Journal of Analytical Toxicology 2014;38:31–38
doi:10.1093/jat/bkt092 Advance Access publication November 22, 2013

Article

A Rapid Assay for the Simultaneous Determination of Nicotine, Cocaine and Metabolites in Meconium Using Disposable Pipette Extraction and Gas Chromatography–Mass Spectrometry (GC–MS)

Dayanne C. Mozaner Bordin[1]*, Marcela N.R. Alves[1], Oscar G. Cabrices[2], Eduardo G. de Campos[3] and Bruno Spinosa De Martinis[3]

[1]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14050-140, Brazil; [2]GERSTEL Inc., 701 Digital Drive, Suite J, Linthicum, MD 21090, USA and [3]Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14050-140, Brazil

*Author to whom correspondence should be addressed. Email: martinis@usp.br

Drug abuse by pregnant women is considered a serious public health problem worldwide. Meconium is the first excretion in newborns and has been used as an alternative matrix to evaluate *in utero* drug exposure. Solid phase extraction (SPE) is widely employed to prepare and clean up samples in the field of forensic analysis. Most SPE products require large volumes of solvent, which culminates in longer sample processing times and increased cost per sample. Disposable pipette extraction (DPX) tips have been used as an alternative to traditional SPE cartridges. They combine efficient and rapid extraction with reduced solvent consumption. The purpose of this study was to develop and validate a method to determine nicotine, cotinine, cocaine, benzoylecgonine, cocaethylene and methyl ester anhydroecgonine in meconium using DPX and gas chromatography–mass spectrometry (GC–MS). Validation results indicated that extraction efficiency ranged 50–98%, accuracy 92–106%, intra-assay precision 4–12% and inter-assay precision 6–12%. Linear calibration curves resulted in R^2 values >0.99, limits of detection ranged from 2.5 to 15 ng/g and the limit of quantitation from 10 to 20 ng/g. The DPX–GC–MS method was shown to selectively analyze trace concentrations of drugs in meconium samples. Finally, the developed and validated method was applied to 50 meconium samples.

Introduction

In 2012, United Nations Office on Drugs and Crime (UNODC) estimated that, in 2010, between 3.4 and 6.6% of the world's population, aged between 15 and 64 years, had used an illicit substance at least once in the previous year; moreover, almost 12% of illicit drug users reported some kind of problems, including drug dependence and drug-use disorders. Among the main illicit drugs, global statistics showed that 0.3–0.4% of the population aged between 15 and 64 years use cocaine (COC) (1).

In Brazil, an increase in COC and tobacco consumption has been noted; according to the Brazilian Center of Information on Psychotropic Drugs (CEBRID), in 2005, the prevalence of the use of COC and crack in the 108 largest cities in Brazil was 2.9 and 0.7%, respectively. Recent statistics on the extent of use of illicit drugs given by the UNODC (2012) report that this consumption by Brazilian women is approximately one-third of the use among men, whereas in other countries the rate is one-tenth (1, 2). Current data indicate an increase in drug use by women of reproductive age. A study by the Ministry of Health of São Paulo showed an increase in 91% of hospital admissions of women in the last 3 years due to COC use. According to a research in the Hospital Faculty of Medicine of Ribeirão Preto, that used interviews and toxicological analyses, it was found that the incidence of COC use in mothers was 6% and tobacco use was 31% during pregnancy (3).

In addition to illicit drugs, licit drugs represent a danger to human health. Tobacco has become a public health concern due to the risks associated with smoking and environmental exposure to tobacco smoke. Today, more than one billion people are smokers worldwide (1). In 2008, the Brazilian Institute of Geography and Statistics (IBGE) reported that 17.2% of the Brazilian population aged over 15 years regularly used tobacco (4).

The prevalence of COC and tobacco use by pregnant women has become a major public health issue. In 2011, the National Survey on Drug Use and Health (NSDUH), in the USA, reported that 5.0% of the pregnant women aged 15–44 years were current illicit drug users; 17.6% of these women had smoked cigarettes in the previous month (5).

Exposure of pregnant women to drugs has serious consequences on the health of the fetus, which manifest themselves before and after birth. COC crosses the placenta without undergoing metabolism with direct action on the fetal vasculature causing vasoconstriction and urogenital, cardiovascular and central nervous system malformations (6). Tobacco use by the mother decreases the fetal growth rate, because it increases the level of carboxyhemoglobin in fetal blood. Moreover, the exposure to tobacco may induce abortion, placental abruption and sudden infant death syndrome during and after pregnancy (7).

In utero drug exposure can be evaluated soon after birth by investigating analytes and metabolites in meconium samples. The assessment of this exposure is crucial to identify, treat and monitor newborns displaying symptoms typical of drug withdrawal. A qualitative or quantitative analysis of drugs, metabolites and biomarkers can be conducted in several types of biological matrices (conventional matrices), such as plasma, serum, whole blood, saliva, urine and tissues (8). Recently, studies have used alternative biological samples, such nails, hair, umbilical cord, amniotic fluid and meconium (9–11).

Meconium, the first fecal matter passed by a neonate, begins to form at ~12 weeks of gestation, which makes the specimen the biological matrix of choice for detecting *in utero* drug exposure. It is a heterogeneous and complex sample consisting of water, desquamated epithelial cells of the gastrointestinal tract and skin and various substances such as acids and bile salts, cholesterol, enzymes, sugars, proteins, pancreatic and intestinal

secretions and swallowed amniotic fluid (12). Meconium is advantageous over conventional matrices when investigating fetal drug exposure: it is easy to collect (noninvasive) and provides a large detection window. Several studies based on meconium have successfully determined and quantified drugs of abuse (11, 13–20).

Considering the complexity of the meconium sample, an effective sample preparation is desirable to obtain clean extracts, remove interferences, avoid matrix effects, reduce deterioration of the chromatographic system and increase analytical sensitivity and specificity (8, 12–14, 21).

Disposable pipette extraction (DPX) is a new solid phase extraction (SPE) method that rapidly extracts sample solutions; it consists of a standard pipette tip, either 1 or 5 mL, 'loaded' with SPE sorbent (22, 23). The sorbent is loosely contained inside the pipette tip, which allows dynamic mixing with solvents, enables rapid equilibration and selective retention of the analyte, reduces the conditioning steps, minimizes the volume of solvent necessary for elution and dismisses the need for vacuum-controlled elution (24).The DPX device requires shorter extraction time, involves less sample manipulation and provides high recovery and efficiency. The whole process can be automated, including sample injection into the chromatographic system (22).

Few literature papers have described GC–MS methods using DPX. This type of extraction has been applied to the analysis of pesticides (22, 25); however, the interest in using DPX to determine drugs/metabolites/biomarkers has grown. DPX has been reported as a sample preparation technique to analyze Δ^9-tetrahydrocannabinol and metabolite in whole blood. It has also been used to estimate nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine (24), and drugs of abuse (amphetamines, opiates, COC and its metabolites, tetrahydrocannabinol metabolite, tricyclic antidepressants, meperidine, methadone and phencyclidine) in urine (26, 27).

The aim of this study was to develop and validate a method based on DPX and GC–MS to identify and quantify COC, nicotine (NIC) and metabolites in meconium.

Experimental section

Standards and reagents

Cocaine (COC), benzoylecgonine (BEG), cocaethylene (COE), anhydroecgonine methyl ester (AME), NIC, cotinine (COT) and the deuterated internal standards (ISTDs) such as cocaine-d3 (COC-d3) and cotinine-d3 (COT-d3) were obtained from Cerilliant (USA) as 1 mg/mL ampoule. Methanol, acetonitrile, hydrochloric acid, dichloromethane and ethyl acetate were purchased from J.T. Baker (USA); 2-propanol and ammonium hydroxide were acquired from Mallinkrodt (USA); and *N*-trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA) was provided by Sigma-Aldrich (USA). The DPX tips (DPX-CX1) were acquired from Gerstel (USA). The solvents were HPLC grade or higher, and the chemicals were ACS grade.

Calibrators

Stock and working solutions of the unlabeled drug standards were prepared in methanol at 1 and 10 μg/mL, respectively. Working solution containing ISTDs was prepared in methanol at 10 μg/mL. These solutions were used to prepare calibrators and quality control (QC) samples and were stored in amber glass at −20°C.

Meconium samples

Meconium specimens screening negative were used to prepare calibrators and QC samples. Meconium samples were collected from newborn babies whose mothers provided an informed consent, at the nursery of Maternidade do Complexo Aeroporto (MATER) in the city of Ribeirão Preto, state of São Paulo, Brazil. The samples were directly obtained from the diapers and stored in a freezer at −20°C until analysis. The samples were stable in this condition for over 6 months. The present study protocol was approved by the research ethics committee of the Pharmaceutical Sciences of the Ribeirão Preto University of São Paulo (CEP/FCFRP) number 256.

Procedures

Specimen preparation

Drug-free meconium (0.30 ± 0.005 g) was weighed into a 10-mL glass tube, and spiked with the ISTD solution, in order to obtain a final concentration of 200 ng/g. Then, 2 mL of methanol was added and the sample was vortex-mixed vigorously, and placed on a horizontal shaker for 20 min at 200 rpm. After agitation, the sample was centrifuged at 2,800 rpm for 20 min. The supernatant, organic layer, was transferred into a clean glass tube containing 100 μL of 0.1 M hydrochloric acid to perform the DPX procedure.

Figure 1 depicts a schematic representation of the procedure of DPX-CX tip extraction. Tips were conditioned with 1 mL of acetonitrile solution (30%, v/v). Then, the sample was submitted to suction and held in contact with the solid phase for 1 min. This allowed a constant air inlet that enables successful contact of the sulfonated polymer present in the solid phase of the tip with the analytes. After this period, continued washing was performed with 1 mL of deionized water. Analytes were eluted with

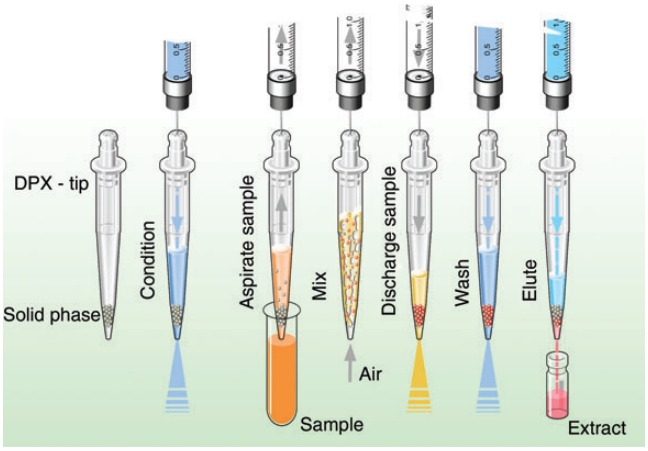

Figure 1. Schematic diagram of a DPX extraction. Diagram obtained from the online publication of GERSTEL, http://www.gerstel.de/pdf/p-gc-an-2009-01.pdf (28).

1 mL (three times) of dichloromethane/2-propanol/ammonium hydroxide (78: 20 : 2, v/v/v). Eluates were dried under a nitrogen stream at 40°C.

Derivatization

The residue was reconstituted with 80 μL of acetonitrile in a glass tube; 60 μL were transferred to an insert containing 20 μL of MSTFA. The insert was placed in an autosampler vial, tightly closed, vortex-mixed (10 s) and heated at 60°C for 20 min. After cooling at room temperature, 1 μL was injected into the GC.

Gas chromatography– mass spectrometry conditions

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out on a 7890 A Series gas chromatograph equipped with an Agilent 7693 autosampler and coupled to a 5975C quadrupole detector mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Data were acquired and analyzed using the standard software supplied by the manufacturer (Agilent Chemstation). The samples were injected in the splitless mode, and the analytes were separated on a capillary column (HP-5MS, column length 30 m × 0.25 mm with a film thickness of 0.25 μm) from J&W Scientific (Agilent Technologies, USA). The column temperature was programmed to rise from an initial temperature of 70°C (2 min), to 160°C (5 min) at 30°C/min, to 170°C at 5°C/min, to 200°C at 20°C/min, to 220°C at 10°C/min and, finally, increased at 30°C/min ramp rate to 320°C (3 min). The injector and the interface were set at 280°C. Helium was used as the carrier gas at a flow rate of 0.8 mL/min.

The mass spectrometer was operated in the electron impact (EI) ionization mode; selective mass detection was achieved by operating it in the selected ion monitoring (SIM) mode. The EI mass spectra of the analyte and ISTDs were recorded in the scan mode (scan range *m/z* 80–400) to determine retention times and characteristic mass fragments. For routine analysis, three characteristic mass fragments were monitored in the SIM mode. Table I summarizes the ions selected for identification and quantification. The ion ratio acceptance criterion was a deviation ≤20% from the mean of the ion ratios from all the calibration samples, and the retention time acceptance criterion was a deviation of ± 2%.

Data analysis

Calibration was conducted by linear regression analysis. Peak area ratios of the target analytes and their respective ISTDs were calculated using the software supplied by the manufacturer (Agilent Chemstation).

Table I

m/z ions selected to identify and quantify the target substances

Compound	SIM ions (*m/z*)	Retention time (min)
NIC	84, 133, *161*	7.13
AME	*152*, 166, 181	7.69
COT	*98*, 118, 176	13.11
COT-d3	*101*, 118, 179	13.17
COC	82, *182*, 303	17.89
COC-d3	85, *185*, 306	17.87
COE	82, *196*, 317	18.16
BEG—TMS	82, *240*, 361	18.23

The italicized ions were selected for the quantification measurement (quantifier ions).

Validation procedures

During the validation process, selectivity, sensitivity, linearity, recovery, precision (intra-day and inter-day) and accuracy, carryover and analyte stability were evaluated.

Selectivity

It is defined as the ability of a method to identify and quantify analyte in the presence or absence of endogenous or exogenous components. Interferences commonly encountered in drugs were added to the drug-free meconium specimens, which were then submitted to the same extraction and analysis procedures. Their retention time and quantifier and qualifier transition peak area ratios were assessed. Retention times had to be within ± 0.2 min of the mean calibrator retention time.

Matrix effects were evaluated by injecting 10 drug-free meconium samples to verify the absence of potential endogenous interferences. To assess potential interferences, drug-free meconium samples were spiked individually, so that they contained 1,000 ng/g of acetylsalicylic acid, alprazolam, amphetamine, methamphetamine, methylenedioxyamphetamine, diazepam, bromazepam, lorazepam, flurazepam, nortriptilyne, phenobarbital, caffeine, dipyrone, ephedrine, phenylephrine, fluoxetine, metoclopramide, iron sulfate and tetrahydrocannabynol.

Sensitivity

It was defined by lower limits of detection (LLOD) and lower limits of quantification (LLOQ); it was determined empirically through extracted meconium specimens fortified with different decreasing concentrations. LLOD and LLOQ were established at a signal-to-noise ratio of at least 3 : 1 and 10 : 1 for all the ions, respectively. Precision and accuracy at LLOQ concentrations were according to acceptable criteria (RSD ≤ 20%).

Linearity

The linearity study was performed using six calibration points.

A sample of drug-free meconium was weighed (0.30 ± 0.005 g) into a 10-mL glass tube and spiked with the ISTDs and standard solution of each analyte, in order to obtain different final concentrations. The concentration of calibration points were: 20, 50, 100, 400, 700 and 900 ng/g for COC, COE, BEG, AME and COT; 100, 200, 300, 500, 800 and 1,000 ng/g for NIC. The ISTDs were added at a concentration of 200 ng/g. The linearity was expressed as the coefficient of determination (R^2), and it was evaluated from a least square regression line calculated from all the standard concentrations. The calibrator concentrations were required to be within ± 20% of the target when calculated against the full calibration curve.

Recovery

Recovery was evaluated in three QC samples for each analyte using the post-extraction addition method: the results from spiked samples obtained prior to modified SPE were compared with the corresponding results achieved after modified SPE. Internal standards were spiked prior to SPE in all the samples.

Precision and accuracy

The precision and accuracy assay was performed using three QC samples (low, medium and high). The QC concentrations were, respectively, 30, 200 and 500 ng/g for COC, 40, 300 and 600 ng/g

for AME, COE, BEG and COT; and 150, 500 and 800 ng/g for NIC. Inter-day precision was determined on five different days ($N_{total} = 15$). Intra-day precision was assessed using five replicates per concentration of QC analyzed in 1 day. Precision was expressed as a percent of the relative standard deviation (RSD%). Accuracy was determined by comparing measured concentrations with target values over runs and expressed as a percent of the target concentration.

Table II

Coefficient of determination (R^2), intra- and inter-day precision, accuracy, recovery, LLOD and LLOQ values obtained for the investigated analytes and metabolites

Analyte	R^2	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)	Recovery (%)	LLOD (ng/g)	LLOQ (ng/g)
		5.50	8.29	97.32	97.86		
COC	0.9958	4.67	9.34	92.57	84.22	2.5	10.0
		4.21	7.74	102.40	73.35		
		7.86	6.37	98.75	98.45		
EMA	0.9974	4.46	7.46	94.43	93.27	6.0	10.0
		6.25	8.34	93.28	84.19		
		10.35	7.32	93.27	98.94		
COE	0.9952	9.12	8.59	105.33	92.43	8.0	10.0
		7.24	7.15	95.24	85.11		
		8.32	10.18	89.67	94.75		
BEG	0.9940	7.56	7.96	103.57	86.14	10.0	15.0
		5.60	8.57	97.42	76.85		
		12.05	8.97	94.37	93.44		
NIC	0.9919	5.42	11.25	101.24	69.45	15.0	20.0
		4.63	9.46	99.47	50.72		
		7.32	8.44	106.38	95.33		
COT	0.9923	5.20	9.23	102.27	71.02	5.0	10.0
		4.78	6.69	98.33	69.90		

Carryover

Carryover was determined by injecting a blank meconium specimen extract immediately after the analysis of the highest point in the calibration curve.

Analyte stability

Analyte stability was evaluated by reinjecting three QC samples in triplicate after standing for 24 h in an autosampler set at room temperature.

Clinical application to the method

The modified SPE procedure was applied to analyze the 50 meconium samples collected from newborns at the nursery of Maternidade do Complexo Aeroporto (MATER) in the city of Ribeirão Preto, state of São Paulo, Brazil, after approval of the

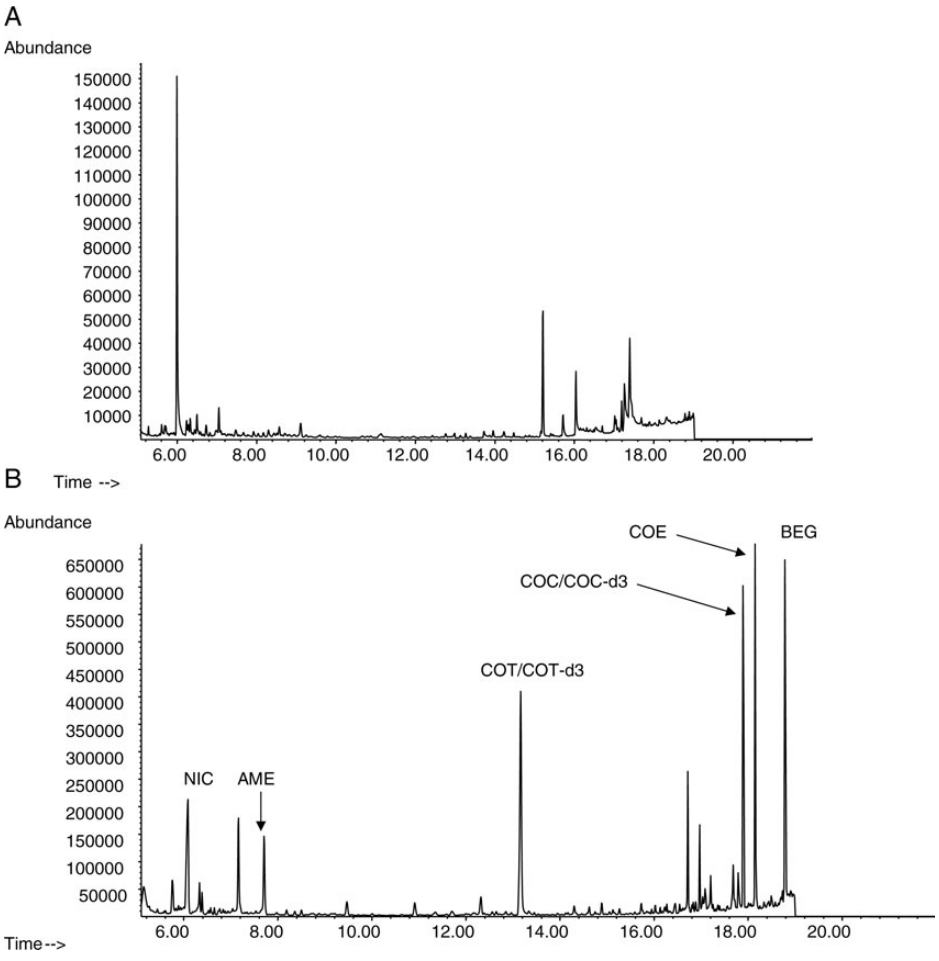

Figure 2. Total ion current chromatograms obtained after DPX extraction of: (A) drug-free meconium and (B) drug-free meconium sample spiked with all the investigated analytes (100 ng/g).

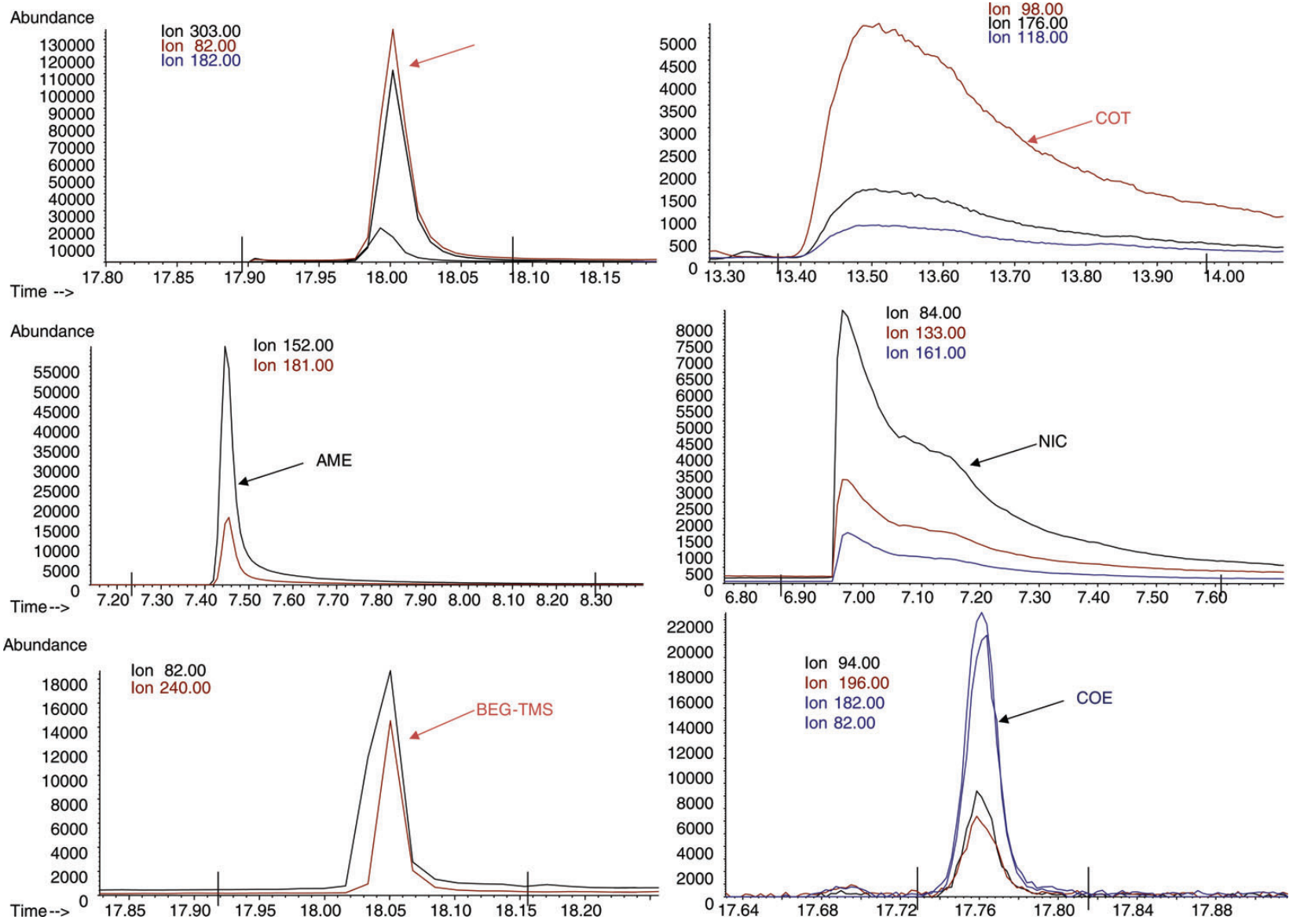

Figure 3. Extraction ion chromatograms (XIC) of analytes.

Table III
Results obtained during maternal interview and meconium drug analysis of 50 samples collected from newborns of mothers who reported using any drugs during their lives

Substance use	Maternal interview	Results of meconium analysis
None (in pregnancy)	20	15
Cocaine (+)	9	13
Crack (+)	0	2
Tobacco (+)	20	25

(+) Positive case.

Table IV
Quantitative results obtained in ng/g of meconium sample analyses

Sample	COC (ng/g)	AME (ng/g)	COE (ng/g)	BEG (ng/g)	NIC (ng/g)	COT (ng/g)
1	nd	nd	nd	nd	nd	**100.2**
2	nd	nd	nd	nd	nd	**90.7**
3	nd	nd	nd	nd	**115.5**	**180.3**
4	nd	nd	nd	nd	nd	**47.8**
5	nd	nd	nd	nd	**130.0**	**65.4**
6	nd	nd	nd	nd	nd	**98.0**
7	nd	nd	nd	nd	nd	**70.5**
8	nd	nd	nd	nd	nd	**40.8**
9	**250.5**	**100.7**	nd	nd	nd	nd
10	nd	nd	nd	nd	nd	**125.3**
11	nd	nd	nd	nd	**175.8**	**140.0**
12	**130.3**	nd	nd	**340.7**	nd	**205.2**
13	nd	nd	nd	nd	nd	**90.4**
14	nd	nd	nd	nd	**198.7**	**220.0**
15	**355.3**	**180.5**	nd	nd	nd	nd
16	**32.6**	nd	nd	**135.6**	nd	nd
17	nd	nd	nd	nd	nd	**47.6**
18	**391.2**	nd	nd	**223.8**	nd	**86.5**
19	nd	nd	nd	nd	**246.9**	**234.3**
20	nd	nd	nd	nd	**374.7**	**95.0**
21	nd	nd	nd	nd	nd	**50.8**
22	nd	nd	nd	nd	**457.0**	**345.3**
23	nd	nd	nd	nd	nd	**60.2**
24	**200.5**	nd	nd	**132.0**	nd	nd
25	nd	nd	nd	**95.6**	nd	nd
26	nd	nd	nd	nd	**569.8**	**105.0**
27	nd	nd	nd	nd	nd	**77.0**
28	nd	nd	nd	nd	**732.6**	**224.5**
29	**170.1**	nd	nd	**53.0**	nd	nd
30	nd	nd	nd	nd	**334.0**	**49.0**
31	**50.8**	nd	nd	nd	nd	nd
32	**137.0**	nd	nd	nd	**802.4**	**191.0**
33	nd	nd	nd	**64.0**	nd	nd
34	**320.0**	nd	nd	**187.0**	nd	nd
35	**97.6**	nd	nd	nd	nd	nd

nd, not detected. Bold values are the quantitative results obtained in ng/g of meconium samples analyses.

Human Research Committee. The questionnaires about drug use and informed consent were obtained after delivery.

Results and discussion

Method validation

Table II summarizes the results obtained during the validation procedure. All the parameters presented acceptable values according to accepted protocols.

The method developed to analyze NIC, COT, COC, BEG, COE and methyl ester anhydroecgonine in meconium samples was validated according to accepted protocols. Linear calibration curves were obtained from the target compounds, and the correlation coefficient (R^2) was higher than 0.99 in all cases. The six points of calibration curves were: 20, 50, 100, 400, 700 and 900 ng/g for COC, cocaethylene, BEG, methyl ester anhydroecgonine and COT, respectively; and 100, 200, 300, 500, 800 and 1,000 ng/g for NIC.

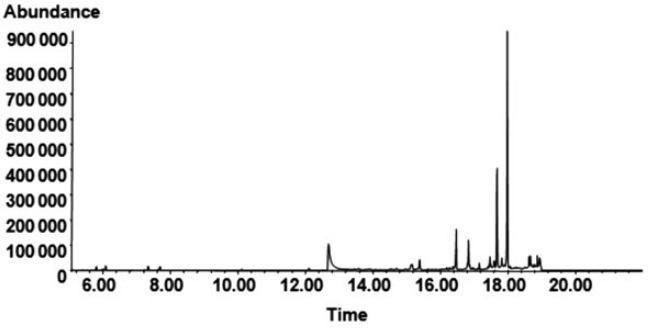

Figure 4. Total ion current chromatogram obtained following DPX extraction of a real meconium sample, positive for COC.

Selectivity was evaluated by injecting 10 drug-free meconium samples and verified the absence of potential endogenous interferents. There were no endogenous interferents at the same retention time of the studied analytes. Furthermore, illicit and common therapeutic drugs were tested as possible interferents, and the results were lower than 20% for the low QC sample. The results of final concentration obtained in this parameter for low QCs were 34.5 ng/g to COC, 32.75 ng/g to AME, 50.25 ng/g to BEG, 33.6 ng/g to COE, 32.2 ng/g to COT and 128.4 ng/g to NIC.

LLOD and LLOQ were considered adequate for the purpose of the study. According to the recovery results, the low QC sample gave higher values compared with the high QC sample. It can be explained by the fact that DPX-CX tips contain less of solid phase than conventional cartridges. Excellent selectivity and sensitivity for all the analytes and satisfactory cleanup of meconium samples was obtained.

Lower levels of percent recoveries would be expected in the case of meconium, considering that some losses should occur at the protein precipitation step, not to mention sample complexity. Nevertheless, improved recoveries of the analytes in meconium were observed.

Results indicated that the addition of hydrochloric acid in to methanol solution before DPX extraction improved sensitivity and reduced the LLOD. Indeed, the resin present in the DPX-CX tip contains a modified cation-exchange polymer backbone with active sulfonic groups, which allows for the selective retention of basic and neutral drugs.

The intra- and inter-day precision ranged from 5.5 to 12.05% and 6.37 to 11.25%, respectively, whereas the accuracies were between 89.67 and 106.38%.

A drug-free meconium sample was injected immediately after the highest point of calibration curve to evaluate the carryover from the previous injection. No carryover or marked matrix-based interferences were detected.

Concentrations of the reinjected extracts measured after standing for 24 h at room temperature showed that the average difference from initial concentrations was lower than 20% .

Figure 2 illustrates the chromatograms recorded after the DPX extraction of the drug-free meconium and drug-free meconium sample spiked with all the investigated analytes. The chromatographic separation of all compounds was achieved in 18.3 min.

Figure 3 illustrates the extraction ion chromatograms (XIC) of analytes.

Application of the developed method in real meconium samples

The method presented were applied to analyze meconium samples collected from the newborns of 50 mothers who stated that they had used drugs during their lives and provided an informed consent. Collections were conducted at the nursery of Maternidade do Complexo Aeroporto (MATER) in the city of Ribeirão Preto, state of São Paulo, Brazil and high correlations were expected among the different estimates of drug usage (maternal interview and meconium analysis). In the end, the results obtained from meconium analysis were compared with data collected during the interview that served as a 'reference' to indicate the use of drugs. Tables III and IV display the results obtained in questionnaires and the results of quantitative analyses, respectively.

Approximately 60% ($n = 30$) of the participating mothers provided self-reported drug use histories. Forty percent ($n = 20$) of the mothers reported no tobacco use during pregnancy, but NIC or COT were measurable in 50% ($n = 25$) of the meconium specimens. As for COC and crack, maternal interviews revealed 18% ($n = 9$) of self-reported use; however, 30% ($n = 15$) of the meconium samples contained COC or metabolites. Figure 4 corresponds to a chromatogram obtained following DPX extraction of a real meconium sample.

Conclusions

The GC–MS modified solid phase extraction DPX method was validated according to accepted international guidelines; it efficiently and sensitively determines COC, cocaethylene, BEG, AME, NIC and COT in meconium samples. Therefore, one can routinely use this method in hospitals to identify mothers who have not admitted to using those substances during pregnancy, which might result in a fast and accurate medical intervention regarding mother and childcare. Despite the fact that meconium requires longer sample preparation time when compared with blood and urine, the long metabolic history combined with the easy and wide window of its collection make this matrix ideal to determine fetal drug exposure.

The scientific literature on the use of DPX for the bioanalysis of drugs in conventional clinical/non-clinical studies is limited; to date, no method using DPX in meconium has been developed. This is the first proposal, and is advantageous for the following reasons: it is simple to operate, is used with a variety of sorbents and can be automated.

Acknowledgments

We thank the nursery of MATER for the collection of meconium samples and the mothers who agreed to participate.

Funding

This research was supported by the Brazilian research foundation of São Paulo's State- FAPESP (Process no. 2011/05196-5).

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