



Confirmatory analysis of buprenorphine, norbuprenorphine, and glucuronide metabolites in plasma by LCMSMS. Application to umbilical cord plasma from buprenorphine-maintained pregnant women

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ABSTRACT

An LCMSMS method was developed and fully validated for the simultaneous quantification of buprenorphine (BUP), norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-Gluc), and norbuprenorphine-glucuronide (NBUP-Gluc) in 0.5 mL plasma, fulfilling confirmation criteria with two transitions for each compound with acceptable relative ion intensities. Transitions monitored were 468.3 > 396.2 and 468.3 > 414.3 for BUP, 414.3 > 340.1 and 414.3 > 326.0 for NBUP, 644.3 > 468.1 and 644.3 > 396.3 for BUP-Gluc, and 590.3 > 414.3 and 590.3 > 396.2 for NBUP-Gluc. Linearity was 0.1–50 ng/mL for BUP and BUP-Gluc, and 0.5–50 ng/mL for NBUP and NBUP-Gluc. Intra-day, inter-day, and total assay imprecision (%RSD) were <16.8%, and analytical recoveries were 88.6–108.7%. Extraction efficiencies ranged from 71.1 to 87.1%, and process efficiencies 48.7 to 127.7%. All compounds showed ion enhancement, except BUP-Gluc that demonstrated ion suppression: variation between 10 different blank plasma specimens was <9.1%. In six umbilical cord plasma specimens from opioid-dependent pregnant women receiving 14–24 mg/day BUP, NBUP-Gluc was the predominant metabolite (29.8 ± 7.6 ng/mL), with BUP-Gluc (4.6 ± 4.8 ng/mL), NBUP (1.5 ± 0.8 ng/mL) and BUP (0.4 ± 0.2 ng/mL). Although BUP biomarkers can be quantified in umbilical cord plasma in low ng/mL concentrations, the significance of these data as predictors of neonatal outcomes is currently unknown.

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1. Introduction

Buprenorphine (BUP) is a semi-synthetic opioid derived from thebaine, prescribed at low doses (0.3–0.6 mg IV or IM) to treat moderate-to-severe pain, and at higher doses (4–24 mg) by the sublingual route as pharmacotherapy for opioid dependence [1]. BUP was recently approved for the treatment of opioid-dependent pregnant women in some countries, and is under consideration for this indication in the US [2–5].

BUP is well absorbed in the gastrointestinal tract, but has low oral bioavailability due to high first-pass hepatic metabolism. BUP is rapidly N-dealkylated to norbuprenorphine (NBUP), mainly by CYP3A4 in the liver [6]. BUP and NBUP are further metabolized by phase II glucuronidation to buprenorphine-glucuronide (BUP-Gluc) and norbuprenorphine-glucuronide (NBUP-Gluc) [7]. The simultaneous determination of BUP, NBUP, BUP-Gluc, and NBUP-Gluc in plasma is important for characterizing BUP phar-

macokinetics, and to interpret clinical and forensic toxicology results.

Umbilical cord plasma is an interesting alternative matrix for identifying in utero drug exposure [8,9]. This specimen is available at the time of birth and reflects concurrent drug concentrations in the newborn. Furthermore, specimen collection is non-invasive, especially in comparison to infant plasma collection. Few data are available on drug concentrations in umbilical cord plasma [10–14], and no data on BUP in this matrix. In adult plasma, the predominant metabolite is NBUP-Gluc [1,15], but BUP and metabolites distribution in umbilical cord plasma are unknown.

Gas chromatography–mass spectrometry (GCMS) [16–18] methods are available for the quantification of BUP and NBUP in plasma. LCMS offers several advantages compared to GCMS for the analysis of these biomarkers. No derivatization is required and glucuronide metabolites can be quantified without the need for costly and time-consuming hydrolysis. LCMS and LCMSMS have been applied for BUP and metabolites' analysis in a wide variety of matrices including, urine [1,19–28], hair [21,23,25,29–32], sweat [33], meconium [34], breast milk [35], placenta [36], and umbilical cord [37]. In plasma, several methods were published for BUP alone

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[18,38], BUP and NBUP [23,39–41]; BUP, NBUP, and glucuronides after hydrolysis [25,42]; BUP, NBUP and BUPG [43]; and all four analytes [1,15,44]. For compound identification by LCMSMS, FDA [45] and European guidelines [46] require chromatographic separation, a minimum of two transitions per analyte or three characteristic ions if LCMS (single MS) is employed, and acceptable relative ion intensities. Despite this recommendation, none of the published LCMS or LCMSMS methods fulfilled these requirements for glucuronides identification; either one ion in single ion monitoring mode (SIM) [44] or only one characteristic transition [1,15,43,44] was monitored. In the literature, several examples of false positive results have been reported utilizing a single transition [47,48], demonstrating the importance of following confirmation guidelines.

We developed and fully validated an LCMSMS method for the simultaneous determination of BUP, NBUP, BUP-Gluc, and NBUP-Gluc in umbilical cord plasma with two characteristic transitions for each analyte with acceptable relative ion intensities, fulfilling FDA and European guidelines requirements for confirmatory analysis. This method was applied to the analysis of umbilical cord plasma specimens from infants of pregnant women receiving observed daily BUP doses as pharmacotherapy for opioid dependence in a randomized, double-blind, controlled clinical study [49].

2. Materials and methods

2.1. Chemicals and materials

BUP, NBUP, BUP-Gluc, NBUP-Gluc, buprenorphine- d_4 (BUP- d_4), and norbuprenorphine- d_3 (NBUP- d_3) ampoules at 100 $\mu\text{g}/\text{mL}$ in methanol were obtained from Cerilliant (Austin, TX). BUP, NBUP, BUP-Gluc, and NBUP-Gluc quality control (QC) samples were prepared from a different lot from Cerilliant, when possible, or from a different vial, with preparation on different days than for calibrators. Reagent grade formic and perchloric acid were from Sigma Chemicals (St. Louis, MO) and Acros Organics (Morris Plains, NJ), respectively. All solvents were HPLC grade. Solid phase extraction (SPE) was performed with Strata-XC columns (60 mg/3 mL) (Phenomenex, Torrance, CA). Different pools of drug-free human plasma were obtained from the Department of Transfusion Medicine, National Institutes of Health (Bethesda, MD).

2.2. Apparatus

LCMSMS analysis was performed with a Shimadzu LC system (Kyoto, Japan) interfaced to a 3200 QTrap (Applied Biosystem/MDS Sciex, Foster City, CA) with an electrospray (ESI) ion source. The Shimadzu system consisted of a binary pump LC-20AD, degasser DGU-20A3, autosampler SIL-20AD, and a column oven CTO-20A. Data acquisition, peak integration and calculation were assigned to a computer work station running AnalystTM Software 1.4.1. A 5804R centrifuge from Eppendorf (Hamburg, Germany) was employed. Solvent evaporation was carried out on a TurboVap LV evaporator from Zymark (Hopkinton, MA).

2.3. Preparation of standard solutions

Solutions containing 10, 1, and 0.1 $\mu\text{g}/\text{mL}$ of BUP, NBUP, BUP-Gluc, and NBUP-Gluc were prepared separately in methanol from 100 $\mu\text{g}/\text{mL}$ stock calibrators. Different working solutions of the four analytes were prepared by appropriate dilution in methanol. The internal standard (IStd) solution, 0.4 $\mu\text{g}/\text{mL}$ BUP- d_4 and NBUP- d_3 , was prepared by the dilution of 100 $\mu\text{g}/\text{mL}$ stock solutions in methanol. QC solutions containing BUP, NBUP, BUP-Gluc, and NBUP-Gluc were prepared in methanol from different stock solutions than those used for calibrators at three

different working concentrations across the linear range of the assay.

2.4. Calibrator, quality control, and specimen preparation

A nine-point calibration curve (0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng/mL) was prepared by adding 25 μL of working calibrator and 20 μL of IStd solution to 0.5 mL blank plasma. 0.1 and 0.2 ng/mL calibrators were not used for NBUP and NBUP-Gluc. Low, medium, and high QC samples were 0.3, 7.5 and 30 ng/mL for BUP and BUP-Gluc, and 1.5, 7.5 and 30 ng/mL for NBUP and NBUP-Gluc. QCs were prepared by adding 25 μL of working QC solution and 20 μL of IStd solution to 0.5 mL blank plasma. For authentic specimens, 20 μL of IStd solution was added to 0.5 mL plasma. After adding 1 mL of 0.1% perchloric acid in water, samples were centrifuged at $5974 \times g$ for 5 min and supernatants were subjected to SPE.

2.5. Extraction

SPE cartridges were preconditioned with 2 mL methanol and 2 mL 0.1% perchloric acid in water. Supernatants were applied followed by washing with 2 mL 2% formic acid in water and 2 mL methanol. Cartridges were dried for 15 min under vacuum before eluting with 3 mL methylenechloride:isopropanol:concentrated ammonium hydroxide (60:35:5, v/v/v). Eluates were dried completely under nitrogen at 45 °C. Dried extracts were reconstituted with 50 μL mobile phase, 85% A (0.1% formic acid in water) and 15% B (0.1% formic acid in acetonitrile), and 20 μL were injected into the LCMSMS.

2.6. Liquid chromatography

Chromatographic separation was achieved with a Synergi Polar-RP 80A (75 mm \times 2 mm, 4 μm) column with a 4 mm \times 2 mm, identically packed guard column (Phenomenex, Torrance, CA) and gradient elution with mobile phase A and B at a 300 $\mu\text{L}/\text{min}$ flow rate. The initial mixture (85% A, 15% B) was maintained for 0.5 min, B was increased from 15% to 65% over 5.5 min, returned to initial conditions over 1 min, followed by a 3 min equilibration for a total run time of 10 min.

2.7. Mass spectrometry

Mass spectrometric data were acquired in positive ion mode with the following ESI-MS parameters: IonSpray voltage: 5500 V; temperature: 600 °C; curtain gas: 10; ion source gas 1: 50; and ion source gas 2: 55. The curtain and ion source gases were nitrogen. Data were recorded in single reaction monitoring (SRM) mode. MSMS optimization was established by infusing 100 ng/mL of each analyte in methanol directly. The precursor ions, product ions, and LCMSMS parameters are displayed in Table 1.

2.8. Validation

Validation parameters included linearity, limits of detection (LOD) and quantification (LOQ), imprecision, analytical recovery, extraction efficiency, process efficiency, matrix effect, selectivity, carryover, dilution integrity, and stability studies. Linearity was determined by least-squares regression with $1/x$ weighting. Acceptable linearity was achieved when the coefficient of determination was at least 0.99 and calibrators quantified within $\pm 20\%$ at the LOQ and $\pm 15\%$ at other concentrations. LOD and LOQ were evaluated with decreasing analyte concentrations in drug-fortified plasma. LOD was defined as the lowest concentration with acceptable chromatography, presence of all qualifier ions with signal-to-noise ratios of at least 3, and a retention time (RT) within

Table 1

LCMSMS parameters, retention times and internal standards (IStd) for buprenorphine (BUP), norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-Gluc), and norbuprenorphine-glucuronide (NBUP-Gluc).

Analyte	SRM transition ^a	DP ^b (V)	EP ^c (V)	CEP ^d (V)	CE ^e (eV)	CXP ^f (V)	RT ^g (min)	IStd
NBUP-Gluc	<u>590.3 > 414.3</u>	96	9.5	34	51	4	3.3	NBUP-d ₃
	590.3 > 396.2	96	9.5	27	50	3		
BUP-Gluc	<u>644.3 > 468.1</u>	101	9.5	30	45	4	4	BUP-d ₄
	644.3 > 396.3	101	9.5	30	71	4		
NBUP	<u>414.3 > 340.1</u>	86	12	26	43	4	4.2	NBUP-d ₃
	414.3 > 326	86	12	26	40	5		
NBUP-d ₃	<u>417.3 > 343.4</u>	86	12	16	39	4	4.2	
	417.3 > 326	86	12	22	43	4		
BUP	<u>468.3 > 396.2</u>	86	12	18	53	6	5	BUP-d ₄
	468.3 > 414.3	86	12	18	45	12		
BUP-d ₄	<u>472.5 > 400.1</u>	86	11	24	61	6	5	
	472.5 > 415.3	86	11	24	45	8		

^a The quantification transition is underlined.

^b Declustering potential.

^c Entrance potential.

^d Collision cell entrance potential.

^e Collision energy.

^f Collision cell exit potential.

^g Retention time.

±0.2 min of the average calibrator RT. LOQ was the lowest concentration that met LOD criteria and a signal-to-noise ratio of at least 10, imprecision lower than 20%, and analytical recovery between 80 and 120%.

Imprecision and analytical recovery were determined at three concentrations by analyzing five replicates on four different days ($n=20$). Imprecision, expressed as % relative standard deviation (%RSD) of the measured values, was expected to be less than 15%. The guidelines given by Krouwer and Rabinowitz [50] were followed for the calculation of pooled intra-day, inter-day, and total imprecision. According to the authors [50], within-run imprecision is subtracted from the standard deviation of the mean run averages to obtain a pure between-run component of imprecision. The estimates of imprecision standard deviation within day (SW), between day (SB), and total (ST), were as follows:

$$SW = \sqrt{\text{MeanSquareWithinDay}}$$

$$SB = \sqrt{\frac{\text{MeanSquareBetweenDay} - \text{MeanSquareWithinDay}}{n}}$$

$$ST = \sqrt{SW^2 + SB^2}$$

In the SB equation, n is the number of replicates and it can happen that mean square between day is less than mean square within day, which means that the quantity under the square-root sign can be negative. In this case, one sets $SB=0$, and then the inter-day imprecision is 0. Analytical recovery was evaluated as the percent of target concentration ($n=20$) with required criteria of 85–115%.

Extraction efficiency for each analyte was measured at each QC concentration. Blank plasma was fortified with QC and IStd solution before and after SPE. Percent extraction efficiency from plasma was expressed as mean analyte area of samples ($n=5$) fortified with control solution before extraction divided by mean area of samples ($n=5$) with control solution added after SPE. Matrix effect was assessed by comparing analyte peak areas in 10 different blank extracted plasma specimens fortified with QC and IStd solutions after SPE to peak areas of samples at the same nominal concentrations prepared in an 85:15 mixture of mobile phase A and mobile phase B (neat). Matrix suppression or enhancement was calcu-

lated as follows: $(100 \times \text{mean peak area of fortified plasma after SPE} / \text{mean peak area of neat}) - 100$. Process efficiency examined the overall effect of SPE extraction efficiency and matrix effect on the quantification of analytes of interest. It was determined by comparing mean analyte peak areas of five samples fortified before SPE with mean peak areas of five neat samples prepared in mobile phase at the same concentration.

Interferences from endogenous matrix components were evaluated by analyzing plasma samples from 10 healthy non-drug-consuming subjects only fortified with IStd solution. Endogenous interferences were considered insignificant if analytes were not detected in these 10 plasma samples. Method specificity was demonstrated by adding high concentrations (500 ng/mL) of potentially interfering licit and illicit drugs to low QC samples. The following drugs and metabolites were examined: cocaine, benzoylecgonine, norcocaine, norbenzoylecgonine, ecgonine ethyl ester, ecgonine methyl ester, anhydroecgonine methyl ester, ecgonine, amphetamine, methamphetamine, p-hydroxyamphetamine, p-hydroxymethamphetamine, norephedrine, hydroxynorephedrine, 3,4-methylenedioxyamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDEA), 3-hydroxy-4-methoxyamphetamine (HMA), 3-hydroxy-4-methoxymethamphetamine (HMMA), methadone, 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), methadol, Δ^9 -tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC, morphine, normorphine, morphine 3- β -glucuronide, morphine 6- β -glucuronide, codeine, norcodeine, 6-acetylmorphine, 6-acetylcodeine, diazepam, lorazepam, oxazepam, alprazolam, imipramine, clomipramine, fluoxetine, norfluoxetine, clonidine, ibuprofen, pentazocine, caffeine, diphenhydramine, chlorpheniramine, brompheniramine, acetylsalicylic acid, acetaminophen, and phencyclidine. Sufficient specificity was achieved if BUP, NBUP, BUP-Gluc, and NBUP-Gluc quantified within $\pm 15\%$ of low QC concentrations.

Lack of carryover was demonstrated by injecting IStd-fortified blank plasma immediately after a sample spiked with all analytes at 100 ng/mL, two times the upper LOQ. Carryover was considered negligible if the measured concentration was below the LOD. Dilution integrity was evaluated by diluting plasma samples ($n=2$) containing 100 ng/mL of each analyte with blank plasma to achieve a 1:4 dilution. IStd was added to diluted samples that

Table 2
Imprecision and analytical recovery for buprenorphine (BUP), and metabolites norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-Gluc), and norbuprenorphine-glucuronide (NBUP-Gluc) in plasma by LCMSMS.

Analyte	Concentration (ng/mL)	Total mean (n = 20, ng/mL)	Imprecision (n = 20 ^a , %RSD)			Analytical recovery (n = 20 ^a , % of target)
			Pooled intra-day	Inter-day	Total	
BUP	0.3	0.3	10.7	0	10.7	98.7
	7.5	8.2	5.4	0	5.4	108.7
	30	31.6	6	0	5.9	105.2
NBUP	1.5	1.5	11	6.8	13	101.3
	7.5	7.6	8.5	0	8.5	100.7
	30	30.3	6.8	0	6.8	100.9
BUP-Gluc	0.3	0.3	0	16.8	16.8	88.6
	7.5	7.3	7.8	4.9	9.2	97.4
	30	30	7.2	4.8	8.6	100
NBUP-Gluc	1.5	1.4	8.1	7.6	11.1	93.9
	7.5	8	8	0	7.8	106
	30	32.5	6.7	0	6.7	108.3

^a 5 replicates on four different days for a total of 20.

were extracted as described. Dilution integrity was maintained if samples quantified within $\pm 15\%$ of 25 ng/mL.

Analyte stability was evaluated for drug-fortified plasma stored at room temperature (22 °C) for 16 h, in the refrigerator (4 °C) for 72 h, and after three freeze–thaw cycles (frozen 24 h, thawed kept at room temperature for 3 h). Plasma samples were fortified with all four analytes at three concentrations (low, medium and high QC) in triplicate. Stability was considered acceptable if QC samples quantified within $\pm 15\%$ of target.

2.9. Identification criteria

Identification criteria included RT within ± 0.2 min of average calibrator RT, presence of two transitions, and relative ion intensities (% of base peak) within $\pm 20\%$, if relative ion intensity was $> 50\%$; $\pm 25\%$ if 20–50%; $\pm 30\%$ if 10–20%; and $\pm 50\%$ if $\leq 10\%$ [46]. Relative ion intensities were calculated based on ion ratios (most abundant transition divided by less abundant) 468.3 > 396.2/468.3 > 414.3 for BUP, 414.3 > 340.1/414.3 > 326 for NBUP, 644.3 > 468.1/644.3 > 396.3 for BUP-Gluc, and 590 > 414.3/590.3 > 396.2 for NBUP-Gluc. These values were compared to mean relative ion intensity of all calibrators.

2.10. Method application

The method was applied to the analysis of umbilical cord plasma and maternal plasma specimens collected at delivery from five

opioid-dependent pregnant women receiving controlled buprenorphine treatment; one set of twins, participant A, and four single births, participants B, C, D, and E. Six umbilical cord plasma (participant A twins, B, C, D, and E), and two maternal plasma at delivery (participants B and E) were collected for research purposes. BUP doses ranged from 14 to 24 mg/day. Specimens were collected as part of a protocol providing BUP pharmacotherapy to opioid-dependent pregnant women approved by the Johns Hopkins Bayview and National Institute on Drug Abuse's Institutional Review Boards. Subjects provided written informed consent to participate.

3. Results

3.1. Analytical method

Linearity of analyte-to-IStd peak area ratio versus theoretical concentration was verified in plasma with $1/x$ -weighted linear regression. Curvature tested on a set of four calibration curves yielded determination coefficients (r^2) above 0.99 (BUP intercept = 0.0042 ± 0.0011 , slope = 0.7970 ± 0.0061 , $r^2 = 0.9952 \pm 0.0033$; NBUP intercept = 0.0246 ± 0.0103 , slope = 0.0446 ± 0.0021 , $r^2 = 0.9949 \pm 0.0010$; BUP-Gluc intercept = 0.0066 ± 0.0131 , slope = 0.2525 ± 0.0606 , $r^2 = 0.9970 \pm 0.0021$; and NBUP-Gluc intercept = 0.0597 ± 0.0252 , slope = 0.3425 ± 0.1157 , $r^2 = 0.9956 \pm 0.0022$), with residuals within $\pm 20\%$ at the LOQ and $\pm 15\%$ at other calibrator concentrations. The linear calibration range included nine

Table 3
Extraction efficiency, process efficiency and matrix effect for buprenorphine (BUP), and metabolites norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-Gluc), and norbuprenorphine-glucuronide (NBUP-Gluc) in plasma by LCMSMS.

Analyte	Concentration (ng/mL)	Extraction efficiency (% , n = 5)	Process efficiency (% , n = 5)	Matrix effect (n = 10)	
				Effect (%)	%RSD
BUP	0.3	87.1	96.3	10.2	7.1
	7.5	83.8	127.7	54.9	6.4
	30	77.9	110.5	44.6	3.9
NBUP	1.5	75.9	93.3	20.8	8.4
	7.5	82.7	105.9	31.3	7.3
	30	77.9	93.6	22.1	4.6
BUP-Gluc	0.3	79.9	53.2	–32.9	9.1
	7.5	81.5	51.7	–35.6	6
	30	73.1	48.7	–32.6	4
NBUP-Gluc	1.5	75.2	118.8	57.1	5.9
	7.5	76	117.7	58.4	6.9
	30	71.1	104.6	50.4	4.1

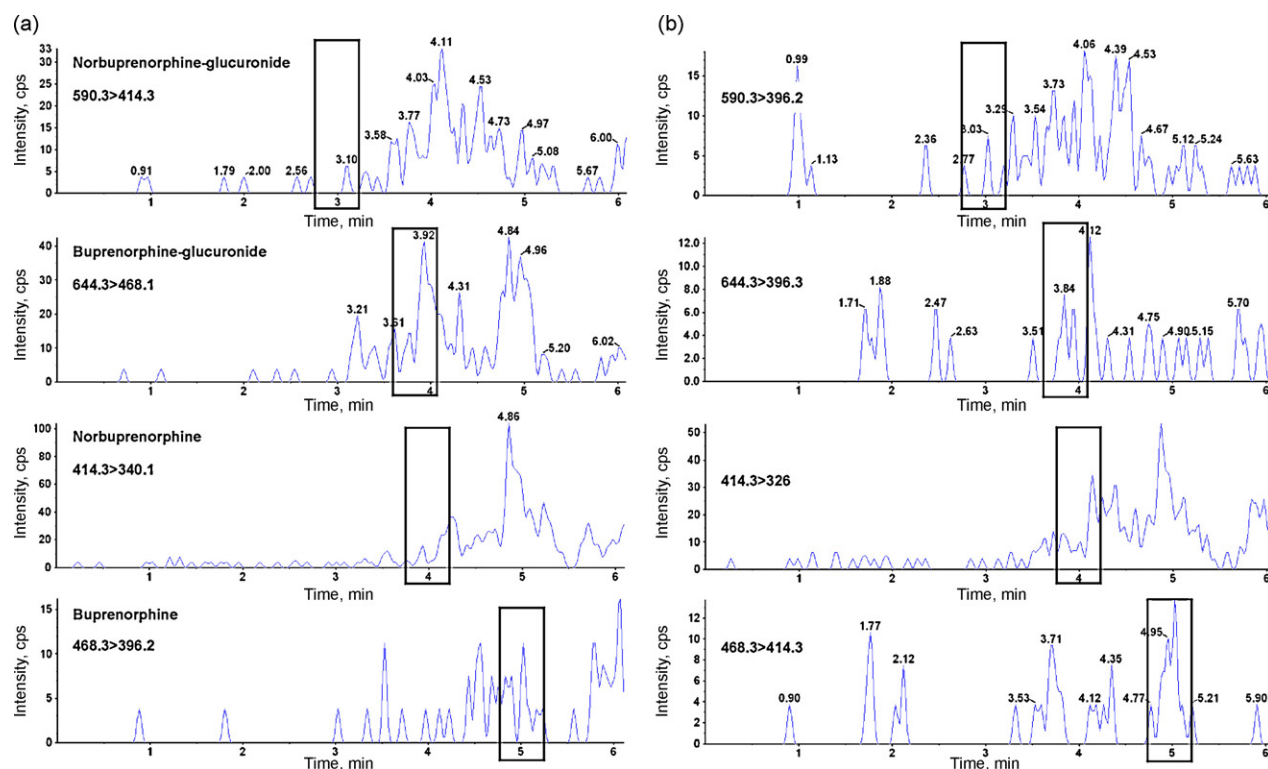


Fig. 1. LCMSMS chromatogram of blank plasma with retention times of analytes of interest noted. Panel (a) shows the quantification transitions and panel (b) qualifier transitions.

concentrations from 0.1 to 50 ng/mL for BUP and BUP-Gluc, and seven concentrations from 0.5 to 50 ng/mL for NBUP and NBUP-Gluc. LODs were 0.075 ng/mL for BUP and BUP-Gluc, 0.4 ng/mL for NBUP, and 0.2 ng/mL for NBUP-Gluc.

Imprecision and analytical recovery results were satisfactory for all tested concentrations (Table 2). Extraction efficiencies for all analytes ranged from 71.1 to 87.1%, and process efficiencies from 48.7 to 127.7%. Matrix effect ranged from 10.2 to 58.4% with variation between 10 different plasma <9.1%. Matrix effect included ion enhancement for all compounds except BUP-Gluc that demonstrated ion suppression. Data are shown in Table 3.

Under described conditions, no interference with any extractable endogenous compound in plasma was observed (Fig. 1). Method selectivity was demonstrated by adding high

concentrations (500 ng/mL) of 52 potentially interfering licit and illicit drugs, and metabolites to low QC samples. All test samples quantified within $\pm 15\%$ of target, indicating no interference with the four analytes of interest. No analyte was detected in a blank sample injected immediately following analysis of a 100 ng/mL sample, indicating no carryover at this concentration. The ability of the method to accurately quantify specimens containing high concentrations of analytes was evaluated by diluting 100 ng/mL samples ($n=2$) with blank plasma; 375 μ L of blank plasma was added to 125 μ L of fortified sample to achieve a 1:4 dilution. Samples quantified within 14% of 25 ng/mL, confirming dilution integrity. Analytes were stable when stored at room temperature (22 $^{\circ}$ C) for 16 h, refrigerated at 4 $^{\circ}$ C for 72 h, and after three freeze–thaw cycles. These results are shown in Table 4.

Table 4

The stability of buprenorphine (BUP) and metabolites norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-Gluc), and norbuprenorphine-glucuronide (NBUP-Gluc) in plasma ($n=3$) under different storage conditions. Room temperature (RT) for 16 h; 4 $^{\circ}$ C for 72 h; three freeze/thaw cycles (F/T); percentage difference from fresh controls (Diff %).

Compound	Fresh ($n=3$)	RT ($n=3$)		4 $^{\circ}$ C ($n=3$)		F/T ($n=3$)	
	Mean (ng/mL)	Mean (ng/mL)	Diff %	Mean (ng/mL)	Diff %	Mean (ng/mL)	Diff %
BUP	0.26	0.31	16.3	0.27	3.0	0.28	5.3
	8.4	8.3	-1.1	8.6	2.5	8.9	5.8
	33.6	31.9	-5.3	34.3	2.0	32.9	-2.1
NBUP	1.4	1.6	16.8	1.3	-6.0	1.4	3.8
	7.8	7.7	-1.0	7.9	1.4	8.2	4.6
	31.6	30.4	-3.7	32	1.4	31.4	-0.4
BUP-Gluc	0.25	0.27	7.9	0.24	-4.5	0.24	-4.7
	8	7.9	-0.8	7.2	-9.7	8.5	6.5
	30.5	32.7	7.3	34.1	12	31.8	4.3
NBUP-Gluc	1.3	1.3	1	1.3	-0.8	1.3	-4
	8.1	7.2	-11.4	8.5	5.1	7.8	-3.6
	34.8	29.8	-14.6	31.6	-9.3	32.4	-6.9

Table 5
Buprenorphine (BUP), norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-Gluc), and norbuprenorphine-glucuronide (NBUP-Gluc) concentrations in umbilical cord plasma from buprenorphine-maintained pregnant women.

Umbilical cord plasma	Dose at delivery (mg)	Time between last dose and delivery (h)	BUP (ng/mL)	NBUP (ng/mL)	BUP-Gluc (ng/mL)	NBUP-Gluc (ng/mL)
A.Baby 1	18	4.3	0.7	0.8	4.4	22.8
A.Baby 2	18	4.3	0.6	0.9	3.8	22.0
B	24	24	ND ^a	1.1	1.6	34.4
C	18	3.3	0.3	1.3	13.9	35.8
D	18	10.5	0.3	2.8	2.2	39.4
E	14	16.5	0.1	1.9	2.0	24.2

^a Not detected.

3.2. Specimen analysis

This plasma method was applied to the analysis of umbilical cord plasma specimens from pregnant women receiving 14–24 mg SL BUP prior to delivery. The predominant compound identified was NBUP-Gluc (29.8 ± 7.6 ng/mL), followed by BUP-Gluc (4.6 ± 4.8 ng/mL), NBUP (1.5 ± 0.8 ng/mL), and BUP (0.4 ± 0.2 ng/mL). Table 5 shows results and Fig. 2 represents the extracted transitions chromatogram of one authentic specimen. Fig. 3 describes BUP and metabolite relative concentrations in each specimen, including also the times between last maternal BUP dose and specimen collection. Two maternal plasma specimens collected at delivery from participants B and E also were analyzed. Participant B's plasma concentrations were NBUP-Gluc 23.1 ng/mL, BUP-Gluc 6.5 ng/mL, NBUP 1.9 ng/mL, and BUP 0.6 ng/mL, and participant's E NBUP-Gluc 42 ng/mL, BUP-Gluc 18.2 ng/mL, NBUP 2.9 ng/mL, and BUP 0.2 ng/mL.

4. Discussion

4.1. Analytical method

A sensitive quantitative method for the simultaneous determination of BUP and three metabolites in plasma was developed and fully validated. Therapeutic BUP plasma levels decrease to 0.3–1.9 ng/mL concentrations within 24 h of a 24 mg dose [18], documenting the need for a highly sensitive method. This assay satisfied sensitivity requirements with limits of quantification of 0.1 ng/mL for BUP and BUP-Gluc, and 0.5 ng/mL for NBUP and NBUP-Gluc. Other published methods achieved similar or better LOQ for BUP and NBUP [1,18,23,39,41,43,51,52]; however, none fulfilled compound identification criteria [45,46] due to monitoring of only a single ion [23,41,42], the surviving transition ion [18,39,43], or only one characteristic transition [1,52]. Favretto et al. [25] achieved 0.1 ng/mL LOQ for BUP and NBUP with an ion trap mass spectrom-

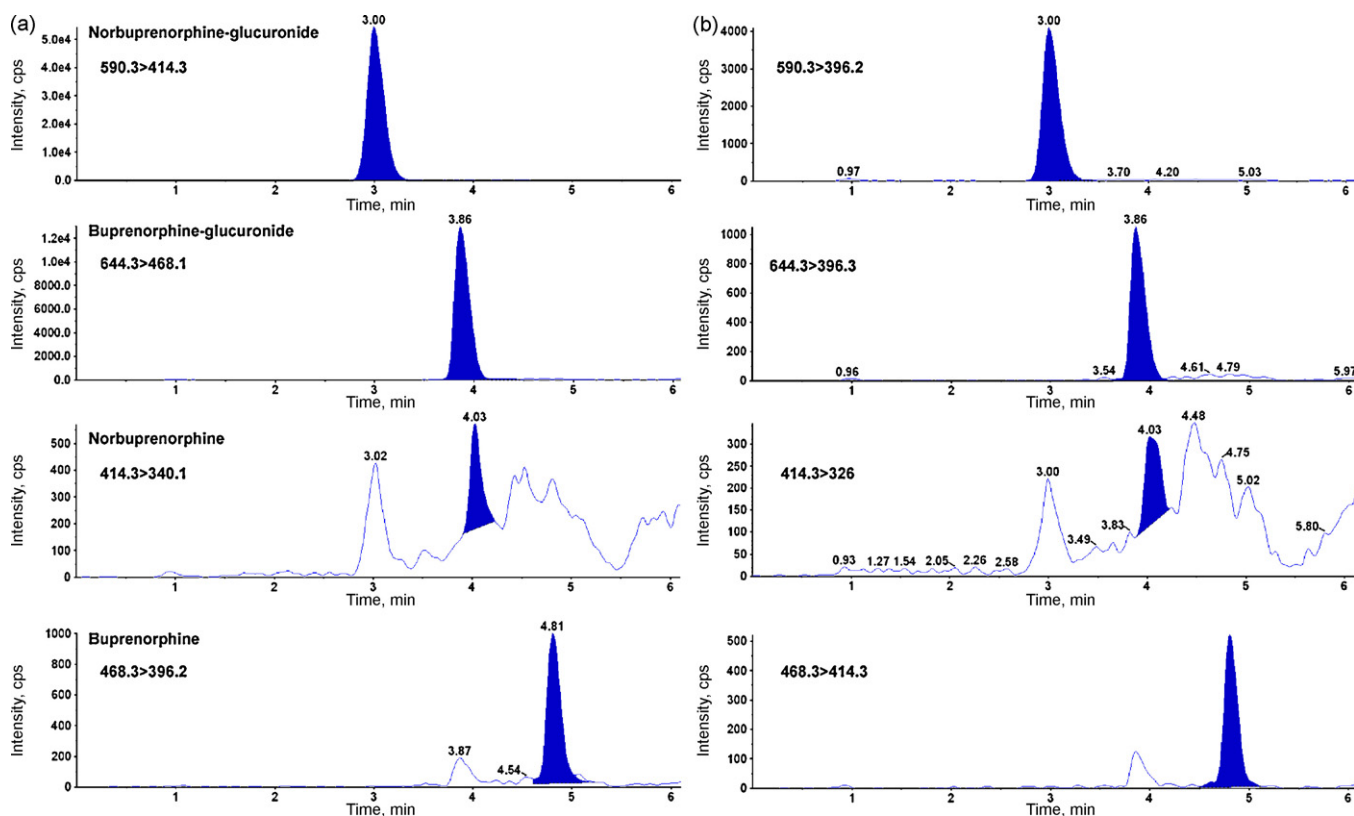


Fig. 2. LCMSMS chromatogram of authentic umbilical cord plasma containing buprenorphine (BUP, 0.7 ng/mL), norbuprenorphine (NBUP, 0.8 ng/mL), buprenorphine-glucuronide (BUP-Gluc, 4.4 ng/mL), and norbuprenorphine-glucuronide (NBUP-Gluc, 22.8 ng/mL). Panel (a) shows the quantification transitions and panel (b) qualifier transitions.

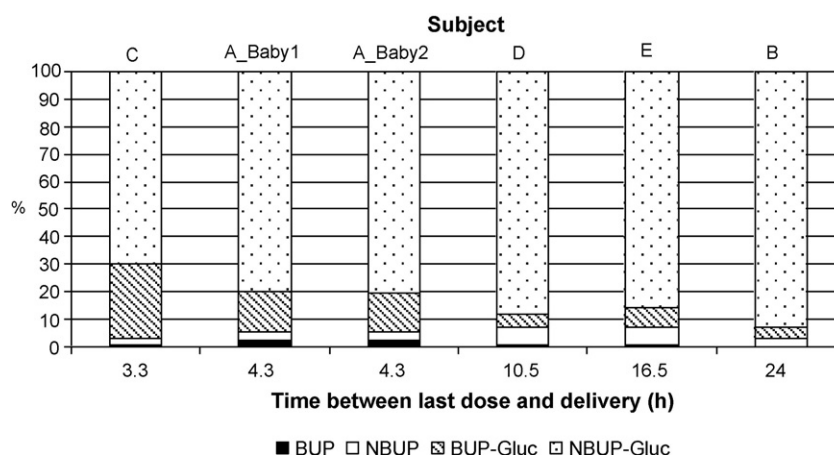


Fig. 3. Graphic representation of relative % concentrations of buprenorphine (BUP), norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-Gluc), and norbuprenorphine-glucuronide (NBUP-Gluc) in six different umbilical cord plasma specimens collected at different times after last BUP dose.

eter performing quantification in SRM mode. Murphy and Huestis [15] and Al-Asmari and Anderson [44] also employed ion trap mass spectrometry, and were able to monitor two transitions for BUP and NBUP, but LOQ were higher than the present method (0.6 ng/mL and 5 ng/mL, respectively), with a required sample volume of 1 mL of plasma compared to 0.5 mL utilized in this method.

Only published methods with ion trap mass spectrometry were able to monitor two characteristic transitions for BUP and NBUP [15,25,44]. With triple quadrupole mass spectrometry, the surviving ion transition [18,39,43,53] or only one characteristic transition [52] was monitored, because at low collision energies there was little fragmentation and at high collision energy many low intensity products were obtained. Musshoff et al. [38] monitored two transitions for BUP with a triple quadrupole mass spectrometer, but selected product ions were not characteristic (468.5 > 55, 468.5 > 83.4).

Glucuronides also have been quantified for BUP and NBUP [1,15,43,44]. Murphy and Huestis [15], and Al-Asmari and Anderson [44] monitored one transition for glucuronides, the cleavage of the glucuronic acid moiety, or just one ion for BUP-Gluc [44], with a higher LOQ (0.6 ng/mL and 5 ng/mL, respectively) than the present method. Polettini and Huestis [43] achieved a LOQ of 0.1 ng/mL for BUP-Gluc, and Huang et al. [1] for NBUP-Gluc, but in both cases only one transition was monitored.

Chromatographic separation of the four compounds was achieved in a reverse-phase column in gradient mode with a total run time of 10 min, whereas in previously published methods for the simultaneous quantification of these analytes a run time of 20 [1] or 30 min [15] was required. Al-Asmari and Anderson [44] developed a method with a run time of 35 min, but analysis of 19 opioids and metabolites were included.

A linear range of 0.1–50 ng/mL for BUP and BUP-Gluc and 0.5–50 ng/mL for NBUP and NBUP-Gluc allowed analysis of most clinical and forensic specimens without dilution. With an upper LOQ of 10 ng/mL [18,25,39–41,52], dilution was unavoidable in many cases. Two methods [15,25] evaluated dilution integrity, but dilution was done with water instead of blank plasma [15] or blood [25]. It is highly recommended to dilute with blank matrix because important parameters such as matrix effect can be modified if the matrix is diluted with water, potentially affecting quantification.

Ion enhancement was detected for all compounds except BUP-Gluc that demonstrated ion suppression. BUP and NBUP deuterated analogs were included as IStd to compensate for these effects. Unfortunately, glucuronide deuterated analogs were not commercially available. Matrix effect was not concentration-dependent, except for BUP that showed no matrix effect at the low QC, but

enhancement at medium and high QC concentrations. Variance in matrix effect in plasma specimens from 10 different sources was <9.1% at low, medium, and high QC concentrations, and all validation parameters satisfied criteria. IStd matrix effect also was tested to check variability among 10 different plasma specimens [54]. IStds showed ion enhancement (BUP-d₄ 62.8%; NBUP-d₃ 35.0%) with low variation among the 10 alternative matrices (BUP-d₄ CV 2.8%; NBUP-d₃ CV 3.1%).

Short- and long-term stability of BUP and NBUP [39,41,52], and their glucuronides [1,15,43,44] were previously reported. No degradation was observed for BUP and NBUP 24 h at room temperature [39,52], up to 238 days at –20 °C [52], and after three freeze/thaw cycles [39,41,52]; however, Murphy and Huestis [15] reported NBUP concentration reduction >40% after three freeze/thaw cycles. BUP-Gluc was stable up to 6 months at –20 °C [43], and NBUP-Gluc up to 1 month at this temperature [44], with both stable for 24 h at room temperature [1,15,55]. After three freeze/thaw cycles, Huang et al. [1] and Al-Asmari and Anderson [44] reported no degradation observed for glucuronides, whereas Murphy and Huestis [15] reported 30% degradation. In this study, no significant degradation of BUP and metabolites was detected under different storage conditions (storage at room temperature for 16 h, refrigerated at 4 °C for 72 h, and after three freeze–thaw cycles).

4.2. Specimen analysis

BUP and metabolites were detected in all umbilical cord plasma specimens, except for BUP in umbilical cord plasma B (24 h between dosing and delivery). Glucuronides were quantified at higher concentrations (NBUP-Gluc from 22 to 39.4 ng/mL, BUP-Gluc from 2 to 13.9 ng/mL) than NBUP (0.8 to 2.8 ng/mL) and BUP (0 to 0.7 ng/mL). Differences in BUP and metabolites' concentrations between specimens could be related to inter-individual variability, different BUP doses (14–24 mg/day), and different times between last dose and specimen collection. Based on these factors, it appears that BUP-Gluc concentrations decrease as time between last dose and delivery increases, whereas the relative percentage of NBUP-Gluc increases (Fig. 3). This suggests that NBUP-Gluc should be monitored if the longest window of drug detection is needed. NBUP-Gluc also was identified as the predominant BUP analyte in adult plasma following BUP administration [1], and in the two maternal plasma specimens collected at delivery from participants B and E. BUP and metabolites showed the same distribution as in umbilical cord plasma. Future comparisons of BUP biomarker concentrations in umbilical cord plasma with those in other newborn matri-

ces (placenta, umbilical cord, meconium) collected at birth will help us to understand drug distribution to and metabolism by the fetus.

One set of monozygotic twins was included in the analysis (one placenta but two umbilical cords); both umbilical cord plasma specimens had similar BUP and metabolite concentrations. In the literature, significant differences in fetal cocaine and cannabinoid exposure between dizygotic twins were reported, whereas cocaine levels were similar in monozygotic twins [56].

5. Conclusion

A method for the simultaneous determination of BUP, NBUP, BUP-Gluc and NBUP-Gluc in plasma by LCMSMS was developed and fully validated, achieving good sensitivity and fulfilling confirmation FDA and European Union Commission criteria with two transitions monitored for each analyte and acceptable relative ion intensities. The method was applied to determining the disposition of BUP and metabolites in umbilical cord plasma specimens from BUP-maintained pregnant women.

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