

# A Sensitive Method for Quantification of Buprenorphine and Norbuprenorphine in Human Whole Blood and Serum by UPLC/MS/MS

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## Abstract

A procedure for the rapid extraction and quantification of buprenorphine and its major metabolite, norbuprenorphine, from human whole blood and serum using liquid-liquid extraction and ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS) is described. Buprenorphine is a Schedule III, semi-synthetic opiate used for the treatment of chronic pain and opiate addiction. It is rarely reported as a sole contributor to cause-of-death, but can be a significant contributor when other psychotropics are present, especially benzodiazepines. Due to poor oral bioavailability combined with its potency, buprenorphine is typically found in low concentrations (0.1-76 ng/mL) in forensic blood samples (therapeutic range for buprenorphine is 2-8 ng/mL). Buprenorphine has been found to cause significant respiratory depression when in combination with benzodiazepines even at therapeutic levels.

Previous methods have required extensive extractions (such as SPE), longer total run-times, derivatization or had reduced selectivity through the use of single-quadrupole MS. The present study introduces a sensitive technique for the quantification of buprenorphine and norbuprenorphine using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The use of UPLC-MS/MS adds an additional layer of selectivity that is important in forensic applications where unknown specimens could contain a myriad array of potential interferences. Subsequent separation and quantification using UPLC/MS/MS was achieved in 4.25 minutes total run-time per sample. Linearity over the range 1 ng/mL to 100 ng/mL was established using deuterated analogs as internal standards. The methodology showed excellent intra-run precision in whole blood (QCs spiked at 20 and 80 ng/mL) with %CV values ranging from 1.27-3.81% for buprenorphine, 1.56-3.56% for norbuprenorphine. Inter-run precision experiments produced %CV values ranging from 4.88-5.02% for buprenorphine, 5.61-6.51% for norbuprenorphine. The reported method proved to be a rapid, robust and sensitive assay for the quantification of buprenorphine and norbuprenorphine in forensic applications and was also found to be relevant in clinical and therapeutic drug monitoring applications such as pain treatment compliance monitoring.

**Keywords:** blood; UPLC/MS/MS; buprenorphine

## Experimental

### Chemicals and reagents

Buprenorphine (100 µg/mL in methanol), norbuprenorphine (100 µg/mL in methanol), buprenorphine-d<sub>4</sub> (100 µg/mL in methanol) and norbuprenorphine-d<sub>3</sub> (100 µg/mL in methanol) standards were obtained from Cerilliant (Round Rock, TX, USA). All solvents were HPLC grade. Hexane and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Formic acid and isoamyl-alcohol were obtained from Sigma (St. Louis, MO, USA). Acetonitrile was purchased from Acros (Geel, Belgium).

### Matrices

Negative blood was prepared by combining a package of red blood cells obtained from Indiana Blood Center with 550 mL of 0.9% saline prepared in-house. The blood was then screened via UPLC-TOF-MS and ELISA for interfering drugs. Negative bovine serum was obtained from Sigma (St. Louis, MO, USA). Negative whole blood was obtained from Utak (Valencia, CA, USA) and screened via UPLC-TOF-MS for interfering drugs before use.

### Calibration curves

Calibration curves for all experiments were prepared according to Table 1.

Table 1. Preparation of buprenorphine and norbuprenorphine calibration curves.

Standard Concentration (ng/mL)	Volume of Working Standard (µL)	Volume of Negative Blood (µL)
100	1000 (Std 1)	0
50	500 (Std 1)	500
25	250 (Std 1)	750
10	1000 (Std 2)	0
5	500 (Std 2)	500
1	100 (Std 2)	900
Negative	0	1000

## Sample Preparation

1 mL of the samples was added to appropriately labeled culture tubes. Deuterated internal standards were added to unknown samples, curves and QC samples, all of which were then vortexed for 10-15 seconds. 8% sodium bicarbonate (pH 12) was added to each tube followed by vortexing, 2% isoamyl-alcohol in hexane was then added to the tubes, which were then vortexed, rocked, and centrifuged. The supernatant was transferred to appropriately labeled culture tubes to which 1% methanolic HCl had been added. The supernatant was evaporated under nitrogen and reconstituted with 0.5% formic acid in water, then transferred to plastic vials and injected on the UPLC-MS/MS.

## Liquid Chromatography

Liquid chromatographic separations were performed on a Waters ACQUITY™ ultra performance liquid chromatograph (UPLC) (Waters Corp., Milford, MA, USA). Separations were achieved on an ACQUITY™ UPLC® HSS T3 column (2.1x 50mm) packed with 1.7µm particles and maintained at 35°C. The mobile phase consisted of deionized water containing 0.1% formic acid (solvent A), and methanol (solvent B). A programmed gradient (listed in Table 2) was used for elution of the compounds. The total run time was 4.25 mins. Samples were maintained at 10°C in the sample organizer and sample injection volumes were 10µL. All flow was directed into the ESI source of the mass spectrometer.

Table 2. UPLC elution gradient

Time (min)	Flow Rate (ml/min)	%A	%B	Curve
Initial	0.5	100	0	6
0.50	0.5	100	0	6
3.50	0.5	20	80	6
3.51	0.5	1	99	6
3.70	0.5	1	99	6
3.71	0.5	100	0	6

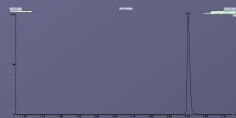
## Mass Spectrometry

Mass spectrometric detection was performed using a Waters Quattro Premier triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). Electrospray ionization (ESI) source conditions were as follows: polarity: positive; capillary voltage: 0.60 kV; extractor voltage: 3.0 V; RF lens voltage: 0.0 V; source temperature: . MS/MS conditions were as follows: capillary voltage: 0.60 kV, cone voltage: 22 V, extractor voltage: 3.1 V, RF lens voltage: 0.1 V. The source temperature was 150°C while the desolvation temperature was set at 425°C. Cone gas was set at a flow of 100 L/Hr while the desolvation gas flow was 900 L/Hr. The collision gas flow was set to 0.25 mL/min. Nitrogen (99.995% purity) was used as the desolvation gas, and ultra-pure argon (99.999% purity) was used as the collision gas. Appropriate quantifier and qualifier mass transitions were identified for each analyte by infusing a 10 µg/mL solution of each compound into the tee-in mobile phase and into the mass spectrometer ionization source (Table 3).

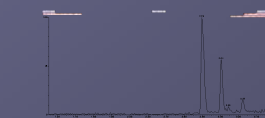
Table 3. MS/MS parameters used for each analyte and deuterated internal standard

Compound	Mass transition	Purpose	Cone (V)	Collision (V)	Dwell (secs)
Buprenorphine	468.24 > 54.83	Quantifying ion	60	45	0.05
Buprenorphine	468.24 > 83.51	Qualifying ion	60	45	0.05
Buprenorphine -d <sub>4</sub>	472.51 > 58.80	Quantifying ion	60	45	0.025
Norbuprenorphine	413.97 > 82.71	Quantifying ion	60	50	0.05
Norbuprenorphine	413.97 > 100.75	Qualifying ion	60	50	0.05
Norbuprenorphine -d <sub>3</sub>	417.82 > 100.83	Quantifying ion	60	50	0.025

Chromatogram 1. Buprenorphine at 1 ng/mL.



Chromatogram 2. Norbuprenorphine at 1 ng/mL. Peak at 3.01 min is from in-source fragmentation of buprenorphine.



## Results and Discussion

### Matrix Correlation

Studies on matrix correlation were performed in order to investigate the equivalence of in-house negative blood to other matrices (serum and whole blood). Calibration points were made from each matrix and compared at each point to the negative blood curve. The deviations from the calibrator true value at each point were used to determine equivalency for each matrix. For buprenorphine, the deviation was found to be less than 12% for both alternative matrices (<6% for negative blood : serum). Norbuprenorphine displayed deviations of <18% (<12% for negative blood : whole blood). This was within our in-house acceptance criteria for matrix correlation.

### Selectivity

To assess selectivity, ten samples that screened negative for buprenorphine/norbuprenorphine by UPLC-TOF-MS were obtained. Three replicates of each sample were aliquoted and treated as follows: one left as negative, one spiked at 20 ng/mL and one spiked at 80 ng/mL. Deviation from target concentration was used to determine whether matrix components could interfere with the quantitative results. For buprenorphine, all samples were within 20% of target concentration. For norbuprenorphine, while the samples were outside of 20% of target, they were within 20% of the calculated values for the quality control samples. Also, serum specimens containing diphenhydramine, cocaine, benzoyllecgonine, cocathylene, chlorpheniramine, doxylamine, dextromethorphan, benzodiazepines, amphetamines, and opiates were run to assess the possibility of false positives; none were found.

### Linearity

Calibration curves were generated for each run and assessed for best-curve fit, percent deviations, and signal-to-noise at the lower limit of quantitation (LLOQ). A quadratic curve fit with 1/x weighting was applied without forcing through the origin. In all curves, the R<sup>2</sup> was 0.99 or greater. Also, all points were within 8% of their nominal value for buprenorphine, and 10.5% for norbuprenorphine.

### Accuracy

Accuracy was assessed by analyzing the relative error of five replicates of low (20 ng/mL) and high (80 ng/mL) quality control samples (QCs) in four different runs. In-run relative error was found to be less than 11% for the buprenorphine low QC and less than 10% for the buprenorphine high QC. For norbuprenorphine these were found to be less than 15% for the low QC and less than 16% for the high QC. Overall relative error was 7.0% for the buprenorphine low QC and 1.9% for the high QC. Norbuprenorphine overall relative error was found to be 10.0% for the low QC and 11.2% for the high QC.

### Precision

Assay precision was also analyzed during the accuracy assessment. In-run % coefficients of variation (CVs) were all less than 4%. Overall CVs were less than 5.0% for buprenorphine and less than 6.5% for norbuprenorphine.

### Matrix Effects

Ion suppression was assessed by first selecting eight samples that screened negative by UPLC-TOF-MS for buprenorphine and norbuprenorphine. Four replicates of each sample were aliquoted and extracted without a calibration curve. Each was reconstituted with a solution that was either negative or spiked at 5 ng/mL, 20 ng/mL, or 80 ng/mL. The responses of each was compared to the responses of the reconstitution solutions themselves, allowing for the calculation of matrix effect as (Response of Reconstitution Solution) ÷ (Response of Drug in Sample). For buprenorphine, this was found to average 84.4% at 5 ng/mL, 84.7% at 20 ng/mL, and 76.1% at 80 ng/mL. Norbuprenorphine showed averages of 81.8% at 5 ng/mL, 83.6% at 20 ng/mL, and 74.3% at 80 ng/mL. The internal standards displayed average matrix effects that were within 6.1% of that displayed by the analytes, so it is believed that they adequately compensated for matrix effect on quantitation.

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