Ultrafast Analysis of Buprenorphine and Norbuprenorphine in Urine Using the Agilent RapidFire High-Throughput Mass Spectrometry System

Application Note

Authors

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Abstract

The analysis of buprenorphine and norbuprenorphine in urine by forensic toxicology laboratories requires a reliable method and accurate detection at low concentrations. Steady increases in the need for greater analytical capacity and higher throughput have placed new demands on traditional analytical technologies. This application note describes a method that uses the Agilent RapidFire/MS/MS system to analyze buprenorphine and norbuprenorphine in urine with much faster sample cycle times and comparable analytical results to typical LC/MS assays. Enzymatic hydrolysis followed by solid phase extraction and analysis by RapidFire/MS/MS allows for the accurate and precise measurement of these analytes in urine over a linear range of 2.5 to 400 ng/mL. Samples were analyzed on the RapidFire/MS/MS system at 15 seconds per sample providing a much higher throughput method of analysis compared to traditional LC/MS/MS protocols.

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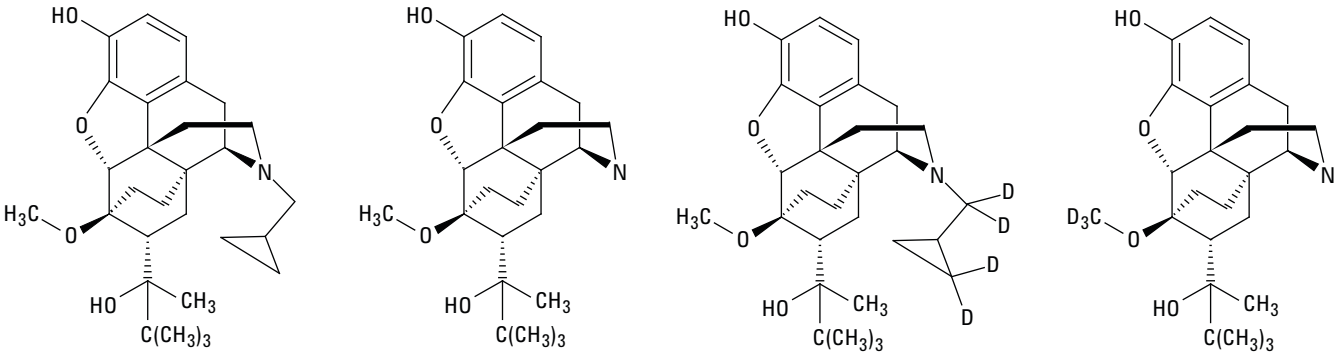
Introduction

We have developed a method which uses the Agilent RapidFire/MS/MS system to analyze buprenorphine and norbuprenorphine in urine with much faster sample cycle times and similar analytical results as compared to traditional LC/MS assays.

Buprenorphine and its dealkylated metabolite, norbuprenorphine, are excreted in urine almost exclusively as glucuronides (Figure 1).[1] Therefore, samples were first hydrolyzed using β-glucuronidase to convert the glucuronide metabolites to buprenorphine and norbuprenorphine.[2,4] Hydrolyzed samples were then extracted by a solid phase extraction procedure using Agilent Bond Elut 96 Plexa PCX plates.[3] Unlike other polymeric sorbents, all members of the Agilent Bond Elut Plexa family possess an amide-free hydroxylated particle surface that excludes protein binding, resulting in minimized ion suppression and maximum sensitivity. This 96-well plate based extraction system allows for automation of the entire sample preparation protocol to increase efficiency and laboratory throughput. The Agilent RapidFire High-throughput Mass Spectrometry System is an ultrafast SPE/MS/MS system capable of analyzing samples with cycle times less than 15 seconds. We have developed an MS/MS based analysis method using this technology in contrast to traditional LC/MS/MS methodology for the extracted samples. This new method, using RapidFire/MS/MS, allows for the rapid, accurate, and precise measurement of buprenorphine and norbuprenorphine in urine over a linear range of 2.5 to 400 ng/mL.



Buprenorphine
$C_{29}H_{41}NO_4$
M.W. 467.65
Log P 4.9-5.0
pKa 8.3

Norbuprenorphine
$C_{25}H_{35}NO_4$
M.W. 413.55
Log P 3.4-3.8

Buprenorphine-D_4
$C_{29}H_{37}D_4NO_4$
M.W. 471.62

Norbuprenorphine-D_3
$C_{25}H_{32}D_3NO_4$
M.W. 416.53

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

Experimental

RapidFire triple quadrupole conditions

The RapidFire/MS/MS system consisted of the following modules: an Agilent RapidFire 360, an Agilent 6460 Triple Quadrupole Mass Spectrometer, and MassHunter Triple Quadrupole Acquisition Software (B.04.01) with Qualitative Analysis (B.04.00), Quantitative Analysis (B.04.00), and RapidFire Integrator Software. Samples were analyzed at a rate of 15 seconds per sample. Quantitative and qualitative ions for buprenorphine, norbuprenorphine, and internal standards were monitored simultaneously in all experiments (Table 1). Agilent Mass Hunter Quantitative software automatically calculated qualifier ion ratios.

Chemicals and reagents

Drug standards were purchased from Cerilliant Corporation (100 µg/mL). All other solvents and reagents were purchased from Sigma Aldrich. Extraction materials were purchased from VWR. Stock buffers were made in the following manner.

Sodium acetate buffer 1.0 M, pH 5.0: Add approximately 200 mL of HPLC grade water to a 250 mL volumetric flask add 34 g of sodium acetate trihydrate and mix until completely dissolved. Adjust pH of the solution to pH 5.0 ± 0.02 using concentrated glacial acetic acid (add approximately 7 mL) adjust volume to 250 mL with HPLC grade water mix well and pH again to 5.0.

Reconstitution solution (5 %): In a 100 mL volumetric flask, add 5 mL of methanol, fill remaining volume with HPLC grade water, and mix well.

Elution solvent: Combine 80 % methylene chloride, 20 % isopropanol, 5 % ammonium hydroxide (make fresh daily).

Table 1. RapidFire/MS/MS conditions.

RapidFire conditions						
Buffer A	Water with 5 mM ammonium acetate, 0.1 % formic acid, 0.01 % trifluoroacetic acid; 1.5 mL/min flow rate					
Buffer B and C	50 % methanol, 25 % isopropanol, 25 % acetonitrile, 0.1 % formic acid, 0.01 % trifluoroacetic acid, 1.25 mL/min flow rate					
Injection volume	10 µL					
Aqueous wash	HPLC grade water					
Organic wash	50 % methanol, 25 % isopropanol, 25 % acetonitrile, 0.1 % formic acid, 0.01 % trifluoroacetic acid					
SPE cartridge	Agilent RapidFire cartridge E (reversed-phase C18 chemistry, G9205A)					
RF State 1	1,000 ms					
RF State 2	3,500 ms					
RF State 3	0 ms					
RF State 4	6,000 ms					
RF State 5	2,000 ms					
Triple quadrupole conditions						
Gas temperature	350 °C					
Gas flow	10 L/min					
Nebulizer	35 psi					
Sheath gas temperature	350 °C					
Sheath gas flow	12 L/min					
Nozzle voltage	0 V					
Capillary voltage	2,800 V					
Analyte	**Q1**	**Q3**	**Dwell**	**Fragmentor**	**CE**	**CAV**
Buprenorphine-d4	472.3	59.1	35	200	62	5
Buprenorphine Quant	468.3	55.1	35	200	62	5
Buprenorphine Qual	468.3	396.2	35	200	45	5
Norbuprenorphine-d3	417.3	83.1	35	188	60	5
Norbuprenorphine Quant	414.3	83.1	35	188	60	5
Norbuprenorphine Qual	414.3	101.1	35	188	50	5

Sample preparation

The samples were pretreated using the following procedure. First, 200 µL of sample was added to a 12×75 mm culture tube. Next, 25 µL of an internal standard was added and the sample was briefly vortexed. Sodium acetate buffer, 50 µL, was added next, followed by another brief vortexing. Next, 10 µL of β-glucuronidase enzyme was added and the sample was briefly vortexed. All tubes were then incubated at 55 °C for 30 minutes. Following incubation, the tubes were removed from the water bath and allowed to reach room temperature.

SPE procedure using a positive pressure manifold

Attach a collection tray to the Plexa PCX plate. Next, condition the Agilent Bond Elut 96 Plexa PCX, 10 mg (p/n: A4968010) by applying 1 mL of methanol to each well. Load the sample into the corresponding well and wash with 1 mL of 2 % formic acid. Then wash with 1 mL of 70 methanol:30 2 % formic acid and dry for 5 minutes under high pressure. Replace the collection tray with a 2 mL deep well collection plate. Elute with 2 × 0.5 mL of the elution solvent by letting the eluate drip into the collection plate under gravity. When the dripping stops, apply low pressure to extract the eluate completely from the packing. Place the 96-well collection plate into the Turbovap 96 and dry with nitrogen at 45 °C (approximately 15-20 minutes).

Reconstitute samples with 200 µL of the reconstitution solution, seal the plate and vortex it for 10 seconds. Inject on the RF/MS/MS system.

In order to monitor the full hydrolysis for buprenorphine glucuronide and norbuprenorphine glucuronide, two levels of quality controls at approximate concentrations of 50 ng/mL and 320 ng/mL were used (Table 2).

Data analysis

Data analysis was performed by using MassHunter Triple Quadrupole Quantitative analysis software. Calibration curves were constructed using linear least squares regression with $1/X^2$ weighting for the multiple reactions monitoring (MRM). The quantitation was performed by comparing the spectral peak area ratio to a known concentration of the internal standards.

Table 2. Quality control specifications.

Drug name	M.W.	Spiked concentration of conjugated drug	Approximate calculated concentration of free drug
Buprenorphine glucuronide	643.8	~320 and ~50	230 and 36.4 ± 10 %
Norbuprenorphine glucurinide	589.7	~330 and ~50	230 and 35.1 ± 10 %

Results and Discussion

Samples were prepared by spiking buprenorphine and norbuprenorphine into drug-free human urine. Samples were then processed using a standard enzymatic protocol for glucuronide hydrolysis. Dilute and shoot analysis of the hydrolyzed sample was initially attempted, but a significant matrix effect greatly reduced sensitivity. Plate-based cation exchange extraction significantly increased sensitivity of the analysis method. Lowering the sample pH to 5.0 during the initial sample preparation allowed the enzymatic hydrolysis to occur and also caused strong retention on the Plexa PCX SPE plate. Eluting with a strong base (NH_4OH) added to the organic mix enhanced the recovery of both analytes from the SPE plate. This plate-based extraction protocol could be automated using a liquid handling instrument capable of sample tracking in order to reduce manual steps and operator variability while generating reproducible results.

Reconstituted samples were then analyzed using the RapidFire/MS/MS system and a hydrophobic C18 cartridge at 15 seconds per sample (Figure 2). This RapidFire/MS/MS methodology is capable of throughputs greater than 240 samples per hour providing a high-throughput and very efficient mode of analysis.

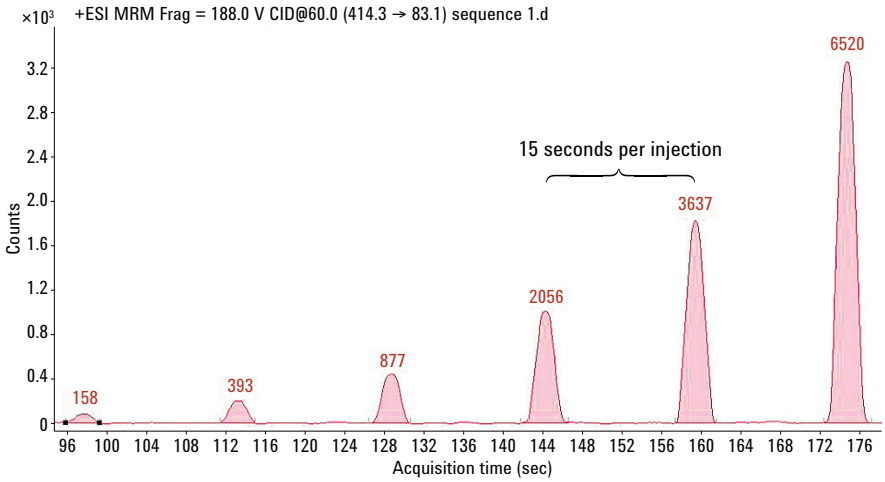


Figure 2. Representative standard curve data for norbuprenorphine showing a 15 seconds per injection interval.

Carryover on the RapidFire/MS/MS system was assessed by analyzing the area count of the blank (baseline noise) calculated as % of the mean peak area count of the 400 ng/mL samples. No significant carryover (0 %) was found for either buprenorphine or norbuprenorphine (Figure 3). Due to the high hydrophobicity of these analytes, when measuring higher concentrations of both buprenorphine and norbuprenorphine (>400 ng/mL) we recommended using one blank injection between wells by injecting a strong organic solution. Matrix effects were also investigated by comparing injections of standard curves prepared in reconstitution solution to those spiked into extracted drug-free urine. Significant differences in the standard curve area results were observed. However, the use of isotopically labeled internal standards compensated for these effects and facilitated accurate and precise results with this method.

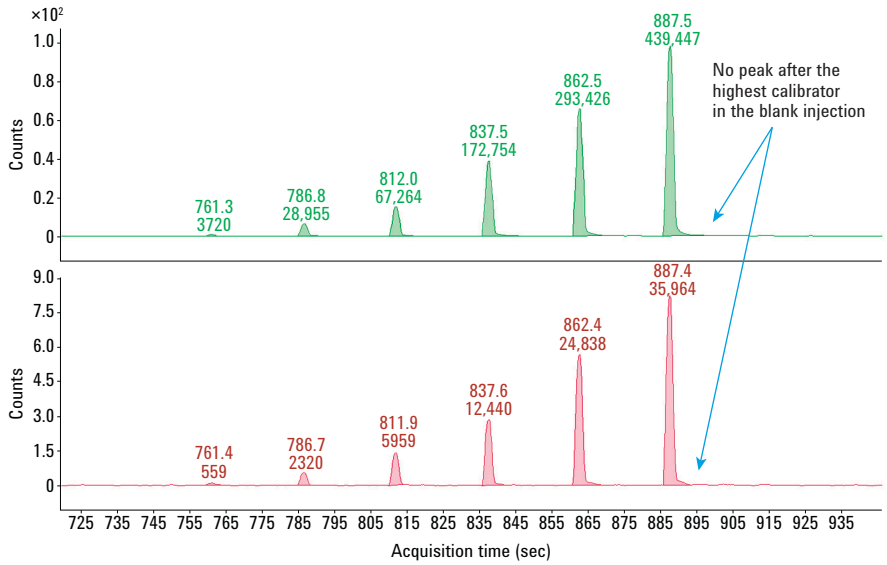


Figure 3. No significant carryover was seen during the matrix blank injection after each calibrator.

Analyte standard curves in urine had excellent linearity within the measured range (2.5-400 ng/mL) with an R^2 value greater than 0.995 (Figure 4). The limit of quantification (LOQ) was determined to be 2.5 ng/mL for both analytes. Quality controls (QC) were analyzed to obtain interday and intraday precision and accuracy values.

Intra and interday accuracies determined were within 10 % and coefficient of variation values were all less than 5 % for concentrations within the measured range (Tables 3 and 4).

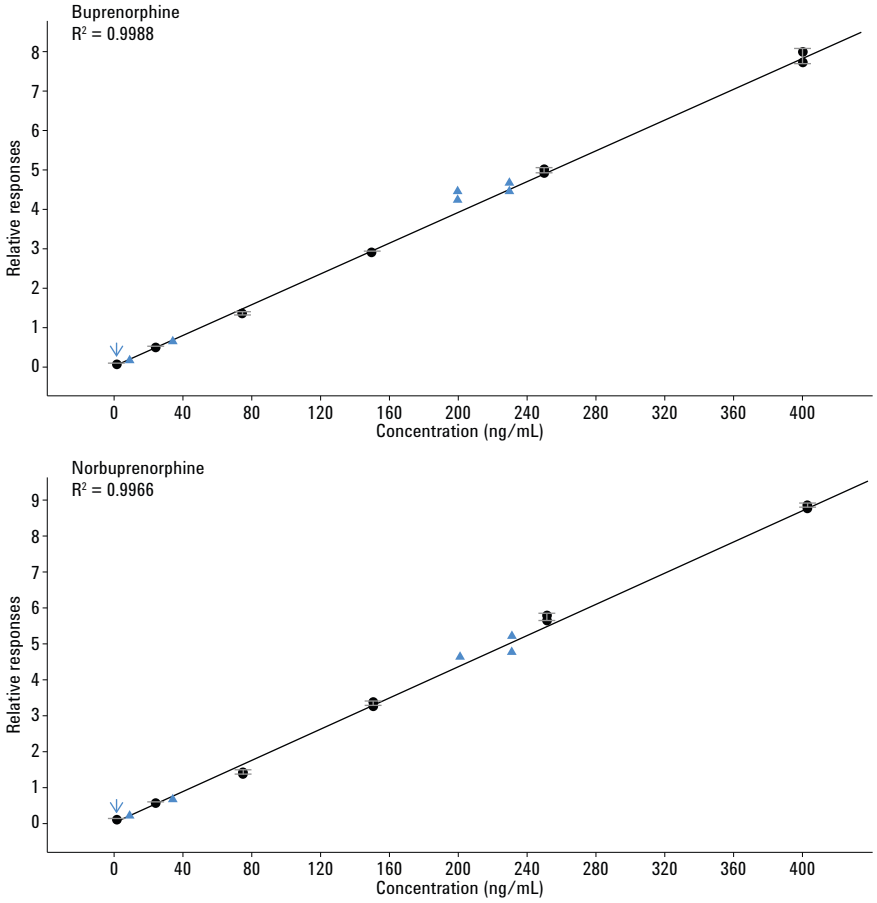


Figure 4. Calibration curves and QCs for buprenorphine and norbuprenorphine in spiked urine extracts. The concentration range is from 2.5 to 400 ng/mL. Black circles are calibrators and blue triangles are QCs for duplicate data sets.

Table 3. Intraday and interday precision and accuracy for buprenorphine.

Buprenorphine (ng/mL)	Intraday % accuracy (n=3)	Intraday % precision (n=3)	Interday % accuracy (n=3)	Interday % precision (n=3)
10 QC	104.6	1.3	102.0	1.6
200 QC	106.6	3.9	105.4	2.0
50 G QC	107.9	1.0	108.3	3.0
320 G QC	104.5	1.2	103.0	3.5

Table 4. Intraday and interday precision and accuracy for norbuprenorphine

Norbuprenorphine (ng/mL)	Intraday % accuracy (n=3)	Intraday % precision (n=3)	Interday % accuracy (n=3)	Interday % precision (n=3)
10 QC	98.3	1.1	96.3	0.8
200 QC	107.6	4.7	101.6	3.1
50 G QC	95.2	2.8	105.0	2.2
330 G QC	100.6	4.9	108.5	4.9

Conclusions

Buprenorphine and its metabolite norbuprenorphine were rapidly, accurately, and precisely measured in hydrolyzed urine using a simple SPE procedure and the Agilent RapidFire/MS/MS System. This forensic method covered a broad linear range of 2.5 to 400 ng/mL for each analyte. Samples were analyzed at 15 seconds per sample, providing a high-throughput method capable of analyzing more than 240 samples per hour. As a result, the Agilent RapidFire/MS/MS system is useful for the fast and efficient detection of similar small molecule analytes in urine.

References

1. Hegstad, S. *et al.* Rapid Quantification of Buprenorphine-Glucuronide and Norbuprenorphine-Glucuronide in Human Urine by LC-MS-MS. *Journal of Analytical Toxicology*, **2007**, 31:214–219.

2. Chang, Y., Moody, D. E., and McCance-Katz, E. F. Novel Metabolites of Buprenorphine Detected in Human Liver microsomes and Human Urine. *Drug Metabolism and Disposition*, **2006**, 34:440-8.

3. LC/MS/MS of Buprenorphine and Norbuprenorphine in Whole Blood Using Agilent Bond Elute Plexa PCX and an Agilent Poroshell 120 Column. *Agilent publication* 5990-9930EN.

4. Kronsdtrand, R., Selden, T. G., and Josefsson, M. Analysis of Buprenorphine, Norbuprenorphine and Their Glucuronides in Urine by Liquid Chromatography-Mass. *Spectrometry, Journal of Analytical Toxicology*, **2003**, 27**:** 465-470.

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