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Validation and Application of a Method for the Determination of Buprenorphine, Norbuprenorphine, and Their Glucuronide Conjugates in Human Meconium

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Abstract

A novel liquid chromatography tandem mass spectrometry method for quantification of buprenorphine, norbuprenorphine, and glucuronidated conjugates was developed and validated. Analytes were extracted from meconium using buffer, concentrated by solid-phase extraction and quantified within 13.5 min. In order to determine free and total concentrations, specimens were analyzed with and without enzyme hydrolysis. Calibration was achieved by linear regression with a $1/x$ weighting factor and deuterated internal standards. All analytes were linear from 20 to 2000 ng/g with a correlation of determination of >0.98 . Accuracy was $\geq 85.7\%$ with intra-assay and interassay imprecision ≤ 13.9 and 12.4% , respectively. There was no interference from 70 licit and illicit drugs and metabolites. Buffer extraction followed by SPE yielded recoveries of $\geq 85.0\%$. There was suppression of ionization by the polar matrix; however, this did not interfere with sensitivity or analyte quantification due to inclusion of deuterated internal standards. Analytes were stable on the autosampler, at room temperature, at 4°C , and when exposed to three freeze/thaw cycles. This sensitive and specific method can be used to monitor in utero buprenorphine exposure and to evaluate correlations, if any, between buprenorphine exposure and neonatal outcomes.

It is estimated that $\sim 5\%$ of pregnant women use illicit drugs during pregnancy.^{1,2} Maternal self-report is the most common mechanism for identifying drug-exposed neonates but is unreliable when compared to biological monitoring of maternal and infant specimens. Toxicological analysis of urine, meconium, or hair is more objective and identifies more cases of in utero drug exposure.^{3–6} Meconium, which begins forming at ~ 12 weeks gestation, is a complex mixture of water, gastrointestinal and skin epithelial cells, lanugo, bile acids and salts, cholesterol and sterol precursors, blood group substances, enzymes, mucopolysaccharides, sugars, lipids, proteins, trace metals, pancreatic and intestinal secretions, and residue of swallowed amniotic fluid.⁷ Since meconium is generally not excreted until after birth, drug in meconium is believed to be cumulative from ~ 12 weeks through birth. Collection of meconium is easy and noninvasive, and meconium analysis has high sensitivity and specificity when compared to other biological specimens, such as maternal and infant urine.⁴

In 2002, buprenorphine (BUP) became the first, and to date, only, drug approved for office-based treatment of opiate addiction under the Drug Treatment Act of 2000.⁸ BUP is a semisynthetic thebaine derivative with low oral bioavailability due to first-pass metabolism. Rapid N-dealkylation by CYP3A4 and CYP2C8 in the liver produces norbuprenorphine

(NBUP).^{9,10} BUP and NBUP can be hydroxylated, and BUP, NBUP, and their hydroxylated metabolites undergo phase II glucuronidation. BUP is excreted primarily in the feces with ~10–30% of the dose excreted in urine, primarily as conjugated NBUP.^{11,12} BUP has been shown to cross the placenta and undergo placental metabolism to NBUP.¹³

Gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry have been used to analyze urine, oral fluid, plasma, whole blood, serum, feces, hair, and breast milk for BUP and metabolites.^{11,14–30} Poletini and Huestis³¹ employed liquid chromatography–tandem mass spectrometry (LC–MS/MS) to quantify BUP, NBUP, and buprenorphine glucuronide (BUP-Gluc), and Murphy and Huestis³² also included norbuprenorphine glucuronide (NBUP-Gluc). Hegstad et al.³³ quantified BUP-Gluc and NBUP-Gluc in human urine by LC–MS/MS, while Kronstrand et al.³⁴ and Huang et al.³⁵ employed LC–MS/MS for the simultaneous quantification of BUP, NBUP, BUP-Gluc, and NBUP-Gluc in urine within a single analytical run. A variety of techniques quantify cocaine, cannabinoids, nicotine, fatty acid ethyl ethers, stimulants, and opiates in meconium,⁶ but to date, there are no published methods for the quantification of BUP and metabolites in meconium. There is one documented case in the literature of BUP and NBUP in meconium; however, the method cited for analysis was for serum, urine, and whole blood, and no information on the application of this method for meconium was provided.³⁶

Although methadone is currently the only recommended pharmacotherapy in the United States for treatment of opiate addiction during pregnancy, there are now published reports of 444 infants exposed to BUP in utero, and it is currently being evaluated as an alternative to methadone maintenance treatment for pregnant opiate-dependent women.^{37–46} Infants exposed to BUP in utero often display neonatal abstinence syndrome (NAS) symptoms shortly after birth. NAS is “a generalized disorder characterized by signs and symptoms of central nervous system hyperirritability, gastrointestinal dysfunction, respiratory distress, and vague autonomic symptoms, which include yawning, sneezing, mottling, and fever”.⁴⁷ The published reports indicate that over 60% of infants exposed to BUP exhibit some signs or symptoms of NAS, with 75% of them requiring pharmacological treatment. Previous studies have shown there is no correlation between maternal BUP dose and NAS, but there are no data available on the ability of meconium BUP concentrations to predict neonatal outcomes.^{45,48,49} There is a strong need for a sensitive and specific method for quantifying BUP and metabolites in meconium.

There are multiple reasons why this analytical method is needed. First, BUP is abused in several countries. This is the first validated chromatographic method for measurement of BUP and NBUP in this complex neonatal matrix. But the most important reason that this method is highly valuable is that quantification of these drugs in meconium provides a model for studying the disposition of drugs to the fetus and for determining whether these concentrations predict neonatal outcomes. The controlled administration of illicit drugs during pregnancy is both unethical and unsafe, and administration of licit medications is recommended only as needed. The administration of BUP to pregnant opiate addicts to reduce illicit drug use and reduce craving provides an important opportunity to study the disposition of this drug in the maternal–fetal dyad. It is as yet unknown whether BUP dose is correlated to BUP or metabolite concentrations in meconium and, more importantly, if concentrations in meconium predict neonatal outcomes. To date, this research has not been possible due to the lack of a validated, quantitative chromatographic method for measuring BUP in this neonatal matrix. This method will enable the question of whether drug doses predict drug concentrations in meconium and whether drug concentrations correlate with the onset, magnitude, and duration of neonatal abstinence syndrome and other neonatal outcomes.

This paper presents the first validated LC–MS/MS method for the quantification of BUP, NBUP, BUP-Gluc, and NBUP-Gluc in human meconium. The validated method was applied to meconium specimens ($N = 10$) collected in an Institutional Review Board approved clinical study evaluating the use of BUP as a pharmacotherapy for pregnant opiate addicts. We provide the first data on the concentrations of BUP and metabolites in infant meconium following maternal daily BUP treatment.

EXPERIMENTAL SECTION

Chemicals

BUP, NBUP, BUP-Gluc, NBUP-Gluc, norbuprenorphine- d_3 (d_3 -NBUP) and buprenorphine- d_4 (d_4 -BUP) standards for calibration and internal standard purposes were purchased from Cerilliant (Austin, TX). NBUP and BUP solutions for preparing controls were purchased from Cambridge Isotope Laboratories (Andover, MA), and BUP-Gluc and NBUP-Gluc controls were purchased from ElSohly Laboratories (Oxford, MS). Reagent grade ammonium acetate and formic acid were purchased from Sigma Chemicals (St. Louis, MO). All solvents were HPLC grade. Glusulase ($>10K$ units of sulfatase, $>90K$ units of glucuronidase, 0.13% thimerazol, and 0.13% N_3NA per milliliter) was obtained from Perkin-Elmer (Waltham, MA). A homogeneous lot of BUP-free meconium was made by combining blank meconium purchased from ElSohly Laboratories with negative meconium donated by United States Drug Testing Laboratories (Des Plaines, IL). The drug-free meconium was mixed well and analyzed to confirm it was free of BUP and metabolites prior to method validation.

Instrumentation

LC–MS/MS analyses were performed on a Thermo Finnegan LCQ Deca XP Plus ion trap mass spectrometer with an atmospheric pressure chemical ionization (APCI) source interfaced with a Surveyor autosampler and LC pump (Thermo Electron, San Jose, CA). Sample homogenization and sonication were achieved with a VCX 750 W Ultrasonic Processor VCX (Cole Parmer, Vernon Hills, IL) and a Bransonic 3510 sonicator (Branson Ultrasonic Corp., Danbury, CT). Samples were centrifuged with an Eppendorf 5804R centrifuge (Eppendorf North America, Westbury, NY). Solid-phase extraction (SPE) was performed with a CEREX System-48 positive-pressure SPE manifold (Hologent Technologies, Baldwin Park, CA). Solvent evaporation was carried out on a TurboVap LV evaporator from Zymark (Hopkinton, MA).

Preparation of Standard Solutions

A solution containing 10 ng/ μ L BUP and NBUP was prepared in methanol from the stock calibrators (100 ng/ μ L). Working calibrators were prepared by appropriate dilution of this solution in methanol. The internal standard (IS) solution containing 1 ng/ μ L each d_4 -BUP and d_3 -NBUP was prepared by diluting 100 ng/ μ L stock solutions with methanol. Deuterated BUP-Gluc and NBUP-Gluc are not commercially available. Quality control (QC) solutions containing BUP, NBUP, BUP-Gluc, and NBUP-Gluc were prepared in methanol at three working concentrations.

A seven-point calibration curve (20, 40, 100, 200, 400, 1000, and 2000 ng/g) was prepared by adding 50 μ L of working calibrator and 50 μ L of IS solution to 0.25 ± 0.01 g of blank meconium. Low, medium, and high QC samples containing 30, 300, or 1600 ng/g free drug, 41.3, 412.5, or 2200 ng/g BUP-Gluc, 42.9, 429, or 2280 ng/g NBUP-GLUC, and 200 ng/g IS also were prepared using 50 μ L of QC solution and 50 μ L of IS in 0.25 ± 0.01 g blank meconium.

Procedures

Specimen Preparation—Analytes were extracted from meconium with buffer and SPE to isolate and concentrate analytes. QC samples and participant specimens were analyzed with and without enzyme hydrolysis to indirectly quantify conjugated analytes.

Approximately 0.25 g of well-mixed meconium was transferred to a polypropylene centrifuge tube. Two aliquots were prepared for each specimen. Fifty microliters of IS (1 ng/ μ L) and 2 mL of 1.1 M sodium acetate buffer (pH 5.1) were added. Meconium was homogenized using ultrasonic disruption for 1 min followed by sonication at 24 °C for 30 min. Fifty microliters of Glusulase was added, and hydrolysis samples were incubated for 3 h in a shaking water bath at 60 °C. After hydrolysis, samples were placed on ice to cool, and hydrolyzed and unhydrolyzed samples were centrifuged at 8000g for 10 min. The supernatant was decanted into a clean test tube.

Solid-Phase Extraction—Clean Screen ZSDAU020 mixed-mode SPE columns (United Chemical Technologies, Bristol, PA) were conditioned with 3 mL of methanol, 3 mL of HPLC water, and 2 mL of sodium acetate buffer. After samples were applied, columns were washed with 3 mL of HPLC water, 2 mL of 100 mM acetic acid, and 3 mL of methanol. Pressure, at 25 psi, was applied to the columns for 1 min following the water wash and 5 min following the acid and methanol washes. Analytes were eluted from the column using 4 mL of freshly prepared methylene chloride/2-propanol/ammonium hydroxide (78:20:2 v/v/v). Eluants were dried under nitrogen at 40 °C and reconstituted in 100 μ L of 20 mM ammonium acetate buffer with 0.05% formic acid at pH 4.6 (mobile phase A). Twenty microliters was injected into the LC–MS/MS.

Liquid Chromatography—Chromatographic separation was achieved using a Synergi Polar-RP 80A (150 $\times$ 2 mm, 4 μ m) column with a 4 $\times$ 2 mm, identically packed, guard column (Phenomenex, Torrance, CA). Gradient elution using mobile phase A and acetonitrile (mobile phase B) separated the analytes. The initial mixture of 60% A was decreased to 40% A over the first 3 min of the run. Solvent A was decreased 15%/min, reaching a ratio of 10% A/90% B at 5 min. This ratio was held for 3 min and then returned to the original mixture over 30 s. The column was allowed to re-equilibrate for 5 min for a total run time of 13.5 min. Flow was maintained at 400 μ L/min throughout the run.

Mass Spectrometry—Mass spectral data were acquired in positive ion mode, with the following APCI-MS parameters: vaporizer temperature, 475 °C; sheath gas flow rate setting, 80; auxiliary gas flow rate setting, 20; corona discharge needle voltage, 5.00 kV; and transfer capillary temperature, 250 °C. Identification of precursor and product ions and MS/MS optimization were established by direct infusion of 1000 ng/mL solutions of single analytes in methanol. Normalized collision energies of 40 and 35% were selected for BUP and NBUP and their deuterated analogues, respectively. Initially the most abundant product ion was selected as the quantifier ion, several product ions were selected for investigation as possible qualifier ions, and the parent ion was monitored. During method optimization, the appropriate qualifier ion was selected based on abundance and reproducibility of qualifier to quantifier ion ratios. In the absence of an appropriate qualifier ion for d₄-BUP, the precursor ion was selected as the qualifier ion. The proposed fragmentation of each analyte and internal standard can be seen in Figure 1. For NBUP and d₃-NBUP, the deuterated portion of the molecule remains intact resulting in quantification and qualification ions that differ by three mass units. However, d₄-BUP is deuterated on the cyclic side chain, which is quickly lost, so fragments differing by an increase of four mass units are not detected.

Validation

The method was validated on the following parameters: sensitivity, carryover, linearity, precision, accuracy, specificity, recovery, hydrolysis efficiency, stability, and matrix effect.

Sensitivity was assessed by establishing the limit of detection (LOD) and limit of quantification (LOQ) for each analyte. These parameters were evaluated using decreasing concentrations of analyte in drug-fortified meconium. LOD was defined as the lowest concentration with acceptable chromatography, acceptable ion ratios, a signal-to-noise ratio of at least 3, and a relative retention time (RRT) within $\pm 2\%$ of the average calibrator RRT. LOQ was the lowest concentration that met LOD criteria with signal-to-noise ratio of at least 10 and acceptable accuracy and precision as defined below. Ratios were acceptable if they were $\pm 10\%$ (absolute) of the average calibrator ratio for d_3 -NBUP, BUP, and d_4 -BUP and $\pm 30\%$ (relative) for NBUP. Lack of carryover was demonstrated by injecting an IS-fortified blank meconium after the highest calibrator and determining analyte concentrations. Carryover was considered negligible up to the concentration of the highest calibrator as long as the calculated analyte concentration of the carryover control specimen was below the method LOQ.

Linearity range was determined using least-squares regression with a $1/x$ weighting factor to compensate for heteroscedasticity. Acceptable linearity was achieved when the coefficient of determination was at least 0.98 and quantification of calibrators was within $\pm 20\%$ of targets.

Accuracy and precision were examined using low-, medium-, and high-concentration QC samples with and without hydrolysis. Targets for low-, medium- and high-QC samples were 30, 300, and 1600 ng/g unhydrolyzed and 60, 600, and 1600 ng/g hydrolyzed. Intra-assay imprecision was computed by quantifying five replicates of each concentration on 1 day and calculating the percent relative standard deviation (RSD). Inter-assay imprecision was calculated using one-way analysis of variance (ANOVA) with Tukey's post-hoc test to determine if there was a significant difference in the average concentrations calculated on each day. Intra-assay imprecision was required to be within $\pm 20\%$. Accuracy was expressed as percent of target concentration and was determined by comparing the mean calculated concentration of 20 replicates evaluated over 4 days to target. Acceptable accuracy was $\pm 20\%$ of target.

Method specificity was demonstrated by adding high concentrations (4000 ng/g) of potentially interfering licit and illicit drugs to low-QC samples. The following drugs and metabolites were evaluated: cocaine, benzoylecgonine, norcocaine, norbenzoylecgonine, ecgonine ethyl ester, ecgonine methyl ester, anhydroecgonine methyl ester, ecgonine, cocaethylene, norcocaethylene, *m*-hydroxycocaine, *p*-hydroxycocaine, *m*-hydroxybenzoylecgonine, *p*-hydroxybenzoylecgonine, methamphetamine, EMDP, EDDP, methadol, φ^9 -tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC, morphine, normorphine, morphine 3- β -D-glucuronide, morphine 6- β -D-glucuronide, codeine, norcodeine, 6-acetylmorphine, 6-acetylcodeine, hydrocodone, hydromorphone, oxycodone, noroxycodone, norhydromorphone, noroxymorphone, (*R*)(+)-cathinone, ($\pm$)-*N*-ethylamphetamine, 4-bromo-2,5-dimethoxyphenethylamine, diazepam, lorazepam, oxazepam, alprazolam, imipramine, clomipramine, fluoxetine, norfluoxetine oxalate, paroxetine maleate, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, clonidine, ibuprofen, pentazocine, caffeine, diphenhydramine, chlorpheniramine, brompheniramine, aspirin, acetaminophen, phenacyclidine, bromazepam, clonazepam, flurazepam, nitrazepam, flunitrazepam, temazepam, nordiazepam, and nicotine. No interference was noted if analyte concentrations quantified within $\pm 20\%$ of low-QC target concentration.

Extraction efficiency for each analyte was measured at each QC concentration. Blank meconium fortified with IS was spiked with QC solution either before extraction or after SPE. Percent recovery from meconium was expressed as mean analyte area to IS area ratio of samples ($N = 5$) fortified with control solution before extraction divided by the mean ratio in samples ($N = 5$) with control solution added after SPE.

Matrix effect was assessed by comparing analyte peak areas in six unique blank extracted meconium samples spiked with QC solution after SPE to peak areas of neat samples, with the same nominal concentrations, prepared in mobile phase A. Matrix suppression or enhancement, expressed as a percent, was calculated using the ratio of average extracted meconium peak area to average neat peak area.

Hydrolysis efficiency was tested at each QC concentration by comparing calculated concentrations of hydrolyzed controls to target. Dilution was necessary to quantify the high hydrolysis control. In order to ensure equivalent matrix effect and a well-homogenized sample, the following technique was employed. Blank meconium, fortified with IS and 50 μL of methanol, was prepared in a manner identical to the hydrolysis control preparation. After the samples were centrifuged, 1 mL of high-hydrolysis QC and 1 mL of IS fortified blank meconium were combined yielding a 1:2 dilution. Two milliliters of 1.1 M sodium acetate buffer was added, and SPE was performed as described above.

Stability of analytes was investigated under a variety of conditions. The 24- and 96-h prepared specimen stability was determined by reinjecting prepared QC samples stored at 10 $^{\circ}\text{C}$ on the autosampler and quantifying from the original calibration curve. In addition, stability was tested on drug-fortified meconium stored at room temperature (22 $^{\circ}\text{C}$) for 16 h, in the refrigerator (4 $^{\circ}\text{C}$) for 72 h, and after three freeze–thaw cycles.

Data Analysis

All LC–MS/MS data were acquired and analyzed using XCalibur 2.0 software. Statistical evaluations were performed using Graphpad Prism 5.

Method Application

The validated method was employed to analyze a meconium specimen obtained from an infant born to a woman participating in an Intramural Research Board approved clinical research study comparing the use of methadone and BUP for the treatment of opiate addiction during pregnancy. The woman was admitted to the double-blind, double-dummy, flexible, randomized, stratified, parallel-group controlled study at 26 weeks estimated gestational age and randomized to methadone or BUP treatment after signing written informed consent. Meconium was collected from the neonates' diapers, combined, and stored at -20°C until analysis.

RESULTS AND DISCUSSION

Validation

We present the first validated LC–MS/MS method for the analysis of BUP, NBUP, BUP-Gluc, and NBUP-Gluc in meconium. Validation of a method is necessary to ensure adequate sensitivity, specificity, reproducibility, and accuracy prior to analysis of valuable clinical specimens.

Calibration results for all analytes can be found in Table 1. LOD was 20 ng/g for all analytes, and the linear range was 20–2000 ng/g with average $R^2 > 0.99$. Retention times for BUP and NBUP during one analytical run varied by less than 0.46 and 0.29% RSD, respectively. While

precursor and product ions were present in 10 ng/g calibrators, ion ratios did not consistently meet required criteria; therefore, method LOD was 20 ng/g for all analytes. A representative total ion chromatogram of blank meconium can be seen in Figure 2A. Selected reaction monitoring chromatograms of quantifier and qualifier transitions in meconium fortified with 20 ng/g BUP and NBUP can be seen in Figure 2B.

Accuracy and precision were evaluated across the dynamic range of the assay using hydrolyzed and unhydrolyzed controls at three concentrations (Table 2). Intra- and interassay imprecision were computed using five replicate analyses a day for 4 days. Intra-assay imprecision was less than 13.9% for each of the four batches, and interassay imprecision was less than 12.4% for all analytes at all concentrations. In addition, ANOVA with Tukey's post-hoc analysis revealed no statistically significant difference between daily mean analyte concentrations ($\alpha = 0.05$.) Accuracy, calculated as percent of target value, was greater than 85.7% across the analytical range. Low-QC samples fortified with 4000 ng/g of potentially interfering abused and over-the-counter drugs all quantified within $\pm 20\%$ of target, indicating no interference with the measurement of BUP and NBUP.

Recovery, matrix effect, and hydrolysis efficiency were evaluated at each control concentration (Table 3). Recovery ranged from 77.0 to 91.5%. Matrix suppression from meconium was observed for the analytes. Use of structurally identical deuterated internal standards allowed for accurate quantification despite ion suppression from the complex matrix.⁵⁰ Average calculated concentrations of neat controls were within $\pm 20\%$ of the average extracted control concentrations, indicating deuterated internal standards were equally affected by the matrix. Hydrolysis efficiency was examined by comparing calculated concentrations of controls containing free and conjugated drug to unhydrolyzed controls with free drug concentrations equivalent to those expected if 100% hydrolysis was achieved. Average hydrolysis ($N = 3$) was 86.9 and 88.1% for BUP and NBUP, respectively. Hydrolysis was examined in every run by including five low-, medium-, and high-hydrolysis controls in each validation run. Hydrolysis controls always quantified within 7.2% of target.

Since calibrators were not hydrolyzed, it was important to document that hydrolysis did not affect free drug concentrations in clinical meconium specimens. Two sets of calibrators were prepared; one underwent hydrolysis and one did not. Calibration curves for the two sets were compared using the procedure described by Motulsky to determine whether hydrolysis treatment had an effect on the curve.⁵¹ Briefly, linear regression was applied to each set of data using Graphpad Prism 5, and the sum-of-squares (SS_{separate}) and degrees of freedom (DF_{separate}) were totaled. Next, the hydrolyzed and unhydrolyzed curve data were combined into one data set, a new curve was fit, and the combined sum-of-squares (SS_{combined}) and degrees of freedom (DF_{combined}) were noted. The F -value was calculated using the following equation, and the p -value was determined using Excel.

$$F = \frac{(SS_{\text{combined}} - SS_{\text{separate}}) / (DF_{\text{combined}} - DF_{\text{separate}})}{SS_{\text{separate}} / DF_{\text{separate}}} \quad (1)$$

Application of this analysis revealed no difference between hydrolyzed and unhydrolyzed curves. Additionally, five replicates of low, medium, and high controls (hydrolyzed and unhydrolyzed) were quantified on each curve. There was no statistically significant difference between the mean concentrations of hydrolyzed and unhydrolyzed samples ($\alpha = 0.05$).

Stability of analytes in meconium was tested under a variety of conditions to mimic those encountered in the laboratory. Drug-fortified meconium was stored at room temperature (22 °C) for 16 h, in the refrigerator (4 °C) for 72 h, or subjected to three freeze/thaw cycles prior to

analysis. In addition, QC samples were reinjected after 24 or 96 h storage on the autosampler (10 °C) and quantified using original calibration data. Stability, reported as a percent, was calculated by dividing the calculated concentration in stability controls to concentrations in fresh controls prepared the day of analysis. Table 4 summarizes the stability of BUP and NBUP in meconium.

Proof of Method

The validated method was used to analyze a meconium specimen from an infant born to a woman treated with up to 20 mg/day BUP for the last 12 weeks of pregnancy. Figure 2C is a representative chromatogram from an extracted participant specimen. Total and free BUP concentrations were 123.8 and 101.6 ng/g, respectively. NBUP concentrations were higher, 719.3 ng/g for total drug and 712.6 ng/g for the unconjugated analyte. Marquet et al. reported meconium BUP and NBUP concentrations of 107 and 295 ng/g, respectively, in a single infant born to a woman treated with 4 mg/day BUP during the last 23 weeks of her pregnancy.³⁶

CONCLUSION

This method is the first fully validated method for the quantification of BUP, NBUP, and glucuronide conjugates in meconium. Extracting analytes of interest from meconium with buffer and SPE, followed by LC–MS/MS, provided a sensitive and specific method for quantifying BUP and NBUP. Analyzing specimens with and without enzymatic hydrolysis permitted estimation of BUP and NBUP glucuronide concentrations.

This method permits quantification of BUP and NBUP in meconium from infants born to BUP maintained women and women self-administering illicit BUP. Quantifying drug concentrations in meconium will help determine whether there is a relationship between maternal BUP dose and meconium drug concentrations and whether drug levels in meconium predict maternal and neonatal outcome measures including neonatal abstinence syndrome.

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Figure 1.

Proposed tandem mass spectrometric fragmentation of (A) buprenorphine, (B) d₄-buprenorphine, (C) norbuprenorphine, and (D) d₃-norbuprenorphine.

Figure 2.

(A) Total ion chromatogram of extracted blank meconium. Selected reaction monitoring chromatogram of quantifier (—) and qualifier transitions (···) for (B) meconium fortified with 20 ng/g buprenorphine and norbuprenorphine, and (C) representative infant meconium containing 123.8, 101.6, 719.3, and 712.6 ng/g total BUP, free BUP, total NBUP, and free NBUP, respectively.

Table 1

Method Calibration Data for Buprenorphine and Norbuprenorphine in Meconium by LC–MS/MS

analyte	buprenorphine	norbuprenorphine
internal standard	d ₄ -buprenorphine	d ₃ -norbuprenorphine
slope ± SE (<i>N</i> = 5)	0.0583 ± 0.0019	0.0171 ± 0.0004
intercept ± SE (<i>N</i> = 5)	−0.0279 ± 0.0384	0.0574 ± 0.0153
coefficient of determination ± SE (<i>R</i> ²), (<i>N</i> = 5)	0.9941 ± 0.0018	0.9937 ± 0.0012
retention time ± SD min, (<i>N</i> = 38)	5.87 ± 0.02	2.74 ± 0.01

Table 2
Precision and Accuracy Results for Buprenorphine and Norbuprenorphine in Meconium by LC-MS/MS

		buprenorphine	norbuprenorphine
Maximum Intra-Assay Imprecision ^a (% RSD), <i>N</i> = 5			
unhydrolyzed	low	9.7	12.6
	medium	11.2	8.2
	high	13.1	6.0
hydrolyzed	low	11.0	8.8
	medium	13.9	7.5
	high	11.7	12.7
Interassay Imprecision ^b (% RSD), <i>N</i> = 20			
unhydrolyzed	low	8.5	12.4
	medium	8.8	4.6
	high	9.9	6.1
hydrolyzed	low	9.3	8.4
	medium	9.3 ^c	6.5
	high	10.3	11.0
Accuracy ^d (% RSD), <i>N</i> = 20			
unhydrolyzed	low	85.7	99.2
	medium	93.3	88.4
	high	95.8	92.3
hydrolyzed	low	93.4	95.2
	medium	93.8 ^c	92.8
	high	102.3	99.2

^a Percent relative standard deviation of five replicates of each concentration analyzed on the same day; maximum imprecision of four different daily batches.

^b Percent relative standard deviation of 20 replicates over 4 days.

^c *N* = 19 due to experimental error

^d Percent of target concentration.

Table 3
Recovery and Matrix Effect of Buprenorphine and Norbuprenorphine in Meconium by LC–MS/MS

	quality control sample	buprenorphine	norbuprenorphine
recovery, ^a <i>N</i> = 5 (%)	low	88.0	77.0
	medium	85.6	85.0
	high	88.4	91.5
matrix effect, ^b <i>N</i> = 5 (% suppression)	low	16.8	14.0
	medium	31.3	44.9
	high	18.4	30.0

^a Mean analyte area to IS area ratio of samples fortified with control solution before extraction divided by the mean ratio in samples with control solution added after solid-phase extraction.

^b Analyte peak areas in six unique blank extracted meconium samples spiked with quality control solution after solid-phase extraction to peak areas of neat samples, with the same nominal concentrations, prepared in mobile phase A.

Table 4
Stability of Buprenorphine and Norbuprenorphine in Meconium by LC–MS/MS^a

storage conditions	buprenorphine			norbuprenorphine		
	low	medium	high	low	medium	high
24 h at 10 °C, <i>N</i> = 5	105.0	105.5	102.7	106.6	101.4	96.4
96 h at 10 °C, <i>N</i> = 5	107.2	98.1	107.3	97.8	103.6	96.4
16 h at 22 °C, <i>N</i> = 3	99.1	103.5	110.0	101.2	108.2	109.6
72 h at 4 °C, <i>N</i> = 3	96.0	88.8	103.5	100.4	89.5	100.3
3 freeze/thaw cycles, <i>N</i> = 3	102.0	96.7	110.0	104.5	96.4	99.7

^aPercent of fresh quality control results.