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Short communication

Application of a validated high-performance liquid chromatography–mass spectrometry assay to the analysis of *m*- and *p*-hydroxybenzoylecgonine in meconium

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Abstract

A high-performance liquid chromatography (HPLC)–mass spectrometry (MS) assay, already validated for opiates and cocaine in meconium, has been re-applied for determination of *m*- and *p*-hydroxybenzoylecgonine, using nalorphine as the internal standard. Methodology included an initial extraction from the matrix by methanol and then a solid-phase extraction (SPE). A reversed-phase chromatography was used with a gradient of 1% acetic acid–acetonitrile coupled to atmospheric pressure ionization electrospray–mass spectrometry single ion monitoring mode. This method, validated in the range 0.005–1.00 μg analytes/g meconium, proved useful to identify and quantify these two metabolites in meconium samples, already tested for the presence of cocaine, benzoylecgonine and cocaethylene. A positivity of range of concentrations varied between 0.007 and 0.338 μg/g, confirming the importance of these two hydroxylated derivatives to monitor fetal exposure to cocaine.

Keywords: *m*-Hydroxybenzoylecgonine; *p*-Hydroxybenzoylecgonine; Meconium; LC–MS

1. Introduction

For the first time in Europe the "Meconium Project" aimed to estimate the prevalence of drug use by pregnant women and the effects of exposure to illicit drugs during pregnancy on the fetus and infant [1]. Within the framework, we recently described a liquid chromatography–mass spectrometry (LC–MS) method for determination of 6-monoacetylmorphine, morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, cocaine, benzoylecgonine and cocaethylene in meconium [2]. The assay was applied to the meconium samples collected at the Hospital del Mar in Barcelona and on the first 830 analyzed specimens an overall 7.9% positivity to drugs was disclosed, with a 3.1% samples positive to cocaine [3]. Nonetheless, without the inclusion of *m*- and *p*-hydroxybenzoylecgonine in our analyses we possibly obtained a more conservative estimate of cocaine exposure than some other studies. Hence, some authors affirmed that these two metabolites, formed through fetal metabolism of cocaine, could be found in specimens resulted negative to cocaine and other principal metabolites [4,5], while others stated that these metabolites were less useful as solely diagnostic indicators of intrauterine exposure to cocaine [6].

In the context of this debate, we decided to test the above-reported LC–MS method for the inclusion of *m*- and *p*-hydroxybenzoylecgonine and reexamine all the samples of the "Meconium Project" including the aryl hydroxylated cocaine metabolites for the eventual enhancement of positive rate. Here, we present the validation parameters obtained for these two substances and the concentrations obtained in meconium specimens from our study cohort.

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2. Experimental

2.1. Solvents and chemicals

m- and *p*-Hydroxybenzoylecgonine were purchased from Sigma–Aldrich (Milan, Italy) and nalorphine–HCl (used as internal standard, I.S.) was obtained from Salars (Como, Italy). Bond Elut Certify solid-phase extraction (SPE) columns were from Varian (Palo Alto, CA, USA). All the reagents were of analytical grade and from Carlo Erba (Milan, Italy).

2.2. Preparation of calibration standards and quality control samples

Stock standard solutions (1 mg/mL) of compounds 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 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 and 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) and samples at LOQ were prepared in drug-free meconium, aliquoted and stored at −20 °C. They were included in each analytical batch to check calibration, analytical recovery, accuracy and precision, and stability of samples under storage conditions.

2.3. Meconium samples

The "Meconium Project" Italian–Spanish joint study aimed to estimate the prevalence of drug use by pregnant women and the effects of exposure to illicit drugs during pregnancy on the mother, fetus, and infant in a European Mediterranean city, such as Barcelona. The study protocol was approved by the local ethical committee (CEIC-IMAS) and was carried out at the Hospital del Mar, the fourth biggest hospital of Barcelona city. Between October 2002 and February 2004, among the 1439 mother–infant dyads attended by the Hospital, 1151 (79%) dyads (singleton births) met eligibility criteria and agreed to participate in the study. Corresponding meconium specimens were collected, aliquoted and stored at −20 °C until analysis.

2.4. Determination of m- and p-hydroxybenzoylecgonine in meconium samples

The detailed methodology is described elsewhere [2]. In brief, analytes were initially extracted from one gram of meconium, added with 10 μl of I.S. working solution, by 4 mL of methanol. The solvent was evaporated and the residue was dissolved in phosphate buffer (pH 6.0) and applied on a Bond Elut Certify solid-phase extraction columns.

Table 1
Mobile phase gradient table in chromatography separation

Start time (min)	%A	%B
0.0	97	3
2.0	97	3
8.0	73	27
10.0	73	27
11.0	97	3
25.0	97	3

A, 1% acetic acid; B, acetonitrile.

Chromatographic separation was achieved by a C_8 reversed-phase column (Zorbax Eclipse XDB-C8 column, 150 mm × 4.6 mm, Agilent Technologies, Palo Alto CA, USA) using a gradient of 1% acetic acid–acetonitrile as a mobile phase (Table 1) at a flow-rate of 1 mL/min.

The mass spectrometer (HPLC–MSD system consisting on an Agilent 1100 series—a G1312A binary pump, a G1322A degasser, a ALS G1329A autosampler, a G1946D mass spectrometry detector from Agilent Technologies, Palo Alto CA, USA) was operated in positive electrospray ionization (ESI) 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 psi, gas temperature 350 °C; capillary voltage at 3000 V and fragmentor (the exit end of the capillary) at 200 V. Qualifying ions were *m*/*z* 306, 186 and 168 for both *m*- and *p*-hydroxybenzoylecgonine and *m*/*z* 312, 212 and 152 for nalorphine. Ion ratio acceptance criterion was a deviation ≤20% of the average of ion ratios of all the calibrators. Ions: *m*/*z* 306 for *m*- and *p*-hydroxybenzoylecgonine and *m*/*z* 312 for nalorphine were selected for quantification.

2.5. Method validation

The method was tested for these two new analytes following a 4-day validation protocol. Selectivity, recovery, linearity, precision, accuracy, limits of detection and quantification were assayed as previously reported [1,2].

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry and validation

Representative chromatograms obtained following the extraction of a drug-free meconium sample (A), 0.01 μg *m*- and *p*-hydroxybenzoylecgonine spiked in 1 g of drug-free meconium (B) and a meconium sample containing 0.085 and 0.086 μg/g *m*- and *p*-hydroxybenzoylecgonine (C), respectively, are shown in Figs. 1 and 2.

It can be noticed that the analytical response of *m*-hydroxybenzoylecgonine is about four-fold higher than that

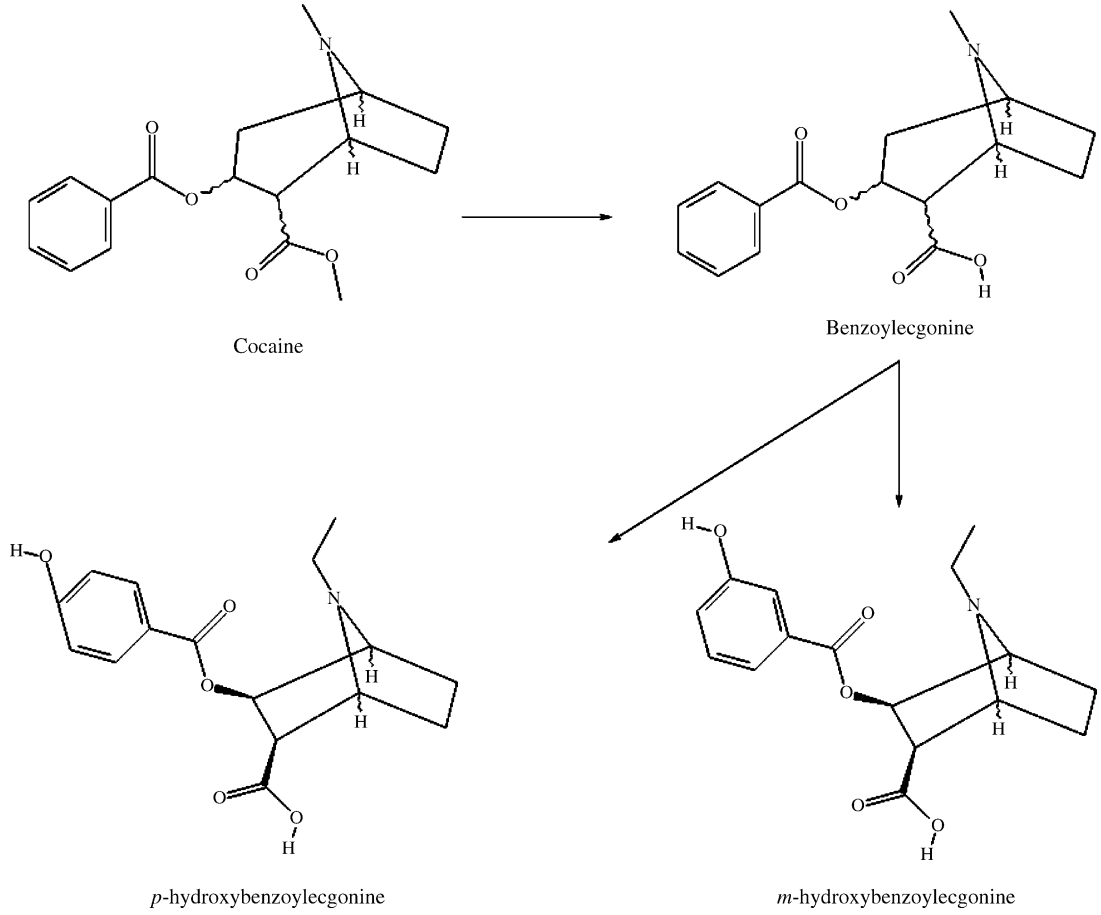

Fig. 1. Cocaine, benzoylecgonine and *m*- and *p*-hydroxybenzoylecgonine.

of the *p*-hydroxybenzoylecgonine. Indeed, at the value of fragmentor (200 V) applied for mass spectrometric analysis, *m*/*z* 306 (protonated molecular ion) resulted the most abundant fragment of *m*-hydroxybenzoylecgonine, while for *p*-hydroxybenzoylecgonine, abundance of fragments *m*/*z* 306, 186 was quite similar with no prevalent fragment. A decrease in the energy of the fragmentor would have minimized *p*-hydroxybenzoylecgonine fragmentation giving a better response at *m*/*z* 306, but would have changed the analytical conditions for all the other compounds and the already validated methodology [2]. Therefore, it was decided to maintain the fragmentor at the value previously established because, in any case using these analytical conditions, validation parameters obtained for *p*-hydroxybenzoylecgonine fitted for purposes of the study.

No additional peak due to endogenous substances that could have interfered with the detection of compounds of interest was observed (Fig. 2A). None of the principal drugs of abuse (6-monoacetylmorphine, morphine, codeine, cocaine, benzoylecgonine, cocaethylene, amphetamine, methylamphetamine, 3,4-methylendioxyamphetamine 3,4-methylendioxymethamphetamine, 9-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol) other than analytes under investigation 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 carryover. Nonetheless, a methanol wash injection (5 min) was introduced between each study samples injection.

Linear calibration curves were obtained for both compounds of interest (for *p*-hydroxybenzoylecgonine calibration line slope of three replicates: 1.344 + 0.017 and calibration line intercept: 0.006 + 0.001; for *m*-hydroxybenzoylecgonine calibration line slope of three replicates: 4.461 + 0.181 and calibration line intercept: 0.0005 + 0.0001) with a goodness of fit (r^2) higher than 0.99 in all cases. Limits of detection and quantification were: 0.0015 and 0.0045 μg/g for *p*-hydroxybenzoylecgonine and 0.0004 and 0.0013 μg/g for *m*-hydroxybenzoylecgonine. Despite the low quantification limits obtained for of both compounds, calibration curves with 0.005 μg/g as lowest point for both substances were used along the study.

The recoveries (mean ± SD) obtained after methanolic and SPE extraction of meconium for three different calibration curve concentrations (0.005, 0.5 and 1 μg/g) varied between 60 and 65%. These results suggested that there was

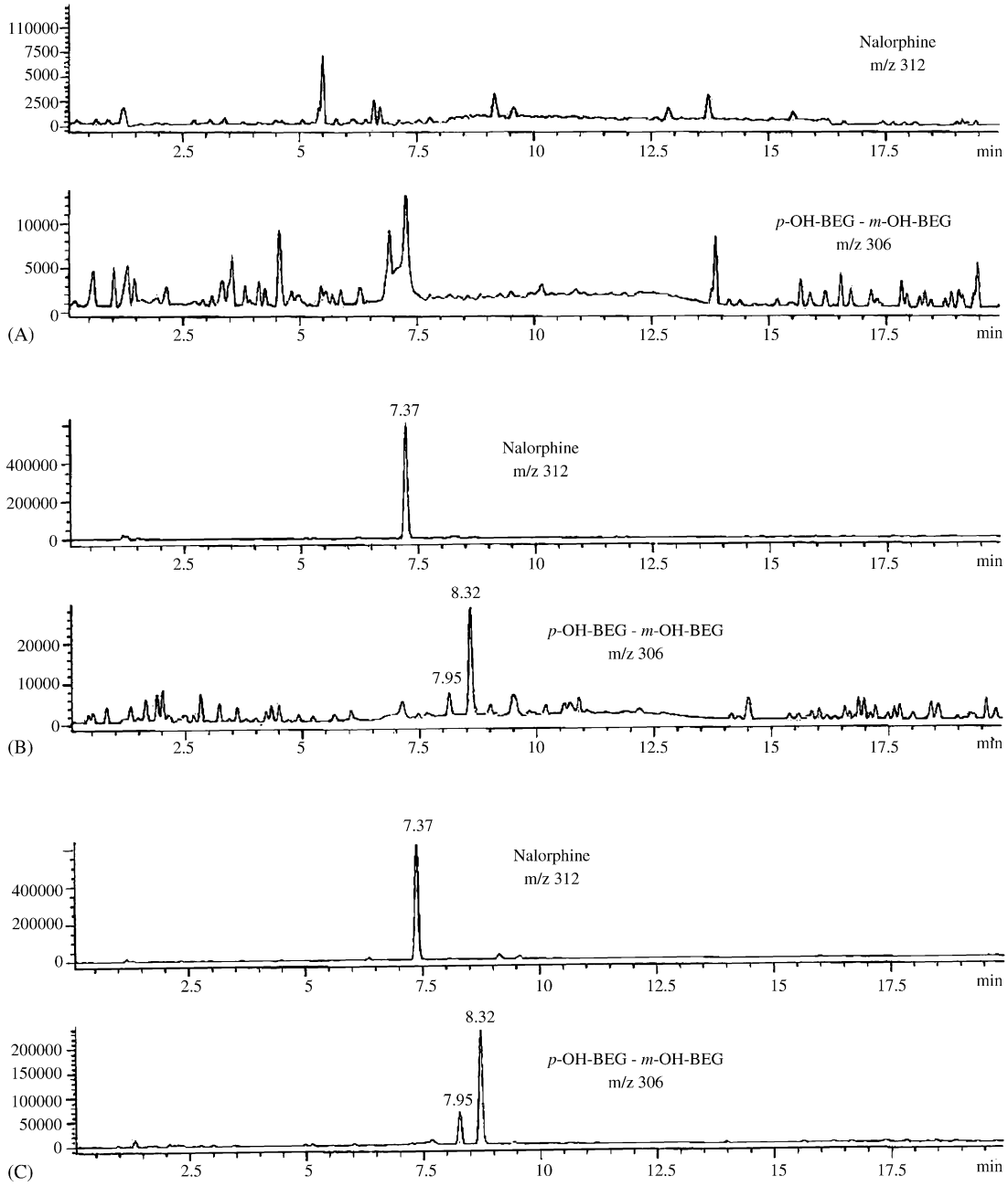

Fig. 2. SIM chromatograms obtained following the extraction of a drug-free meconium sample (A), 0.01 μg *m*- and *p*-hydroxybenzoylecgonine spiked in 1 g of drug-free meconium (B) and a meconium sample containing 0.085 and 0.086 μg/g *m*- and *p*-hydroxybenzoylecgonine (C).

no relevant difference in extraction recovery at different concentration levels for the analytes under investigation.

Table 2 shows intra- and inter-assay precision and accuracy calculations for the two analytes. The results obtained met the internationally established acceptance criteria [7,8] and were considered adequate for the purposes of the present study.

With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted drug-free meconium samples versus those for pure diluted standards showed less than 10% analytical signal suppression due to coeluting endogenous substances. No relevant degradation was observed after any of the three freeze/thaw cycles, with differences in the initial concentration less than 10%.

3.2. Application to meconium samples analysis

The method validated for *m*- and *p*-hydroxybenzoylecgonine was applied to meconium samples, already ana-

Table 2
Intra- ($n = 5$) and inter-assay ($n = 15$) precision and accuracy

Analytes	Intra-assay precision (RSD)			Intra-assay accuracy (error%)[a]			Inter-assay precision (RSD)			Inter-assay accuracy (error%)[a]		
	0.005 μg/g	0.12 μg/g	0.85 μg/g	0.005 μg/g	0.12 μg/g	0.85 μg/g	0.005 μg/g	0.12 μg/g	0.85 μg/g	0.005 μg/g	0.12 μg/g	0.85 μg/g
p-OH-BEG	5.2	7.8	4.9	7.7	5.6	3.8	5.8	5.1	2.9	4.6	3.3	2.9
m-OH-BEG	8.3	6.5	5.8	12.2	7.1	5.6	4.4	3.9	3.3	14.5	5.9	6.9

p-OH-BEG: *p*-hydroxybenzoylecgonine; *m*-OH-BEG: *m*-hydroxybenzoylecgonine.
[a] Reported as absolute value.

Table 3
Analytes concentration in meconium samples (ng analyte/g meconium)

Samples	*p*-OH-BEG	*m*-OH-BEG	BEG	Cocaine	Cocaethylene
0053	0.009	0.045	0.017	0.023	ND
0060	ND	ND	0.004	0.004	ND
0063	0.030	0.045	0.527	0.878	0.015
0066	0.319	0.312	0.847	0.903	0.051
0067	ND	ND	ND	0.003	ND
0071	ND	ND	ND	0.022	ND
0077	ND	ND	ND	0.003	ND
0086	ND	ND	ND	0.003	ND
0088	0.104	0.161	0.134	0.072	ND
0097	0.007	0.007	0.010	ND	ND
0098	ND	ND	ND	0.01	ND
0127	0.086	0.085	0.035	0.003	ND
0174	0.053	0.171	0.089	0.069	ND
0213	ND	ND	ND	0.003	ND
0292	0.076	0.163	1.577	0.697	ND
0348	0.022	0.249	0.273	0.359	ND
0365	0.074	0.220	0.819	0.407	ND
0568	0.062	0.338	0.084	ND	ND
0604	0.009	0.018	0.887	0.282	0.013
0932	0.009	0.008	0.041	0.003	ND
0945	0.008	0.037	0.629	0.194	ND
0988	ND	ND	0.004	0.003	0.006
1016	ND	ND	0.004	ND	ND
1024	ND	0.018	ND	0.003	0.004
1025	0.162	0.216	1.303	0.858	ND
1330	0.012	0.119	0.042	0.013	ND
1395	0.009	0.015	0.004	0.009	ND
1480	ND	ND	ND.	0.003	0.004

p-OH-BEG: *p*-hydroxybenzoylecgonine; *m*-OH-BEG: *m*-hydroxybenzoylecgonine; BEG: benzoylecgonine; ND: not detected.

lyzed by the same methodology for opiates, cocaine, benzoylecgonine and cocaethylene (Table 3). On 28 samples positive to cocaine and/or benzoylecgonine, 18 and 17 samples were also positive to *m*- and *p*-hydroxybenzoylecgonine, respectively. Of the two aryl hydroxylated derivatives, the *m*-metabolite showed the highest concentrations, being the most abundant compound in 8 samples. These findings are in agreement with what reported by other authors [4,5,9], which firstly characterized these two metabolites. Conversely, in contrast with some of these authors [5], which affirmed that *m*-hydroxybenzoylecgonine was the only metabolite found in a 20% of meconium specimens, resulted negative to cocaine and other principal metabolites, we detected this metabolite together with *p*-hydroxybenzoylecgonine in meconium when also cocaine itself, or benzoylecgonine were present in the same specimen. This finding was consistent with those of Xia et al. [6]. However, Xia only examined 22 meconium samples from newborns, whose mothers showed a positive screening test for urinary cocaine, while we examined 1151 "blind" meconium samples from all the newborns of the study population. The presented results could likely be due to limits of quantification reported for our LC–MS assay, which were 5–10 times lower than those used in previous studies. As a confirmation of that hypothesis, none of the specimens, negative to cocaine and any other drug

of abuse showed the presence of *m*- and *p*-hydroxybenzoylecgonine.

4. Conclusion

The LC–MS method, already reported to analyze opiates and cocaine in meconium, was validated for the inclusion of *m*- and *p*-hydroxybenzoylecgonine. These two compounds were investigated in all the specimens of the "Meconium Project" and were found only in samples positive to cocaine and principal metabolites. These results confirm that *m*- and *p*-hydroxybenzoylecgonine proved useful as additional markers of cocaine exposure during fetal life.

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