Anal. Chem. **2000,** *72,* 764−771

An LC−MS−MS Method for the Comprehensive Analysis of Cocaine and Cocaine Metabolites in Meconium

Yang Xia,[†] PingPing Wang,[‡] Michael G. Bartlett,*[,‡] Harvey M. Solomon,[§] and Kenneth L. Busch[†,⊥]

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0400, Department of Pharmaceutical & Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, Georgia 30602-2352, and Quintiles Laboratories, Ltd., 5500 Highlands Parkway, Suite 600, Smyrna, Georgia 30082

A sensitive, precise, and accurate liquid chromatography−mass spectrometry (LC−MS−MS) method was developed to quantitate cocaine and cocaine metabolites, which were simultaneously extracted from suspected drug-positive meconium samples using solid-phase extraction. The ability to analyze cocaine and multiple cocaine metabolites in meconium makes this method a powerful tool for the study of cocaine exposure and metabolism in neonates. Of 22 samples, only 1 did not show the presence of cocaine or any metabolite of cocaine. The identified metabolites varied both qualitatively and quantitatively between samples. Ecgonine appears to hold the most promise as a diagnostic marker compound for neonatal cocaine exposure as this metabolite was present in 21 of 21 of the positive samples tested, and at a relatively high median concentration. However, a core group of eight metabolites (present in at least 20 of 21 positive samples) was identified that appears to possess the greatest utility for determining cocaine exposure. Finally, the use of this method for assessment of the magnitude of fetal cocaine exposure was demonstrated.

Cocaine has long been one of the most widespread illicit drugs of abuse in the United States.[1] In 1984, The National Institute on Drug Abuse estimated that as many as 20−30 million Americans were users of cocaine, although more recent surveys have shown a modest reduction in the population of drug users.[2] Many of these drug users are women of childbearing age, and some of them will use drugs of abuse during pregnancy.[3] The impacts of prenatal exposure to cocaine on neonates include neuroteratogenicity,[4] a significant dose-related decrease in fetal weight and head size,[5] increased problems in the infant's early life,[6] and extended costs and lengths of stay in the hospital.[7] Therefore, it is desirable to diagnose the degree of the neonate's drug exposure since it provides the basis for appropriate treatment and adequate follow-up for the long-term development of the child. In addition to serving as a diagnostic tool, a method capable of detecting and determining the concentrations of cocaine and its major metabolites would be a powerful tool for elucidating cocaine transport from the maternal to the fetal compartment and for studies of the metabolism of cocaine.[8,9]

One of the first problems encountered in the analysis of cocaine and its metabolites in neonates is the selection of an appropriate specimen for testing. The specimens examined to date include whole blood,[10] plasma,[11] serum,[12] urine,[13] meconium, amniotic fluid,[14] and even hair.[15] Urine (maternal and/or neonatal) has been most widely used for the determination of cocaine exposure during pregnancy. However, it is difficult to collect urine from newborns, and the cocaine-derived compounds found in urine only correspond to a short period of drug exposure history (only within a few days of birth). Meconium, the baby's first bowel movement after birth, is a better choice for the determination of a more extensive period of fetal drug exposure. Meconium starts to accumulate in the fetal intestine from between the 12th and 16th week of gestation. Therefore, meconium provides an integrated history of drug exposure during the second and third trimesters of pregnancy.[16−19] Collection of meconium from newborns is easy, and the samples are stable. The major disadvantage of meconium

[†] Georgia Institute of Technology.
[‡] University of Georgia.
[§] Quintiles Laboratories, Ltd.
[⊥] Current address: Office of Sponsored Programs, Kennesaw State University, 1000 Chaistain Road, Kennesaw, GA 30144.

(1) Long, R. E., Ed. *Drugs in America*; H. W. Wilson: New York, 1993.
(2) Ellis, J. E.; Byrd, L. D.; Sexson, W. R.; Patterson-Barnett, C. A. *Southern Med. J.* **1993**, *86*, 725−731.
(3) *Substance Abuse Among Women and Parents*; CSR, Inc.: Washington, DC; National Institute On Drug Abuse: Rockville, MD, July 1994.
(4) Chen, W. A.; West, J. R. *Dev. Brain Res.* **1997**, *100*, 220−229.
(5) Church, M. W.; Morbach, C. A.; Subramanian, M. G. *Neurotoxicol. Teratol.* **1995**, *17*, 559−567.
(6) Oro, A. S.; Dixon, S. D. *J. Pediatr.* **1987**, *111*, 571−578.
(7) Joyce, T.; Racine, A. D.; McCalla, S.; Wehbeh, H. *Health Services Res.* **1995**, *30*, 341−358.
(8) Booze, R. M.; Lehner, A. F.; Wallace, D. R.; Welch, M. A.; Mactutus, C. F. *Neurotoxicol. Teratol.* **1997**, *19*, 7−15.
(9) Bourget, P.; Roulot, C.; Fernandez, H. *Clin. Parmacokinet.* **1995**, *28*, 161−180.
(10) Smirnow, D.; Logan, B. K. *J. Anal. Toxicol.* **1996**, *20*, 463−467.
(11) Tagliaro, F.; Antonioli, C.; Battisti, Z. D.; Ghielmi, S.; Marigo, M. *J. Chromatogr., A* **1994**, *674*, 207−215.
(12) Lau, C. E.; Ma, F.; Falk, J. L. *J. Chromatogr.* **1990**, *532*, 95−103.
(13) Clauwaert, K. M.; Bocxlaer, J. F. V.; Lambert, W. E.; Leenheer, A. P. *Anal. Chem.* **1996**, *68*, 3021−3028.
(14) Garcia, D. C.; Romero, A.; Garcia, G. C.; Ostera, E. M., Jr. *Pediatrics* **1996**, *98* (2), 291−293.
(15) Kwong, T. C.; Ryan, R. M. *Clin. Chem.* **1997**, *43*, 235−242.
(16) Lewis, D. E.; Moore, C. M.; Leikin, J. B. *Clin. Toxicol.* **1994**, *32*, 697−703.
(17) Ryan, R. M.; Wagner, C. L.; Schultz, J. M.; Varley, J.; DiPreta, J.; Sherer, D. M.; Phelps, D. L.; Wong, T. *J. Pediatr.* **1994**, *125* (3), 435−440.
(18) Lewis, D. E.; Moore, C. M.; Leikin, J. B.; Koller, A. *J. Anal. Toxicol.* **1995**, *19*, 148−150.

10.1021/ac990201p CCC: $19.00
Published on Web 01/19/2000

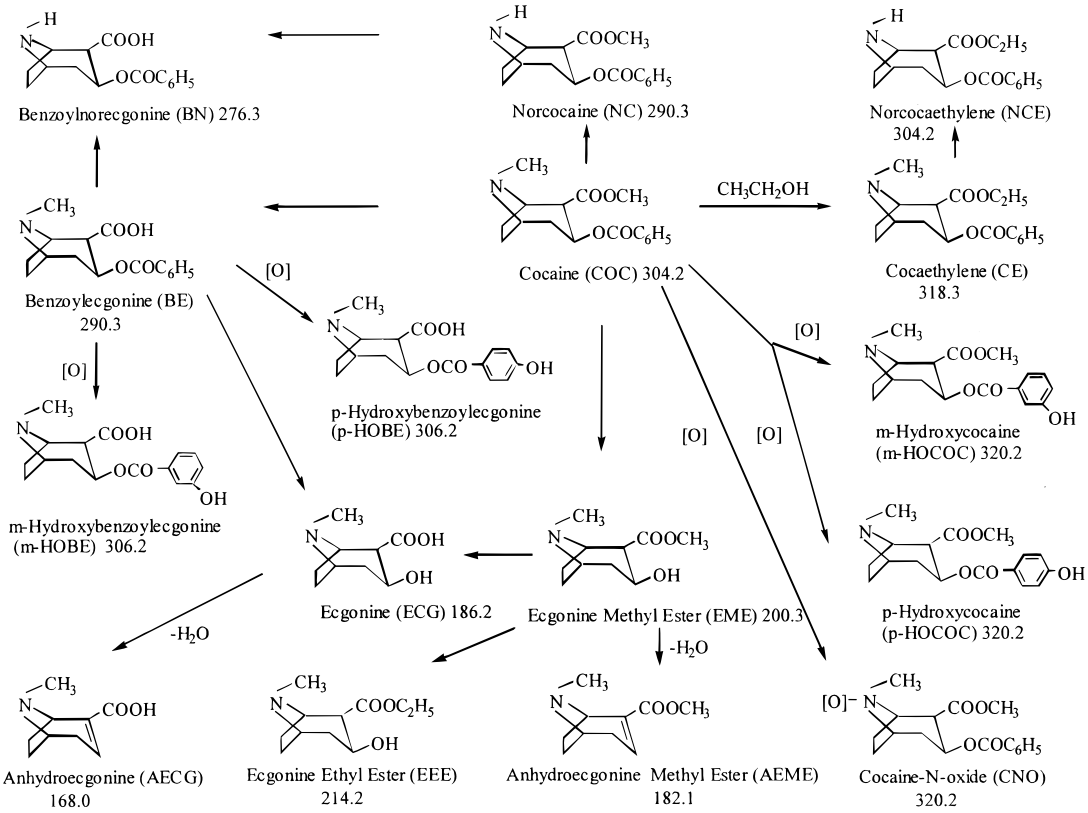

Figure 1. General metabolic pathway of cocaine. Each compound is displayed with the corresponding weight of the protonated molecular species.

analysis is the increased labor and time required for sample treatment and recovery of drugs and drug metabolites from this complex matrix.

GC−MS and LC−MS are often used to confirm the results from enzyme-related immunoassays for clinical screening for cocaine and cocaine metabolites.[20] More recently, LC−MS has increased in popularity due to the fact that it does not require sample derivatization, therefore simplifying sample preparation. However, the sample preparation needed for the analysis of meconium is still quite extensive.

In this study, a solid-phase extraction method was developed to simultaneously extract cocaine and 15 cocaine metabolites from meconium. The recovery and precision of this method were determined. A sensitive, precise, and accurate liquid chromatography−mass spectrometry (LC−MS−MS) method was used to quantitate cocaine and cocaine metabolites for 22 meconium samples from newborn infants whose mothers' urine screened positive for benzoylecgonine.

EXPERIMENTAL SECTION

Chemicals. Standards of (−)-anhydroecgonine methyl ester (AEME) fumarate, (−)-ecgonine (ECG) hydrochloride, (−)-ecgonine methyl ester (EME) hydrochloride, (−)-benzoylnorecgonine (BN) hydrochloride, (−)-benzoylecgonine (BE), (−)-cocaine (COC) hydrochloride, (−)-*N*-norcocaethylene (NCE) fumarate, (−)-cocaethylene (CE) fumarate, (−)-*N*-norcocaine (NC), (−)-cocaine *N*-oxide (CNO) hydrochloride, and [*N*-C^2H_3]cocaine ([D_3]-COC) hydrochloride were provided by the National Institute on Drug Abuse (Bethesda, MD). *m*-Hydroxybenzoylecgonine (*m*-HOBE), *p*-hydroxybenzoylecgonine (*p*-HOBE), *m*-hydroxycocaine (*m*-HOCOC), and *p*-hydroxycocaine (*p*-HOCOC) were purchased from Research Biochemicals International (Natick, MA). Anhydroecgonine (AECG) hydrochloride and ecgonine ethyl ester (EEE) were obtained from Radian International (Austin, TX). AECG and EEE were provided as 1 mg/mL methanol solutions. All other standards were powders, and were dissolved and diluted to a known concentration in methanol. The structures of cocaine and its metabolites are shown in Figure 1.

HPLC-grade methanol, acetonitrile, water, methylene chloride, 2-propanol, and ammonium hydroxide were purchased from J. T. Baker (Philipsburg, NJ). Ammonium acetate (97+%; Aldrich, Milwaukee, WI) was used without further purification.

Instrumentation. LC−MS−MS analysis was performed using a Hewlett-Packard (Palo Alto, CA) model 1100 LC system interfaced to a Micromass Quattro II (Beverly, MA) triple quadrupole mass spectrometer. HPLC separation was achieved using a Zorbax Eclipse XDB-C8 narrow-bore column (2.1 × 150 mm, 5 μm) obtained from MAC-MOD Analytical (Chadds Ford, PA). The column temperature was maintained at 38 °C, and the flow rate was 0.27 mL/min. The mobile phase used in the separation consisted of A (20 mM ammonium acetate, pH 2.7) and B (1:1 methanol/acetonitrile). The gradient program is shown in Table 1.

(19) Oyler, J.; Darwin, W. D.; Preston, K. L.; Suess, P.; Cone, E. J. *J. Anal. Toxicol.* **1996**, *20*, 453−462.
(20) Moore, C.; Lewis, D.; Leikin, J. *Clin. Chem.* **1995**, *41*, 1614−1616.

Table 1. Mobile-Phase Gradient Table in HPLC Separation

A (%), 20 mM ammonium acetate, pH 2.7	B (%), 50%/50% methanol/acetonitrile	start time (min)	A (%), 20 mM ammonium acetate, pH 2.7	B (%), 50%/50% methanol/acetonitrile	start time (min)
92	8	0	15	85	15
90	10	2	5	95	20
78	22	8	92	8	25

Table 2. LC−MS−MS Quantification Traces of Cocaine and Its Metabolites

analyte	MRM	collision energy (eV)	analyte	MRM	collision energy (eV)
AECG	168.0 > 91.0	23	COC	304.2 > 182.2	21
AEME	182.1 > 118.2	22	NCE	304.2 > 182.2	19
ECG	186.2 > 168.1	20	*m*-HOBE	306.2 > 168.1	22
EME	200.3 > 182.2	19	*p*-HOBE	306.2 > 168.1	22
[D_3]EME	203.3 > 185.2	19	[D_3]COC	307.2 > 185.1	20
EEE	214.2 > 196.3	20	CE	318.3 > 196.2	19
BN	276.3 > 154.1	17	*m*-HOCOC	320.2 > 182.2	20
BE	290.3 > 168.0	20	*p*-HOCOC	320.2 > 182.2	20
[D_3]BE	293.3 > 168.0	20	CNO	320.2 > 82.1	24
NC	290.3 > 168.0	19			

The electrospray ionization source of the mass spectrometer was operated with a source temperature of 120 °C. The capillary and cone voltages were optimized to 3.5 kV and 35 V, respectively. All LC−MS−MS data were recorded in MRM (multiple reaction monitoring) mode. The analytical quadrupole was set to unit mass resolution. Precursor ion selection channels had a 0.2 m/z span and a 0.02 s interchannel delay in switching. MS−MS detection channels for the 17 ions monitored in these experiments are shown in Table 2.

Immunoassay Screening. Maternal urine samples were screened for benzoylecgonine by immunoassay using the Emit II cocaine metabolite assay (Syva Co., Dade Behring Inc., California). The Grady Memorial Hospital Pathology Laboratory performed all Emit II assays, which detect benzoylecgonine, the major metabolites of cocaine, in concentrations equal to or greater than 300 ng/mL. Meconium samples from infants whose mothers' urine was positive for benzoylecgonine were collected and analyzed by LC−MS−MS for confirmation of cocaine exposure.

Solid-Phase Extraction. A solid-phase extraction (SPE) method was developed for the extraction of cocaine and its metabolites from meconium. Diapers containing meconium from human neonates were stored at −20 °C. For generation of calibration curves, approximately 0.5 g of a known drug-free "blank" meconium was defrosted in a culture tube. A 1 mL sample of a standard solution containing all the components of interest and 1 mL of the 0.2 μg/mL [D_3]COC internal standard solution were spiked into the meconium, followed by the addition of 2 mL of methanol into the culture tube. For the extraction of suspected drug-positive meconium samples, 1 mL of 0.2 μg/mL [D_3]cocaine internal standard solution and 3 mL of methanol were added to the meconium. The tube was then vortexed and sonicated thoroughly for 20 min and then centrifuged at 3000 rpm for 20 min. The supernatant was removed, and 2 × 1 mL of methanol was used to further treat the meconium. Three portions of supernatant were recombined into a clean culture tube and dried under vacuum at room temperature. The residue was reconstituted in 1 mL of pH 5.93 phosphate buffer and then loaded into a Bont Elut Certify SPE cartridge (Varian, Harbor City, CA) preconditioned with 2 mL of methanol, followed by 2 mL of pH 5.93 phosphate buffer. The reconstituted solution was drawn through the cartridge without vacuum. The cartridge was then washed under vacuum (10 mmHg) with 1 mL of water followed by 1 mL of methanol. After the washing was discarded, the analytes were eluted with 5 × 3 mL of methylene chloride/2-propanol/ammonium hydroxide (78/20/2). The eluent was then dried using a vacuum centrifuge (SC110A SpeedVac Plus and RVT400 refrigerated vapor trap, Savant Inc., Farmingdale, NY). The residue was reconstituted in 1 mL of deionized water. The sample extract was filtered through a 0.2 μm Acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI), and then 100 μL of the extract was injected into the LC.

Generation of the Calibration Curve. The 1 mL of standards and 1 mL of internal standard solution prepared in distilled water were spiked into the blank meconium matrix prior to solid-phase extraction. The final concentrations of the spiked meconium samples were 0.005, 0.01, 0.1, 1, and 5 μg/g. [D_3]Cocaine was used as the internal standard at a concentration of 0.2 μg/g. To generate a calibration curve, the peak area ratios between the standards and the internal standard were plotted against the concentration. For each curve five different concentrations were used. The correlation coefficients so obtained for calibration curves for cocaine and its metabolites were calculated.

RESULTS AND DISCUSSION

Precision and Accuracy of the Assay. The intraday precision (n = 6) of the assay was determined by the analysis of two sets of blank meconium samples spiked at 0.005, 0.01, 0.1, 1, and 5 μg/g concentrations for all of the components of interest. Three replicate runs were performed at each concentration level for each data set. The two sets were completely analyzed within 24 h. The interday precision (n = 9) was evaluated within a month by preparing three independent calibration curves using the same SPE procedure as used for the intraday study. The peak area ratios between the standards and the internal standard were taken to calculate the mean for each concentration. RSDs for each concentration level at different days were obtained through replicate runs. At low concentration RSDs were less than 20%, ranging from 0.60% (COC) to 19.88% (CE). At high concentration

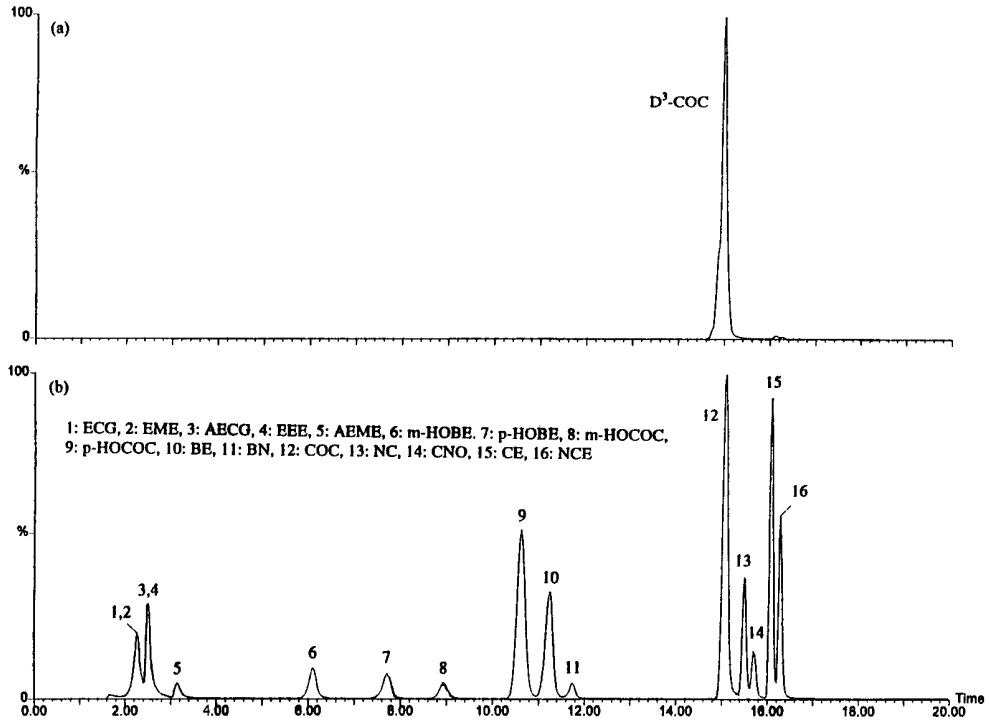

Figure 2. LC−MS−MS total ion chromatograms of (a) [D_3]COC as internal standard and (b) cocaine and its metabolites from spiked blank meconium.

RSDs were less than 10%, ranging from 0.24% (COC) to 9.88% (*p*-HOCOC). The accuracy of the assay was determined using six samples spiked at known concentrations of 0.012 and 2.5 μg/g in meconium. The percent errors for cocaine and its metabolites at a concentration of 0.012 ug/g were between 1.37% and 19.78%, and the percent errors at the high concentration were between 3.85% and 17.43%. These accuracy and precision results for the analytes in meconium are slightly higher than those reported for the analysis of urine,[21] plasma,[22] and serum[23] samples. The complex mixture of components present in meconium and the extensive sample-handling requirements (both liquid−liquid and SPE steps) for sample preparation may account for this increase. In bioanalytical assays of serum or urine samples, a single lot of certified drug-free serum or urine is used to generate calibration curves and the resultant precision and accuracy studies. This practice removes patient-to-patient variability and potential unsuspected interferences. In the current results, each data point has been generated from a separate meconium sample, and each patient has an unknown medical history. Therefore, the current meconium-based assay has been validated more rigorously than most, and the process used is similar to that recently recommended by Matuszewski et al.[24] for the validation of bioanalytical assays from serum and urine.

The correlation coefficients (r^2) were also calculated for the calibration curves produced on four different days for the analyte range of 0.005−5 μg/g. The calculation was based on the peak area ratios of the analytes to the internal standard. Values from replicate runs were used to obtain the mean. The response was linear for each analyte throughout the calibration range, with all r^2 values falling within the range between 0.999 and 0.982. The limit of quantitation for each analyte was between 1 and 5 ng/g.

Percent recoveries were calculated by comparing the absolute peak areas between a water standard of the same concentration and the extracted sample. The data were derived from replicate injections ($n = 6$), and the percent recoveries were determined on the basis of a linear regression calibration equation. At the concentration of 2.5 μg/g, the highest recovery was for benzoylnorecgonine (49.43%) and the lowest recovery was for anhydroecgonine (36.86%). At the low concentration (0.012 μg/g), the recovery for the metabolites in meconium ranged from 42.29% (anhydroecgonine) to 59.11% (*p*-hydroxybenzoylecgonine).

Chromatography and Electrospray Mass Spectrometry. The separation achieved for cocaine and its metabolites with liquid chromatography is displayed in Figure 2 as a "total" ion chromatogram (TIC), with summation of data from all of the monitored ion channels. The gradient program for this separation (Table 1) is the result of an optimization of the LC separation. MS−MS experiments are based on precursor ion to product ion transitions. The ion transitions monitored are shown in Table 2 and follow those suggested for use by Wang and Bartlett.[25] MS−MS experimental conditions, such as collision energy and collision cell pressure, were optimized from flow injection sample introduction of standard solutions. The multiple reaction monitoring channels were grouped such that ions of appropriate masses were monitored during the proper time window. Early eluting compounds included anhydroecgonine, anhydroecgonine methyl ester,

(21) Nishikawa, M.; Nakajima, K.; Tatsuno, M.; Kasuya, F.; Igarashi, K.; Fukui, M.; Tsuchihashi, H. *Forensic Sci. Int.* **1994**, *66*, 149−158.
(22) Pan, W.; Hedaya, A. *J. Chromatogr., B* **1997**, *703*, 129−138.
(23) Ma, F.; Zhang, J.; Lau, C. E. *J. Chromatogr., B* **1997**, *693*, 307−312.
(24) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. *Anal. Chem.* **1998**, *70*, 882−889.
(25) Wang, P. P.; Bartlett, M. G. *J. Mass Spectrom.* **1998**, *33*, 961−967.

ecgonine, ecgonine methyl ester, and ecgonine ethyl ester, all of which eluted between 2 and 4 min, and these are designated as group 1. The metabolites benzoylnorecgonine, benzoylecgonine, *m*-hydroxybenzoylecgonine, *p*-hydroxybenzoylecgonine, *m*-hydroxybenzoylecgonine, and *p*-hydroxybenzoylecgonine (group 2) eluted with retention times between 5 and 14 min. Finally, norcocaine, cocaine, norcocaethylene, cocaethylene, and cocaine *N*-oxide (group 3) eluted after 14 min. In addition to the individual ion channels for the compounds mentioned above, two extra channels were assigned to monitor [*N*-C^2H_3]ecgonine methyl ester ([D_3]EME) and [*N*-C^2H_3]benzoylecgonine ([D_3]BE). Monitoring of these channels allows a check to see if experimental conditions result in any decomposition of the internal standard [D_3]cocaine. No degradation of the internal standard is observed using the LC conditions described. The standard solutions of cocaine and its metabolites showed no degradation when stored at 5 °C or less during the month of analysis.

Solid-Phase Extraction. The procedure used to isolate, purify, or concentrate cocaine and its metabolites from a biological matrix substantially affects the outcome of immunoassay techniques, especially when they are used for meconium screening.[26] Cocaine and its metabolites exhibit a wide range of polarities, from the nonpolar compound norcocaethylene to ionic compounds such as benzoylecgonine. At a pH of 5.93 the amine group in each of the compounds in the study is protonated. In addition, the carboxylic groups in compounds such as benzoylecgonine, *m*-hydroxybenzoylecgonine, and *p*-hydroxybenzoylecgonine would be uncharged. The SPE cartridge employed in this study was a mixed-phase cartridge, which utilized a packed bed consisting of a nonpolar C_8 sorbent interspersed with a strong cation exchange (SCX) resin. This combination is effective for retaining both nonpolar and cationic analytes. Analytes are strongly retained by the sorbent at a pH close to 6 by the combination of Coulombic and nonpolar mechanisms,[27] which allowed washings with water and methanol (as described) to remove other nonpolar, polar, and anionic matrix contaminants.

Clinical Study. The protocol for the clinical study was approved by the Human Investigations Committee of Emory University, School of Medicine, Atlanta, GA. Neonatologists and members of the nursing staff at Grady Memorial Hospital, Atlanta, GA, assisted in the collection of meconium samples from infants whose mothers had a positive urine test for benzoylecgonine by enzyme-mediated immunoassay screening. Of the 22 screened samples tested, 21 were confirmed positive by the clear presence of multiple cocaine metabolites as determined by LC−MS−MS. One sample was analyzed by the standard protocol and found to be negative for cocaine and all of its metabolites.

For sample extracts identified as "positive", ecgonine had the highest mean concentration, and was the most frequently detected metabolite.[28] The metabolite CE was also frequently detected (Table 3). Metabolites that are listed as nondetectable are those for which signals were not present with $S/N = 3$ for the individual ion peak. Analytes with determined concentrations either below 0.005 μg/g or above 5 μg/g were out of the calibration range and are so designated by having the determined concentration value contained within parentheses. All reported values are subject to the uncertainties used for accuracy and precision assessments for this study. A small number of samples had relatively high concentrations of cocaine metabolites. It is more useful to use the median concentrations for comparison of metabolite concentrations than the average concentrations, as these values more closely reflect the common concentrations found in subjects.

Table 3 summarizes the quantitative results determined for all 22 meconium sample extracts. For sample 3, neither cocaine nor cocaine metabolites were detected, although the mother was identified as drug positive (benzoylecgonine) in the original enzyme-mediated immunoassay screening. However, the LC−MS−MS data are exculpatory, and this sample was excluded from further statistical analysis of the data set. Of the remaining 21 samples, 3 metabolites (ecgonine, *p*-hydroxycocaine, and cocaethylene) were detected in all samples (although at various levels) and 5 metabolites (*p*-hydroxybenzoylecgonine, norcocaine, benzoylecgonine, ecgonine methyl ester, and anhydroecgonine methyl ester) were detected in 20 of 21 samples. Cocaine itself was also detected in the same 20 of 21 samples. The levels of all 15 metabolites will vary with metabolism and time after exposure, as well as other uncontrolled parameters. However, these eight targeted metabolites form a core group, and the presence of any of these metabolites constitutes confirmatory evidence of neonatal cocaine exposure.

Conversely, the metabolite cocaine *N*-oxide was detected in only 12 of 21 samples. This metabolite was only recently identified in a single sample of meconium.[29] Thus, this study establishes that cocaine *N*-oxide is a relatively commonly encountered metabolite (57%) in meconium. Other metabolites (cocaine *N*-oxide, *m*-hydroxybenzoylecgonine, *m*-hydroxybenzoylcocaine, ecgonine ethyl ester, benzoylnorecgonine, and anhydroecgonine) are sporadically distributed among the meconium samples, and would seem to be less useful if relied upon solely as diagnostic indicators of cocaine exposure.

Although these are not control experiments designed to elucidate details of cocaine metabolism, the data derived from this study are of importance. Ecgonine was the metabolite with the highest median concentration, but the range of values was also broad. Median concentrations of the *m*-hydroxylated metabolites (*m*-hydroxybenzoylecgonine and *m*-hydroxycocaine) exceed those of the corresponding para-hydroxylated metabolites (*p*-hydroxybenzoylecgonine and *p*-hydroxycocaine). In contrast, the metabolites *p*-hydroxybenzoylecgonine and *p*-hydroxycocaine were found in more subjects (21 and 20, respectively) than *m*-hydroxybenzoylecgonine and *m*-hydroxycocaine (13 and 16, respectively), even though the median concentrations of the meta-hydroxylated metabolites were higher than the median concentration of the corresponding para-hydroxylated metabolites. Finally, the median concentrations of all hydroxylated metabolites exceeded that of cocaine, emphasizing the significance of this metabolic pathway.

The data in Table 3 support the suggestion that ecgonine is one of the most important metabolites to monitor for the detection of cocaine exposure during pregnancy. Ecgonine is the metabolite formed only through metabolic processes, thereby eliminating

(26) Moore, C.; Negrusz, A.; Lewis, D. *J. Chromatogr.* **1998**, *713*, 137−146.
(27) Thurman, E. M.; Mills, M. S. *Solid-Phase Extraction Principles and Practice*; John Wiley & Sons: New York, 1998; p 11.
(28) Abusada, G. M.; Abukhalaf, I. K.; Alford, D. D.; Vinzon-Bautlsta, I.; Framanik, A. K.; Ansarl, N. A.; Manno, J. E.; Manno, B. R. *J. Anal. Toxicol.* **1993**, *17*, 353−358.
(29) Wang, P. P.; Bartlett, M. B. *J. Anal. Toxicol.* **1999**, *23*, 62−66.

Table 3. Detected Cocaine and Its Metabolites Profile (μG/G) in the Meconium of Neonates[a]

	1	2	3	4	5	6	7	8	9	10	11
AECG	2.4222	0.0976	ND	0.0262	0.0445	(0.0038)	0.2519	ND	0.0476	0.0872	0.0224
AEME	0.0689	(0.0017)	ND	(0.0043)	0.0066	(0.0002)	0.0051	(0.0008)	(0.0019)	0.4194	(0.0001)
ECG	0.9219	2.7792	ND	0.3124	0.1277	0.0495	0.3210	0.3048	0.1021	(8.8020)	0.0756
EME	0.0896	0.2112	ND	(0.0005)	0.0142	(0.0008)	0.1208	0.0338	(0.0024)	(6.8854)	0.0287
EEE	0.0567	0.0457	ND	(0.0013)	(0.0015)	(0.0003)	(0.0008)	(0.0023)	(0.0005)	0.1457	(0.0006)
BN	0.0060	0.0366	ND	(0.0019)	(0.0021)	ND	ND	ND	(0.0003)	ND	ND
BE	0.0104	0.0431	ND	0.0124	0.0412	(0.0001)	0.0148	(0.0005)	0.0065	0.6571	(0.0002)
NC	0.1332	0.0052	ND	0.0075	0.0181	0.0491	0.0606	0.3391	0.0756	0.6623	0.0812
COC	0.0930	0.0191	ND	(0.0040)	0.0090	(0.0003)	(0.0022)	(0.0028)	(0.0009)	0.4002	(0.0003)
NCE	0.4119	0.0247	ND	(0.0034)	(0.0049)	0.0348	0.0450	0.2403	0.0333	0.8406	0.0574
m-HOBE	0.0109	0.1143	ND	0.0063	0.0128	(0.0001)	(0.0012)	ND	(0.0007)	0.1590	ND
p-HOBE	0.0330	0.2838	ND	0.0439	0.0904	0.0194	0.0379	0.1155	0.0312	0.4625	0.0270
CE	0.1729	0.0113	ND	(0.0031)	(0.0023)	0.0077	0.0108	0.0498	0.0071	0.5828	0.0115
m-HOCOC	0.0674	0.4256	ND	(0.0030)	(0.0020)	0.0390	ND	0.2690	ND	ND	ND
p-HOCOC	0.0375	0.0060	ND	0.0143	0.0199	(0.0007)	0.0108	(0.0038)	0.0074	0.6833	(0.0008)
CNO	0.0378	(0.0017)	ND	0.0107	0.0352	0.0549	0.0669	ND	0.0520	0.5382	ND

	12	13	14	15	16	17	18	19	20	21	22
AECG	0.0160	0.0251	ND	0.0342	1.5419	ND	(12.905)	0.2737	(12.100)	3.3525	0.7867
AEME	ND	(0.0022)	(0.0008)	(0.0033)	0.0307	0.0254	0.2221	0.0091	0.0939	0.0532	0.0477
ECG	0.1518	0.1529	0.0551	0.2585	1.3908	1.8024	4.2616	0.9765	(129.58)	1.3733	1.7537
EME	0.0189	0.0336	(0.0015)	0.0608	0.6428	ND	2.2165	0.1928	0.0295	0.9490	0.1500
EEE	(0.0010)	(0.0005)	(0.0004)	(0.0011)	0.0316	ND	0.0119	0.0078	0.0404	ND	ND
BN	(0.0003)	0.0118	ND	(0.0009)	0.1370	ND	0.3999	0.0157	(59.995)	0.1477	(0.0014)
BE	(0.0003)	0.1124	(0.0002)	0.1396	0.5002	ND	4.0916	0.2997	3.1418	0.9452	0.2741
NC	0.1611	0.0638	0.0644	ND	1.4106	2.1452	0.1113	0.9211	0.9046	0.1780	0.1262
COC	(0.0006)	(0.0011)	(0.0003)	(0.0018)	0.0057	ND	0.2026	0.0112	0.4058	0.5704	0.0843
NCE	0.1136	0.0453	0.0454	0.0454	0.0530	1.5160	0.0496	0.0547	0.1844	ND	0.0576
m-HOBE	ND	0.0238	ND	0.0066	1.0814	ND	3.4977	0.1352	4.3373	2.2277	0.0470
p-HOBE	ND	0.0399	0.0214	0.0367	0.6226	0.7118	3.4644	0.1288	1.0177	0.8425	0.1210
CE	0.0229	0.0095	0.0091	0.0108	0.0137	0.3175	0.0164	0.0170	0.0805	0.0078	0.0144
m-HOCOC	ND	ND	ND	ND	0.0624	1.7066	0.1472	0.0686	0.8026	0.2590	0.0737
p-HOCOC	(0.0016)	0.0051	(0.0006)	(0.0033)	0.0062	0.0279	0.5847	0.0207	0.6404	0.2959	0.0257
CNO	ND	ND	ND	0.0688	0.0886	ND	ND	0.0915	0.4370	ND	ND

[a] ND = not detected. Parentheses indicate estimated values. The numbers in the column heads are the subject numbers.

concerns of sample contamination. Further ecgonine is found at higher median concentrations than other metabolites, making it easier to detect. However, ecgonine is not normally determined in GC−MS analysis due to the difficulty associated with its isolation. In fact, it is only by decreasing the number of rinse steps in sample preparation that we were able to isolate this metabolite from meconium with the reported recoveries. The recommended SPE method by the cartridge manufacturer for cocaine and benzoylecgonine extraction from urine suggests that the cartridge be rinsed with 6 mL of deionized water, 3 mL of 0.1 M HCl, and 9 mL of methanol. Under these conditions all of the ecgonine is lost prior to analysis.

It has not been determined if the manner in which cocaine is ingested has any influence on its possible adverse effects on the fetus/neonate. However, information involving the route of ingestion may be obtained. Anhydroecgonine and anhydroecgonine methyl ester are formed as pyrolytic products of cocaine, and their presence is used to determine whether the neonate was exposed to cocaine via smoking of the "crack" form of cocaine. Additionally, the concurrent use of cocaine and alcohol can be observed as this combination results in the formation of cocaethylene, ecgonine ethyl ester, and norcocaethylene,[30] and these metabolites can be used as an indicator of such use. The method we report allows stratification of the manner of exposure and may permit the identification of subgroups of infants who are at particular risk.

Surveys have shown that 60−80% of cocaine abusers also concurrently consume alcohol.[31] An independent study of patients at Grady Memorial Hospital in Atlanta showed 50% of cocaine-abusing mothers were also alcohol users.[32] The frequency with which CE was detected in meconium in this study (15/21 samples above 10 ng/g) is slightly higher than, but still consistent with, these reported statistics, especially considering the integrated nature of meconium analysis. Additionally, norcocaethylene is formed in a large number of samples, and levels exhibit a positive correlation with cocaethylene. The presence of ecgonine ethyl ester among the samples is sporadic, and thus its correlation with cocaethylene and norcocaethylene is lower.

Differences in the median concentration levels of the hydroxylated metabolites present in meconium are particularly noteworthy. The hydroxylated benzoylecgonines have received much attention due to their identical responses in immunoassays based on the detection of benzoylecgonine. It has been proposed that *m*-hydroxybenzoylecgonine may be more useful than BE for confirmation of cocaine use. In one study, *m*-hydroxybenzoylecgonine was present in 95% of samples that gave a positive result for enzyme immunoassay, as compared to 77% for benzoylecgonine.[33]

(30) Torres, G.; Horowitz, J. M.; Lee, S.; Rivier, C. *Mol. Brain Res.* **1996**, *43*, 225−232.

(31) Snodgrass, S. R. *J. Child Neurol.* **1994**, *9*, 227−233.

(32) Sexson, W. R.; Carson D. *Pediatr. Res.* **1989**, *25*, 230A.

Table 4. Concentrations of Detected Cocaine and Its Metabolites in the Meconium of Neonates (μ/g)

metabolite	valid *N*	mean	median	min	max	range	std dev[a]
AECG	21	1.5644	0.0342	0.0000	12.9050	12.9050	3.7490
AEME	21	0.0475	0.0051	0.0000	0.4194	0.4194	0.0996
ECG	21	7.4073	0.3211	0.0495	129.5800	129.5305	28.0665
EME	21	0.5563	0.0338	0.0000	6.8854	6.8854	1.5374
EEE	21	0.0139	0.0011	0.0000	0.1457	0.1457	0.0332
BN	21	2.8871	0.0014	0.0000	59.9950	59.9950	13.0854
BE	21	0.4949	0.0431	0.0000	4.0916	4.0916	1.0784
NC	21	0.3547	0.0812	0.0000	2.1452	2.1452	0.5607
COC	21	0.0821	0.0028	0.0000	0.5704	0.5704	0.1670
NCE	21	0.1612	0.0454	0.0000	1.5160	1.5160	0.3591
m-HOBE	21	0.5492	0.0128	0.0000	4.3373	4.3373	1.2418
p-HOBE	21	0.3885	0.0904	0.0000	3.4664	3.4644	0.7691
CE	21	0.0579	0.0113	0.0023	0.5828	0.5805	0.1381
m-HOCOC	21	0.1834	0.0030	0.0000	1.7006	1.7006	0.3992
p-HOCOC	21	0.1126	0.0074	0.0006	0.6833	0.6827	0.2284
CNO	21	0.0688	0.0017	0.0000	0.5382	0.5382	0.1438

[a] Standard deviation based on all values.

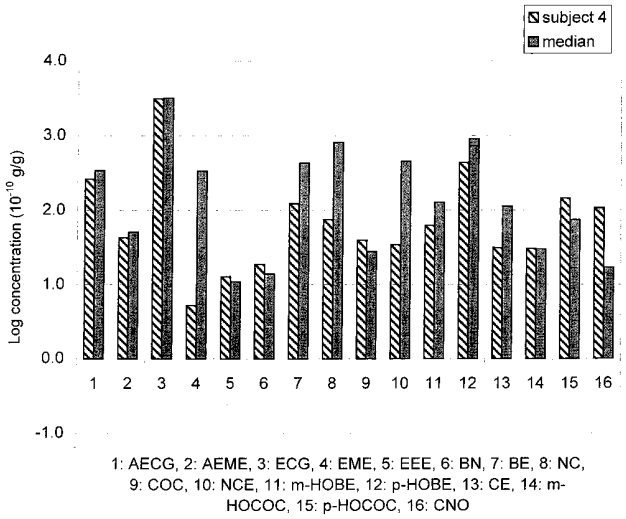

Figure 3. Measured levels of cocaine and its metabolites in meconium from subject 4.

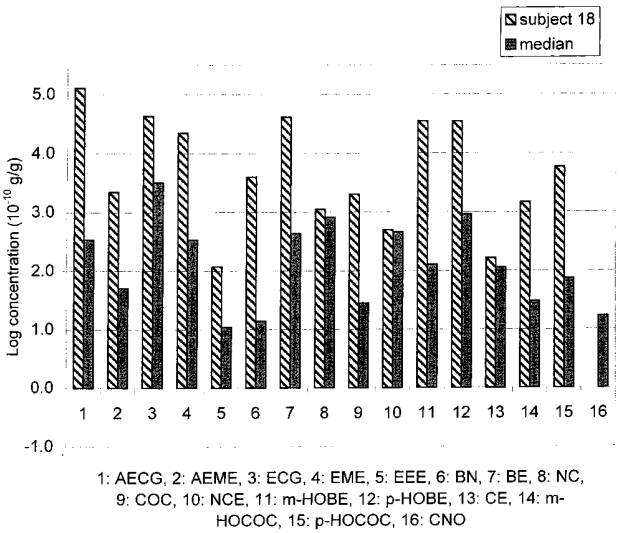

Figure 4. Measured levels of cocaine and its metabolites in meconium from subject 18.

However, our results show that while the meta isomer exists at a higher median concentration (Table 4), the para isomer is more frequently detected in these meconium samples.

Selected Patient Profiles. The benefit to analytical monitoring for a larger number of cocaine metabolites is the additional information about the patient that can be derived, even beyond suggestions of use profiles discussed above. Figure 3 displays the levels of cocaine and cocaine metabolites identified for subject 4. The presence of every cocaine metabolite provides a striking demonstration of the complexity of the metabolism of cocaine and of the versatility of the analytical technique. However, since most of the metabolites (12 of 16) were observed below the median concentrations for the 21 samples studied, one can suggest that the level of exposure to cocaine was either relatively low or remote. The concentrations of the three ethyl esters (cocaethylene, norcocaethylene, and ecgonine ethyl ester) were below 0.005 μg/g, indicating little concurrent alcohol abuse. The levels of the pyrolytic reaction products are also low, indicating that there was not significant maternal use of crack cocaine.

Figure 4 documents the exposure pattern reflected in the metabolites identified from subject 18. This neonate has received a more significant exposure, as 15 of 16 measured metabolites were in excess of the median values for the study. The only compound that was not in excess of the study median value was cocaine *N*-oxide, which was not observed in this sample. Three of the compounds measured from this subject (anhydroecgonine, benzoylecgonine, and *p*-hydroxybenzoylecgonine) were the highest measured from any sample in this study. Levels of the pyrolytic reaction products were extremely high, indicating recent and/or extensive use of crack cocaine. The levels of all three ethanol reaction products were elevated, indicating significant concurrent alcohol use. Therefore, we would characterize subject 18 as a neonate who had received substantial fetal exposure to crack cocaine and alcohol. Subjects with this pattern of drug exposure, which can only be established by chemical analysis, should be targeted for programs to aid in future physical and intellectual development to counter the effects of in utero cocaine exposure.[34]

(33) Lewis, D. E.; Moore, C. M.; Leikin, J. B. *Neonatal Intensive Care* **1994**, *7* (5), 24−27.

(34) Eyler, F. D.; Behnke, M.; Conlon, M.; Woods, N. S.; Wobie, K. *Pediatrics* **1998**, *101* (2), 229−237.

CONCLUSION

This study documents the use of meconium as the sample for cocaine and cocaine metabolite analysis, describes a SPE sample cleanup method that provides acceptable recoveries for a wide range of metabolites, characterizes an LC−MS−MS method with multiple reaction monitoring for the analysis of the extracts, and identifies the cocaine metabolites in terms of a core group of eight metabolites that can be used to determine neonatal cocaine exposure, and groups of metabolites that profile the pattern of cocaine usage. The established method can be used for an in-depth analysis of the metabolism of cocaine in the human fetus. It can also be used to study the patterns of excretion of cocaine metabolites that demonstrate unique features different from those observed in adult humans and qualitatively or quantitatively different between individuals.

The advantage of using meconium as a sample for analysis of cocaine and cocaine metabolites is known, as it is an integrating medium for neonatal exposure to cocaine during gestation. Difficulties in the use of this material were centered on sample preparation. We developed an SPE-based sample preparation method that minimizes SPE column washings and preserves the ability to recover ecgonine. Additionally, the use of MS−MS increases the specificity and allows analysis of ecgonine, which otherwise requires additional derivatization for GC−MS. The method described herein is an example of the manner in which a superior analytical method can be applied to understand a timely and complex problem in human pharmacology/toxicology, which has important medical and social implications.

ACKNOWLEDGMENT

We thank the National Institute on Drug Abuse and the University of Georgia Research Foundation for support. We also thank Susan T. Butcher, M.D., of Grady Memorial Hospital, Atlanta, GA, and Richard E. Mullins, Ph.D., of Emory University Hospital, Atlanta, GA, for their help in obtaining the meconium samples.

Received for review February 19, 1999. Accepted December 8, 1999.

AC990201P