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Simultaneous quantification of buprenorphine, naloxone and phase I and II metabolites in plasma and breastmilk by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Opioid abuse during pregnancy is associated with fetal growth restriction, placental abruption, preterm labor, fetal death, and Neonatal Abstinence Syndrome. Current guidelines for medication-assisted opioid addiction treatment during pregnancy are methadone or buprenorphine monotherapy. Buprenorphine/naloxone combination therapy (Suboxone®) has not been thoroughly evaluated during pregnancy and insufficient naloxone safety data exist. While methadone- and buprenorphine-treated mothers are encouraged to breastfeed, no studies to date investigated naloxone concentrations during breastfeeding following Suboxone administration. For this reason, we developed and fully validated a liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of buprenorphine, buprenorphine–glucuronide, norbuprenorphine, norbuprenorphine-glucuronide, naloxone, naloxone-glucuronide and naloxone-*N*-oxide in 100 μL human plasma and breastmilk in a single injection following protein precipitation and solid-phase extraction. Lowest limits of quantification were 0.1–2 μg/L with 20–100 μg/L upper limits of linearity. Bias and imprecision were <±16%. Matrix effects ranged from −57.9 to 11.2 and −84.6 to 29.3% in plasma and breastmilk, respectively. All analytes were stable (within ±20% change from baseline) under all tested conditions (24 h room temperature, 72 h at 4 °C, 3 freeze/thaw cycles at −20 °C, and in the autosampler for 72 h at 4 °C). For proof of concept, buprenorphine and its metabolites were successfully quantified in authentic positive maternal and infant plasma and paired breastmilk specimens. This comprehensive, highly sensitive and specific method detects multiple buprenorphine markers in a small specimen volume.

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1. Introduction

According to the 2014 National Survey on Drug Use and Health, 5.3% of pregnant women self-reported last month illicit drug use [1]. Overall drug use prevalence in pregnant women has remained consistent over the last ten years; however, from 2000 to 2009, the number of pregnant women using opiates increased from 1.19 to 5.63 per 1000 births annually due to the current prescription opioids and heroin epidemic in the US [2]. Opioid abuse during pregnancy is associated with fetal growth restriction, placental abruption, preterm labor, fetal death, and Neonatal Abstinence Syndrome (NAS) [3]. Current guidelines for medication-assisted opioid addiction treatment during pregnancy recommend methadone or buprenorphine [4]. Buprenorphine monotherapy is advantageous compared to methadone, as it does not require daily clinic visits or frequent dose adjustments and is associated with milder NAS symptoms [4,5]. Suboxone® is 4:1 buprenorphine:naloxone in sublingual films or tablets. The combination therapy prevents abuse, as naloxone will precipitate withdrawal if insufflated or injected [5]; however, there are insufficient naloxone data to recommend Suboxone during pregnancy [4]. Physicians recommend that women receiving Suboxone be changed to methadone or buprenorphine. Postpartum, methadone- or buprenorphine-treated mothers are encouraged to breastfeed [4], but few studies investigated buprenorphine safety during breastfeeding [6–9] and

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none are yet available for naloxone concentrations in neonates, as no analytical method is available for naloxone and metabolites in breastmilk.

Buprenorphine, a semi-synthetic opiate susceptible to high first-pass hepatic metabolism, has low oral bioavailability (~40%) [10]. Sublingual buprenorphine doses are typically 2–24 mg [4]. Buprenorphine is highly protein bound (95–98%) [11], and undergoes *N*-dealkylation to norbuprenorphine via CYP3A4 in the liver [12]. Buprenorphine and norbuprenorphine are extensively conjugated to form buprenorphine-glucuronide (BUP-Gluc) and norbuprenorphine-glucuronide (NBUP-Gluc) [13]. In pregnant women during buprenorphine therapy, median plasma buprenorphine, norbuprenorphine, and BUP-Gluc concentrations are similar; however, NBUP-Gluc concentrations are nearly 10-fold higher [14]. Naloxone, when co-administered with buprenorphine, is typically a low dose (2–6 mg) [15] and is poorly absorbed when taken orally (~10% bioavailability) [10]. Naloxone is susceptible to *N*-dealkylation to nornaloxone and conjugation to glucuronide to form naloxone-glucuronide (NAL-Gluc) [10].

In order to assess safety of opioid-addiction treatment therapies during pregnancy and breastfeeding, sensitive analytical techniques are required to monitor buprenorphine, naloxone, and metabolites. Several liquid chromatography–tandem mass spectrometry (LC–MS/MS) assays exist for the detection of BUP analytes [8,16–19], NAL analytes [15], and BUP + NAL combination [20–23]. However, these techniques require 0.5–1 mL plasma and analysis of infant specimens is limited by low sample volume and requires low limits of quantification (LOQ). Wide linear ranges and high upper limits of linearity (ULOL) are needed to simultaneously quantify low infant concentrations and higher maternal concentrations in the same method. Incorporating buprenorphine and naloxone phase I and phase II metabolites into an analytical method provides comprehensive pharmacokinetic profiling without time-consuming hydrolysis procedures and assesses exposure to active non-conjugated buprenorphine and naloxone concentrations. Previously published methodologies utilized liquid-liquid extraction (LLE) [8,18,21,22] and solid-phase extraction (SPE) [15–17,19,20] for analyzing buprenorphine and naloxone markers in plasma or breastmilk. With the advent of polymeric sorbent beds for SPE, extractions are improved over silica-based cartridges used by Huang et al. [16] and Al-Asmari et al. [20]. However, SPE is not always sufficient for minimizing matrix effects and maximizing sensitivity in low volume specimens; we also incorporated protein precipitation and optimized all steps of extraction and analyte detection. The most comprehensive LC–MS/MS assay quantifies buprenorphine, norbuprenorphine, naloxone and three glucuronides in postmortem blood with 0.85–4.1 μg/L LOQs [20], but required 1 mL blood and two 36-min injections, failing to achieve required sensitivity for neonatal studies. To the best of our knowledge, no published studies investigated buprenorphine or naloxone phase II metabolites in breastmilk. We developed and validated a LC–MS/MS method for the simultaneous quantification of buprenorphine, BUP-Gluc, norbuprenorphine, NBUP-Gluc, naloxone, NAL-Gluc and naloxone-*N*-oxide in 100 μL plasma or breastmilk in a single LC injection following protein precipitation and SPE. This method is applicable for quantifying infant buprenorphine and naloxone exposure from in utero and breastmilk exposure.

2. Materials and methods

2.1. Chemicals and materials

Buprenorphine, buprenorphine-d4, norbuprenorphine, norbuprenorphine-d3, BUP-Gluc, NBUP-Gluc, naloxone, naloxone-d5 and naloxone-*N*-oxide were purchased from Cerilliant Corporation (Round Rock, TX). NAL-Gluc was obtained from Lipomed (Cambridge, MA) and NAL-Gluc-d5 from Toronto Research Chemicals (Toronto, Ontario).

Formic acid (Optima® LC–MS grade), methanol (HPLC and Optima® LC–MS grade), acetonitrile (Optima® LC–MS grade), glacial acetic acid, and ammonium hydroxide were acquired from Fisher Scientific (Fair Lawn, NJ). Methylene chloride and phosphoric acid were purchased from J.T. Baker (Phillipsburg, NJ) and 2-propanol (LC–MS grade) from Sigma-Aldrich (St. Louis, MO). Water was purified with an ELGA Purelab Ultra Scientific purifier (Siemens Water Technologies, Lowell, MA). Strata-X-C 33 μm polymeric strong cation SPE cartridges (60 mg/3 mL) were from Phenomenex (Torrance, CA).

Blank plasma and breastmilk were obtained from the NIH blood bank and Mother's Milk Bank of Ohio, respectively. Blank plasma and breastmilk for calibrators and quality control samples (QC) were evaluated for absence of analytes in our method. Method applicability was demonstrated by analyzing authentic positive plasma and breastmilk specimens obtained through an Institutional Review Board (IRB) approved protocol at Johns Hopkins University School of Medicine through the Center for Addiction and Pregnancy (CAP).

2.2. Instrumentation

SPE was performed with a CEREX-48 positive-pressure manifold (SPEware Corporation, Baldwin Park, CA), and nitrogen evaporation with a Zymark Turbovap® LV evaporation system (Biotage, Charlotte, NC). Analytes were quantified on a SCIEX 5500 QTRAP® mass spectrometer equipped with a TurboV electrospray ionization (ESI) source (AB Sciex, Foster City, CA), interfaced to a Shimadzu UFLCXR system with two LC-20ADXR pumps, a CTO-20 AC column oven, and a SIL-20ACXR autosampler (Shimadzu Corporation, Columbia, MD). Data were acquired and analyzed with Analyst (version 1.6.1) and Multiquant (version 3.0.1), respectively.

2.3. Preparation of standard solutions

Primary stock solutions containing individual analytes were prepared at 10,000 μg/L in methanol. Mixed analyte calibrator solutions were prepared via dilution in methanol creating calibrators at 0.1, 0.2, 0.5, 1, 2.5, 5, 10, and 20 μg/L for buprenorphine and BUP-Gluc, 0.25, 0.5, 1.25, 2.5, 6.25, 12.5, 25, and 50 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 1, 2, 5, 10, 25, and 50 μg/L for NAL-Gluc, and 2, 4, 10, 20, 50, and 100 μg/L for norbuprenorphine. QC stock solutions were prepared with separate ampules than for preparing calibrators. Mixed analyte QC solutions were prepared via dilution in methanol creating low, medium, and high QC solutions at 0.3, 3 and 16 μg/L for buprenorphine and BUP-Gluc, 0.75, 7.5 and 40 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 3, 30 and 40 μg/L for NAL-Gluc, and 6, 60 and 80 μg/L for norbuprenorphine. Primary stock solutions of buprenorphine-d_4, norbuprenorphine-d_3, naloxone-d_5, and NAL-Gluc-d_5 were diluted in methanol to produce a mixed internal standard (IS) solution of 50 μg/L each (except 25 μg/L for buprenorphine-d_4). Negative, calibrator, QC and specimen samples were fortified with 10 μL internal standard solution. No commercially available deuterated internal standards were available for BUP-Gluc, NBUP-Gluc, or naloxone-*N*-oxide. All standard solutions were stored at −20 °C.

2.4. Sample preparation and extraction

Blank plasma or breastmilk (100 μL) was aliquoted into 1.5 mL microcentrifuge tubes and fortified with calibrator (10 μL) or QC (10 μL) and IS (10 μL). Specimens (100 μL) were fortified with

Table 1
Optimized liquid chromatography–tandem mass spectrometry parameters for buprenorphine, buprenorphine–glucuronide (BUP-Gluc), norbuprenorphine, norbuprenorphine–glucuronide (NBUP-Gluc), naloxone, naloxone–glucuronide (NAL-Gluc), naloxone-*N*-oxide and deuterated internal standards.

Analyte	Q1 mass (*m*/*z*)	Q3 mass (*m*/*z*)	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)	RT (min)	IS
Buprenorphine	*468.17*	*396.2*	15	156	10	51	32	3.97	BUP-d_4
	468.17	414.2	15	156	10	45	32		
BUP-Gluc	*644.18*	*468.3*	15	96	10	55	40	3.59	NAL-Gluc-d_5
	644.18	396.2	15	96	10	75	36		
Norbuprenorphine	*414.12*	*187.1*	15	171	10	49	16	3.69	NBUP-d_3
	414.12	152.1	15	171	10	125	18		
NBUP-Gluc	*590.17*	*414.2*	65	171	10	49	38	3.14	NAL-Gluc-d_5
	590.17	396.3	65	171	10	51	44		
Naloxone	*328.05*	*212.1*	45	76	10	51	18	2.53	NAL-d_5
	328.05	268.1	45	76	10	35	22		
NAL-Gluc	*504.04*	*328.1*	45	81	10	35	28	1.87	NAL-Gluc-d_5
	504.04	486.2	45	81	10	31	44		
Naloxone-*N*-oxide	*343.99*	*185.1*	65	120	10	47	13	3.20	NAL-d_5
	343.99	128.1	65	101	10	95	10		
BUP-d_4	*472.21*	*400.2*	15	141	10	55	34	3.96	–
	472.21	415.2	15	120	10	47	13		
NBUP-d_3	*417.12*	*187.1*	15	56	10	49	16	3.69	–
	417.12	151.9	15	56	10	129	14		
NAL-d_5	*333.04*	*315.2*	45	101	10	27	28	2.51	–
	333.036	273.2	45	101	10	37	24		
NAL-Gluc-d_5	*509.02*	*333.0*	45	120	10	47	13	1.84	–
	509.02	491.1	45	101	10	31	50		

Masses in italics indicate quantifying transition.
Q1 = quadrupole 1, Q3 = quadrupole 3, DP = declustering potential, EP = entrance potential, CE = collision energy, CXP = exit potential, RT = retention time, IS = internal standard.

IS and processed identically. 300 μL acetonitrile was added to precipitate proteins and centrifuged at 8000 *g* for 10 min at 4 °C. Supernatants were transferred to 10 mL conical polypropylene tubes containing 2 mL 0.1 M phosphoric acid, vortexed, and placed in an ice bath until application onto SPE cartridges.

Acidified samples were decanted onto SPE cartridges preconditioned with 2 mL each of methanol, water and 0.1 M phosphoric acid. Cartridges were washed with 2 mL each of water, 0.1 M acetic acid and methanol and dried at 137.9 kPa for 5 min. Analytes were eluted into clean 10 mL glass centrifuge tubes with 3 mL methylene chloride:isopropanol:NH_4OH (70:26:4, v/v/v). Eluates were dried under nitrogen at 35 °C and reconstituted in 125 μL mobile phase (85:15, A:B, v/v). Samples were vortexed prior to centrifugation at 1800 *g* for 5 min at 4 °C and transferred to 1.5 mL polypropylene microcentrifuge tubes. After centrifugation at 15,000 *g* for 5 min at 4 °C, 110 μL were transferred to autosampler vials and 50 μL injected onto the LC–MS/MS.

2.5. Liquid chromatography

Analytes were separated via gradient elution at 0.5 mL/min with 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) on a Restek Raptor Biphenyl column (100 × 2.1 mm, 2.7 μm) fitted with a 5 × 2.1 mm matching guard column (Restek, Bellefonte, PA). Gradient elution included 0.5 mL/min for 30s at 15% B, increasing to 33% B in 1 min, and 98% B in 2.6 min. Following a 2.5 min hold at 98% B, the column was re-equilibrated to 15% B over 0.1 min and held for 1.4 min (total run time 8 min). To minimize carryover, flow rate was increased to 0.7 mL/min after 4.1 min and held for 2.8 min before returning to 0.5 mL/min. The HPLC eluent was diverted to waste for the first 0.7 and final 4 min of analysis. Autosampler and column oven temperatures were 4 and 40 °C, respectively.

2.6. Mass spectrometry

Multiple reaction monitoring MS/MS transitions were acquired via positive ESI. MS/MS parameters were optimized via direct analyte infusion (20 μg/L in 50:50 A:B) at 10 μL/min (Table 1). The nitrogen collision gas was set to high, curtain gas to 310.3 kPa, and source temperature to 650 °C. Gas1 was set to 482.6, 448.2, and 413.7 kPa, gas2 was set to 448.2, 482.6 and 413.7 kPa, and ion spray voltage to 4500, 5000 and 5500 V for periods 1 (naloxone and NAL-Gluc), 2 (naloxone-*N*-oxide and NBUP-Gluc), and 3 (norbuprenorphine, buprenorphine and BUP-Gluc), respectively. Quantifier and qualifier transitions were monitored for each analyte and internal standard utilizing unit resolution.

The following identification criteria were employed: retention time (RT) within ±0.1 min of average calibrator RT, Gaussian peak

Table 2
Linearity, limits of detection (LOD), lowest limits of quantification (LOQ), and upper limits of linearity (ULOL) for buprenorphine, buprenorphine–glucuronide (BUP-Gluc), norbuprenorphine, norbuprenorphine–glucuronide (NBUP-Gluc), naloxone, naloxone–glucuronide (NAL-Gluc), and naloxone-*N*-oxide in plasma and breastmilk.

Analyte	LOD (μg/L)	LOQ (μg/L)	ULOL (μg/L)	Plasma			Breastmilk		
				y-intercept (mean ± SD, n = 5)	Slope (mean ± SD, n = 5)	R^2 (range, n = 5)	y-intercept (mean ± SD, n = 5)	Slope (mean ± SD, n = 5)	R^2 (range, n = 5)
Buprenorphine	0.05	0.1	20	0.005 ± 0.003	0.377 ± 0.016	0.996–1.000	0.011 ± 0.005	0.383 ± 0.019	0.991–0.998
BUP-Gluc	0.05	0.1	20	0.005 ± 0.054	5.229 ± 0.592	0.993–0.999	0.085 ± 0.205	14.444 ± 2.517	0.991–0.997
Norbuprenorphine	1	2	100	0.024 ± 0.025	0.193 ± 0.005	0.994–1.000	0.026 ± 0.018	0.189 ± 0.008	0.996–0.999
NBUP-Gluc	0.125	0.25	50	−0.001 ± 0.019	0.969 ± 0.127	0.994–0.999	−0.011 ± 0.061	2.069 ± 0.367	0.990–0.998
Naloxone	0.125	0.25	50	0.011 ± 0.001	0.059 ± 0.002	0.995–1.000	0.001 ± 0.001	0.057 ± 0.004	0.993–1.000
NAL-Gluc	0.5	1	50	0.009 ± 0.026	0.278 ± 0.009	0.995–1.000	−0.011 ± 0.024	0.269 ± 0.014	0.998–1.000
Naloxone-*N*-oxide	0.125	0.25	50	0.000 ± 0.001	0.013 ± 0.001	0.997–0.999	0.001 ± 0.001	0.017 ± 0.001	0.991–0.997

Table 3
Bias data for buprenorphine, buprenorphine–glucuronide (BUP-Gluc), norbuprenorphine, norbuprenorphine–glucuronide (NBUP-Gluc), naloxone, naloxone–glucuronide (NAL-Gluc), and naloxone-*N*-oxide in plasma and breastmilk at three concentrations over the dynamic range of the assay.

Analyte	Plasma						Breastmilk					
	Mean between-run bias (%, n = 25)			Maximum within-run bias (%, n = 5)			Mean between-run bias (%, n = 20)			Maximum within-run bias (%, n = 5)		
	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]
Buprenorphine	102.7	99.6	96.6	101.3	108.5	96.2	99.0	96.6	93.9	94.0	95.3	95.0
BUP-Gluc	105.1	106.9	96.0	101.3	99.3	95.6	101.2	108.2	100.2	90.7	115.8	92.0
Norbuprenorphine	103.4	98.7	96.3	101.5	95.6	99.2	99.4	97.6	94.3	94.9	97.3	94.1
NBUP-Gluc	107.5	105.6	99.6	101.6	103.6	96.0	102.3	107.7	103.1	91.7	109.4	89.8
Naloxone	112.4	113.6	105.3	112.3	110.7	104.6	107.9	110.8	106.9	106.4	108.1	107.8
NAL-Gluc	104.6	99.4	98.9	101.2	98.2	101.3	103.4	100.0	99.7	101.5	98.5	98.3
Naloxone-*N*-oxide	103.0	101.2	99.9	98.7	102.7	102.4	101.0	101.4	100.9	100.8	97.2	97.1

[a] Low concentrations were: 0.3 μg/L for buprenorphine and BUP-Gluc, 0.75 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 3 μg/L for NAL-Gluc, and 6 μg/L for norbuprenorphine.
[b] Med concentrations were: 3 μg/L for buprenorphine and BUP-Gluc, 7.5 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 30 μg/L for NAL-Gluc, and 60 μg/L for norbuprenorphine.
[c] High concentrations were: 16 μg/L for buprenorphine and BUP-Gluc, 40 μg/L for naloxone, NBUP-Gluc, naloxone-*N*-oxide, and NAL-Gluc, and 80 μg/L for norbuprenorphine.

shape, presence of both product ions, and ion ratio of quantifying ion/qualifier ion within ±20% of the average calibrator ion ratio.

2.7. Plasma validation

The method was fully validated for plasma and cross-validated for breastmilk, in accordance with Scientific Working Group for Forensic Toxicology guidelines [24]. Sensitivity was defined by limits of detection (LOD) and quantification (LOQ) and empirically determined by decreasing concentrations of drug-fortified matrix. LOD was evaluated in three different sources, in duplicate, over 3 runs and was defined as the lowest concentration with acceptable chromatography, signal/noise ratio ≥3, and proper analyte identification. LOQ was the lowest concentration meeting LOD criteria and quantifying within ±20% of target. Linearity (R^2) was evaluated by least squares regression with $1/x^2$ weighting using ≥6 nonzero calibrators on 5 separate days. Acceptable linearity was achieved when $R^2 \geq 0.99$ and calibrators quantified within 15% of target (±20% for LOQ).

Bias and imprecision were determined from 5 replicates at 3 QC concentrations over 5 days (n = 25). Bias was determined as % target concentration and imprecision expressed as % CV. Within-run imprecision was determined each day from 5 QC replicates and the largest % CVs reported. Bias was acceptable if within ±20%, and imprecision if % CV was within 15%.

Matrix effect and extraction recovery were determined as per Matuszewski et al. [25] using 10 plasma sources. Extraction recovery was calculated from mean analyte peak areas and expressed as a percent. Matrix effect was assessed by dividing mean analyte peak areas, converting to a percentage, and subtracting from 100; positive and negative values indicate ion enhancement and suppression, respectively.

Endogenous interferences were evaluated in 10 blank plasma pools. Interferences from common therapeutic and illicit drugs (n = 90) were evaluated at 10,000 μg/L by fortifying into low QC samples (Supplemental Table 1).

Dilution integrity (10-fold) was assessed by fortifying 3 plasma specimens at twice the ULOL. 10 μL sample was added to 90 μL blank plasma and fortified with IS and prepared and extracted. Dilution was acceptable if analyte concentrations were within ±20% of target. Carryover (n = 3) was assessed by injecting an extracted blank plasma fortified with IS immediately after a sample containing analytes at twice the ULOL. Absence of carryover was documented by failure of LOD criteria.

Analyte stability was evaluated with blank plasma samples fortified at low and high QC concentrations under the following conditions: 24 h at room temperature (n = 4), 72 h at 4 °C (n = 3), and after three freeze/thaw cycles at −20 °C (n = 3). Stability samples were fortified with IS immediately prior to protein precipitation. Autosampler stability was assessed by injecting extracted QC

Table 4
Precision data for buprenorphine, buprenorphine–glucuronide (BUP-Gluc), norbuprenorphine, norbuprenorphine–glucuronide (NBUP-Gluc), naloxone, naloxone–glucuronide (NAL-Gluc), and naloxone-*N*-oxide in plasma and breastmilk at three concentrations over the linear dynamic range of the assay.

Analyte	Plasma						Breastmilk					
	Between-run imprecision (%CV, n = 25)			Maximum within-run imprecision (%CV, n = 5)			Between-run imprecision (%CV, n = 20)			Maximum within-run imprecision (%CV, n = 5)		
	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]
Buprenorphine	6.3	4.5	6.0	8.6	5.5	5.6	7.5	5.7	3.7	9.5	3.9	4.2
BUP-Gluc	8.2	6.9	9.4	5.5	2.7	4.9	10.9	8.7	11.7	7.1	5.2	12.0
Norbuprenorphine	5.7	4.8	5.9	2.0	6.0	4.5	6.0	4.9	4.4	4.2	3.1	5.2
NBUP-Gluc	8.3	6.0	7.7	5.9	4.4	5.9	10.3	7.7	10.4	2.8	5.5	8.8
Naloxone	4.3	3.9	5.4	3.1	4.8	3.6	5.3	5.4	3.8	6.9	5.7	4.1
NAL-Gluc	5.5	4.7	6.2	4.2	5.7	5.9	5.7	8.8	4.9	8.5	5.7	4.2
Naloxone-*N*-oxide	8.0	7.8	7.2	6.2	4.5	4.9	6.9	10.6	7.3	5.5	9.1	5.3

[a] Low concentrations were: 0.3 μg/L for buprenorphine and BUP-Gluc, 0.75 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 3 μg/L for NAL-Gluc, and 6 μg/L for norbuprenorphine.
[b] Med concentrations were: 3 μg/L for buprenorphine and BUP-Gluc, 7.5 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 30 μg/L for NAL-Gluc, and 60 μg/L for norbuprenorphine.
[c] High concentrations were: 16 μg/L for buprenorphine and BUP-Gluc, 40 μg/L for naloxone, NBUP-Gluc, naloxone-*N*-oxide, and NAL-Gluc, and 80 μg/L for norbuprenorphine.

samples (n = 5) after 72 h at 4 °C. Mean analyte concentrations of stability QCs were compared to mean fresh QC sample concentration prepared on the same day. Differences within ±20% were considered acceptable.

2.8. Breastmilk validation

For cross-validation in breastmilk, the same calibrator and control concentrations were monitored, and all validation experiments pertaining to matrix were re-evaluated. Pre-validation investigations of LOD, LOQ, and linearity were performed in breastmilk, using the same acceptance criteria. LOQ was assessed daily during validation and specimen analysis by demonstrating acceptable quantification of lowest non-zero calibrator (n = 5). Similarly, linearity was assessed using ≥6 non-zero calibrators on 5 separate days. Bias and imprecision were determined from 5 replicates of 3 QC concentrations analyzed over 4 days (n = 20). Matrix effect and extraction recovery were determined with 5 blank breastmilk sources. Freeze/thaw stability was evaluated with triplicate blank breastmilk samples fortified at low and high QC concentrations.

2.9. Method application

Method applicability was demonstrated by analyzing authentic plasma and breastmilk specimens from one lactating woman receiving buprenorphine monotherapy, and will be utilized for other women enrolled in an ongoing IRB approved study. Unfortunately, we were unable to obtain breastmilk specimens from women taking Suboxone, the buprenorphine-naloxone combination product, as naloxone safety during pregnancy and breastfeeding is not yet fully established. However, obstetricians and pediatricians are beginning to evaluate Suboxone in this population. Having this method available will enable evaluation of buprenorphine and naloxone and their metabolites' concentrations in plasma and breastmilk and contribute to the drug safety review.

Maternal blood was collected into a sodium fluoride/potassium oxalate vacutainer tube, mixed, and stored on ice for no more than 2 h before centrifugation (1200 *g*, 10 min, 4 °C). Plasma was transferred to CryoTubes and stored frozen (−20 °C) prior to analysis. Infant blood was collected via heel stick into a microcentrifuge tube containing sodium fluoride and potassium oxalate, mixed, and stored on ice for no more than 2 h before centrifugation (5000 *g*, 5 min, 4 °C). Plasma was transferred to a microcentrifuge tube and stored frozen prior to analysis.

Breastmilk was pumped into a 50 mL Falcon tube and stored on ice for no more than 2 h before transferring to a 10 mL conical polypropylene tube and stored at −20 °C prior to analysis.

3. Results

LODs were 0.05 (buprenorphine, BUP-Gluc), 0.125 (naloxone, NBUP-Gluc, naloxone-*N*-oxide), 0.5 (NAL-Gluc), and 1 (norbuprenorphine) μg/L in plasma and breastmilk. Linear ranges are presented in Table 2. Calibration curves for matrices yielded correlation coefficients (R^2) above 0.99 (Table 2).

Bias and imprecision results for plasma and breastmilk are presented in Tables 3 and 4, respectively. In plasma, between- and within-run bias were 96.0–113.6% and 95.6–112.3% of target concentration, while between- and within-run imprecision were 3.9–9.4% CV and 2.0–8.6% CV. For breastmilk between- and within-run bias were 93.9–110.8% and 89.8–115.8%, while between- and within-run imprecision were 3.7–11.7% CV and 2.8–12.0% CV. Extraction recoveries and matrix effects in plasma were 38.0–125.1% and −57.9 to 11.2%, respectively, (Table 5). In breastmilk, extraction recoveries and matrix effects were 13.6–105.0% and −84.6- to 29.3%, respectively. Matched deuterated internal standards had similar matrix effects and extraction recoveries to their d0 counterparts (Table 5).

Ten plasma and 5 breastmilk sources contained no interfering peaks. 90 potential exogenous interferences were examined at 10,000 μg/L. The hydroxycocaine (OH–COC) mixture (containing *m*-hydroxycocaine, *p*-hydroxycocaine, *m*-hydroxybenzoylecgonine, and *p*-hydroxybenzoylecgonine) and 7-aminonitrazepam interfered with norbuprenorphine quantifier and qualifier ions, respectively, in neat low QC samples. Similarly, hydrocodone exhibited an interference with naloxone-*N*-oxide in both transitions, but at a slightly different RT (3.17 vs 3.20 min). Upon extraction of these analytes at 100 μg/L, no interferences >LOD were observed. When individually extracted at high QC concentrations, trace naloxone impurity was detected in the naloxone-*N*-oxide standard and trace NBUP-Gluc was detected in the BUP-Gluc standard. However, these peaks quantified <LOQ.

Dilution integrity was acceptable; specimens fortified at twice the ULOL before dilution and analysis quantified within ±10% of expected diluted concentrations. There was no carryover in negative specimens injected after samples containing twice the ULOL.

In plasma, all analytes quantified within −10.8 to 18.4% of target when fortified at low and high QC concentrations for 24 h at room temperature, 72 h 4 °C, 3 freeze/thaw cycles, and 72 h post-extraction in the autosampler at 4 °C as seen in Table 6. In breastmilk, all analytes quantified within −7.5 to 13.2% of target in QC samples undergoing 3 freeze/thaw cycles.

As proof of method, breastmilk and maternal and infant plasma samples from a buprenorphine-treated breastfeeding woman were collected over 30-days. Median (range) maternal plasma specimens (n = 5) contained buprenorphine 2.7 (2.4–4.0), BUP-Gluc 3.3 (0.8–9.3), norbuprenorphine 3.6 (3.2–4.0), and NBUP-Gluc 32.5 (24.9–34.5) μg/L. In a single infant plasma specimen collected on day 14, only buprenorphine was detected (0.7 μg/L). Buprenorphine and to a lesser extent its three metabolites were also detected in paired breastmilk specimens (n = 5). Median (range) breastmilk concentrations (μg/L) were 4.6 (2.7–9.9) buprenorphine, 0.2 (<LOQ-0.3) BUP-Gluc, 2.3 (<LOQ-3.7) norbuprenorphine, and 1.3 (0.8–2.6) NBUP-Gluc. Fig. 1 shows a chromatogram of blank plasma, analytes at the LOQ in plasma, authentic plasma and breastmilk specimens.

4. Discussion

A sensitive and specific LC–MS/MS method for buprenorphine, naloxone and phase I and II metabolites quantification in plasma and breastmilk was developed and fully validated. Previous studies required larger sample volumes (0.5–1 mL) and did not offer sufficient sensitivity for detecting buprenorphine, naloxone and metabolites in infant specimens. Our LOQs were 0.1–2 μg/L with 100 μL plasma or breastmilk. Lower LOQs may be achieved with larger specimen volume; however, infant plasma specimens are volume limited. Traditional approaches to monitoring buprenorphine and naloxone often include monitoring each analyte independently with different chromatography and extraction procedures. Our goal was to establish a robust method that would simultaneously incorporate analytes and metabolites in a single injection across a wide linear range applicable to maternal and infant specimens. This approach was less costly in terms of solvents, consumables, and instrument and personnel time.

Sample preparation required protein precipitation and SPE. Infant plasma specimens were hemolyzed and initial SPE was insufficient to eliminate chromatographic interferences. A combination of precipitation and SPE allowed for the cleanest samples while maintaining sufficient LOQs and limiting interferences in infant specimens. Two-centrifugation steps also were incorporated to

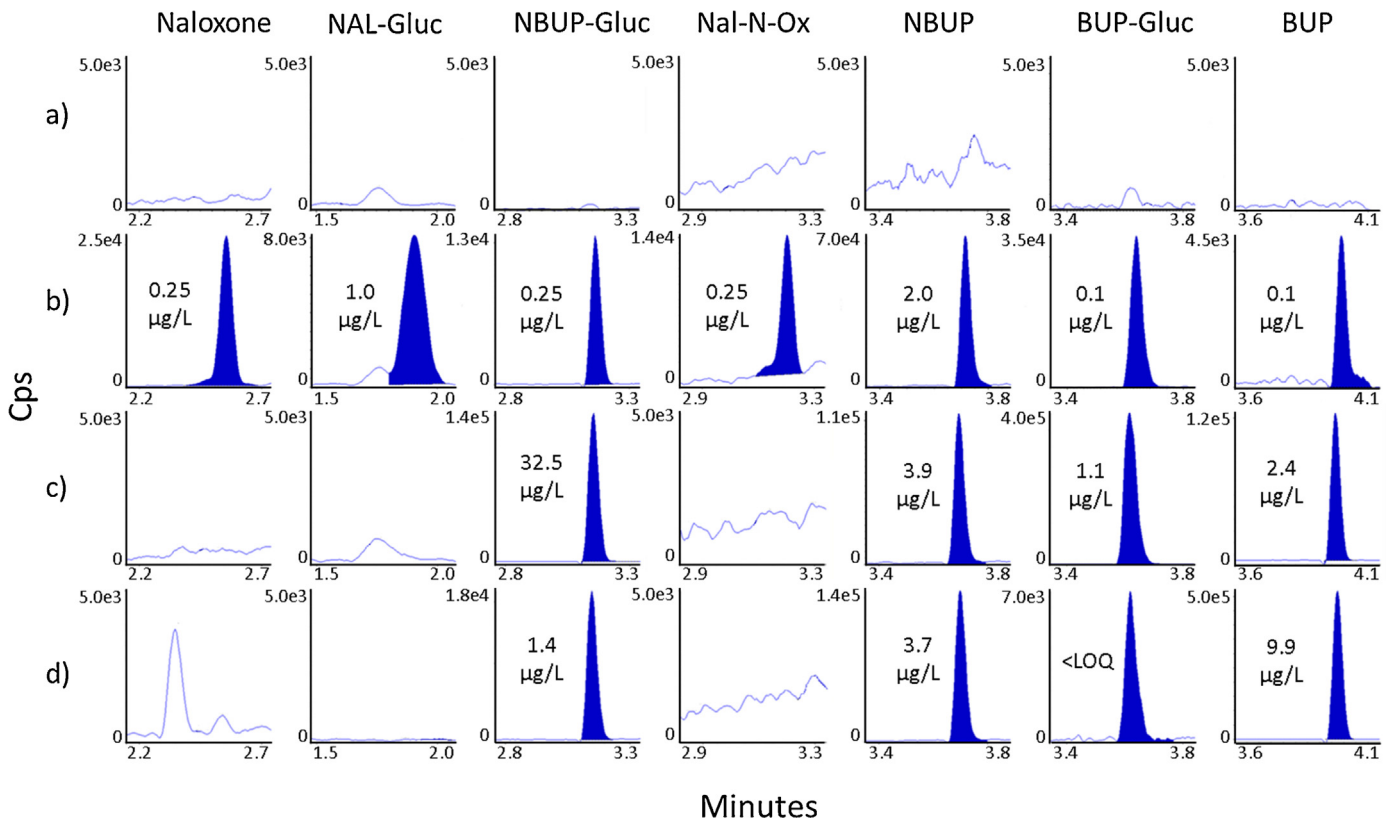

Fig. 1. Multiple reaction monitoring chromatograms for naloxone (NAL), NAL-glucuronide (NAL-Gluc), naloxone-*N*-oxide (Nal-*N*-Ox), buprenorphine (BUP), norbuprenorphine (NBUP), BUP-Gluc, and NBUP-Gluc quantifier ions in (a) blank plasma, (b) blank plasma fortified at analyte limits of quantification (LOQ), c) authentic maternal plasma, and (d) authentic breast milk. Concentrations listed for each analyte in μg/L. Cps = counts per second (intensity).

Table 5
Mean extraction recovery and matrix effect (with % coefficient of variance (CV)) for buprenorphine, buprenorphine–glucuronide (BUP-Gluc), norbuprenorphine, norbuprenorphine–glucuronide (NBUP-Gluc), naloxone, naloxone–glucuronide (NAL-Gluc), and naloxone-*N*-oxide in plasma and breastmilk at low and high concentrations.

Analyte	Plasma				Breastmilk			
	Extraction efficiency (%, n = 10)		Matrix effect (%, n = 10)		Extraction efficiency (%, n = 5)		Matrix effect (%, n = 5)	
	Low[a]	High[b]	Low[a] (%CV)	High[b] (%CV)	Low[a]	High[b]	Low[a] (%CV)	High[b] (%CV)
Buprenorphine	87.8	85.1	−33.5 (4.3)	−41.8 (5.9)	87.3	102.6	−56.7 (6.1)	−69.6 (9.7)
BUP-Gluc	58.6	63.2	5.1 (3.8)	−7.6 (6.7)	39.1	47.8	−29.3 (16.3)	−38.9 (14.2)
Norbuprenorphine	88.5	89.9	−21.5 (2.9)	−23.3 (6.5)	85.3	105.0	−33.2 (3.3)	−44.4 (8.6)
NBUP-Gluc	56.9	57.2	−12.0 (4.2)	−15.4 (6.8)	33.1	35.8	−41.9 (13.2)	−46.0 (19.3)
Naloxone	77.0	79.6	−57.9 (7.2)	−41.5 (6.3)	72.2	77.8	−84.6 (11.8)	−78.0 (11.1)
NAL-Gluc	38.0	41.2	−15.7 (6.7)	−36.4 (10.0)	14.9	15.6	−75.1 (14.2)	−80.3 (19.2)
Naloxone-*N*-oxide	81.1	86.2	−53.0 (4.6)	−45.5 (9.2)	75.2	77.5	−78.3 (16.0)	−74.7 (15.2)
BUP-d_4	113.2	116.3	−13.5 (0.7)	−36.5 (2.4)	93.7	95.0	−64.1 (2.6)	−62.5 (7.5)
NBUP-d_3	112.8	125.1	−17.5 (3.8)	−27.2 (4.2)	94.6	96.9	−38.3 (3.4)	−29.7 (6.6)
NAL-d_5	100.7	110.0	−32.6 (7.0)	−39.6 (5.6)	79.7	74.2	−80.3 (9.7)	−75.0 (9.7)
NAL-Gluc-d_5	51.0	54.1	11.2 (3.9)	−24.6 (1.4)	15.6	13.6	−74.2 (19.0)	−73.3 (15.3)

[a] Low concentrations were: 0.3 μg/L for buprenorphine and BUP-Gluc, 0.75 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 3 μg/L for NAL-Gluc, and 6 μg/L for norbuprenorphine.

[b] High concentrations were: 16 μg/L for buprenorphine and BUP-Gluc, 40 μg/L for naloxone, NBUP-Gluc, naloxone-*N*-oxide, and NAL-Gluc, and 80 μg/L for norbuprenorphine.

maximize analyte recovery and pellet remaining particulate matter. Methanol and cold acetonitrile also were evaluated for protein precipitation; however, analyte recoveries were lower and interferences and background noise increased. Our extraction method allowed for simultaneous glucuronide conjugate quantification, eliminating time-consuming hydrolysis. During method development, other techniques were investigated; including supported liquid extraction, phospholipid depletion tubes, and various other SPE chemistries. Once the Strata X-C column was selected, recoveries were optimized across a broad range of analyte polarity particularly for glucuronides, by investigating several different sample preparation solutions and elution solvents. We optimized the acidic conditioning and wash solutions and examined multiple possibilities, pHs, and combinations to provide the best sample cleanup for our extracts. Phosphoric acid provided the best extracts when used for sample preparation. In order to reduce lengthy and costly instrument maintenance procedures, we attempt to keep our instruments as clean as possible. We utilized the acetic acid solution during the wash step of the extraction to avoid phosphate salts from entering the mass spectrometer. Optimal chromatography

Table 6
Fortified and extracted stability (% difference) for buprenorphine, buprenorphine glucuronide (BUP-Gluc), norbuprenorphine, norbuprenorphine glucuronide (NBUP-Gluc), naloxone, naloxone glucuronide (NAL-Gluc), and naloxone-*N*-oxide in plasma and breastmilk under different conditions at two concentrations.

Analyte	Fortified								Extracted	
	24 h room temp. (% difference, n = 4) Plasma		72 h 4 °C (% difference, n = 3) Plasma		3 freeze/thaw cycles (% difference, n = 3) Plasma		3 freeze/thaw cycles (% difference, n = 3) Breastmilk		72 h autosampler (% difference, n = 5) Plasma	
	Low[a]	High[b]	Low[a]	High[b]	Low[a]	High[b]	Low[a]	High[b]	Low[a]	High[b]
Buprenorphine	12.6	6.3	−2.8	−0.1	−0.8	−1.0	−4.3	−7.5	4.1	3.2
BUP-Gluc	18.4	6.0	6.8	2.9	11.1	2.3	−6.9	−6.1	0.0	−6.8
Norbuprenorphine	2.3	7.0	7.0	2.1	8.2	−0.8	7.1	0.3	10.8	5.4
NBUP-Gluc	10.0	11.0	−3.9	5.7	−10.8	3.3	−0.2	13.2	2.8	4.8
Naloxone	4.4	3.3	4.7	0.8	2.7	−3.6	−0.6	−2.7	7.2	14.1
NAL-Gluc	6.2	5.4	8.1	−1.2	8.1	−2.4	−2.5	9.5	10.4	17.4
Naloxone-*N*-oxide	−4.3	2.1	−4.0	6.6	1.0	1.9	−3.0	4.1	−4.0	3.4

[a] Low concentrations were: 0.3 μg/L for buprenorphine and BUP-Gluc, 0.75 μg/L for naloxone, NBUP-Gluc, and naloxone-*N*-oxide, 3 μg/L for NAL-Gluc, and 6 μg/L for norbuprenorphine.
[b] High concentrations were: 16 μg/L for buprenorphine and BUP-Gluc, 40 μg/L for naloxone, NBUP-Gluc, naloxone-*N*-oxide, and NAL-Gluc, and 80 μg/L for norbuprenorphine.

was achieved after exploring different column chemistries (Synergi Polar-RP, Kinetex Phenyl-Hexyl, Raptor Biphenyl) and mobile phase preparations (acetonitrile versus methanol; ammonium formate + formic acid, ammonium acetate + acetic acid, formic acid alone). The Raptor Biphenyl column provided the best separation and least interference while improving early eluting compound peak shape. LC column chemistry offered enhanced selectivity with methanolic mobile phase and the LC gradient (flow rate and high organic wash time) was modified in order to minimize carryover. During method development, mass spectrometric source settings (gases, temperature, and ion spray voltage) were optimized for each compound via infusion and incorporated into the acquisition windows to maximize sensitivity.

Validation results for buprenorphine, naloxone and metabolites were acceptable in plasma and breastmilk. As LOQs, linear ranges, sample preparation, and data acquisition were the same for both matrices, validation parameters pertaining solely to matrix and sample storage were evaluated in breastmilk, following full validation in plasma. Bias and imprecision were acceptable for both. Extraction recoveries in plasma were >77% for unconjugated analytes and lower (38–63%) for glucuronides. Extraction recoveries >100% were for internal standards, not d0 analytes. Unfortunately, no commercially available deuterated internal standards were available for BUP-Gluc or NBUP-Gluc; d5-NAL-Gluc was selected to most closely match recovery and matrix effects.

In contrast to plasma, human breastmilk has significantly higher protein and lipid content. In breastmilk, extraction recoveries for naloxone, naloxone-*N*-oxide, norbuprenorphine, and buprenorphine were similar to plasma but significantly decreased for glucuronides (including d5-NAL-Gluc). However, despite decreased recoveries, acceptable LOQs were achieved. Despite employing protein precipitation and SPE, matrix effects showed high ion suppression. Interestingly, ion suppression was less for glucuronides. Matrix effect and recovery were not concentration-dependent. Despite ion suppression exceeding 25%, results were highly reproducible (<19.2% CV) in 10 blank plasma sources and 5 blank breastmilk sources, and did not affect other critical validation parameters (LOD, LOQ, bias and imprecision).

No endogenous interferences were observed in blank plasma or breastmilk. The OH–COC metabolites, 7-aminonitrazepam, and hydrocodone, if present in authentic specimens, are not anticipated to be problematic, as interferences were not observed at clinically relevant plasma concentrations. There were trace impurities in the standards, or potentially minor conversion or contamination, but concentrations were clinically insignificant.

Analytes were stable in plasma at room temperature, refrigeration, after three freeze/thaw cycles, and on the autosampler. As all authentic breastmilk specimens were stored frozen, only freeze/thaw stability was investigated, with no degradation observed. Long-term stability studies in plasma were previously reported for buprenorphine, norbuprenorphine, [21,26,27] and their glucuronides [16,20,28,29]. Buprenorphine and norbuprenorphine were stable for 238 days at −20 °C [21], while BUP-Gluc was stable for 6 months [29] and NBUP-Gluc for 1 month frozen [20]. Naloxone and NAL-Gluc were stable for 1 month frozen [20] and naloxone and nornaloxone stable in plasma for up to 468 days at −20 °C [15].

Buprenorphine, BUP-Gluc, norbuprenorphine, and NBUP-Gluc were successfully quantified in paired maternal plasma and breastmilk specimens collected over 30-days from a buprenorphine-treated breastfeeding woman. Only buprenorphine was detected at a low concentration (0.7 μg/L) in the single infant plasma specimen. Norbuprenorphine concentrations and two glucuronides in breastmilk were low (<3.7 μg/L), so it is not surprising that infant plasma concentrations were also low (<0.7 μg/L). Grimm et al. [8] and Ilett et al. [18] reported similar BUP and NBUP concentrations in breastmilk; however, to our knowledge, these are the first BUP-Gluc and NBUP-Gluc concentration data in breastmilk following maternal buprenorphine monotherapy. Also, naloxone and metabolites were not previously characterized in breastmilk. This method provides for analysis of naloxone and metabolites' concentrations when prescribed to breastfeeding women to collect safety data. Buprenorphine and metabolites, including glucuronides, are well studied in plasma. However, without detailed dosing information, we are unable to make comparisons to pharmacokinetic studies. This is the first study to examine paired maternal plasma, infant plasma, and breastmilk collected simultaneously.

A method for the simultaneous, direct quantification of buprenorphine, BUP-Gluc, norbuprenorphine, NBUP-Gluc, naloxone, NAL-Gluc, and naloxone-*N*-oxide in plasma or breastmilk by LC–MS/MS was developed and fully validated with low sample volume and increased sensitivity over previously published methods. The method is the first, to our knowledge, to quantify BUP-Gluc, NBUP-Gluc, naloxone, and NAL-Gluc in human breastmilk. The low LOQ and dynamic linear ranges are applicable to maternal and infant specimen analysis to improve understanding of drug transfer to the fetus and infant during medication-assisted treatment of opioid addiction in pregnant and breastfeeding women.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.03.076.

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