitable to the procedure, taking into consideration the analytes under study. After finding the most appropriate sorbent [17], the choice of solvent, as well as the percentages of acid or base, was based on the study by Rosado *et al.* [33], since the class of drugs in both studies was the same.

The statistical tool (DOE) was used to quickly evaluate the decisive factors that could affect the extraction procedure. Therefore, a full factorial design was used at two-levels (2^k). Five factors were considered, each at the lowest and highest level. These factors were number of strokes of the sample load step [5 (lowest) and 15 (highest)], number of wash cycles (1–3 × 50 μL) and number of elution cycles [4 (lowest) and 8 (highest) × 100 μL]. This evaluation was performed with blank urine samples spiked at 10 μg/mL. After extraction, 50 μL of IS solution was added. None of the variables was considered significant in terms of response (data not shown), and a response surface methodology was used. Hence, the final and best conditions to perform this clean-up procedure were three cycles of 250 μL of methanol followed by three cycles of 250 μL 2% (vol.) formic acid for the conditioning step; 5 cycles of 150 μL for sample loading; wash with 1 × 50 μL of 3.36% (vol.) formic acid; finally, four cycles of 100 μL of 2.36% (vol.) ammonium hydroxide in methanol for the elution step.

Table 3
Intra-day. inter-day and intermediate precision (CV%) and accuracy (bias %)) of the proposed method for the target drugs spiked in urine samples.

Analyte	Concentration (ng/mL)	Inter-day (n = 5)			Intra-day (n = 5)			Intermediate (n = 15)		
		Measured	CV (%)	Bias (%)	Measured	CV (%)	Bias (%)	Measured	CV (%)	Bias(%)
TRM	1	1.0 ± 0.1	13.5	97.0	–	–	–	–	–	–
	10	9.9 ± 1.2	11.8	101.0	11.26 ± 1.3	10.5	87.4	10.5 ± 0.9	8.2	95.5
	25	22.6 ± 1.9	8.4	109.8	–	–	–	–	–	–
	50	52.7 ± 5.8	11.0	94.5	–	–	–	–	–	–
	250	215.0 ± 13.6	6.3	114.0	229.8 ± 11.7	5.09	108.1	–	–	–
	400	–	–	–	–	–	–	391.3 ± 38.	9.7	102.2
	500	516.1 ± 30.7	5.9	96.8	–	–	–	–	–	–
	750	786.1 ± 53.3	6.8	95.2	651.1 ± 73.6	11.30	113.2	–	–	–
	800	–	–	–	–	–	–	794.6 ± 116.4	14.7	100.7
	1000	967.2 ± 57.8	6.0	103.3	–	–	–	–	–	–
COD	1	0.9 ± 0.1	11.7	106.0	–	–	–	–	–	–
	10	9.4 ± 0.9	10.0	106.2	8.8 ± 0.7	7.7	111.9	9.8 ± 1.4	13.8	101.7
	25	27.5 ± 1.4	4.9	89.9	–	–	–	–	–	–
	50	51.4 ± 5.4	10.5	97.2	–	–	–	–	–	–
	250	243.6 ± 17.8	7.3	102.5	253.7 ± 3.4	1.4	98.5	–	–	–
	400	–	–	–	–	–	–	391.8 ± 40.0	10.3	102.0
	500	523.2 ± 13.9	2.7	95.4	–	–	–	–	–	–
	750	766.9 ± 12.2	1.6	97.8	773.2 ± 71.2	9.3	96.9	–	–	–
	800	–	–	–	–	–	–	780.9 ± 115.6	14.8	102.4
	1000	967.8 ± 32.8	3.4	103.2	–	–	–	–	–	–
MOR	1	1.0 ± 0.0	0.8	100.0	–	–	–	–	–	–
	10	9.6 ± 0.9	9.4	103.3	10.6 ± 0.9	8.8	94.5	10.1 ± 1.1	11.1	99.0
	25	25.6 ± 1.5	6.0	97.5	–	–	–	–	–	–
	50	45.9 ± 1.8	3.9	108.3	–	–	–	–	–	–
	250	243.0 ± 24.3	10.0	102.8	235.3 ± 8.7	3.7	105.9	–	–	–
	400	–	–	–	–	–	–	378.2 ± 48.6	12.8	105.5
	500	512.5 ± 27.9	5.4	97.5	–	–	–	–	–	–
	750	721.9 ± 79.0	10.9	103.6	703.4 ± 87.9	12.5	106.2	–	–	–
	800	–	–	–	–	–	–	926.2 ± 50.2	13.6	84.2
	1000	1118.5 ± 47.1	4.2	88.2	–	–	–	–	–	–
6-AC	1	1.1 ± 0.0	2.6	89.0	–	–	–	–	–	–
	10	9.3 ± 0.7	7.0	107.3	8.7 ± 0.7	7.7	113.5	10.2 ± 1.3	12.7	98.4
	25	21.9 ± 2.1	9.5	112.4	–	–	–	–	–	–
	50	50.8 ± 6.6	12.9	98.3	–	–	–	–	–	–
	250	269.4 ± 23.3	8.7	92.2	288.1 ± 11.8	4.1	84.8	–	–	–
	400	–	–	–	–	–	–	436 ± 51.7	11.9	91.0
	500	511.8 ± 31.6	6.2	97.6	–	–	–	–	–	–
	750	787.1 ± 22.8	2.9	95.1	875.8 ± 22.2	2.5	83.2	–	–	–
	800	–	–	–	–	–	–	773.5 ± 111.9	14.5	103.3
	1000	948.7 ± 48.1	5.1	105.1	–	–	–	–	–	–
6-MAM	1	1.0 ± 0.1	11.3	99.0	–	–	–	–	–	–
	10	10.2 ± 0.6	6.0	98.5	11.6 ± 0.1	0.9	84.0	10.3 ± 1.0	9.4	97.1
	25	24.5 ± 1.2	5.7	102.0	–	–	–	–	–	–
	50	48.3 ± 4.2	8.8	103.3	–	–	–	–	–	–
	250	253.5 ± 9.1	3.7	98.6	286.6 ± 14.9	5.2	85.4	–	–	–
	400	–	–	–	–	–	–	368.8 ± 31.0	8.4	107.8
	500	509.6 ± 20.3	4.0	98.1	–	–	–	–	–	–
	750	753.1 ± 60.7	8.1	99.6	859.4 ± 18.6	2.2	85.4	–	–	–
	800	–	–	–	–	–	–	769 ± 48.3	6.3	103.9
	1000	986.4 ± 29.2	3.0	101.4	–	–	–	–	–	–
FNT	10	11.7 ± 0.4	3.1	82.7	11.5 ± 0.5	4.3	84.9	11.5 ± 0.4	3.8	84.6
	25	22.9 ± 2.7	11.8	108.4	–	–	–	–	–	–
	50	46.8 ± 5.9	12.5	106.4	–	–	–	–	–	–
	250	239.4 ± 19.2	8.0	104.2	207.1 ± 17.8	8.6	117.2	–	–	–
	400	–	–	–	–	–	–	438.4 ± 18.6	4.3	90.4
	500	495.2 ± 22.1	4.5	101.0	–	–	–	–	–	–
	750	780.8 ± 15.5	2.0	95.9	782.4 ± 35.7	4.6	95.7	–	–	–
	800	–	–	–	–	–	–	887.0 ± 37.1	2.2	89.1
	1000	996.8 ± 16.2	1.6	100.3	–	–	–	–	–	–

All concentrations in ng/mL; Mean values ± standard deviation CV - coefficient of variation.

3.2. Method validation

3.2.1. Selectivity

The selectivity of an analytical method is the ability of detecting the target analyte while assessing the presence of endogenous interferences that could conflict at the retention times and selected transitions of the target analytes. Selectivity was studied considering the ANSI/ASB Standard 036 recommendations for acceptance [35], and the described method was considered selective given that no interferences were observed at the retention time and respective monitored ions. Fig. 2 represents a comparison between a blank urine sample for the target opiates and a sample spiked at the lower limit of quantification (LLOQ). Both samples were analysed by the herein described method.

3.2.2. Calibration curves and limits

The method was found linear in the range of 1–1000 ng/mL for all compounds, except for FNT (10–1000 ng/mL). Spiked samples were analysed using the above-described MEPS clean-up procedure and

Table 4
MEPS recovery (%) of the target opiates in urine samples (n = 3).

Analyte	Concentration (ng/mL)*		
	10	100	800
TRM	61.3 ± 9.4	52.6 ± 7.3	50.1 ± 8.2
COD	64.3 ± 6.8	46.3 ± 4.5	31.4 ± 2.7
MOR	17.1 ± 2.5	17.0 ± 0.8	12.3 ± 1.2
6-AC	57.4 ± 11.1	72.3 ± 15.5	79.9 ± 27.0
6-MAM	57.0 ± 6.5	51.5 ± 4.7	36.4 ± 4.4
FNT	85.0 ± 32.3	107.8 ± 47.7	74.5 ± 26.5

*Mean values ± standard deviation.

linearity was evaluated using eight calibrators (seven calibrators in the case of FNT) with five replicates. A determination coefficient (R^2) higher than 0.99 and the accuracy of the calibrators in the range of ± 15% from the nominal value (except for the LLOQ, where ± 20% range was accepted) were adopted as acceptance criteria. The adopted calibration ranges were wide, and as such weighted least squares regressions had to be used to compensate for heteroscedasticity (1/x for all compounds, except for MOR, $1/x^2$). Table 2 shows the calibration obtained data. The limits obtained in this method can be considered adequate, when comparing these results with those obtained in other studies. For example, Bévalot et al. [11] have obtained LLOQs of 12.5 ng/mL for MOR and 6-MAM using SPE as extraction technique, hence supporting the advantages of MEPS in this case. On a different study [13], where Dispersive Liquid-Liquid Microextraction (DLLME) was used, a LOD of 5 ng/mL was obtained for 6-MAM, while in the presented method, the value is five times lower. Shamsipur *et al.* [12] have obtained higher LODs for MOR and COD, namely 7.0 and 10.0 ng/mL, using 5 mL of urine, while the LODs herein presented are, at least 1 ng/mL for these compounds. A published method using MEPS to determine COD, MOR and oxycodone in urine samples [30] has obtained LOD values of 2 and 5 ng/mL for COD and MOR, respectively. However, it is important to note that in this work LOD was not systematically studied, but since the LOQs are within 1–10 ng/mL we can infer that, at minimum, the LODs are also in this same range, or lower than the compared studies made herein, proving this method's suitability. It should be stated, however, that the LOQ obtained for fentanyl (10 ng/mL) can be considered quite high, particularly considering the concentrations of this analyte usually present in biological samples. It was not possible to obtain linearity for

Table 7
Analysis of authentic urine samples.

Sample	Analyte (s)	Concentration (ng/mL)
1	MOR	6275.0
	6-MAM	10.7
2	MOR	182.2
	6-MAM	6.03
3	MOR	30.6
	6-MAM	1.1
4	COD	536.0
	MOR	14507.9
	6-MAM	3.6
5	MOR	533.7
	6-MAM	7.9
6	MOR	2736.1
7	MOR	734.9

Table 5
Autosampler, room temperature and freeze/thaw stability and accuracy (n = 3) of each opiate in urine samples.

Analyte	Concentration (ng/mL)	Autosampler stability (n = 3)			Room temperature stability (n = 3)			Freeze/thaw (n = 3)		
		Measured	Bias (%)	CV (%)	Measured	Bias (%)	CV (%)	Measured	Bias (%)	CV (%)
TRM	10	10.4 ± 0.1	95.8	14.1	11.3 ± 0.4	86.9	3.3	10.9 ± 1.1	8.5	10.5
	400	410.5 ± 60.8	97.4	14.8	404.9 ± 5.1	98.8	1.3	376.7 ± 48.1	−0.1	12.8
	800	720.3 ± 93.4	110.0	12.9	783.8 ± 31.7	102.0	4.1	693.2 ± 50.5	−13.4	7.1
COD	10	8.9 ± 0.8	110.7	8.8	n.d	n.d	n.d	8.8 ± 0.2	−12.3	1.8
	400	411.1 ± 36.5	97.2	8.9	367.0 ± 15.2	108.3	4.2	374.3 ± 49.8	−6.4	13.3
	800	677.9 ± 13.6	115.3	2.0	702.0 ± 17.4	112.1	2.5	718.0 ± 54.2	−10.3	7.6
MOR	10	9.7 ± 0.9	102.5	9.0	10.0 ± 2.0	100	20.4	11.5 ± 0.3	14.9	2.3
	400	382.0 ± 43.2	104.5	11.3	389.2 ± 67.6	102.7	17.4	446.3 ± 15	11.6	3.4
	800	885.4 ± 38	89.3	4.3	811.3 ± 142.2	98.6	17.5	846.0 ± 104	5.8	12.1
6-AC	10	10.9 ± 1.5	91.4	14.0	8.4 ± 0.4	115.9	4.32	8.2 ± 0.1	−17.8	1.5
	400	409.0 ± 49.8	97.8	9.8	350.1 ± 13.3	112.5	3.8	387.6 ± 58.4	0.0	15.1
	800	845.2 ± 102.6	94.4	12.1	794.0 ± 16.2	108.3	2.1	703.2 ± 8.1	−0.1	8.1
6-MAM	10	10.5 ± 0.8	95.4	8.0	9.3 ± 0.8	107.2	8.5	9.8 ± 1.2	0.0	11.7
	400	410.0 ± 13.1	97.5	3.2	452.7 ± 5.9	86.8	1.3	379.9 ± 11.2	−0.1	3.0
	800	760.1 ± 53.6	105.0	7.1	893.4 ± 28.1	88.3	3.1	749 ± 81.7	−0.1	11.0
FNT	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	400	412.7 ± 59.9	96.8	14.5	339.1 ± 24.8	100.2	7.3	441.1 ± 17.4	10.3	4.0
	800	744.5 ± 74.4	106.9	9.6	767.9 ± 74	104	9.6	810.7 ± 49	1.3	6.0

All concentrations in ng/mL; Mean values ± standard deviation CV: coefficient of variation; n.d. not detected.

Table 6
Evaluation of the dilution integrity (n = 3) at 2000 ng/mL of the target compounds.

Compounds	Dilution factor								
	1:2			1:5			1:10		
	Measured (ng/mL)	CV (%)	Accuracy (%)	Measured (ng/mL)	CV (%)	Accuracy (%)	Measured (ng/mL)	CV (%)	Accuracy (%)
TRM	1982.1 ± 140.5	7.1	100.9	2148.0 ± 166.9	7.8	92.6	2180.6 ± 118.9	5.5	91.0
COD	1770.3 ± 100.7	5.7	111.5	1779.4 ± 82.4	4.6	111.0	1756.1 ± 47.0	2.7	112.2
MOR	2188.9 ± 125.0	5.7	90.6	2058.67 ± 89.0	4.3	97.1	2082.4 ± 225.0	10.8	95.9
6-AC	2185.1 ± 49.0	2.2	90.8	2224.56 ± 4.6	0.2	88.8	2262.8 ± 32.6	1.4	86.9
6-MAM	1971.3 ± 28.2	1.4	101.4	2046.6 ± 49.1	2.4	97.7	2103.6 ± 152.2	7.2	94.8
FNT	2127.7 ± 90.7	4.3	93.6	2254.5 ± 128.5	5.7	87.3	2123.9 ± 74.6	3.5	93.8

All concentrations in ng/mL; Mean values ± standard deviation; CV: coefficient of variation.

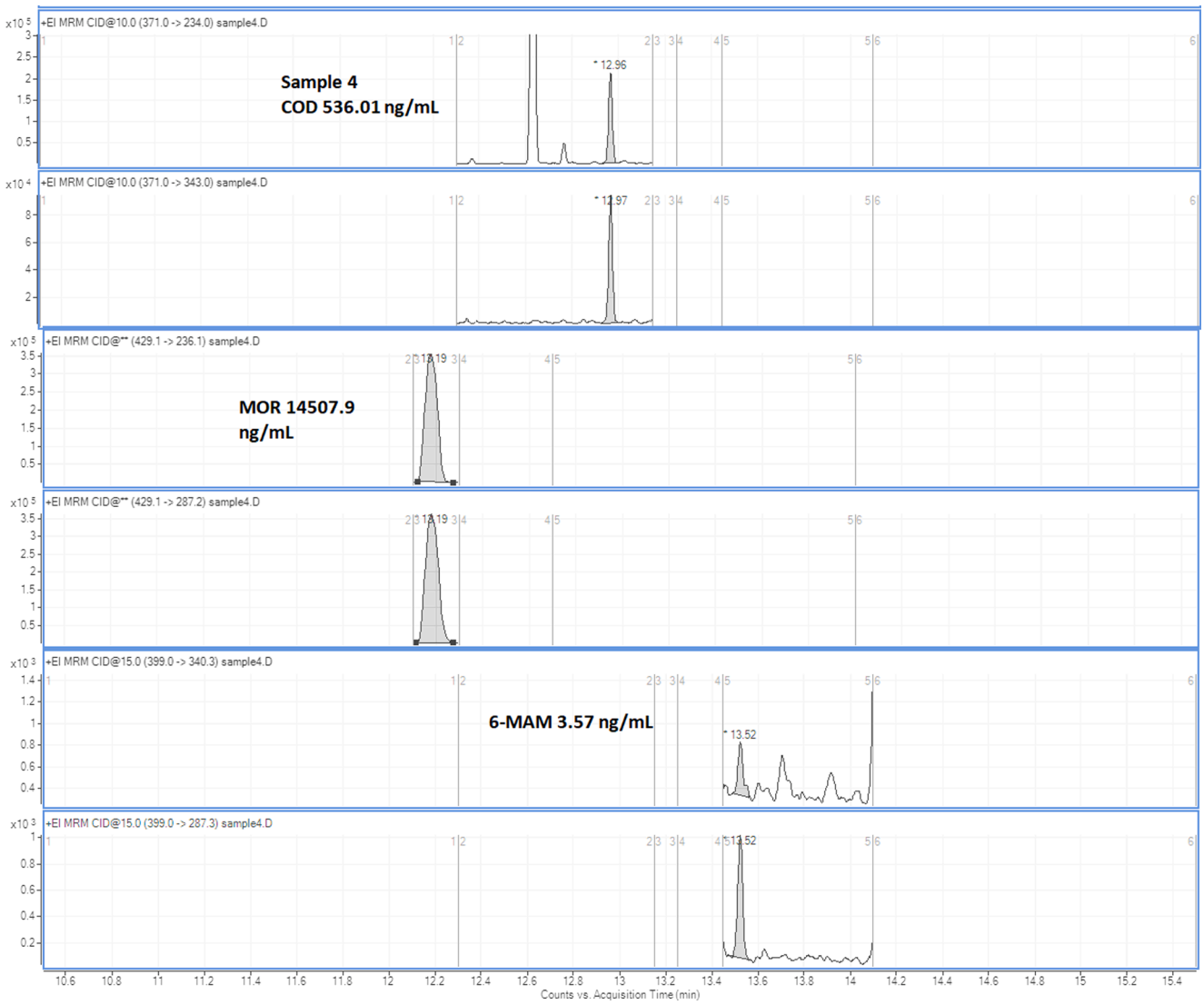


Fig. 3. Extracted ion chromatograms obtained after analysis of authentic urine sample positive for MOR, 6-MAM and COD (sample number 4) by the proposed method.

this analyte starting from 1 ng/mL, despite the fact that this concentration is detectable by our method. This would imply that in some authentic samples the determination of fentanyl would be qualitative in nature, rather than quantitative. Da Cunha *et al.* [31] developed a method to determine synthetic fentanyl opioids. The LLOQ was also 10 ng/mL. However, there are no published methods for the determination of 6-MAM and TRM in urine using MEPS. It is important to note the research and interest in all the other compounds of this work, since they are still some of the most consumed opiates worldwide, or are used as markers of heroin consumption [36–38].

3.2.3. Precision and accuracy

The acceptance criteria for precision were coefficients of variation (CV) equal or lower than 15% for all concentration levels, whilst accuracy was identified in terms of mean relative error (RE) / BIAS in the range of ± 15% for all concentrations, aside from the LLOQ (±20%).

Accuracies between 83 and 114% and 83 and 117%, were obtained for the inter and intraday precision studies, respectively.

Intermediate precision and accuracy were studied using quality control (QCs) samples at three concentrations levels (10; 400 and 800 ng/mL) in triplicates during the 5-day validation protocol (n = 15), simultaneously with the calibration curve. Accuracy was once again satisfactory, with values between 84 and 108%. Table 3 presents these data.

3.2.4. Recovery studies

According to the followed guidelines, it is usually required that analyte recoveries ought to be evaluated at a low and a high concentration level. Nonetheless, in this work we have used three concentration levels: 10, 100, and 800 ng/mL for all opiates. The obtained recoveries are presented in Table 4. The average extraction recovery (n = 3) was higher than 57% at the lowest concentrations, 46 % at the intermediate concentrations and 31% at the highest concentrations, except for morphine, for which the average extraction efficiency was 15 % at all tested concentrations. Bévalot *et al.* [11] obtained similar results regarding MOR recovery using SPE, both at low and high concentration levels; however, the used matrix was bone marrow, hence no assumption can be made. Prata *et al.* [26] have used MEPS to determine MOR, COD, and 6-MAM in blood matrices, and have obtained even lower percentages of absolute recovery for all compounds. Moreover, Li *et al.* [39] have obtained absolute recoveries of 8 % and 17 % for MOR and COD, respectively, using MEPS. Abdel-Rehim *et al.* [20] studied and developed MEPS and all factors associated with it. In this study, it is shown that solvent percentage in the washing step plays an important role on the extraction

efficiency. It is likely that the use of 3.36% (vol.) formic acid was responsible for the low recovery percentage of MOR, albeit all other compounds had acceptable recoveries.

3.2.5. Stability

In this work, stability evaluation was divided into autosampler, room temperature and freeze/thaw stability. Stability samples were compared to freshly prepared samples, and the analytes would be considered stable as long as the CVs between the two sets of samples were below 15%.

Autosampler stability was assessed by analysing the extracts at three different concentration levels (10; 400 and 800 ng/mL) ($n = 3$) after kept in the autosampler for 24 h. CVs were lower than 15%. As for room temperature stability, samples were left at this temperature and 24 h later the extraction was performed. COD and FNT were not stable at 10 ng/mL. However, for all other analytes, the presented CVs were lower than ± 20 %. Concerning freeze–thaw stability, samples were subjected to three cycles of freeze (stored at − 20 °C) and thaw (at room temperature) before extraction. All opiates demonstrated CVs values lower than 16%, except FNT, which was not stable at 10 ng/mL. This data is shown in Table 5.

3.2.6. Dilution integrity

Whenever the quantification of authentic samples does not meet the calibration curve (e.g. concentration values exceeding the upper limit of quantification (ULOQ)), it is necessary to evaluate the effect of sample dilution [35]. Thus, three dilution factors (1:2, 1:5, and 1:10) were tested, using a concentration of 2000 ng/mL for all analytes (the dilution was prepared with blank urine samples). The results are presented in Table 6, and CVs were below 11% for all compounds with an accuracy between 87 and 112%.

3.2.7. Method applicability

Method applicability was verified by analysing ~~of~~ several authentic urine samples obtained from actual opioid users. It was possible to analyse 7 different samples and find positive results for COD, MOR and 6-MAM. The latter indicate the consumption of heroin [40]. Overall, 6-MAM concentration levels found in urine samples ranged from 1.1 to 10.7 ng/mL, whereas MOR from 30.6 to 14507.9 ng/mL and finally COD was only found in sample number 4 at a concentration of 536.0 ng/mL.

Table 7 shows some of these results and the chromatogram obtained from the analysis of sample number 4 is present in Fig. 3.

4. Conclusion

A high-throughput GC–MS/MS method for the determination of selected opiates in urine samples was developed and fully validated. MEPS procedure was fully optimized, proving to be highly efficient for the extraction of opiates from urine samples, allowing overall good recoveries, requiring a small amount of sample volume (250 µL). The limits of quantification were 1 ng/mL for all analytes except for fentanyl (10 ng/mL). This is the first developed method coupling MEPS to GC–MS/MS to determine heroin metabolites and TRM in urine samples. Overall, the cost effectiveness, rapidity, easiness and the re-utilization of the sorbent of the MEPS procedure, alongside with the use of GC–MS/MS instrumentation allowed the development of a method for application in clinical toxicology laboratory analysis.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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