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Application Note

A Comprehensive Method for the Analysis of Pain Management Drugs and Drugs of Abuse Incorporating Simplified, Rapid Mixed-Mode SPE with UPLC-MS/MS for Forensic Toxicology

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For research use only. Not for use in diagnostic procedures.

Abstract

This application note describes a complete method for the solid phase extraction and UPLC-MS/MS analysis of pain management drugs and drugs of abuse for Clinical Research.

A number of advantages are highlighted.

Sample preparation is optimized to efficiently extract all analytes with a simplified procedure that reduces the number of manual steps. The water wettable nature of the sorbent enables in-well sample pretreatment and direct loading without conditioning and equilibration, eliminating sample transfer and potential transcription errors. The efficient and reproducible extraction is evident in the high recoveries, consistent matrix effects, and accurate and precise quantitative data.

The use of the ACQUITY UPLC BEH C_{18} Column results in rapid analysis of a large panel while maintaining all required baseline separations for accurate quantification.

The Waters Xevo TQ-S micro, with features such as StepWave Technology and XDR Detector ensures extremely rapid and accurate quantification of all compounds over wide dynamic ranges. This enables the simultaneous quantification of 6-MAM at 2 ng/mL and methamphetamine at 2500 ng/mL.

This combination of sample preparation, UPLC separation, and MS/MS detection optimizes the workflow and results in a rapid, accurate, and precise method.

Benefits

- Rapid, simplified sample preparation of a comprehensive drug panel
- Efficient and consistent recovery for all analytes
- Consistent matrix effects
- All sample pretreatment and extraction performed in-well, eliminating transfer steps
- LC-MS/MS analysis of 80 compounds in four minutes
- Accurate and precise quantitative data for all compounds

Introduction

Analyte panels for use in clinical research analysis typically include illicit drugs and common drugs of abuse. Often, multiple clinical research methods are used to obtain a comprehensive view of the multiple drug classes. These methods may include immunoassay, GC-MS, LC-MS/MS, or a combination of methods. Waters has developed a method for the quantification of a comprehensive drug panel to achieve the appropriate analytical sensitivity, selectivity, and accuracy for unambiguous identification for clinical Research.

This method employs a simple sample extraction procedure using Oasis MCX µElution Plates coupled with a rapid and reproducible chromatographic method using an ACQUITY UPLC BEH C_{18} Column that achieves baseline separation for all critical pairs of potentially interfering analytes. A Waters Xevo TQ-S micro with Xtended Dynamic Range (XDR) capablilities provided the analytical sensitivity and dynamic range capabilities

required for this diverse group of compounds.

Experimental

All standards were obtained from Cerilliant (Round Rock, TX) and Cayman Chemical (Ann Arbor, MI). A mixed stock solution was prepared in methanol at concentrations of 2, 10, and 25 µg/mL, depending upon the analyte. An internal standard stock solution was prepared in methanol at a concentration of 1 µg/mL. Stable isotope labeled internal standards were used for all compounds except naltrexone, methedrone, dehydronorketamine, m-OH-benzoylecgonine, α-Pyrrolidinovalerophenone (alpha-PVP) metabolite 1, meprobamate, flurazepam, norpropoxyphene, and clonazepam. In those cases, either the internal standard interfered with the quantification of one of the other analytes (naltrexone and clonazepam) or the stable labeled IS was not readily available. Samples were prepared by diluting stock solutions into pooled, blank urine. External quality control material was obtained from UTAK Laboratories (Valencia, CA). All analytes, along with their retention times and calibration ranges are listed in Table 1.

Name	RT	Concentration range (ng/mL)	Name	RT	Concentration range (ng/mL)
Morphine	0.86	25–2500	Tapentadol	1.71	10–1000
Oxymorphone	0.91	25–2500	alpha-PVP	1.77	10–1000
Hydromorphone	0.98	25–2500	7-aminoflunitrazepam	1.69	10–1000
Dihydrocodeine	1.15	10–1000	Cocaine	1.81	10–1000
Naloxone	1.15	10–1000	Normeperidine	1.82	10–1000
Codeine	1.17	25–2500	Meperidine	1.83	10–1000
Pregabalin	1.20	10–1000	Zolpidem	1.85	10–1000
Gabapentin	1.20	10–1000	alpha-PVP Metabolite 1	1.88	10–1000
Methylone	1.21	10–1000	Norbuprenorphine	1.90	2–200
Noroxycodone	1.25	10–1000	Chlordiazepoxide	1.93	10–1000
6-beta Naltrexol	1.26	10–1000	Trazodone	1.99	10–1000
Naltrexone	1.26	10–1000	Cocaethylene	2.01	10–1000
Amphetamine	1.28	25–2500	Fenfluramine	2.03	10–1000
Oxycodone	1.28	25–2500	PCP	2.09	10–1000
6-MAM	1.28	2–200	Meprobamate	1.96	10–1000
MDA	1.30	25–2500	Fentanyl	2.15	2–200
Norhydrocodone	1.31	10–1000	alpha-OH Midazolam	2.13	10–1000
Ethylone	1.32	10–1000	Midazolam	2.17	10–1000
O-desmethyl Tramadol	1.32	10–1000	Flurazepam	2.23	10–1000
Methedrone	1.33	10–1000	Buprenorphine	2.27	2–200
Hydrocodone	1.34	25–2500	EDDP	2.29	10–1000
Dehydronorketamine	1.33	10–1000	Norprpoxyphene	2.51	25–2500
Methamphetamine	1.36	25–2500	Verapamil	2.52	10–1000
MDMA	1.37	25–2500	Propoxyphene	2.56	10–1000
m-OH BZE	1.34	10–1000	Methadone	2.60	10–1000
Butylone	1.41	10–1000	alpha-OH Alprazolam	2.51	10–1000
Phentermine	1.43	25–2500	alpha-OH Triazolam	2.51	10–1000
Mephedrone	1.47	10–1000	Nitrazepam	2.52	10–1000
Norketamine	1.47	10–1000	Oxazepam	2.59	10–1000
MDEA	1.48	25–2500	Clonazepam	2.65	10–1000
Ritalinic Acid	1.48	25–2500	Lorazepam	2.66	10–1000
Ketamine	1.52	10–1000	Carisoprodol	2.67	10–1000
Norfentanyl	1.54	2–200	Alprazolam	2.68	10–1000
BZE	1.52	10–1000	2-OH Ethyl Flurazepam	2.68	10–1000
7-aminoclonazepam	1.51	10–1000	Nordiazepam	2.68	10–1000
N-desmethyl Zopiclone	1.58	10–1000	Triazolam	2.73	10–1000
Zopiclone	1.61	10–1000	Desalkylflurazepam	2.78	10–1000
Tramadol	1.68	10–1000	Flunitrazepam	2.77	10–1000
N-desmethyl Tramadol	1.69	10–1000	Temazepam	2.87	10–1000
Methylphenidate	1.70	25–2500	Diazepam	3.05	10–1000

Table 1. Retention times and calibration ranges of all compounds.

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC BEH $C_{18,}$ 1.7 µm, 2.1 x 100 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.6 mL/min.
Mobile phase A (MPA):	0.1% Formic acid in MilliQ water
Mobile phase B (MPB):	0.1% Formic acid in acetonitrile (ACN)
Purge solvent:	50:50 MeOH:H_2O
Wash solvent:	25:25:25:25 MeOH:H_2O:IPA:ACN

UPLC Gradient Program:

Time (min)	Flow (mL/min)	% MPA	% MPB
0	0.6	98	2
3.33	0.6	33	67
3.5	0.6	10	90
3.6	0.6	98	2

Time (min)	Flow (mL/min)	% MPA	% MPB
4	0.6	98	2

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI positive
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/hr
Cone gas flow:	150 L/hr
Acquisition range:	MRM transitions optimized for individual compounds
Capillary voltage:	1.0 kV
Collision energy:	Optimized for individual compounds (See Appendix 1)
Cone voltage:	Optimized for individual compounds (See Appendix 1)
Data management:	MS software: MassLynx Quantification software: TargetLynx XS

Analyte recoveries and matrix effects were calculated as described previously.[1] Internal standard corrected matrix effects were calculated using the response factor of the analyte.

SPE Extraction

100 µL of urine was added to individual wells of an Oasis MCX µElution Plate, followed by 100 µL of a solution containing hydrolysis buffer, 10 µg/mL of β-glucuronidase enzyme, and 100 ng/mL internal standards and mixed by several aspirations. After incubation, 200 µL of 4% H_3PO_4 was added and mixed by several aspirations. All samples were drawn directly into the sorbent bed by vacuum and subsequently washed with 200 µL of 80:20 H_2O:MeOH. The plate was dried under high vacuum (~15 inch Hg) for one minute to remove as much of the wash solution as possible. Samples were eluted using 2 x 25 µL of 50:50 ACN:MeOH containing 5% strong ammonia solution (Fisher, 28–30%). All samples were diluted with 150 µL of sample diluent (2% ACN:1% formic acid in MilliQ water) prior to LC-MS/MS analysis. A graphical workflow of the extraction procedure is shown in Figure 1.

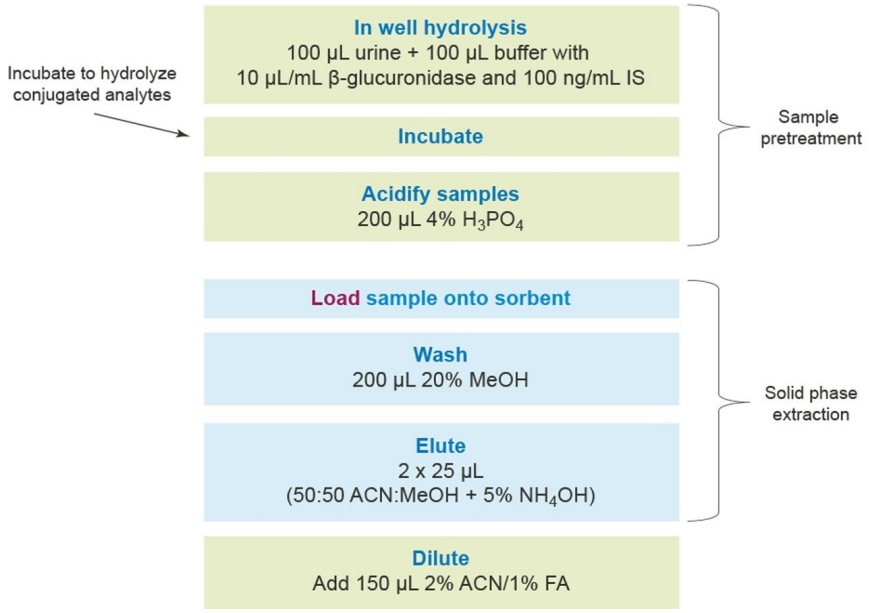

Figure 1. Details of the extraction method for the analysis of a comprehensive drug panel using Oasis MCX µElution Plates. Enzymatic hydrolysis and sample pretreatment are performed in the wells of the extraction plate, minimizing transfer steps. Conditioning and equilibration steps are eliminated and a single wash step is used instead of two, significantly simplifying the procedure.

Results and Discussion

Chromatography

All test compounds are listed in Table 1, along with their retention times and calibration ranges. Figure 2 shows the chromatography of all compounds included in the panel on the ACQUITY UPLC BEH C_{18} Column. Meprobamate and norpropoxyphene were included in the panel but were only monitored qualitatively, as they are not fully compatible with the sample preparation procedures. As with any multi-analyte panel, care must be taken to ensure that compounds and internal standards do not interfere with each other. Figures 3A and 3B highlight the chromatography of several groups of analytes with the potential to interfere with each other. In each case, either baseline separation is achieved (see naloxone vs. 6-MAM, Figure 3B) or the MRMs do not interfere with each other (see dehydronorketamine and ethylone, Figure 3A). In some cases, certain internal standards were not used.

For example, clonazepam-d4 was not used as it interfered with the quantification of lorazepam. The high efficiency of the UPLC Column enabled all compounds to elute in just over three minutes, without any compromise in resolution for this large panel, with a total run time of four minutes.

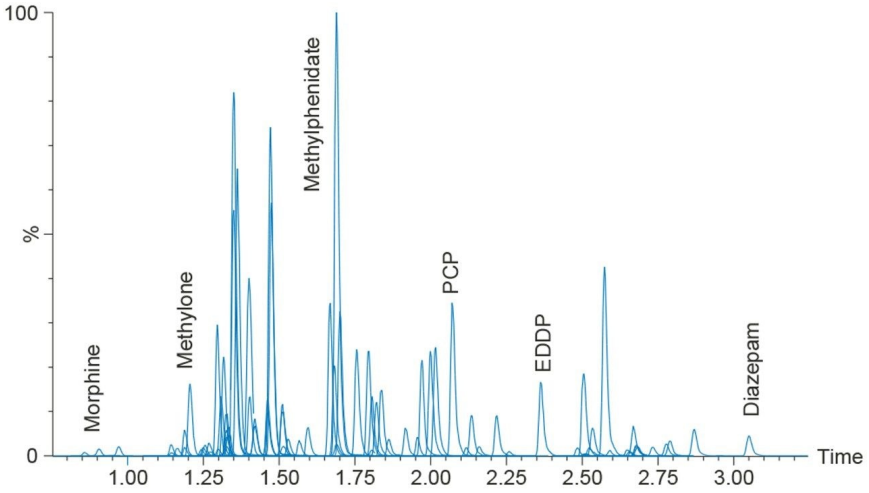

Figure 2. Chromatography of all compounds on the ACQUITY UPLC BEH C $_{18}$ Column. The earliest eluting compound is morphine at 0.86 minutes and the latest eluting compound is diazepam at 3.05 minutes.

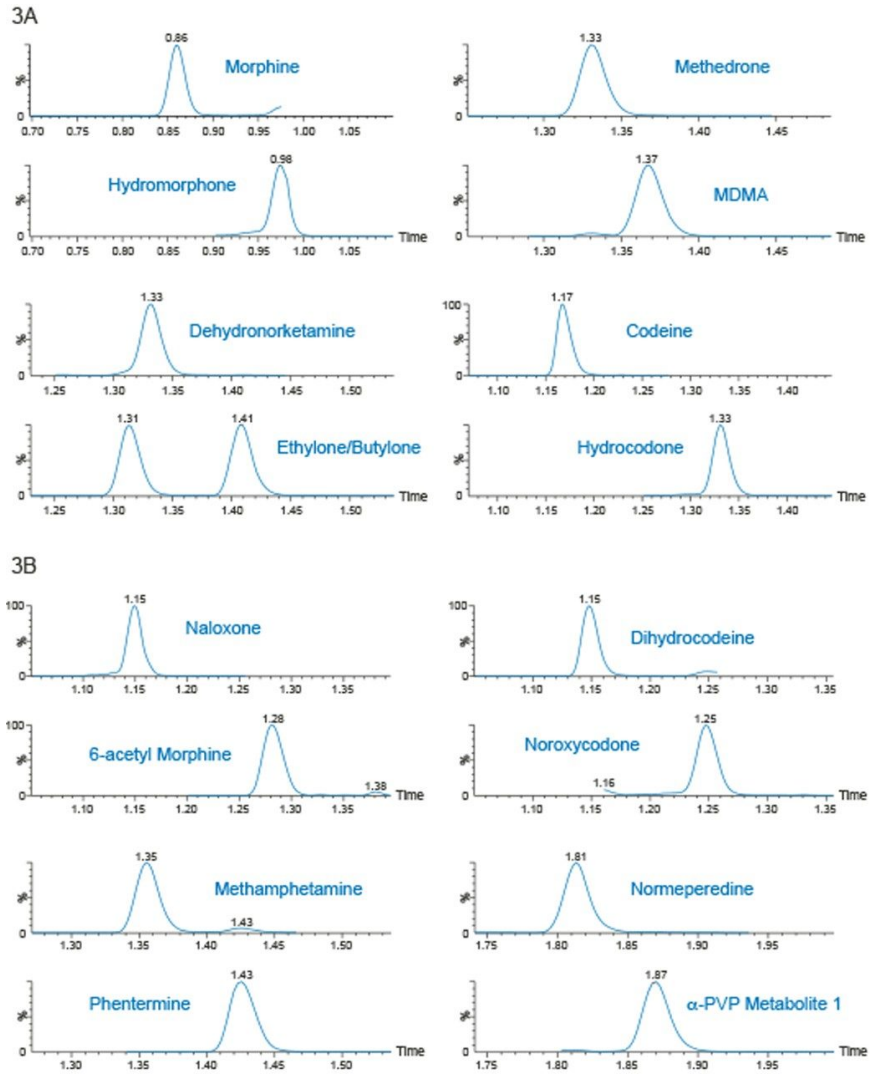

Figure 3A and 3B. Selected chromatography of compounds with the potential to interfere with each other. In each case, compounds are either baseline separated or else did not contain any product ions that caused interference. Column: ACQUITY UPLC BEH C_{18} , 1.7 µm, 2.1 x 100 mm.

Recovery and Matrix Effects

The goal of any extraction technique is to achieve efficient and reproducible recovery for all relevant analytes. As in previous work, the wash protocol was modified from the traditional MCX technique to accommodate the benzodiazepines.[2] Figure 4 shows the mean extraction recoveries of the entire panel of compounds from six

different lots of urine. With the exception of meprobamate and norpropoxyphene, all compounds but two (MDMA and EDDP) had recoveries greater that 70%. Extraction efficiencies were also consistent. Coefficients of variation (%CV) were less than 10% for all quantitative compounds. Recovery data for individual batches followed the same pattern. These highly efficient recoveries across different matrix lots demonstrate the robustness of the extraction technique and are important for quantification of these compounds in samples from different sources.

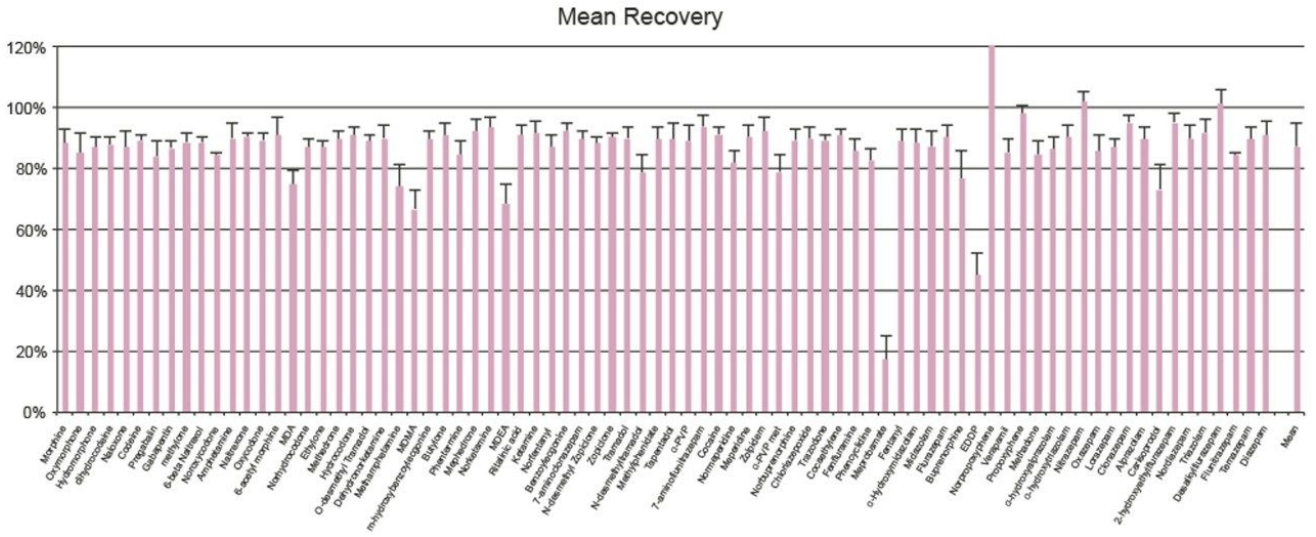

Figure 4. Mean extraction recovery for the compounds in this application. Values represent the mean of six different lots of urine. N = 4 for each lot.

Matrix effects were also evaluated using multiple lots of urine. As with recovery, consistent matrix effects are essential for accurate quantification. Figure 5A shows the aggregate matrix effects from six lots of urine. Ion suppression was observed for the majority of analytes, with up to 60% ion suppression was observed for morphine and hydroxymorphone. However, with only two exceptions (m-OH BZE and α-OH midazolam) standard deviations from matrix effects were less than 20% indicating consistent matrix lot to lot performance. Figure 5B shows the matrix effects when corrected using the internal standards. In this case 75/78 of the corrected matrix effects were less than 20%.

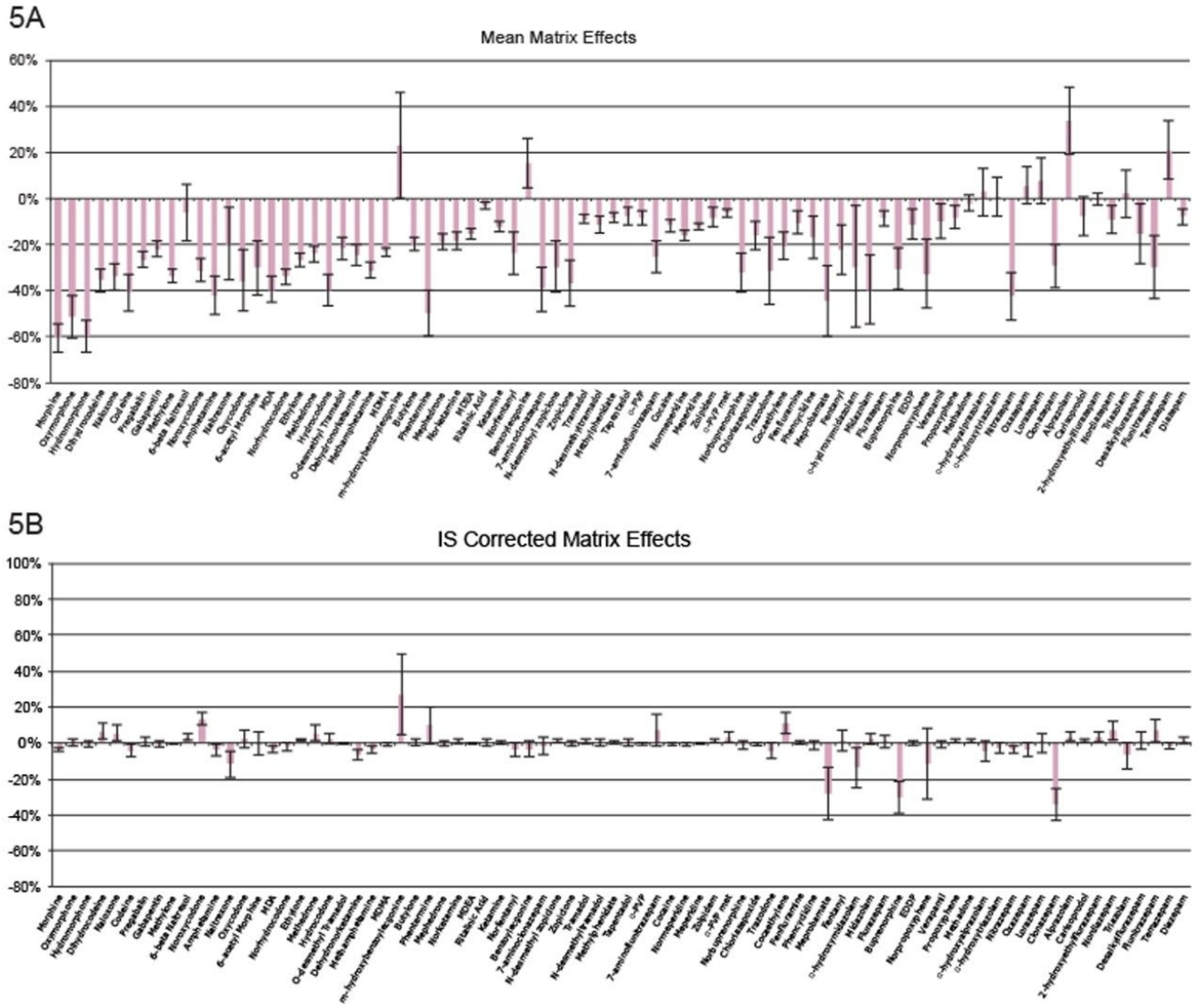

Figure 5A. Mean matrix effects from all compounds from six lots of urine. Bars indicate mean matrix effects and errors indicate standard deviations. Figure 5B. Internal standard corrected matrix effects from six lots of urine. In this graph the matrix effects from figure 5A have been corrected using the internal standards. Of all compounds assessed, only two had standard deviations exceeding 20% and only three of the quantitatively assessed compounds had corrected matrix effects greater than 20%.

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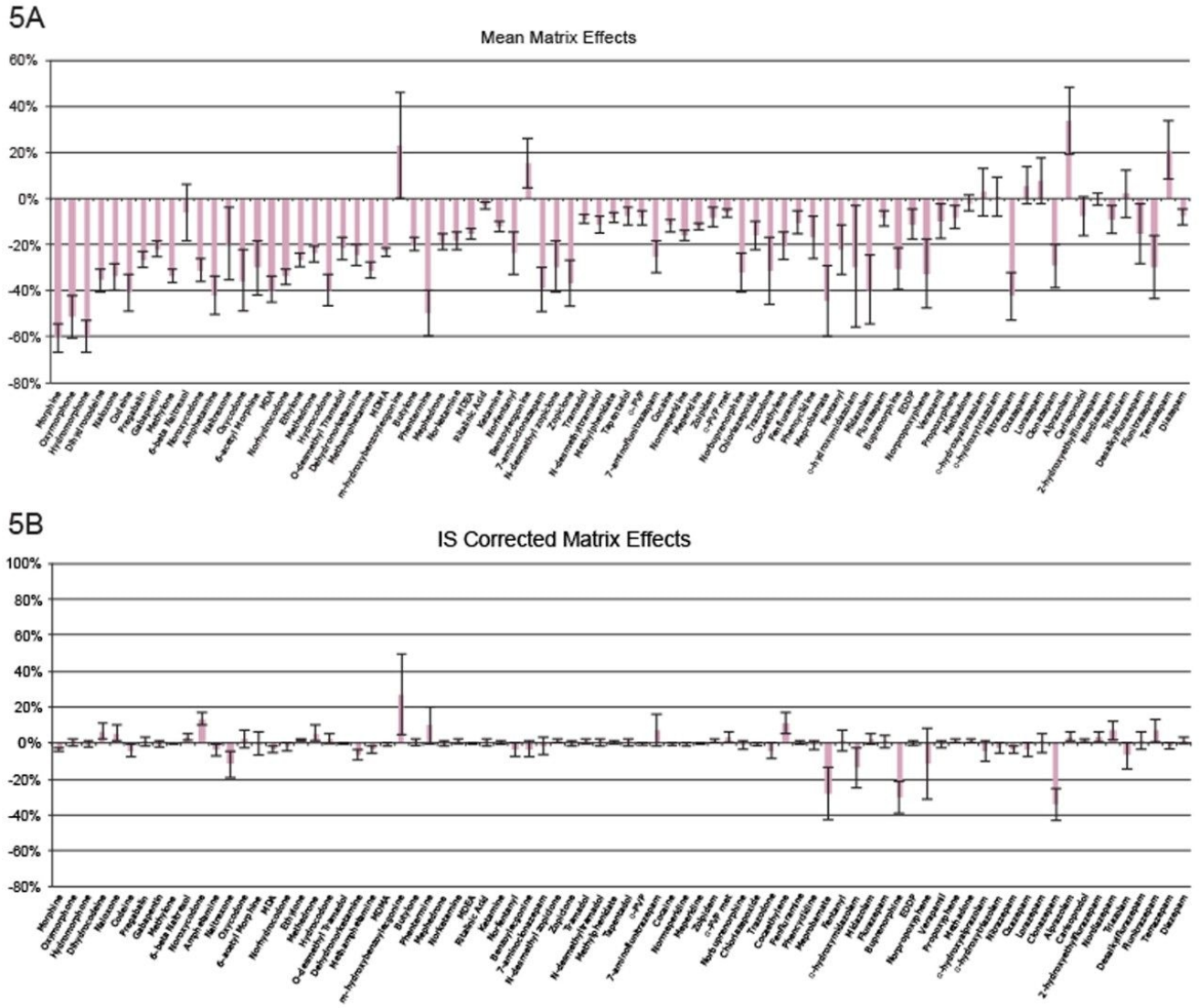

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Quantitative Analysis

Seven point calibration curves were extracted across the concentration ranges shown in Table 1. Calibration

ranges were tailored to reflect the expected concentrations of various compounds. Quality control samples were prepared at 4 concentrations spanning the range of the calibrators, with the lowest at 1.5x the lowest calibrator and the highest at 75% of the highest calibrator. For most compounds, these QC levels were 15, 75, 250, and 750 ng/mL. The compounds at the lower concentrations had QC levels at 3, 15, 50, and 150 ng/mL and the analytes at the higher concentration range had QC levels at 37.5, 187.5, 625, and 1875 ng/mL. Quantitative method validation involved extracting full curves and QC samples over five different days. Calibration curves were extracted in duplicate and six replicates of QC samples were prepared each day. Control limits for individual calibrators and QC samples were ±15% of target values, with the exception of the lowest points, which were required to be within 20%. Precision limits for QC samples were 20% for the lowest QC point and 15% for the other points. Meprobamate and norpropoxyphene were assessed qualitatively only and were not subject to these controls. A summary of the five independent extractions and analyses met all of these criteria and can be seen in Appendix 2. The majority of compounds were within 10% of their target values with %CVs under 10%. For within batch results, all compounds met the accuracy criteria, and the only compound that had precision results greater than 15% was the high amphetamine QC at 18%.

All calibration curves conformed to FDA bioanalytical method validation requirements,[3] which dictate that all calibrators be within 15% of target values except the lowest point, which must be within 20% of its target value and that 75% of calibrators meet this criteria. All compounds met these criteria and all curves had R^2 values of 0.99 or greater.

Limits of quantification were defined as those points in which the signal was 5X greater than that of an extracted matrix blank, signal to noise ratios were >10, and both bias and %CV were both less than 20%. To evaluate this, six replicates of the lowest calibrator were extracted in one of the validation batches. All compounds met these criteria.

On instrument stability was also assessed. A single batch was extracted and analyzed five times over an eight day period. Through four days, all compounds met the quantitative validation criteria described above.

In order to assess accuracy, external quality control samples from UTAK Laboratories were evaluated. These results can be seen in Tables 2A–2D. Analytes assessed using external quality control samples included opioids, benzodiazepines, stimulants, and synthetic cathinones. These results show that 91/98 (93%) of the results were within 20% of the target value. The larger deviations for analytes such as fentanyl, norfentanyl, and buprenorphine could be a result of slight errors in the preparation of the master stock mix, as these compounds were spiked using low volumes (20 µL of stock solution). In addition, 7-aminoclonazepam may have stability

issues in the urine matrix which could account for its low bias. All results had %RSD values <10%.

Name	Mean (ng/mL)	Acc.	%RSD	Mean (ng/mL)	Acc.	%RSD
Morphine	55.0	110.0%	2.4%	404.5	101.1%	0.4%
Oxymorphone	50.0	100.0%	1.9%	405.1	101.3%	1.2%
Hydromorphone	49.8	99.6%	3.6%	405.1	101.3%	1.2%
Codeine	52.0	104.1%	9.8%	411.5	102.9%	3.4%
Oxycodone	48.5	97.0%	8.3%	419.9	105.0%	6.8%
6-AM	5.4	109.0%	7.7%	43.5	108.7%	2.5%
Norhydrocodone	52.0	104.1%	4.6%	384.6	96.1%	4.9%
Hydrocodone	44.0	88.0%	2.9%	336.7	84.2%	3.1%
O-desmethyl-tram	49.1	98.1%	2.0%	375.5	93.9%	2.3%
Norfentanyl	6.1	121.4%	4.1%	45.8	114.4%	2.5%
Tramadol	53.8	107.5%	2.7%	396.1	99.0%	1.3%
Tapentadol	49.3	98.6%	2.7%	388.8	97.2%	1.4%
Normeperidine	53.4	106.8%	2.6%	385.0	96.3%	1.3%
Meperidine	48.7	97.4%	2.8%	372.7	93.2%	1.5%
Norbuprenorphine	55.1	110.2%	5.2%	392.4	98.1%	3.0%
Fentanyl	6.6	131.5%	2.2%	49.4	123.4%	1.0%
Buprenorphine	71.4	142.8%	1.9%	389.5	97.4%	3.0%
EDDP	50.6	101.3%	2.6%	391.5	97.9%	1.1%
Methadone	54.6	109.2%	1.5%	399.3	99.8%	2.0%

Table 2A. Opioid results from external quality control material. Each sample was analyzed in replicates of four. Highlighted cells represent bias values >20%.

Name	Mean (ng/mL)	Acc.	%RSD	Mean (ng/mL)	Acc.	%RSD
Amphetamine	321.7	91.9%	3.3%	678.9	97.0%	0.8%
MDA	319.7	91.3%	1.5%	664.9	95.0%	3.8%
Methamp	331.1	94.6%	1.4%	656.4	93.8%	3.7%
MDMA	313.2	89.5%	0.6%	667.5	95.4%	2.3%
Phentermine	300.7	85.9%	2.0%	638.8	91.3%	5.1%
MDEA	309.4	88.4%	1.8%	593.2	84.7%	3.0%

Table 2B. Amine stimulant results for external quality control samples. Each sample was analyzed in replicates of four.

Name	Mean (ng/mL)	Acc.	%RSD	Mean (ng/mL)	Acc.	%RSD
7-aminoclonazepam	70.1	70.1%	3.6%	317.5	79.4%	1.6%
7-aminoflunitrazepam	85.9	85.9%	2.8%	353.0	88.2%	1.7%
Zolpidem	93.9	93.9%	2.6%	372.1	93.0%	0.6%
Chlordiazepoxide	87.2	87.2%	2.1%	352.3	88.1%	1.6%
a-OH-midazolam	128.3	128.3%	3.0%	471.2	117.8%	3.2%
Midazolam	92.0	92.0%	1.0%	371.0	92.7%	1.5%
Flurazepam	107.1	107.1%	4.1%	402.8	100.7%	3.7%
alpha-OH Alprazolam	96.4	96.4%	4.4%	366.7	91.7%	3.8%
a-OH-triazolam	108.5	108.5%	8.9%	395.8	99.0%	1.7%
Nitrazepam	95.8	95.8%	4.9%	366.7	91.7%	0.7%
Oxazepam	98.7	98.7%	2.7%	398.2	99.5%	0.7%
Lorazepam	102.5	102.5%	4.4%	382.1	95.5%	2.2%
Clonazepam	96.2	96.2%	1.1%	379.5	94.9%	1.5%
Alprazolam	103.0	103.0%	4.5%	464.8	116.2%	4.8%
2-OH Ethyl Flurazepam	100.6	100.6%	4.3%	364.2	91.0%	1.4%
Nordiazepam	99.9	99.9%	2.5%	379.5	94.9%	4.2%
Triazolam	96.8	96.8%	3.1%	382.4	95.6%	2.4%
Desalkylflurazepam	89.2	89.2%	2.2%	393.6	98.4%	2.6%
Flunitrazepam	98.5	98.5%	2.5%	390.5	97.6%	1.7%
Temazepam	100.0	100.0%	1.4%	383.9	96.0%	1.2%
Diazepam	88.7	88.7%	2.4%	379.6	94.9%	2.7%

Table 2C. Benzodiazepine results for external quality control samples. Each sample was analyzed in replicates of four. Highlighted cells represent bias values >20%.

Name	Mean (ng/mL)	Acc.	%RSD
Methylone	16.9	112.5%	2.8%
Ethylone	15.6	103.9%	2.7%
Methedrone	16.7	111.2%	2.2%
Butylone	16.2	107.9%	1.5%
Mephedrone	17.7	117.9%	2.4%
alpha-PVP	16.2	107.7%	2.7%

Table 2D. Synthetic cathinone results for external quality control samples. Each sample was analyzed in replicates of four.

Conclusion

This application note describes a complete method for the solid phase extraction and UPLC-MS/MS analysis of illicit drugs and drugs of abuse for clinical research. A number of advantages are highlighted.

- Sample preparation is optimized to efficiently extract all analytes with a simplified procedure that reduces the number of manual steps. The water wettable nature of the sorbent enables in-well sample pretreatment and direct loading without conditioning and equilibration, eliminating sample transfer and potential transcription errors. The efficient and reproducible extraction is evident in the high recoveries, consistent matrix effects, and accurate and precise quantitative data.
- The use of the ACQUITY UPLC BEH C_{18} Column results in rapid analysis of a large panel while maintaining all required baseline separations for accurate quantification.
- The Waters Xevo TQ-S micro, with features such as StepWave Technology and XDR Detector ensures extremely rapid and accurate quantification of all compounds over wide dynamic ranges. This enables the simultaneous quantification of 6-MAM at 2 ng/mL and methamphetamine at 2500 ng/mL.

This combination of sample preparation, UPLC separation, and MS/MS detection optimizes the workflow and results in a rapid, accurate, and precise method.

References

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2. Danaceau, J.P., E. Chambers: LC-MS/MS analysis of urinary benzodiazepines and Z-drugs via a simplified, mixed-mode sample preparation strategy. Waters Application Note 720005973, 1–9 (2017).
3. Bansal, S., A. DeStefano: Key elements of bioanalytical method validation for small molecules. *The AAPS Journal* 9(1), E109–E114 (2007).

Appendix 1

Name	RT	$M+H^+$	MRM product ions	Cone voltage	Collision energy
Morphine	0.86	286.1	201.1 165.1	25 25	25 35
Oxymorphone	0.91	302.1	227.1 242.1	25 25	25 25
Hydromorphone	0.98	286.1	185.1 157.1	25 25	30 40
Dihydrocodeine	1.15	302.2	199.1 128.1	25 25	30 60
Naloxone	1.15	328.2	253.1 212.1	25 25	28 38
Codeine	1.17	300.2	215.1 165.1	25 25	25 40
Pregabalin	1.20	160.1	125.1 107.1	25 25	12 15
Gabapentin	1.20	172.1	137.1 95.0	25 25	15 20
Methylone	1.21	208.1	160.1 132.1	25 25	15 25
Noroxycodone	1.25	302.1	187.1 227.1	25 25	22 28
6-beta Naltrexol	1.26	344.2	308.2 254.1	10 10	26 30
Naltrexone	1.26	342.2	324.2 270.1	25 25	18 26
Amphetamine	1.28	136.1	119.1 91.1	25 25	15 40
Oxycodone	1.28	316.2	241.1 256.2	25 25	25 25
6-MAM	1.28	328.2	165.1 211.1	25 25	45 30
MDA	1.30	180.1	163.1 105.1	22 22	8 20
Norhydrocodone	1.31	286.1	199.1 128.1	25 25	25 50
Ethylone	1.32	222.2	174.1 146.1	25 25	15 25
O-desmethyl Tramadol	1.32	250.2	58.1	25	15
Methedrone	1.33	194.1	161.1 146.1	25 25	15 30
Hydrocodone	1.34	300.2	199.1 171.1	20 20	28 36
Dehydronorketamine	1.33	222.1	142.1 177.1	25 25	25 15
Methamphetamine	1.36	150.1	119.1 91.1	24 24	9 15
MDMA	1.37	194.1	163.1 105.1	26 26	10 22
m-OH BZE	1.34	306.1	168.1 121.1	25 25	20 25
Butylone	1.41	222.1	174.1 146.1	25 25	15 25
Phentermine	1.43	150.1	133.1 91.1	24 24	9 15
Mephedrone	1.47	178.1	145.1 91.1	25 25	15 30
Norketamine	1.47	224.1	125.0 179.1	25 25	20 15
MDEA	1.48	208.1	163.1 105.1	26 26	10 24
Ritalinic Acid	1.48	220.1	84.0 56.0	25 25	40 40
Ketamine	1.52	238.1	125.0 179.1	25 25	25 15
Norfentanyl	1.54	233.2	84.1 177.1	25 25	15 15

*MS Parameters for all analytes. *Chlorine isotopes were used for the precursor icons for Clonazepam and Lorazepam.*

Name	RT	M+H+	MRM product ions	Cone voltage	Collision energy
BZE	1.52	290.1	168.1 105	36 36	18 32
7-aminoclonazepam	1.51	286.1	121.1 222.1	25 25	30 26
N-desmethyl Zopiclone	1.58	375.1	245.0 331.0	8 8	12 8
Zopiclone	1.61	389.1	245.0 112.0	6 6	14 58
Tramadol	1.68	264.2	58.1	25	15
N-desmethyl Tramadol	1.69	250.2	44.0 232.2	25 25	10 7
Methylphenidate	1.70	234.2	84.1 91.1	25 25	15 40
Tapentadol	1.71	222.2	121.1 107.1	25 25	20 25
alpha-PVP	1.77	232.2	91.1 126.1	25 25	20 25
7-aminoflunitrazepam	1.69	284.1	135.1 227.1	34 34	26 22
Cocaine	1.81	304.2	182.2 82.1	25 25	34 20
Normeperidine	1.82	234.1	160.1 131	25 25	15 28
Meperidine	1.83	248.2	174.1 220.2	25 25	20 20
Zolpidem	1.85	308.2	235.1 92.1	34 34	32 52
alpha-PVP Metabolite 1	1.88	234.2	117.1 173.1	25 25	25 20
Norbuprenorphine	1.90	414.3	101.3 83.3	20 20	48 48
Chlordiazepoxide	1.93	300.1	227.0 283.1	34 34	20 12
Trazodone	1.99	372.2	176.1 148.1	25 25	20 35
Cocaethylene	2.01	318.2	196.1 105.1	42 42	20 38
Fenfluramine	2.03	232.1	159.0 109.0	25 25	20 40
PCP	2.09	244.2	86.1 159.1	25 25	12 12
Meprobamate	1.96	219.1	158.1 96.9	25 25	5 10
Fentanyl	2.15	337.2	188.1 105.1	25 25	22 35
alpha-OH Midazolam	2.13	342.1	168.1 203.1	20 20	40 24
Midazolam	2.17	326.1	291.1 223.1	16 16	24 36
Flurazepam	2.23	388.2	315.1 100.1	25 25	26 28
Buprenorphine	2.27	468.3	55.1 101.3	25 25	50 40
EDDP	2.29	278.2	234.1 249.2	25 25	30 25
Norprpoxyphene	2.51	326.2	252.2 118.0	10 10	5 5
Verapamil	2.52	455.3	165.1 303.2	25 25	25 25
Propoxyphene	2.56	340.2	266.2 143.1	25 25	7 25
Methadone	2.60	310.2	265.2 105.0	25 25	15 25
alpha-OH Alprazolam	2.51	325.1	297.1 243.1	25 25	25 30
alpha-OH Triazolam	2.51	359.1	176.1 141.0	28 28	24 38
Nitrazepam	2.52	282.1	236.1 180.1	25 25	20 36

*MS Parameters for all analytes. *Chlorine isotopes were used for the precursor icons for Clonazepam and*

Lorazepam.

Name	RT	M+H+	MRM product ions	Cone voltage	Collision energy
Oxazepam	2.59	289.1*	243.1 104.1	25 25	20 30
Clonazepam	2.65	316.0	270.1 241.1	25 25	25 35
Lorazepam	2.66	323.0*	277 229.1	25 25	20 30
Carisoprodol	2.67	261.2	176.1 158.1	25 25	8 8
Alprazolam	2.68	309.1	205.1 281.1	25 25	40 26
2-OH Ethyl Flurazepam	2.68	333.1	109.0 194.0	25 25	25 20
Nordiazepam	2.68	271.1	140.0 165.0	30 30	30 28
Triazolam	2.73	343.1	308.1 239.1	28 28	24 38
Desalkylflurazepam	2.78	289.1	140.0 226.1	25 25	30 25
Flunitrazepam	2.77	314.1	268.1 239.1	25 25	25 30
Temazepam	2.87	301.1	255.1 177.1	25 25	20 46
Diazepam	3.05	285.1	154.0 193.1	25 25	26 30

*MS Parameters for all analytes. *Chlorine isotopes were used for the precursor icons for Clonazepam and Lorazepam.*

Appendix 2

	QC 15			QC 75			QC 250			QC 750		
Compound	Mean	% Dev	%CV	Mean	% Dev	%CV	Mean	% Dev	%CV	Mean	% Dev	%CV
Morphine	94.3%	-5.7%	3.2%	99.4%	-0.6%	3.8%	99.0%	-1.0%	5.2%	100.7%	0.7%	2.5%
Oxymorphone	94.6%	-5.4%	3.2%	101.2%	1.2%	2.7%	101.4%	1.4%	2.5%	100.3%	0.3%	2.8%
Hydromorphone	92.3%	-7.7%	6.3%	101.5%	1.5%	3.9%	100.9%	0.9%	3.0%	100.8%	0.8%	2.2%
Dihydrocodeine	96.6%	-3.4%	2.5%	101.3%	1.3%	0.7%	98.4%	-1.6%	1.0%	97.8%	-2.2%	3.8%
Naloxone	92.5%	-7.5%	8.5%	96.5%	-3.5%	1.9%	97.3%	-2.7%	5.6%	100.4%	0.4%	2.5%
Codeine	96.9%	-3.1%	2.1%	101.2%	1.2%	3.5%	100.2%	0.2%	3.3%	101.1%	1.1%	2.7%
Pregabalin	92.9%	-7.1%	5.8%	101.9%	1.9%	3.0%	101.7%	1.7%	2.9%	100.3%	0.3%	1.8%
Gabapentin	93.9%	-6.1%	6.8%	101.4%	1.4%	0.7%	101.2%	1.2%	1.5%	99.3%	-0.7%	2.4%
Methylone	92.8%	-7.2%	3.0%	103.1%	3.1%	1.6%	102.6%	2.6%	2.9%	100.6%	0.6%	2.3%
6-beta-Naltrexol	94.2%	-5.8%	3.7%	100.5%	0.5%	4.1%	100.4%	0.4%	4.7%	102.2%	2.2%	6.4%
Noroxycodone	95.0%	-5.0%	4.1%	102.8%	2.8%	3.8%	101.5%	1.5%	5.4%	98.6%	-1.4%	3.7%
Amphetamine	91.5%	-8.5%	5.5%	103.8%	3.8%	3.8%	97.9%	-2.1%	3.5%	97.3%	-2.7%	4.7%
Naltrexone	100.6%	0.6%	9.7%	97.0%	-3.0%	6.7%	98.4%	-1.6%	9.7%	104.1%	4.1%	8.0%
Oxycodone	96.6%	-3.4%	2.4%	99.3%	-0.7%	4.6%	98.2%	-1.8%	5.0%	98.6%	-1.4%	4.6%
6-AM	90.4%	-9.6%	15.0%	98.3%	-1.7%	2.9%	100.7%	0.7%	5.5%	98.7%	-1.3%	4.1%
Norhydrocodone	95.4%	-4.6%	4.5%	101.1%	1.1%	3.6%	101.0%	1.0%	5.3%	101.4%	1.4%	4.3%
MDA	95.5%	-4.5%	4.1%	102.9%	2.9%	2.5%	100.0%	0.0%	2.8%	97.8%	-2.2%	0.9%
Ethylone	95.0%	-5.0%	4.1%	99.0%	-1.0%	2.4%	99.0%	-1.0%	3.0%	100.3%	0.3%	1.7%
Methedrone	97.4%	-2.6%	3.5%	103.8%	3.8%	2.4%	100.2%	0.2%	1.3%	98.7%	-1.3%	3.3%
Hydrocodone	93.7%	-6.3%	4.0%	102.0%	2.0%	2.4%	99.0%	-1.0%	3.4%	101.4%	1.4%	3.7%
O-Dm-Tramadol	95.0%	-5.0%	3.1%	99.8%	-0.2%	3.0%	99.0%	-1.0%	2.7%	100.1%	0.1%	3.0%
Dehydronorketamine	90.4%	-9.6%	5.2%	100.7%	0.7%	3.1%	101.9%	1.9%	2.0%	99.9%	-0.1%	1.8%
Methamphetamine	92.2%	-7.8%	3.5%	102.7%	2.7%	1.7%	100.2%	0.2%	5.8%	98.7%	-1.3%	1.8%
MDMA	95.4%	-4.6%	2.8%	100.0%	0.0%	2.2%	100.1%	0.1%	3.2%	100.5%	0.5%	2.5%
m-OH BZE	91.8%	-8.2%	4.2%	104.3%	4.3%	2.0%	98.8%	-1.2%	2.2%	98.9%	-1.1%	2.8%
Butylone	94.3%	-5.7%	1.0%	102.0%	2.0%	3.8%	102.1%	2.1%	4.2%	101.6%	1.6%	3.4%
Phentermine	99.5%	-0.5%	7.5%	96.9%	-3.1%	4.9%	93.1%	-6.9%	2.4%	99.5%	-0.5%	1.3%
Mephedrone	94.5%	-5.5%	5.1%	100.4%	0.4%	3.6%	98.4%	-1.6%	3.0%	98.8%	-1.2%	2.0%
Norketamine	93.6%	-6.4%	7.5%	98.6%	-1.4%	2.6%	98.1%	-1.9%	1.7%	100.2%	0.2%	2.8%
MDEA	95.3%	-4.7%	2.0%	100.1%	0.1%	2.2%	99.7%	-0.3%	2.7%	100.6%	0.6%	1.9%
Ritalinic Acid	95.2%	-4.8%	4.2%	101.3%	1.3%	4.4%	99.6%	-0.4%	2.8%	98.8%	-1.2%	1.2%
Ketamine	93.1%	-6.9%	3.4%	100.5%	0.5%	2.2%	100.9%	0.9%	3.1%	100.6%	0.6%	1.0%
Norfentanyl	95.5%	-4.5%	5.6%	100.7%	0.7%	4.0%	100.3%	0.3%	2.5%	101.0%	1.0%	2.6%
BZE	94.1%	-5.9%	5.0%	103.1%	3.1%	1.9%	98.2%	-1.8%	2.6%	99.1%	-0.9%	1.7%
7-aminoclonazepam	93.0%	-7.0%	3.7%	99.8%	-0.2%	3.9%	100.2%	0.2%	4.4%	99.9%	-0.1%	3.4%
N-Dm Zopiclone	93.6%	-6.4%	6.6%	100.8%	0.8%	1.8%	100.5%	0.5%	3.9%	101.3%	1.3%	1.5%
Zopiclone	96.1%	-3.9%	4.9%	99.0%	-1.0%	1.9%	99.3%	-0.7%	2.4%	100.8%	0.8%	2.2%
Tramadol	94.9%	-5.1%	2.5%	102.1%	2.1%	4.2%	99.3%	-0.7%	2.8%	99.0%	-1.0%	2.8%
N-Dm Tramadol	91.9%	-8.1%	2.2%	102.6%	2.6%	5.4%	103.5%	3.5%	4.8%	101.4%	1.4%	2.4%
Methylphenidate	95.6%	-4.4%	1.7%	105.7%	5.7%	2.7%	100.0%	0.0%	3.4%	94.2%	-5.8%	4.7%
Tapentadol	94.9%	-5.1%	7.7%	98.2%	-1.8%	7.0%	99.5%	-0.5%	4.1%	101.2%	1.2%	1.8%
alpha-PVP	92.1%	-7.9%	4.2%	101.7%	1.7%	6.4%	102.5%	2.5%	6.4%	100.4%	0.4%	1.5%
7-aminoflunitrazepam	90.2%	-9.8%	9.1%	103.9%	3.9%	5.9%	97.2%	-2.8%	6.0%	99.1%	-0.9%	6.4%
Cocaine	95.3%	-4.7%	1.5%	100.6%	0.6%	4.4%	100.7%	0.7%	5.3%	101.6%	1.6%	2.7%
Normeperidine	96.2%	-3.8%	4.3%	102.3%	2.3%	4.9%	102.5%	2.5%	6.1%	102.0%	2.0%	3.3%
Meperidine	96.0%	-4.0%	2.8%	101.0%	1.0%	4.3%	101.5%	1.5%	4.1%	101.9%	1.9%	3.0%
Zolpidem	97.1%	-2.9%	5.3%	101.9%	1.9%	2.2%	100.0%	0.0%	2.4%	100.7%	0.7%	1.0%
alpha-PVP Met	98.5%	-1.5%	4.2%	98.5%	-1.5%	4.6%	97.4%	-2.6%	10.3%	101.7%	1.7%	2.6%
Norbuprenorphine	104.2%	4.2%	10.5%	98.9%	-1.1%	7.3%	99.9%	-0.1%	7.1%	103.7%	3.7%	4.3%
Chlordiazepoxide	97.5%	-2.5%	5.6%	101.5%	1.5%	4.8%	100.7%	0.7%	3.7%	101.6%	1.6%	2.2%
Trazodone	100.3%	0.3%	5.4%	102.5%	2.5%	5.7%	102.6%	2.6%	5.8%	103.7%	3.7%	4.3%
Cocaethylene	94.2%	-5.8%	1.4%	102.3%	2.3%	4.2%	102.7%	2.7%	5.4%	101.3%	1.3%	3.3%
Fenfluramine	94.7%	-5.3%	3.0%	101.2%	1.2%	5.1%	101.8%	1.8%	5.2%	101.7%	1.7%	1.3%
PCP	96.1%	-3.9%	2.8%	101.8%	1.8%	2.3%	100.2%	0.2%	2.2%	99.3%	-0.7%	1.7%
Meprobamate	—	—	—	—	—	—	—	—	—	—	—	—
Fentanyl	99.8%	-0.2%	5.1%	100.5%	0.5%	2.7%	100.2%	0.2%	3.4%	101.9%	1.9%	2.6%

Between run quantitative summary (N = 5 days).

	QC 15			QC 75			QC 250			QC 750		
Compound	Mean	% Dev	%CV	Mean	% Dev	%CV	Mean	% Dev	%CV	Mean	% Dev	%CV
α-OH-midazolam	102.1%	2.1%	5.5%	105.2%	5.2%	2.0%	101.8%	1.8%	2.4%	100.5%	0.5%	2.9%
Midazolam	99.6%	-0.4%	7.1%	103.1%	3.1%	4.6%	102.1%	2.1%	4.3%	103.6%	3.6%	3.1%
Flurazepam	97.6%	-2.4%	4.5%	103.6%	3.6%	4.2%	100.6%	0.6%	4.6%	96.9%	-3.1%	2.5%
Buprenorphine	99.1%	-0.9%	10.3%	105.6%	5.6%	8.0%	103.3%	3.3%	11.3%	105.8%	5.8%	6.2%
EDDP	99.5%	-0.5%	4.4%	99.0%	-1.0%	2.4%	98.6%	-1.4%	3.6%	102.2%	2.2%	3.1%
Norprpoxyphene	—	—	—	—	—	—	—	—	—	—	—	—
Verapamil	109.6%	9.6%	12.7%	109.2%	9.2%	7.0%	107.6%	7.6%	6.6%	106.2%	6.2%	4.7%
Propoxyphene	103.8%	3.8%	8.2%	101.9%	1.9%	5.2%	103.0%	3.0%	4.5%	104.8%	4.8%	2.4%
Methadone	106.0%	6.0%	11.0%	104.1%	4.1%	6.5%	104.1%	4.1%	6.3%	105.3%	5.3%	2.7%
alpha-OH Alprazolam	98.6%	-1.4%	8.7%	101.9%	1.9%	2.7%	99.8%	-0.2%	3.1%	98.3%	-1.7%	1.7%
α-OH-triazolam	95.5%	-4.5%	4.6%	101.1%	1.1%	3.8%	100.3%	0.3%	3.4%	100.1%	0.1%	2.8%
Nitrazepam	94.3%	-5.7%	4.2%	100.5%	0.5%	2.9%	99.0%	-1.0%	2.7%	99.4%	-0.6%	2.2%
Oxazepam	100.0%	0.0%	5.6%	100.5%	0.5%	3.8%	99.8%	-0.2%	1.8%	101.5%	1.5%	2.6%
Lorazepam	94.3%	-5.7%	3.6%	100.2%	0.2%	3.8%	101.0%	1.0%	3.2%	101.4%	1.4%	2.9%
Clonazepam	95.5%	-4.5%	3.4%	102.9%	2.9%	3.1%	103.5%	3.5%	3.5%	101.8%	1.8%	3.1%
Alprazolam	96.7%	-3.3%	3.4%	102.3%	2.3%	2.7%	98.6%	-1.4%	4.5%	100.5%	0.5%	5.5%
Carisoprodol	95.2%	-4.8%	6.3%	100.8%	0.8%	2.6%	99.7%	-0.3%	3.0%	100.6%	0.6%	2.1%
2-OH Ethyl Flurazepam	96.4%	-3.6%	2.4%	103.9%	3.9%	1.9%	99.9%	-0.1%	4.0%	100.3%	0.3%	3.1%
Nordiazepam	99.8%	-0.2%	4.3%	111.7%	11.7%	3.5%	101.5%	1.5%	1.3%	101.1%	1.1%	1.9%
Triazolam	97.8%	-2.2%	6.1%	103.0%	3.0%	4.5%	99.6%	-0.4%	3.5%	100.1%	0.1%	1.2%
Desalkylflurazepam	97.3%	-2.7%	4.9%	104.7%	4.7%	1.7%	100.3%	0.3%	3.0%	102.6%	2.6%	2.3%
Flunitrazepam	96.3%	-3.7%	4.6%	101.6%	1.6%	1.8%	100.6%	0.6%	2.2%	99.6%	-0.4%	2.5%
Temazepam	96.0%	-4.0%	3.9%	103.1%	3.1%	2.4%	99.7%	-0.3%	3.3%	100.0%	0.0%	1.8%
Diazepam	97.2%	-2.8%	3.7%	108.2%	8.2%	1.3%	99.8%	-0.2%	2.7%	100.5%	0.5%	2.9%

Between run quantitative summary (N = 5 days).

Featured Products

Xevo TQ-S micro Mass Spectrometer <https://www.waters.com/134798856>

ACQUITY UPLC I-Class System (FTN) <https://www.waters.com/134613317>

MassLynx Software <https://www.waters.com/513164>

TargetLynx Application Manager <https://www.waters.com/513791>

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