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Development and validation of a liquid chromatography–mass spectrometry assay for the determination of opiates and cocaine in meconium

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Abstract

A procedure based on liquid chromatography–mass spectrometry (LC–MS) is described for determination of 6-monoacetylmorphine, morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, cocaine, benzoylecgonine and cocaethylene in meconium using nalorfine as the internal standard. The analytes are initially extracted from the matrix by methanol (6-monoacetylmorphine, morphine, codeine, cocaine, benzoylecgonine and cocaethylene) or 0.01 *M* ammonium hydrogen carbonate buffer (morphine-3-glucuronide, morphine-6-glucuronide). Subsequently a solid-phase extraction with Bondelut Certify columns (6-monoacetylmorphine, morphine, codeine, cocaine, benzoylecgonine and cocaethylene) or ethyl solid-phase extraction columns (morphine-3-glucuronide, morphine-6-glucuronide) was applied. Chromatography was performed on a C_8 reversed-phase column using a gradient of acetic acid 1%–acetonitrile as a mobile phase. Analytes were determined in LC–MS single ion monitoring mode with atmospheric pressure ionisation-electrospray (ESI) interface. The method was validated in the range 0.005–1.00 μg/g using 1 g of meconium per assay and applied to analysis of meconium in newborns to assess fetal exposure to opiates and cocaine.
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1. Introduction

Drug abuse during pregnancy is a major problem because of the associated high incidence of perinatal complications and high morbidity and mortality rates of newborns. Various neonatal birth defects are thought to be related to fetal exposure to drugs, chemical agents and other xenobiotics [1].

In utero exposure to heroin can result in a fluctuating cycle of fetal intoxication and withdrawal and neonatal abstinence syndrome [2]. In particular increased startle reflex, tremors, inability to self-quiet, abnormal sleep patterns, fever and seizures characterize this syndrome [3]. On the other hand, neonatal complications following in utero cocaine exposure include intrauterine growth retardation,

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hypertension, small head size, reduced birth weight and fetal death [4].

Accurate assessment of fetal exposure to drugs of abuse through the objective measure of biomarkers could be of major importance since it provides the basis for appropriate treatment and adequate follow-up of the newborn, which can present symptoms of drug withdrawal. Furthermore, information regarding the real prevalence of drugs of abuse use during pregnancy could also be disclosed.

During the last decades, urine has been the specimen of choice for screening drugs of abuse at delivery [5]. However, since drugs present in the urine reflect consumption or exposure during the preceding 1–4 days [3], abstinence from drug use by the mothers for several days prior to delivery may produce a negative result [5]. Recently, investigators have reported the utility of meconium as a test specimen in the screening of newborns for drug abuse [6–8]. Meconium is the first fecal matter passed by a neonate. Its formation starts between the 12th and 16th week of gestation and usually accumulates in fetal bowel until birth and is passed by the neonate 1–5 days after birth. For this reason, meconium analysis extends the window of detection of drug use to approximately the last 20 weeks of gestation, being more informative than urine for the detection of drug exposure in pregnancy [9,10].

Immunochemical assays have been described as useful analysis methods for screening for the presence of opiates and cocaine in meconium specimens [1]. On the other hand, chromatographic methods have been applied for confirmation purposes and to investigate the disposition of parent drugs and metabolites in meconium [1].

In particular, two assays based on high-performance liquid chromatography coupled with UV detection [11,12] analyzed a limited number of analytes (i.e. cocaine, benzoylecgonine and morphine). Other studies suggested the use of gas chromatography–mass spectrometry (GC–MS) [13–16] or liquid chromatography–tandem mass spectrometry (LC–MS–MS) [17] and a broader spectrum of heroin and cocaine metabolites was examined. The linearity of these methods ranging between 0.005 and 10 μg/g for cocaine and metabolites and between 0.005 and 2 μg/g for opiates, was adequate for concentrations of drugs and metabolites found in meconium specimens. However, none of these methods simultaneously determined opiates and cocaine, using the same extraction and analysis procedure, nor included the detection of morphine glucuronides.

Within the framework of a pilot study aimed at estimating chronic fetal exposure to pharmaceuticals, drugs of abuse and tobacco smoke in Italy and Spain, the development of more easily used, sensitive and specific methods for the determination of different analytes in this biological matrix were found to be necessary. A combined HPLC–mass spectrometric method was the favorite choice due to its versatility, feasibility for simultaneous determination of lipophilic parent drugs and hydrophilic metabolites and because of simplified sample preparation.

The present paper describes a sensitive and selective analytical method based on HPLC–MS using electrospray ionization detection for the determination of 6-monoacetylmorphine, morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, cocaine, benzoylecgonine and cocaethylene in meconium using nalorfine as the internal standard. This analytical method meets the accepted criteria for bioanalytical method validation [18,19].

2. Experimental

2.1. Solvents and chemicals

Heroin free base, 6-monoacetylmorphine–HCl (6-MAM), morphine–HCl, codeine–HCl, nalorphine–HCl, cocaine–HCl, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and benzoylecgonine tetrahydrate (BEG) were purchased from Salars (Como, Italy). Cocaethylene metanolic solution (100 μg/ml) was a gift from Professor J. Segura (IMIM, Barcelona, Spain). Bond Elut Certify solid-phase extraction (SPE) columns were from Varian (Palo Alto, CA, USA) and ethyl solid-phase extraction columns were from J.T. Baker (Milan, Italy). Acetonitrile was obtained from Panreac Quimica (Barcelona, Spain) and methyl alcohol was obtained from Riedel-de Haën (Germany). All other reagents were of analytical grade and from Carlo Erba (Milan, Italy).

2.2. Meconium samples

Meconium samples came from the Hospital del Mar in Barcelona (the fourth largest hospital in the city), Spain as part of the ‘‘Meconium Project’’ Italian–Spanish joint study. The study protocol, which was approved by the local ethical committee (CEIC-IMAS) and by the Spanish Ministry of Health, started at the beginning of 2002, and meconium specimens from at least 1000 newborns will be collected and analyzed. Once collected, samples are aliquoted and stored at −20 °C until analysis.

2.3. Instrumentation

The HPLC/MSD system consisted of an Agilent 1100 series (a G1312A binary pump, a G1322A degasser, a ALS G1329A autosampler, a G1946D mass spectrometry detector; all from Agilent Technologies, Palo Alto CA, USA). Masses were acquired on a Agilent spectrometer equipped with an atmospheric pressure ionisation-electrospray (ESI) interface.

2.4. Liquid chromatography–mass spectrometry

Chromatographic separation was achieved at ambient temperature using a Zorbax Eclipse XDB-C_8 column (150×4.6 mm; Agilent Technologies, Palo Alto, CA, USA) and linear gradient elution consisting of 97% acetic acid (1% aqueous solution) and 3% acetonitrile at the start of the run, changing to 73% acetic acid (1% aqueous solution) and 27% acetonitrile in 11 min, and returning to initial conditions in 14 min. The flow-rate was 1 ml/min. All chromatographic solvents were degassed with helium before use. The injection volume was 20 μl and the column temperature was set at 30 °C.

The mass spectrometer was operated in positive electrospray ionization mode and selected ion monitoring (SIM) acquisition mode. The following ESI conditions were applied: drying gas (nitrogen), 13.0 l/min; nebulizer gas (nitrogen), 40 p.s.i.; gas temperature, 350 °C; capillary voltage at 3000 V and fragmentor (the exit end of the capillary) at 250 V for opiates and 200 V for cocaine and metabolites. Qualifying ions were: m/z 328, 268 and 172 for 6-MAM; m/z 286, 227 and 209 for morphine; m/z 462, 286 and 257 for M3G and M6G; m/z 300, 241 and 181 for codeine; m/z 312, 212 and 152 for nalorphine; m/z 304, 212 and 182 for cocaine; m/z 290, 190 and 168 for BEG; and m/z 318, 196 and 327 for cocaethylene. Ion ratio acceptance criterion was a deviation ≤20% of the average of ion ratios of all the calibrators. Ions m/z 328 for 6-MAM, m/z 286 for morphine, m/z 462 for M3G, m/z 462 for M6G, m/z 300 for codeine, m/z 312 for nalorphine, m/z 304 for cocaine, m/z 290 for BEG, and m/z 318 for cocaethylene were selected for quantification.

2.5. Preparation of calibration standards and quality control samples

Stock standard solutions (1 mg/ml) were prepared in methanol. Working solutions at concentrations of 10 and 1 μg/ml were prepared by dilution of the stock standards with methanol and stored at −20 °C until analysis. The internal standard (I.S.) working solution was used at a concentration of 10 μg/ml.

Calibration standards containing 1, 0.5, 0.1, 0.05, 0.01, 0.005 μg/g meconium were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 1 g of pre-checked drug-free meconium pool. Quality control samples of 0.85 μg/g (high control), 0.12 μg/g (medium control), 0.012 μg/g (low control 1) and samples at the limit of quantification (LOQ) of each analyte were prepared in drug-free meconium, aliquoted and stored at −20 °C. They were included in each analytical batch to check calibration, accuracy and precision, and stability of samples under storage conditions.

2.6. Sample preparation

A 1-g amount of meconium with 10 μl of I.S. working solution was transferred into 15-ml screw-capped glass tubes and 4 ml of methanol were added. The tubes were placed in a horizontal shaker for 20 min. After centrifugation at 2000 rpm for 10 min the organic layer was transferred to another tube and the solvent was evaporated to dryness at 30 °C under a

nitrogen stream. The residue was dissolved in 2 ml 0.1 *M* phosphate buffer, pH 6.0 and applied on a Bond Elut Certify solid-phase extraction (SPE) column, which had been preconditioned with 3 ml methanol, 3 ml water and 1 ml 0.1 *M* phosphate buffer, pH 6.0.

The column was washed with 3 ml water, 1 ml potassium acetate buffer (pH 3), 3 ml ethyl acetate: hexane (1:1) and 3 ml methanol. The analytes (6-MAM, morphine, codeine, cocaine, benzoylecgonine and cocaethylene) were eluted with 3 ml dichloromethane:isopropyl alcohol (80:20) with 2% ammonium hydroxide. The eluent was evaporated to dryness under a stream of nitrogen and redissolved in 50 μl 1% acetic acid. In case of M3G and M6G, the same portion of meconium left from methanol extraction was added with 2 ml 0.01 *M* ammonium hydrogen carbonate buffer, pH 9.3 and mixed in a horizontal shaker for 20 min. After centrifugation at 2000 rpm for 10 min, the buffer layer was directly applied to ethyl solid-phase extraction columns using a procedure described in the literature [23]. The eluent was evaporated to dryness under a stream of nitrogen and redissolved in 50 μl 1% acetic acid. The two portions of 1% acetic acid, coming from the two different extractions, were combined and a 20-μl volume was injected into HPLC column.

2.7. Method validation

Prior to application to real samples, the method was tested following a 3-day validation protocol. Selectivity, recovery, linearity, precision, accuracy, limits of detection and quantification were assayed.

2.7.1. Selectivity

A total of 20 meconium samples from newborns, whose mothers had a negative history of illicit drug exposure during pregnancy, were extracted and analysed for assessment of potential interferences from endogenous substances. The apparent response at the retention times of the analytes under investigation was compared to the response of analytes at the limit of quantification. Furthermore, potential interferences from principal amphetamines and related substances (amphetamine, methylamphetamine, 3,4-methylendioxyamphetamine 3,4-methylendioxymethamphetamine, ephedrine, norephedrine), cannabinoids (9-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol), benzodiazepines (clorazepate, diazepam, lorazepam, oxazepam, alprazolam, triazolam), and antidepressants (imipramine, desipramine, clomipramine, desmethylclomipramine, amitriptyline, nortriptyline, fluoxetine, norfluoxetine, paroxetine) were also evaluated by spiking 1 g of pre-checked drug-free meconium pool with 1 μg of the aforementioned substances and carrying out the entire procedure.

The potential for carry-over was investigated by injecting a blank meconium, with added internal standard, immediately after the highest point of the calibration curve on each of the 3 days of validation protocol and measuring the area of eventual peaks present at the retention times of analytes under investigation.

2.7.2. Recovery

Analytical recoveries were calculated by comparing the peak areas obtained when calibration samples were analyzed by adding the reference substances and the internal standards in the extract from drug-free meconium prior to and after the extraction procedure. The recoveries were assessed at three concentration levels (1, 0.05 and 0.005 μg/g) using four replicates at each level.

For an evaluation of the matrix effect, the peak areas of extracted blank samples spiked with standards after the extraction procedure were compared to the peak areas of pure diluted substances.

2.7.3. Calibration and sample quantification

Calibration curves were tested over the quantification limit: 1 μg/g range for all the analytes. Peak area ratios between compounds and I.S. were used for calculations. A weighted (1/concentration) least-square regression analysis was used (SPSS, version 9.0.2 for Windows). Five replicates of blank samples were used for calculating the limits of detection (LOD) and quantification. Standard deviation (SD) of the analytical background response was used to determine the detection limit (LOD: 3 SD) and the quantification limit (LOQ: 10 SD).

2.7.4. Precision and accuracy

A total of five replicates at three different concentration standards (LOQ, 0.12, and 0.85 μg/g)

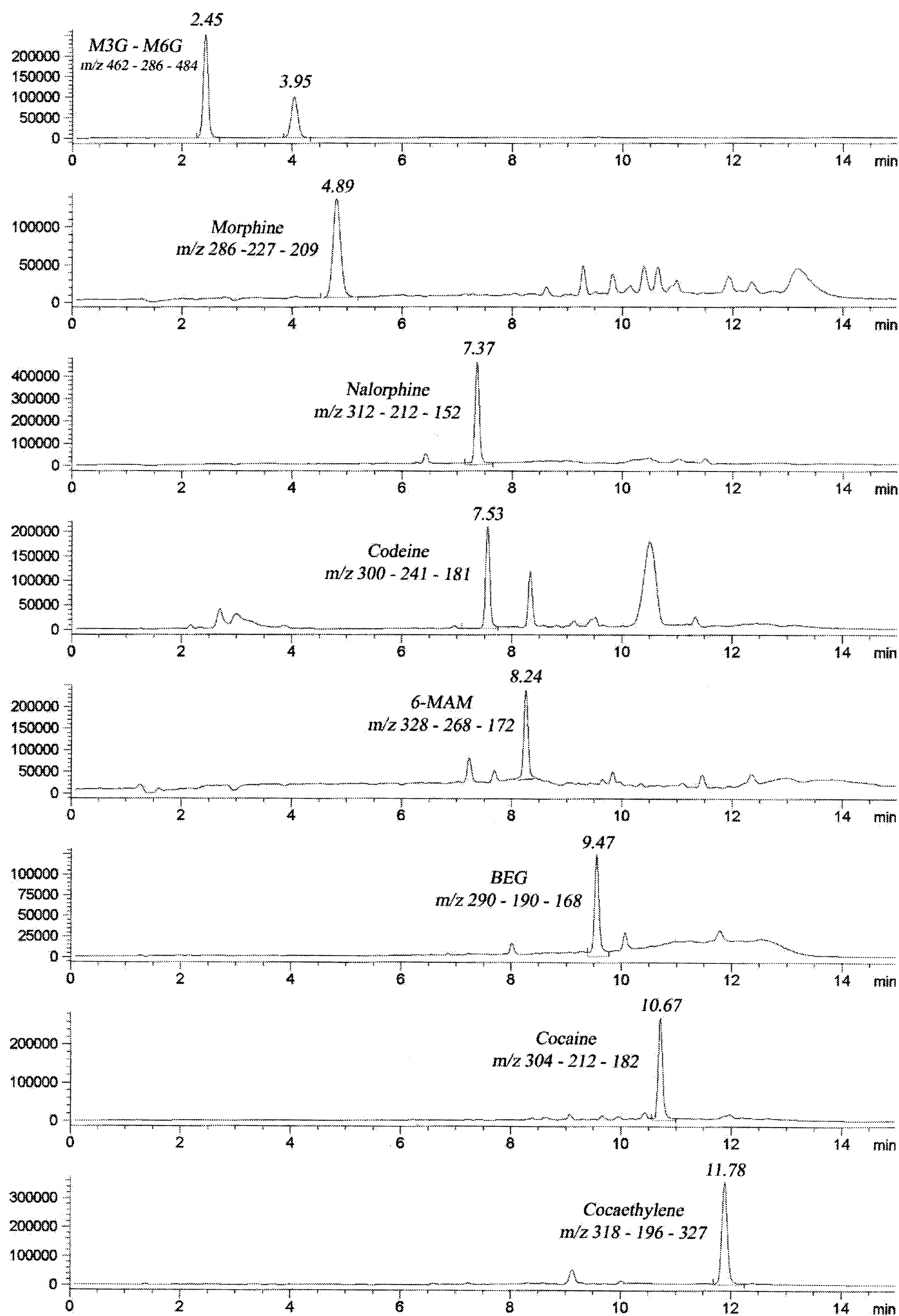

Fig. 1. SIM chromatogram of an extract of 1 g drug-free meconium sample spiked with 0.05 μg 6-MAM, morphine, M3G, M6G, codeine, cocaine, BEG and cocaethylene.

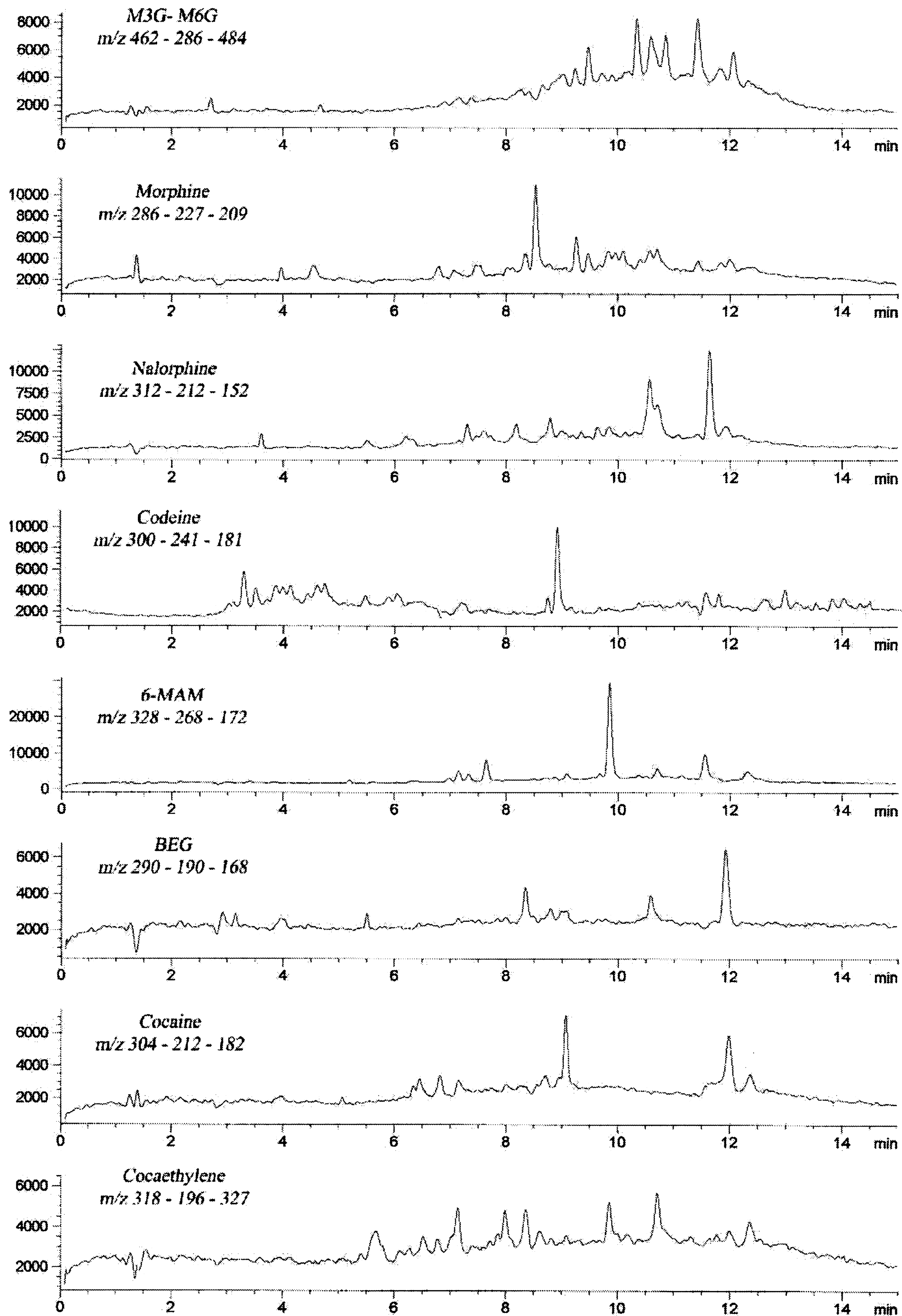

Fig. 2. SIM chromatogram of an extract of 1 g drug-free meconium sample.

added to drug-free meconium samples and extracted as reported above were analyzed for the determination of intra-assay precision and accuracy, while the inter-assay precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Inter-run precision was expressed as the relative SD (RSD) of concentrations calculated for quality control samples. Inter-run accuracy was expressed as the relative error of the calculated concentrations.

2.7.5. Stability

The effects of three freeze–thaw cycles (storage at −20 °C on the compound stability in meconium were evaluated by repeated analysis ($n=3$) of quality control samples (0.012, 0.12, and 0.85 μg/g for all the analytes). The stability was expressed as a percentage of the initial concentration of the analytes spiked in meconium and quantified just after preparation.

3. Results and discussion

3.1. Mass spectra analysis and selectivity

A representative chromatogram obtained following the extraction of 0.05 μg 6-MAM, morphine, M3G, M6G, codeine, cocaine, BEG and cocaethylene spiked in 1 g of drug-free meconium is shown in Fig. 1.

A chromatographic run was completed in 12 min, and initial conditions were restored in 25 min. No additional peak due to endogenous substances that could have interfered with the detection of compounds of interest was observed (Fig. 2). None of the drugs of abuse other than analytes under investigation or aforementioned medications carried through the entire procedure interfered with the assay. Blank samples injected after the highest point of the calibration curve did not present any traces of carry-over. Nonetheless, a 5-min run of methanol was introduced between each injection of study samples.

3.2. Recovery

The recoveries (mean±SD) obtained after methanolic and SPE extraction of meconium are presented in Table 1. These results suggested that there was no relevant difference in extraction recovery at different concentration levels for the analytes under investigation.

Table 1
Recovery of analytes under investigation

Compound	n	Concentration (μg/g)	Mean recovery (%)	SD
6-MAM	4	0.005	89.9	1.0
	4	0.05	90.6	2.3
	4	1	89.5	5.3
Morphine	4	0.005	83.0	1.8
	4	0.05	84.7	5.0
	4	1	83.5	2.8
M3G	4	0.005	71.9	9.5
	4	0.05	70.1	5.7
	4	1	79.9	1.0
M6G	4	0.005	84.3	2.4
	4	0.05	88.6	5.2
	4	1	89.2	2.4
Codeine	4	0.005	84.2	6.7
	4	0.05	83.3	6.5
	4	1	83.7	4.6
Cocaine	4	0.005	82.1	7.8
	4	0.05	83.2	8.0
	4	1	82.8	0.4
BEG	4	0.005	80.9	2.2
	4	0.05	86.8	5.0
	4	1	85.0	2.0
Cocaethylene	4	0.005	88.6	9.6
	4	0.05	85.4	5.4
	4	1	88.9	1.8

With respect to the matrix effect, comparison between peak areas of analytes spiked in extracted blank meconium samples versus those for pure diluted standards showed less than 10% analytical signal suppression due to coeluting endogenous substances.

3.3. Linearity and calculation of limits of detection and quantification

Linear calibration curves were obtained for the compounds of interest with a correlation coefficient

Table 2
Method calibration

Analyte	Calibration line, slope[a]	Calibration line, intercept[a]	Correlation coefficient[a] (r^2)	LOD[b] (μg/g)	LOQ[b] (μg/g)
6-MAM	8.098±0.6141	0.022±0.0342	0.999±0.0010	0.0003	0.001
Morphine	10.139±1.0352	0.058±0.0212	0.999±0.0004	0.0012	0.004
Codeine	9.270±0.2530	0.014±0.0451	0.998±0.0021	0.0012	0.004
M3G	0.938±0.2147	0.010±0.0040	0.996±0.0015	0.0012	0.004
M6G	0.467±0.0268	0.009±0.0027	0.985±0.01724	0.0003	0.001
Cocaine	11.654±2.1838	0.094±0.1070	0.994±0.0071	0.0009	0.003
BEG	4.215±0.4164	0.006±0.0005	0.9990±0.0001	0.0012	0.004
Cocaethylene	19.209±1.517	0.162±0.0519	0.9990±0.0002	0.0012	0.004

[a] Mean±SD of three replicates.
[b] LOD and LOQ are calculated from SD of the blank samples (see text).

Table 3
Intra-day precision and accuracy obtained for analytes under investigation

Compound	n	Concentration (μg/g)	Estimated mean±SD (μg/g)	Precision (RSD)	Accuracy (error %)
6-MAM	5	0.001	0.0009±0.0001	11.1	10.0
	5	0.12	0.112±0.008	7.3	6.6
	5	0.85	0.843±0.136	16.1	1.0
Morphine	5	0.004	0.0036±0.0004	11.1	10.0
	5	0.12	0.110±0.005	4.5	8.3
	5	0.85	0.810±0.095	11.7	4.7
M3G	5	0.004	0.0037±0.0003	8.1	7.5
	5	0.12	0.116±0.005	4.3	3.3
	5	0.85	0.835±0.022	2.6	1.7
M6G	5	0.001	0.0009±0.0001	11.1	10.0
	5	0.12	0.111±0.007	6.3	7.5
	5	0.85	0.839±0.020	2.3	1.2
Codeine	5	0.004	0.0035±0.0004	11.4	12.5
	5	0.12	0.117±0.006	5.1	2.5
	5	0.85	0.843±0.021	2.4	1.5
Cocaine	5	0.003	0.0027±0.0003	11.1	10.0
	5	0.12	0.110±0.003	2.7	8.3
	5	0.85	0.755±0.057	7.5	11.1
BEG	5	0.004	0.0036±0.0005	13.8	10.0
	5	0.12	0.103±0.005	4.8	10.3
	5	0.85	0.756±0.031	4.1	11.0
Cocaethylene	5	0.004	0.0038±0.0004	10.5	5.0
	5	0.12	0.105±0.008	7.6	12.5
	5	0.85	0.749±0.044	5.8	11.8

(r^2) higher than 0.99 in all cases, and limits of detection and quantification were considered adequate for the purpose of the study (Table 2).

3.4. Precision and accuracy

Tables 3 and 4 show the results obtained for

Table 4
Inter-run precision and accuracy obtained for analytes under investigation

Compound	n	Concentration (μg/g)	Estimated mean±SD (μg/g)	Precision (RSD)	Accuracy (error %)
6-MAM	15	0.001	0.0009±0.0001	11.1	10.0
	15	0.12	0.108±0.007	6.4	10.0
	15	0.85	0.821±0.100	12.2	3.4
Morphine	15	0.004	0.011±0.0003	2.7	8.3
	15	0.12	0.109±0.005	4.6	9.2
	15	0.85	0.806±0.065	8.0	5.1
M3G	15	0.004	0.0035±0.0003	8.5	12.5
	15	0.12	0.106±0.005	4.7	11.6
	15	0.85	0.825±0.022	2.6	2.9
M6G	15	0.001	0.0009±0.0001	11.1	10.0
	15	0.12	0.107±0.007	6.5	10.8
	15	0.85	0.829±0.020	2.4	2.4
Codeine	15	0.004	0.0036±0.0004	11.1	10.0
	15	0.12	0.105±0.005	4.8	12.5
	15	0.85	0.759±0.103	13.5	10.7
Cocaine	15	0.003	0.0027±0.0002	7.4	10.3
	15	0.12	0.108±0.002	1.9	10.0
	15	0.85	0.795±0.051	6.4	6.5
BEG	15	0.004	0.0037±0.0005	13.5	7.5
	15	0.12	0.108±0.005	4.6	10.0
	15	0.85	0.786±0.033	4.2	7.5
Cocaethylene	15	0.004	0.0038±0.0004	10.5	5.0
	15	0.12	0.109±0.007	6.4	9.2
	15	0.85	0.768±0.036	4.6	9.6

intra-assay and inter-assay precision and accuracy calculations for all analytes. Precision and accuracy of analytes under investigation at reported concentrations satisfactorily met the internationally established acceptance criteria [18,19].

3.5. Stability

With reference to freeze–thaw stability assays for quality control samples, no relevant degradation was observed after any of the three freeze–thaw cycles, with differences in the initial concentration being less than 10%.

3.6. Application to meconium sample analysis

The method here presented is being applied to meconium samples collected at the Hospital del Mar in Barcelona. Up to 117 samples have been analyzed to date with only three providing positive results for cocaine and/or opiates (Table 5; Fig. 3). These preliminary findings seem to disclose a prevalence of

Table 5
Analytes concentration in meconium samples positive for cocaine and/or opiates

Analyte	Sample 063 (μg/g)	Sample 066 (μg/g)	Sample 088 (μg/g)
6-MAM	0.005	0.142	0.006
Morphine	ND	0.397	ND
Codeine	0.015	0.048	ND
M3G	ND	0.120	ND
M6G	ND	0.091	ND
Cocaine	0.878	0.903	0.072
BEG	0.527	0.847	0.134
Cocaethylene	0.015	0.051	ND

ND, not detected.

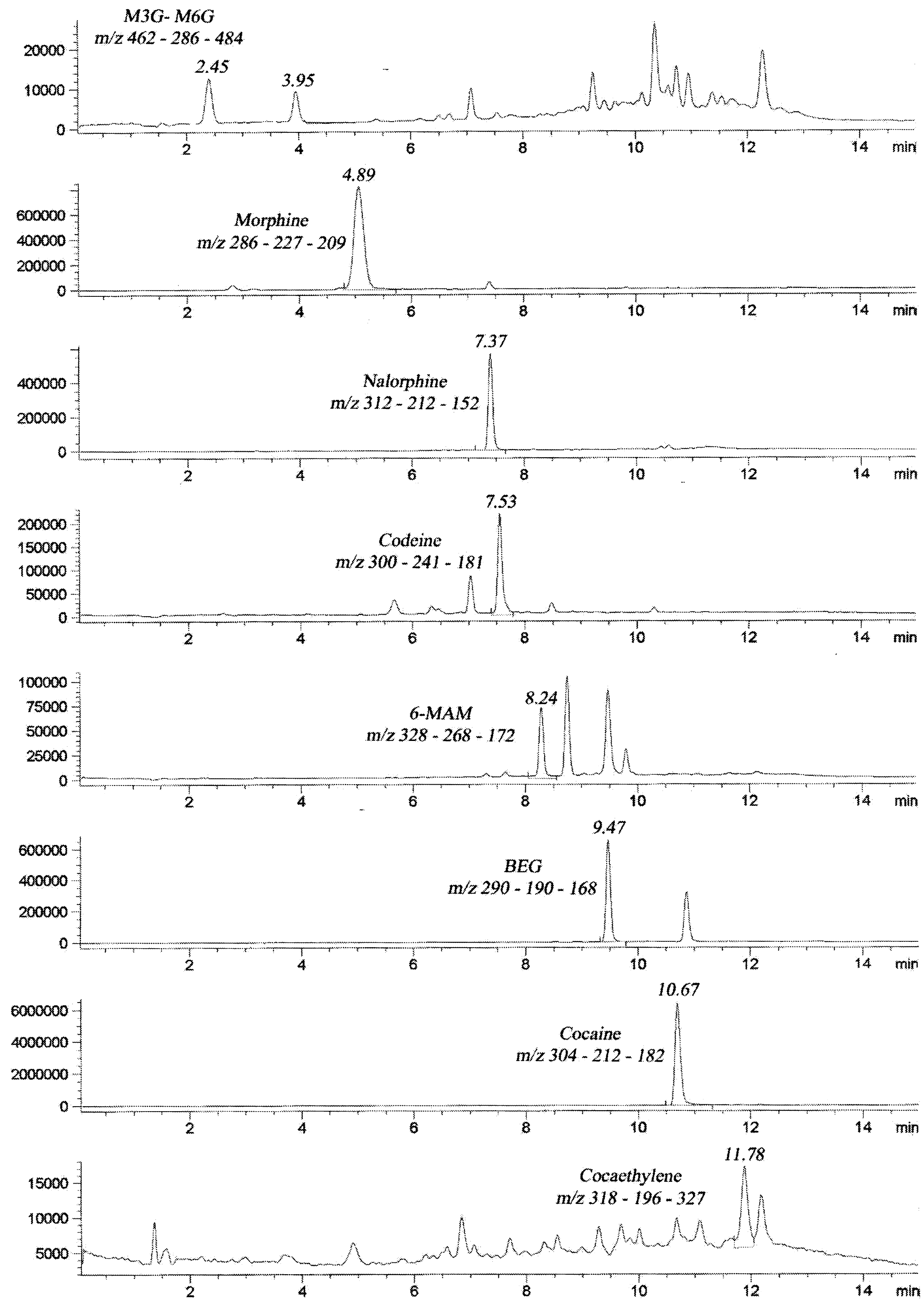

Fig. 3. SIM chromatogram of an extract of sample 066 containing 0.142 μg/g 6-MAM, 0.397 μg/g morphine, 0.120 μg/g M3G, 0.091 μg/g M6G, 0.05 μg/g codeine, 0.88 μg/g cocaine, 0.85 μg/g BEG and 0.05 μg/g cocaethylene.

opiates and cocaine consumption during pregnancy lower than that reported in the north American population [5–20], but figures have to be confirmed in a larger number of samples.

Regarding the panel of analytes under investigation, for the first time morphine glucuronides have been detected in meconium specimens positive for opiates and the presence of 6-MAM, only reported by Salem et al. [16], has been confirmed. In case of cocaine metabolites, this study investigated the presence of cocaine, benzoylecgonine and cocaethylene in meconium. Some authors affirmed that *m*-hydroxybenzoylecgonine is the only metabolite found in 20% of meconium specimens with negative results for cocaine and other principal metabolites [21]. However, other authors detected this metabolite in meconium when benzoylecgonine, cocaethylene or cocaine itself are also present in the same specimen [22] and stated that this metabolite is less useful if relied upon solely as a diagnostic indicator of cocaine exposure [17]. Furthermore, the same authors supporting the importance of this hydroxy metabolite, in another publication [15] only confirm benzoylecgonine in meconium specimens positive for cocaine with immunoassays. Thus, in our opinion, the affirmation that this metabolite is the only one present in some meconium specimens requires further evaluation.

4. Conclusion

The LC–MS method to analyze opiates and cocaine in meconium reported in this paper was validated according to internationally accepted criteria [18,19]. The method consists of sample preparation by liquid and solid-phase extraction, followed by chromatographic separation on a C_8 column and detection in SIM mode. The method proved to be sensitive enough for determination of all the compounds of interest using 1 g of meconium and was validated in the range 0.005–1.0 μg/g. Because meconium is a repository of substances to which the fetus is exposed in utero, meconium analysis by LC–MS—a versatile analytical tool—can provide a wide window for the detection of fetal exposure to various drugs and xenobiotic agents.

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