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Short Communication

Quantitation of benzoylnorecgonine and other cocaine metabolites in meconium by high-performance liquid chromatography

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ABSTRACT

A method for simultaneous extraction of cocaine and metabolites benzoylnorecgonine, benzoylecgonine and norcocaine from meconium was developed. The procedure uses solid-phase extraction columns with both cation-exchange and hydrophobic properties after vortex-mixing meconium with methanol. Chromatography utilizes reversed-phase high-performance liquid chromatography with a C_{18} column and phosphate buffer–acetonitrile as mobile phase. The method is specific and sensitive to 50 ng/g meconium for all compounds. Standard curves are linear from 0.05 to 5.0 μg/g ($r^2 \geq 0.989$). Intra-assay coefficients of variation were ≤6.9%. Meconium from infants exposed to cocaine *in utero* contained varying combinations of the four drugs.

INTRODUCTION

The analysis of meconium (first passed stool) for cocaine and other drugs of abuse is an increasingly prevalent method to identify infants exposed to these substances *in utero* [1–5]. Meconium begins to form early between ten to twelve weeks of gestation and continues to be formed throughout intrauterine life [6]. It may serve as a reservoir for abused substances and/or their metabolites [1,2] and therefore forms a record of fetal drug exposure for up to the last twenty weeks of gestation [4]. Screening of urine for such compounds, however, is sensitive only if the fetus is exposed in the days preceding birth [1–5].

In the case of cocaine, current methods in the literature analyze for only cocaine and the metabolite benzoylecgonine (BE) [1–4]. Benzoylnorecgonine (BN) is the hydrolysis product of norcocaine (NOR) [7], which itself is the N-demethylated metabolite of cocaine. BN causes seizures in rats following intracerebroventricular administration [8], chelates calcium [9] and accumulates

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in the guinea pig fetus following maternal cocaine administration [10]. A preliminary study in humans [11] reported large concentrations of BN (μg/ml range) in the urine of pregnant women who had taken cocaine, suggesting a similar accumulation of BN in the human fetus may be possible. Quantitation of this biologically active metabolite as well as the active metabolites BE and NOR is of interest in trying to establish a correlation between fetal drug exposure and neonatal effects.

No methods for the detection and quantitation of BN in meconium are currently described. Therefore, a solid-phase extraction for the simultaneous determination of BN as well as cocaine, BE and NOR from meconium was developed. Extracted samples were analyzed by high-performance liquid-chromatography (HPLC).

EXPERIMENTAL

Chemicals

Cocaine · HCl and lidocaine · HCl were purchased from Sigma (St. Louis, MO, USA). BN · HCl, BE and NOR were obtained from the Research Triangle Institute (Research Triangle Park, NC, USA) through the National Institute on Drug Abuse (Bethesda, MD, USA). Stock solutions of 100 ng/μl were prepared by dissolving BN, cocaine or BE in water and NOR in ethanol and were stored in 1.5-ml aliquots at −15°C until use. Acetonitrile and methanol (Malinckrodt, Paris, KY, USA) were HPLC grade. Water was purified with a Milli-Q Plus water system for analytical applications (Millipore, Bedford, MA, USA). All other chemicals were reagent grade.

Solid-phase extraction

Clean Screen columns (World Wide Monitoring, Horsham, PA, USA) were used to extract cocaine, BN, BE and NOR from meconium. This column has a combination of hydrophobic and cation-exchange properties. Columns were attached to a Vac Elut manifold system (Varian Assoc., Harbor City, CA, USA) and were conditioned with 2 × 3 ml of methanol, 1 × 3 ml of water and 1 × 3 ml of 0.01 *M* NaH_2PO_4, pH 2.0 using ≤ 10 kPa of vacuum, which allowed passage of solvent but was low enough to prevent sorbent drying. Meconium (0.50–0.75 g) was vortex-mixed with 2 ml of methanol for 60 s followed by centrifugation at 10 000 *g* for 10 min. The resulting supernatant was transferred to a clean test tube, and 3 ml of 0.01 *M* NaH_2PO_4, pH 2.0 containing 1.0 μg lidocaine as internal standard were added. The mixture was vortex-mixed for 10 s and placed in the reservoir of the conditioned extraction column and drawn through under vacuum at 0.5–1.0 ml/min, after which the sorbent was dried with ≥ 35 kPa vacuum for 5 min. The column was then washed with 1 ml of water at a flow-rate of approximately 1 ml/min and dried with 15 kPa vacuum for 3 min, followed by 1 ml of 0.1 *M* HCl with the same rate and drying procedure as the water wash. A final wash of 3 ml of methanol was rapidly applied and the sorbent again dried with ≥ 35 kPa vacuum for 5 min. Drugs were eluted with 10 ml of a 78:20:2 mixture of dichloromethane–isopropyl alcohol–ammonium hydroxide. Due to the volume of elution solvent used, eluate was collected in two 5-ml test tubes and was dried at 45°C under nitrogen. Samples in each 5-ml aliquot were reconstituted in 500 μl of mobile phase and combined for a final volume of 1000 μl.

Column liquid chromatography

A modification of the method of Sandberg and Olsen [12] was used to chromatograph extracted samples. A Model AS-100 HRLC automatic sampling system (Bio-Rad, Richmond, CA, USA) was added to the Model 334 liquid chromatographic system (Beckman Instruments, Berkeley, CA, USA). Separation of compounds was accomplished on a Microsorb C_{18} column (Rainin, Woburn, MA, USA), 100 mm × 4.6 mm I.D., 3 μm particle size. A Model 783A programmable absorbance detector (Applied Biosystems, Ramsey, NJ, USA) was set at 0.01 a.u.f.s. with drugs detected at 233 nm. The detector response was integrated by a Model 3396 II integrator (Hewlett-Packard, Los Angeles, CA, USA) and archived on an IBM-AT computer using Peak-96 software (Hewlett-Packard, Avondale, PA,

USA). Mobile phase consisted of 0.01 *M* NaH_2PO_4, pH 2.0 with 58 μM of the modifier tetrabutylammonium hydroxide and 13% (v/v) acetonitrile. The flow-rate was 1.0 ml/min and the injection volume was 200 μl using a 1000-μl injection loop. Standard curves were determined by least-squares linear regression analysis of blank samples spiked with known amounts of drug.

Human samples

Meconium (first passed stool) from infants of mothers known to have abused cocaine while pregnant was collected, after obtaining informed consent. Meconium from infants with no history of maternal drug abuse was obtained from the newborn nursery of a local hospital. After a negative test for cocaine and metabolites, this meconium was used for the preparation of standard curves.

RESULTS

A solid-phase extraction method for the simultaneous determination of BN as well as the other active cocaine metabolites, BE and NOR, was developed. The described method provides an acceptable blank with no interfering peaks (Fig. 1A). The chromatographic conditions are specific for cocaine, BN, BE and NOR [12] (Fig. 1B and C).

Standard curves constructed from the analysis of meconium spiked with known amounts of drug were linear with r^2 values ≥ 0.989, $F \geq 422$ and $p < 0.01$. Concentrations of BN, cocaine, BE and NOR for the standard curves ranged from 0.05 to 5.0 μg drug per g meconium. Samples were diluted as necessary to fit on the standard curve. The limit of quantitation was 0.05 μg/g for all four compounds. Intra-assay coefficients of variation for the internal standard lidocaine, cocaine and metabolites ranged from 3.1 to 6.9% and was 6.9% for BN. Variation was determined at a concentration of 1 μg/g for BN, cocaine, BE and NOR and at 1 μg per extraction for lidocaine. Recoveries of the drugs, at the above concentration, were 71% for BN, 88% for cocaine, 89% for BE and 90% for NOR. Accuracy for BN, cocaine, BE and NOR at the level of 1 μg/g was 91, 87, 93 and 85%, respectively. While 1 μg of lidocaine is added to a 0.5-g sample of meconium (equal to 2 μg/g), the assay is linear for lidocaine from 0.10 to 5.0 μg/g ($r^2 = 0.989$, $F = 290$, $p < 0.01$). The limit of detection for lidocaine is 0.05 μg/g.

Analysis of meconium from eleven infants of mothers known to have abused cocaine during pregnancy revealed the presence of cocaine and/

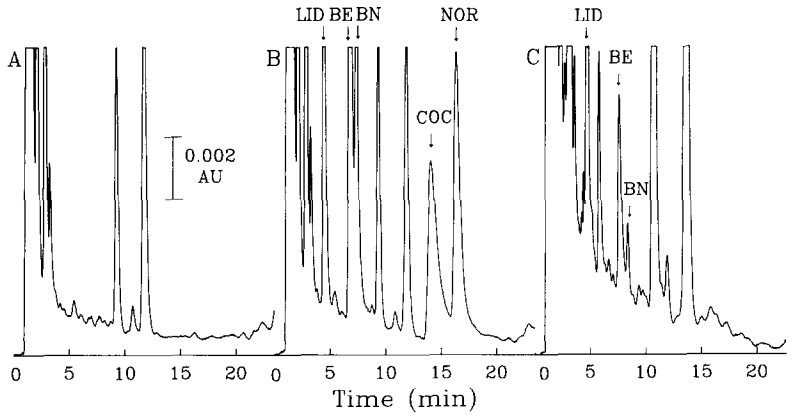

Fig. 1. Chromatograms of drug-free meconium (A), meconium spiked with 1 μg/g cocaine (COC), BN, BE, NOR and 1.0 μg per extraction of lidocaine (LID) (B) and a typical sample meconium with BE and BN (C). This sample did not contain cocaine or NOR. As seen in panel A, two peaks were consistently eluted from the extraction cartridge, but these peaks did not interfere with cocaine or any of the metabolite peaks.

or one or more of the three metabolites (Fig. 1C). BN was detected in seven of eleven samples, while ten of eleven were positive for BE. Neither BN nor BE were present in one sample but cocaine was detected in that sample. BN concentrations ranged from 100 ng/g to 5.1 μg/g. In one neonate, all four drugs were detected and were found to be 5.1, 4.6, 3.0 and 2.4 μg/g for BN, cocaine, BE and NOR, respectively.

An interesting observation was made when analyzing 0.5-g aliquots of large meconium samples (>2 g). In some infants BN, cocaine or BE could be detected in some aliquots, but not in all. This suggests that cocaine and metabolites are not uniformly distributed throughout the meconium, unlike urine or plasma.

DISCUSSION

A method for the solid-phase extraction of the biologically active cocaine metabolite BN from meconium is described. This procedure allows for the simultaneous determination of cocaine, BN, BE and NOR.

The use of 0.025–0.1 *M* NaH_2PO_4, pH 3.0 to 6.0 has been described for the extraction of cocaine and BE [3,4], however this proved inadequate for BN. A pH 2.0, 0.01 *M* NaH_2PO_4 buffer for application of sample was found necessary to retain BN on the Clean Screen column. Several elution solvent combinations were tested. The 78:20:2 mixture of dichloromethane–isopropyl alcohol–ammonium hydroxide has been utilized by others [4], but with a lesser volume than used here. A 3-ml volume of this mixture was tried initially, but found to give only 15–20% recovery of BN. However, increasing the polarity of the elution solvent by using basic methanol or any mixture of basic methanol–dichloromethane from 50:50 to 10:90 resulted in the co-elution of interfering meconium constituents. Aqueous buffers also resulted in contamination by meconium constituents. Increasing the volume of the original dichloromethane–isopropyl alcohol–ammonium hydroxide mixture to 10 ml resulted in an adequate recovery of BN, as well as the more easily eluted cocaine, BE and NOR.

Clark *et al.* [4] reported using a similar extraction column to assay for BE and cocaine and reported recoveries of 30% for BE and 99% for cocaine. The low recovery of BE may be explained by the use of a pH 6.0 buffer to apply the sample to the column, as this pH was found to allow both BN and BE to pass through the Clean Screen column with minimal retention when these drugs were extracted from blank methanol. Browne *et al.* [3] reported 98–100% of BE and cocaine using a strong cation-exchange extraction method, however, it is unknown if such a column would be useful for the extraction of BN.

The sensitivity of 50 ng/g for the method described here compares favorably with other published procedures. Browne *et al.* [3] report 50 ng/g as the lower end of calibration, and Clark *et al.* [4] report sensitivity to 250 and 500 ng/g for cocaine and BE. No current procedures have similar values for comparison to BN.

The discovery of BN in the meconium of infants exposed to cocaine *in utero* is of great interest. BN causes seizures and behavioral changes in developing and adult rats [8,13] and chelates calcium [9]. Both of these activities may have detrimental effects on the developing fetal central nervous system. Due to the inability of the liver to N-demethylate BE to BN, the presence of BN is an indication of prior NOR formation and subsequent hydrolysis [7]. NOR, which has similar sympathomimetic and local anesthetic properties as cocaine [14,15], may also in part be responsible for the fetal effects of maternal cocaine use. The finding of BN in seven of eleven meconium samples indicates the importance of an oxidative N-demethylation metabolic pathway for cocaine in the maternal–fetal unit. These metabolites may explain the "cocaine baby" syndrome observed in the first month of life of infants exposed to cocaine *in utero* [16].

The observation that the distribution of cocaine and metabolites is not uniform throughout large meconium samples needs to be considered in future analyses. This could lead to false negative reports if meconium analysis becomes a widely accepted screening method to determine maternal drug abuse during pregnancy.

It has been reported in one study [17], that lidocaine, the compound used as the internal standard, may be an occasional contaminant of street-cocaine. There are no reports in the literature, at this time, of lidocaine accumulating in meconium, and lidocaine was not detected when samples were extracted and analyzed without internal standard present. Further review of the data from this study strengthens the conclusion that lidocaine is not a contaminant of meconium. The lidocaine peak areas in the samples were not different from the peak areas of lidocaine added to known drug-free meconium which were extracted, suggesting that all eleven samples were lidocaine-free. Reanalysis of chromatograms using standard curves calculated from cocaine and metabolite peak area alone, instead of ratios with lidocaine, was done to remove any possible effect of illicit lidocaine contamination from the final results. This resulted in an average 12% difference in cocaine and metabolite concentrations, which was not statistically significant. Laboratories conducting forensic analyses for the State of Oregon were contacted and reported that lidocaine is not seen in the urine of known cocaine users, including instances when cocaine is present, indicating that even if lidocaine is present in street samples it does not accumulate biological samples. However, if contamination of meconium by lidocaine is suspected, samples should be confirmed lidocaine-free by extraction without lidocaine before further analyses are undertaken.

In conclusion, a solid-phase extraction method for the analysis of BN, cocaine, BE and NOR from meconium is described. This is the first method for the detection and quantitation of the biologically active cocaine metabolite BN from meconium. The method is sensitive, specific and provides adequate recoveries of all compounds. BN was quantitated from the meconium of infants who were exposed to cocaine *in utero*.

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