應用手冊

Simultaneous Quantitative Determination of Opioid Dependency Treatment Drugs in Human Urine Using UPLC-MS/MS

Russell Watts, Allan Traynor, Michelle Wood

Waters Corporation, Concateno plc

For forensic toxicology use only.

Abstract

In this application note we develop and validate a single simple and rapid UPLC-MS/MS method for the simultaneous quantitative determination of the opioid dependency treatment drugs methadone, buprenorphine, and dihydrocodeine in human urine.

Introduction

- Across the developed countries of the world, 0.4–0.8% of adults develop a dependence on illicit opioids.[1]
- Common treatment methods include detoxification by supervised withdrawal and tapered doses of replacement drugs.[2]
- Buprenorphine, methadone and more recently, dihydrocodeine have been shown to be effective as replacement drugs for the treatment of opioid dependency.[3–5]
- Urine analysis of these compounds is essential to monitor abstinence and detect or confirm relapses.
- The associated overdose risk, potential for abuse and links with criminal activity has made the analysis of these compounds widespread in other areas of toxicology such as post-mortem and forensic.

Figure 1. Waters ACQUITY TQD System.

Experimental

Specimens

Validation was performed using human urine samples obtained from Concateno (London, UK) and Salford Royal NHS Foundation Trust Hospital (Manchester, UK). All samples were stored at -20 °C until analysis. Blank urine obtained from volunteers was used as the control material to prepare all the calibrators and quality controls (QC).

Standards

Standard reference material, deuterated analogues and drug metabolites were purchased from LGC Promochem (Teddington, UK). A mixed standard stock solution containing buprenorphine (BUP) and norbuprenorphine (NBUP) at 12.5 µg/mL and methadone (METH), 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) and dihydrocodeine (DHC) at 125 µg/mL was prepared in methanol. A mixed internal standard (IS) stock solution containing buprenorphine-D4 (BUP-D4) and norbuprenorphine-D3 (NBUP-D3) at 1.25 µg/mL and methadone-D9 (METH-D9), 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine-D3 (EDDP-D3) and dihydrocodeine- D6 (DHC-D6) at 2.5 µg/mL was prepared in methanol.

Sample Preparation

Enzymatic hydrolysis was performed on all samples, calibrators and QC’s (250 µL) after the addition of the IS (10 µL). 1 M Sodium acetate, pH 5 (20 µL) and β-glucuronidase (10 µL – Helix pomatia, 100,000 units/mL, Sigma-Aldrich, Gillingham, UK) were added to the samples which were then heated at 56 °C for 1 hour. After hydrolysis, saturated disodium tetraborate buffer (250 µL) was added to all samples and a liquid/liquid extraction (LLE) using a mixture of dichloromethane, hexane, diethyl ether and isoamyl alcohol (30:20:50:0.5) was performed. The supernatant was taken to dryness using a sample concentrator block (50 °C) under nitrogen, before being reconstituted with a 50/50 mix of methanol and mobile phase A (250 µL).

LC Conditions

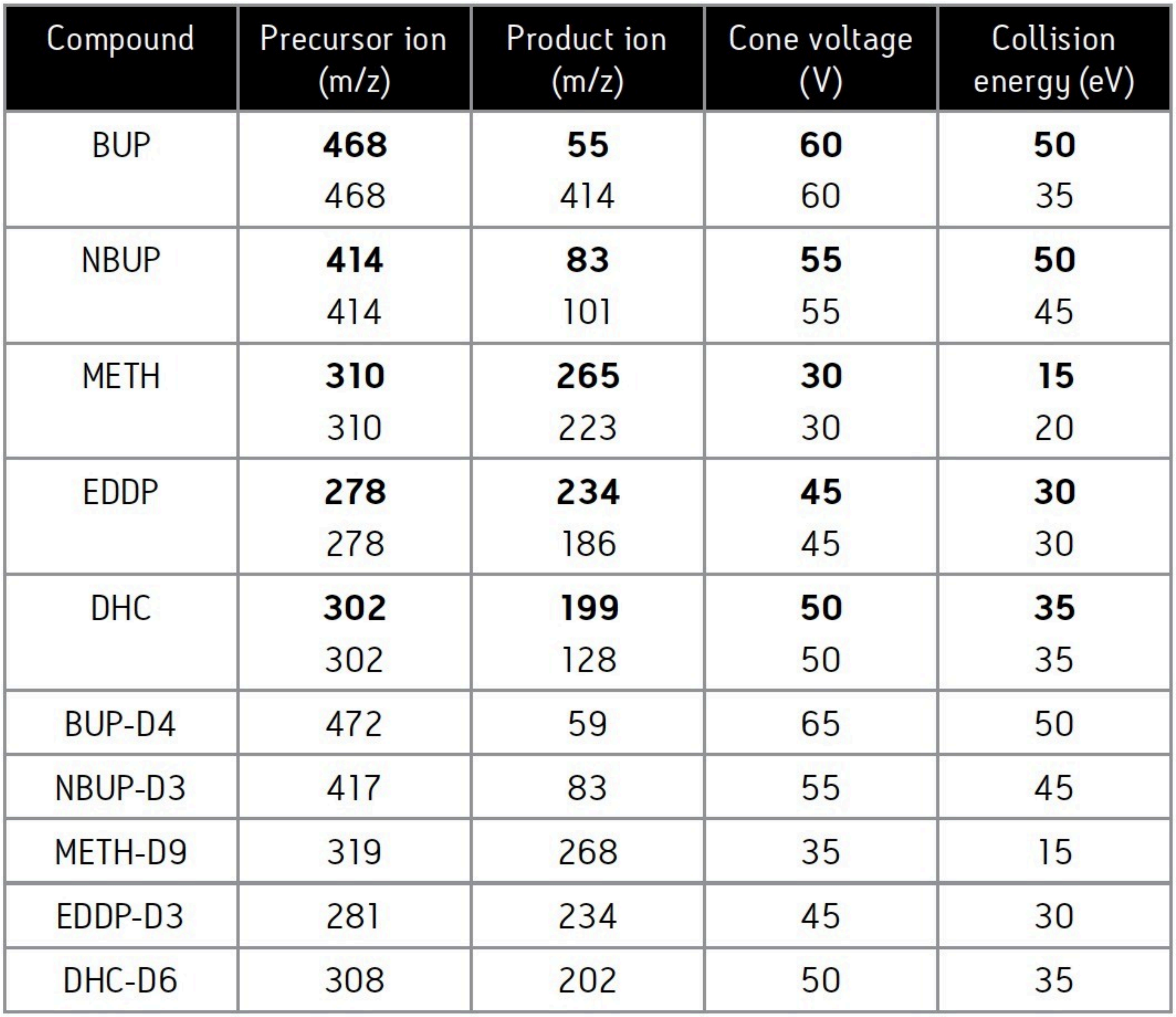
LC system:	Waters ACQUITY UPLC
Column:	ACQUITY UPLC HSS T3 column (2.1 x 100 mm, 1.8 µm)
Column temp:	30 °C
Flow rate:	300 L/min.
Mobile phase A:	5 mM Ammonium acetate containing 0.025 % formic acid in water
Mobile phase B:	Methanol
Gradient:	15-95% B over 5 min.
Injection volume:	10 L
Strong Wash Solvent:	Mobile phase B (800 µL)
Weak Wash Solvent:	Mobile phase A (2400 µL)

MS Conditions

MS system:	Waters TQ Detector
Ionization mode:	ESI Positive
Capillary voltage:	3 kV
Collision Gas Pressure:	4.5×10^{-3} mbar
Acquisition Mode:	Multiple reaction monitoring (MRM)
Data Processing:	MassLynx v4.1 with TargetLynx

Results and Discussion

The MRM conditions used for the measurement of all compounds of interest and their respective internal standards are summarised in Table 1.



Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
BUP	**468** 468	**55** 414	**60** 60	**50** 35
NBUP	**414** 414	**83** 101	**55** 55	**50** 45
METH	**310** 310	**265** 223	**30** 30	**15** 20
EDDP	**278** 278	**234** 186	**45** 45	**30** 30
DHC	**302** 302	**199** 128	**50** 50	**35** 35
BUP-D4	472	59	65	50
NBUP-D3	417	83	55	45
METH-D9	319	268	35	15
EDDP-D3	281	234	45	30
DHC-D6	308	202	50	35

Table 1. MRM conditions used for all compounds and their internal standards.

Bold transitions used as the quantifier ion.

Figure 2 shows the MRM chromatograms obtained from a 5 µL injection of a low level urine calibrator (50 ng/mL for METH, EDDP, and DHC, 5 ng/mL for BUP and NBUP). The quantifier/qualifier ion ratios for all compounds were monitored for all calibrators, QC's and samples and were found to be within ±20% of the target ion ratios.

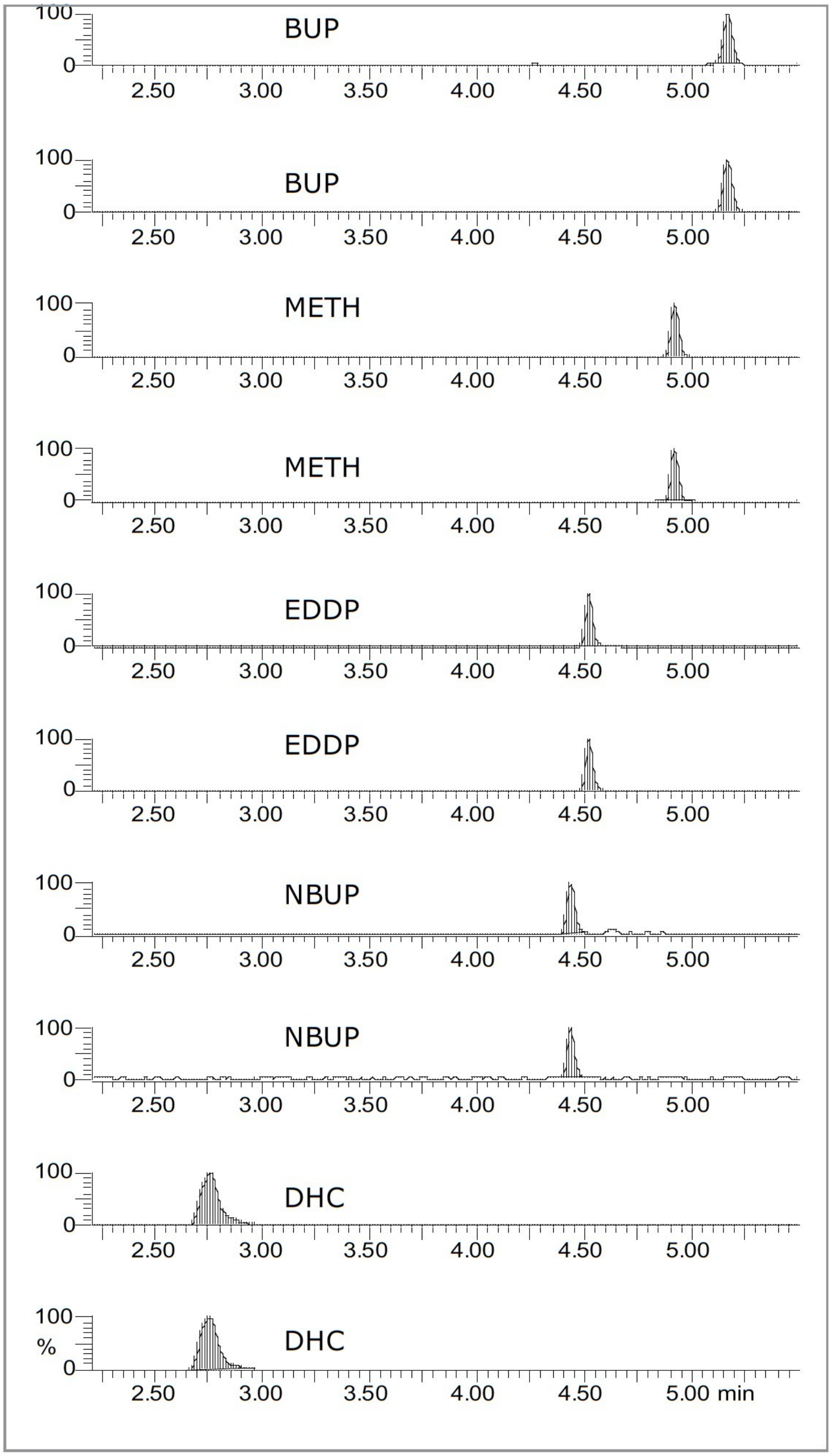


Figure 2. MRM chromatograms for qualifier and quantifier ions obtained from a low level calibrator. The upper trace is the qualifier ion and the lower trace is the quantifier ion chromatogram for each compound.

A calibration curve (25–2500 ng/mL for METH, EDDP, and DHC, 2.5–250 ng/mL for BUP and NBUP) was prepared by adding all the compounds to blank urine. Calibrators and QC's were prepared by the same procedure as previously described for the samples. Quantitation was performed by the integration of the area under the peak of the specific MRM chromatogram. Figure 3 shows a typical standard curve for all compounds in urine. Calibrators were plotted using 1/x weighting and found to be linear for all compounds, over the investigated range (coefficient of determination r^2 = >0.997).

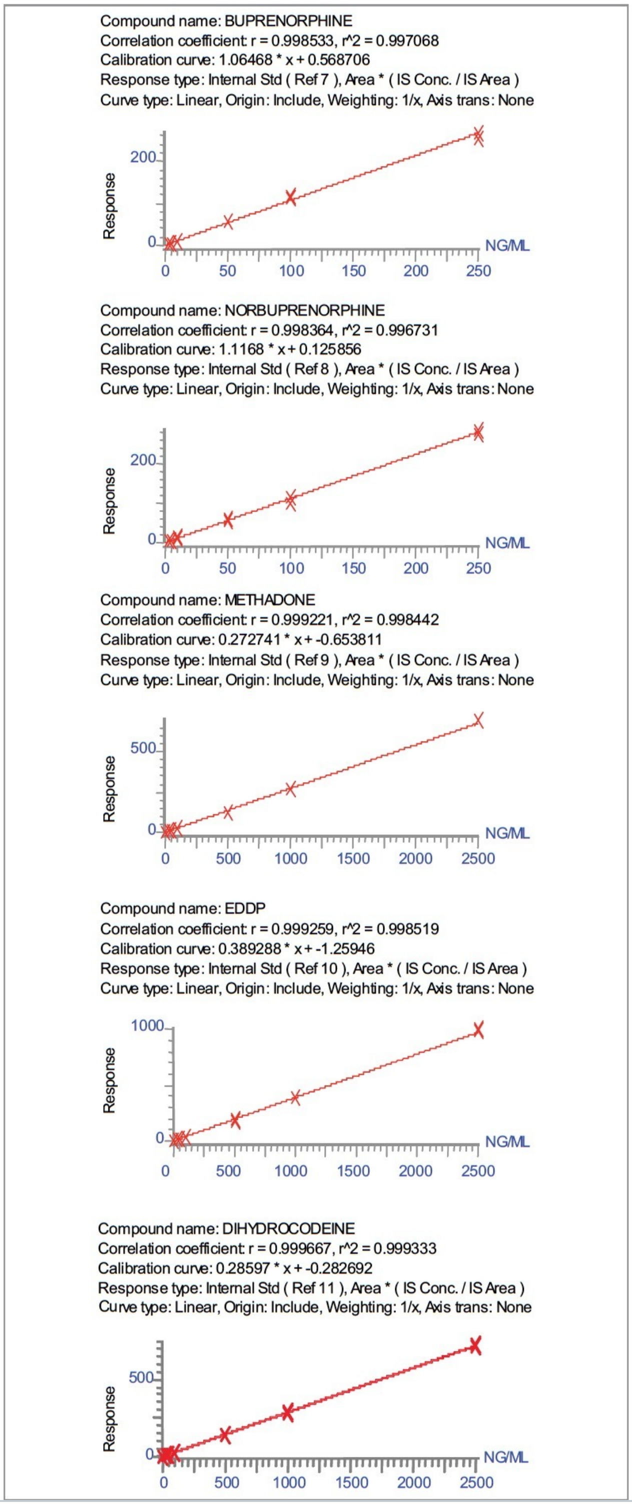
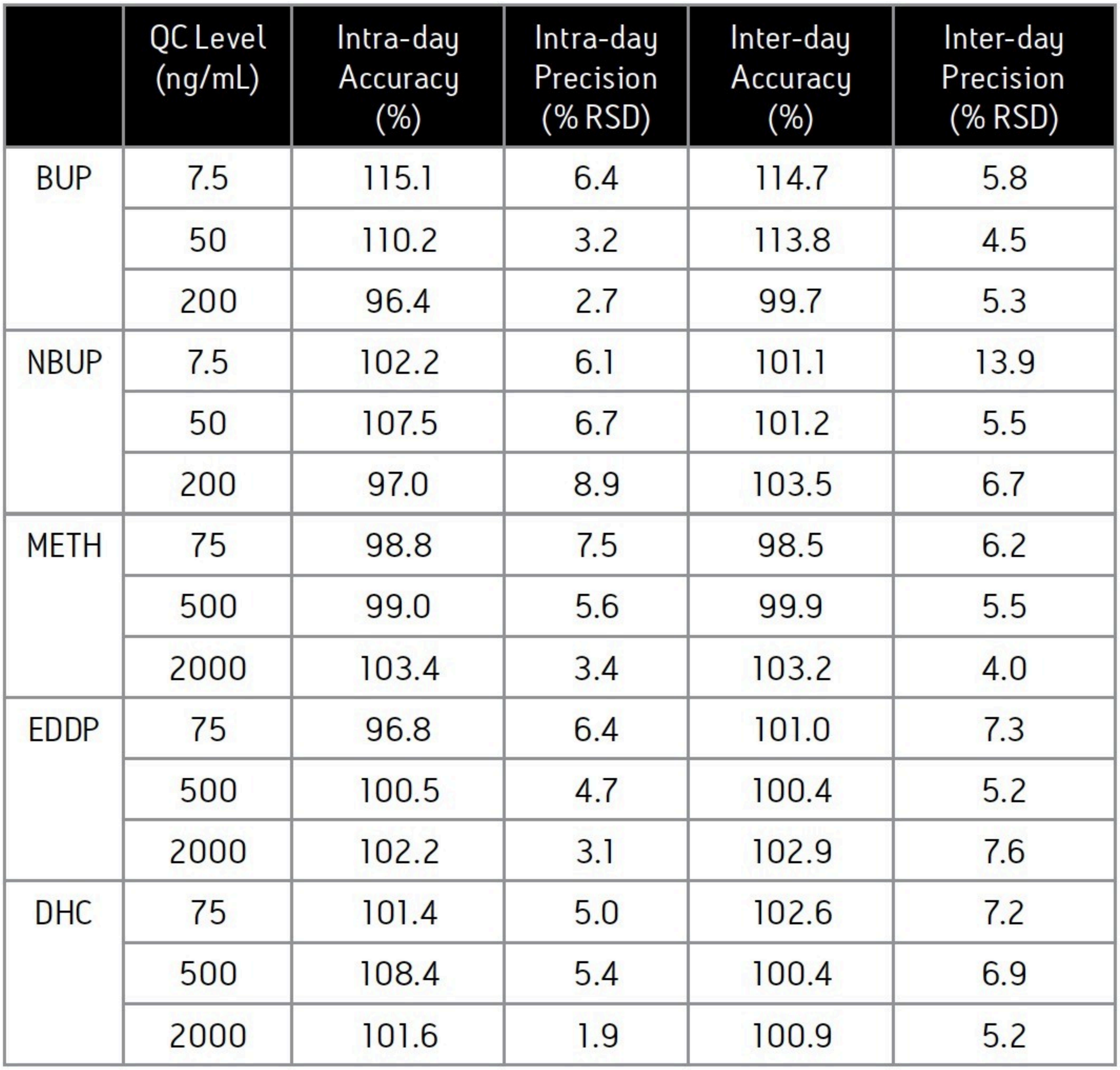


Figure 3. Typical calibration curves obtained for all compounds of interest.

Limits of detection were 0.5 ng/mL for EDDP, 1ng/mL for METH and DHC and 2 ng/mL for BUP and NBUP, all of which were acceptable for this analysis.

Intra-day precision and accuracy were assessed by adding all the compounds to blank urine (n=5) at three QC concentrations (7.5, 50 and 200 ng/mL for BUP and NBUP and 75,500 and 2000 ng/mL for METH, EDDP, and DHC). Inter-day precision was assessed by analysing the QC samples in duplicate on five different days. Intra and inter-day precision and accuracy were found to be good, with precision CV's <14% and accuracy between 96-115 %, as shown in Table 2.



	QC Level (ng/mL)	Intra-day Accuracy (%)	Intra-day Precision (% RSD)	Inter-day Accuracy (%)	Inter-day Precision (% RSD)
BUP	7.5	115.1	6.4	114.7	5.8
	50	110.2	3.2	113.8	4.5
	200	96.4	2.7	99.7	5.3
NBUP	7.5	102.2	6.1	101.1	13.9
	50	107.5	6.7	101.2	5.5
	200	97.0	8.9	103.5	6.7
METH	75	98.8	7.5	98.5	6.2
	500	99.0	5.6	99.9	5.5
	2000	103.4	3.4	103.2	4.0
EDDP	75	96.8	6.4	101.0	7.3
	500	100.5	4.7	100.4	5.2
	2000	102.2	3.1	102.9	7.6
DHC	75	101.4	5.0	102.6	7.2
	500	108.4	5.4	100.4	6.9
	2000	101.6	1.9	100.9	5.2

Table 2. Intra and inter-day precision and accuracy for all the compounds of interest at 3 QC levels across the calibration range.

The stability of prepared samples and calibrators were assessed over 24 hours. A prepared calibrator (50/500 ng/mL, BUP and NBUP/ METH, EDDP, and DHC) was stored at 5°C in the dark in the ACQUITY sample manager and an injection performed every hour. No significant changes in absolute peak area were found for any of the compounds over the investigated time period.

Matrix effects were assessed in 2 ways, firstly a post-column infusion of all compounds was performed during the injection of a solvent blank and prepared urine (n=5). An example is shown in Figure 4. BUP and NBUP showed some areas of ion suppression in all samples at the same retention time as the compounds. To investigate this further, a second type of experiment was performed by spiking blank prepared patient samples (n=7) with all compounds and comparing the absolute peak areas against the equivalent concentration of calibrator in solvent. The average matrix effects were found to be acceptable for METH, EDDP, and DHC but BUP and NBUP showed a significant degree of matrix effects; -39% and +19.3%, respectively. Therefore, deuterated internal standards were used in all calibrator, QC's and samples to minimise the impact of the measured matrix effects on the data quality.

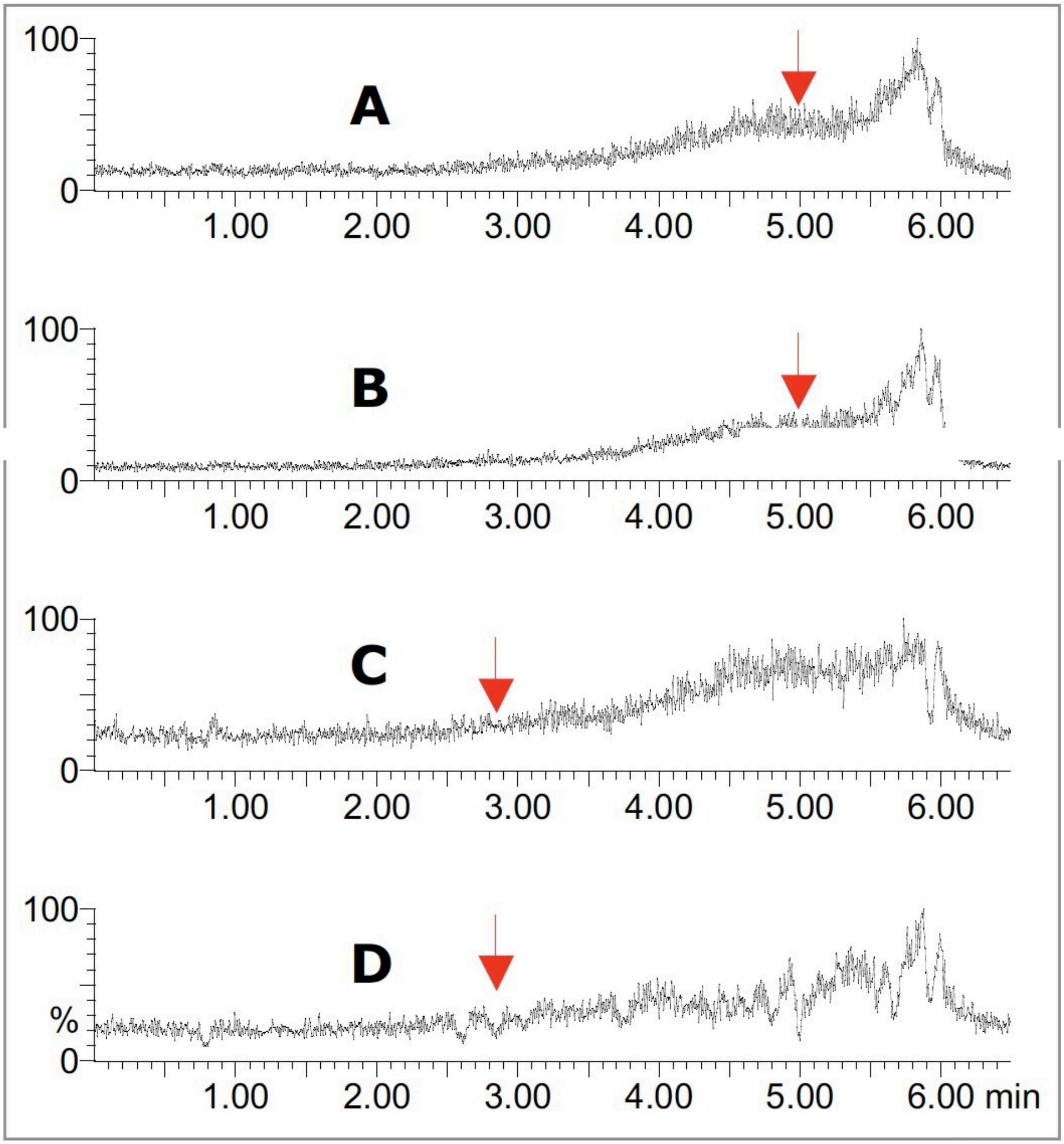


Figure 4. Chromatograms showing the post-column infusion of METH (A+B) and DHC (C+D) at 100 ng/mL, during the injection of solvent blank (A+C) and a prepared urine blank (B+D). Red arrows show the elution position of both compounds.

The utility of the newly-developed UPLC-MS/MS method was demonstrated by the analysis of 58 authentic samples. These samples had been previously analysed at Concateno plc, using two separate LC-MS/MS assays i.e., one assay for BUP and its main metabolite (NBUP) and one for METH and its main metabolite (EDDP). The BUP, NBUP assay comprised a fairly lengthy sample preparation step including enzymatic hydrolysis and solid-phase extraction (SPE); total preparation time was approximately 3.5 hours/batch. Final analysis was then performed using a 7 minute LC-MS/MS method6. The in-house METH, EDDP assay comprised a simple urine dilution with 0.5 hour total preparation time for a sample batch followed by final analysis using a 5 minute LC-MS/MS method.

These urine samples were subsequently analysed using the newlydeveloped technique. The same preparation protocol (enzymatic hydrolysis followed by LLE; total preparation time estimated at 2 hours/batch) was suitable for all of the analytes of interest and was followed by the UPLC-MS/MS assay which monitored all compounds simultaneously in a single 6.5 minute run time. Preliminary results showed that many samples contained METH and EDDP concentrations which were above the calibration range used. For accurate quantification, these samples required dilution and reanalysis. In order to reduce the need for reanalysis, additional experiments were performed to assess the linearity of the method for METH and EDDP over an extended analytical range. The responses for these two analytes were found to be linear to 25,000 ng/mL. When all the high samples were re-quantified using the extended calibration series, quantitative results were comparable to those diluted and reanalysed, thus minimising reanalysis.

Overall, the single UPLC-MS/MS method showed very good agreement with the results obtained from the previously described procedures (Figure 5). The correlations ranged from $r^2=0.956 – 0.995$ for METH, EDDP, BUP, and NBUP. In respect to DHC, no suitably quantified patient samples could be obtained. Samples (n=20) containing DHC that had been qualitatively analysed using thin layer chromatography were obtained and analysed using the newly developed method. The qualitative results showed good comparison.

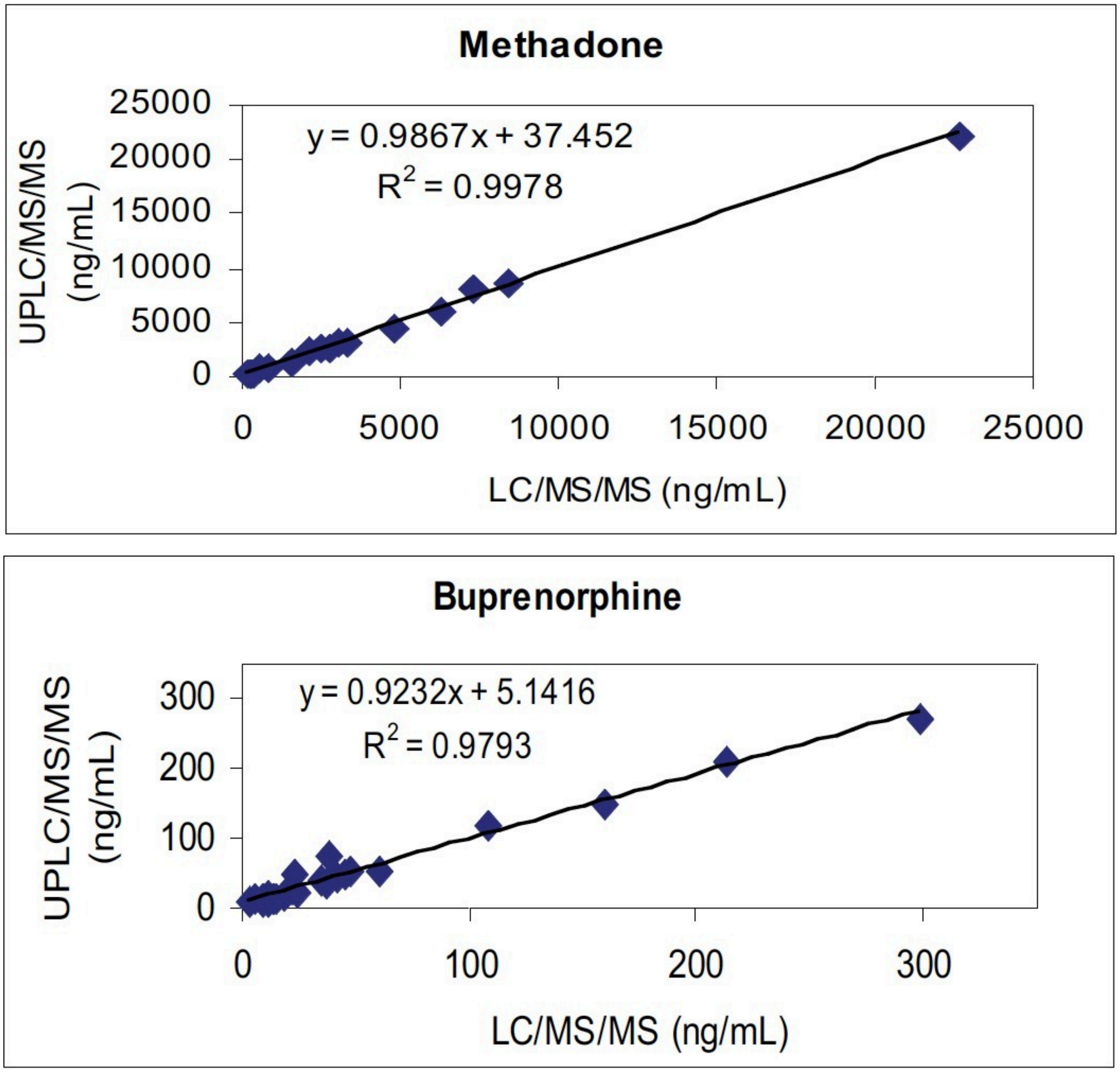


Figure 5. Analysis of METH and BUP concentrations in patient samples by Concateno plc and the developed Waters UPLC-MS/MS method.

Conclusion

The developed method has been shown to be accurate and precise in the measurement of all the compounds of interest with a single analysis that takes only 6.5 minutes.

The method was successfully applied to the analysis of patient samples and quantitative results showed good correlation to an established method for methadone, EDDP, buprenorphine, and norbuprenorphine.

This method allows the rapid urine analysis of patients in opioid dependency therapy and having all treatment drugs in one single method, removes the issue of changing between multiple methodologies in the determination of three prescribed treatment drugs. The use of one assay for the analysis of these treatment drugs offers significant time saving benefits, including a fifty percent reduction in sample preparation time, which provides increased productivity and profitability for the analytical laboratory.

References

1. Degenhardt L, Hall MD, Warner-Smith M, Lynskey M. Geneva: World Health Organisation, 2004; 1109–75.
2. Mattick RP, Hall WD. Lancet 1996; 347: 97–100.
3. Mattick RP, Kimber J, Breen C, Davoli M. Cochrane Database Syst Rev 2008; 2: CD002207.
4. NICE technology appraisal guidance 114. www.nice.org.uk/TA114.
5. Robertson JR, Raab GM, Bruce M, McKenzie JS, Storkey HR, Salter A. Addiction 2006; 101: 1752–59.
6. Polettini A, Huestis MA. *J. Chromatogr. B*, 2001; 754: 447–459.

Acknowledgements

George Waite, Salford Royal NHS Foundation Trust Hospital (Manchester, UK) for supplying anonymous patient samples containing dihydrocodeine.

Featured Products

- ACQUITY UPLC System <https://www.waters.com/514207>
- MassLynx MS Software <https://www.waters.com/513662>
- TargetLynx <https://www.waters.com/513791>

720003005, July 2009

© 2021 Waters Corporation. All Rights Reserved.