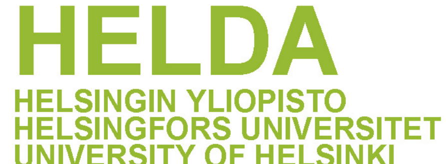

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Mariottini, Claudia

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RESEARCH ARTICLE

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Determination of buprenorphine, norbuprenorphine, naloxone, and their glucuronides in urine by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of buprenorphine (BUP), norbuprenorphine (NBUP), naloxone (NAL), and their glucuronide conjugates BUP-G, NBUP-G, and NAL-G in urine samples was developed. The method, omitting a hydrolysis step, involved non-polar solid-phase extraction, liquid chromatography on a C18 column, electrospray positive ionization, and mass analysis by multiple reaction monitoring. Quantification was based on the corresponding deuterium-labelled internal standards for each of the six analytes. The limit of quantification was 0.5 μg/L for BUP and NAL, 1 μg/L for NAL-G, and 3 μg/L for NBUP, BUP-G, and NBUP-G. Using the developed method, 72 urine samples from buprenorphine-dependent patients were analysed to cover the concentration ranges encountered in a clinical setting. The median (maximum) concentration was 4.2 μg/L (102 μg/L) for BUP, 74.7 μg/L (580 μg/L) for NBUP, 0.9 μg/L (85.5 μg/L) for NAL, 159.5 μg/L (1370 μg/L) for BUP-G, 307.5 μg/L (1970 μg/L) for NBUP-G, and 79.6 μg/L (2310 μg/L) for NAL-G.

KEYWORDS

buprenorphine, glucuronides, LC-MS/MS, naloxone, norbuprenorphine

1 | INTRODUCTION

Opioid use disorder (OUD) is a chronic relapsing disorder associated with significantly increased rates of morbidity and mortality; 26.8 million people were estimated to be living with OUD globally in 2016, with more than 100,000 opioid overdose deaths annually.[1]

Buprenorphine (BUP) is a semi-synthetic opioid, widely used in moderate to severe and usually chronic pain therapy. It is also commonly used as a medication for OUD patients attending opioid agonist treatment (OAT), which has the aim of minimizing the social and health harms related to opioid misuse.[2,3] Well-conducted trials have demonstrated that long-term OAT with methadone and BUP have great efficacy for OUD treatment and can save lives.[1] BUP is a partial μ-opioid receptor agonist and a κ-opioid receptor antagonist, showing higher affinity but lower intrinsic activity than full μ-opioid agonists.[4–8] BUP binds to the opioid receptor and displaces lower affinity opioids, without activating the receptor to a similar extent, thus showing less respiratory depressant activity and lower addiction potential. The dissociation of BUP from the opioid receptors is very slow, resulting in a long duration of action.

In OAT, BUP is delivered transmucosally as a sublingual or buccal formulation in immediate-release formulations and as an injection or

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implant in extended-release formulations.[1] To prevent parenteral misuse, BUP-based OAT is often implemented using a sublingual combination product containing BUP and naloxone (NAL).[9–11] NAL is a full non-selective antagonist at all three opioid receptors, with a higher affinity for the μ-receptor and lacking any μ-receptor efficacy. BUP has a poor oral bioavailability due to extensive first-pass metabolism, but because of high lipid solubility, it has an excellent sublingual bioavailability.[10,12,13] The presence of NAL does not influence the effect of BUP when the combination is taken sublingually, as it metabolizes rapidly to inactive metabolites during the hepatic first pass. However, when injected intravenously, NAL becomes pharmacologically active, produces a fast onset of action, and induces withdrawal symptoms, thus limiting parenteral use of the product.

BUP is extensively metabolized in the liver to norbuprenorphine (NBUP) by *N*-dealkylation, primarily through the cytochrome P450 enzyme CYP3A4 and secondarily through CYP2C8, CYP3A5, and CYP3A7.[14–18] BUP, NBUP, and NAL are further metabolized primarily by glucuronidation to produce their major active metabolites buprenorphine-glucuronide (BUP-G), norbuprenorphine-glucuronide (NBUP-G), and naloxone-glucuronide (NAL-G), respectively.[7,19–22]

Due to its lower capacity for inducing respiratory depression, clinical outcomes in cases of BUP toxicity are significantly better than in cases of methadone toxicity.[23] A study of the relative safety of BUP and methadone for OAT revealed that BUP was six times safer than methadone regarding overdose risk in the general population.[24] Despite its relative safety, accidental and intentional BUP toxicity deaths still occur among both OAT patients and opioid users outside of treatment, with intravenous injection and concomitant sedative drug use being recognized as risk factors.[25,26] Even the combination product BUP/NAL is liable to misuse and fatal poisonings.[27,28]

Within therapeutic drug monitoring and toxicology, laboratory analysis of BUP and its metabolites is used to monitor adherence and to reveal BUP toxicity. Blood toxicology may not, however, be sufficiently informative in all aspects, as there is no clinically relevant difference between therapeutic and toxic levels of BUP in blood, while NAL cannot generally be detected due to its very low concentration.[26,29,30] Urine analysis seems to offer a better means of clinical interpretation in various contexts of clinical and forensic toxicology and in monitoring compliance in OAT patients. BUP metabolite and NAL analysis in urine, involving metabolite concentration ratios, has been applied to estimate the time of BUP intake,[31] differentiating between therapeutic and illicit use of BUP products[32] and identifying urine adulterated by submerging BUP medication.[33,34]

Liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based methods, with or without hydrolysis of conjugates, are commonly used to analyse one or more of the analytes BUP, NBUP, NAL, BUP-G, NBUP-G, and NAL-G.[35–55] However, to our knowledge, to date, there is no published LC-MS/MS method for the simultaneous quantification of all six analytes, including NAL-G, in human urine.

The objective of this study was to create a practical urine analysis method for improved monitoring of adherence to BUP treatment and for estimating toxicity. In the following, an LC-MS/MS method for the simultaneous quantification of BUP, NBUP, NAL, and their glucuronide conjugates in non-hydrolysed human urine is described and validated. For proof of concept, the concentration ranges obtained for these drugs in clinical patient urine specimens by the developed method are presented.

2 | EXPERIMENTAL

2.1 | Materials

BUP was purchased from Chiron AS (Trondheim, Norway), BUP-d_4 and NBUP-d_3 from Cerilliant (Round Rock, TX, USA), NBUP from LGC (Leads, UK), NAL from U.S.P. Inc. (Rockville, MD, USA), and NAL-d_5 from TRC (North York, ON, Canada). BUP-G, NBUP-G, NAL-G, BUP-G-d_4, NBUP-G-d_3, and NAL-G-d_5 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All inorganic chemicals and organic solvents were of analytical grade.

2.2 | Instrumentation

The LC separation was carried out with a system consisting of a 1312A pump, a 1367B autosampler, and a 1316A column oven (all from Agilent Technologies, Santa Clara, CA, USA). A Gemini-NX C18 column (100 × 2.1 mm; 4 μm) and a C18 guard column (4 × 2 mm) were used in the LC separation (all from Phenomenex, Torrance, CA, USA). The mass spectrometric analysis was performed using a 4000 QTRAP® LC-MS/MS instrument equipped with a Turbo V™ source and a TurboIonSpray® probe (all from AB Sciex, Concord, ON, Canada) in triple quadrupole mode. The software used was Analyst 1.6.3 (Sciex, Framingham, MA, USA).

2.3 | Methods

2.3.1 | Standard solutions

A standard mixture containing all six analytes in methanol at a concentration of 10 μg/ml was prepared from stock solutions. Stock solution concentrations were 1000 μg/ml for BUP, NAL, and NAL-G and 100 μg/ml for NBUP, BUP-G, and NBUP-G. Working mixtures were diluted from the standard mixture in methanol/water (50/50, v/v) to prepare nine different concentrations of calibration standards (0.3, 0.5, 1, 3, 5, 10, 30, 50, and 100 μg/L). BUP-d_4, NBUP-d_3, NAL-d_5, BUP-G-d_4, NBUP-G-d_3, and NAL-G-d_5 were used as internal standards. Commercial stock solutions of the internal standards were diluted with methanol to prepare an internal standard mixture with a concentration of 10 μg/L. The internal standard mixture was diluted with methanol/water (50/50, v/v) to obtain a working mixture with a concentration of 1 μg/ml for each standard.

2.3.2 | Sample preparation

Urine samples (0.5 ml) were spiked with 25 μl of the internal standard working mixture. The samples were vortex-mixed for 1 min and then centrifuged at 5000 rpm for 5 min. Isolute C18(EC) (200 mg/6 ml; Biotage, Uppsala, Sweden) extraction columns were placed into a Pressure+ 48® (Biotage, Uppsala, Sweden) positive pressure manifold. The columns were activated and conditioned with 1 ml of methanol, followed by 1 ml of deionized water, using a flow speed of 2 ml/min. The samples were introduced into the columns, which were subsequently washed with 1 ml of deionized water, followed by drying for 2 min. The analytes were then eluted using 1 ml of a mixture of acetonitrile and methanol (50/50, v/v), and the eluate was evaporated to dryness in a dry block heater at 40°C. The residue was reconstituted with 100 μl of ammonium acetate buffer (10 mmol/L, 0.1% formic acid, pH 3.2), vortex-mixed for 2 min, and centrifuged at 12,000 rpm for 3 min. The extract was finally transferred into an autosampler vial equipped with a 300-μl insert.

2.3.3 | Liquid chromatography conditions

The analytical column was stabilized at 40°C. The mobile phase gradient consisted of methanol and ammonium acetate buffer (both containing 0.1% formic acid) as follows: the methanol proportion was held at 10% during the equilibrium time of 5 min, with a flow rate of 250 μl/min. After injection, the methanol proportion was increased to 15% in 2 min, to 45% in 10 min, to 85% in 2 min, and then to 95% in 6 min. The methanol proportion was finally decreased back to 10% in 2 min. The total run time was 28 min (equilibration time 5 min + run time 23 min). The injection volume was 20 μl.

2.3.4 | Mass spectrometry conditions

The total flow from the LC was directed to the ion source without splitting. The needle voltage was 5.2 kV, and the ion spray heater temperature was 450°C. The nebulizer gas (nitrogen) was set at 40 psi (276 kPa) and turbo heater gas (nitrogen) at 60 psi (414 kPa). The values for collision energy, cell exit potential, and declustering potential were optimized for each compound (Table 1). A multiple reaction monitoring (MRM) method was used for monitoring three ion transitions for each analyte and one for the internal standards (Figure 1).

The chromatographic run was split into two time periods to enhance sensitivity, the first period (11 min) containing NAL and NAL-G, and the second period (11 min) containing BUP, BUP-G, NBUP, and NBUP-G (Figure 2). A dwell time of 60 ms was used for BUP, BUP-G, and NBUP-G, while a dwell time of 80 ms was used for NBUP, NAL, and NAL-G. Table 1 shows the experimental conditions in the final MS/MS method.

2.4 | Method validation

Method validation was performed as per general guidelines,[56,57] including the determination of selectivity, limit of detection (LOD), limit of quantification (LOQ), measurement range, accuracy and precision, combined variation from matrix effects, measurement uncertainty, and stability.

2.4.1 | Selectivity

Selectivity was assessed by analysing 10 urine extracts from patient samples, which had been previously analysed with a reference

TABLE 1 Experimental conditions in MS/MS: internal standards (ISTD), protonated molecules $[M + H]^+$, monitored fragments, retention times (RT), collision energies (CE) for target ions*, and declustering potentials (DP) of studied analytes

Analyte	ISTD	$[M + H]^+$ *m/z*	Fragments *m/z*	RT min	CE eV	DP V
BUP	BUP-d_4	468	396*, 414, 101	17.4	50	90
BUP-d_4		472	400	17.4	50	90
NBUP	NBUP-d_3	414	187*, 340, 101	15.2	50	90
NBUP-d_3		417	343	15.2	40	90
NAL	NAL-d_5	328	310*, 212, 268	3.9	25	90
NAL-d_5		333	315	3.9	25	90
BUP-G	BUP-G-d_4	644	468*, 414, 396	15.2	55	110
BUP-G-d_4		648	472	15.2	55	120
NBUP-G	NBUP-G-d_3	590	414*, 340, 364	11.7	50	110
NBUP-G-d_3		593	417	11.7	50	110
NAL-G	NAL-G-d_5	504	310*, 328, 268	1.8	35	110
NAL-G-d_5		509	333	1.8	35	110

Abbreviations: BUP, buprenorphine; NBUP, norbuprenorphine; NAL, naloxone; BUP-G, buprenorphine-glucuronide; NBUP-G, norbuprenorphine-glucuronide; NAL-G, naloxone-glucuronide; and the corresponding deuterated internal standards.

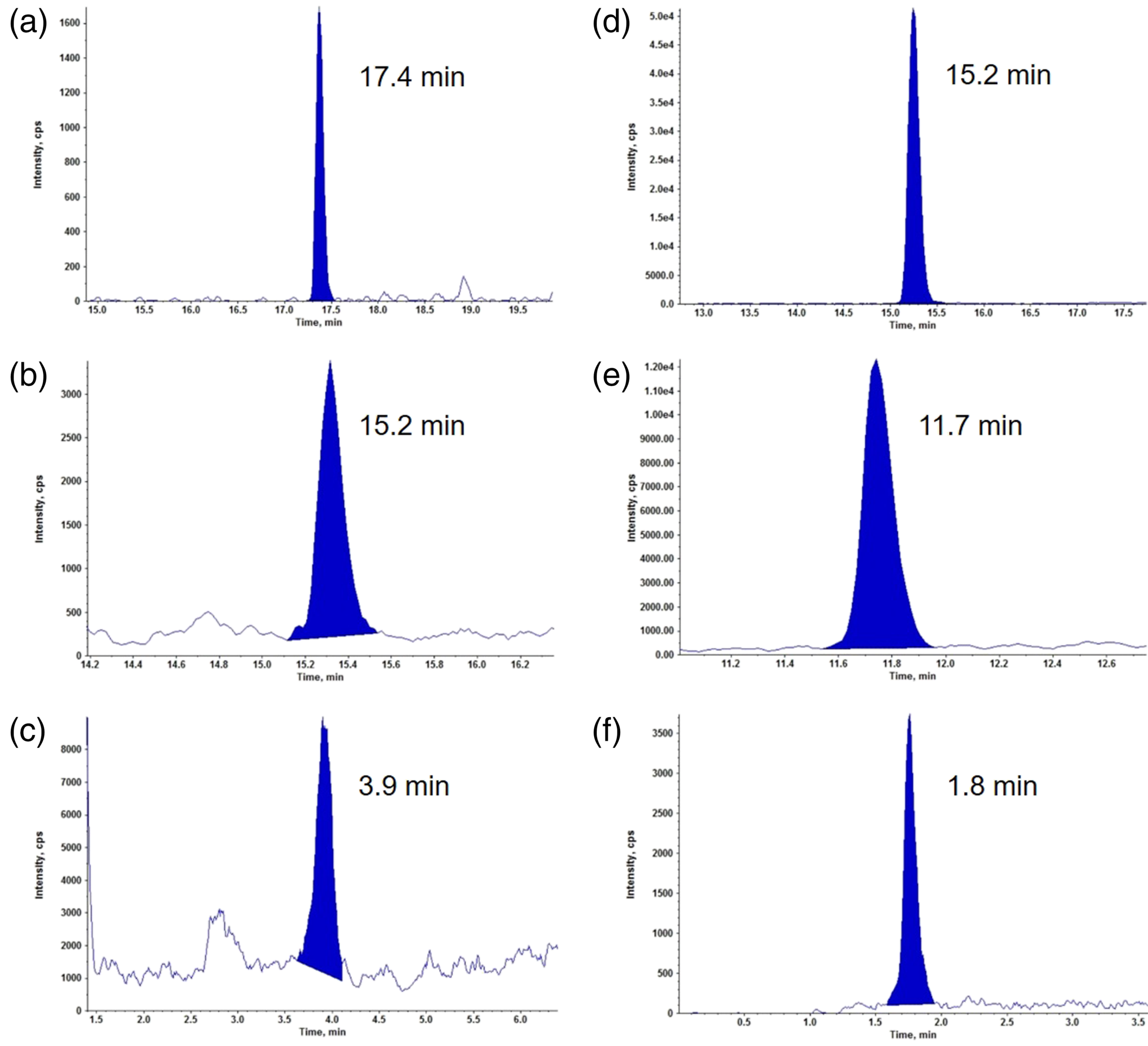

FIGURE 1 Multiple reaction monitoring (MRM) chromatograms for (a) buprenorphine (BUP), (b) norbuprenorphine (NBUP), (c) naloxone (NAL), (d) buprenorphine-glucuronide (BUP-G), (e) norbuprenorphine-glucuronide (NBUP-G), and (f) naloxone-glucuronide (NAL-G) at their limits of quantification. Cps = counts per second (intensity) [Colour figure can be viewed at wileyonlinelibrary.com]

method[32] and were negative for BUP, NBUP, and NAL, but not necessarily for other drugs.

2.4.2 | Limits of detection and quantification

LOD was defined as the lowest concentration at which the peak area was at least three times as large as the background noise, that is, signal-to-noise ratio (S/N) ≥ 3. LOQ was established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve lower than 20%, (2) relative standard deviation (R.S.D.) of six replicates lower than 20%, (3) peak shape acceptable, and (4) S/N ≥ 10.

2.4.3 | Measurement range

Measurement range was determined by analysing six replicates of processed samples at 10 concentration levels between 0 and 100 μg/L. Measurement range was defined by plotting the peak area ratio of the analyte to the internal standard versus drug concentration. The following criteria for defining the measurement range were applied: (1) quadratic regression through zero with a correlation coefficient greater than 0.990, (2) bias from the calibration curve lower than 15% for all individual calibration points, and (3) R.S.D. of six replicates lower than 15% (at LOQ 20%). The lower limit of the measurement range was defined as the LOQ.

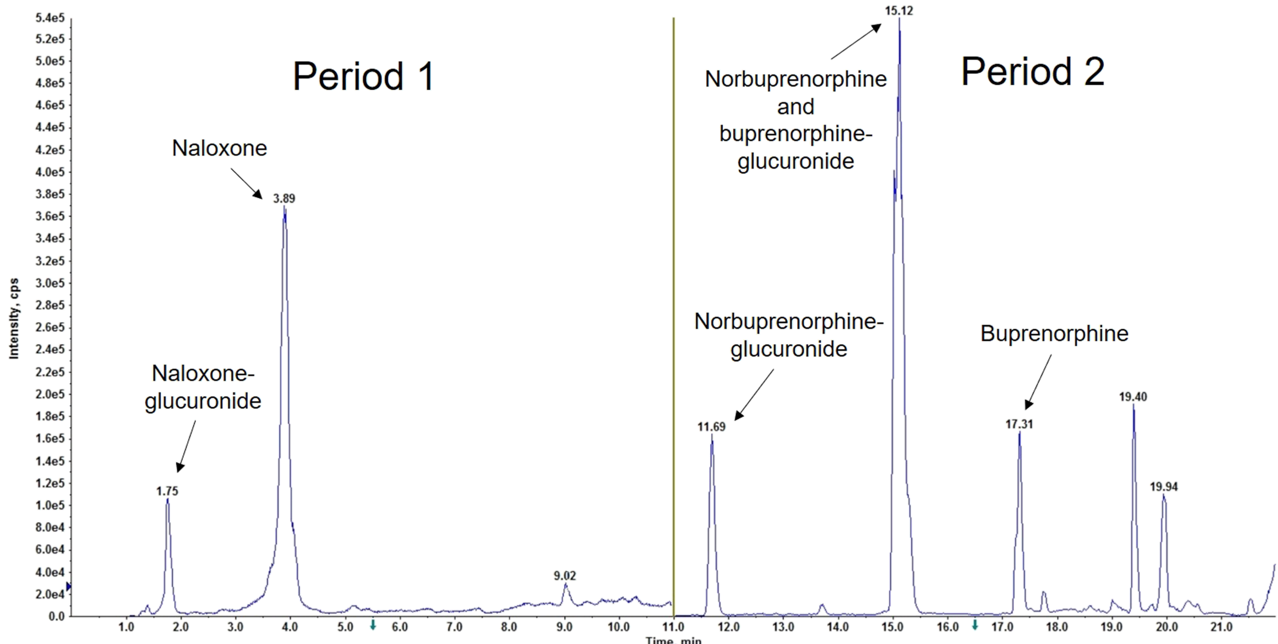

FIGURE 2 Total ion chromatogram (TIC) of a urine sample at a concentration level of 3 μg/L for each analyte. Peaks in period 1: naloxone-glucuronide (NAL-G) and naloxone (NAL). Peaks in period 2: norbuprenorphine-glucuronide (NBUP-G), norbuprenorphine (NBUP), buprenorphine-glucuronide (BUP-G), and buprenorphine (BUP) [Colour figure can be viewed at wileyonlinelibrary.com]

2.4.4 | Intra-day accuracy and precision

Intra-day accuracy and precision were calculated as average values across the measurement range. Spiked urine samples were prepared in six replicates at 10 concentration levels. Accuracy was expressed as average bias from the corresponding theoretical value and precision as R.S.D. at each concentration level within the measurement range. The criterion for accuracy was a bias lower than 15% and for precision an R.S.D. lower than 15% (at LOQ 20%).

2.4.5 | Intermediate precision

Intermediate precision was calculated from nine patient urine samples, analysed by different persons and over five consecutive days. The criterion for intermediate precision was an R.S.D. lower than 30%.

2.4.6 | Matrix effect

The overall matrix effects were estimated by comparing the R.S.D. within and between patient samples and by calculating the average bias of the spiked samples. A significantly higher R.S.D. between samples together with high bias indicate combined effects of matrix and variation of extraction efficiency.[45] Three replicates of 15 randomly chosen negative urine samples were spiked with the six analytes at two different concentration levels (5 and 30 μg/L). The R.S.D. within samples was calculated as average R.S.D. of the replicates, whereas the R.S.D. between samples was calculated from the 15 averaged concentrations. As stated by Peters and Remane,[57] this approach does cover the most important property, namely, the variability of matrix effects from sample to sample. However, it does not enable differentiation of effects caused by extraction from those caused by ion suppression or enhancement.

2.4.7 | Measurement uncertainty

Measurement uncertainty (U) was calculated using the equation $U = (U_1{}^2 + U_2{}^2)^{1/2}$, where U_1 is the proportion of systematic error and U_2 the proportion of random error. U_1 was estimated based on the average bias from theoretical concentration in the calibration experiments and in the matrix effect experiments. U_2 was estimated based on the average imprecision in the calibration experiments, in the intermediate precision experiments, and in the matrix effect experiments. The expanded measurement uncertainty $U95\% = 2 \times U$ defines an interval having a level of confidence of approximately 95%.

2.4.8 | Stability

To investigate stability, a set of five urine extracts was stored at 4°C and analysed again after 3, 7, and 14 days. Another set of five urine extracts was stored at 4°C and analysed again after 7, 14, and 21 days.

2.5 | Patient urine sample collection for LC-MS/MS analysis

Altogether 72 patient urine samples were analysed using the developed LC-MS/MS method. This is a substudy of a previous clinical study[58] for which the samples had been collected from BUP-dependent patients either being assessed for need for OAT or already enrolled in OAT at the outpatient clinic for opioid-dependent patients of Helsinki University Central Hospital (HUCH). The study was approved by the HUCH Ethics Committee for Gynaecology and Obstetrics, Paediatrics, and Psychiatry (HUS/1518/2016).

3 | RESULTS AND DISCUSSION

A method for the simultaneous quantification of BUP, NBUP, NAL, BUP-G, NBUP-G, and NAL-G in 500-μl human urine samples by LC-MS/MS was developed. Following non-polar solid-phase extraction, these analytes were separated by LC on a C18 reversed phase column and subsequently introduced into a triple quadrupole mass spectrometer. Ionization was carried out with a turbo ion spray source in positive mode, and the mass analyser was operated using an MRM method. Quantification was based on a corresponding deuterium-labelled internal standard for each analyte. Using the developed method, 72 urine samples from BUP-dependent patients in different phases of OAT were analysed to determine the actual concentration ranges involved.

3.1 | Method development and validation

The method was designed to be compatible with regular LC-MS/MS operating conditions for drug analysis in the authors' laboratory, involving a methanolic mobile phase buffered to acidic pH and a multipurpose C18 column. The mobile phase gradient was tailored to meet the chromatographic requirements of both the polar (NAL and NAL-G) and the less polar analytes. However, co-elution of NBUP and BUP-G was inevitable despite efforts to separate them. For sample preparation, a non-polar C18 SPE phase was chosen after discouraging experiments with mixed mode cation exchange phases. Use of C18 did not require adjustment of the pH of urine samples. A mixture containing acetonitrile and methanol (50/50, v/v) was chosen as the elution solvent in SPE for an optimal balance between elution strength (methanol stronger) and interference from impurities.

To evaluate the influence of other exogenous and endogenous species in urine extracts on the method's selectivity, 10 patient urine samples were analysed for interference. The study showed that additional peaks were detected at clearly lower intensities than the analytes at the LOQ level, and they did not impair selectivity.

Table 2 shows the main validation results for each analyte. The LOQ was 0.5–3 μg/L depending on the analyte. The intra-day accuracy (bias) and precision (R.S.D.) was always better than 5% and 20%, respectively, for parallel measurements at the different concentration levels across the measurement range.

The overall matrix effects were estimated by comparing the R.S.D. within and between patient samples and by calculating the average bias of the spikes.[45] For all analytes, the between-sample R.S.D. was higher than the within-sample R.S.D., suggesting that the sample matrix causes some variation in the results. The within-sample R.S.D. was always below 10% and the between-sample R.S.D. always below 20%, indicating that the variation was at an acceptable level.

The intermediate precision (R.S.D.) was better than 20% for all analytes. The uncertainty *U95%* (U, U_1, $U_{2)}$ values were 25% (12%, 2.1%, 12%) for BUP, 35% (18%, 4.1%, 17%) for NBUP, 24% (12%, 2.6%, 12%) for NAL, 23% (12%, 2.2%, 12%) for BUP-G, 25% (12%, 2.6%, 12%) for NBUP-G, and 30% (15%, 3.4%, 15%) for NAL-G.

Table 3 indicates that the analytes were stable in urine extracts stored at 4°C in both the 14-day and 21-day stability tests, as no changes or trends in concentrations were evident within the level of confidence defined by *U95%*.

3.2 | Analysis of urine samples from OAT patients

For proof of concept, 72 urine samples previously collected from BUP-dependent patients in different phases of OAT were analysed by

TABLE 2 Validation results: limit of detection (LOD), limit of quantification (LOQ), measurement range, correlation coefficient, intra-day accuracy (bias), and precision (R.S.D.)

Analyte	LOD (μg/L)	LOQ (μg/L)	Measurement range (μg/L)	Correlation coefficient (R^2)	Bias (%)	R.S.D. (%)
BUP	0.3	0.5	0.5–100	0.9968	1.4	9.2
NBUP	1	3	3–100	0.9951	1.6	7.6
NAL	0.5	0.5	0.5–100	0.9976	−1.9	5.6
BUP-G	0.3	3	3–100	0.9969	2.1	5.9
NBUP-G	1	3	3–100	0.9959	1.0	8.4
NAL-G	1	1	1–100	0.9976	1.0	7.2

Abbreviations: BUP, buprenorphine; BUP-G, buprenorphine-glucuronide; NAL, naloxone; NAL-G, naloxone-glucuronide; NBUP, norbuprenorphine; NBUP-G, norbuprenorphine-glucuronide.

the LC-MS/MS method to determine the concentrations encountered. The median (maximum) concentration was 4.2 μg/L (102 μg/L) for BUP, 74.7 μg/L (580 μg/L) for NBUP, 0.9 μg/L (85.5 μg/L) for NAL, 159.5 μg/L (1370 μg/L) for BUP-G, 307.5 μg/L (1970 μg/L) for NBUP-G, and 79.6 μg/L (2310 μg/L) for NAL-G. The glucuronide metabolites thus showed considerably higher urinary concentrations than the parent compounds, and due to large concentration differences several samples had to be diluted in order to remain in the measurement range.

3.3 | Comparison with previous studies

The LOQs reported in the previous literature for LC-MS/MS methods in human urine were 0.1–5 μg/L for BUP, 0.1–25 μg/L for NBUP, 1–50 μg/L for NAL, 0.1–5 μg/L for BUP-G, and 0.1–25 μg/L for NBUP-G.[39,41–43,49,50,53]

Table 4 compares in more detail previously reported LC-MS/MS methods that include at least five of the six analytes in any sample matrix. As with the present method, all methods listed involved a

TABLE 3 Stability test results

Analyte	Change in concentration relative to day 0 (%)					
	14-day stability test			21-day stability test		
	Day 3	Day 7	Day 14	Day 7	Day 14	Day 21
BUP	−7.6	−6.0	−6.6	−6.9	2.4	1.8
NBUP	9.1	0.2	−22.1	−15.6	−12.0	−26.2
NAL	−3.9	−9.4	−7.8	−8.5	−6.8	−37.7
BUP-G	28.3	−6.1	−0.1	−21.9	−16.1	−16.2
NBUP-G	−1.0	1.3	−6.1	5.2	7.1	6.8
NAL-G	−2.8	4.0	−0.3	8.0	−0.2	−0.1

Abbreviations: BUP, buprenorphine; BUP-G, buprenorphine-glucuronide; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantification; NAL, naloxone; NAL-G, naloxone-glucuronide; NBUP, norbuprenorphine; NBUP-G, norbuprenorphine-glucuronide.

TABLE 4 Comparison of previous LC-MS/MS quantification methods

Reference	Cohier et al.[55]	Ransohoff et al.[59]	Joshi et al.[52]	Swortwood et al.[51]	McMillin et al.[49]	Al-Asmari and Anderson[40]
Sample matrix	Whole blood (rat)	Urine	Plasma (rat)	Plasma and breast milk	Urine	Whole blood
Sample size (μl)	50	100	25	100	1000	1000
Sample preparation	PP	Dilute-and-shoot	LLE	SPE	SPE	SPE
SPE stationary phase	-	-	-	Polymeric strong cation exchange	Polymeric strong cation exchange	C18
LC stationary phase	Biphenyl	C18	HILIC	Biphenyl	C18	Phenyl-ether
LOD (μg/L)	BUP: 1.4 NBUP: 0.5 NAL: 0.4 BUP-G: 1.1 NBUP-G: 0.4 NAL-G: 17.5	*N/A*	BUP: 1 NBUP: 5 NAL: 5 BUP-G: 1 NBUP-G: 1 NAL-G: 1	BUP: 0.05 NBUP: 1 NAL: 0.125 BUP-G: 0.05 NBUP-G: 0.125 NAL-G: 0.5	*N/A*	BUP: 0.55 NBUP:1.2 NAL: 0.39 BUP-G: 0.39 NBUP-G: 0.49 NAL-G: 0.25
LOQ (μg/L)	BUP: 6.9 NBUP: 6.2 NAL: 1.3 BUP-G: 3.6 NBUP-G: 3.3 NAL-G: 57.7	BUP: 5 NBUP: 5 NAL: 100 BUP-G: 5 NBUP-G: 5	BUP: 1 NBUP: 5 NAL: 5 BUP-G: 1 NBUP-G: 1 NAL-G: 1	BUP: 0.1 NBUP: 2 NAL: 0.25 BUP-G: 0.1 NBUP-G: 0.25 NAL-G: 1	BUP: 2 NBUP: 2 NAL: 50 BUP-G: 5 NBUP-G: 5	BUP: 1.84 NBUP: 4.09 NAL: 1.32 BUP-G: 1.28 NBUP-G: 1.65 NAL-G: 0.85

Abbreviations: BUP, buprenorphine; BUP-G, buprenorphine-glucuronide; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantification; NAL, naloxone; NAL-G, naloxone-glucuronide; NBUP, norbuprenorphine; NBUP-G, norbuprenorphine-glucuronide; PP, protein precipitation; SPE, solid-phase extraction.

triple quadrupole mass analyser operated with positive electrospray ionization and MRM. However, a variety of different analytical approaches was taken in terms of sample preparation and chromatographic separation methods. The LOQ ranges in Table 4 were 0.1–6.9 μg/L for BUP, 2–6.2 μg/L for NBUP, 0.25–50 μg/L for NAL, 0.1–5 μg/L for BUP-G, 0.25–5 μg/L for NBUP-G, and 0.85–57.7 μg/L for NAL-G. With the present method, generally lower LOQs were obtained than in the two previous methods applied to urine samples.[49,59] However, endogenous interferences in blood or plasma samples are generally less prominent than in urine samples, allowing for lower LOQs.

Previously published comprehensive methods for urine analysis[49,59] report high LOQs, especially for NAL, while NAL-G is completely lacking in their repertoire. The benefit of the NAL assay in these studies was mainly related to assessing compliance with BUP/NAL therapy and revealing urine adulteration with this product.

This study confirms previous findings by showing that free BUP concentrations in urine are generally quite low, free NBUP and BUP-G concentrations are higher, and NBUP-G concentrations are the highest. NAL-G concentrations were presented here for the first time for BUP-dependent patients. A detailed analysis of NAL metabolites in future studies will provide improved diagnostic data, especially regarding BUP-dependent patients with frequent intravenous use of various BUP preparations. One such situation is monitoring the compliance of OAT patients, where the patient's medication includes a BUP/NAL combination product regularly administered as a sublingual resoriblette, but the patient has also intermittent parenteral use of BUP/NAL.[60] An important goal of the present method is the differentiation between medically supervised use and misuse of OAT medication for the safety of the patient.

4 | CONCLUSIONS

A straightforward LC-MS/MS method was developed for the simultaneous quantification of BUP, NBUP, NAL, and their glucuronide metabolites in human urine. The novelty of the method lies in all six substances, including NAL-G, being measured simultaneously. The omission of a hydrolysis step allows for a more accurate monitoring of the excreted drug species, providing a better clinical interpretation when assessing the timing and type of drug intake as well as revealing adulteration.

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