

LC/MS/MS of Buprenorphine and Norbuprenorphine in Whole Blood Using Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 Column

Application Note

Forensic Toxicology

Author

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Abstract

Determination of buprenorphine and norbuprenorphine in whole blood by forensic toxicology laboratories requires an analytical method capable of reliable detection of these compounds at concentrations below 1 ng/mL. A simple sample cleanup procedure coupled with an LC/MS/MS method using mass transitions 468.2 → 55.1 and 414.2 → 83.1 allows for a limit of detection (LOD) below 0.1 ng/mL for both analytes. Typical calibration curves are linear in the range of 0.2 to 20 ng/mL for each analyte, with R^2 values equal or higher than 0.999. High sensitivity is achieved by using Agilent products, including an Agilent Bond Elut Plexa PCX mixed mode polymeric SPE sorbent, an Agilent Poroshell 120 EC-C18 2.7 μm superficially porous LC column, an Agilent 1200 Infinity LC system, and an Agilent 6460 Triple Quadrupole LC/MS System with Agilent Jet Stream Technology (AJST) enhanced electrospray source.



Agilent Technologies

Introduction

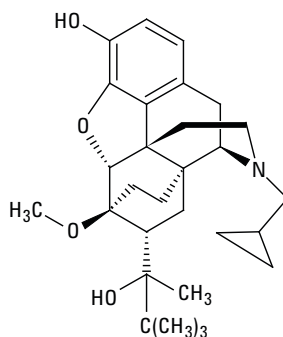
Buprenorphine is a semisynthetic opioid with a structure similar to morphine, although buprenorphine is much more hydrophobic (Figure 1). Buprenorphine is converted to norbuprenorphine, its major active metabolite [1,4]. Concentrations of buprenorphine and norbuprenorphine in blood are very similar, and in more than 50% cases, are below 1 ng/mL [9], presenting a challenge for an analyst. In addition, MS/MS detection of these compounds is complicated by the rigidity of the molecular structures of the analytes, resulting in very low amounts of collision-induced fragments. To achieve sensitivity below 1 ng/mL, analytical methods for determination of these compounds need not only excellent MS performance, but also an efficient sample cleanup procedure providing high recoveries and low ion suppression. We used an extraction method that delivered detection limits below 0.1 ng/mL, easily achieved due to the cleanliness of SPE-processed whole blood extracts. Unlike other polymeric sorbents, all members of the Agilent Bond Elut Plexa family possess an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

Good separation of analytes and excellent peak shapes achieved with this method are distinctive features of the Agilent Poroshell 120 column family. With superficially porous 2.7 μm particles, these columns provide similar efficiency to sub-2 μm UHPLC columns, but with approximately 40% less backpressure. This allows users of even 400 bar LC systems to increase resolution and to shorten analysis and re-equilibration times by applying a higher flow rate.

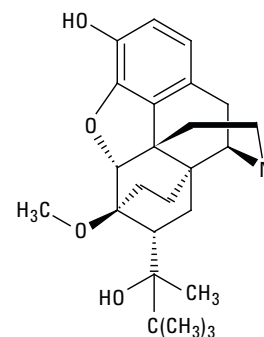
New ion transitions identified as the most abundant and used in this work for quantitation are 468.2 > 55.1 (buprenorphine) and 414.2 > 83.1 (norbuprenorphine). With only 0.5 mL of blood, a low sample injection volume of 10 μL and preconcentration of only 5 \times at the extraction step, the method demonstrates excellent signal-to-noise ratios at 0.2 ng/mL:84:1 for buprenorphine and 20:1 for norbuprenorphine (Figure 2).

Experimental

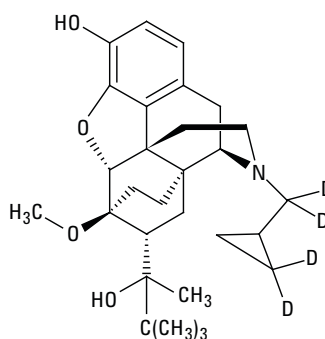
Analytes



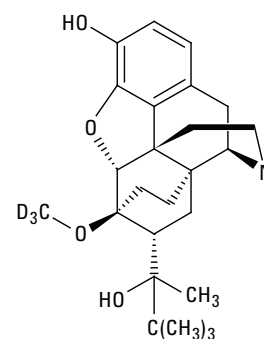
Buprenorphine
 $\text{C}_{29}\text{H}_{41}\text{NO}_4$ M.W. 467.65
Log P 4.9-5.0
pKa 8.3



Norbuprenorphine
 $\text{C}_{25}\text{H}_{35}\text{NO}_4$ M.W. 413.55
Log P 3.4-3.8



Buprenorphine-D₄
 $\text{C}_{29}\text{H}_{37}\text{D}_4\text{NO}_4$ M.W. 471.62



Norbuprenorphine-D₃
 $\text{C}_{25}\text{H}_{32}\text{D}_3\text{NO}_4$ M.W. 416.53

Figure 1. Buprenorphine and norbuprenorphine analytes and their structures. Log P –pKa values are from SRC and PubChem.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (buprenorphine, norbuprenorphine) and 100 $\mu\text{g}/\text{mL}$ (buprenorphine-D₄ and norbuprenorphine-D₃) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent silanized autosampler vials 2 mL (p/n 5183-2072)
- Agilent vial inserts, 250 µL, deactivated glass, with polymer feet (p/n 5181-8872)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column (p/n 699975-302)
- Agilent 1260 Infinity LC system (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Pretreatment

1. Spike 0.5 mL of blood with ISTD at 10 ng/mL, or prepare 10 ng/mL solution of ISTD in 0.1 M phosphate buffer (pH 6.0) and add 0.5 mL of this buffer to each blood sample. Use of methanol-rinsed and air-dried glass tubes 12 × 75 mm is recommended.
2. After adding ISTD, add 2 to 2.5 mL phosphate buffer (so that blood is diluted at least 1:5).
3. Vortex and centrifuge to obtain a good pellet.

Extraction

1. Condition Bond Elut Plexa PCX cartridge with 0.5 mL methanol, soak, then let drip.
2. Load sample/supernatants with a Pasteur glass pipette.
3. Wash 1: 2 × 2 mL 2% formic acid.
4. Wash 2: 3 mL of 70 MeOH:30 of 2% formic acid.
5. Dry 5-10 minutes under vacuum (10-15 in Hg).
6. Elute with 1.5 mL of 80 ethyl acetate:20 isopropanol: 5 NH₄OH eluent. Add NH₄OH shortly before elution. Apply eluent in 2 aliquots and soak the sorbent bed with each aliquot. Soak for approximately 0.5 minute with the

stopcock valves closed, then let the eluate drip into the collection vials under gravity. When the dripping stops, apply low vacuum to extract eluate from the smallest pores.

7. Evaporate to dryness under a stream of nitrogen at 45 °C.
8. Reconstitute in 0.1 mL initial mobile phase (15% methanol, 85% water, 0.1% formic acid), vortex, and transfer into vial inserts with polymer feet.

LC/MS/MS

LC conditions

Mobile phase A:	0.1% formic acid in water	
Mobile phase B:	0.1% formic acid in methanol	
Flow rate:	0.8 mL/min	
Gradient:	Time (min)	% B
	0.0	15
	2.0	70
	2.1	95
	5.5	95
	5.51	15
Stop time:	5.6 min	
Post time:	2 min	
Max pump pressure:	400 bar	
Injection volume:	10 µL	

Injection with needle wash

Needle wash:	Flush port 95 methanol:5 water for 10 s
Disable overlapped injection:	
No automatic delay volume reduction:	

MS conditions

ES source parameters

Ionization mode:	positive
Capillary voltage:	2,800 V
Drying gas flow:	10 L/min
Drying gas temperature:	350 °C
Nebulizer gas:	35 psi
Sheath gas flow:	12 L/min
Sheath gas temperature:	350 °C
Nozzle voltage:	0 V

MS parameters

Scan type:	MRM
Prerun script:	SCP_MS DiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments:	#1: 1.8 min - diverter valve to MS
Delta EMV (+):	400 V

Table 1 shows the MRM transitions for one quantifier and one qualifier product ion for the target compounds, and their deuterated internal standards.

Table 1. MRM Transitions.

Compound name	Precursor	MS1 Res	Product	MS2 Res	Fragmentor	Collision energy
Buprenorphine	468.3	Unit	55.1	Wide	200	62
Buprenorphine	468.3	Unit	396.2	Wide	200	45
Buprenorphine-D ₄	472.3	Unit	59.1	Wide	200	62
Buprenorphine-D ₄	472.3	Unit	400.2	Wide	200	45
Norbuprenorphine	414.3	Unit	83.1	Wide	188	60
Norbuprenorphine	414.3	Unit	57.1	Wide	188	50
Norbuprenorphine-D ₃	417.3	Unit	83.1	Wide	188	60
Norbuprenorphine-D ₃	417.3	Unit	57.1	Wide	188	50

Results and Discussion

At low pH, buprenorphine and norbuprenorphine are protonated at the tertiary amine group and strongly retained on Agilent Bond Elut Plexa PCX polymeric sorbent by a combination of hydrophobic retention and strong cation exchange.

A 100% methanol wash led to partial loss of analytes from the SPE column. The optimum wash that efficiently removed most matrix interferences without loss of analytes proved to be 70 MeOH:30 2% formic acid. A strong base is added to the organic eluent to break the ionic interaction between the analytes and the strong cation-exchange sorbent. The recovery of buprenorphine and norbuprenorphine was optimized with 5% NH₄OH added to the combination eluent (80 ethyl acetate: 20 isopropanol) shortly before sample elution. Two-step elution with a soaking procedure is recommended to enhance the solvent-analyte interaction and improve analyte recoveries.

Due to high hydrophobicity, buprenorphine and norbuprenorphine can adhere to glassware, LC tubing, and injector parts, which is why we recommend a 95% MeOH column rinse in the LC method and 95 MeOH:5 water flushing solution for the flushport needle rinse. Deactivated vials/inserts and MeOH-rinsed/air-dried glassware (both tubes and bottles for STD/ISTD dilutions) also ensure reproducible results.

The LC separation intentionally begins with a relatively low fraction of organic solvent (15%) to allow salts and other polar components of blood to elute at the beginning of the sample run. A flow rate of 0.8 mL/min allows for a short retention and re-equilibration time. Each sample run begins with diverting a first portion of flow (0 to 1.8 minutes) to waste to minimize source contamination. Data collection begins at 1.8 minutes, immediately after the diverter valve switch.

Chromatograms for buprenorphine and norbuprenorphine at the LOQ of 0.2 ng/mL and corresponding deuterated internal standards at 10 ng/mL are shown in Figure 2.

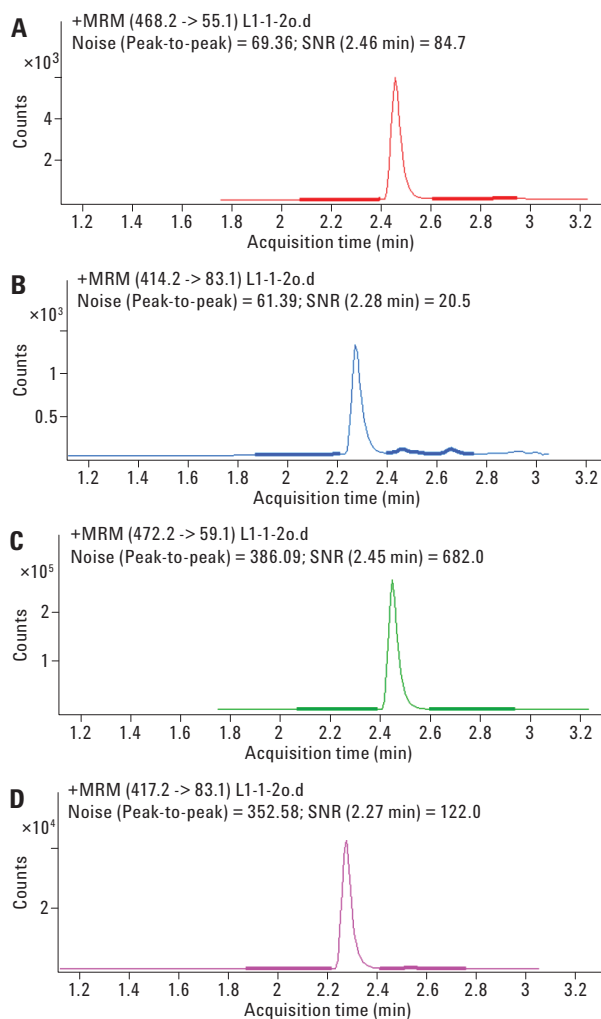


Figure 2. MRM extracted ion chromatograms: A: buprenorphine, B: norbuprenorphine (both at 0.2 ng/mL), C: buprenorphine-D₄, and D: norbuprenorphine-D₃ (both at 10 ng/mL) in whole blood extract processed on Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 μm column. Noise regions are shown in bold.

The high stability of molecular ions of both buprenorphine and norbuprenorphine presents a challenge for MS/MS detection [3,9]. It led many researchers to quantitation in SIM mode [2,8], or in SRM mode by monitoring a molecular ion > molecular ion transition at relatively high collision energy without fragmentation [3,9]. Compared to a more selective quantitation by a parent-product transition, this approach is less reliable. It results in a much higher signal-to-noise (S/N) ratio and, therefore, in a higher lower limit of quantification (LLOQ). MS-MS transitions most commonly used for buprenorphine/norbuprenorphine quantification were 468 to 414, 396 *m/z* for buprenorphine, and 414 to 396, 340 and 101 *m/z* for norbuprenorphine [2, 3, 4, 5, 6, 7]. A new stable fragmentation pattern achieved with an Agilent 6460 Triple Quadrupole LC/MS System (Table 1) at high collision energy levels allows for a reliable quantitation with an LLOQ of 0.2 ng/mL for both analytes. The most abundant fragment of buprenorphine is the methylocyclopropyl (C₄H₇) group with *m/z* 55.1. Its identification is confirmed by a fragment of buprenorphine-D₄ with *m/z* 59.1. The most abundant product of norbuprenorphine fragmentation (*m/z* 83.1) probably comes from the branched side chain of the parent ion and includes the tert-butyl group (CH₃)₃C. Compared to most commonly used fragmentation products obtained at their optimum collision energies, *m/z* 55.1 is a 8× more abundant product of buprenorphine than *m/z* 396.2, while *m/z* 83.1 is a 2× more abundant product of norbuprenorphine than *m/z* 101.1.

MRM transitions listed in Table 1 are for one quantifier and one qualifier product ion for both target compounds and their deuterated ISTDs. Agilent MassHunter software automatically calculates qualifier ion ratios, highlighting those out of the acceptable range. Either normal or dynamic MRM acquisition modes can be used with this method.

S/N ratios at the LLOQ level of 0.2 ng/mL were 84:1 for buprenorphine and 20:1 for norbuprenorphine (Figures 2, A and B). This illustrates the efficiency of a sample cleanup procedure and the excellent sensitivity of the 6460 Triple Quadrupole, capable of detecting these analytes with LODs way below 0.1 ng/mL.

Figure 3 shows typical calibration curves for buprenorphine and norbuprenorphine in extracted whole blood standards at five concentration levels. Calibration standards were prepared by spiking whole blood with analytes at 0.2, 1, 5, 10, and 20 ng/mL. Deuterated internal standards were added at 10 ng/mL. Excellent linear fit ($R^2 > 0.999$) to each of the curves demonstrates linearity of the method. No weighting was applied, and the origin was included in the curve fit.

Table 2 shows recovery (accuracy) and precision (CV, or RSD) data collected for five samples of whole blood fortified with 1 ng/mL of each analyte. Quantitation was performed against calibration curves obtained from the spiked matrix standards (Figure 3).

Conclusions

A simple, solid phase extraction procedure coupled with an LC/MS/MS detection method allows determination of buprenorphine and norbuprenorphine in whole blood at concentrations below 0.2 ng/mL. This method is intended for users of Agilent 1100 and 1200 LC series since the backpressure in the LC system does not exceed 400 bar.

Table 2. Method Validation at 1 ng/mL of Each Analyte, n = 5.

	Accuracy (%)	CV %
Buprenorphine	94.2	0.6
Norbuprenorphine	103	0.2

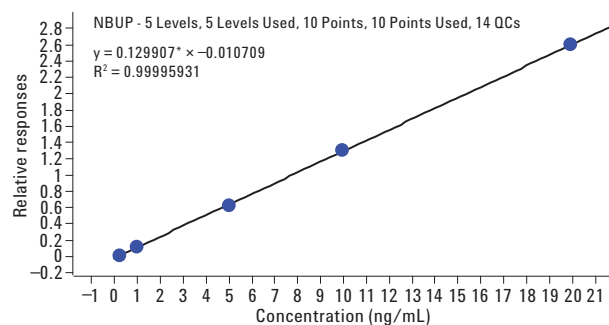
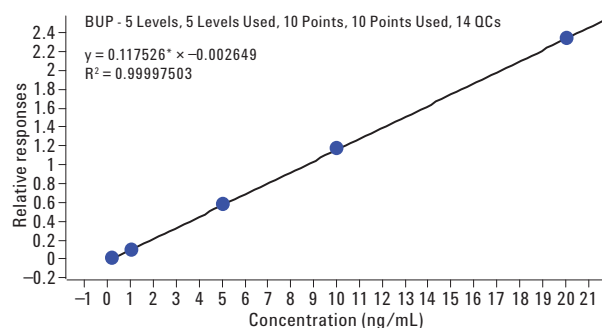


Figure 3. Typical calibration curves for buprenorphine and norbuprenorphine in whole blood extract. Concentration range 0.2 to 20 ng/mL. Linear fits $R^2 > 0.999$.

Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS System instruments. Low detection limits are achieved due to cleanliness of sample extracts and robust MS detection using newly identified ion transitions with abundant fragmentation products.

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