[APPLICATION NOTEBOOK]

Forensic Toxicology Application Notebook

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[TECHNOLOGY BRIEF]

No Compromise! Improved Sensitivity for Negatively-Ionizing Substances

Rob Lee[1], Scott Freeto[2], Mike Wakefield[3], and Michelle Wood[1]
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GOAL

To assess the impact of alternate mobile phases on the sensitivity for a series of negatively-ionizing substances.

BACKGROUND

In 2014, Waters released the first version of the Forensic Toxicology Screening Application Solution with UNIFI,®[1] which comprised acquisition of MS^E data using a time-of-flight mass spectrometer operated in electrospray positive ionization mode (ESI+). Data were subsequently compared with a comprehensive library containing more than 1,000 toxicologically-relevant substances.[2,3]

Since this time, on-going efforts have been underway to further improve the forensic solution by continuing to expand the library content, to include novel psychoactive substances and their metabolites, but also to include substances that may preferentially ionize in negative mode (ESI-), such as the barbiturates, cannabinoids, diuretics, and the non-steroidal anti-inflammatory drugs (NSAIDs).[4,5] For convenience, some screening approaches employ the same chromatographic conditions for both positive and negative ionization modes however, the impact of this approach should be evaluated, particularly with regards to the effect on sensitivity.

Altered mobile phases provide improved sensitivity when using the Waters Forensic Toxicology Screening Application Solution with UNIFI in negative ionization mode.

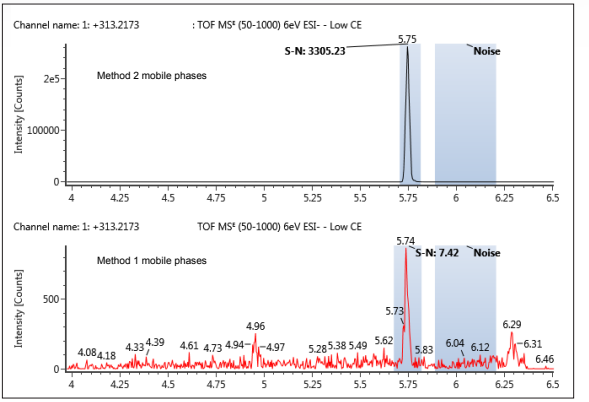

Figure 1. Improvement in both 3D peak response and signal-to-noise ratio for 2500 ng/mL injection of THC standard when using the altered mobile phases (upper chromatogram) in comparison with the original mobile phases (lower chromatogram).

Consequently, the aim of this study was to compare the sensitivity obtained for a series of negatively-ionizing substances when analyzed using the mobile phases that are usually employed in ES+ mode (Method 1), with some alternative chromatographic conditions, that are based on a previously-reported method for barbiturates (Method 2).[6]

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EXPERIMENTAL

Sample preparation

Individual standards were prepared at 1 mg/mL in methanol, then diluted in 10% acetonitrile in water for injection. The final concentrations ranged from 20 ng/mL to 2,500 ng/mL.

LC conditions

LC system:	ACQUITY UPLC® I-Class (FTN)
Column:	ACQUITY UPLC HSS C_{18}, 2.1 x 150 mm, 1.8 µm
Vials:	Waters Maximum Recovery Vials
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A method 1:	5 mM ammonium formate pH 3.0
Mobile phase A method 2:	Water containing 0.001% formic acid
Mobile phase B method 1:	Acetonitrile containing 0.1% formic acid
Mobile phase B method 2:	Acetonitrile containing 0.001% formic acid
Gradient:	Isocratic at 87% A for 0.5 min then to 5% A at 4.5 min, hold for 1 min before switching to 87% A
Run time:	7.5 min

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI-
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M-H]^-$ *m/z* = 554.2620
Acquisition range:	*m/z* 50-1000
Scan time:	0.1 s
Capillary voltage:	1.5 KV
Cone voltage:	20 V
Collision energy:	Function 1: 6 eV Function 2: Ramped 10 to 40 eV

RESULTS

Sixty-two compounds, including barbiturates, cannabinoids, diuretics, NSAIDs, and steroids, were analyzed in triplicate. The retention times for a selection of the compounds, under both sets of mobile phases evaluated, are listed in Table 1, together with the observed increase in 3-dimensional (3D) peak response and signal-to-noise ratio, with the alternative mobile phases.

Sixty of the sixty-two compounds evaluated showed an increase in 3D peak response when using the alternate mobile phases, with 75% of the compounds tested showing a greater than two-fold increase, and only two compounds showing a reduced 3D peak response. The greatest increase in 3D response was for THC, which is illustrated in Figure 1, and showed an increase of more than 50-fold alongside a dramatic increase in signal-to-noise ratio.

A small number of compounds were only identified, at the concentrations investigated, when using the altered mobile phases. The signal-to-noise ratio comparison between the two methods, for a selection of compounds, is highlighted in Table 1, and complements the increase in 3D response. Only small differences in retention time were observed when switching between the two mobile phases.

Analyte	Drug class	Injection conc.	Degree of improvement (method 2/1)		Retention time (min)	
		ng/mL	3D peak response	Signal-to-noise ratio	Method 1	Method 2
Phenobarbital	barbiturate	100	15	17	2.9	2.9
Secobarbital	barbiturate	100	11	11	3.5	3.5
Carboxy-THC	cannabinoid	20	3	3	5.0	5.0
THC	cannabinoid	2500	67	445	5.7	5.8
Amiloride	diuretic	250	3	3	1.1	1.0
Furosemide	diuretic	250	7	3	3.1	3.1
Naproxen	NSAID	400	23	20	3.8	3.8
Ibuprofen	NSAID	1000	52	73	4.4	4.5
Hydrocortisone	steroid	400	6	5	3.0	3.0
Triamcinolone	steroid	400	5	3	2.6	2.6

Table 1. Improvement in 3D peak response and sensitivity for a selection of analytes, using the two sets of mobile phases along with their retention times.

SUMMARY

While it is certainly possible to use the same mobile phases for screening analysis in both positive and negative mode, this simple study clearly demonstrates that these compromises in chromatographic conditions can influence analytical performance and sensitivity, and in some cases, this can be significant. In a toxicological screening this can yield false negative results, particularly for the cannabinoids which are the most-commonly encountered illicit drug substances. For this reason the expanded Waters Forensic Toxicology Screening Application Solution with UNIFI includes a fully-optimized chromatographic method for more efficient, more accurate toxicological screening of negatively-ionizing substances.

References

1. Forensic Toxicology Screening Application Solution with UNIFI. Waters Brochure (P/N 720004830EN).
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5. Lee R and Wood M. Screening for Cannabinoids using the Waters Forensic Toxicology Application Solution with UNIFI. Waters Application Note 2015 (P/N 720005413EN).
6. Lee R, et al. Quantitative Analysis of Barbiturates in Urine Using UPLC/MS/MS. Waters Application Note 2014 (P/N 720004466EN).

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Analysis of Plant Alkaloids Through Accurate Mass Screening and Discovery

Jeff Goshawk and Michelle Wood
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

Analyze plant alkaloids using the Forensic Toxicology Application Screening Solution with UNIFI[1] to demonstrate the simplicity of library creation and expansion. This application note also showcases the power of the latest suite of discovery tools within the UNIFI Scientific Information System v1.8.

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Forensic Toxicology Application Solution with UNIFI®

ACQUITY UPLC® I-Class System (FTN)

ACQUITY UPLC HSS Column

Xevo® G2-S QTof

Maximum Recovery Vials

KEY WORDS

Plant alkaloids, forensic toxicology, UPLC-QTof-MS^E, UNIFI, identification, discovery

INTRODUCTION

Over the last decade there has been a significant increase in the popularity of time-of-flight mass spectrometry (Tof-MS) for multi-residue analysis. Accurate mass imparts high specificity for substance identification and, together with the isotopic data, can provide the user with the opportunity to propose likely elemental compositions. The proposal of elemental formulae is often the starting point for a complex multi-stage process to elucidate chemical structures.

For screening, accurate mass instrumentation presents a significant, and key, advantage over its nominal mass counterpart, i.e., an ability to implement screening methodologies without the requirement of reference material. In this particular workflow the theoretical (expected) exact mass can be determined empirically from the elemental formula. In a toxicological setting this can provide a valuable means with which the analyst may 'prospectively' target novel psychoactive drugs, or new substances and metabolites where reference material may not yet, be available.

An on-going initiative to expand the UNIFI Toxicology Scientific Library led to the analysis of a series of plant alkaloids. These nitrogen-containing compounds are derived from plants and plant material. They are pharmacologically active and have been used for many centuries for both medicinal and recreational purposes. Consequently, their analysis is of forensic importance. Analysis of these substances provided an opportunity to evaluate the tools within the UNIFI Scientific Information System for both target assignment and structural elucidation.

EXPERIMENTAL

Materials

The following plant alkaloids were obtained from Sigma-Aldrich (Poole, UK) as solid material: amygdalin, berberine chloride, bufalin, coumarin, digitoxin, gitoxin, lanatocide C, neriifolin, and α-solanine.

Sample preparation

Individual stock solutions of the plant alkaloids were initially prepared, by dilution with methanol, to a concentration of 10 µg/mL; these solutions were stored at -20 °C until further use. Prior to Tof-MS analysis, the stock solutions were further diluted with mobile phase A to yield samples for injection at a concentration of 1 µg/mL.

LC-MS method conditions

ACQUITY UPLC conditions

System:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY HSS C_{18}, 2.1 x 150 mm, 1.8 µm
Run time:	15 min
Vials:	Waters Maximum Recovery Vials
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM aqueous ammonium formate, adjusted to pH 3.0
Mobile phase B:	Acetonitrile containing 0.1% formic acid

Gradient:

Time	%A	%B
0.00	87	13
0.50	87	13
10.00	50	50
10.75	5	95
12.25	5	95
12.50	87	13
15.00	87	13

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI+
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M+H]^+ = m/z$ 556.2766
Acquisition range	m/z 50–1000
Scan time:	0.1 s
Capillary voltage:	0.8 kV
Cone voltage:	25 V
Collision energy:	Function 1: 6 eV Function 2: Ramped 10 to 40 eV

Data management

Forensic Toxicology Screening Application Solution with UNIFI v1.8

RESULTS AND DISCUSSION

Prior to analysis, a new UNIFI Scientific Library was created specifically for plant alkaloids, by simply entering the names of the nine alkaloids. A MOL file describing the structure of each substance was added to each entry in the library (Figure 1). Individual solutions of the plant alkaloids were injected and data were acquired using the standard screening conditions supplied with the Forensic Toxicology Screening Application Solution with UNIFI.[1] These data were subsequently processed using the UNIFI Scientific Information System and screened against the new plant alkaloid library.

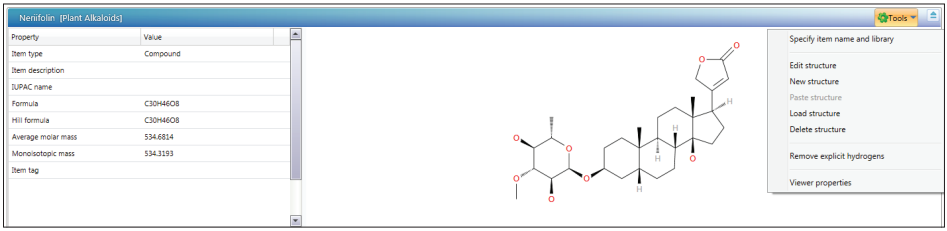


Figure 1. Creating a library entry for neriifolin. Existing MOL file structures can be appended (Load structure) or created by standard chemical drawing packages and subsequently appended (New structure).

Identification through the application of *in-silico* fragmentation techniques

The presence of each plant alkaloid was confirmed through the mass accuracy of the protonated precursor ion in combination with theoretical fragment ions that were automatically generated from the structure of each substance and matched to ions in the high-energy spectrum.

Figure 2 shows the identification of α-solanine as presented in UNIFI. The Component Summary table presents the information related to the identification of this alkaloid and includes; the observed *m/z* value together with the deviation from the expected *m/z* value, the difference between measured and theoretical isotope patterns in terms of both *m/z* and intensity distributions, the observed retention time, the number of theoretical fragment ions found, and the detector counts, which represents the abundance of all the low-energy ions associated with the detected compound.

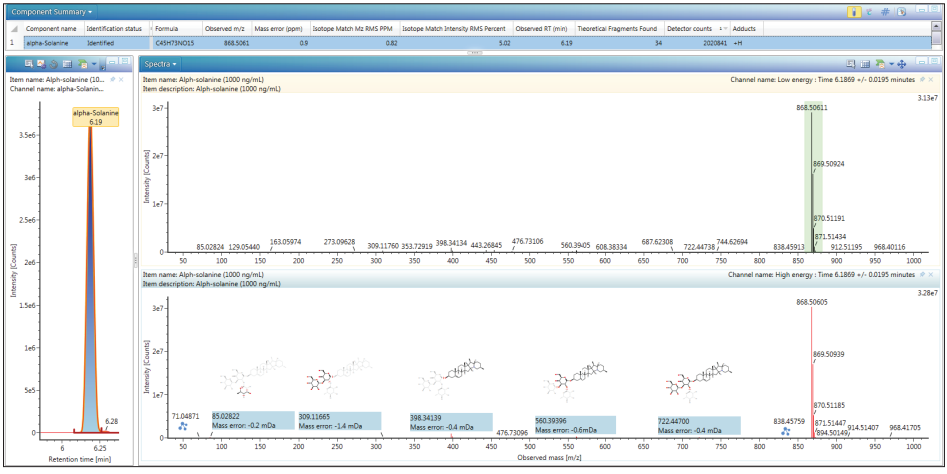
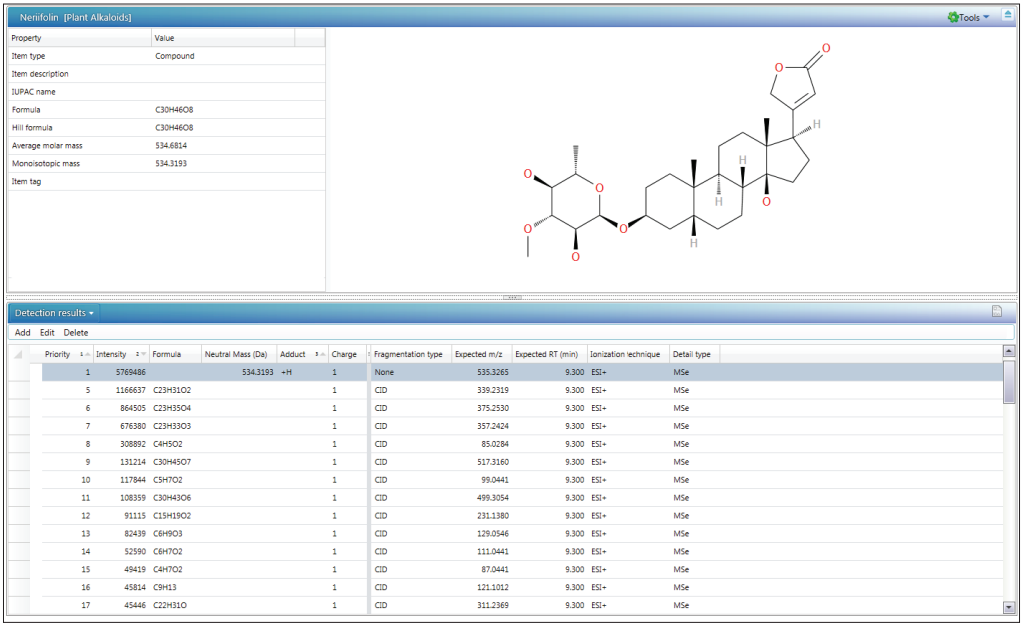


Figure 2. Identification of α-solanine in the UNIFI Scientific Information System.

Updating library entries

All of the alkaloids were identified on the basis of the mass accuracy of the precursor ion and theoretical fragment ions generated during processing. Upon identification, a retention time was associated with each substance. With UNIFI, the library entries can be updated directly from the analysis such that they contain the expected retention time and the expected *m/z* value for each assigned adduct and fragment ion. Following the update, a typical library entry has information similar to that shown for neriifolin in Figure 3. This additional information can be used to target the substance in subsequent analyses.



Priority	Intensity	Formula	Neutral Mass (Da)	Adduct	Charge	Fragmentation type	Expected m/z	Expected RT (min)	Ionization technique	Detail type
1	5769486		534.3193	+H	1	None	535.3265	9.300	ESI+	MSe
5	1166637	C23H31O2			1	CID	339.2319	9.300	ESI+	MSe
6	864505	C23H35O4			1	CID	375.2530	9.300	ESI+	MSe
7	676380	C23H33O3			1	CID	357.2424	9.300	ESI+	MSe
8	308892	C4H5O2			1	CID	85.0284	9.300	ESI+	MSe
9	131214	C30H45O7			1	CID	517.3160	9.300	ESI+	MSe
10	117844	C5H7O2			1	CID	99.0441	9.300	ESI+	MSe
11	108359	C30H43O6			1	CID	499.3054	9.300	ESI+	MSe
12	91115	C15H19O2			1	CID	231.1380	9.300	ESI+	MSe
13	82439	C6H9O3			1	CID	129.0546	9.300	ESI+	MSe
14	52590	C6H7O2			1	CID	111.0441	9.300	ESI+	MSe
15	49419	C4H7O2			1	CID	87.0441	9.300	ESI+	MSe
16	45814	C9H13			1	CID	121.1012	9.300	ESI+	MSe
17	45446	C22H31O			1	CID	311.2369	9.300	ESI+	MSe

Figure 3. Library entry for neriifolin. The lower section of the composite is now populated with expected retention time and the expected m/z values of precursor and fragment ions.

Multiple adducts

Data for gitoxin, one of the other alkaloids investigated in this study, is shown in Figure 4. The low-energy ions assigned to this substance are highlighted in green within the spectrum and correspond to the protonated isotope cluster. The detector counts determined for the protonated isotope cluster of gitoxin is 568. The high-energy spectrum is annotated with sub-structures of gitoxin, as determined automatically by UNIFI and associated to the high-energy spectral peaks as fragment ions.

Figure 4. Identification of gitoxin in the UNIFI Scientific Information System.

Further examination of the low-energy spectrum for this substance revealed that some of the ions may correspond to other adducts of gitoxin. Consequently the data was reprocessed to target the $[NH_4]^+$, $[Na]^+$, and $[K]^+$ adducts in addition to the protonated species. Figure 5 details the isotope clusters in the low-energy data assigned to each adduct following reprocessing. The assignment of the additional adducts to gitoxin has been reflected in the detector counts which has increased from 568, determined from the isotope cluster of the protonated adduct, to 118680. Similar results were obtained for the other substances in this analysis.

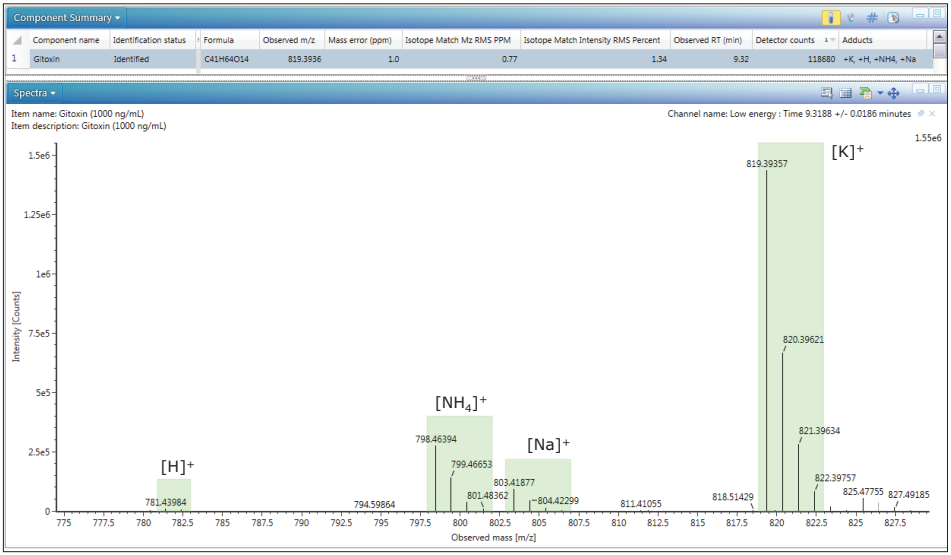


Figure 5. Multiple adduct assignment for gitoxin.

The discovery tool

Another new feature in the UNIFI Scientific Information System v1.8 is the discovery tool, which chains together elemental composition, library searching and fragment match functionality into a single step process making it easier to obtain the identity of unexpected substances within a sample. The parameters used to run the discovery tool are detailed in Figure 6A–D.

The first set of parameters, displayed in Figure 6A, control the maximum number of elemental compositions returned for each component, and the number of library hits returned for each elemental composition. For each component selected, the measured *m/z* value is submitted to the elemental composition application, the parameters of which are displayed in Figure 6B. Each scientific formula returned by the elemental composition application is then automatically submitted to the list of selected libraries. The libraries can either belong to the UNIFI Scientific Library or, if connected to the internet, ChemSpider. The dialog showing the selection of ChemSpider libraries is presented in Figure 6C.

Every hit for each scientific formula that is returned from the library search is then automatically submitted to the fragment match application, provided the library hit has an associated structure in the form of a MOL file.

The fragment match application performs a systematic bond disconnection of each structure, applying the parameters selected through the dialog displayed in Figure 6D, and matches the *m/z* values of theoretical sub-structures to measured high-energy fragment ions. The number of fragment ions matched and the percentage of the intensity of the high-energy spectrum accounted for by those matches are both determined.

For the purposes of illustration, the candidate component identified as amygdalin in the targeted analysis was submitted to the discovery tool. The results, upon running the application with respect to the parameters shown in Figure 6A–D, are presented in Figure 7.

The component submitted to the discovery tool was Candidate Mass *m/z* 458.1649. The results show that one elemental composition, $C_{20}H_{27}NO_{11}$, with an i-FIT™ confidence of 89% was determined for *m/z* 458.1649. This elemental composition, was automatically submitted to the FDA UNII – NLM library within ChemSpider and a hit for amygdalin was returned with a list of synonyms, a structure and the number of citations. The structure was used automatically in conjunction with fragment match and appropriate sub-structures were assigned to the high-energy spectrum associated with Candidate Mass *m/z* 458.1649, as shown in Figure 7. The number of high energy fragments matched by sub-structures and the percentage of the intensity of the high energy spectrum accounted for by those fragment matches are displayed for the library hit.

Access to this information for a range of components, elemental compositions, and library hits enables the analyst to make an informed decision with respect to the identity of unexpected substances in their samples.

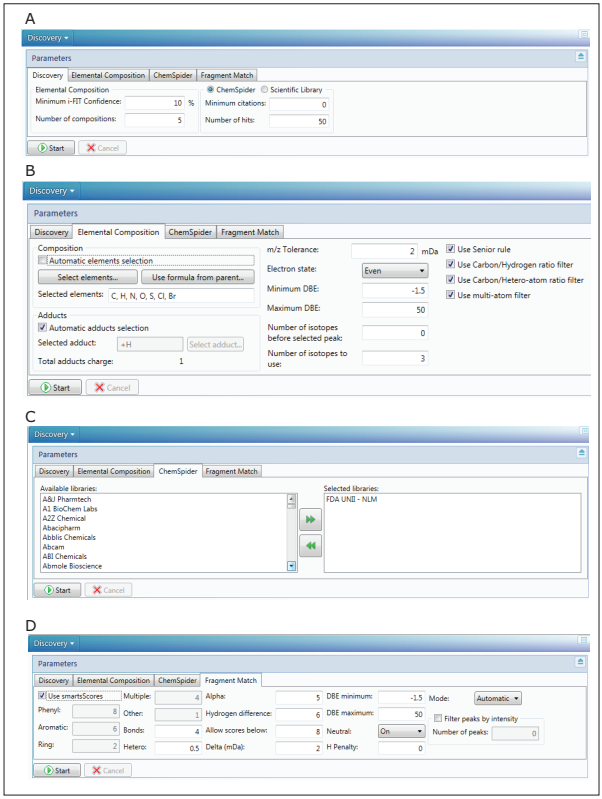


Figure 6. Discovery tool in UNIFI. A) General discovery tool parameters. B) Elemental composition parameters. C) ChemSpider parameters. D) Fragment match parameters.

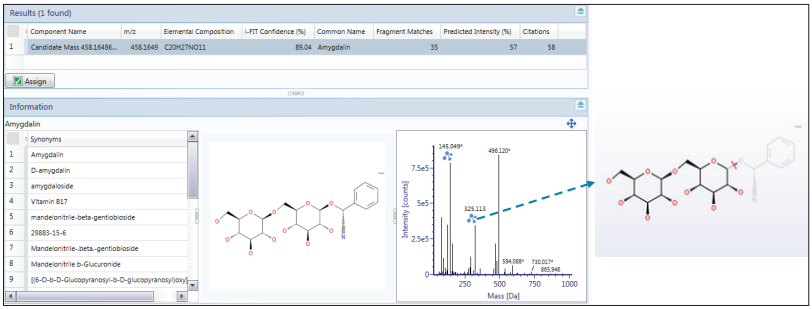


Figure 7. A typical result in the discovery tool.

CONCLUSIONS

In this study, the Forensic Toxicology Application Screening Solution with UNIFI[1] was applied to a selection of plant alkaloids. The ease by which the scientific library items can be created and updated has been clearly demonstrated. The UNIFI Scientific Information System v1.8 was used to process the MS^E data and for these plant alkaloids multiple adducts were detected. The fragment match functionality was also able to assign sub-structures to high-energy ions. Additionally, the new discovery tool has been shown to enhance the elucidation of unknown components.

Reference

1. Forensic Toxicology Screening Application Solution. Waters Brochure (P/N 720004830EN).

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Screening for Cannabinoids Using the Waters Forensic Toxicology Application Solution with UNIFI

Rob Lee and Michelle Wood
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

Expanded Forensic Toxicology Application Solution with UNIFI enabling the detection and quantitation of negative ionizing cannabinoids in urine.

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System (FTN)

Xevo® G2-S QTof Mass Spectrometer

ACQUITY UPLC HSS Column

Forensic Toxicology Application Solution with UNIFI®

Maximum Recovery Vial

KEY WORDS

Cannabinoids, workplace drug testing, urine, UPLC-QTof-MS^E, UNIFI

INTRODUCTION

Cannabis is the most widely used illicit substance in the world and long-term use can lead to dependency. Cannabinoids are one of the most commonly detected classes of illegal drugs; consequently their analysis is of key importance in both forensic and workplace testing. Δ-9 tetrahydrocannabinol (THC) and cannabinol (CBN) are psychoactive elements present in the plant *Cannabis sativa*.[1] THC produces a number of metabolites such as 11-nor-9-hydroxy-Δ9 tetrahydrocannabinol (THC-OH), but the most significant metabolite for urine drug testing is 11-nor-9-carboxy-Δ9 tetrahydrocannabinol (cTHC), which is the major metabolite eliminated in urine, as the free acid or the ester-linked β-glucuronide.[2] Cannabidiol (CBD) is a major constituent of cannabis resin but is believed to have limited psychoactive properties.

The Waters Forensic Toxicology Application Solution with UNIFI currently comprises acquisition of accurate mass MS^E data on an orthogonal acceleration time-of-flight mass spectrometer operating in electrospray positive ionisation mode (ESI+), followed by comparison of the data with a comprehensive library containing more than 1000 toxicologically-relevant substances.[3,4,5] A number of compounds, such as the cannabinoids, also ionise in negative electrospray mode (ESI-) and the aim of the recent work was to further extend the Forensic Toxicology Application Solution to include those compounds. The new method was used to determine the presence of cannabinoids in urine, particularly at concentrations, below the current screening cut-off,[6] and to compare the values obtained using this method with a recently published fully validated UPLC-MS/MS assay.[7]

EXPERIMENTAL

ACQUITY UPLC conditions

UPLC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC HSS, 100Å, 1.8 µm, C_{18}, 2.1 mm x 150 mm (p/n 186003534)
Vials:	Maximum Recovery Vials (p/n 186000327C)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water containing 0.001% formic acid
Mobile phase B:	Acetonitrile containing 0.001% formic acid
Gradient:	Isocratic at 87% A for 0.5 min, then to 5% A at 4.5 min, hold for 1 min before switching to 87% A
Run time:	7.5 min

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI-
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M-H]^-$ *m/z* = 554.2620
Acquisition range	*m/z* 50–1000
Scan time:	0.1 s
Capillary voltage:	1.5 KV
Cone voltage:	20 V
Collision energy:	Function 1: 6 eV Function 2: ramped 10 to 40 eV

Materials

Reference standards THC, CBD, CBN, THC-OH, and cTHC (1 mg/mL) were purchased from LGC Standards (Teddington, UK); cTHC-glucuronide and the deuterated (d-3) analogue of cTHC (for use as internal standard; ISTD), were obtained from the same supplier at 0.1 mg/mL.

Prior to use the individual standards were diluted to 5000 ng/mL in acetonitrile and the internal standard was diluted to 100 ng/mL in 0.001% formic acid.

Bio-Rad normal control urine and Bio-Rad Liquichek Urine Toxicology Controls Level C2 and Level S10 reference urines were obtained from Bio-Rad Laboratories (Hemel Hempstead, UK).

All other chemicals used were of the highest grade available and stored according to the supplier's instructions.

Standards preparation

Standards (0.1 mL) were added to 0.9 mL 0.001% formic acid in a Maximum Recovery Vial and vortex-mixed to give a concentration of 500 ng/mL.

Sample preparation

Acetonitrile (0.1 mL) was added to 0.2 mL urine and ISTD (0.7 mL). The sample was vortex-mixed, for 5 min at 1200 rpm, and then centrifuged at 8000 *g* for 10 min. Supernatant was transferred to a Maximum Recovery Vial.

RESULTS AND DISCUSSION

The cannabinoids investigated in this analysis are listed in Table 1, along with their exact neutral mass, high energy fragment ions, and UPLC retention times.

Analyte	Neutral monoisotopic mass	Fragment ions (*m/z*)		Retention time (min)
cTHC-glucuronide	520.2308	343.1915	299.2017	4.7
		245.1547	175.0248	
cTHC	344.1988	299.2017	245.1547	5.0
		191.1078	179.1078	
THC-OH	330.2190	311.2017	281.1547	5.1
		268.1469	267.1391	
Cannabidiol	314.2246	245.1547	229.1234	5.4
		179.1078	135.1179	
Cannabinol	310.1933	279.1391	252.1156	5.6
		222.0686	159.0815	
THC	314.2246	245.1547	229.1234	5.8
		191.1078	149.0972	
cTHC-d3	347.2176	302.2191	248.1739	5.0

Table 1. Analyte neutral mass, high energy fragment ions, and retention times.

The acceptance criteria for a positive identification of each analyte was as follows: three dimensional (3D) low energy ion count intensity greater than 250, retention time to be within 0.35 min of reference, and the observed precursor mass to be within 5 ppm of expected. For additional confirmation, a minimum of one diagnostic fragment ion had to be found in the high energy function. Chromatographic separation of cannabinoid standards at 100 ng/mL (50 ng/mL for cTHC-glucuronide and 500 ng/mL for cannabinol) is shown in Figure 1.

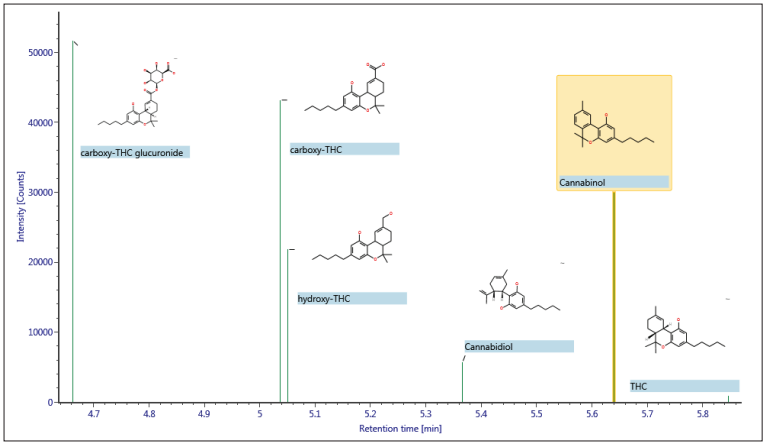

Figure 1. Component plot showing positively identified cannabinoids in a mixture of standards (ISTD not shown). The plot shows chromatographic separation for the isomers cannabidiol and THC.

To investigate linearity, Bio-Rad control urine was spiked with cTHC and cTHC-glucuronide over a range from 0 to 500 ng/mL (250 ng/mL for cTHC-glucuronide) and prepared in duplicate, as described above. The 3D peak response for each positively identified analyte trace was generated automatically during processing and referenced to the ISTD response; calibration curves were plotted using a 1/x weighting and a linear fit was applied to both analytes. The correlation coefficient (r^2 value) was ≥0.995 for both analytes; a calibration curve for cTHC is shown in Figure 2. The lowest calibrator positively identified, using the criteria detailed above, was 6.25 ng/mL for cTHC-glucuronide and 12.5 ng/mL for cTHC.

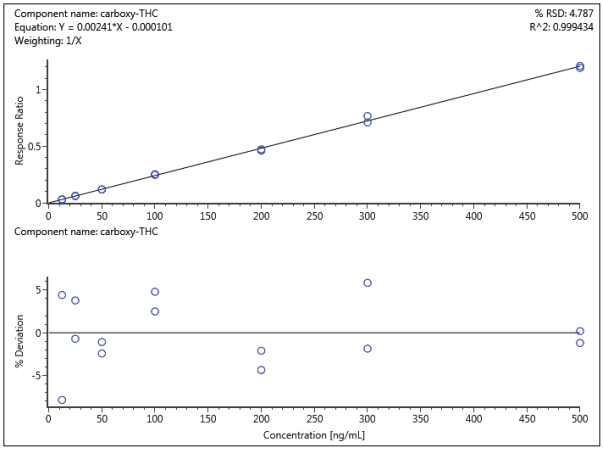

Figure 2. A spiked urine calibration curve for cTHC over the range 0 to 500 ng/mL using a linear fit with 1/x weighting applied.

Analysis of authentic urine samples

Twenty-six authentic urine samples and two commercial reference urines (Bio-Rad Liquichek Level C2 and S10) were analyzed following the sample preparation method described here. The authentic samples comprised anonymized samples that had previously been quantified using a fully validated UPLC-MS/MS assay.[7] The UPLC-MS/MS results for the 26 authentic samples are shown in Table 2, along with the results from the UPLC-QTof-MS^E assay, showing whether cTHC or cTHC-glucuronide were positively identified based on the criteria stated previously.

	cTHC		cTHC-glucuronide	
Sample	UPLC-MS/MS (ng/mL)	UPLC-QTof-MS^E (positive ID)	UPLC-MS/MS (ng/mL)	UPLC-QTof-MS^E (positive ID)
001	20	YES	40	YES
002	0	NO	33	YES
003	68	YES	285	YES
004	0	NO	0	NO
005	0	NO	0	NO
006	0	NO	0	NO
007	0	NO	0	NO
008	12	YES	70	YES
009	3	NO	16	YES
010	6	NO	33	YES
011	0	NO	0	NO
012	18	YES	49	YES
013	7	NO	21	YES
014	14	YES	206	YES
015	11	YES	10	YES
016	0	NO	12	YES
017	54	YES	14	YES
018	15	YES	76	YES
019	10	YES	177	YES
020	0	NO	0	NO
021	0	NO	0	NO
022	67	YES	120	YES
023	0	NO	0	NO
024	83	YES	233	YES
025	0	NO	0	NO
026	0	NO	7	NO

Table 2. Comparison of individual sample results for either the UPLC-MS/MS or UPLC-QTof-MS^E methods. Positive identification criteria for the UPLC-QTof-MS^E assay were 3D low energy ion count intensity greater than 250, retention time to be within 0.35 min of reference, observed precursor mass to be within 5 ppm of expected and a minimum of one high energy fragment ion detected.

The UPLC-QTof-MS^E method positively identified cTHC in 11 samples and cTHC-glucuronide in 16 samples, and overall demonstrated excellent concordance with the UPLC-MS/MS data (Table 3). This demonstrated that this method can consistently detect cTHC and cTHC-glucuronide in urine, using a simple fivefold dilution, at concentrations below the current European Workplace Drug Testing Society (EWDTS) screening cut-off of 50 ng/mL for cTHC.[6]

Furthermore the method detected and correctly assigned cTHC in both commercial reference urines. The semi-quantitative results obtained using this method for the analysis for the Bio-Rad Liquichek Level C2 and S10 reference urines were in accordance with the manufacturer's stated reference values, and are shown in Table 4. The additional confirmation provided by the presence of 4 high energy fragments for cTHC in the Bio-Rad Liquichek level S10 reference urine is shown in Figure 3.

cTHC			cTHC-glucuronide		
UPLC-MS/MS		UPLC-QTof-MS^E	UPLC-MS/MS		UPLC-QTof-MS^E
Blank	12	15 NEG	Blank	9	10 NEG
Positive <10 ng/mL	3		Positive <10 ng/mL	1	
Positive ≥10 ng/mL	11	11 POS	Positive ≥10 ng/mL	16	16 POS

Table 3. Summary of results for 26 authentic urine samples obtained using the quantitative UPLC-MS/MS methodology[7] and the described UPLC-QTof-MS^E assay.

Reference urine	GC/MS (ng/mL)	UPLC-QTof-MS^E (ng/mL)
Bio-Rad Liquichek Level C2	11.5	11.1
Bio-Rad Liquichek Level S10	35.3	40.5

Table 4. Comparison between the values obtained using the UPLC-QTof-MS^E method for the analysis of the Bio-Rad Liquichek reference urines and the values stated by the manufacturer.

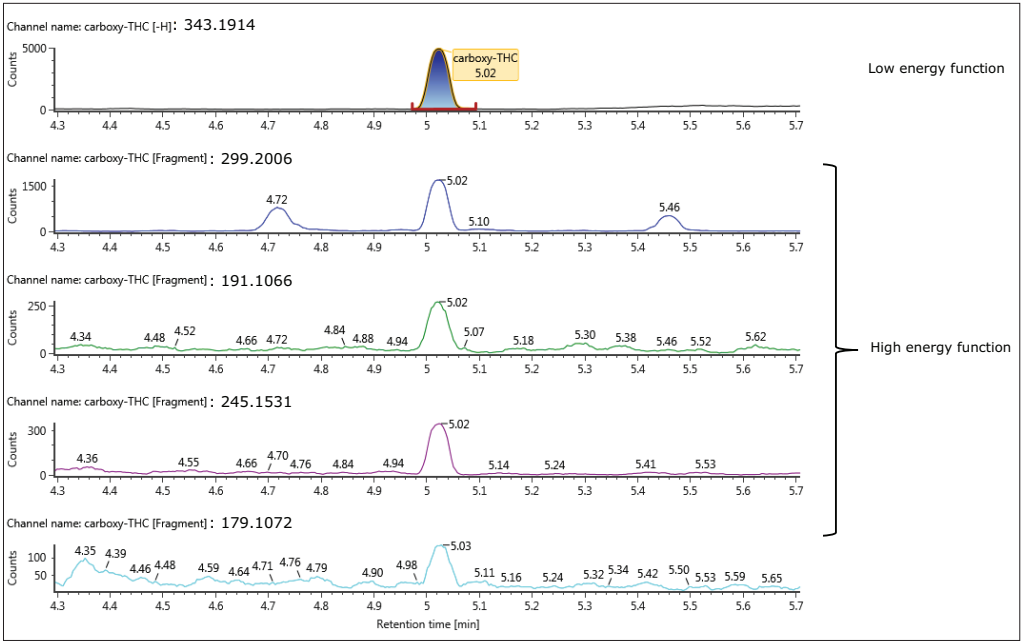

Figure 3. Data for cTHC in the Bio-Rad Liquichek Level S10 reference urine; additional confirmation is achieved by the presence of 4 fragment ions in the high energy function.

CONCLUSIONS

This application note demonstrates the sensitivity and selectivity of the expanded Forensic Toxicology Application Solution with UNIFI using negative ionisation in providing a consistent comprehensive determination of cannabinoids. It can be applied as both a screen for selected cannabinoids and a method suitable for quantifying these analytes, at levels below the current EWDTS urine screening cut-off (50 ng/mL for cannabis metabolites), using a simple five-fold dilution. The excellent linear dynamic range of this system is demonstrated by simple automatically generated calibration plots.

Acknowledgements

CEDAM Italia, Bresso, Italy and Bianalisi Analisi Mediche, Carate Brianza, Italy for supplying the anonymized authentic urine samples.

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A full validation by the user would be necessary prior to adoption in a laboratory.

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Using the Waters Forensic Toxicology Screening Application Solution With UNIFI to Determine Diuretics in Urine

Rob Lee and Michelle Wood
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

Expanded Forensic Toxicology Screening Application Solution with UNIFI,® enabling the detection and quantitation of negative ionising diuretics in urine.

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System (FTN)

Xevo® G2-S QTof Mass Spectrometer

ACQUITY UPLC HSS Column

Forensic Toxicology Screening Application Solution with UNIFI

KEY WORDS

Diuretics, World Anti-Doping Agency (WADA), sports doping, urine, UPLC-QTof-MS,^{E} UNIFI

INTRODUCTION

Diuretics are a class of pharmaceutical compounds whose primary aim is to promote urine production. As such they can be used to treat a number of medical conditions including congestive heart failure and hypertension. In sports, diuretics can be abused by athletes to generate rapid weight loss or to maintain low body weight. They can also be used to mask the presence of banned or illegal substances by facilitating dilution of the urine and aiding excretion. Consequently the use of diuretics is prohibited for athletes by the World Anti-Doping Agency (WADA). The list of banned compounds can be found in the WADA technical document[1] and includes at least 25 compounds with diuretic properties. To ensure consistency of measurement amongst doping control laboratories, WADA defines the minimum required performance level (MRPL), which is the concentration of a prohibited substance that laboratories are expected to detect; currently this is set at 200ng/mL.[2]

The Forensic Toxicology Screening Application Solution with UNIFI currently comprises acquisition of accurate mass data on an orthogonal acceleration time-of-flight mass spectrometer, operating in MS^E mode using electrospray positive ionisation mode (ESI+), followed by comparison of the data with a comprehensive library containing more than 1000 toxicologically-relevant substances.[3-5] However, as a number of the diuretics only ionise in negative electrospray mode (ESI-), the aim of the recent work was to further extend the Forensic Toxicology Screening Application Solution with UNIFI to include compounds that ionise in negative mode and to use the method to determine the presence of diuretics in urine, particularly at concentrations below the WADA MRPL.

EXPERIMENTAL

ACQUITY UPLC conditions

UPLC System:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC HSS C_{18}, 100Å, 1.8 µm, 2.1 mm x 150 mm, (p/n 186003534)
Vials:	Maximum Recovery Vials, 12 x 32mm, screw neck (p/n 186000327c)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water containing 0.001% formic acid
Mobile phase B:	Acetonitrile containing 0.001% formic acid
Gradient:	Isocratic at 87% A for 0.5 min then to 5% A at 4.5 min, hold for 1 min before switching to 87% A
Run time:	7.5 min

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI-
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M-H]^-$ m/z = 554.2620
Acquisition range:	m/z 50-1000
Scan time:	0.1 s
Capillary voltage:	1.5 KV
Cone voltage:	20 V
Collision energy:	Function 1: 6 eV
	Function 2: ramped 10 to 40 eV

Materials

Ioxinyl, for use as internal standard (ISTD), was purchased from Sigma-Aldrich (Poole, UK). A stock solution was prepared at 1 mg/mL in methanol and stored at -20 °C. Prior to use, the stock was diluted to 100 ng/mL in 0.001% formic acid.

All other chemicals used were of the highest grade available and stored according to the supplier's instructions.

Bio-Rad normal control urine was obtained from Bio-Rad Laboratories (Hemel Hempstead, UK).

Sample preparation

Acetonitrile (0.1 mL) was added to 0.2 mL urine and ISTD (0.7 mL). The sample was vortex-mixed, for 5 min at 1200 rpm, and then centrifuged at 8000 *g* for 10 min. Supernatant was transferred to a Maximum Recovery Vial (p/n 186000327c).

RESULTS

Sixteen diuretics that ionise in ESI- were included in this analysis and are listed in Table 1, along with their exact neutral mass and UPLC® retention times. The list includes five compounds that solely ionise in negative mode (bendroflumethiazide, benzthiazide, furosemide, hydrochlorothiazide, and hydroflumethiazide).

The acceptance criteria for a positive identification of each analyte was as follows: retention time to be within 0.35 min of reference and the observed precursor mass to be within 5 ppm of expected. For additional confirmation, a minimum of one diagnostic fragment ion had to be found in the high energy function.

Analyte	Neutral monoisotopic mass	Retention time (min)
Acetazolamide	221.9881	1.7
Chlorothiazide	294.9488	1.8
Hydrochlorothiazide	296.9645	1.9
Hydroflumethiazide	330.9908	2.4
Chlorthalidone	338.0128	2.6
Furosemide	330.0077	3.1
Metolazone	365.0601	3.2
Benzthiazide	430.9835	3.4
Indapamide	365.0601	3.4
Cyclothiazide	389.0271	3.5
Bendroflumethiazide	421.0378	3.5
Canrenoic acid	358.2144	3.4
Xipamide	354.0441	3.7
Bumetanide	364.1093	3.8
Probenecid	285.1035	3.9
Tolvaptan	448.1554	4.1

Table 1. Analyte retention times and neutral mass.

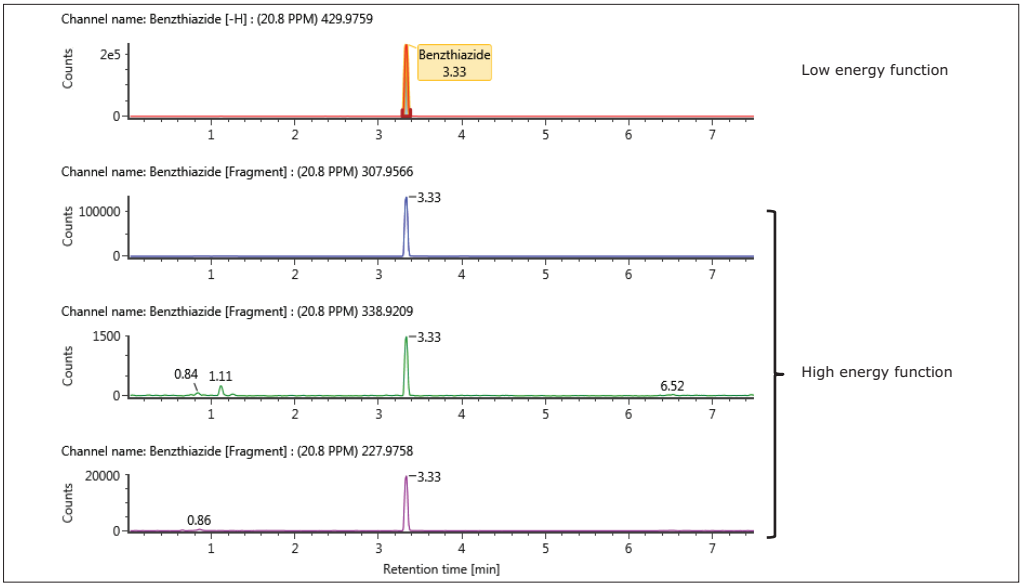

Figure 1. Data for benzthiazide in a blank urine sample that has been spiked at the WADA MRPL (200 ng/mL); additional confirmation is achieved by the presence of three fragment ions in the high energy function.

The utility of the MS^E approach and associated fragment ion data generated under the higher energy condition is further demonstrated in Figure 2. The figure displays the high energy data for metolazone and indapamide which have identical elemental composition ($C_{16}H_{16}ClN_3O_3S$) and, under the chromatographic conditions employed here, are also closely-eluting i.e., within 0.2 min. Under these conditions it could be challenging to differentiate between the two diuretics; however the figure shows clear differentiatiation when the diagnostic fragment ions are taken into account.

To investigate linearity, control urine was spiked with the diuretics over a range from 0 to 2000 ng/mL and prepared, in duplicate, as described above. The response for each analyte trace was generated automatically during processing and referenced to the ISTD response. Semi-quantitative calibration curves were plotted using a 1/x weighting and a quadratic fit was applied to all the analytes. The correlation coefficient of determination was >0.99 for each analyte. A calibration curve from 0 to 2000 ng/mL for furosemide is shown in Figure 3. Urine spiked at the WADA MRPL was quantified against the relevant calibration curves; all analytes were positively identified at this level.

A comparison between the high energy fragments identified in an analytical standard and those identified in a blank urine sample spiked with furosemide is shown in Figure 4.

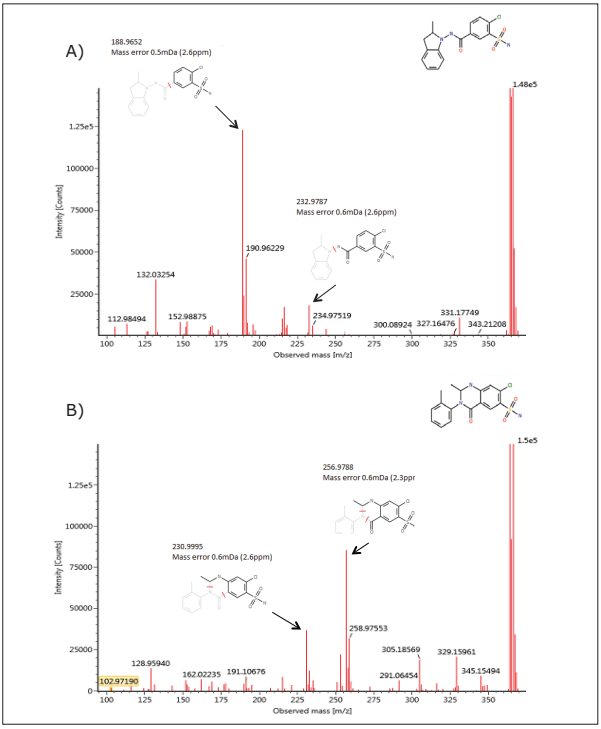

Figure 2. Spectra showing the high energy data for indapamide (Figure 2A) and metolazone (Figure 2B) highlighting the differences in the high energy fragments detected.

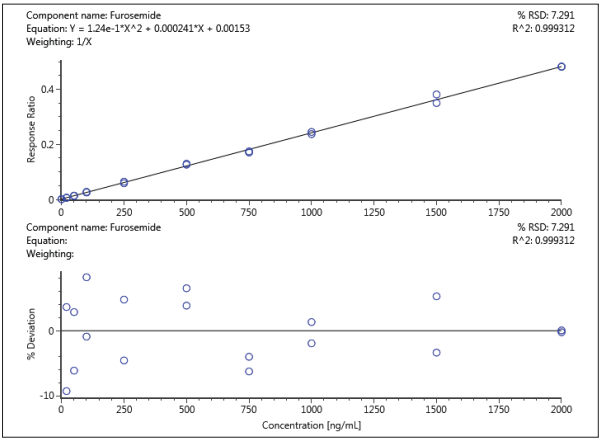

Figure 3. A spiked urine calibration curve for furosemide over the range 0 to 2000 ng/mL using a quadratic fit with 1/x weighting applied.

Analysis of Authentic Urine Sample

An authentic urine sample was analysed following the sample preparation method described and was shown to contain the diuretic furosemide. Further investigation of the data using UNIFI's metabolite identification (Met ID) tools indicated the presence of both phase 1 and phase 2 metabolites. The retention times for the parent molecule and the most prevalent metabolite, a glucuronide conjugate, (2.69 min) are shown in Figure 5. The software highlights metabolic transformation with observed retention time along with observed *m/z* and the mass error in ppm.

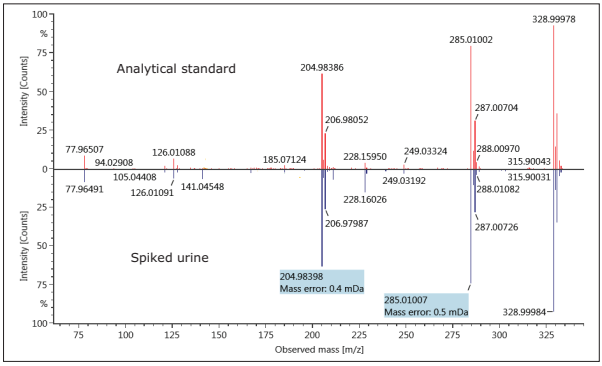

Figure 4. Results of the binary comparison analysis for furosemide (the plot is scaled to the relative percentages of the identified fragments). The data have been generated using the binary compare tool in UNIFI and highlight the mass error between the standard (upper-trace) and the sample data (lower-trace).

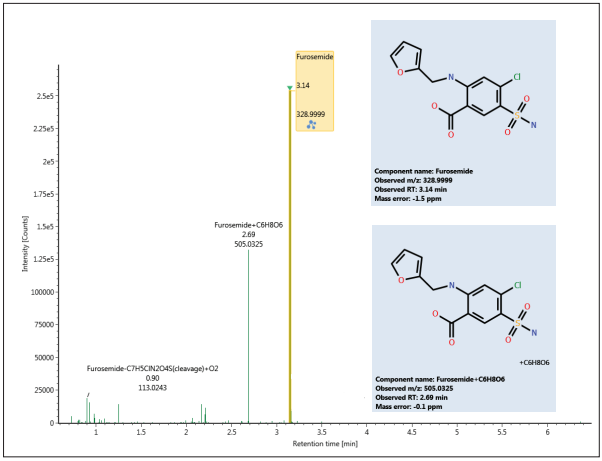

Figure 5. A selection of UNIFI Met ID proposed metabolites (showing observed m/z, mass error in ppm, retention time, and transformation) detected in an authentic urine sample.

CONCLUSIONS

The rise of sports doping testing has highlighted the need for a quick, accurate, reliable, and robust method to initially screen large numbers of samples. Expanding the Forensic Toxicology Screening Application Solution with UNIFI to determine negative ionising compounds enables the determination of diuretics in diluted urine at levels which will allow this method to be applied to anti-doping labs that comply with the WADA guidelines.

The use of the binary compare and metabolite identification tools within UNIFI can increase the confidence in the data by highlighting high energy fragment matches and facilitates discovery of metabolites in the sample which are not present in the database. These metabolites can subsequently be added to the library.

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A full validation by the user would be necessary prior to adoption in a laboratory.

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Analysis of Beta-Blockers Using UPLC with Accurate-Mass Screening

Mark Roberts and Michelle Wood
Waters Corporation, Manchester, UK

APPLICATION BENEFITS

The Forensic Toxicology Application Solution with UNIFI® enables analysts to confidently screen and identify beta-blocker drugs in urine; data is processed automatically and presented to the user with fully customizable workflows and reports.

WATERS SOLUTIONS

Forensic Toxicology Application Solution with UNIFI

ACQUITY UPLC® I-Class System

Xevo® G2-S QTof

KEY WORDS

Beta-blockers, UPLC, QTof, MS^E, UNIFI, toxicology, anti-doping, mass spectrometry, UPLC-MS

INTRODUCTION

Propranolol was the first, clinically successful beta-blocker. Synthesized by JW Black in the early 1960s,[1] it revolutionized the management of angina pectoris and spawned the development of additional beta-blockers. Beta-blockers competitively block the action of β-adrenergic agonists at the β-receptors in the cells of heart muscle and other tissues of the sympathetic nervous system. They are legally prescribed and used primarily for the management of hypertension, angina, and cardiac arrhythmias. These substances however, can be abused by athletes who want to decrease their heart rate, lower their blood pressure, or improve their fine motor skills. Consequently, the World Anti-Doping Agency (WADA) includes beta-blockers in its 2014 Prohibited List[2] (Category P2), limiting the prohibition to sports like archery, golf, and shooting.

Recent advances in liquid chromatography and mass spectrometry can help determine the presence of beta-blockers in urine.

Sample preparation

A mixed, methanolic standard containing the following beta-blockers was prepared at a concentration of 50 µg/mL: acebutolol, alprenolol, atenolol, bunolol, bisoprolol, carazolol, celiprolol, levobunolol, metipranolol, metoprolol, nadolol, nebivolol, oxprenolol, pindolol, sotalol, and timolol. Blank human urine was spiked with the mixed standard, resulting in final concentrations of 50, 100*, 250, and 500 ng/mL. A simple five-fold dilution with mobile phase A was used to prepare each spiked urine sample for injection.

* Minimum required performance level (MRPL) for a WADA-accredited laboratory.

EXPERIMENTAL

Method conditions

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Run time:	15 min
Column:	ACQUITY UPLC HSS C_{18} 2.1 x 150 mm, 1.8 µm
Vials:	Waters® Maximum Recovery Vials
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM aqueous ammonium formate, adjusted to pH 3.0
Mobile phase B:	Acetonitrile with 0.1% formic acid
Gradient:	87% A to 50% A over 10 min, reduce to 5% A and hold for 1.5 min before returning to 87% A

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI+
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M+H]^+ = 556.2766$
Acquisition range:	*m/z* 50–1000
Scan time:	0.1 s
Capillary voltage:	0.8 kV
Cone voltage:	25 V
Collision energy:	Function 1: 6 eV Function 2: ramped 10 to 40 eV

RESULTS AND DISCUSSION

The diluted spiked urine samples were injected and data was acquired using the standard MS^E-based toxicology screen.[3,4] Data were subsequently processed using the UNIFI Forensic Toxicology Library comprising more than 1,000 toxicologically-relevant substances. Qualitative identification was achieved through a combination of mass accuracy, retention time (RT) and the presence/absence of expected fragment ions. In the same processing step, UNIFI Scientific Information System also generates and displays any quantitative data.

UNIFI uses a simple workflow approach to guide the user through the sample results; data is automatically filtered and presented to the user according to the degree of confidence in the identification, thereby decreasing the requirement for analyst's review. Workflows are fully customizable - an example of the criteria that may be used is shown in Figure 1.

All of the beta-blockers were successfully identified at the lowest concentration investigated in this study (50 ng/mL) and met the user-defined criteria for a "Positive" drug finding. Figures 1 through 4 provide an illustrative example of some of the data that is automatically-displayed or available to the user on a "single-click" from the Review pane.

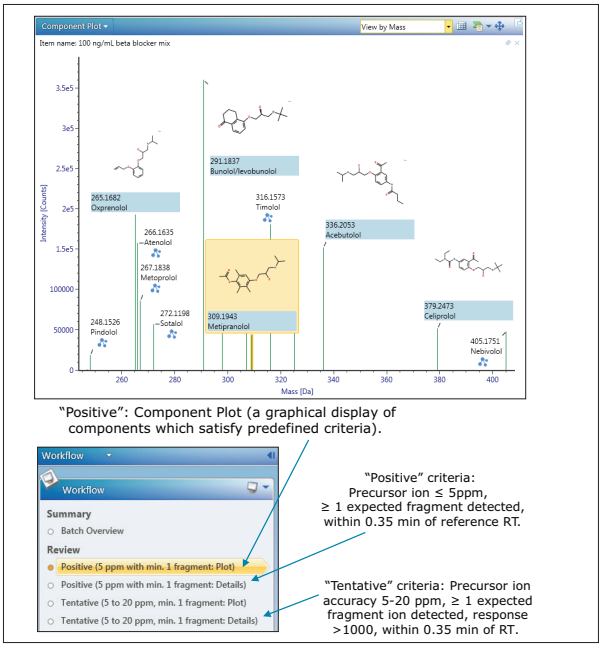


Figure 1. Example of a workflow based on two differing categories of identification ("Positive" and "Tentative") together with a summary of the criteria used for each.

In contrast to the plot view, which provides a very simple graphical display of detected components, full details of each identification can be viewed by selection of the Component Summary (Figure 2). This is a user-friendly table that summarizes key characteristics of identified peaks including mass accuracy, confirmatory fragment ions and isotope information (in this example, only those components that matched the "Positive" criteria are shown).

The extracted mass chromatograms for the precursor ion and all of the high collision energy fragment ions for a particular component can also be displayed if required, as shown in the Chromatograms window (lower left of Figure 2).

Further information is available by viewing the low and high energy spectra for a component as shown in the Spectra window. This view highlights the precursor ion in the top trace and the found fragment ions in the bottom trace. UNIFI provides improved three-dimensional (3D) chromatographic peak detection with its integrated ApexTrack™ algorithm, which facilitates the generation of cleaner mass spectra, enabling better library matching of fragment ions.

In addition to viewing the spectra, it is often useful to display a summary of the confirmatory fragment ion data. Figure 2 also shows the Fragments table which contains details for the expected fragments for acebutolol, the mass error associated with each detected fragment, and the detected fragment intensity.

Component Summary: User-friendly table that summarizes key characteristics of identified peaks including information related to: mass accuracy; RT; presence of confirmatory fragment ions and isotope information.

Figure 2. Details for a 50 ng/mL spiked urine sample. The Component Summary shows details for the first five analytes present in alphabetical order. The F v E (%) column displays the number of found vs number of expected fragments, expressed as a percentage. The Isotope Match Intensity RMS Percent and Isotope Match Mz RMS PPM indicate the degree of matching between the theoretical isotopic pattern and the observed pattern for the precursor ion cluster. The chromatogram's window contains the extracted mass chromatograms for the selected precursor i.e., acebutolol (m/z 337.212) and fragment ions (m/z 116, 319, 98, and 260). The Spectra window displays the low collision energy (upper spectrum) and high collision energy (lower spectrum) for acebutolol and the Fragments table shows each of the expected fragments for acebutolol.

The isotopic pattern obtained for each component can also be an aid in identification. Figure 3 shows the mass spectrum of the low collision energy trace for sotalol, a sulphur-containing compound. The two most abundant stable isotopes of sulphur are ^{32}S and ^{34}S which are present at a ratio of 95:4. An algorithm within UNIFI can be used to indicate the degree of matching between the theoretical and observed isotopic patterns for a component, with a low score indicating a good match. This "Isotope Match Intensity RMS Percent" column can be added to the Component Summary table as an extra point of confirmation. A further UNIFI algorithm is used to evaluate the level of agreement between the expected *m/z* and found *m/z* of each isotopic peak and these results are shown in the Isotope Match Mz RMS PPM, again with a low score indicating a good match as shown in the rightmost columns of the Component Summary window of Figure 2.

Isotopic information: UNIFI includes both a graphical display as well as calculated comparisons of measured isotopic data against the theoretical (see Figure 2).

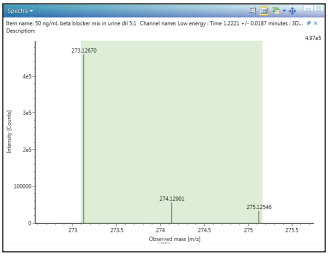


Figure 3. The low collision energy spectrum for sotalol showing the m/z 273 and 275 ions corresponding to the sulphur isotopes ^{32}S and ^{34}S. UNIFI includes algorithms to automatically compare the isotopic data of the measured component with the theoretical for the proposed substance; this data is included in the last two columns of the Component Summary table.

In particular, Figure 4 shows, for each beta-blocker, a semi-quantitative calibration plot that draws data from three replicate injections made at each concentration (50, 100, 250, and 500 ng/mL). The calibrations are calculated from the response value for each analyte, a value that originates with the 3D integration of the monoisotopic precursor-ion peak. Because no internal standards were used in this study, this semi-quantitative data demonstrates only the typical dynamic range of the instrument.

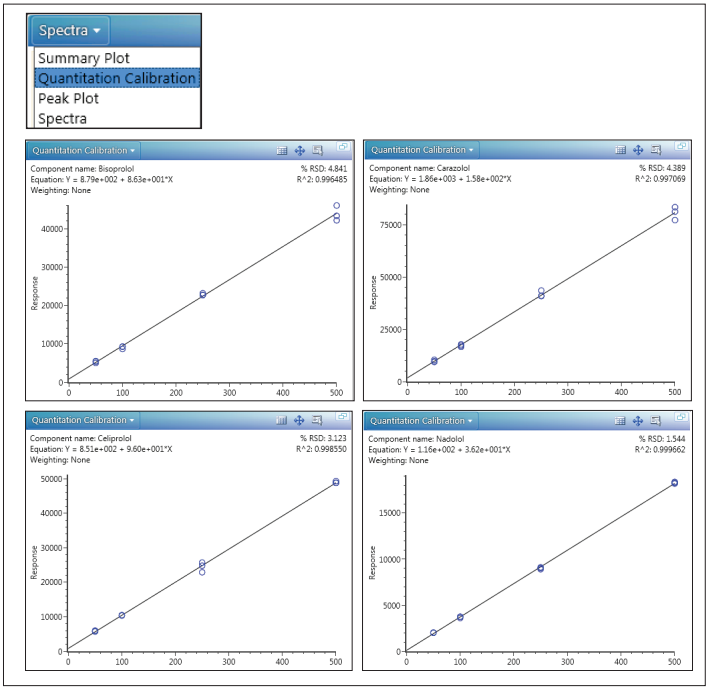


Figure 4. Calibration plots for four beta-blocker analytes spiked into urine (triplicate injections) at 50, 100, 250, and 500 ng/mL using a linear fit with no weighting applied.

Figure 5 illustrates a fully customizable report generated by the UNIFI Software from the results that provided the key details of the identifications made for this sample. A section from this report is shown in Figure 5 and provides a Component Plot as well as a Component Summary for each identification category.

Sample Information

Item name: 50 ng/mL beta blocker mix in urine dil 5:1

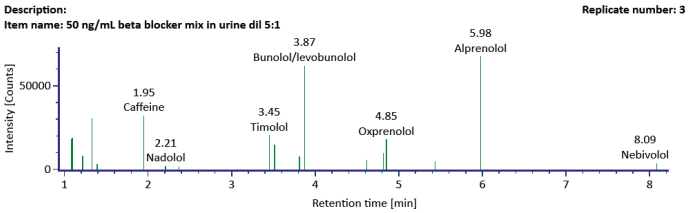
Sample type:

Description:

Replicate number: 3

Sample position: 1:B,6

Positive Identifications (Mass error <5ppm, at least one diagnostic fragment ion)



Item name: 50 ng/mL beta blocker mix in urine dil 5:1

Component name	m/z	Mass error (ppm)	Expected RT (min)	Observed RT (min)	Identified High Energy Fragments	Expected Fragments Count	F v E (%)	Response	Isotope Match Mz RMS PPM	Isotope Match Intensity RMS Percent
Acebutolol	337.2121	-0.27	3.7	3.5	4	4	100.00	14684	0.48	1.84
Alprenolol	250.1804	1.07	6.2	6.0	2	3	66.00	67513	0.99	1.50

Figure 5. A fully customizable report showing the Component Plot and the first two lines of the Component Summary for this injection.

CONCLUSIONS

This application note demonstrates the sensitivity and selectivity of the Forensic Toxicology Application Solution with UNIFI in providing comprehensive screening of beta-blockers at low levels of concentration in human urine and achieving the MRPL with minimal sample preparation. Despite the complex nature of accurate mass MS^E data, the UNIFI Software enables user-friendly, comprehensive data analysis, interpretation, and reporting. The excellent linear dynamic range of this system is demonstrated in four, simple, automatically generated calibration plots.

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The Utility of MS^E for Toxicological Screening.

Michelle Wood

GOAL

To investigate the utility of the innovative data acquisition mode MS^E for the screening of toxicants in human urine.

BACKGROUND

Laboratories are frequently required to perform broad screening techniques on complex biological samples to identify drugs of abuse and other toxicants. In recent years there has been an increased interest in the use of Time-of-Flight (Tof) instruments for this purpose owing to the high level of specificity offered by exact mass data.

Whilst exact mass libraries can be automatically generated without reference material *i.e.,* from molecular formulae, the lack of additional information can lead to false positive results in the analysis of authentic samples. Thus, where possible, additional information *e.g.,* an associated retention time (RT) and confirmatory fragment ions should be used to increase confidence in drug identification and to improve the ease and speed of data review and reporting.

MS^E is a novel, patented mode of data acquisition that permits the seamless collection of a comprehensive catalog of information for both precursor and fragment ions in a single analysis. This is achieved by rapidly alternating between two functions *i.e.,* the first, acquired at low energy provides exact mass precursor ion spectra; the second, at elevated energy provides high energy exact mass of the fragment ions. In addition to providing increased confidence in identification, fragmentation can help to differentiate between isobaric compounds.

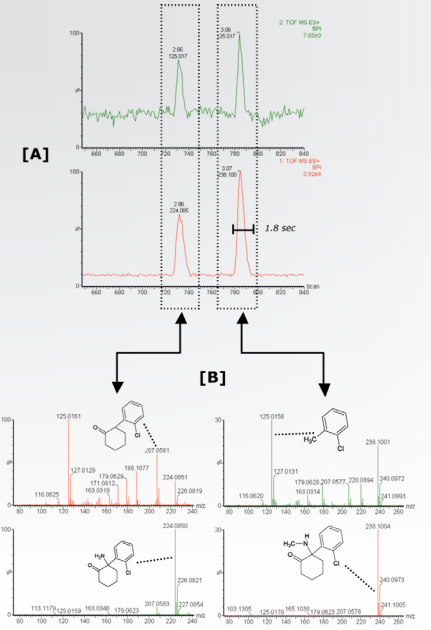

Figure 1. MS^E analysis of an authentic urine sample.

Panel A shows the chromatograms for the low (lower-trace) and high (higher-trace) energy data. The displayed data focuses on two unknown analytes eluting at 2.9 and 3.1 min, respectively. A minimum of 12 data points are collected for each analyte and for each energy condition.

Panel B shows the underlying exact mass spectra for the low (lower-traces) and high energy (upper-traces) condition. If desired, any ions within these spectra can be submitted for Elemental Composition Analysis which uses a combination of exact mass and isotope patterns to propose likely elemental formulae. MassFragment™ can be used to verify and assign logical molecular structures for a given measured mass.

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In contrast to the more conventional data dependent acquisition (DDA), MS^E has no duty cycle restrictions and therefore better addresses the problems associated with co-elution in complex biological mixtures and the challenges of the sharper, narrower peaks associated with UPLC separation.

THE SOLUTION

LC/MS System Configuration

ACQUITY UPLC® System in combination with the Xevo™ G2 QTof Mass Spectrometer

LC/MS Conditions

Column:	ACQUITY UPLC® HSS C_{18} Column
Run time:	15 min gradient elution
Ionization Mode:	ESI Positive
Acquisition range:	50-1200
Resolution:	20,000 FWHM
MS^E conditions:	Collision energy ramp 10-40 eV

Software and Database

ChromaLynx™ XS application manager was used in targeted mode for automated comparison with an in-house database comprising more than 700 toxicologically-relevant compounds/metabolites. The database includes precursor ion mass and RT and is supplemented with fragment ion information (Figure 3).

Doxepine	C19H21NO	6.93	f:107.0497	f:141.0704	f:235.1123
Ecgonine methyl ester	C10H17NO3	0.82	f:182.1128	f:82.0657	f:150.0919
EDDP	C20H23N	7.34	f:249.1517	f:234.1283	f:186.1283

Figure 3. Excerpt from the in-house toxicology database. Data includes the following information (from left to right): elemental formula; RT and exact mass information of specific fragment(s).

RESULTS

Diluted urine samples were analysed using UPLC/TOF analysis in MS^E mode. The increased speed and resolution associated with UPLC separation results in a significant reduction in peak width. Figure 1 shows an example of a typical peak, where widths (half-height) can be less than 2 sec, but data quantity and quality remains uncompromised. With MS^E, data is collected continually at both low, and elevated, energy thus a full data set for both precursors and fragment ions is always acquired. Even in the case of closely or co-eluting analytes, full exact mass spectra are available.

The data was processed automatically using ChromaLynx XS and compared to an in-house database. The compounds eluting at 2.9 and 3.1 min were identified as norketamine and ketamine respectively. The sample was also positive for theophylline, another ketamine metabolite i.e., dehydronorketamine, the recreational drug MDMA (Ecstasy) and two of its metabolites (HMMA and MDA) (Figure 4.). The combination of MDMA and ketamine are commonly-abused in a drug practice known as 'kitty-flipping'.

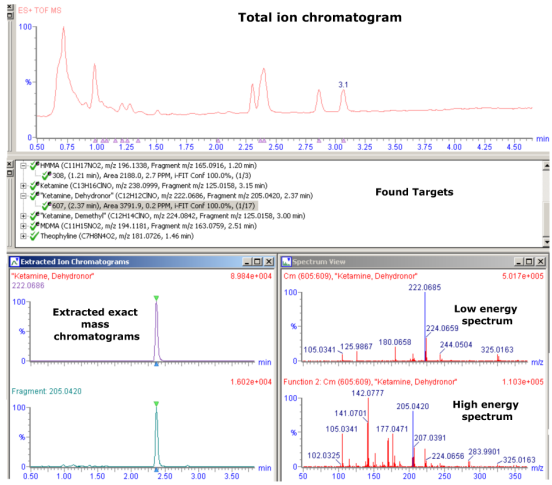

Figure 4. ChromaLynx browser showing details for identified drugs. With the exception of theophylline all analytes were identified and confirmed with additional fragment ions (denoted by the bold 'e'). Where extra confirmatory ions are not specified in the target list (as in the case of theophylline), identification is based on exact mass, isotope ratios and RT.

SUMMARY

MS^E was successfully used to analyse authentic urine samples. Fragment ion confirmation provides superior confidence in analyte identification and minimizes the opportunity for false positives thus improving the ease and speed of review and reporting.

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Screening for a Panel of 12 Toxicologically-Relevant Drugs in Urine using Tof-MRM

Jeff Goshawk and Michelle Wood
Waters Corporation, Wilmslow, UK

The use of Tof-MRM to confirm the presence, or absence, of 12 drugs in authentic urine using the Xevo G2-XS QTof and UNIFI.

GOAL

The application of Tof-MRM using a Xevo® G2-XS QTof with UNIFI® Scientific Information System for the analysis of forensically-relevant drugs in urine.

BACKGROUND

Laboratory testing for illicit drug substances frequently employs a combination of immunoassay-based screening, for common drug classes, followed by confirmatory testing using targeted LC-MS/MS based techniques. Some service providers have successfully replaced this multiple step approach with a single LC-MS/MS procedure targeted for a selection of key analytes.[1] While both of these strategies represent an effective procedure for a limited panel of analytes, the approach does not provide information for a broad range of drug substances. Additionally, these methods are also unlikely to include some of the newer, emerging drug substances.

Previously we have described a time-of-flight (Tof) screening method with the potential to screen for an unlimited number of toxicologically-relevant substances within 15 minutes.[2,3] This technique employs Tof-MS^E, a non-targeted data acquisition mode which yields a complete dataset from which thousands of substances may be screened. The same mass spectrometer may also be used in targeted mode i.e., multiple reaction monitoring mode (Tof-MRM), providing enhanced sensitivity; this mode allows isolation of a precursor mass using the quadrupole followed by Tof detection of specific fragment ions.[4]

Tof-MRM conditions

Compound	Precursor ion *m/z*	Product ions *m/z*	Acquisition time (min)
Cotinine	177.1022	**98.0601**, 146.0601	0.57–1.57
Morphine	286.1437	**201.0911**, 165.0699	0.60–1.60
Norcodeine	286.1437	**268.1333**, 225.0911	1.10–2.10
Codeine	300.1593	**215.1067**, 225.0911	1.20–2.20
Dihydrocodeine	302.1750	**199.0754**, 201.0911	1.20–2.20
6-monoacetylmorphine	328.1543	**211.0754**, 165.0699	1.60–2.60
Amphetamine	136.1120	**91.0543**, 119.0856	1.75–2.75
Benzoylecgonine	290.1386	**168.1020**, 105.0335	2.45–3.45
Norbuprenorphine	414.2638	**396.2534**, 101.0961	4.58–5.58
Buprenorphine	468.3108	**414.2639**, 396.2170	6.47–7.47
EDDP	278.1903	**234.1278**, 249.1512	6.79–7.79
Methadone	310.2165	**265.1587**, 105.0335	7.94–8.94

Table 1. Tof-MRM conditions for the included analytes; the quantifier ion is shown in bold.

Here we present the analysis of 12 common drugs in urine using Tof-MRM. The technique uses the same well-established chromatographic separation as that used for the Forensic Toxicology Screening Application Solution with UNIFI[3] and as such, provides the user with ability to perform screening and confirmation on a single platform.

THE SOLUTION

Sample preparation

Authentic drug-free urine was collected from volunteers and pooled. The pooled urine was spiked with a mixture of 12 drug substances to yield a series of samples at the following concentrations: 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 ng/mL. A blank urine sample was also prepared. Samples were further diluted 5 five-fold with mobile phase prior to analysis.

LC-MS method conditions

Data was acquired using the ACQUITY UPLC® I-Class (FTN) together with the Xevo G2-XS QTof in Tof-MRM mode. Two product ions (quantifier and qualifier) were monitored for each of the 12 drugs shown in Table 1. All transitions were acquired with a collision energy ramp from 10 eV to 40 eV, with the exception of norbuprenorphine, for which a constant collision energy of 40 eV was used to monitor of the *m/z* 101.0961 fragment ion.

RESULTS

Data were acquired and processed using UNIFI. Processing comprised automatic extraction of the mass chromatogram, for each transition, followed by peak integration. Figure 1 shows representative qualifier chromatograms for three of the substances investigated. The corresponding data for the blank urine sample is included for comparison. Similar results were obtained for the other nine drugs in the study. The standard curve for the quantifier ion of 6-monoacetylmorphine is shown in Figure 2 and demonstrates excellent linearity over the entire concentration range.

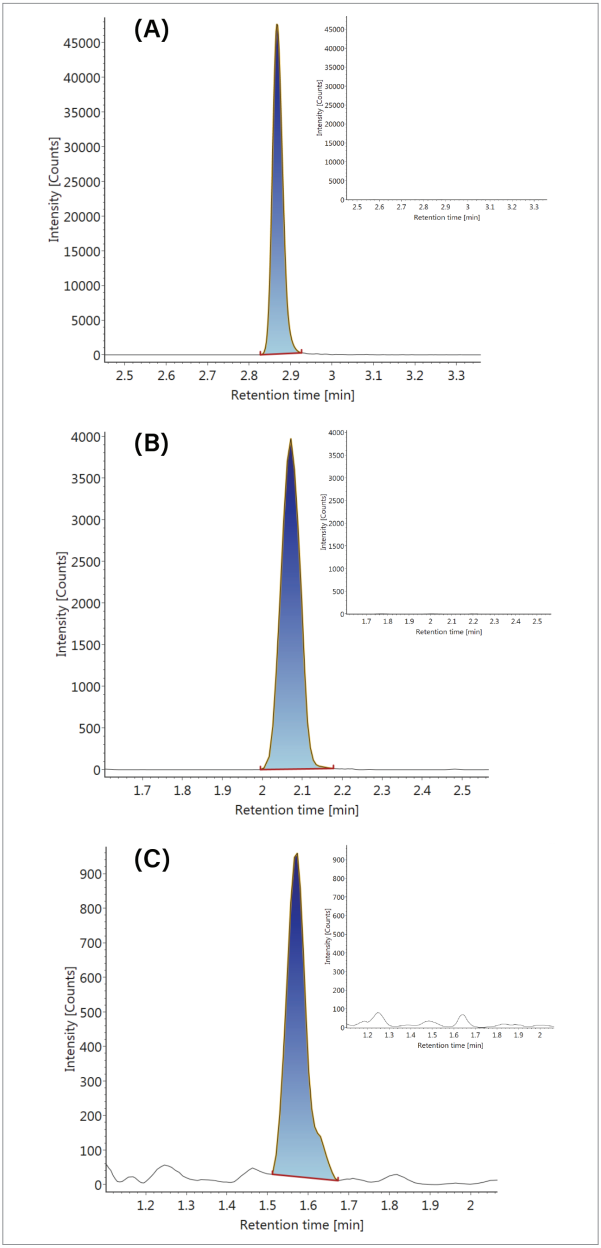

Figure 1. Chromatograms of the quantifier ion for (A) benzoylecgonine (B) 6-monoacetylmorphine and (C) norcodeine. The chromatograms are from the urine sample spiked at a concentration of 10 ng/mL and diluted prior to injection. For comparison, the inset chromatograms provide responses for a blank urine.

SUMMARY

Tof-MRM has been successfully used, on the Xevo G2-XS QTof, to analyze a panel of 12 drug substances. The enhanced selectivity of Tof-MRM permitted detection of the analytes in urine spiked at low ng/mL concentrations and prepared by a simple dilution.

Tof-MRM combined with the ability to perform Tof-MS^E enables comprehensive non-targeted screening and confirmation on a single platform.

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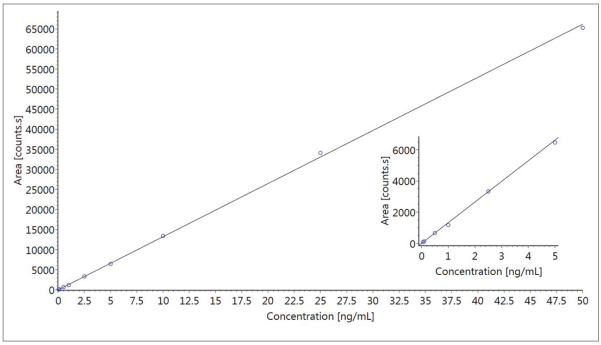

Figure 2. The standard curve for the quantifier ion of 6-monoacetylmorphine. The inset graph shows the detail for the samples at the lower concentrations.

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Systematic Toxicological Screening Using the ACQUITY UPLC I-Class/Xevo TQ-S micro

Rob Lee and Michelle Wood
Waters Corporation, Wilmslow, UK

GOAL

To evaluate the performance of previously published methodology using the Xevo® TQ-S micro.

BACKGROUND

Forensic toxicology laboratories require reliable screening techniques that can detect a wide variety of toxicants in highly complex biological matrices, such as ante and postmortem specimens. The original Waters® systematic toxicological screening method used the Waters Alliance® 2695 Separations Module in conjunction with the Waters/Micromass® ZQ™ Single Quadrupole Mass Spectrometer.[1] In 2009, this approach was migrated to the ACQUITY® TQD System to deliver the same comprehensive toxicological screening capabilities in half the time.[2] The solution was further developed over subsequent years to provide a full scan screening method and associated toxicology libraries, capable of screening for >950 drug substances and metabolites in 15 minutes. This method has been successfully and routinely used in toxicology laboratories worldwide.[3,4] Owing to the popularity of this methodology, in 2013, this solution was transferred to the ACQUITY UPLC® I-Class System and Xevo TQD.[5] The release of the Xevo TQ-S micro allows for further evolution of this successful solution.[6]

A simple, sensitive UPLC-MS method for forensic toxicology screening of compounds in various biological matrices.

Figure 1. ACQUITY UPLC I-Class System and Xevo TQ-S micro.

THE SOLUTION

Combining the ACQUITY UPLC I-Class System with the Xevo TQ-S micro allows this established UPLC-MS screening methodology to be used on the latest generation of Waters mass spectrometers.

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EXPERIMENTAL

Test substance

Bio-Rad S10 Liquichek Urine Toxicology Quality Control human urine was obtained from Bio-Rad, Hemel Hempstead, UK.

Sample preparation

The reference urine (250 µL) was extracted using a simple liquid–liquid extraction protocol. Following removal of the upper organic layer and evaporation of the organic solvent, samples were reconstituted in 50 µL of mobile phase A and transferred to a Waters Total Recovery vial.

LC conditions

System:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS C_{18}, 100Å, 1.8 µm, 2.1 mm x 150 mm (P/N 186003534)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Wash solvent:	Acetonitrile/water (95:5 v/v)
Purge solvent:	5 mM ammonium formate pH 3.0
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM ammonium formate pH 3.0
Mobile phase B:	Acetonitrile containing 0.1% formic acid

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	3.0 KV
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Cone gas:	20 L/Hr
Cone voltages:	50 V to 125 V in 15 V increments (preconfigured in provided MS method)
Acquisition range:	*m/z* 80–650

RESULTS

The technique uses in-source collision induced fragmentation at various cone voltages followed by library matching using the ChromaLynx™ Application Manager. Previous analysis of mixtures of drug substances using the Xevo TQ-S micro indicated that the cone voltages required to produce comparable fragmentation patterns were higher than those used with the previous generation mass spectrometers (e.g. Xevo TQD), therefore modified libraries were prepared and evaluated. Figure 2 shows a comparison of spectra obtained on the two platforms, highlighting the additional 30 V applied to the cone for each function on the Xevo TQ-S micro.

A selection of the information available in the ChromaLynx results browser for the extracted urine sample is shown in Figure 3.

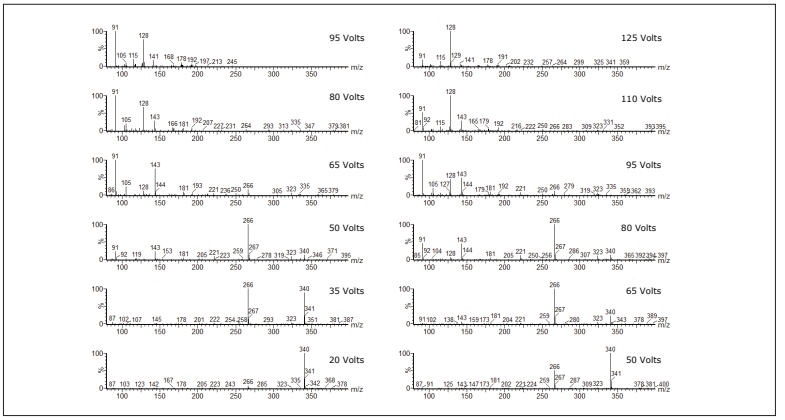

Figure 2. Comparison between the fragmentation patterns obtained using the Xevo TQD (left panel) and the Xevo TQ-S micro (right panel) for propoxyphene in the Bio-Rad S10 Liquichek Urine Toxicology Quality Control reference urine.

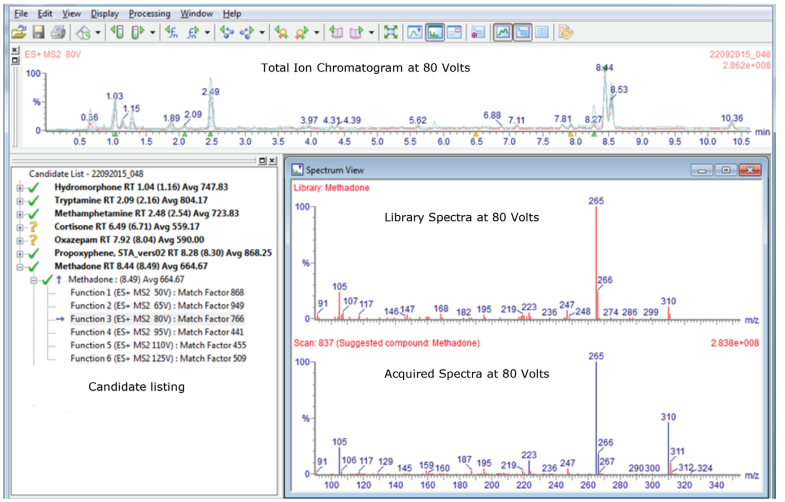

Figure 3. ChromaLynx browser displaying selected information for the analysis of the Bio-Rad S10 Liquichek Urine Toxicology Quality Control urine, highlighting the identification of methadone.

SUMMARY

The Xevo TQ-S micro is a tandem mass spectrometer designed to provide rapid, reliable, and reproducible data to deliver consistent low levels of quantitation over a wide dynamic range. We have shown that the highly successful systematic toxicological screening method can be transferred to the Xevo TQ-S micro by altering the acquisition method to take into account the different energy applied in the source. In conjunction with amended libraries, the Xevo TQ-S micro platform performs to the same high level as previous Waters MS platforms. The Xevo TQ-S micro is a highly versatile instrument for use in toxicology, providing the user with both broad qualitative full scan MS and targeted MRM-based screening capabilities as well as high sensitivity quantitative detection on the same instrument platform.

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6. Xevo TQ-S micro Product Brochure. 2014. Waters Marketing Brochure, 720005046EN.

This is a proof of principle demonstration of an analytical method, which may include examples of typical results that can be achieved with the stated configuration. This method represents a basic starting point from which users should perform their own in-house validation.

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Targeted MRM Screening Using the ACQUITY UPLC I-Class/ Xevo TQ-S micro

Rob Lee and Michelle Wood
Waters Corporation, Wilmslow, UK

GOAL

To evaluate the performance of previously published methodology using the Xevo® TQ-S micro.

A simple, sensitive UPLC-MS/MS method for targeted forensic toxicology screening of compounds in various biological matrices.

BACKGROUND

Forensic toxicology laboratories require reliable screening techniques that can detect a wide variety of toxicants in highly complex biological matrices, such as ante and postmortem specimens. The Waters® targeted toxicology screening application using the ACQUITY® TQD System was released in 2009.[1] This approach has been used by Rosano *et al* to compare screening methodologies for postmortem blood samples.[2] Following its success, this solution was transferred in 2013 to the ACQUITY UPLC® I-Class and Xevo TQD system.[3] The release of the Xevo TQ-S micro allows for further evolution of this solution.[4]

Figure 1. ACQUITY UPLC I-Class and Xevo TQ-S micro configuration.

THE SOLUTION

Combining the ACQUITY UPLC I-Class with the Xevo TQ-S micro allows this established UPLC-MS/MS screening methodology to be used on the latest generation of Waters mass spectrometers.

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EXPERIMENTAL

Test substances

The following commercial human urine reference controls were obtained: Basis-line U from Medidrug (40201); Blankcheck urine (UR015) and DCT -25% (UR22020A) both from ACQ Science; Urine Toxicology Control DAU HC2 (50701) from UTAK; and the following Liquichek Urine Toxicology Quality Controls from Bio-Rad: Negative Control (460), C2 (442), and S10 (673).

Sample preparation

The commercial reference urines were diluted 5-fold with mobile phase A and vortex-mixed. Following centrifugation the supernatant was transferred to a Waters Maximum Recovery vial and triplicate injections were analyzed.

LC conditions

System:	ACQUITY UPLC I-Class with FTN
Column:	ACQUITY UPLC HSS C_{18}, 100Å, 1.8 µm, 2.1 mm x 150 mm (P/N 186003534)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Wash solvent:	Acetonitrile/water (95:5 v/v)
Purge solvent:	5 mM ammonium formate pH3.0
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM ammonium formate pH3.0
Mobile phase B:	Acetonitrile containing 0.1% formic acid

MS conditions

System:	Xevo TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	3.0 KV
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Cone gas:	20 L/Hr
Cone voltages:	Preconfigured in provided MRM method
Collision energies:	Preconfigured in provided MRM method

RESULTS

The data was collected using the supplied MRM method which contains two transitions (qualifier and quantifier) per compound, with associated preconfigured parameters for cone voltage and collision energies for 178 compounds. The three negative control reference urines (Basis-line U, Blankcheck, and Negative Control) and four positive control reference urines (C2, S10, DAU HC2, and DCT -25%) containing certified levels of analytes, were assayed using the method described above. The data was automatically processed using the TargetLynx™ Application Manager, following a slight increase in the area threshold reject parameter, as a result of the increased response of the TQ-S micro. Screening results were compared for equivalence to the data obtained from the Xevo TQD platform.

A number of compounds were detected in the negative control reference urines on both platforms, i.e. caffeine and other substances associated with over-the-counter medications, which are routinely detected in urine screens.

For the certified positive control reference urines, both platforms detected the same number of expected compounds in the S10 reference urine. The Xevo TQ-S micro also found the same analytes as the Xevo TQD in the C2 and DAU HC2 urine samples, but in addition was able to detect α-hydroxyalprazolam in the C2 urine and lorazepam in the DAU HC2 urine.

Additional sensitivity for the benzodiazepines was also confirmed through analysis of the ACQ Science DCT -25% sample. Figure 2 details five additional benzodiazepines which were detected using the Xevo TQ-S micro. This commercial reference urine has certified levels of analytes at a concentration equivalent to 25% lower than the maximum cut-off concentration currently recommended by the European Workplace Drug Testing Society (EWDTS) for confirmation tests in urine;[5] the benzodiazepines detected here are present in this urine at 75 ng/mL.

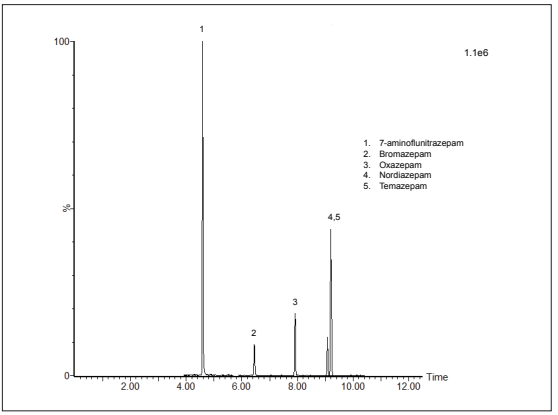

Figure 2. Chromatogram showing benzodiazepines in the ACQ DCT -25% commercial reference urine detected by the Xevo TQ-S micro using the supplied targeted MRM method but not the Xevo TQD. The quantifier ion transition is displayed.

The average number of scans per function has increased as the method has evolved from the ACQUITY TQD System through the ACQUITY UPLC I-Class/Xevo TQD and now to the ACQUITY UPLC I-Class/Xevo TQ-S micro; because the dwell time (10 msec) in the supplied MRM method has not changed this increase can be attributed to the improvements in electronic design that have accompanied each new MS platform. This increased number of scans per function improves precision, reproducibility, and sensitivity.

SUMMARY

The Xevo TQ-S micro is a tandem mass spectrometer designed to provide rapid, reliable, and reproducible quantitative data and to deliver consistent low levels of quantitation over a wide dynamic range. This technical brief has highlighted the increased response of the Xevo TQ-S micro when using a preconfigured targeted MRM qualitative screening method; when compared to data collected from the same samples on the Xevo TQD an improvement in the number of true positives was observed. The ACQUITY UPLC I-Class/Xevo TQ-S micro is a highly-versatile instrument for use in toxicology, providing the user with both broad qualitative screening capabilities as well as high sensitivity quantitative detection on the same instrument platform.

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4. Xevo TQ-S micro Product Brochure. 2014 Waters Marketing Brochure, 720005046EN.
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Forensic Toxicology Screening Using the ACQUITY UPLC I-Class System with the Xevo TQD

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APPLICATION BENEFITS

Two complementary methodologies for comprehensive toxicological screening using the latest generation of instrumentation. The ACQUITY UPLC® I-Class System and Xevo® TQD MS:

- A targeted MRM method utilizing two transitions per compound that can screen for a panel of 178 key analytes with excellent sensitivity and selectivity
- A full scan MS method incorporating a spectral library for over 950 toxicologically relevant substances that can be easily appended by the user

WATERS SOLUTIONS

ACQUITY UPLC I-Class System

Xevo TQD Mass Spectrometer

RADAR Technology ™

PIC® Scanning

ACQUITY UPLC HSS C_{18} Column

Waters® Total Recovery Vials

KEY WORDS

Forensic toxicology, targeted analysis, non-targeted screening, toxicants, UPLC®/MS/MS

INTRODUCTION

Forensic toxicology laboratories require broad screening techniques that can detect toxicants in highly complex biological matrices, such as ante- and post-mortem specimens. Two alternative toxicological screening methods, each utilizing the ACQUITY UPLC System with ACQUITY® TQD, have been previously described.[1-3] These two complementary approaches allow the user to take full advantage of the benefits associated with full scan data acquisition and the improved sensitivity associated with targeted MRM screening.[4]

These methods have been successfully implemented in over one hundred laboratories worldwide, including those with little or no previous LC/MS experience. With the availability of a new generation of instruments offering improved functionality and performance, there is an interest in applying these powerful and proven screening methods to the new systems. This application note presents a preliminary evaluation of the applicability of the existing two screening methods to the ACQUITY UPLC I-Class System and Xevo TQD Mass Spectrometer.

The goals of this study were to evaluate the utility of the ACQUITY UPLC I-Class System and Xevo TQD for forensic toxicology screening and to assess the applicability of the existing chromatographic method combined with the existing full scan MS and MRM methodologies[1-3] on this new platform.

EXPERIMENTAL

Sample Description

Drug standards were obtained from Sigma-Aldrich® (Poole, Dorset, UK) and LGC Standards (Teddington, Surrey, UK) and were either solid chemicals or certified solutions at concentrations of 1 mg/mL. Ammonium formate and formic acid were from Sigma-Aldrich. Acetonitrile was obtained from Greyhound Chromatography (Birkenhead, UK). Authentic urine samples were obtained from collaborators for routine screening.

Authentic urine samples were extracted by liquid/liquid extraction and transferred to Waters Total Recovery Vials. Extracts were injected onto both the ACQUITY UPLC I-Class/Xevo TQD and the original configuration of the classic ACQUITY UPLC/TQD.

UPLC conditions

System:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS C_{18}, 2.1 x 150 mm, 1.8 µm, part number 186003534
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	5 µL (MRM) or 10 µL (full scan)
Needle wash solvent:	5 mM ammonium formate, pH 3.0
Purge solvent:	5 mM ammonium formate, pH 3.0
Flow rate:	400 µL/min
Mobile phase A:	5 mM ammonium formate, pH 3.0
Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	13% mobile phase B increasing to 95%, with 15-minute analysis time

These are the same conditions that were previously used with the ACQUITY UPLC System.[1,2]

MS conditions

Mass spectrometer:	Xevo TQD
Ionization mode:	ESI positive (and ESI negative in full scan)
Capillary voltage:	3.0 kV
Cone voltage:	Varies according to method
Collision energy:	Varies according to method
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Cone gas:	20 L/h
Acquisition mode:	Multiple Reaction Monitoring (MRM) or full scan MS
Data management:	MassLynx® incorporating TargetLynx™ and ChromaLynx™ application managers

These are the same conditions that were previously used on the ACQUITY TQD.[1,2]

INNOVATIVE TECHNOLOGIES

The ACQUITY UPLC I-Class System with Flow-Through Needle (FTN)[5] design, shown in Figure 1, ensures that the sample comes into contact with only the needle and the direct flow path to the UPLC column. The sample is not transferred to a loop, which minimizes sample carryover; therefore, confidence in results is improved. In addition, the system volume has been reduced to 100 µL, which produces less analyte dispersion and improves peak shape. The column heater has also been improved with the inclusion of the active pre-heater (APH) reducing gradient delay and extra-column band-spreading.

The latest Xevo TQD,[6] shown in Figure 1, incorporates additional instrument features which could be highly advantageous to forensic analysis, such as RADAR and PICs. RADAR offers the capability to acquire full scan information while performing MRM analysis, which is a very useful tool for method development and troubleshooting. Product Ion Confirmation scanning (PICs) provides the option to automatically trigger a product ion scan when a particular MRM peak is detected. This allows the analyst to view additional confirmatory data and improve analyte identification.

Figure 1. The ACQUITY UPLC I-Class with Xevo TQD Mass Spectrometer.

RESULTS AND DISCUSSION

Overview of the MRM and full scan MS techniques

The original MRM method screened for 178 commonly encountered substances. The acquisition method was arranged into 30 time windows over the chromatographic elution range in order to improve the efficiency of data collection and to ensure sufficient data points for peak characterization. Each MRM time window was configured so that the start of the window was 0.5 minutes before the first eluting compound, and the end of the window was 0.5 minutes after the last eluting compound. Therefore, a key element of this study was an evaluation of the transferability of retention time (RT) and to assess whether the original MRM time windows were still applicable on the ACQUITY UPLC I-Class System.

The original full scan MS method generated a comprehensive catalogue of mass spectral data by acquiring data at seven different cone voltages (+20 V, +35 V, +50 V, +65 V, +80 V, +95 V, and -20 V). This data was automatically compared to a library comprised of spectral data for more than 950 substances using the ChromaLynx Application Manager.

In this study, ChromaLynx was used to compare spectral data acquired on the new MS system with the spectra contained in the original ACQUITY TQD library.

System suitability mixture injections using MRM and full scan acquisition modes

It is good laboratory practice to verify the performance of any analytical system prior to acquiring authentic sample data. This is commonly achieved by injecting a system suitability mixture (SSM) that contains a combination of substances that elute over the entire chromatographic range.

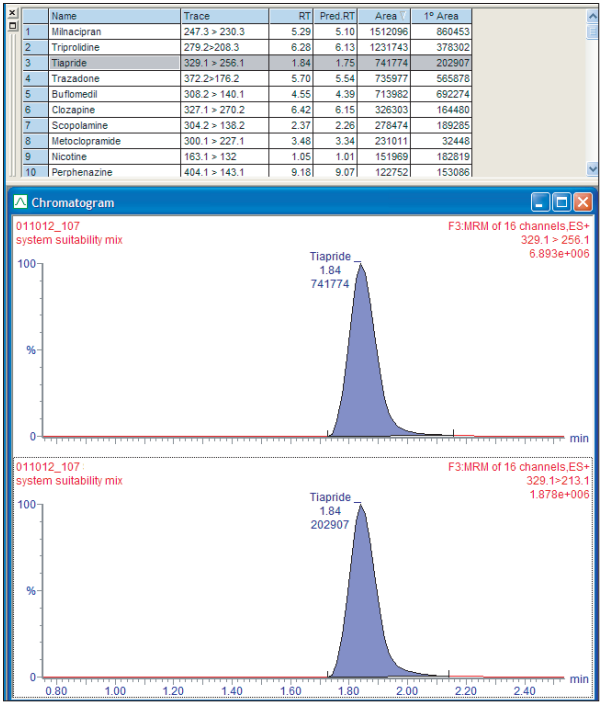

Figure 2. TargetLynx browser displaying the results obtained with a typical SSM following targeted MRM screening using an ACQUITY UPLC I-Class/Xevo TQD. The RTs for all ten components detected were within 0.3 minutes of the expected RT and well within the original MRM time windows. This indicates successful transfer of the chromatography method to the ACQUITY UPLC I-Class System.

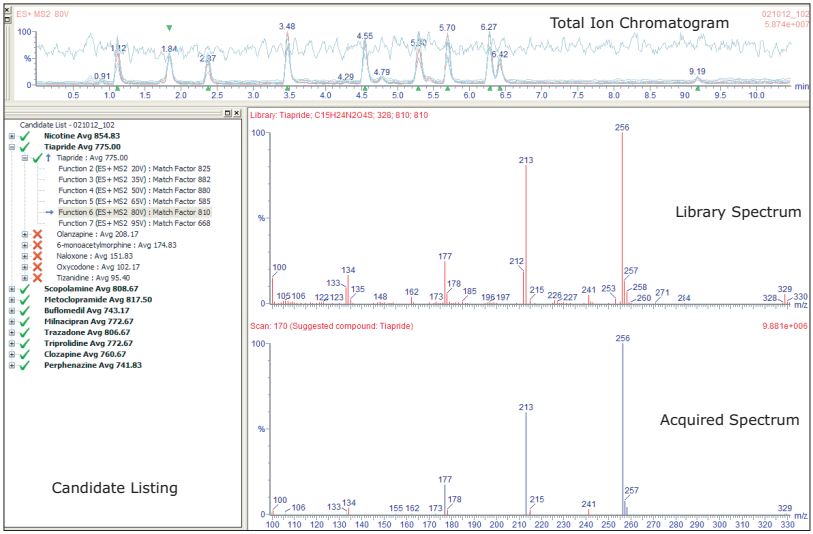

Figure 3. ChromaLynx browser displaying results of the full scan screening analysis of the SSM. Again, all ten components were detected, each one with a high average (Avg) match factor, indicating excellent agreement with the existing spectral library.

Analysis of an authentic urine sample by targeted MRM screening

The results for a urine sample submitted for routine forensic toxicological screening are shown in Table 1. The RTs of all identified substances were within 0.19 minutes of the expected RT, again supporting excellent transferability of the existing chromatographic method to the ACQUITY UPLC I-Class System.

The identification of drug metabolites within a biological specimen can be highly beneficial for the following reasons: they can be used to extend the window of drug detection; provide additional confirmation of drug intake; and generally assist in data interpretation. In this sample, methadone and its metabolite EDDP, as well as cocaine and its metabolite, benzoylecgonine, were identified by both systems. Figure 4 shows the TargetLynx browser detailing the results from the ACQUITY UPLC I-Class/Xevo TQD MRM analysis. Some additional substances were detected with the new system configuration suggesting enhanced sensitivity; this was also supported by the observation that the peak areas obtained with the new configuration were generally larger than those seen with the ACQUITY UPLC/TQD. This may be as a result of the new ACQUITY UPLC I-Class FTN, designed to minimize peak dispersion and maximize peak response.

		ACQUITY UPLC/TQD (original configuration)		ACQUITY UPLC I-Class/Xevo TQD (new configuration)	
Compound Name	**Predicted RT**	**Found RT**	**Peak Area**	**Found RT**	**Peak Area**
Methadone	8.61	8.77	434734	8.80	463948
EDDP	7.46	7.67	145310	7.65	149118
Paracetamol	1.50	1.58	5703	1.55	55966
Cocaine	4.61	4.80	11644	4.75	11702
Benzoylecgonine	2.97	3.13	10500	3.09	11261
Nicotine	1.01	1.02	3284	1.05	6143
Caffeine	2.10	2.19	1499	2.16	3866
Temazepam	9.34	–	–	9.46	3683
Oxazepam	8.07	8.17	1853	8.18	2528
Theophylline	1.46	–	–	1.45	2255
Nordiazepam	9.14	–	–	9.31	1297

Table 1. Results for an authentic urine sample analyzed by MRM targeted screening using both original and newer instrument configurations.

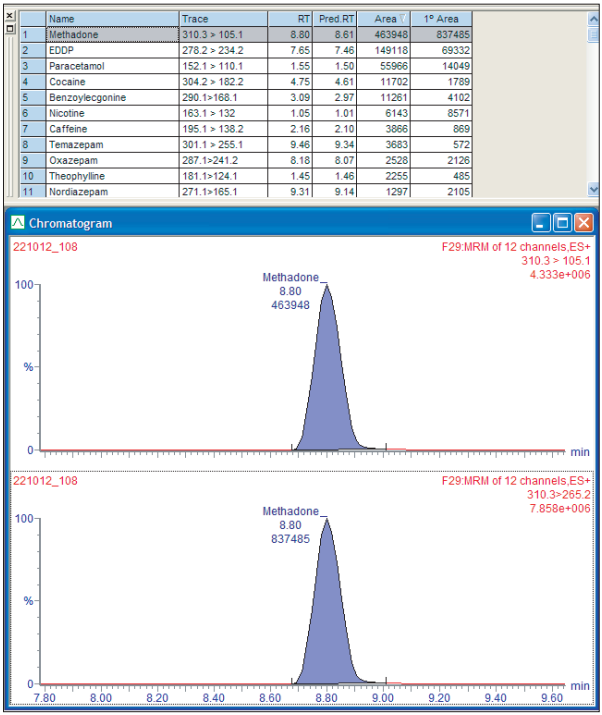

Figure 4. TargetLynx browser displaying processed results from a urine sample extract showing result for methadone.

Analysis of an authentic toxicology urine sample by full scan MS

A major advantage of the full scan MS approach is the ability to simultaneously screen for extremely large numbers of substances (limited only by the size of the spectral library). This is in contrast to the targeted MRM approach which is restricted to a panel of key analytes. At this time, a library comprising spectral data for more than 950 substances is available from Waters. Additionally, this library can be very easily expanded by the user. Alternatively, new libraries may be created and multiple libraries searched using the ChromaLynx Application Manager. Another benefit of this approach is that the data is not restricted to specific channels; thus the complete data set is available for retrospective analysis.

Figure 5 shows the results for another urine sample screened using the full scan MS method on the new system. In this sample the metabolite mirtazapine N-desmethyl was found, as well as the parent compound, mirtazapine. The spectrum window within the ChromaLynx browser clearly shows a good match for the fragments of mirtazapine N-desmethyl compared with the library spectrum that was originally acquired using the ACQUITY TQD. This indicates that the existing library acquired on ACQUITY TQD can be used with the newer Xevo TQD.

The full scan MS method also detects another compound, xylometazoline. This nasal decongestant would not be identified by the targeted MRM method because the substance is not currently included in the targeted panel.

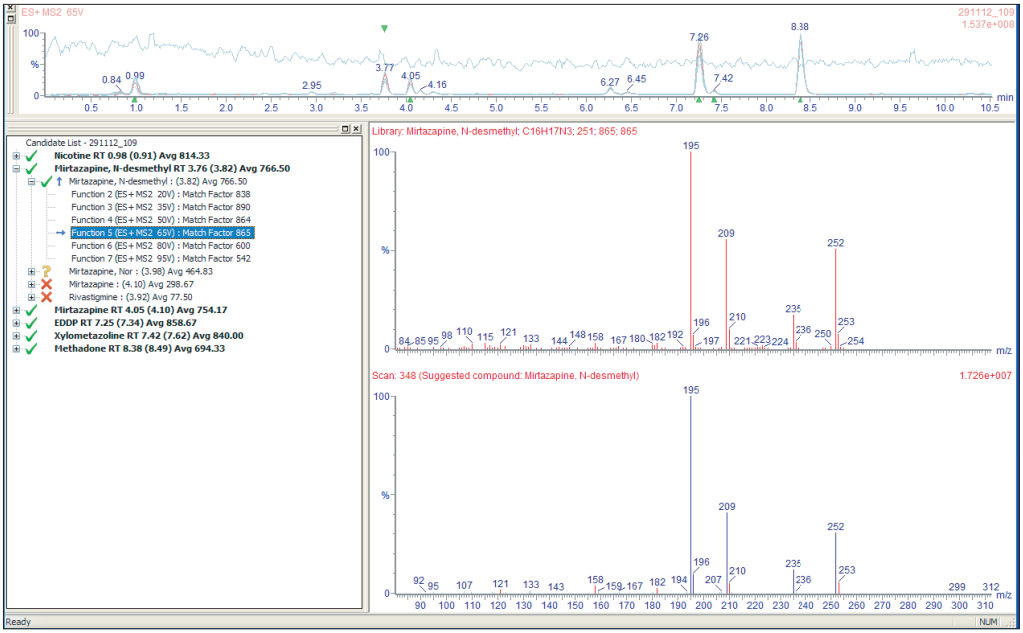

Figure 5. ChromaLynx browser displaying results from an analysis of urine sample extract showing results for the N-desmethyl metabolite of mirtazapine.

Using PICs to distinguish between hydromorphone and morphine

A useful feature of the new Xevo TQD is the ability to trigger data-directed Product Ion Confirmation scanning (PICs) during MRM analysis. This enables very similar compounds to be distinguished from one another by the pattern of their fragmentation from precursor into product ions.

Figure 6 shows the data obtained following analysis of hydromorphone using the MRM screening method. Responses were obtained in the two MRM channels for hydromorphone but also for morphine. This is not surprising, as these two substances are isomers sharing several MRM transitions. Furthermore, they elute within 0.2 minutes of one another using the chromatography described in this application note. Data generated from PIC scanning, however, may be used to provide additional information, which may assist in the differentiation of similar substances.

In this example, the PICs data produced a better match with a previously saved reference spectrum for hydromorphone than the one for morphine. The analyst is able to select both reference spectra from within the TargetLynx method to allow visual comparison of the spectra.

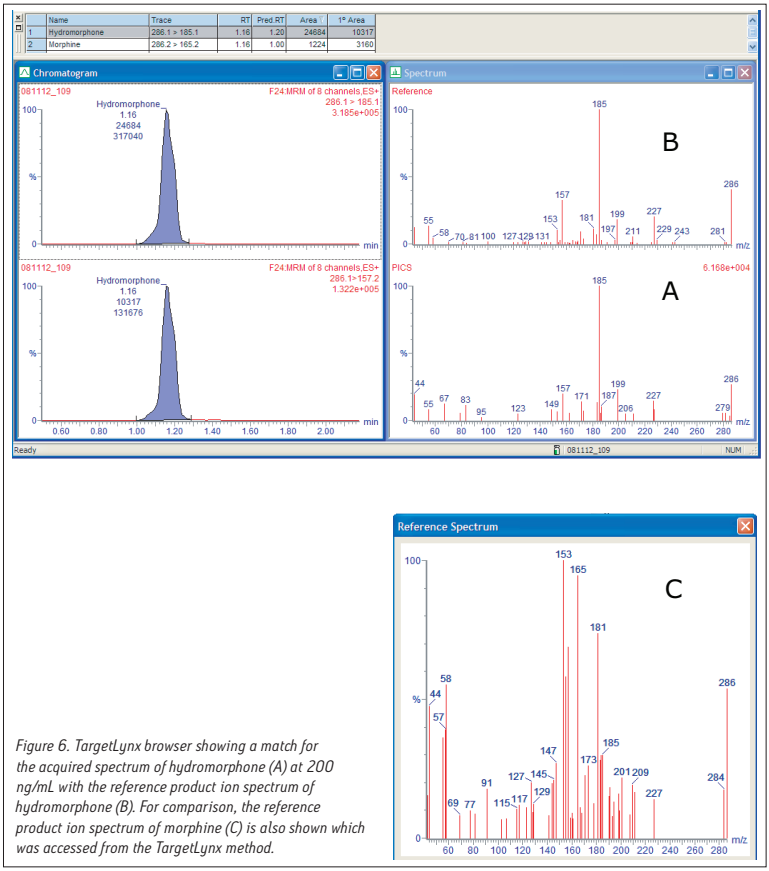

Figure 6. TargetLynx browser showing a match for the acquired spectrum of hydromorphone (A) at 200 ng/mL with the reference product ion spectrum of hydromorphone (B). For comparison, the reference product ion spectrum of morphine (C) is also shown which was accessed from the TargetLynx method.

Using RADAR to indicate the presence of extra analytes

The urine sample analyzed by full scan MS was also analyzed using the MRM targeted screening method with RADAR enabled. This allows full scan data to be collected while performing conventional MRM analysis and will show peaks that would potentially be missed if the analytes were not in the MRM method. Figure 7 shows the full scan data from this analysis with a selection of the MRM data that was simultaneously acquired. The full scan peaks for the metabolites N-desmethyl mirtazapine, desmethyl citalopram, and the drug, xylometazoline, are clearly visible at 3.79, 6.47, and 7.42 minutes, respectively; however, they were not detected by the MRM method as these substances were not included in this targeted assay. Figure 8 shows the mass spectra of the two metabolite peaks acquired at a cone voltage of 30 V. This extra information can be very useful, particularly in complex biological specimens, as it can indicate the presence of unknown components that would typically remain undetectable in a targeted screening scenario. Moreover, it can be an invaluable tool for troubleshooting during method development and validation as it may be used to identify and resolve issues with co-eluting compounds.

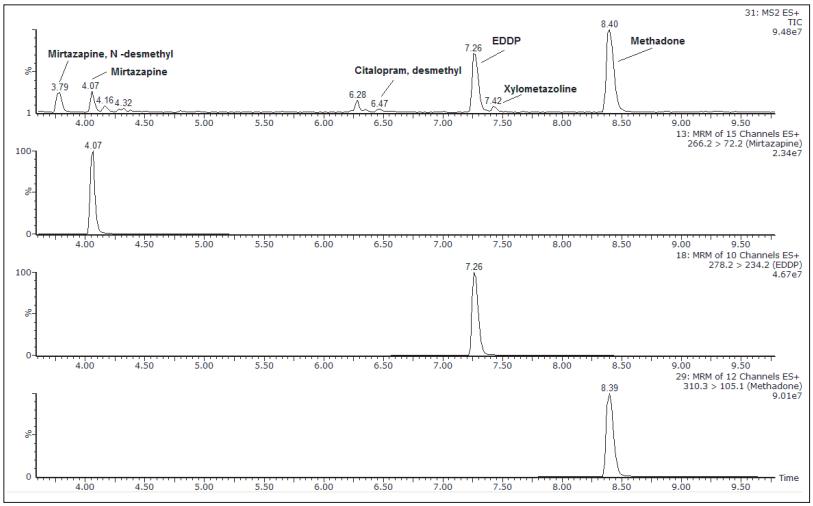

Figure 7. Full scan data (A) that was acquired simultaneously with MRM data for the hydrolyzed urine sample using RADAR. The full scan peak at 3.79 minutes was N-desmethyl mirtazapine, at 6.47 minutes desmethyl citalopram, and at 7.42 minutes was xylometazoline which are not currently included in this targeted MRM method.

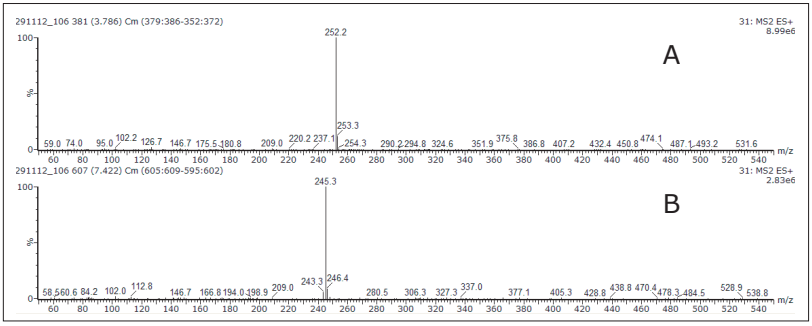

Figure 8. Full scan mass spectra (cone voltage 30 V) collected in RADAR mode for N-desmethyl mirtazapine at 3.79 minutes (A) and xylometazoline at 7.42 minutes (B).

CONCLUSIONS

Successful transfer of the toxicology screening methods to the next generation of the UPLC/MS system enables forensic analysts to access the latest analytical tools for their laboratories. The ACQUITY UPLC I-Class System has reduced peak dispersion and small system volume, thereby improving the sensitivity of the MS used in the assay. The Xevo TQD has the new features of RADAR and PICs, which can be used to enhance the information that is available to the analyst.

Starter projects are available that contain all the necessary methods to both acquire and process the data. The preconfigured methods contain a large number of the most commonly encountered toxicants and are ready for laboratory implementation with minimal user intervention.

The methods are fully customizable and can be easily appended to meet the scientists' needs. For example, additional compounds can be added to the databases. This ensures that the methods are versatile and will remain relevant for the future.

A full validation by the user would be necessary prior to adoption in a laboratory.

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[TECHNOLOGY BRIEF]

Using UPLC-MS/MS for Workplace Drug Testing

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GOAL

To develop a UPLC®-MS/MS method for the analysis of 21 substances, commonly measured in workplace drug testing (WPDT) schemes, using a simple dilution of the urine prior to analysis.

BACKGROUND

In recent years WPDT laws have been implemented in certain geographies for workers employed in specific industry sectors, particularly those in safety-critical job roles such as transportation (pilots, train/bus drivers), nuclear–safety employees and construction. Random drug testing in the workplace is aimed, not only at reducing costs in terms of lost productivity and absenteeism, but also at ensuring safety for the individual and the wider community[1].

After prior notification workers provide a urine sample which is commonly screened for a variety of drugs including; opiates, methadone, buprenorphine, cocaine, amphetamines, and cannabinoids by a technique such as immunoassay. Any samples containing analytes above a pre-defined cut-off level (putative positives) are then confirmed by a different technique, often GC-MS or LC-MS/MS.

A simple sensitive UPLC-MS/MS method for substances commonly measured in workplace drug testing schemes.

For some analytes immunoassay is not sufficiently specific and can only indicate the presence of a certain class of compounds rather than pinpoint the actual compound present. In contrast, the use of UPLC-MS/MS for screening can provide a specific, semi-quantitative tool for determining the samples that are positive and improves overall efficiency of the testing process by reducing the number of false positives sent for confirmation.

THE SOLUTION

Combining the ACQUITY UPLC® I-Class System with the Xevo® TQD allows these compounds to be detected at levels lower than the currently applied cut-offs and permits a compound specific semi-quantitative determination of the relevant analytes.

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EXPERIMENTAL

Sample preparation

Internal standard (ISTD) mixture (0.05 mL) was added to 0.2 mL urine (either sample or calibrator), which was then vortex-mixed for 5 min at 1200 rpm then centrifuged at 8000 g for 10 min. Supernatant (0.125 mL) was added to 0.375 mL deionized water in a Waters® Maximum Recovery Vial.

Assay concentration of ISTDs was 25 ng/mL.

Chromatography conditions

Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 100 mm with BEH C_{18} 1.7 µm VanGuard™
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	8 µL
Wash solvent:	95% acetonitrile/5% water
Purge solvent:	0.1% formic acid
Flow rate:	400 µL/min
Mobile phase A:	0.1% formic acid
Mobile phase B:	acetonitrile

Gradient

Time (min)	%B	Curve
0	2	Initial
1.5	13	6
1.8	13	6
2.65	36	6
3.00	36	6
3.40	50	6
3.45	95	6
4.75	95	6
4.80	2	6

Mass Spectrometry conditions

MS system:	Xevo TQD
Ionization mode:	ESI with polarity switching
Capillary voltage:	1.0 kV positive, 2.95 kV negative
Source temp.	150°C
Desolvation temp.	500°C
Desolvation gas:	800 L/Hr
Cone gas:	20 L/Hr

Multiple reaction monitoring conditions

Peak #	Compound	RT (min)	Time window (min)	MRM transitions	Cone voltage (V)	Collision energy (eV)	ISTD
1	Normorphine	1.32	1.0–1.80	**272.1>201.1** 272.1>165.1	50	**24** 35	Morphine-d6
2	Morphine	1.45	1.0–1.90	**286.1>201.1** 286.1>153.1	50	**24** 38	Morphine-d6
3	Norcodeine	2.10	1.90–2.35	**286.1>165.1** 286.1>153.1	45	**40** 34	Morphine-d6
5	Dihydrocodeine	2.12	1.95–2.35	**302.1>199.0** 302.1>171.0	50	**39** 32	Morphine-d6
6	Codeine	2.17	1.95–2.35	**300.1>215.1** 300.1>165.0	55	**25** 38	Morphine-d6
8	6-Monoacetylmorphine (6-MAM)	2.48	2.30–2.70	**328.0>165.1** 328.0>211.1	52	**35** 25	Morphine-d6
4	Ephedrine	2.11	1.90–2.30	**166.1>117.0** 166.1>133.1	25	**18** 18	Amphetamine-d11
7	Amphetamine	2.39	2.20–2.60	**136.0>119.0** 136.0>91.0	20	**8** 12	Amphetamine-d11
9	MDA	2.49	2.30–2.70	**180.1>163.0** 180.1>133.0	18	**8** 16	Amphetamine-d11
10	Methamphetamine	2.63	2.45–2.85	**150.0>119.0** 150.0>91.0	25	**10** 16	Amphetamine-d11
11	MDMA	2.69	2.50–2.90	**194.1>163.0** 194.1>105.0	24	**11** 24	Amphetamine-d11
12	MDEA	2.90	2.70–3.10	**208.1>163.0** 208.1> 72.0	22	**12** 12	Amphetamine-d11
15	MBDB	3.02	2.85–3.25	**208.1>177.1** 208.1> 135.0	25	**11** 13	Amphetamine-d11
13	Benzoylecgonine (BZE)	2.95	2.75–3.15	**290.1>168.1** 290.1>82.0	36	**18** 28	BZE-d8
14	Ketamine	2.95	2.75–3.15	**238.0>207.1** 238.0>125.0	28	**12** 24	BZE-d8
16	Cocaine	3.22	3.05–3.45	**304.0>182.1** 304.0>82.1	40	**18** 28	BZE-d8
17	Norbuprenorphine-glucuronide	2.90	2.70–3.10	**590.3>414.3** 590.3>101.0	70	**35** 55	Buprenorphine-d4
18	Norbuprenorphine	3.30	3.15–3.55	**414.1>101.0** 414.2> 83.0	55	**38** 48	Buprenorphine-d4
19	Buprenorphine-glucuronide	3.20	3.00–3.40	**644.3 >468.2** 644.3 >101.0	65	**40** 65	Buprenorphine-d4
20	Buprenorphine	3.61	3.35 - 3.80	**468.2>396.2** 468.2>101.0	60	**38** 42	Buprenorphine-d4
21	EDDP	3.70	3.50 - 3.95	**278.1>249.2** 278.1>234.2	50	**22** 28	Methadone-d9
22	Methadone	3.98	3.80–4.25	**310.1>265.2** 310.1>105.1	30	**16** 28	Methadone-d9
23	Carboxy-THC (cTHC)	4.45	4.20–4.80	**343.1>299.2** 343.1>245.2	45	**22** 28	cTHC-d3
24	cTHC-glucuronide	4.70	4.20–5.20	**519.2 >343.2** 519.2>299.2	40	**22** 34	cTHC-d3
	Morphine-d6	1.45	1.00–1.90	292.1>201.1	50	24	
	Amphetamine-d11	2.35	2.25–2.70	147.0>130.0	20	8	
	BZE-d8	2.95	2.75–3.25	298.1>171.1	36	18	
	Buprenorphine-d4	3.60	3.35–3.80	472.2>400.2	60	38	
	Methadone-d9	4.00	3.80 - 4.25	319.1>268.2	30	16	
	cTHC-d3	4.45	4.20–4.80	346.3>302.2	40	22	

RESULTS

The acceptance criteria for a positive identification of analytes were the retention time to be within 0.1 min of predicted and the quantifier/qualifier ion ratio to be within 20% of the predicted ratio, which was based on the average of the ratios across the entire calibrator range. Figure 1 shows a chromatogram of a urine calibrator spiked at 25 ng/mL.

To investigate linearity for all analytes, spiked urine calibrators were prepared from 1 ng/mL to 500 ng/mL, except for norbuprenorphine, buprenorphine, their respective glucuronides and cTHC-glucuronide which were from 1 to 250 ng/mL; calibrators were prepared and analysed over five consecutive days.

Peak areas for each MRM trace were generated using the TargetLynx™ Application Manager and referenced to the appropriate ISTD peak area. Semi-quantitative calibration curves were plotted using a 1/x weighting. A quadratic fit was applied to all analytes except the following where a linear fit was applied; normorphine, morphine, norcodeine, cocaine, buprenorphine, EDDP, and methadone. Interday correlation coefficient or coefficient of determination (assessed over five days) was >0.995 for each analyte.

The limit of detection (LOD) was defined as the lowest concentration which gave a signal-to-noise ratio >10:1 (for both transitions) in spiked urine. The lower limit of quantitation (LLOQ) was defined as the lowest concentration which gave a signal to noise ratio >10:1 (for both transitions) and ion ratios within 20% of expected and a %RSD <20% in spiked urine. The LOD and LLOQ for each analyte are summarized in Table 1 along with the assay calibration range.

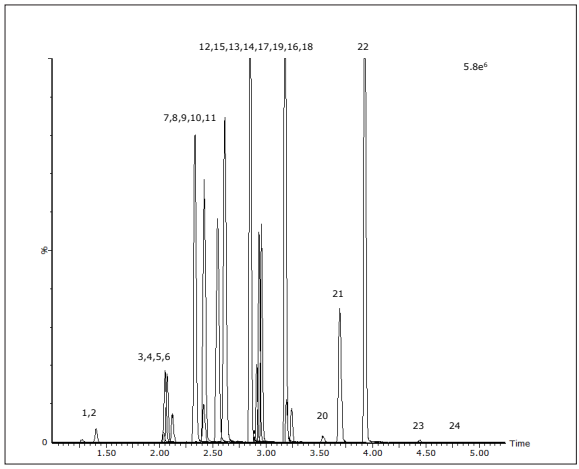

Figure 1. Chromatogram of a urine calibrator spiked at 25 ng/mL. Peak assignments are listed in Table 1.

Peak #	Compound	LOD spiked urine (ng/mL)	LLOQ spiked urine (ng/mL)	Assay range (ng/mL)
1	Normorphine	2	4	4-500
2	Morphine	2	2	2-500
3	Norcodeine	2	2	2-500
5	Dihydrocodeine	0.25	0.5	2-500
6	Codeine	0.25	2	2-500
8	6-MAM	0.5	2	2-500
4	Ephedrine	0.25	1	1-500
7	Amphetamine	0.5	0.5	1-500
9	MDA	1	1	1-500
10	Methamphetamine	0.25	0.5	1-500
11	MDMA	1	1	1-500
12	MDEA	0.25	0.5	1-500
15	MBDB	0.25	0.25	1-500
13	BZE	0.5	0.5	1-500
14	Ketamine	0.25	0.5	1-500
16	Cocaine	0.25	0.25	1-500
17	Norbuprenorphine-glucuronide	2	5	5-250
18	Norbuprenorphine	1	1	1-250
19	Buprenorphine-glucuronide	2	2	2-250
20	Buprenorphine	0.5	2	2-250
21	EDDP	0.25	0.25	1-500
22	Methadone	0.25	0.25	1-500
23	cTHC	2	4	4-500
24	cTHC-glucuronide	4	5	5-250

Matrix effects were investigated at the following concentrations: 10 ng/mL (low), 50 ng/mL (medium) and 250 ng/mL (high), except for norbuprenorphine, buprenorphine, their respective glucuronides and cTHC-glucuronide which were determined at 5 ng/mL (low), 25 ng/mL (medium) and 125 ng/mL (high). The results showed the matrix effect to be less than 20% for the majority of analytes.

Interday accuracy and precision were assessed by analysing three quality control (QC) concentrations (15, 150 and 300 ng/mL, except for norbuprenorphine, buprenorphine, their respective glucuronides and cTHC-glucuronide which were determined at 7.5, 75, and 150 ng/mL) over five different days. The mean achieved values for the quality control replicates over the five day period at the three concentration levels were within 15% of target and the % RSD was <15%.

Analysis of authentic urine samples

Two commercial quality control reference urines and 114 authentic urine samples were analyzed using the described UPLC-MS/MS method.

The method detected and correctly assigned the analytes in both commercial reference urines. The semi-quantitative results obtained using this UPLC-MS/MS method for the analysis of the Bio-Rad Liquichek™ level C2 reference urine were in accordance with the manufactured stated reference values (Figures 2 and 3).

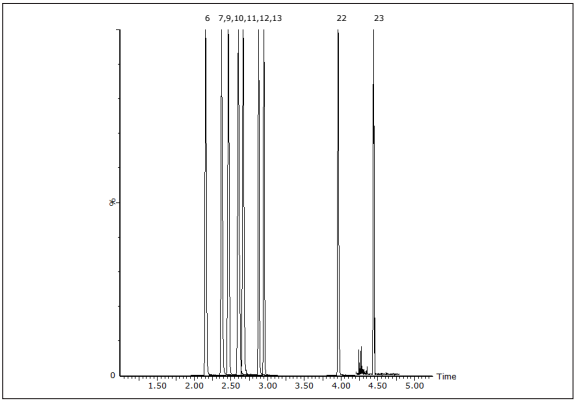

Figure 2. Chromatogram of Bio-Rad Liquichek level C2 reference urine (peaks are scaled to maximum response). Peak assignments are listed in Table 1.

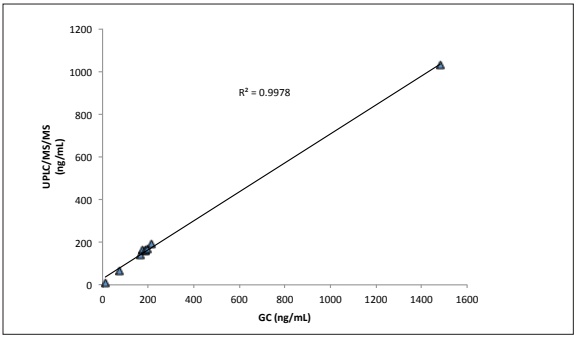

Figure 3. Comparison between the semi quantitative UPLC-MS/MS analysis of Bio-Rad Liquichek level C2 reference urine and GC analysis.

The authentic samples comprised anonymised samples that had previously been screened using either the Beckman Olympus AU400 or the Abbott Architect 4000 immunoassay system. Any sample that had screened positive by either immunoassay technique had been sent for subsequent confirmation by GC-MS. Eleven of these samples gave putative positives for buprenorphine, methadone, amphetamines and cTHC, but were not confirmed by the subsequent GC-MS assays; all of these samples were negative by the UPLC-MS/MS based screen. Sixty samples were shown to contain at least one and, in some instances, multiple analytes; this was in agreement with the GC-MS confirmation data. Some additional analytes were found that had not been confirmed by the various GC-MS assays as their concentration was below the applied immunoassay cut-offs (opiates 300 ng/mL, amphetamines 500 ng/mL, BZE 300 ng/mL, methadone 500 ng/mL, buprenorphine 5 ng/mL and cannabinoids 50 ng/mL). Figure 4 shows the chromatogram of a sample that screened positive for cTHC yet negative for BZE. In this sample set BZE was the most commonly detected analyte by this UPLC-MS/MS method and was detected in 25 of the 114 samples.

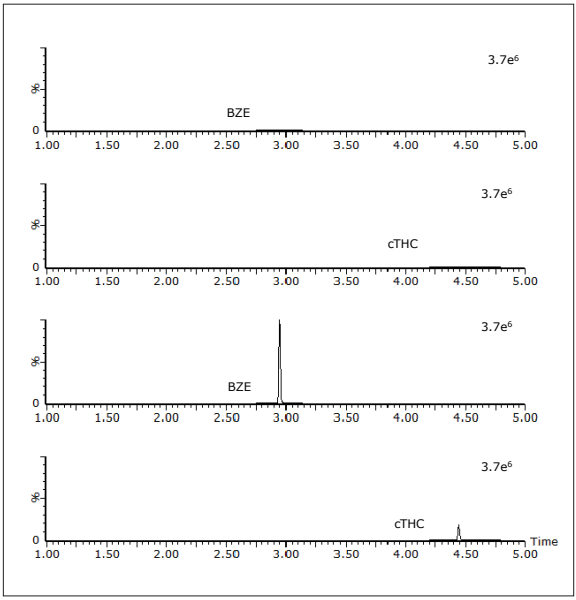

Figure 4. Chromatogram of a sample that screened positive for cTHC yet negative for BZE by immunoassay but by UPLC-MS/MS was shown to contain BZE at 50 ng/mL (lower than the 300 ng/mL cut-off). A negative sample is shown for comparison (upper traces). The traces show the quantifier ions for both analytes only (ISTDs not shown).

SUMMARY

The rise of workplace drug testing has highlighted the need for a quick, accurate, reliable and robust method to initially screen the large number of samples. The developed approach meets these requirements and demonstrates excellent correlation with GC-MS methods.

The use of the ACQUITY UPLC® I-Class system allows for a quick and robust analytical method that can detect all the analytes in a single run, with an injection to injection time of 7 min combined with the simple sample dilution used allows for high sample throughput. Furthermore the superior sensitivity of the Xevo TQD permits detection of the analytes from a simple dilution of the sample at levels lower than the currently applied cut-offs and minimizes false positives.

Acknowledgements

CEDAM Italia, Bresso, Italy and Bianalisi Analisi Mediche, Carate Brianza, Italy for supplying the anonymised authentic urine samples.

References

1. Kazanga I *et al.* Prevalence of drug abuse among workers: Strengths and pitfalls of the recent Italian workplace Drug Testing (WPDT) legislation. *Forensic Science International.* 2012; 215 (1-3): 46–50.

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A Simplified, Mixed-Mode Sample Preparation Strategy for Urinary Forensic Toxicology Screening by UPLC-MS/MS

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APPLICATION BENEFITS

- Rapid, simplified sample preparation of a comprehensive forensic toxicology panel in 30 minutes or less
- Significant savings in solvent usage and disposal costs
- Greater than 90% recovery for 36 out of 38 compounds
- Analysis of 38 compounds in 4 minutes
- Enhanced retention of polar compounds for the analysis of glucuronidated metabolites
- All sample pretreatment and extraction performed in-well, eliminating transfer steps

WATERS SOLUTIONS

- Xevo® TQD Mass Spectrometer
- ACQUITY UPLC® I-Class System (fixed loop injector)
- ACQUITY UPLC BEH Phenyl, 1.7 µm, 2.1 x 100 mm Column (p/n 186002352)
- Oasis® MCX µElution Plate (p/n 186001830BA)
- MassLynx® Software
- TargetLynx™ Application Manager

KEY WORDS

SPE, sample preparation, forensic toxicology, pain panel, opioids, benzodiazepines, amphetamines, urine, stimulants

INTRODUCTION

In forensic toxicology, drug-screening panels often include commonly used substances such as opiates, benzodiazepines, and stimulants. Often, multiple screening 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. Regardless of the methods used, the goal is to achieve sufficient sensitivity, specificity, and accuracy to proceed with the appropriate confirmation, or alternatively, to be confident a sample tests negative.

Sample preparation is as important a consideration in forensic toxicology screening as the choice of instrumentation technique. While many laboratories use a "dilute and shoot" approach for urinary toxicology panels, the presence of matrix components, buffers, residual enzymes, and other substances in the sample can result in excessive matrix effects, significantly reduced column lifetimes, and increased instrument downtime resulting from contaminant buildup on electrospray sources in LC-MS.

Though solid phase extraction (SPE) is often perceived as difficult or time-consuming, a judicious choice of method can simplify this process significantly. The most selective method of sample preparation, SPE results in cleaner samples than most other techniques, making it ideal for obtaining accurate results.

Here we detail a single sample preparation and UPLC®-MS/MS analysis strategy for a comprehensive panel of compounds often analyzed in forensic toxicology screens. In an abbreviated, modified extraction method, Waters' Oasis MCX µElution Plates are used to rapidly extract a panel that includes opioids, amine stimulants, benzodiazepines, benzoylecgonine (BZE), and phencyclidine (PCP). UPLC-MS/MS analysis is achieved using a Waters® ACQUITY UPLC BEH Phenyl Column and a Xevo TQD. All sample preparation steps, including enzymatic hydrolysis, are performed within the wells of the micro-elution plates, and the extraction method is simplified, eliminating conditioning and equilibration steps, and consolidating the wash procedures into a single step.

EXPERIMENTAL

All standards were obtained from Cerilliant (Round Rock, TX). Stock solutions were prepared in methanol. Samples were prepared by diluting stock solutions into pooled, blank urine. All analytes are listed in Table 1.

SPE extraction

50 µL of urine was added to individual wells of an Oasis MCX µElution Plate (p/n 186001830BA), along with 50 µL of 0.5 M ammonium acetate buffer and 10 µL of β-glucuronidase enzyme (Roche, *E. coli*) to simulate all the reagents added for enzymatic hydrolysis. 200 µL of 4% H_3PO_4 was then added and each sample was mixed by several aspirations. The samples were then drawn into the sorbent bed by vacuum. All samples were subsequently washed with 200 µL of 20% MeOH containing 0.02 N HCl. After washing, the plate was dried under high vacuum (~15 inches Hg) for 5–10 minutes, to remove as much of the wash solution as possible. Samples were then eluted with 2 x 50 µL of 60:40 ACN:MeOH containing 5% strong ammonia solution (Fisher, 28–30%). All samples were evaporated to dryness under nitrogen at 40 °C and reconstituted with 50 µL of sample diluent (2% ACN:1% formic acid in MilliQ water). Figure 1 depicts the workflow of the extraction procedure.

Method conditions

LC conditions

LC system:	ACQUITY UPLC I-Class (FL)
Column:	ACQUITY UPLC BEH Phenyl, 1.7 µm, 2.1 x 100 mm (p/n 186002352)
Column temp.:	40 °C
Sample temp.:	10 °C
Injection vol.:	15 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)
Gradient:	Initial conditions were 95:5 MPA/MPB. The percentage of MPB was increased to 62.5% over 5 minutes, returned to 5% over 0.1 minutes, and remained at 5% for 0.9 minutes. The entire cycle time was 6.0 minutes.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI positive
Acquisition range:	MRM transitions optimized for individual compounds
Capillary voltage:	1.0 kV
Collision energy:	Optimized for individual compounds (see Table 2)
Cone voltage:	Optimized for individual compounds (see Table 2)

Data management

MassLynx with TargetLynx Application Manager

Analyte recovery was calculated according to the following equation:

$$\%\text{Recovery} = \left(\frac{\text{Area A}}{\text{Area B}}\right) \times 100\%$$

Where A = the peak area of an extracted sample and B = the peak area of an extracted matrix sample to which the compounds were added post-extraction.

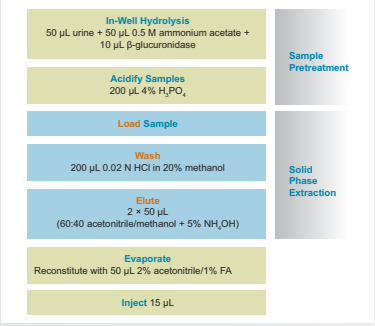

Figure 1. Details of the extraction method for the analysis of a comprehensive forensic toxicology panel using Oasis MCX µElution Plates. Enzymatic hydrolysis and sample pretreatment are performed in the wells of the extraction plate, minimizing transfer steps. Conditioning and equilibration steps are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

RESULTS AND DISCUSSION

All test compounds are listed in Table 1, and Figure 2 shows their chromatography. The compounds are grouped into related classes to facilitate viewing. Table 2 lists the retention times and MS conditions of all compounds in their elution order. Previous work[1] demonstrates the increased ability, compared with other columns, of the ACQUITY UPLC BEH Phenyl Column to retain polar opiate compounds. Combined with the narrow peak shape of UPLC, this chromatographic method retains and separates even the most polar analytes, maintaining the resolution of isobaric compounds, while still allowing all compounds to elute within four minutes. Baseline resolution was readily obtained between all isobaric groups, including morphine-3-glucuronide, hydromorphone-glucuronide, and morphine-6-glucuronide, supporting the ability to accurately identify and quantify all compounds. Such resolution is useful for monitoring these compounds to ensure complete hydrolysis or in cases where direct quantification of these metabolites is desired. Baseline separation was also achieved between methamphetamine and phentermine, which share a major product ion and can interfere with each other.

Peak number	Compound	LOD (ng/mL)
1	Morphine-3-gluc	10
2	Morphine-6-gluc	10
3	Morphine	10
4	Oxymorphone	5
5	Hydromorphone	5
6	Amphetamine	1
7	Naloxone	5
8	Dihydrocodeine	1
9	Codeine	5
10	MDA	1
11	Noroxycodone	5
12	Methamphetamine	1
13	Phentermine	1
14	O-desmethyl tramadol	1
15	6-MAM	2
16	Oxycodone	1
17	MDMA	1
18	Hydrocodone	1
19	MDEA	1

Peak number	Compound	LOD (ng/mL)
20	Norfentanyl	1
21	7-Aminoclonazepam	5
22	BZE	1
23	Tramadol	1
24	Tapentadol	1
25	Norbuprenorphine	1
26	PCP	1
27	Fentanyl	1
28	Flurazepam	1
29	Buprenorphine	2
30	EDDP	1
31	Alpha-OH alprazolam	5
32	Methadone	1
33	Oxazepam	1
34	Lorazepam	5
35	Clonazepam	1
36	Alprazolam	1
37	Temazepam	1
38	Diazepam	1

Table 1. Compound list and limits of detection.

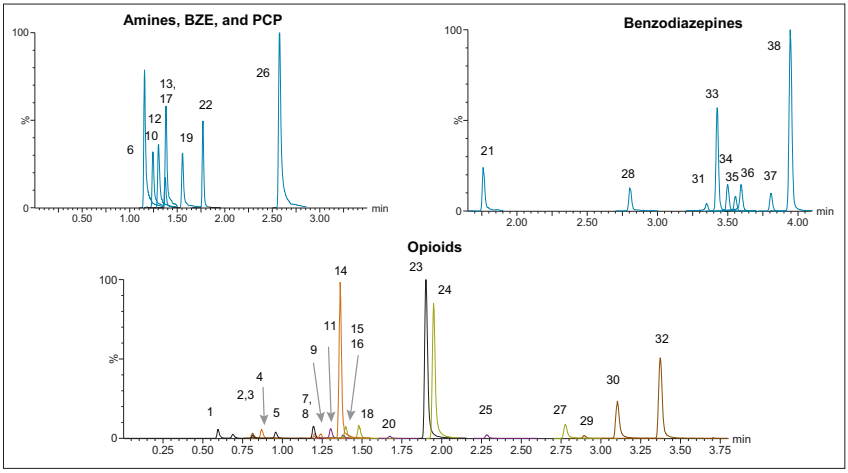

Figure 2. Chromatography of the comprehensive panel of forensic toxicology compounds analyzed in this application. Compounds are grouped into related categories for ease of viewing. See Table 1 for compound key.

Column: ACQUITY UPLC BEH Phenyl, 1.7 µm, 2.1 x 100 mm.

Compound	RT	M+H+	MRM product ions	Cone voltage	Collision energy
3-beta-morphine glucuronide	0.59	462.17	286.1 201.1	58 58	52 30
6-beta-morphine glucuronide	0.81	462.17	286.1 201.1	58 58	52 30
Morphine	0.81	286.2	201.1 165.1	54 54	28 34
Oxymorphone	0.86	302.2	284.2 227.1	44 44	30 37
Hydromorphone	0.95	286.1	185.0 157.0	65 65	46 62
Amphetamine	1.16	136	119.0 91.0	22 22	8 10
Dihydrocodeine	1.18	302.2	199.1 128.1	60 60	45 75
Naloxone	1.19	328.2	253.2 212.0	40 40	32 52
Codeine	1.23	300.2	165.1 199.1	58 58	54 42
MDA	1.25	180.1	163.0 105.0	22 22	110 22
Noroxycodone	1.29	302.1	187.1 227.1	36 36	26 30
Methamphetamine	1.31	302.1	91.0 119.1	24 24	20 10
Phentermine	1.37	150.0	91.0 133.1	24 24	20 10
O-desmethyl tramadol	1.35	250.2	58.2	30	20
6-acetyl morphine	1.36	328.2	165.1 211.1	56 56	58 40
Oxycodone	1.37	316.2	298.2 241.1	44 44	25 44
MDMA	1.39	194.1	163.0 105.0	26 26	12 22
Hydrocodone	1.48	300.2	199.1 171.1	56 56	40 58
MDEA	1.57	208.1	105.0 135.1	26 26	24 20
Norfentanyl	1.65	233.2	177.2 150.1	38 38	18 24
7-aminoclonazepam	1.77	286.1	121.1 222.1	48 48	26 26
Benzoylecgonine	1.78	290.1	168.1 105.0	36 36	18 32
Tramadol	1.87	264.2	58.2	30	25
Tapentadol	1.92	222.2	107.0 121.0	40 40	24 24
Norbuprenorphine	2.25	414.3	101.1 187.2	94 70	55 55
PCP	2.58	244.2	86.0 159.1	22 22	10 16
Fentanyl	2.74	337.3	105.1 188.2	50 50	56 36
Flurazepam	2.80	388.2	315.1 100.0	40 40	26 28
Buprenorphine	2.85	468.4	101.1 396.3	82 82	68 55
EDDP	3.05	278.2	234.2 249.2	60 60	40 33
Alpha-hydroxyalprazolam	3.35	325.1	297.1 243.1	50 50	26 38
Methadone	3.33	310.3	105.0 265.2	32 32	38 20
Oxazepam	3.43	287.0	104.0 241.1	44 44	30 32
Lorazepam	3.51	321.0	229.1 194.0	40 40	28 50
Clonazepam	3.56	316.0	214.1 241.1	54 54	42 40
Alprazolam	3.60	309.1	205.1 274.1	60 60	42 26
Temazepam	3.82	301.1	177.1 255.1	36 36	46 10
Diazepam	3.96	285.1	154.0 193.1	54 54	26 34

Table 2. Retention times and MS conditions of all compounds.

Figure 3 shows the extraction recoveries of the entire panel of compounds. With the exception of morphine-3-glucuronide, all compounds had recoveries greater than 80%. The average recovery was 100% for all compounds. Extraction efficiencies were also consistent. Coefficients of variation (%CV) were less than 10% for 37 of 38 compounds and only 12.5% for the remaining compound. A series of experiments performed during method development revealed that more than 20% methanol in the wash step resulted in loss of the acidic benzodiazapines, such as oxazepam, clonazepam, lorazepam, and temazepam. Thus, the single wash step consisted of 20% methanol containing 0.02 N HCl. This simple modification enabled the highly efficient extraction of the entire panel of compounds in a single method.

In addition to the benefit of extracting multiple drug classes using a single SPE method, the traditional six-step mixed-mode SPE method was simplified into just three steps. The conditioning and equilibration steps were eliminated, and the two wash steps (aqueous and organic) were combined into one. Eliminating these steps did not effect the extraction efficiency of the method (data not shown), a result consistent with the water-wettable nature of the sorbent. Unlike traditional silica-based sorbents, Oasis sorbent does not lose retentivity if allowed to dry out. This property also enables all sample pretreatments to be performed within the wells of the 96-well plate, eliminating individual transfer steps that can be time-consuming or error-prone. Combining the wash steps into a single wash also helps to accelerate the workflow. Compared to a traditional mixed-mode SPE workflow, which includes conditioning, equilibration, and two wash steps, half of these steps are eliminated. This reduces a six-step procedure to a three-step procedure, reducing processing time by 50%. An entire plate can be processed within 30 minutes.

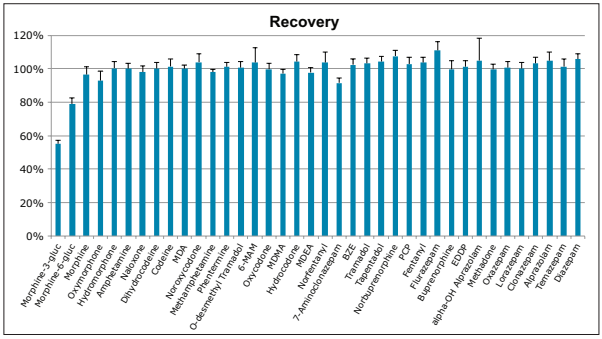

Figure 3. Extraction recovery for the compounds in this application. Values represent the mean of six individual extractions performed over 3 days (2 per day; N = 4 for each extraction).

Five-point calibration curves at 1, 5, 10, 50, and 100 ng/mL (0.2, 1.0, 2.0, 10, and 20 ng/mL for fentanyl, norfentanyl, 6-acetyl morphine, norbuprenorphine, and buprenorphine) were extracted in order to estimate limits of detection for the assay. Limits of detection were defined as those points in which the signal was five times greater than that of an extracted matrix blank, and both bias and %CV were less than 20%. Table 1 shows the calculated LODs for each compound. These values ranged from 1 to 10 ng/mL depending on the compound. This demonstrates that the method has the sensitivity and specificity required for semi-quantitative screening of this expanded toxicology panel.

CONCLUSIONS

We have described a rapid and broadly applicable SPE protocol and UPLC-MS/MS method for analyzing a comprehensive panel of compounds common to forensic toxicology screens. The unique, water-wettable nature of the Oasis sorbent allowed us to eliminate the common conditioning and equilibration steps without any loss in recovery or reproducibility for any of the 38 compounds in this testing panel. This property also enables the entire hydrolysis step to be conducted within the wells of the Oasis MCX µElution Plate, eliminating the time-consuming and error-prone transfer steps. Combining this with the consolidation of two wash steps into a single one further facilitates the reduction of a six-step extraction process into only three steps. Though this procedure remains slightly more time-consuming than sample dilution, it nonetheless can be completed in 30 minutes. Moreover, it offers the additional added benefits of increased sensitivity, reduced matrix interferences, increased analytical column lifetimes, and reduced risk of ion source-fouling.

This method was designed for the analysis of enzymatically hydrolyzed samples. Yet the use of the ACQUITY UPLC BEH Phenyl Column also enables the resolution and analysis of morphine-3-glucuronide and morphine-6-glucuronide, allowing the method's use for direct analysis without hydrolysis. It also allows monitoring of the metabolites of these glucuronides. Because these compounds have been shown to be difficult to fully hydrolyze using beta glucuronidase,[2] monitoring their presence can be an important factor in ensuring complete conversions to the free drugs.

This method enables the rapid extraction and analysis of a large panel of drugs for forensic toxicology screening. When combined with the chromatography of the ACQUITY UPLC BEH Phenyl Column, it provides a rapid, specific method with the sensitivity and reproducibility required to accurately screen for this panel of compounds.

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Evaluation of the Potential of the ACQUITY QDa Mass Detector for Use in Forensic Chemistry and Drug Control Laboratories

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Application of an existing toxicology library to the qualitative screening of medicines using the ACQUITY QDa — a promising tool for drug control.

GOAL

Assess the transferability of an existing toxicology library to the ACQUITY® QDa® Mass Detector.

BACKGROUND

In recent years, a comprehensive spectral library for use in analytical toxicology has been developed. The library was originally generated using the Waters® ACQUITY TQD Mass Spectrometer and was prepared by acquiring full scan mass spectra over multiple cone voltages, to yield compound-specific fragmentation patterns by the process of in-source collision-induced dissociation.[1] Since the first application of this methodology, over a decade ago, the approach has been applied to newer generation instruments[2] and the library has been expanded; it now contains data for over 950 toxicologically-relevant substances. The purpose of the current work was to evaluate the feasibility of using the existing library in combination with the ACQUITY QDa[3] to provide a simple, low-cost, qualitative screening and identification system for use in forensic chemistry and drug control laboratories. For this study, a selection of over-the-counter and prescribed medicines were analyzed as representative agents.

The ACQUITY UPLC® I-Class System and ACQUITY QDa Mass Detector.

THE SOLUTION

Data for a selection of medicines were acquired using the ACQUITY UPLC® I-Class System combined with the ACQUITY QDa according to a well-established technique in which samples are screened against a library comprising reference retention time and multiple spectra.[1,2,4]

Materials

Preliminary spectral testing was performed for a range of licit and illicit drug substances, using mixtures prepared from certified reference material (Sigma-Aldrich). Eight mixtures, each containing ten compounds, were analyzed.

Authentic samples for subsequent testing were prepared using a selection of over-the-counter and prescribed medicines in tablet, capsule, or liquid form.

Sample preparation

Individual tablets/capsules or 250 µL of medicines supplied in liquid form, were added to 25 mL of a methanol and water mixture (70:30) and sonicated at room temperature for 30 minutes. One millilitre of the resulting solution was transferred to a 2 mL microcentrifuge tube and centrifuged at 13000 rpm for 5 minutes. Fifty microlitres of the supernatant was diluted with 950 µL of water in a maximum recovery vial and vortex-mixed. LC-MS analysis was performed using 10 µL of the resulting solution.

LC-MS method conditions

Chromatographic separation was achieved within 15 minutes using an ACQUITY UPLC I-Class (FTN) and an established toxicology screening gradient.[1,2,4] The ACQUITY QDa was operated in ESI+, and full scan data were acquired over a *m/z* range of 80–650 at the following five cone voltages: 10 V, 20 V, 35 V, 45 V, and 55 V.

RESULTS

A preliminary assessment of voltages was performed using mixtures of drug standards at a concentration of 200 ng/mL. Data were acquired using the ACQUITY QDa at identical voltages to those used in the preparation of the original ACQUITY TQD library, i.e. from 20 V to 95 V in increments of 15 V. This initial data indicated that for the same cone voltages, the ACQUITY QDa exhibited increased fragmentation; consequently library voltages were adjusted to achieve parity with the ACQUITY QDa. The modified library was subsequently applied to the analysis of eight pharmaceuticals.

The multi cone voltage data acquired for each of the eight samples were processed using MassLynx® Software with ChromaLynx™ Application Manager which detects the components within each sample and provides an identification through library matching. The confidence with which a substance is identified is presented as an average library match factor which has a maximum value of

Medicine	Active ingredients detected (match factor)
LEMSIP® Max Cold and Flu Remedy	Phenylephrine 6.1 mg (707), paracetamol 500 mg (804), caffeine 25 mg (897)
Galpharm™ Hayfever and Allergy Relief	Cetirizine 10 mg (832)
Entrolax®	Bisacodyl 5 mg (889)
Galpharm Extra Power Pain Relief	Paracetamol 200 mg (816), caffeine 45 mg (880)
Buscopan®	Scopolamine butylbromide 10 mg (902)
Prozac®	Fluoxetine 20 mg (883)
Benylin®	Guaifenesin 100 mg/5 mL (701)
Imuran®	Azathioprine 50 mg*

Table 1. The active ingredients in each of the medicines analyzed, together with the disclosed amount and the average match factors to the library entries.

**Azathioprine was not present in the library and the measured spectra were used to create a library entry.*

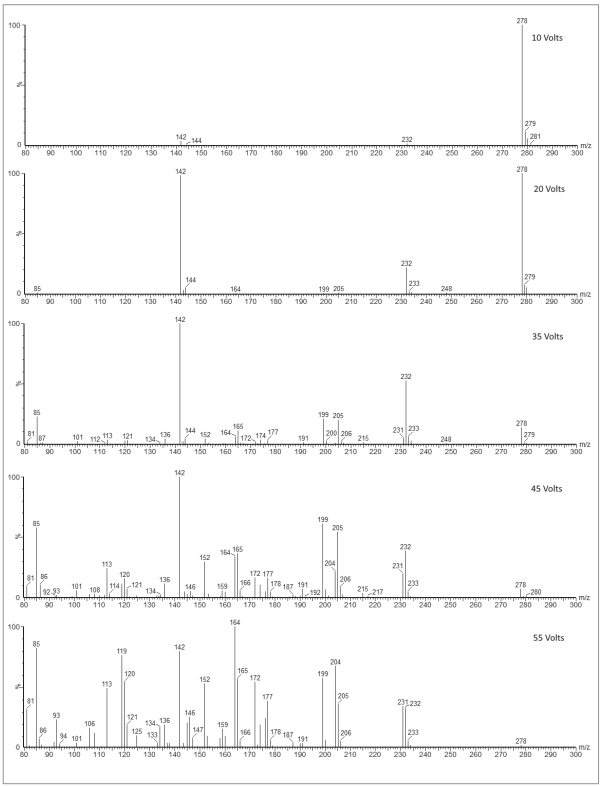

Figure 1. ESI+ spectra for azathioprine (precursor mass m/z 278) on the ACQUITY QDa at the following cone voltages: 10V, 20V, 35V, 45V, and 55V.

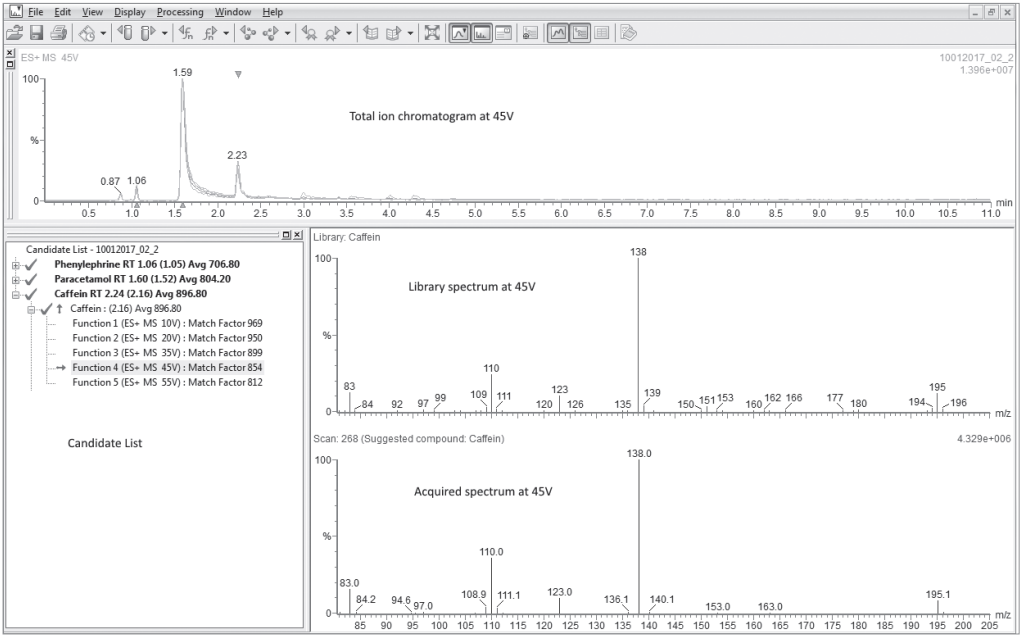

Figure 2. The ChromaLynx browser displaying the results of the analysis of the LEMSIP Max Cold and Flu Remedy, highlighting the identification of caffeine with a precursor ion at m/z 195.

1000. The average match factor is determined by comparing the measured and library spectra acquired over the five cone voltages.

The active ingredients detected in each of the eight samples are listed in Table 1 together with the average match factors as determined by ChromaLynx. For one of the medicines, Imuran, there was no match with the library, however, a large response was observed at the same retention time for each of the five cone voltages. The package insert for the product indicated that the active ingredient in Imuran is azathioprine, and this was consistent with the spectral data shown in Figure 1. The acquired data was subsequently used to generate library entries for azathioprine.

An example of the information available in the browser of the ChromaLynx Application Manager is shown in Figure 2 for the analysis of the LEMSIP MAX Cold and Flu Remedy.

SUMMARY

In this study, a series of representative medicines were used to assess the feasibility of applying an existing toxicology library to the ACQUITY QDa Mass Detector. Application of an established chromatographic method together with the qualitative screen demonstrated very good agreement between library spectra and acquired data, leading to the identification of the active ingredients in the medicines. Therefore, the modified toxicology library, in combination with the ACQUITY QDa, appears promising as a low-cost solution for forensic chemistry and drug control testing.

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Simple, Fast, and Clean Extraction of Synthetic Cannabinoids from Whole Blood Using Oasis PRiME HLB

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APPLICATION BENEFITS

- A simple SPE protocol for that eliminates conditioning and equilibration steps.
- Efficient recoveries and low matrix effects for all tested compounds.
- Rapid, universal extraction method for analysis of synthetic cannabinoids and metabolites from whole blood.
- >95% reduction in phospholipids compared to protein precipitation.
- Excellent quantitative accuracy and precision for a wide variety of synthetic cannabinoids and metabolites.

WATERS SOLUTIONS

Oasis PRiME HLB 30 mg Plate

CORTECS® UPLC C_{18}, 90Å, 1.6 µm, 2.1 x 100 mm Column (p/n 186007095)

ACQUITY® I-Class System

Xevo® TQD Mass Spectrometer

2 mL 96-well collection plates (p/n 186002482)

KEY WORDS

Synthetic cannabinoids, UPLC,® forensic toxicology, whole blood, solid phase extraction, CORTECS, solid core, OASIS PRiME HLB, phospholipid removal

INTRODUCTION

Oasis® PRiME HLB is a novel reversed phase solid phase extraction (SPE) sorbent developed to enable simpler and faster SPE protocols, while at the same time generating cleaner extracts than other sample preparation methods. With Oasis PRiME HLB, a 3-step load-wash-elute SPE protocol eliminating conditioning and equilibration was successfully employed to extract 22 synthetic cannabinoids and metabolites from whole blood samples. Excellent analyte recoveries and modest matrix effects (ME) were achieved across the entire panel of compounds. These results were consistent, with low variability for all compounds. In addition, Oasis PRiME HLB removed more than 95% phospholipids from the whole blood samples compared to protein precipitation (PPT). The 22 synthetic cannabinoids and metabolites were extracted using Waters Oasis PRiME HLB 30 mg plates. Calibration curves for all compounds ranged from 0.2–100 ng/mL. Quantitative results from quality control samples were accurate and precise across the entire calibration range. The analysis of several different classes of these drugs and metabolites, which includes neutral molecules, acids and bases, demonstrates the utility of this method across the different chemotypes and should render this method applicable to newly developed related compounds with little, if any, modification necessary.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC® I-Class
Column:	CORTECS UPLC C_{18}, 90Å, 1.6 µm; 2.1 x 100 mm (p/n 186007095)
Column temp.:	30 °C
Injection volume:	5 µL
Flow rate:	0.6 mL/min
Mobile phase A:	0.1% formic acid in MilliQ water
Mobile phase B:	0.1% formic acid in ACN
Gradient:	Initial conditions started at 30% B. The %B was increased to 50% over 2 minutes, and held at 50% B for 1 minute, increased to 90% B over 4 minutes and then returned to 30% over 0.2 minutes. The system was allowed to re-equilibrate for 1.3 min. The entire cycle time was 8.5 min. The solvent gradient is listed in Table 1.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI Positive
Acquisition mode:	MRM (See Tables 2 and 3 for transitions)
Capillary voltage:	1 kV
Collision energy (eV):	Optimized for individual compounds (See Table 2)
Cone voltage (V):	Optimized for individual compounds (See Table 2)

Data management

All data were acquired and analyzed using Waters MassLynx® software v.4.1 (scn 855) and quantified using TargetLynx™ Software. MS conditions were optimized using Intellistart.™

Materials

AM2233, JWH-015, RCS-4, JWH-203, RCS-8, JWH-210, JWH-073, and JWH-018 were purchased from Cerilliant (Round Rock, TX). All other compounds and metabolites were purchased from Cayman Chemical (Ann Arbor, MI)

Individual stocks (1 mg/mL) were prepared in methanol, DMSO, or 50:50 DMSO: methanol. A combined stock solution of all compounds (10 µg/mL) was prepared in methanol. Working solutions were prepared daily in 40% methanol.

Calibrators and quality control (QC) samples were prepared by spiking working standards at various concentrations and into matrix (whole blood). Calibrator concentrations ranged from 0.2–100 ng/mL for all analytes. Quality control samples were prepared at 2.5, 7.5, and 75 ng/mL, in whole blood.

The 22 compounds analyzed are listed in Table 1 and constitute a panel that includes various classes of forensically relevant synthetic cannabinoids. These include adamantoylindoles (AM 1248 and AKB48), napthoylindoles (JWH 022), phenylacetyl indoles (RCS-4 and RCS-8), and tetramethylcyclopropylindoles (UR-144 and XLR11). Major metabolites of JWH-073 and JWH-018 were also included, as some of these compounds are structural isomers with identical mass spectral fragments that require adequate chromatographic separation for accurate quantitation.

Sample preparation

Samples were extracted using Oasis PRiME HLB 30 mg Plates. 0.1 mL of a solution of 0.1 M zinc sulfate/ammonium acetate was added to 0.1 mL whole blood, and vortexed for 5 seconds to lyse the cells. All samples were then precipitated by adding 400 µL ACN. The entire sample was vortexed for 10 seconds and centrifuged for 5 min at 7000 rcf. The supernatant was then diluted with 1.2 mL water prior to loading. The sample was directly loaded on the Oasis PRiME 30 mg Plate without conditioning or equilibration. All wells were then washed with 2 x 500 µL 25:75 MeOH:water, and eluted with 2 x 500 µL 90/10 ACN/MeOH. The eluate was then evaporated under Nitrogen and reconstituted with 100 µL 30% ACN. 5 µL was injected onto the UPLC system.

Analyte recovery was calculated according to the following equation:

$$\%\ \text{Recovery} = \left(\frac{\text{Area } A}{\text{Area } B}\right) \times 100\%$$

Where A equals the peak area of an extracted sample and B equals the peak area of an extracted matrix sample in which the compounds were added post-extraction.

Matrix effects were calculated according to the following equation:

$$\text{Matrix Effects} = \left(\frac{\textit{Peak area in the presence of matrix}}{\textit{Peak area in the absence of matrix}}\right) \times 100\%$$

The peak area in the presence of matrix refers to the peak area of an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

RESULTS AND DISCUSSION

Chromatography

The design of the solid-core CORTECS Particle, combined with optimal packing in the column, results in excellent chromatographic performance. A representative chromatogram of all compounds from a 20 ng/mL calibration standard is shown in Figure 1. Using a CORTECS UPLC C_{18} Column (90Å, 1.6 µm, 2.1 x 100 mm), all analytes were analyzed within 7.5 minutes with a total cycle time of 8.5 minutes. Peak shape was excellent for all compounds, with no significant tailing or asymmetries, and all peak widths were under 3 seconds at 5% of baseline.

Recovery and matrix effects

The synthetic cannabinoids and metabolites in this application include compounds that are neutral, acidic and basic. Use of the Oasis PRiME HLB Sorbent enabled the simultaneous extraction of all of the compounds and metabolites tested, regardless of their functionality. Recoveries and matrix effects (ME) were calculated according to the equations described in the experimental section and the results are shown in Figure 2. This extraction protocol results in nearly complete recovery for all compounds and minimizes matrix effects for the majority of analytes. All but one compound had recoveries of 80% or greater with an overall average recovery of 91%. Recoveries were consistent with an average %RSD at 5% across all compounds. Matrix effects across the panel were excellent. Only two compounds had matrix effects that slightly exceeded 40%, and all remaining compounds had matrix effects less than 25%. The average magnitude of matrix effects was only 17%. The high recoveries and minimal matrix effects for this panel of synthetic cannabinoids indicate that Oasis PRiME HLB should give similar results for other related compounds with a simple load-wash-elute protocol.

Time (min.)	Flow (mL/min.)	%A	%B
0	0.6	70	30
2.0	0.6	50	50
3.0	0.6	50	50
7.0	0.6	10	90
7.2	0.6	70	30
8.5	0.6	70	30

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Methods section.

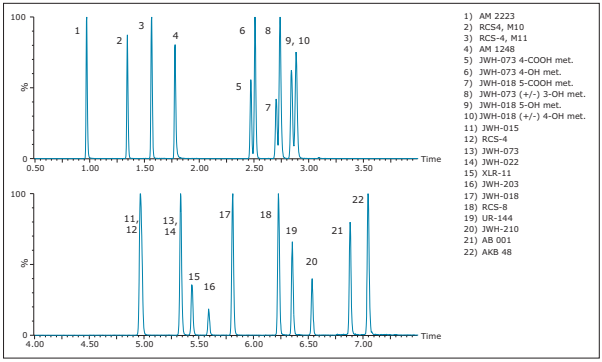

Figure 1. UPLC-MS/MS chromatogram for 22 synthetic cannabinoids and metabolites.

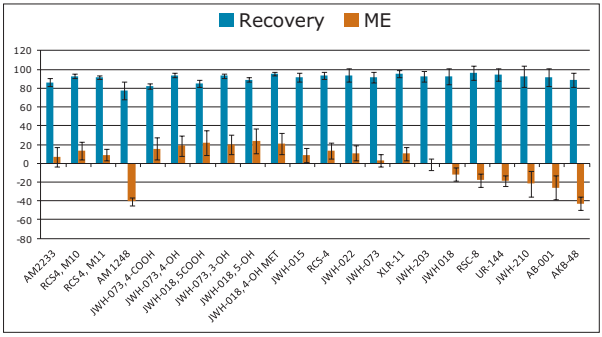

Figure 2. Recovery and matrix effects of synthetic cannabinoid compounds from whole blood following extraction with Oasis PRiME HLB µElution plates. Bars and error bars represent means and standard deviations (N=6), respectively.

Phospholipid removal

One of the key attributes of Oasis PRiME HLB, is its ability to deliver cleaner extracts than other sample preparation methods. One way that this is achieved is by removing endogenous phospholipids. Figure 3 shows chromatograms of combined phospholipid traces from an Oasis PRiME HLB extract and an identical sample subject to protein precipitation. Compared with protein precipitation (PPT), Oasis PRiME HLB removes over 95% phospholipids (Figure 3) resulting in a much cleaner extraction. This can translate to reduced matrix effects, longer column lifetimes, and less mass spectrometer source maintenance.

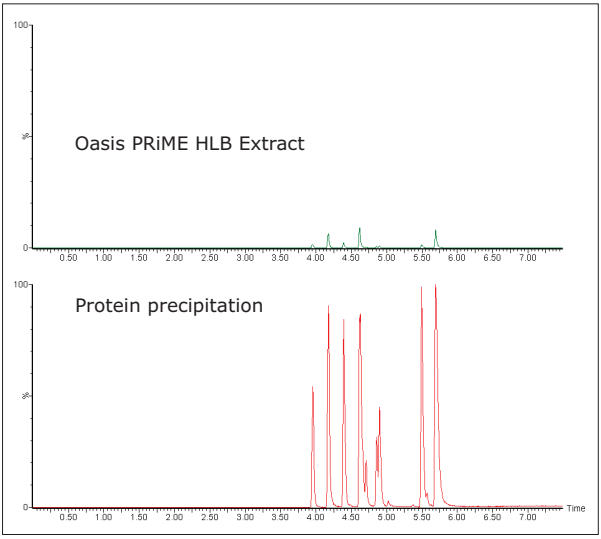

Figure 3. Chromatograms of phospholipids remaining in Oasis PRiME HLB extraction vs. protein precipitation. Scales are linked.

Standard curve performance, accuracy, precision, and sensitivity

In order to assess linearity and analytical sensitivity, calibration curves were extracted at concentrations ranging from 0.2–100 ng/mL for all components. Quality control samples (N=4) at 2.5, 7.5, and 75 ng/mL were also extracted and analyzed. Table 2 summarizes R^2 values from the calibration curves and QC summary data for all compounds. Quality control (QC) results were accurate and precise at low, medium and high concentrations. Accuracies for low level QC samples (2.5 ng/mL) ranged from 95–110% (except one compound, AM2233) with an average of 102%. The results for the medium and high QC levels were excellent for all analytes except one, with all accuracies within 15% of expected values. Analytical precision was excellent with most % RSDs less than 10% and none greater than 15%. When accuracy was assessed over all levels (low, medium, and high), the means ranged from 93% to 104%. Limits of quantification of 0.1 ng/mL were reached for most of the analytes and were no greater than 1 ng/mL. These results were achieved without the use of deuterated internal standards, once again demonstrating the consistency associated with Oasis PRiME HLB.

		QC concentrations (ng/mL)						
		2.5		7.5		75		
	R^2	%Acc	%RSD	%Acc	%RSD	%Acc	%RSD	Mean
AM2233	0.994	74.53	7.78	85.52	5.29	93.18	4.96	84.41
RCS4, M10	0.995	107.75	6.91	95.72	5.43	95.93	3.33	99.80
RCS 4, M11	0.993	106.60	3.15	92.52	5.09	92.92	3.34	97.34
JWH-073, 4-COOH	0.998	108.00	2.44	96.00	5.43	107.07	3.20	103.69
JWH-073, 4-OH	0.996	106.90	5.01	95.88	3.76	94.12	2.15	98.97
JWH-018, 5COOH	0.996	107.58	5.98	97.98	2.32	99.30	2.65	101.62
JWH-073, 3-OH	0.996	110.00	6.22	94.43	2.24	91.95	2.38	98.79
JWH-018, 5-OH	0.996	103.17	9.39	83.55	4.01	101.77	3.90	96.16
JWH-018, 4-OH MET	0.996	106.40	5.45	85.15	2.87	104.70	4.17	98.75
JWH-015	0.998	108.85	6.19	99.57	3.49	100.95	2.48	103.12
RCS-4	0.998	109.80	4.06	94.63	3.47	94.62	5.25	99.68
JWH-022	0.999	107.32	3.23	97.27	4.95	96.90	5.65	100.50
JWH-073	0.999	111.28	3.11	97.38	4.96	99.62	7.47	102.76
XLR-11	0.998	101.32	8.07	98.90	4.12	103.78	5.71	101.33
JWH-203	0.999	101.60	4.34	99.72	6.55	101.24	7.13	100.85
JWH 018	0.998	102.30	9.31	99.08	8.86	102.00	7.62	101.13
RSC-8	0.995	95.92	8.61	93.02	9.10	100.98	11.55	96.64
UR-144	0.997	99.22	9.81	99.58	9.55	104.60	7.24	101.13
JWH-210	0.993	110.60	10.42	94.02	12.92	100.35	8.71	101.66
AB-001	0.995	107.02	7.82	92.54	11.04	101.98	6.22	100.51
AKB-48	0.974	97.07	11.76	89.95	13.76	107.63	13.50	98.21
	Mean	**102.43**		**92.27**		**98.24**		

Table 2. R^2 values and quality control results for all compounds. Mean values at the bottom indicate averages of all compounds at particular concentrations. Values to the right indicate averages of individual compounds across all QC concentrations.

No.	Compound	RT	Mol. Formula	Cone voltage	MRM Transitions	Coll. energy
1	AM2233	0.97	$C_{22}H_{23}IN_2O$	48	459.2⇒98.05	50
				48	459.2⇒112.1	40
2	RCS-4, M10	1.34	$C_{20}H_{21}NO_3$	40	324.2⇒121.0	36
				40	324.2⇒93.0	72
3	RCS-4, M11	1.57	$C_{20}H_{19}NO3$	42	322.2⇒121.0	32
				42	322.2⇒93.0	60
4	AM 1248	1.78	$C_{26}H_{34}N_2O$	62	391.4⇒135.1	42
				62	391.4⇒112.1	50
5	JWH-073 4-butanoic acid met.	2.47	$C_{23}H_{19}NO_3$	52	358.2⇒155.1	32
				52	358.2⇒127.1	70
6	JWH-073 4-hydroxybutyl met.	2.51	$C_{23}H_{21}NO_2$	52	344.2⇒155.1	32
				52	344.2⇒127.1	70
7	JWH-018 5-pentanoic acid met.	2.71	$C_{24}H_{21}NO_3$	54	372.2⇒155.1	32
				54	372.2⇒127.1	72
8	JWH-073 (+/-) 3-hydroxybutyl met.	2.74	$C_{23}H_{21}NO_2$	54	344.2⇒155.1	36
				54	344.2⇒127.1	64
9	JWH-018 5-hydroxypentyl met.	2.84	$C_{24}H_{23}NO_2$	50	358.2⇒155.1	24
				50	358.2⇒127.1	48
10	JWH-018 (+/-) 4-hydroxypentyl met.	2.89	$C_{24}H_{23}NO_2$	50	358.2⇒155.1	34
				50	358.2⇒127.1	64
11	JWH-015	4.97	$C_{23}H_{21}NO$	48	328.2⇒155.1	32
				48	328.2⇒127.1	62
12	RCS-4	4.98	$C_{21}H_{23}NO_2$	48	322.2⇒135.1	40
				48	322.2⇒92.0	68
14	JWH-022	5.34	$C_{24}H_{21}NO$	52	340.2⇒155.1	34
				52	340.2⇒127.1	60
13	JWH-073	5.34	$C_{23}H_{21}NO$	48	328.2⇒155.1	36
				48	328.2⇒127.1	56
15	XLR-11	5.44	$C_{21}H_{28}FNO$	52	330.3⇒125.1	34
				52	330.3⇒83.0	42
16	JWH-203	5.59	$C_{21}H_{22}ClNO$	44	340.2⇒188.1	32
				44	340.2⇒125.0	42
17	JWH-018	5.82	$C_{24}H_{23}NO$	50	342.2⇒155.1	34
				50	342.2⇒127.1	60
18	RCS-8	6.23	$C_{25}H_{29}NO_2$	44	376.3⇒121.1	36
				44	376.3⇒91.0	66
19	UR-144	6.36	$C_{21}H_{29}NO$	48	312.3⇒214.2	35
				48	312.3⇒125.1	34
20	JWH-210	6.54	$C_{26}H_{27}NO$	54	370.2⇒214.2	34
				54	370.2⇒183.1	36
21	AB 001	6.88	$C_{24}H_{31}NO$	62	350.3⇒135.1	44
				62	350.3⇒93.0	62
22	AKB 48	7.05	$C_{23}H_{31}N_3O$	36	366.3⇒135.1	28
				36	366.3⇒93.1	68

Table 3. Molecular formulae, retention times, and MS/MS conditions for the synthetic cannabinoid compounds and metabolites in this application. Quantification transitions are listed first, followed by confirmatory transitions

Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cone Voltage (V)	Collison Energy (eV)
496.40	184.40	35	30
520.40	184.40	35	30
522.40	184.40	35	30
524.40	184.40	35	30
704.40	184.40	35	30
758.40	184.40	35	30
760.40	184.40	35	30
784.40	184.40	35	30
786.40	184.40	35	30
806.40	184.40	35	30
808.40	184.40	35	30

Table 4. MS/MS conditions for the Phospholipids.

CONCLUSIONS

This application note highlights the use of Oasis PRiME HLB, a novel reversed-phase SPE sorbent which is designed to enable simple and fast SPE protocols while nearly eliminating endogenous phospholipids. Employing a simple load-wash-elute strategy, without any sorbent conditioning or equilibration, a panel of 22 synthetic cannabinoids was extracted from whole blood samples. Extraction recoveries averaged 91% across the entire panel, with an average matrix effect magnitude of only 17%. These results were consistent with mean %RSDs of 5% for all compounds. In addition, greater than 95% of phospholipids were removed vs. protein precipitation. Quantitative results were also excellent. Even without the use of deuterated internal standards, calibration curves were linear, with R^2 values of 0.99 for 21/22 compounds. 97% of QC results were within 15% of target values and all %RSDs were less than 15%. In conclusion, Oasis PRiME has been used to achieve consistent, high recoveries with low matrix effects while virtually eliminating endogenous phospholipids from whole blood samples. The enabled excellent quantitiative results, even without the use of deuterated internal standards.

References

1. Danaceau, J. P, Chambers, E. E., and Fountain, K. J, Analysis of synthetic cannabinoids from urine for forensic toxicology using Oasis HLB µElution Plates and CORTECS UPLC Columns, Waters Application note p/n 720004780EN

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Analysis of "Bath Salt" Compounds from Urine for Forensic Toxicology Using µElution Mixed-mode SPE Combined with UPLC/MS/MS Detection

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APPLICATION BENEFITS

- Single method for comprehensive panel of synthetic cathinones
- Linear response for all analytes and metabolites
- Greater than 90% recovery for most analytes
- Matrix effects under 15% for all compounds
- Baseline resolution of structural isomers with identical mass spectra
- No evaporation or reconstitution steps needed for final SPE eluate

WATERS SOLUTIONS

Oasis® MCX µElution plates

96-Well Plate

ACQUITY UPLC® BEH Column

ACQUITY UPLC System

Xevo® TQD Mass Spectrometer

KEY WORDS

Bath salts, cathinones, UPLC®, forensic toxicology, SPE

INTRODUCTION

Synthetic cathinones, commonly marketed as "bath salts," are variations of the chemical cathinone, naturally found in the Khat plant (*Catha edulis*). These drugs are central nervous system stimulants, mimicking the effects of drugs such as amphetamine, methamphetamine, cocaine, and methylphenidate. Often labeled as "not for human consumption," their popularity and use have increased substantially in the last several years[1]. In addition, new drugs with modifications to existing cathinone structures are constantly being developed and marketed in order to circumvent drug of abuse legislation aimed at specific compounds. This current application note details a strategy for the successful extraction and analysis of representatives of several different classes of synthetic cathinones from human urine samples for forensic toxicology. Using mixed-mode solid phase extraction (SPE) followed by UPLC/MS/MS analysis, a panel of 10 synthetic cathinones was extracted with excellent recovery and analytical sensitivity. Matrix effects were minimal for all compounds, and calibration curves were linear from one to 500 ng/mL. The analysis of several different classes of these drugs should render this method applicable to newly developed related compounds with minimal, if any, modification necessary.

EXPERIMENTAL

Method conditions

LC conditions

System:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH C_{18} 1.7 µm, 2.1 x 100 mm (p/n 186002352)
Column temp.:	30 °C
Injection volume:	10 µL
Flow rate:	0.5 mL/min
Mobile phase A:	0.1% formic acid in MilliQ water
Mobile phase B:	0.1% formic acid in ACN
Gradient:	Initial hold at 20% B for 0.5 minute, increased to 30% over 2 minutes, then returned to 20% over 0.1 minute. The system was allowed to re-equilibrate for 1.4 minutes. The entire cycle time was 4.0 minutes.
Vials/plates:	96-well sample collection plates, 700 µL (p/n 186005837)

MS conditions

Mass spectrometer:	Xevo TQD
Ionization mode:	ESI positive
Acquisition mode:	MRM (See Table 1 for transitions)
Capillary voltage:	1 kV
Collision energy (eV):	Optimized for individual compounds (See Table 1)
Cone voltage (V):	Optimized for individual compounds (See Table 1)

Data management

All data was acquired and analyzed using Waters® MassLynx® Software

Materials

4-methylmethcathinone (Mephedrone, 4-MMC), 3,4-methylenedioxy-N-methylcathinone (methylone), and methylenedioxypyrovalerone (MDPV) were purchased from Cerilliant (Round Rock, TX). All other compounds and metabolites were purchased from Cayman Chemical (Ann Arbor, MI).

A combined stock solution of all compounds (5 µg/mL) was prepared in methanol. Working solutions were prepared daily by preparing standards in matrix (urine), and performing serial dilutions to achieve the desired concentrations. Calibrator concentrations ranged from five to 500 ng/mL for all analytes.

The 10 compounds analyzed, listed in Table 1, constitute a panel which includes forensically relevant cathinones such as pyrrolidiniophenones (α-PPP, α-PVP), methylenedioxycathinones (methylone, ethylone), and methoxymethcathinones (methedrone). All are weak bases of moderate hydrophobicity that are well suited to extraction by mixed-mode ion exchange. Cone voltages, MRM transitions, and respective collision energies are listed for all compounds in Table 1.

Sample preparation

Samples were extracted using mixed-mode, strong cation SPE. For each sample, 100 µL of urine was pre-treated by adding an equal volume of 4% H_3PO_4. Wells of the 96-well Oasis MCX µElution Plate (p/n 186001830BA) were conditioned with 200 µL MeOH, followed by 200 µL MilliQ water. 200 µL of each diluted sample was then added to each well, resulting in a sample load of 100 µL urine. After loading, the wells were washed with 200 µL of aqueous 2% formic acid, followed by 200 µL MeOH. All samples were then eluted with 2 x 50 µL of 60:40 ACN/IPA containing 5% by volume of a concentrated NH_4OH solution (Fisher, 20% to 22%). Samples were then neutralized with 5 µL of concentrated formic acid, and diluted with 100 µL of water. 10 µL was injected onto the LC/MS/MS system.

Matrix effects were calculated according to the following equation:

$$\textit{Matrix Effects} = \left(\left(\frac{\textit{Peak area in the presence of matrix}}{\textit{Peak area in the absence of matrix}}\right) - 1\right) \times 100\%$$

RESULTS AND DISCUSSION

Chromatography

A representative chromatogram of all compounds from a 50-ng/mL calibration standard is shown in Figure 1. Peak assignments are listed in Table 1. Using an ACQUITY UPLC BEH C_{18} 1.7 µm, 2.1 x 100 mm Column, all analytes were analyzed within two minutes with a total cycle time of four minutes. Ethylone and butylone (peaks 2 and 6), an isobaric pair of compounds with identical precursor and product ions, demonstrate baseline resolution despite the short analysis time, enabling unambiguous identification that would not be possible if the two compounds co-eluted. Peak shape was excellent for all compounds, with no significant tailing or asymmetries, and all peak widths were under four seconds.

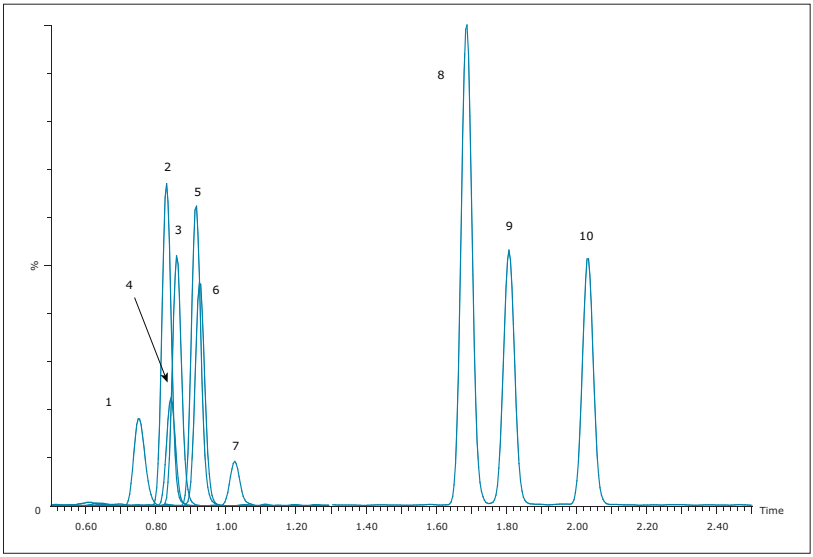

Figure 1. UPLC/MS/MS chromatogram for 10 "bath salt" compounds. Peak assignments are listed in Table 1.

	Drug	Alt name	RT	Formula	Mass	Cone voltage	MRM transitions	Coll. energy
1	Methylone	3,4-methylenedioxy-N-methylcathinone	0.75	$C_{11}H_{13}NO_3$	207.23	28 28	208.2→132.0 208.2→160.0	28 18
2	Ethylone	MDEC, bk-MDEA	0.83	$C_{12}H_{15}NO_3$	221.26	30 30	222.3→174.1 222.3→204.1	20 14
3	Methedrone	4-methoxymethcathinone	0.84	$C_{11}H_{15}NO_2$	193.25	28 28	194.2→161.0 194.2→146.0	22 30
4	α-PPP	*alpha*-Pyrrolidinopropiophenone	0.86	$C_{13}H_{17}NO$	203.28	42 42	204.3→105.0 204.3→98.0	24 28
5	MDPPP	3',4'-Methylenedioxy-α-pyrrolidinopropiophenone	0.92	$C_{14}H_{17}NO_3$	247.29	42 42	248.3→98.0 248.3→147.0	26 24
6	Butylone	Bk-MBDB	0.92	$C_{12}H_{15}NO_3$	221.26	32 32	222.3→174.1 222.3→204.1	18 15
7	Mephedrone	4-methylmethcathinone, 4-MMC	1.02	$C_{11}H_{15}NO$	177.24	26 26	178.2→145.0 178.2→91.0	22 34
8	α-PVP	*alpha*-Pyrrolidinopentiophenone	1.66	$C_{15}H_{21}NO$	231.34	38 38	232.4→91.0 232.4→105.0	26 28
9	MDPV	Methylenedioxypyrovalerone	1.78	$C_{16}H_{21}NO_3$	275.35	38 38	276.4→175.0 276.4→205.0	22 20
10	α-PVP Met 1	*alpha*-Pyrrolidinopentiophenone metabolite 1	2.00	$C_{15}H_{23}NO$	233.35	30 30	234.4→72.0 234.4→173.0	20 24

Table 1. MS/MS conditions and retention times for the cathinone compounds in this application.

Recovery and matrix effects

For this application, elution from Oasis MCX µElution plates was initially performed with a solution of 60:40 ACN/MeOH containing 5% concentrated NH_4OH. Recoveries were good for most compounds, averaging approximately 80%. However, the α-PVP hydroxyl metabolite was recovered at only 40%. In addition, significant matrix effects, mostly in the form of ion suppression, were seen for many of the compounds tested, especially the three earliest eluting compounds, methylone, ethylone, and methedrone. Replacing the MeOH in the elution solvent with water did not reduce the matrix effects seen, and reduced the recovery of the α-PVP hydroxyl metabolite to only 4%. However, when the MeOH was replaced with isopropanol (IPA), the recovery of the α-PVP hydroxyl metabolite was increased to 87.4%, and the recoveries for the other nine analytes were improved from an average of 81% to nearly 98%. In addition, matrix effects were nearly eliminated for all compounds. Figure 2 summarizes the recoveries and matrix effects seen with the final extraction method. Recoveries range from 87.4% to 100.5% with matrix effects ranging from -3.6% to 12.4% at an average of 6.2%. This extraction protocol results in almost complete recovery, and minimizes matrix effects for the compounds tested.

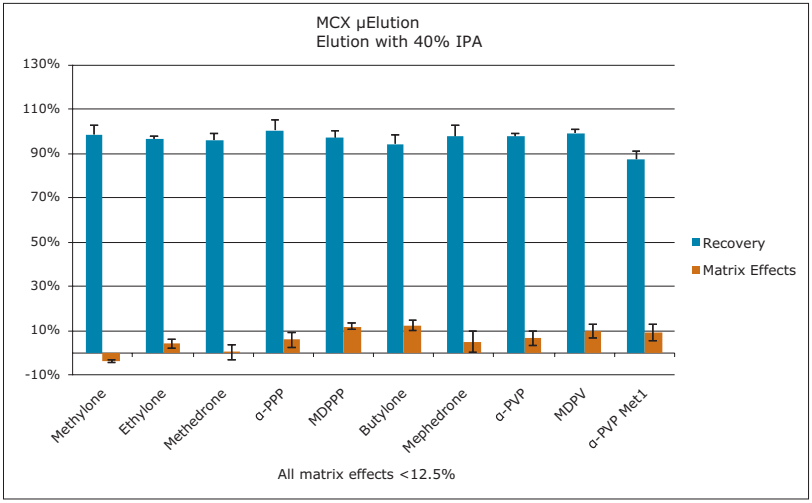

Figure 2. Recovery and matrix effects of "bath salt" compounds from urine following SPE using MCX µElution plates. Bars and error bars represent mean and standard deviations (N=4), respectively.

Linearity and analytical sensitivity

In order to assess linearity and analytical sensitivity, calibration curves were extracted at concentrations ranging from 1 to 500 ng/mL for all components. Table 2 summarizes R^2 values and average deviations (N=3) from nominal values for all compounds. With the exception of the 10 ng/mL point for the α-PVP metabolite, nearly all calibration points were within 15% of their target values. At the 1 ng/mL level, peak areas for all compounds were at least five-fold higher than that of blank, extracted urine samples, and all were within 5% of the nominal value.

	Concentration (ng/mL)						
	1	5	10	50	100	500	R^2
Methylone	-3.80	9.85	10.13	2.83	-7.43	-15.87	0.990
Ethylone	-2.60	8.13	10.43	2.20	-8.37	-9.80	0.990
Methedrone	-3.80	9.85	10.13	2.83	-7.43	-15.87	0.987
α-PPP	-1.37	4.33	5.67	-0.40	-6.80	-1.43	0.997
MDPPP	-1.70	7.33	3.30	-0.93	-6.53	-1.43	0.996
Butylone	-2.07	8.90	13.85	2.47	-6.13	-9.43	0.989
Mephedrone	-1.53	7.90	5.23	1.60	-6.33	-4.20	0.994
α-PVP	-1.70	4.60	8.20	-3.80	-9.27	0.80	0.994
MDPV	-1.20	3.60	3.20	-1.37	-9.23	2.33	0.997
α-PVP Met1	4.15	-9.60	-30.70	-4.97	9.47	11.07	0.983
All values indicate % deviation from nominal values							

Table 2. Accuracy of standard curve points and R^2 values of calibration curves for "bath salt" compounds.

CONCLUSIONS

A panel of 10 synthetic cathinone drugs were extracted from urine by mixed-mode SPE, and analyzed by UPLC/MS/MS. The use of Waters MCX µElution plates resulted in excellent recoveries for all analytes while minimizing matrix effects. Furthermore, no evaporation or reconstitution steps were necessary, saving time and eliminating the risk of sample loss by evaporation or adsorption that can accompany such procedures. Separation by UPLC enabled the analysis of all compounds in two minutes with baseline resolution of a critical isobaric pair. Calibration curves were linear from one to 500 ng/mL with limits of quantitation of 1 ng/mL for all compounds. This method enables the rapid and reliable extraction and analysis of this critical class of compounds for forensic toxicology.

Reference

1. Coppola M, Mondola R. Synthetic cathinones: Chemistry, pharmacology and toxicology of a new class of designer drugs of abuse marketed as "bath salts" or "plant food." *Toxicology Letters.* 2012; 211(2): 144-149.

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Analysis of Synthetic Cannabinoids from Urine for Forensic Toxicology using Oasis HLB µElution plates and CORTECS UPLC Columns

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APPLICATION BENEFITS

- Rapid, universal extraction method for analysis of synthetic cannabinoids and metabolites from urine
- Baseline resolution of structural isomers with identical mass spectra
- Increased separation efficiency and resolution on CORTECS™ UPLC® Columns vs. fully porous particle columns
- Sample concentration without evaporation using the µElution plate format
- Excellent linearity, accuracy, and precision for a wide variety of synthetic cannabinoids and metabolites

WATERS SOLUTIONS

Oasis® HLB µElution plates

CORTECS UPLC C_{18}, 1.6 µm, 2.1 x 100 mm Column

ACQUITY UPLC® I-Class System

Xevo® TQD Mass Spectrometer

KEY WORDS

Synthetic cannabinoids, designer drugs, "Spice" compounds, UPLC, forensic toxicology, urine, Solid-Phase Extraction, CORTECS, solid core, Oasis

INTRODUCTION

Synthetic cannabinoids, often referred to or marketed as "Spice" compounds, constitute a growing challenge for law enforcement agencies and forensic laboratories. These designer drugs mimic the psychoactive effects of natural cannabinoids. Often labeled as "not for human consumption" and marketed as a legal alternative to natural cannabis, their popularity and use have risen substantially in the last several years.[1,2] While recent legislation has banned some of these compounds, minor modifications to existing structures have resulted in a proliferation of substances designed to circumvent existing laws. This application note details a strategy for the successful extraction and analysis of representatives of several different classes of synthetic cannabinoids from urine samples for forensic toxicology. Twenty-two synthetic cannabinoids and metabolites were extracted from urine using Waters Oasis HLB µElution plates. Analytical separation was achieved using Waters' newly developed solid-core particle UPLC Column (CORTECS) with optimally packed 1.6 µm particles, resulting in excellent chromatographic performance and separation efficiency. Calibration curves for all compounds were linear from 1-100 ng/mL. Quantitative results from quality control samples were accurate and precise across the calibration range. The analysis of several different classes of these drugs and metabolites should render this method applicable to newly developed related compounds with little, if any, modification necessary.

EXPERIMENTAL

Final method conditions

LC Conditions

LC System:	ACQUITY UPLC I-Class
Column:	CORTECS UPLC C_{18}, 1.6 µm, 2.1 x 100 mm (p/n 186007095)
Column temp.:	30 °C
Injection volume:	5 µL
Flow rate:	0.6 mL/min.
Mobile phase A:	0.1% formic acid in MilliQ water
Mobile phase B:	0.1% formic acid in ACN
Gradient:	Initial conditions started at 30% B. The %B was increased to 50% over 2 minutes, held at 50% B for 1 minute, increased to 90% B over 4 minutes and then returned to 30% over 0.2 minutes. The system was allowed to re-equilibrate for 1.3 min. The entire cycle time was 8.5 min.
Vials/plates:	96-well collection plates with 700 µL deactivated glass inserts (p/n 186000349DV)

MS conditions

MS system:	Xevo TQD Mass Spectrometer
Ionization mode:	ESI Positive
Acquisition mode:	MRM (See Table 1 for transitions)
Capillary voltage:	1 kV
Collision energy (eV):	Optimized for individual compounds (See Table 1)
Cone voltage (V):	Optimized for individual compounds (See Table 1)

Data Management

All data were acquired and analyzed using Waters MassLynx® Software v.4.1 and quantitated using TargetLynx™ Software. MS conditions were optimized using IntelliStart.™

No.	Compound	RT	Mol. formula	Cone voltage	MRM transitions	Coll. energy
1	AM2233	0.97	$C_{22}H_{23}IN_{20}$	48 48	459.2 → 98.0 459.2 → 112.1	50 40
2	RCS-4, M10	1.34	$C_{20}H_{21}NO_3$	40 40	324.2 → 121.0 324.2 → 93.0	36 72
3	RCS-4, M11	1.57	$C_{20}H_{19}NO_3$	42 42	322.2 → 121.0 322.2 → 93.0	32 60
4	AM 1248	1.78	$C_{26}H_{34}N_2O$	62 62	391.4 → 135.1 391.4 → 112.1	42 50
5	JWH-073 4-butanoic acid met.	2.47	$C_{23}H_{19}NO_3$	52 52	358.2 → 155.1 358.2 → 127.1	32 70
6	JWH-073 4-hydroxybutyl met.	2.51	$C_{23}H_{21}NO_2$	52 52	344.2 → 155.1 344.2 → 127.1	32 70
7	WH-018 5-pentanoic acid met.	2.71	$C_{24}H_{21}NO_3$	54 54	372.2 → 155.1 372.2 → 127.1	32 72
8	JWH-073 (+/-) 3-hydroxybutyl met.	2.74	$C_{23}H_{21}NO_2$	54 54	344.2 → 155.1 344.2 → 127.1	36 64
9	JWH-018 5-hydroxypentyl met.	2.84	$C_{24}H_{23}NO_2$	50 50	358.2 → 155.1 358.2 → 127.1	24 48
10	JWH-018 (+/-) 4-hydroxypentyl met.	2.89	$C_{24}H_{23}NO_2$	50 50	358.2 → 155.1 358.2 → 127.1	34 64
11	JWH-015	4.97	$C_{23}H_{21}NO$	48 48	328.2 → 1 55.1 328.2 → 127.1	32 62
12	RCS-4	4.98	$C_{21}H_{23}NO_2$	48 48	322.2 → 135.1 322.2 → 92.0	40 68
14	JWH-022	5.34	$C_{24}H_{21}NO$	52 52	340.2 → 155.1 340.2 → 127.1	34 60
13	JWH-073	5.34	$C_{23}H_{21}NO$	48 48	328.2 → 155.1 328.2 → 127.1	36 56
15	XLR-11	5.44	$C_{21}H_{28}FNO$	52 52	330.3 → 125.1 330.3 → 83.0	34 42
16	JWH-203	5.59	$C_{21}H_{22}ClNO$	44 44	340.2 → 188.1 340.2 → 125.0	32 42
17	JWH-018	5.82	$C_{24}H_{23}NO$	50 50	342.2 → 155.1 342.2 → 127.1	34 60
18	RCS-8	6.23	$C_{25}H_{29}NO_2$	44 44	376.3 → 121.1 376.3 → 91.0	36 66
19	UR-144	6.36	$C_{21}H_{29}NO$	48 48	312.3 → 214.2 312.3 → 125.1	35 34
20	JWH-210	6.54	$C_{26}H_{27}NO$	54 54	370.2 → 214.2 370.2 → 183.1	34 36
21	AB 001	6.88	$C_{24}H_{31}NO$	62 62	350.3 → 135.1 350.3 → 93.0	44 62
22	AKB 48	7.05	$C_{23}H_{31}N_{30}$	36 36	366.3 → 135.1 366.3 → 93.1	28 68

Table 1. Molecular formulae, retention times, and MS/MS conditions for the synthetic cannabinoid compounds and metabolites in this application.

Materials

AM2233, JWH-015, RCS-4, JWH-203, RCS-8, JWH-210, JWH-073, and JWH-018 were purchased from Cerilliant (Round Rock, TX). All other compounds and metabolites were purchased from Cayman Chemical (Ann Arbor, MI)

Individual stocks (1 mg/mL) were prepared in methanol, DMSO, or 50:50 DMSO:methanol. A combined stock solution of all compounds (10 µg/mL) was prepared in methanol. Working solutions were prepared daily by spiking standards into matrix (urine) and performing serial dilutions to achieve the desired concentrations. Calibrator concentrations ranged from 0.5-100.0 ng/mL for all analytes. Quality control samples were prepared at 2.5, 7.5, and 75.0 ng/mL, in urine.

The 22 compounds analyzed are listed in Table 1 and constitute a panel that includes various classes of forensically relevant synthetic cannabinoids. These include adamantoylindoles (AM 1248 and AKB48), napthoylindoles (JWH 022), phenylacetyl indoles (RCS-4 and RCS-8), and tetramethylcyclopropylindoles (UR-144 and XLR11). Major metabolites of JWH-073 and JWH-018 were also included, as some of these compounds are structural isomers with identical mass spectral fragments that require adequate chromatographic separation for accurate quantitation.

Sample Preparation

Samples were extracted using Oasis HLB µElution plates (p/n 186001828BA). 0.5 mL of 0.8 M potassium phosphate buffer (pH 7.0) was added to 1.0 mL of urine, followed by 10 µL of β-glucuronidase (>140 IU/mL; Roche). After incubation at 40 °C for 1 hr, 1.5 mL of 4% H_3PO_4 was added to all samples. 600 µL of the final prepared sample (equivalent to 200 µL urine) was then extracted using the Oasis HLB µElution plate. All wells were conditioned with 200 µL methanol (MeOH) and 200 µL H_2O. 600 µL of the hydrolyzed, pretreated urine sample was then loaded in each well. All wells were washed with 200 µL water and 200 µL 50:50 H_2O:MeOH. Samples were eluted with 2 x 25 µL aliquots of 60:40 ACN:isopropanol (IPA). All samples were diluted with 75 µL H_2O and 5 µL was injected onto the UPLC System.

Analyte recovery was calculated according to the following equation:

$$\% \text{ Recovery} = \left(\frac{\text{Area A}}{\text{Area B}}\right) \times 100\%$$

Where A equals the peak area of an extracted sample and B equals the peak area of an extracted matrix sample in which the compounds were added post-extraction.

Matrix effects were calculated according to the following equation:

$$\text{Matrix Effects} = \left(\left(\frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}}\right) - 1\right) \times 100\%$$

The peak area in the presence of matrix refers to the peak area of an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

RESULTS AND DISCUSSION

Chromatography

The design of the solid-core CORTECS particle combined with optimal packing in the column results in excellent chromatographic performance. A representative chromatogram of all compounds from a 20 ng/mL calibration standard is shown in Figure 1. Peak assignments are listed in Table 1. Using a CORTECS UPLC C_{18} Column (2.1 x 100 mm; 1.6 µm), all analytes were analyzed within 7.5 minutes with a total cycle time of 8.5 minutes. Peak shape was excellent for all compounds, with no significant tailing or asymmetries, and all peak widths were under 3 seconds at 5% peak height. Peaks 9 and 10, an isobaric pair of metabolites with identical precursor and product ions, were nearly baseline resolved, with a calculated resolution of 1.04, enabling unambiguous identification and quantitation that would not be possible if the two compounds co-eluted. When the same mix of compounds was analyzed on an ACQUITY UPLC BEH C_{18} Column (also 2.1 x 100 mm), adequate separation was not achieved for these two compounds. Co-elution of compound pairs 5 and 6 and 7 and 8 were also seen on the BEH C_{18} Column. Figure 2 highlights the improvements in chromatography seen for compounds 5-10 when using the CORTECS C_{18} Column vs. the BEH C_{18} Column. A more thorough comparison revealed that peak widths on the CORTECS Columns were reduced and peak capacities were improved compared with two analogous, fully porous UPLC Columns of matched dimensions (BEH C_{18} and HSS T3). On average, peak widths on the CORTECS Columns were 12% and 23% narrower, respectively, than those on the BEH and HSS T3 columns. This improvement in performance would also be expected to be seen over other commercially available fully porous UHPLC columns of similar particle sizes as a result of the performance advantages of the solid-core particles and the optimal packing of the CORTECS Columns.

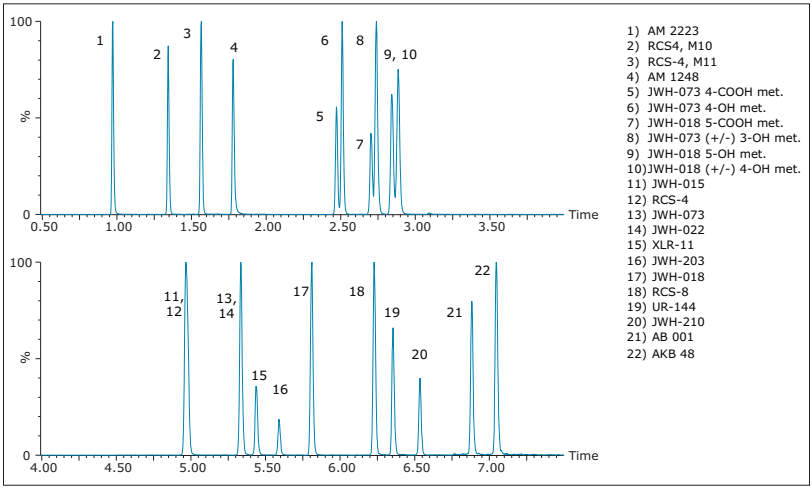

Figure 1. UPLC-MS/MS chromatogram for 22 synthetic cannabinoids and metabolites. Peak assignments are listed in Table 1.

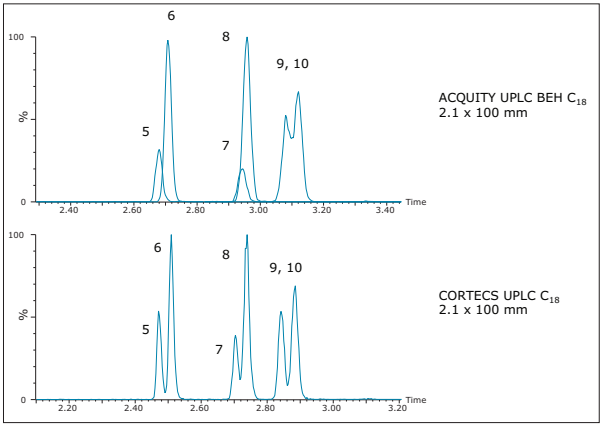

Figure 2. Comparison of the chromatography of peaks 5-10 on the CORTECS UPLC C_{18} Column and the ACQUITY UPLC BEH C_{18} Column. Peak assignments are listed in Table 1.

Recovery and Matrix Effects

The synthetic cannabinoids and metabolites in this application include compounds that are neutral, acidic and basic. Use of the Oasis HLB sorbent enabled the simultaneous extraction of all of the compounds and metabolites tested, regardless of their functionality. Recoveries and matrix effects were calculated according to the equations described in the experimental section and the results are shown in Figure 3. Recoveries ranged from 44-102% with an average of 74%. Matrix effects ranged from -49% (ion suppression) to 32% (enhancement), although most were less than 20%. Even in instances in which recovery was comparatively low, there was more than adequate sensitivity for the purposes of this assay.

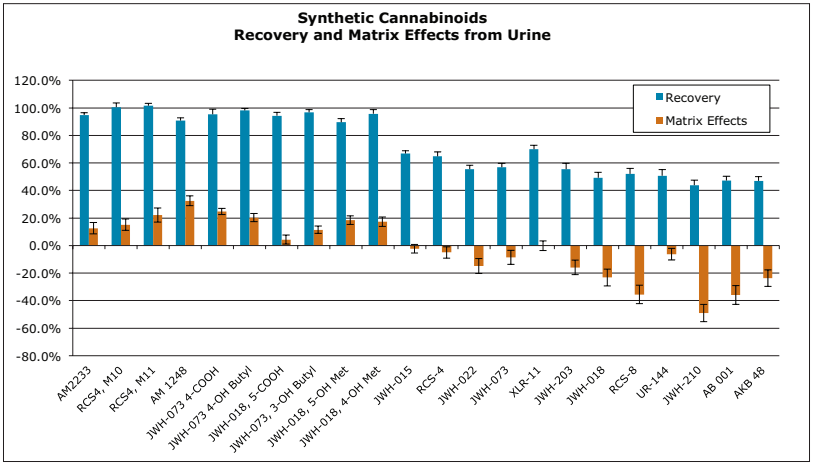

Figure 3. Recovery and matrix effects of synthetic cannabinoid compounds from urine following extraction with Oasis HLB µElution plates. Bars and error bars represent means and standard deviations (N=4), respectively.

Standard curve performance, accuracy, precision, and sensitivity

In order to assess linearity and analytical sensitivity, calibration curves were extracted at concentrations ranging from 0.5-100.0 ng/mL for all components. Figure 4 shows representative calibration curves from acidic, neutral and basic compounds (JWH-073 4-COOH metabolite, JWH-018 5-OH metabolite, and AM2233, respectively). These three example curves demonstrate that the Oasis HLB µElution plate can be used to extract a diverse range of synthetic cannabinoids and metabolites alike with a high degree of accuracy. This is important as both the terminal hydroxylated and carboxylic acid metabolites of JWH-018 and JWH-073 have been shown to be present in substantial amounts in human urine.[3,4] Quality control samples (N=4) at 2.5, 7.5, and 75 ng/mL were also extracted and analyzed. Table 2 summarizes R^2 values from the calibration curves and QC summary data for all compounds. Quality control (QC) results were accurate and precise at low, medium and high concentrations. Accuracies for low level QC samples (2.5 ng/mL) ranged from 90-111% with an average of 101%. The results for the medium and high QC levels were excellent for all analytes, with all accuracies within 15% of expected values. Analytical precision was excellent with most % RSDs less than 10% and none greater than 15%. When accuracy was assessed over all levels (low, medium, and high), the means ranged from 92% to 107%. Limits of detection were as low as 0.1 ng/mL for some of the analytes and none were greater than 2 ng/mL. This was sufficient for the performance requirements of the assay, as these compounds are typically measured in the ng/mL range.

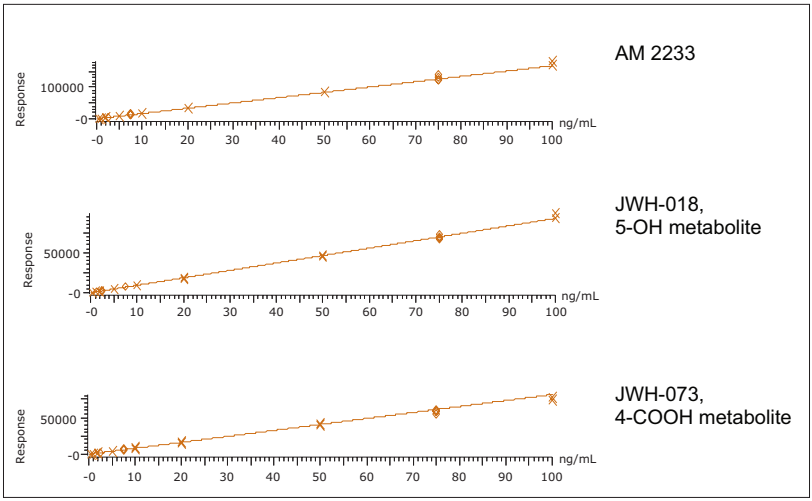

Figure 4. Example calibration curves of AM2233, JWH-018 5-OH metabolite, and JWH-073 4-COOH metabolite, which represent acidic, neutral and basic compounds, respectively.

		QC concentration (ng/mL)						
		2.5		7.5		75		
	R^2	%Acc	%RSD	%Acc	%RSD	%Acc	%RSD	Mean
AM2233	0.996	95.25	7.59	109.78	8.45	102.48	6.52	**102.50**
RCS4, M10	0.998	99.00	3.65	103.13	1.97	96.63	4.33	**99.58**
RCS4, M11	0.999	102.30	2.40	103.53	0.68	96.43	4.11	**100.75**
AM 1248	0.987	111.43	3.79	110.70	1.73	98.98	2.97	**107.03**
JWH-073 4-COOH	0.997	104.68	3.29	108.43	1.13	94.58	3.87	**102.56**
JWH-073 4-OH Butyl	0.999	105.40	2.53	110.30	0.99	93.78	2.59	**103.16**
JWH-018, 5-COOH	0.998	102.10	4.94	104.53	1.62	97.53	4.61	**101.38**
JWH-073, 3-OH Butyl	0.999	103.63	3.89	108.00	0.35	98.95	2.47	**103.53**
JWH-018, 5-OH Met	0.999	103.40	4.65	107.40	1.88	100.58	3.50	**103.79**
JWH-018, 4-OH Met	0.999	103.63	2.11	108.60	1.15	100.70	2.75	**104.31**
JWH-015	0.994	96.65	3.39	99.53	1.81	93.23	3.60	**96.47**
RCS-4	0.992	98.05	2.27	97.88	2.24	91.85	3.02	**95.93**
JWH-022	0.993	100.80	3.69	93.50	5.63	93.28	5.68	**95.86**
JWH-073	0.982	95.48	7.19	88.30	4.51	103.23	6.01	**95.67**
XLR-11	0.987	105.20	8.37	103.55	1.96	90.85	2.87	**99.87**
JWH-203	0.990	97.35	5.39	85.65	2.85	93.65	3.00	**92.22**
JWH-018	0.996	98.48	2.1	86.60	9.38	95.95	6.25	**93.68**
RCS-8	0.992	98.58	4.09	93.48	10.85	96.23	6.38	**96.09**
UR-144	0.989	114.30	9.22	94.35	4.15	94.65	2.31	**101.10**
JWH-210	0.991	89.95	10.86	90.78	14.52	99.80	8.28	**93.51**
AB 001	0.988	100.28	4.02	86.38	9.66	97.45	5.96	**94.70**
AKB 48	0.985	104.28	3.58	87.55	5.79	94.35	5.07	**95.39**
	Mean	**101.37**		**99.18**		**96.60**		

Table 2. R^2 values and quality control results for all compounds. Mean values at the bottom indicate averages of all compounds at particular concentrations. Values to the right indicate averages of individual compounds across all QC concentrations.

CONCLUSIONS

A panel of 22 synthetic cannabinoid drugs and metabolites were extracted from urine and analyzed by UPLC-MS/MS. The use of Oasis HLB µElution plates enabled the simultaneous extraction of acidic, basic and neutral compounds, ensuring that a wide variety of compounds and metabolites could be analyzed. Separation using the CORTECS UPLC C_{18} Column enabled the analysis of all compounds in a short analysis time with baseline resolution of critical isobaric pairs. Separation efficiency and peak widths were superior to fully porous columns of matching dimensions and particle size. This method enables the rapid and reliable extraction and analysis of this critical class of compounds for forensic toxicology. The excellent performance seen on this variety of compounds and the universal nature of the extraction method should allow its use on other synthetic cannabinoids and metabolites, an important feature given the rapid development of new, related compounds.

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High Sensitivity Analysis of Opioids in Oral Fluid Using ionKey/MS

Gregory T. Roman, Robert Lee, James P. Murphy, and Michelle Wood

GOAL

To separate and quantify opioids at very low levels in oral fluid with superior sensitivity compared to 2.1 mm I.D. chromatography.

BACKGROUND

Over the past three decades oral fluid has emerged as a highly valuable biological specimen and is commonly used in numerous settings, including therapeutic drug monitoring, and workplace and roadside drug testing. Oral fluid analysis has many advantages compared with other matrices such as blood or urine. These include collection convenience and reduced sample collection overheads.

Whilst oral fluid itself is a relatively clean matrix, comprising mostly of water and a small percentage of proteins, the popularity of the specimen has led to the development of a large variety of collection devices, aiming to further simplify and standardize collection. Typically these devices will include additives and preservatives to improve the stability of the collected sample. One of the key analytical challenges with oral fluid analysis is the limited amount of sample available for testing compared with blood or urine, requiring very sensitive instrumentation to reach the low levels of detection and quantification. The ionKey/MS™ System offers the capability of improving sensitivity in sample-limited situations, making it ideally suited for this application.

The ionKey/MS System facilitates high sensitivity and robust analysis of opioids in oral fluid.

Figure 1. The ionKey/MS System with the ACQUITY UPLC® M-Class System and the Xevo® TQ-S Mass Spectrometer.

Time (min)	Flow rate (µL/min)	Composition A (%)	Composition B (%)	Curve
0	3	95	5	Initial
6	3	55	45	6
8	3	15	85	6
11	3	95	5	6
Mobile phase A: Water, 0.1% formic acid Mobile phase B: Acetonitrile, 0.1% formic acid				
Trapping conditions				
1 min	15	99.50%	0.50%	6

Table 1. LC-MS conditions.

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Performing analysis of oral fluid samples taken directly from a collection device provides a streamlined method for reducing workflow and increasing throughput. The use of a trapping column prior to analytical separation allows the removal of additives (e.g., surfactants) that can cause suppression in LC-MS and lead to reduced sensitivity. Trapping can also provide improved peak shape for hydrophilic small molecules, and crucially enables enhanced loading for ionKey/MS. Furthermore, the trapping column adds a layer of protection similar to that of a guard column, for precious downstream consumables, making the analytical method more robust.

THE SOLUTION

Here we demonstrate the separation and high sensitivity detection of opioids in oral fluid with the ionKey/MS System comprised of an ACQUITY UPLC M-Class System in combination with a Xevo TQ-S Mass Spectrometer. Preliminary trapping was achieved using an ACQUITY UPLC M-Class HSS T3, 100Å, 1.8 µm, 300 µm x 50 mm Trap Column (P/N 186008029) in combination with a 5 µL loop (Figure 1 and Table 1). Analytical separation was

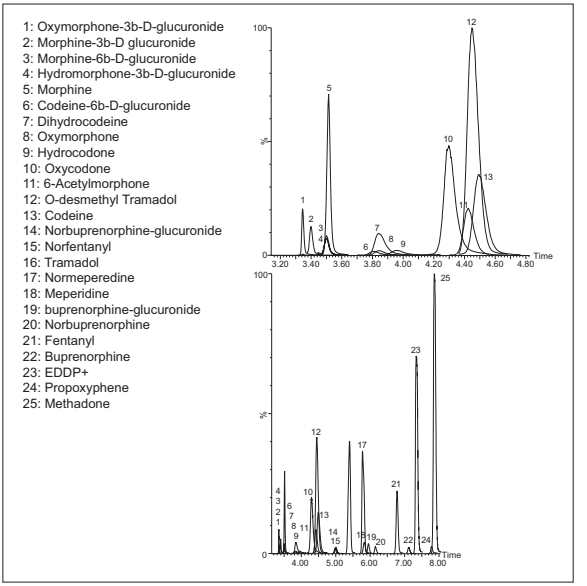

Figure 2. MRM chromatograms for 25 opioids and metabolites.

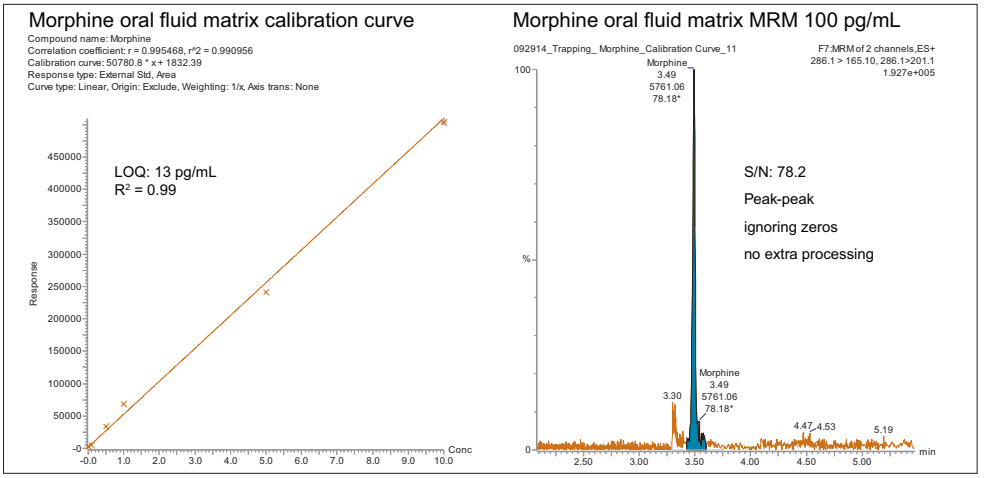

Figure 3. Calibration curve and LOQ of morphine spiked into oral fluid.

performed using an iKey™ HSS T3, 100Å, 1.8 µm, 150 µm x 100 mm (P/N 186007261) Separation Device. Trapping-enabled quantitative analysis of 25 early eluting opioids and metabolites in oral fluid is illustrated in Figure 2.

Figure 3 shows the calibration curve and LOQ of morphine spiked into oral fluid. The LOQ (based on a signal to noise ratio of 10:1) was calculated to be 13 pg/mL. The peak width of morphine, at 10% peak height, was 3.0 seconds and is shown in the insert in Figure 2. Figure 4 compares morphine detected using an ionKey/MS System to an ACQUITY UPLC 2.1 mm I.D. Column. The ionKey/MS System shows an improvement of 9X over the analogous 2.1 mm column format. The trapping column and separation device used in these studies were specifically chosen for their increased retentivity for hydrophilic opioids and metabolites. These comparisons were performed with equivalent injection volumes of 5 µL.

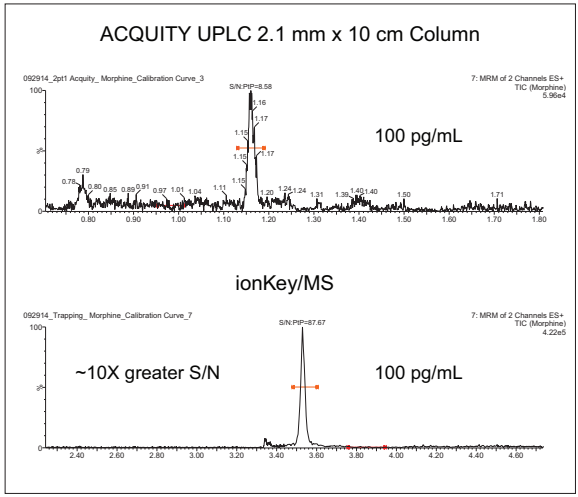

Figure 4. Comparison of morphine detection using an ionKey/MS System to an ACQUITY UPLC 2.1 mm I.D. Column.

SUMMARY

The utilization of the ionKey/MS System enabled a 9X improvement in sensitivity for morphine compared to an ACQUITY UPLC 2.1 mm I.D. Column when injecting the same volume. In addition to morphine, many of the glucuronides were also identified as having improved sensitivity.

Specifically, morphine-3b glucuronide was demonstrated to be baseline separated from morphine, with a sensitivity improvement of 11X. Whilst this particular glucuronide may not be relevant for oral fluid, the increased sensitivity presents a clear advantage for analysis in other biological specimens.

The assay described here is robust and reproducible using oral fluid matrix injections of over 500 injections. Retention time reproducibilities were <1% RSD, while peak area reproducibility of morphine was 11.1% RSD.

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Direct Analysis of Urinary Opioids and Metabolites by Mixed-Mode µElution SPE Combined with UPLC/MS/MS for Forensic Toxicology

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Analysis of all metabolites without enzymatic hydrolysis
- Comprehensive panel of 26 opiate and opioid analgesic compounds
- Rapid and simple sample preparation
- Linear response for all analytes and metabolites
- Improved linearity, accuracy and precision vs. dilution protocol
- Reduced matrix effects

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ACQUITY UPLC® System

ACQUITY UPLC BEH, 2.1 x 100 mm, 1.7 µm Column

Oasis® MCX µElution Plate

Xevo® TQD Mass Spectrometer

MassLynx™ Software

KEY WORDS

Opiates, opioids, UPLC, toxicology, SPE, sample preparation

INTRODUCTION

Meet the scientist behind the application and hear a short summary of his work.*

The analysis of natural and synthetic opioid drugs continues to be an important aspect of forensic toxicology. In the past, analyses were typically conducted by GC/MS after first subjecting the samples to acid or enzymatic hydrolysis to liberate glucuronide metabolites.[1] With the advent of LC/MS/MS techniques, glucuronide metabolites can now be analyzed directly.[2-5] Direct analyses of glucuronide metabolites can eliminate the risk of false negatives due to incomplete hydrolysis, as enzymatic efficiency can vary greatly depending upon the enzyme used and the drug substrate analyzed.[6]

Urine samples, unlike some other matrices, can be analyzed by "dilute and shoot" methods in which samples are diluted with an internal standard mix and directly injected onto an LC/MS/MS system.[2,4] Disadvantages to this type of technique, however, include the fact that urine contains many matrix components that can interfere with MS signals. In addition, this technique does not allow for any sample concentration. This can potentially affect the quantification of some of the glucuronide metabolites that elute under high aqueous conditions, where desolvation efficiency is reduced, as well as many of the opioid drugs, since many of them do not produce intense MS/MS product fragments.

This application note highlights a method for the analysis of 26 opioid drugs and metabolites by mixed-mode SPE followed by UPLC®/MS/MS. Glucuronide metabolites are directly analyzed, eliminating the need for enzymatic or chemical hydrolysis. Direct comparison demonstrates that mixed-mode SPE has improved linearity, greater accuracy and precision, and fewer matrix effects than a simple dilute and shoot method. Previously confirmed, incurred samples were also analyzed, allowing for additional evaluation of this method.

EXPERIMENTAL

LC Conditions

LC system:	ACQUITY UPLC
Column:	BEH C_{18}, 2.1 x 100 mm, 1.7 µm (p/n 186002352)
Column temp.:	30 °C
Injection volume:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% formic acid in MilliQ® water
Mobile phase B:	0.1% formic acid in ACN
Gradient:	Initial conditions were 2% B. The %B was increased to 52.8% over 6.0 min and then returned to 2% over 0.5 min. The system was allowed to reequilibrate for 1.5 min. The entire cycle time was 8.0 min.

MS Conditions

MS system:	Xevo TQD Mass Spectrometer
Ionization mode:	ESI^+
Acquisition mode:	MRM (See Table 1 for transitions)
Capillary voltage:	1 kV
Collision energy (eV):	Optimized for individual compounds (See Table 1)
Cone voltage (V):	Optimized for individual compounds (See Table 1)
Data Management:	All data were acquired and analyzed using MassLynx Software v.4.1

Materials

All compounds and internal standards (IS) were purchased from Cerilliant® (Round Rock, TX). Complementary, deuterated internal standards were used for all compounds with the exception of hydromorphone-3-glucuronide, codeine-6-glucuronide, norbuprenorphine-glucuronide, norfentanyl, and buprenorphine-glucuronide. For these compounds, a deuterated IS with the most similar response was chosen as a surrogate.

A combined stock solution of all compounds (10 µg/mL; 2.5 µg/mL for fentanyl and norfentanyl) was prepared in methanol. Working solutions were made daily by preparing high standards and QCs in matrix (urine) and performing serial dilutions to achieve the desired concentrations. Calibrator concentrations ranged from 5 to 500 ng/mL for all analytes with the exception of fentanyl and norfentanyl, which were prepared at 25% of the concentration of the other analytes (1.25 to 125 ng/mL). A combined internal standard stock solution (5 µg/mL; 1.25 µg/mL for fentanyl and norfentanyl) was prepared in methanol. Working IS solutions were prepared daily in MilliQ water at 50 ng/mL.

Sample Preparation

Sample preparation consisted of either simple dilution or mixed-mode SPE. For the dilution method, 100 µL of urine was diluted 1:1 with MilliQ water containing internal standards. The samples were vortexed and then loaded into individual wells in the collection plate. For mixed-mode SPE, urine samples (method blanks, standards, QCs and unknowns) were pretreated by adding equal amounts of 4% H_3PO_4 and a working IS mixture (50 ng/mL) prepared in MilliQ water. Wells in the Oasis MCX µElution 96-well plate (p/n 186001830BA) were conditioned with 200 µL MeOH followed by 200 µL MilliQ water. 300 µL of each prepared sample was then added to each well, resulting in a sample load of 100 µL urine. After loading, the wells were washed with another 200 µL water followed by 200 µL MeOH. All samples were then eluted with 2 x 50 µL of 60:40 MeOH/ACN containing 5% of a concentrated NH_4OH solution (Fisher, 20-22%). After elution, all samples were evaporated under N_2 to dryness (approximately 5 min) and reconstituted with a solution of 98:2 water/ACN containing 0.1% formic acid and 0.1% human plasma. 10 µL was injected onto the LC/MS/MS system.

RESULTS AND DISCUSSION

The 26 compounds and metabolites screened are listed in Table 1 and constitute a comprehensive panel of natural opiate drugs, semi-synthetic opioids, and synthetic narcotic analgesic compounds. Most all of the compounds are weak bases, with pKa values of approximately 8 to 9. They have a wide range of polarities, with LogP values ranging from -3.48 for morphine-3β-d-glucuronide to 5.00 for methadone, as shown in Table 1; MRM transitions used are also listed there.

	Compound	RT	Formula	Molecular Mass	LogP (predicted)	MRM Transitions	Cone Voltage	Coll. Energy
1	Morphine-3β-D-glucuronide	1.21	$C_{23}H_{27}NO_9$	461.17	-3.48	462.1>286.1 462.1>201.1	58 58	30 52
2	Oxymorphone-3β-D-glucuronide	1.21	$C_{23}H_{27}NO_{10}$	477.16	–	478.1>284.1 478.1>227.1	46 46	28 50
3	Hydromorphone-3β-D- glucuronide	1.34	$C_{23}H_{27}NO_9$	461.17	–	462.1>286.1 462.1>185.1	58 58	28 56
4	Morphine-6β-D-glucuronide	1.47	$C_{23}H_{27}NO_9$	461.17	-2.98	462.2>286.2 462.2>201.2	64 64	38 40
5	Morphine	1.50	$C_{17}H_{19}NO_3$	285.14	0.90	286.2>201.1 286.2>165.1	54 54	28 34
6	Oxymorphone	1.61	$C_{17}H_{19}NO_4$	301.13	0.78	302.1>227.1 302.1>242.1	44 44	28 24
7	Hydromorphone	1.76	$C_{17}H_{19}NO_3$	285.13	1.62	286.2>185.1 286.2>157.1	66 66	32 42
8	Codeine-6β-D-glucuronide	2.00	$C_{24}H_{29}NO_9$	475.18	-2.84	476.2>300.2 476.2>165.2	60 60	36 40
9	Dihydrocodeine	2.07	$C_{18}H_{23}NO_3$	301.17	1.55	302.2>199.1 302.2>128.1	52 52	34 58
10	Codeine	2.14	$C_{18}H_{21}NO_3$	299.15	1.34	300.2>215.2 300.2>165.1	54 54	26 38
11	Oxycodone	2.37	$C_{18}H_{21}NO_4$	315.15	1.03	316.2>256.2 316.2>241.1	44 44	26 26
12	6-Acetylmorphone (6-AM)	2.41	$C_{19}H_{21}NO_4$	327.15	1.31	328.2>165.1 328.2>211.1	60 60	26 36
13	O-desmethyl Tramadol	2.46	$C_{15}H_{23}NO_2$	249.17	1.72	250.2>58.0	26	18
14	Hydrocodone	2.50	$C_{18}H_{21}NO_3$	299.15	1.96	300.2>199.1 300.2>171.0	60 60	30 44
15	Norbuprenorphine-glucuronide	2.83	$C_{31}H_{43}NO_{10}$	589.29	–	590.3>414.3 590.3>101.0	70 70	34 54
16	Norfentanyl	2.93	$C_{14}H_{20}N_2O$	232.16	1.42	233.2>177.2 233.2>150.1	30 30	14 18
17	Tramadol	3.21	$C_{16}H_{25}NO_2$	263.19	2.45	264.2>58.0	24	16
18	Normeperedine	3.58	$C_{14}H_{19}NO_2$	233.10	2.07	234.1>160.1 234.1>188.2	36 36	12 18
19	Meperidine	3.60	$C_{15}H_{21}NO_2$	247.16	2.46	248.2>174.1 248.2>220.2	48 48	22 20
20	Buprenorphine-glucuronide	3.64	$C_{35}H_{45}NO_{10}$	643.34	–	644.3>468.3 644.3>187.1	66 66	42 62
21	Norbuprenorphine	3.77	$C_{24}H_{31}NO_4$	413.26	2.30	414.3>101.0 414.3>187.2	66 66	42 34
22	Fentanyl	4.29	$C_{22}H_{28}N_2O$	336.22	3.82	337.2>188.2 337.2>105.1	48 48	22 38
23	Buprenorphine	4.55	$C_{29}H_{41}NO_4$	467.30	3.55	468.3>101.0 468.3>396.3	72 72	40 48
24	EDDP$^+$	4.79	$C_{20}H_{24}N^+$	278.19	–	278.3>234.2 278.3>249.2	50 50	24 32
25	Propoxyphene	5.18	$C_{22}H_{29}NO_2$	339.30	4.90	340.3>266.2 340.3>143.1	22 22	8 32
26	Methadone	5.25	$C_{21}H_{27}NO$	309.20	5.01	310.2>105.0 310.2>223.1	32 32	22 28

Table 1. Chemical properties and MS conditions of test compounds.

Chromatography

During the initial chromatographic method development, two types of acidic additives (buffers) were evaluated. One was 0.1% formic acid and the second was a combination of 2 mM ammonium acetate with 0.1% formic acid, a mobile phase similar to one used in a related application.[7] No substantial differences in chromatography were seen. However, the analytical sensitivity of several compounds was significantly suppressed when using the combination of ammonium acetate and formic acid. The peak area of all of the glucuronide metabolites and norbuprenorphine were reduced by 60% to 80% compared to those seen with formic acid alone. Thus, the remaining experiments were conducted with the mobile phases containing 0.1% formic acid alone.
A representative chromatogram of all compounds from a 50 ng/mL calibration standard is shown in Figure 1. Peak assignments can be found in Table 1. Using an ACQUITY UPLC BEH C_{18}, 2.1 x 100 mm, 1.7 µm Column we were able to analyze all analytes in under 5.5 min with baseline separation between all critical pairs of isomers, such as between morphine-3-glucuronide, morphine-6-glucuronide, and hydromorphone-3-glucuronide (compounds 1, 3, and 4, respectively) and near baseline separation between morphine-6-glucuronide and morphine.

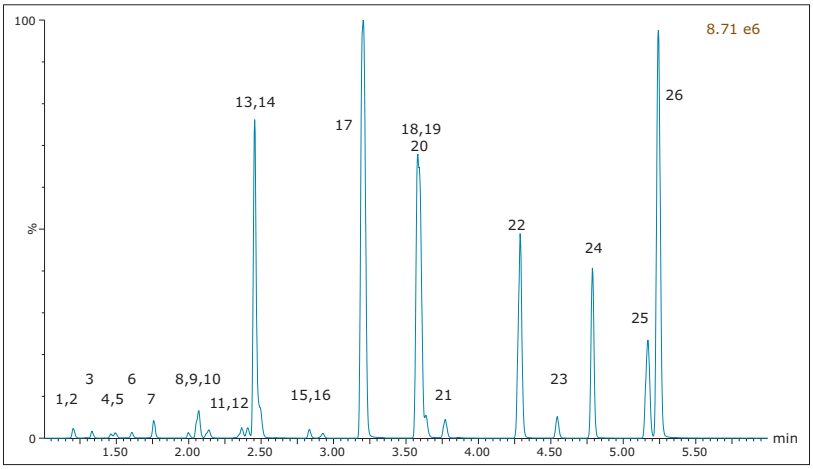

Figure 1. Chromatography of opiate and synthetic analgesic compounds. Peak assignments are listed in Table 1.

Recovery and Matrix Factors

Both mixed-mode SPE and simple dilution were evaluated as possible sample preparation methods. Sample dilution has the advantages of being very simple, inexpensive, and, in the case of urine samples, compatible with reversed-phase chromatographic conditions. Disadvantages include reduced analytical sensitivity resulting from sample dilution and potential interference from matrix components remaining in the sample. SPE, on the other hand, can reduce potential matrix effects because of its selective nature. In addition, the ability of SPE to concentrate the sample can help improve analytical sensitivity of the assay. For this application, evaporation of the organic eluate and reconstitution in a high aqueous solution (2% ACN) was necessary to prevent solvent effects that otherwise interfered with the chromatography of the glucuronide metabolites. Figure 2 shows the average recovery of all compounds from six different lots of urine using the Oasis MCX µElution protocol detailed above. With the exception of the four earliest eluting glucuronide metabolites, all compounds demonstrated recoveries of 89% or greater. In addition, when peak areas from extracted 50 ng/mL samples were compared, the areas for the Oasis MCX µElution protocol ranged from 2.1 to more than six times greater than the dilution protocol. Thus, the ability to concentrate the samples more than made up for the limited recovery seen for a few analytes.

In addition to recovery, matrix factors were evaluated for both protocols. Matrix factors were caclulated according to the following equation:

Matrix Factor (MF) = (peak area in the presence of matrix)/(peak area in the absence of matrix)

In the case of SPE, blank urine was subjected to the extraction protocol, and standards (dissolved in methanol) were added to the final eluate. For the solvent standard, the same methanolic standard solution was combined with 50 µL of the elution solution. Both groups of samples were then evaporated and reconstituted as previously described. For dilution samples, diluted urine samples spiked with drug standards were compared to samples consisting of the reconstitution solution spiked with drug standards.

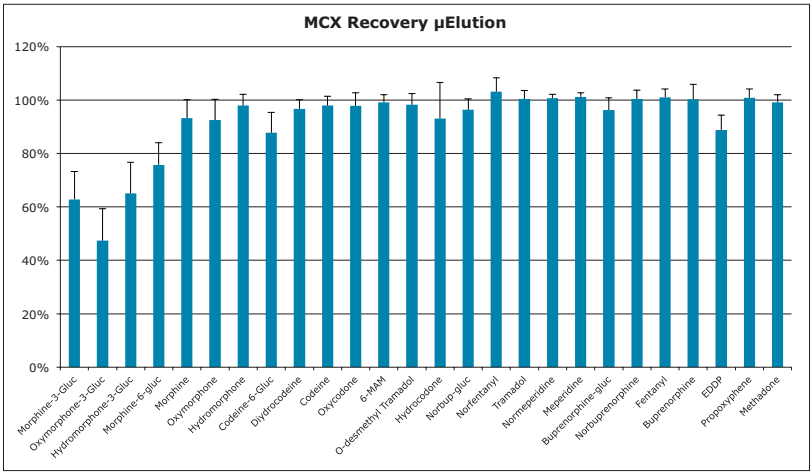

Figure 2. Recovery of opioid compounds from urine using Oasis MCX µElution Plates. Bars represent the mean recovery from six lots of urine.

Figure 3 shows the results of the matrix factor experiments conducted with six different lots of urine. While both protocols show the trend toward suppression of the earlier eluting compounds, statistical analysis reveals that nearly half of the compounds (12 of 26) demonstrated significantly less matrix interference when the Oasis MCX µElution protocol was used. The asterisks in the figure indicate those compounds in which matrix factors were significantly different between the two protocols. In every case in which a significant difference was observed, mixed-mode SPE resulted in matrix factors closer to the ideal value of 1 (no matrix effect). In addition, matrix factors were more consistent when using the mixed-mode SPE protocol. With the exception of oxymorphone (17.0%), oxycodone (15.9%), and fentanyl (20.6%), all compounds in the SPE prepared samples had coefficients of variation (CVs) of less than 15.0%. By contrast, only 12 of the compounds prepared by sample dilution had CVs less than 15.0%. Thus, the use of mixed-mode SPE resulted in not only reduced matrix effects, but also resulted in less variability among different lots of urine.

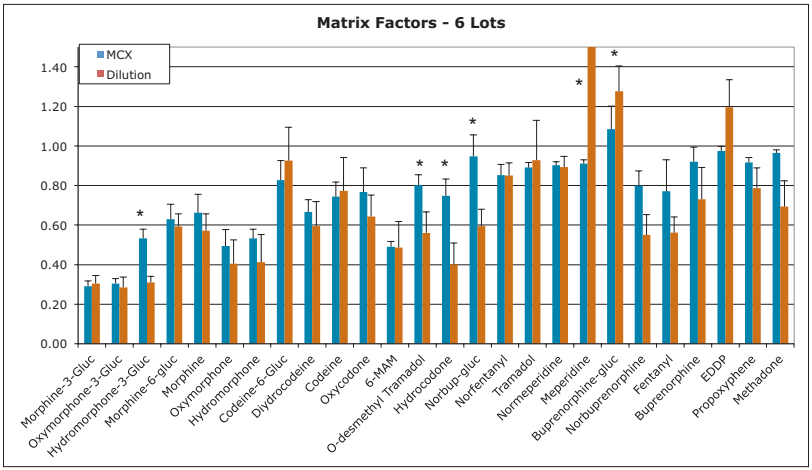

Figure 3. Mean matrix effects of opioid compounds from six lots of urine. Blue bars indicate matrix effects measured from Oasis MCX µElution Plates. Red bars indicate matrix effects resulting from sample dilution. Asterisks indicate compounds in which the difference between the two protocols was significantly different.

Linearity

The two sample preparation protocols were also evaluated for linearity and accuracy. Calibration standards were prepared in urine at concentrations ranging from 5 to 500 ng/mL (1.25 to 125 ng/mL for fentanyl and norfentanyl). Quality control samples (N=4) were prepared at four concentrations: 7.5, 75, 250 and 400 ng/mL. These samples were then prepared by either mixed-mode SPE or sample dilution. The mean accuracies and R^2 values for the calibration curves are shown in Tables 2 and 3. For the SPE prepared samples, the means of all calibration points were within 10% of their expected values. The American Association of Clinical Chemistry (AACC) suggests that %CVs be less than 10%, a criterion which is met by all points with the exception of morphine at 10 and 500 ng/mL and morphine-6-glucuronide at 5 ng/mL. All compounds show excellent linearity, with R^2 values of 0.992 or greater.

Table 3 summarizes calibration data for the samples prepared by dilution. Despite good linearity and accuracy for most compounds, it is clearly evident that a greater number of calibration points exceed the recommended %CV of 10%. Morphine, in particular, shows unacceptable precision throughout the calibration range.

AACC requirements for LLOQs also require that %CVs be under 10%. For the SPE prepared samples, only morphine-6-glucuronide at 5 ng/mL misses this requirement, while six compounds in the dilution prepared samples fail to meet this requirement at the 5 ng/mL level.

		Curve Point (ng/mL)																	
		5		10		20		40		50		100		200		400		500	
	R^2	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV
Morphine-3-β-d-glucuronide	0.996	98.8	8.9%	99.0	7.9%	103.7	5.0%	103.2	4.7%	104.7	5.6%	99.5	1.1%	100.7	4.9%	95.9	3.4%	96.2	2.7%
Oxymorphone-3-b-d-glucuronide	0.997	101.7	0.1%	97.3	4.9%	97.6	3.6%	101.5	1.0%	103.3	7.6%	103.4	3.6%	101.2	2.3%	98.5	5.6%	95.7	7.1%
Hydromorphone-3-b-d-glucuronide	0.998	98.5	1.1%	100.7	2.9%	103.4	4.4%	102.3	1.0%	98.5	7.1%	102.9	3.0%	100.9	3.1%	98.7	4.7%	95.6	3.0%
Morphine-6-gluc	0.994	97.3	11.6%	104.0	7.3%	95.9	6.3%	107.5	2.2%	104.5	2.8%	104.4	2.2%	101.6	6.5%	94.1	5.8%	92.3	2.9%
Morphine	0.992	102.0	5.1%	93.9	11.3%	102.2	8.3%	107.0	9.9%	99.6	2.6%	99.0	4.9%	92.5	4.4%	104.8	9.7%	100.8	12.3%
Oxymorphone	0.998	99.7	0.7%	98.9	2.3%	103.1	2.9%	100.5	2.8%	101.1	3.2%	102.0	7.7%	102.0	1.3%	97.9	3.0%	95.9	3.3%
Hydromorphone	0.998	98.9	7.7%	101.3	2.9%	97.2	6.2%	106.2	0.9%	100.5	0.8%	101.3	2.5%	99.3	0.6%	98.7	3.1%	97.4	2.0%
Codeine-6-β-d-glucuronide	0.998	100.5	0.5%	100.9	4.7%	96.8	2.0%	102.1	0.4%	96.5	2.8%	99.1	6.6%	100.9	3.1%	100.9	2.3%	102.4	1.3%
Dihydrocodeine	0.997	96.7	6.4%	102.0	1.0%	101.5	0.2%	107.0	0.0%	103.5	0.7%	102.0	0.7%	100.6	1.8%	95.3	1.0%	93.1	1.5%
Codeine	0.995	95.6	4.1%	102.2	3.5%	105.8	0.9%	108.0	2.1%	101.4	1.5%	104.8	0.9%	100.3	2.2%	93.6	0.5%	91.4	2.3%
Oxycodone	0.996	96.9	4.4%	101.6	3.0%	101.7	5.0%	105.7	0.1%	104.8	1.0%	102.9	1.4%	100.1	1.2%	96.0	3.6%	91.8	4.2%
6-Acetylmorphone (6-AM)	0.997	95.5	4.9%	105.7	1.3%	99.5	3.1%	103.6	4.0%	100.1	2.4%	98.8	2.9%	101.6	0.9%	100.1	0.9%	94.7	4.5%
O-desmethyl Tramadol	0.999	99.2	3.3%	100.2	0.2%	99.1	0.2%	105.0	1.3%	101.0	1.6%	102.0	0.4%	100.4	0.5%	97.6	1.0%	96.6	0.5%
Hydrocodone	0.999	99.4	0.6%	101.5	2.5%	96.7	1.1%	103.5	1.4%	98.8	0.6%	101.7	1.5%	101.2	0.5%	98.0	1.5%	99.1	1.2%
Norbuprenorphine-glucuronide	0.998	99.6	7.7%	100.6	2.1%	98.6	2.5%	103.1	1.9%	100.7	3.4%	96.8	3.6%	101.0	5.8%	101.0	1.1%	99.0	1.3%
Norfentanyl	0.998	97.9	5.6%	102.4	6.3%	99.9	3.5%	100.9	3.9%	101.8	0.5%	100.2	1.2%	101.7	1.3%	98.0	2.1%	96.8	1.1%
Tramadol	0.995	95.0	0.1%	103.0	0.4%	104.0	1.8%	109.7	0.1%	104.4	0.9%	103.3	1.0%	99.0	0.5%	92.6	1.0%	92.1	0.7%
Normeperedine	0.997	97.0	1.9%	101.2	1.7%	102.1	3.4%	107.4	0.7%	104.3	1.1%	102.8	0.5%	99.7	2.3%	94.2	0.9%	93.5	1.2%
Meperidine	1.000	98.7	0.1%	100.8	1.3%	101.3	0.6%	103.2	1.1%	99.9	0.6%	100.9	1.1%	100.1	1.4%	97.6	1.0%	98.5	1.2%
Buprenorphine-gluc	0.997	104.1	2.2%	97.9	5.0%	94.5	0.8%	95.4	2.4%	94.8	2.4%	100.2	2.3%	101.5	2.3%	105.2	2.4%	104.5	1.0%
Norbuprenorphine	0.997	96.5	0.7%	102.0	1.8%	102.7	1.7%	109.3	1.6%	102.7	2.1%	99.6	2.4%	101.2	3.1%	95.8	0.6%	93.1	3.0%
Fentanyl	0.998	98.1	1.8%	100.9	1.6%	100.7	1.0%	105.8	1.1%	102.0	0.9%	102.7	0.4%	101.1	0.4%	95.9	1.8%	94.4	0.5%
Buprenorphine	0.998	100.9	0.5%	98.1	2.6%	98.3	1.5%	105.1	1.1%	101.4	0.8%	103.7	0.9%	101.8	1.3%	97.4	0.5%	94.9	1.0%
EDDP+	0.999	99.5	0.2%	100.6	0.9%	98.2	0.8%	104.2	0.8%	99.6	1.1%	101.3	0.3%	101.8	0.9%	97.9	0.3%	97.6	0.4%
Propoxyphene	0.996	96.9	2.6%	101.0	0.8%	102.2	0.0%	108.7	0.3%	105.2	0.4%	103.0	0.9%	100.2	1.6%	94.6	1.1%	90.8	1.2%
Methadone	0.998	99.3	1.8%	99.3	1.5%	100.0	0.2%	106.5	2.0%	102.8	0.9%	102.4	1.8%	100.4	1.6%	97.4	0.4%	93.9	1.0%

■ *%CV values > 10%*

Table 2. Accuracy and coefficients of variation (%CV) from opiate calibration curves extracted using Oasis MCX µElution Plates. The concentrations of fentanyl and norfentanyl were ¼ that of the other compounds.

		Curve Point (ng/mL)																	
		5		10		20		40		50		100		200		400		500	
	R^2	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV
Morphine-3-β-d-glucuronide	0.986	102.9	9.8%	91.2	14.9%	102.0	1.4%	111.2	0.8%	93.9	3.7%	106.9	5.7%	95.4	4.0%	102.5	8.2%	94.0	9.7%
Oxymorphone-3-b-d-glucuronide	0.985	102.7	7.5%	100.2	3.1%	86.3	2.2%	105.7	11.0%	98.7	8.5%	100.0	6.9%	97.9	6.0%	102.5	7.9%	106.0	20.1%
Hydromorphone-3-b-d-glucuronide	0.987	96.8	8.1%	100.8	4.0%	110.2	4.4%	109.1	8.1%	92.8	5.3%	101.3	6.5%	94.1	4.8%	101.9	12.9%	93.1	9.8%
Morphine-6-gluc	0.979	94.8	18.4%	109.9	3.2%	96.7	10.5%	110.7	16.3%	100.5	3.3%	98.7	6.5%	91.2	4.3%	100.4	2.9%	97.1	16.8%
Morphine	0.954	89.5	29.2%	98.6	18.9%	119.2	28.6%	92.3	15.4%	97.5	29.7%	93.0	10.8%	115.7	20.5%	99.7	16.3%	100.0	27.5%
Oxymorphone	0.989	89.4	2.5%	95.0	8.7%	96.3	8.3%	109.3	3.2%	100.5	11.1%	98.4	2.4%	94.5	9.7%	99.5	12.7%	97.3	17.1%
Hydromorphone	0.996	97.2	1.2%	110.8	8.4%	114.4	14.2%	102.8	3.6%	98.1	9.1%	100.0	1.8%	98.8	6.4%	97.3	1.6%	98.5	4.9%
Codeine-6-β-d-glucuronide	0.99	94.6	2.3%	107.8	15.2%	106.3	0.9%	104.2	5.8%	96.4	4.5%	98.0	7.5%	95.4	6.0%	98.9	3.2%	98.4	0.3%
Dihydrocodeine	0.997	97.6	1.7%	102.3	6.6%	105.1	6.6%	102.0	2.0%	97.3	2.6%	100.3	4.4%	95.9	4.1%	100.1	5.2%	99.3	5.4%
Codeine	0.99	93.4	11.3%	109.7	2.9%	104.4	8.4%	108.2	10.3%	99.7	5.8%	97.3	5.1%	94.8	6.1%	97.1	3.6%	95.3	2.8%
Oxycodone	0.993	98.6	8.2%	104.1	8.3%	98.0	11.6%	98.3	3.5%	99.4	4.1%	104.6	9.6%	97.0	0.7%	100.7	3.2%	99.3	8.6%
6-Acetylmorphone (6-AM)	0.99	98.4	10.6%	105.1	11.4%	95.8	5.4%	106.9	2.9%	90.6	2.5%	105.2	6.8%	98.1	8.8%	101.9	6.5%	112.6	25.2%
O-desmethyl Tramadol	0.997	96.8	9.0%	104.3	5.0%	102.4	4.1%	104.4	2.1%	100.1	1.0%	101.9	2.1%	94.8	3.8%	99.2	3.5%	96.1	3.0%
Hydrocodone	0.995	95.1	0.4%	113.3	6.0%	103.6	3.7%	105.6	6.0%	100.4	2.1%	99.0	2.1%	96.7	4.8%	97.4	6.8%	95.3	3.7%
Norbuprenorphine-glucuronide	0.992	94.6	13.4%	105.9	5.3%	105.8	5.4%	102.9	1.5%	108.0	6.6%	103.7	1.6%	93.8	5.0%	93.9	2.8%	91.5	1.4%
Norfentanyl	0.995	95.6	4.1%	106.0	4.1%	102.9	6.4%	103.1	1.5%	102.5	3.0%	104.2	2.8%	95.8	4.3%	95.8	5.7%	94.1	3.4%
Tramadol	0.996	95.9	1.6%	104.6	3.0%	103.5	0.8%	107.4	1.4%	101.6	1.1%	101.6	1.4%	95.7	2.1%	96.3	0.4%	93.4	1.8%
Normeperedine	0.996	97.0	3.6%	102.8	3.8%	102.7	3.4%	105.9	2.2%	101.7	1.9%	104.5	1.7%	97.5	3.1%	96.0	1.6%	91.9	5.2%
Meperidine	0.997	96.5	1.5%	105.7	6.0%	100.4	3.1%	104.8	1.6%	100.0	2.0%	100.9	2.9%	96.2	1.8%	98.8	1.8%	96.6	3.9%
Buprenorphine-gluc	0.991	93.3	13.3%	110.0	6.4%	103.4	8.7%	103.9	2.0%	105.8	5.1%	100.0	2.6%	97.4	5.2%	93.8	8.2%	92.4	1.7%
Norbuprenorphine	0.995	95.4	5.5%	104.8	1.4%	105.2	7.5%	105.2	3.9%	103.3	3.6%	102.5	2.7%	94.9	4.5%	94.7	3.8%	94.0	1.6%
Fentanyl	0.997	97.2	0.4%	102.9	3.9%	101.9	4.8%	105.9	0.6%	102.6	1.0%	101.1	3.2%	96.0	3.5%	97.4	5.6%	95.1	1.6%
Buprenorphine	0.994	97.2	8.6%	102.8	9.4%	102.0	8.8%	102.9	0.9%	105.6	4.9%	102.2	2.9%	100.1	5.6%	94.7	7.9%	92.3	1.0%
EDDP+	0.998	97.3	1.2%	103.5	4.3%	101.3	1.2%	104.2	0.8%	101.4	0.9%	100.8	1.7%	97.2	3.2%	98.3	1.1%	95.9	1.7%
Propoxyphene	0.995	95.8	1.0%	105.3	3.0%	101.1	1.1%	105.9	1.7%	105.7	1.0%	102.2	3.1%	99.7	2.7%	94.8	0.8%	89.4	2.4%
Methadone	0.997	98.8	0.9%	101.1	2.1%	98.5	3.4%	105.1	0.5%	103.1	2.5%	102.8	4.0%	101.0	3.0%	98.0	6.4%	91.6	1.2%

■ *%CV values > 10% or differ from expected values by > 15%*

Table 3. Accuracy and coefficients of variation (%CV) from opiate calibration curves prepared using a simple sample dilution protocol. The concentrations of fentanyl and norfentanyl were ¼ that of the other compounds.

Accuracy and Precision

A similar pattern seen in the calibration curves is observed when looking at quality control results for both methods. Table 4 reveals that, with the exception of morphine at 7.5 ng/mL, %CVs for all compounds prepared by mixed-mode SPE fall within the suggested precision requirements of < 10% at all four QC concentrations. With very few exceptions, nearly all accuracy and precision values are less than 10%. In addition, only three QC points show a deviation from expected values of more than 10% and all are within 15%. By contrast, the results for samples prepared by the dilution protocol show that many compounds fail precision (%RSD) requirements, especially at the lower concentration of 7.5 ng/mL, as shown in Table 5, and many values deviate from their expected concentrations by more than 15%, especially at the low QC concentration.

	QC Concentration (ng/mL)											
	7.5			75			250			400		
	Mean	%CV	Bias	Mean	%CV	Bias	Mean	%CV	Bias	Mean	%CV	Bias
Morphine-3-β-d-glucuronide	7.10	8.3%	-5.3%	74.5	5.2%	-0.7%	250.0	2.2%	0.0%	386.3	3.6%	-3.4%
Oxymorphone-3-b-d-glucuronide	7.43	9.7%	-1.0%	76.9	3.0%	2.5%	239.9	4.9%	-4.0%	372.1	3.7%	-7.0%
Hydromorphone-3-b-d-glucuronide	7.98	7.8%	6.3%	76.4	5.8%	1.9%	252.4	2.9%	0.9%	398.1	3.7%	-0.5%
Morphine-6-gluc	8.30	8.7%	10.7%	74.9	6.7%	-0.1%	240.9	5.1%	-3.7%	376.8	4.0%	-5.8%
Morphine	8.15	10.1%	8.7%	75.6	7.7%	0.8%	217.1	5.1%	-13.2%	391.2	4.3%	-2.2%
Oxymorphone	7.85	5.1%	4.7%	73.3	4.2%	-2.3%	243.6	4.7%	-2.6%	385.5	4.5%	-3.6%
Hydromorphone	7.93	1.6%	5.7%	75.7	3.0%	0.9%	247.8	3.7%	-0.9%	388.9	1.2%	-2.8%
Codeine-6-β-d-glucuronide	7.78	4.0%	3.7%	73.6	3.8%	-1.9%	257.3	5.0%	2.9%	421.7	2.6%	5.4%
Dihydrocodeine	7.65	0.8%	2.0%	75.8	1.1%	1.1%	243.8	0.6%	-2.5%	377.9	2.8%	-5.5%
Codeine	7.68	4.7%	2.3%	75.8	0.6%	1.1%	245.2	1.9%	-1.9%	385.4	0.9%	-3.7%
Oxycodone	7.58	5.2%	1.0%	75.5	2.3%	0.7%	244.5	3.4%	-2.2%	378.0	2.8%	-5.5%
6-Acetylmorphone (6-AM)	7.70	5.3%	2.7%	76.2	4.3%	1.6%	245.9	2.3%	-1.7%	391.5	0.7%	-2.1%
O-desmethyl Tramadol	7.83	1.9%	4.3%	75.0	1.3%	0.0%	247.1	0.7%	-1.2%	384.6	0.7%	-3.8%
Hydrocodone	7.60	1.9%	1.3%	74.5	1.3%	-0.7%	244.2	1.6%	-2.3%	381.3	0.9%	-4.7%
Norbuprenorphine-glucuronide	7.80	3.6%	4.0%	76.4	3.1%	1.8%	255.0	3.9%	2.0%	401.9	1.3%	0.5%
Norfentanyl	1.90	0.0%	1.3%	19.4	2.3%	3.3%	62.7	1.2%	0.4%	101.7	2.2%	1.7%
Tramadol	7.60	0.0%	1.3%	76.8	0.3%	2.4%	240.5	0.8%	-3.8%	369.2	0.5%	-7.7%
Normeperedine	7.48	2.0%	-0.3%	75.3	1.6%	0.4%	238.7	1.2%	-4.5%	371.4	1.4%	-7.2%
Meperidine	7.43	0.7%	-1.0%	73.2	0.5%	-2.5%	242.4	2.4%	-3.1%	388.1	1.7%	-3.0%
Buprenorphine-gluc	8.08	2.7%	7.7%	77.8	1.8%	3.7%	267.0	1.6%	6.8%	441.1	1.3%	10.3%
Norbuprenorphine	7.73	1.2%	3.0%	77.7	3.8%	3.6%	246.1	1.5%	-1.6%	377.2	1.0%	-5.7%
Fentanyl	1.90	0.0%	1.3%	19.2	1.1%	2.4%	60.8	1.0%	-2.7%	96.8	1.0%	-3.2%
Buprenorphine	7.55	2.3%	0.7%	77.2	1.9%	2.9%	247.2	1.9%	-1.1%	397.1	1.3%	-0.7%
EDDP+	7.65	1.3%	2.0%	75.0	1.1%	0.0%	243.2	0.9%	-2.7%	387.7	1.1%	-3.1%
Propoxyphene	7.55	0.8%	0.7%	78.4	0.5%	4.5%	243.4	0.9%	-2.6%	378.9	1.9%	-5.3%
Methadone	7.58	0.7%	1.0%	78.2	1.5%	4.3%	246.4	1.0%	-1.4%	386.4	1.2%	-3.4%

■ %RSD > 10% or %deviation >15%

Table 4. Quality control statistics for opioid compounds extracted using Oasis MCX µElution Plates. For each concentration, mean, %CV and % bias are listed (N=4).

	QC Concentration (ng/mL)											
	7.5			75			250			400		
	Mean	%RSD	Bias	Mean	%RSD	Bias	Mean	%RSD	Bias	Mean	%RSD	Bias
Morphine-3-β-d-glucuronide	7.08	10.3%	-5.7%	73.3	6.1%	-2.3%	239.4	2.3%	-4.2%	380.0	6.2%	-5.0%
Oxymorphone-3-b-d-glucuronide	6.85	18.1%	-8.7%	72.9	6.8%	-2.8%	229.7	4.0%	-8.1%	365.9	7.0%	-8.5%
Hydromorphone-3-b-d-glucuronide	7.75	14.5%	3.3%	78.1	4.5%	4.1%	236.5	6.9%	-5.4%	362.7	5.8%	-9.3%
Morphine-6-gluc	7.85	23.1%	4.7%	74.0	17.5%	-1.4%	249.1	9.3%	-0.4%	358.6	3.5%	-10.4%
Morphine	5.28	26.9%	-29.7%	76.0	7.9%	1.3%	267.4	9.4%	7.0%	410.7	16.6%	2.7%
Oxymorphone	8.98	23.3%	19.7%	82.4	9.7%	9.9%	251.9	5.8%	0.7%	360.1	5.4%	-10.0%
Hydromorphone	8.13	14.1%	8.3%	79.3	5.0%	5.7%	251.8	5.1%	0.7%	381.1	3.4%	-4.7%
Codeine-6-β-d-glucuronide	6.45	11.5%	-14.0%	71.6	7.0%	-4.5%	226.7	8.0%	-9.3%	358.6	4.4%	-10.4%
Dihydrocodeine	8.25	9.4%	10.0%	86.1	8.0%	14.8%	244.8	5.6%	-2.1%	387.3	5.3%	-3.2%
Codeine	7.90	10.5%	5.3%	76.5	4.7%	2.0%	236.2	8.0%	-5.5%	366.0	3.9%	-8.5%
Oxycodone	7.53	20.4%	0.3%	79.2	6.8%	5.6%	243.0	3.4%	-2.8%	380.3	3.4%	-4.9%
6-Acetylmorphone (6-AM)	6.50	7.7%	-13.3%	68.3	9.5%	-8.9%	215.6	2.8%	-13.8%	371.6	5.2%	-7.1%
O-desmethyl Tramadol	7.45	3.6%	-0.7%	79.5	4.9%	5.9%	240.2	3.3%	-3.9%	369.0	2.5%	-7.8%
Hydrocodone	6.75	8.2%	-10.0%	71.9	3.6%	-4.2%	227.2	6.4%	-9.1%	341.2	5.8%	-14.7%
Norbuprenorphine-glucuronide	7.25	5.3%	-3.3%	77.1	2.7%	2.8%	234.5	5.0%	-6.2%	350.2	3.0%	-12.4%
Norfentanyl	1.53	11.2%	-18.7%	20.1	3.7%	6.9%	60.3	3.7%	-3.6%	92.1	0.6%	-7.9%
Tramadol	6.53	1.5%	-13.0%	69.8	3.6%	-6.9%	218.1	1.3%	-12.8%	335.5	0.8%	-16.1%
Normeperedine	7.45	4.6%	-0.7%	79.3	5.1%	5.7%	234.6	3.1%	-6.2%	356.8	0.7%	-10.8%
Meperidine	7.33	1.7%	-2.3%	77.4	7.0%	3.2%	236.3	2.1%	-5.5%	367.0	2.7%	-8.2%
Buprenorphine-gluc	4.80	4.5%	-36.0%	65.8	3.6%	-12.3%	211.1	4.9%	-15.6%	327.1	2.1%	-18.2%
Norbuprenorphine	7.15	9.2%	-4.7%	79.6	2.8%	6.2%	242.6	5.6%	-3.0%	364.2	1.7%	-9.0%
Fentanyl	1.75	3.3%	-6.7%	19.5	2.9%	3.9%	60.0	3.9%	-4.1%	91.9	1.4%	-8.2%
Buprenorphine	6.80	6.4%	-9.3%	75.5	3.8%	0.6%	231.1	3.7%	-7.6%	356.6	2.3%	-10.9%
EDDP+	7.45	1.7%	-0.7%	78.3	3.3%	4.4%	239.2	1.0%	-4.3%	365.2	2.1%	-8.7%
Propoxyphene	7.00	8.2%	-6.7%	75.9	2.2%	1.2%	229.7	2.8%	-8.1%	349.9	4.5%	-12.5%
Methadone	6.98	6.0%	-7.0%	75.6	2.5%	0.7%	232.8	3.4%	-6.9%	349.5	4.4%	-12.6%

■ *%RSDs > 10% or %deviation > 15%*

Table 5. Quality control statistics for opioid compounds prepared using a simple sample dilution protocol. For each concentration, mean, %CV and % bias are listed (N=4).

Analysis of Incurred Samples

In order to test this method in a real-world context, 32 urine samples (two negative, 30 positive) previously confirmed for opiate compounds were obtained and analyzed by the current method. These samples had been analyzed for 6-MAM (heroin metabolite), codeine, hydrocodone, hydromorphone, morphine, oxycodone, and oxymorphone. Among the differences in analysis was the fact that these samples had been hydrolyzed to release the conjugated metabolites from the glucuronide moieties. Figure 4 compares the results obtained from the current method to those reported from the laboratory that provided the samples for oxycodone and hydrocodone. These two compounds both lack hydroxyl groups at positions three and six. This renders them incapable of undergoing phase two glucuronidation,[6,8] eliminating any discrepancies in the data due to incomplete hydrolysis. These two figures show fairly good correlation when comparing the two methods, with R^2 values of 0.956 and 0.985 for hydrocodone and oxycodone, respectively. With a slope of near 1 (m=0.962), the oxycodone results between the two methods are in good agreement. For hydrocodone, there is a bias towards higher concentrations in the method presented here (m=0.689). This could be due to the influence of two highly concentrated samples with measured concentrations of 6574 and 7032 ng/mL by the current method that had previously reported results of 3750 and 4610 ng/mL, respectively. For the current analysis, these samples were diluted to concentrations within the reported linear range of 5 to 500 ng/mL.
It is unknown if the previously reported results represented samples that had been properly diluted or not.

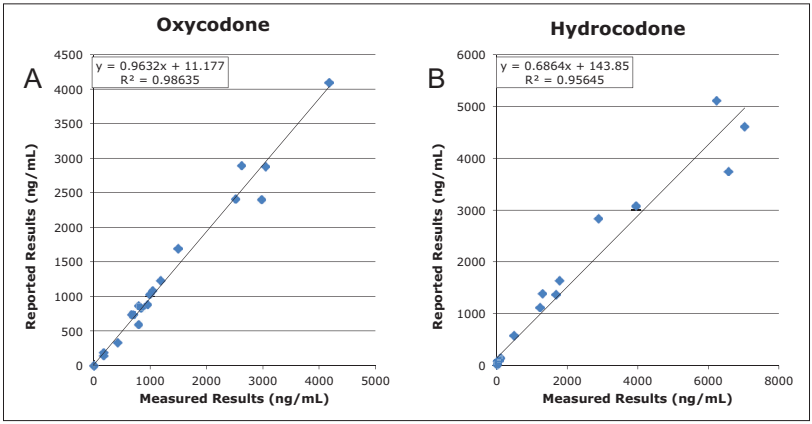

Figure 4. Comparison of results obtained using the current method vs. an alternative LC/MS/MS confirmation method for previously analyzed incurred samples.

A significant difference was seen when the samples were analyzed for compounds such as morphine, oxymorphone, and hydromorphone that undergo significant glucuronidation prior to excretion. Many methods used to analyze opioid drugs rely on enzymatic hydrolysis. However, the degree of hydrolysis is greatly dependent upon not only the β-glucuronidase enzyme used (ex: *Patella vulgate, Helix pomata, Escherichia coli*), but also on the substrate (morphine-6-gluc vs. morphine-3-gluc, morphine-3-gluc vs. hydromorphone-3-gluc).[6] Analysis of the same group of samples by the current and previously reported methods revealed that the reliance on enzymatic hydrolysis dramatically underestimates the total amount of glucuronidated metabolites. Regression analysis of reported released oxymorphone and hydromorphone vs. the actual measured totals of each compound using the current method (glucuronide conjugate + free drug) yielded slopes of 0.20 and 0.25, respectively, indicating that 75% to 80% of the drug was not hydrolyzed. Analysis with this current method reveals that > 85% of total oxymorphone and hydromorphone exist as glucuronide conjugates. Thus, any inefficiencies in glucuronide hydrolysis could result in significant underestimation of total compound concentration. The current method, obviously, is not subject to this limitation, since glucuronide metabolites are measured directly.

CONCLUSIONS

The method presented here demonstrates the advantages of mixed-mode µElution SPE combined with UPLC/MS/MS for the analysis of 26 opioid compounds and metabolites of interest. All compounds were analyzed in under 5.5 min with complete resolution of all isobaric compound pairs. The use of Oasis MCX µElution Plates resulted in improved linearity, and significantly reduced matrix effects compared to a simple dilution method. Accuracy and precision for quality control samples and calibration standards were also improved using mixed-mode SPE. The ability to achieve LOQs of 5 ng/mL for nearly all analytes and the ability to measure glucuronide metabolites directly without hydrolysis make this method well suited for the analysis of these compounds.

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Direct Analysis of Opioids and Metabolites from Whole Blood Using Ostro Sample Preparation Plates Combined with UPLC-MS/MS for Forensic Toxicology

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APPLICATION BENEFITS

- Analysis of glucuronide metabolites without enzymatic hydrolysis
- Comprehensive panel of 22 opiate and opioid analgesic compounds
- Rapid and simple sample preparation compared to traditional LLE, SPE, or protein precipitation
- Removal of endogenous phospholipids
- Linear response for all analytes and metabolites

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ACQUITY UPLC® BEH Column

ACQUITY UPLC System

Xevo® TQD Mass Spectrometer

KEY WORDS

Opiates, opioids, UPLC, forensic toxicology, whole blood

INTRODUCTION

The analysis of natural and synthetic opioid drugs continues to be an important aspect of forensic toxicology. A substantial percentage of arrests and/or deaths are attributed to the misuse or abuse of narcotic pain relievers such as oxycodone and hydrocodone, as well as the illegal opiate, heroin. Forensic laboratories often need to analyze whole blood specimens for the presence of different drugs to determine the precise cause of death, in cases of driving under the influence of drugs, or other criminal or research purposes. In the past, opioid analyses were typically conducted by GC/MS after first subjecting the samples to acid or enzymatic hydrolysis to liberate glucuronide metabolites.[1] This step of using enzymatic hydrolysis to convert glucuronide metabolites to their free form adds time and expense to analysis, and complete and consistent hydrolysis is not always assured.[2] With the advent of modern UPLC®/MS/MS techniques, glucuronide metabolites can now be analyzed directly.[3-6]

Many sample preparation strategies have been used for whole blood analysis, including liquid-liquid extraction (LLE) and solid phase extraction (SPE). One of the simplest involves cell lysis followed by protein precipitation. The method presented in this study describes a rapid and straightforward sample preparation strategy using Ostro Sample Preparation Plates whereby whole blood samples can be pre-treated to lyse the cells, precipitated with acetonitrile, and eluted using a simple 96-well format. All sample pre-treatment is conducted within the wells of the Ostro Plate, without the need for centrifugation or sample transfer from individual tubes.

Following sample preparation, 22 opioid drugs and metabolites are subsequently analyzed by UPLC-MS/MS. Glucuronide metabolites are directly analyzed, eliminating the need for enzymatic or chemical hydrolysis. Calibration curves are linear with appropriate limits of detection easily reached.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 100 mm, 1.7 µm (p/n 186002352)
Column temp.:	30 °C
Injection volume:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% formic acid in MilliQ water
Mobile phase B:	0.1% formic acid in ACN
Gradient:	Initial conditions were 2% B. The %B was increased to 52.8% over 6 minutes, then returned to 2% over 0.5 minute. The system was allowed to re-equilibrate for 1.5 minutes. The entire cycle time was 8.0 minutes.

MS conditions

Mass spectrometer:	Xevo TQD
Ionization mode:	ESI positive
Acquisition mode:	MRM (see Table 1 for transitions)
Capillary voltage:	1 kV
Collision energy (eV):	Optimized for individual compounds (see Table 1)
Cone voltage (V):	Optimized for individual compounds (see Table 1)

Data management

Waters® MassLynx® Software *v*4.1

Sample description

All compounds and internal standards (IS) were purchased from Cerilliant (Round Rock, TX). Complementary, deuterated internal standards were used for all compounds with the exception of hydromorphone-3-glucuronide, codeine-6-glucuronide, and norbuprenorphine-glucuronide. For these compounds, a deuterated IS with the most similar response was chosen as a surrogate.

A combined stock solution of all compounds (10 µg/mL; 2.5 µg/mL for fentanyl) was prepared in methanol. Working solutions were prepared daily with high standards and QCs in matrix (blood) and performing serial dilutions to achieve the desired concentrations. Calibrator concentrations ranged from 5 to 500 ng/mL for all analytes except fentanyl, which was prepared at 25% of the concentration of the other analytes (1.25 to 125.00 ng/mL). A combined internal standard stock solution (5 µg/mL; 1.25 µg/mL for fentanyl) was prepared in ACN.

Sample preparation

Whole blood samples were prepared by adding 150 µL of aqueous 0.1 M $ZnSO_4$/0.1 M NH_4CH_3COOH to the wells of an Ostro Pass-through Sample Preparation Plate. 50 µL of whole blood was added to the $ZnSO_4$/NH_4CH_3COOH solution and mixed briefly (5 s) to lyse the cells. 600 µL of ACN containing internal standards was then added to the prepared samples. After vortexing for 3 minutes, the samples were eluted into a 96-well collection plate, evaporated to dryness under N_2, and reconstituted in 50 µL of 0.1% formic acid in 2% ACN. 10 µL was injected onto the LC/MS/MS system.

RESULTS AND DISCUSSION

The 22 compounds and metabolites screened, listed in Table 1, constitute a comprehensive panel of natural opiate drugs, semi-synthetic opioids, and synthetic narcotic analgesic compounds. Most of the compounds are weak bases, with pKa values of approximately 8 to 9. They have a wide range of polarities, with LogP values ranging from -3.48 for morphine-3β-d-glucuronide to 5.0 for methadone. MRM transitions used are also listed in Table 1. With the exception of tramadol and O-desmethyl tramadol, primary and confirmatory MRM transitions are listed along with their respective collision energies.

Chromatography

A representative chromatogram of all compounds from a 50 ng/mL calibration standard is shown in Figure 1. Peak assignments can be found in Table 1. Using an ACQUITY UPLC BEH C_{18} 2.1 x 100 mm, 1.7 µm Column, all analytes were analyzed in less than 5.5 minutes with a baseline separation between the critical isomer pairs of morphine-3-glucuronide and hydromorphone-3-glucuronide (compounds 1 and 3), morphine and hydromorphone (compounds 4 and 6), and codeine and hydrocodone (compounds 9 and 13). Total cycle time was 8.0 minutes.

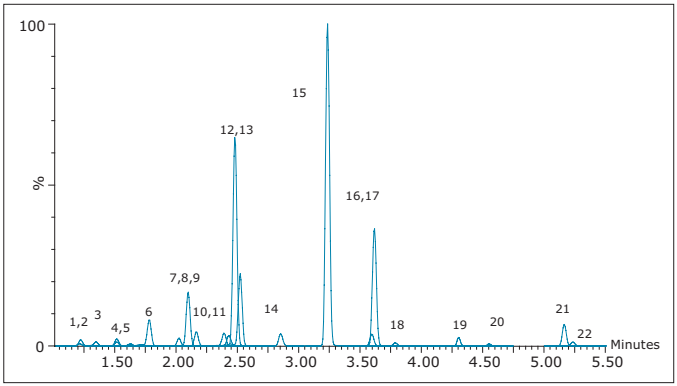

Figure 1. Chromatography of opiate and synthetic analgesic compounds. Peak assignments are listed in Table 1.

Compound		RT	Formula	Molecular mass	MRM transitions	Cone voltage	Collision energy
1	Morphine-3β-D-glucuronide	1.21	$C_{23}H_{27}NO_9$	461.17	462.1>286.1, 201.1	58	30, 52
2	Oxymorphone-3β-D-glucuronide	1.21	$C_{23}H_{27}NO_{10}$	477.16	478.1>284.1, 227.1	46	28, 50
3	Hydromorphone-3β-D- glucuronide	1.34	$C_{23}H_{27}NO_9$	461.17	462.1>286.1, 185.1	58	28, 56
4	Morphine	1.50	$C_{17}H_{19}NO_3$	285.14	286.2>201.1, 165.1	54	28, 34
5	Oxymorphone	1.61	$C_{17}H_{19}NO_4$	301.13	302.1>227.1, 242.1	44	28, 24
6	Hydromorphone	1.76	$C_{17}H_{19}NO_3$	285.13	286.2>185.1, 157.1	66	32, 42
7	Codeine-6β-D-glucuronide	2.00	$C_{24}H_{29}NO_9$	475.18	476.2>300.2, 165.2	60	36, 40
8	Dihydrocodeine	2.07	$C_{18}H_{23}NO_3$	301.17	302.2>199.1, 128.1	52	34, 58
9	Codeine	2.14	$C_{18}H_{21}NO_3$	299.15	300.2>215.2, 165.1	54	26, 38
10	Oxycodone	2.37	$C_{18}H_{21}NO_4$	315.15	316.2>256.2, 241.1	44	26, 26
11	6-Acetylmorphone (6-AM)	2.41	$C_{19}H_{21}NO_4$	327.15	328.2>165.1, 211.1	60	26, 36
12	O-desmethyl tramadol	2.46	$C_{15}H_{23}NO_2$	249.17	250.2>58.0	26	18
13	Hydrocodone	2.50	$C_{18}H_{21}NO_3$	299.15	300.2>199.1, 171.0	60	30, 44
14	Norbuprenorphine-glucuronide	2.83	$C_{31}H_{43}NO_{10}$	589.29	590.3>414.3, 101.0	70	34, 54
15	Tramadol	3.21	$C_{16}H_{25}NO_2$	263.19	264.2>58.0	24	16
16	Normeperedine	3.58	$C_{14}H_{19}NO_2$	233.10	234.1>160.1, 188.2	36	12, 18
17	Meperidine	3.60	$C_{15}H_{21}NO_2$	247.16	248.2>174.1, 220.2	48	22, 20
18	Norbuprenorphine	3.77	$C_{25}H_{35}NO_4$	413.26	414.3>101.0, 187.2	66	42, 34
19	Fentanyl	4.29	$C_{22}H_{28}N_2O$	336.22	337.2>188.2, 105.1	48	22, 38
20	Buprenorphine	4.55	$C_{29}H_{41}NO_4$	467.30	468.3>101.0, 396.3	72	40, 48
21	Propoxyphene	5.18	$C_{22}H_{29}NO_2$	339.30	340.3>266.2, 143.1	22	8, 32
22	Methadone	5.25	$C_{21}H_{27}NO$	309.20	310.2>105.0, 223.1	32	22, 28

Table 1. Chemical properties and MS conditions of test compounds.

Recovery

For this application, evaporation of the organic eluate and reconstitution in a high aqueous solution (2% ACN) was necessary to prevent solvent effects that, otherwise, would interfere with the chromatography of the glucuronide metabolites. Figure 2 shows the average recovery of all compounds from whole blood using the Ostro Pass-through protocol detailed above. With the exception of the three earliest eluting glucuronide metabolites, all compounds demonstrated recoveries of 60% or greater, and the majority of compounds were recovered at 80% or greater.

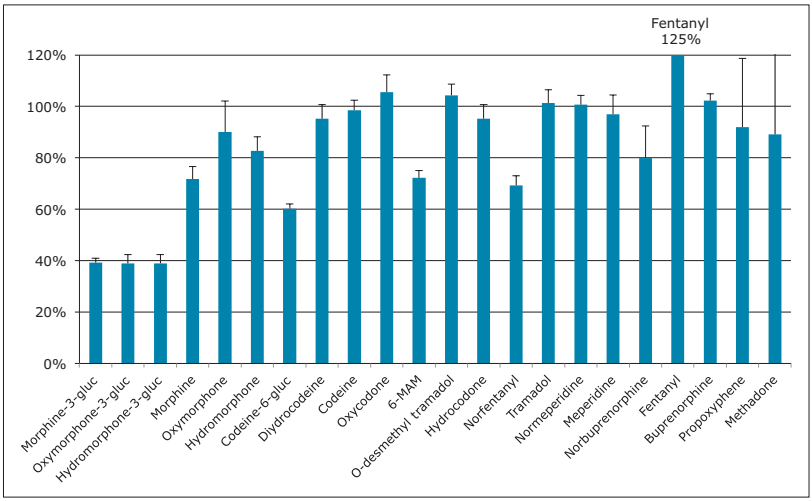

Figure 2. Recovery of opioid compounds from whole blood using Ostro Pass-through Sample Preparation Plates. Bars represent the mean recovery from whole blood samples (N=4).

Linearity and sensitivity

The whole blood extraction method described herein was evaluated for linearity. Calibration standards were prepared in urine at concentrations ranging from 5 to 500 ng/mL (1.25 to 125.00 ng/mL for fentanyl). Table 2 summarizes the R^2 values, % deviations, and %CVs for all of the compounds. The average deviation of all calibration points from the curve was less than 2% for all compounds, and mean %CV values were less than 10% for all but three compounds. Individually, >93% of all calibrators were within 15% of their nominal concentration and >95% of the %CVs for individual calibration points (N=3) were less than 15%. Individual R^2 values were all greater than 0.973, and all but 2 were greater than 0.98. In addition to the good linearity seen over the calibration range, all compounds demonstrated excellent sensitivity. At 5 ng/mL, the UPLC-MS/MS signal for oxymorphone-3-glucuronide, the least sensitive compound, was at least 20x greater than that of a blank extracted whole blood sample. In most cases, the signal for this low calibrator well exceeded 100x the method blank signal.

			Accuracy		%CV	
Compound		R^2	Mean	S.D.	Mean	S.D.
1	Morphine-3-β-d-glucuronide	0.985	99.7	4.9	9.6%	4.6%
2	Oxymorphone-3-b-d-glucuronide	0.983	100.7	4.7	8.4%	6.6%
3	Hydromorphone-3-b-d-glucuronide	0.986	100.8	8.3	8.2%	3.8%
4	Morphine	0.986	101.0	7.4	10.0%	2.9%
5	Oxymorphone	0.989	98.9	6.5	5.7%	3.3%
6	Hydromorphone	0.988	99.4	8.1	4.6%	2.1%
7	Codeine-6-β-d-glucuronide	0.973	99.8	13.5	6.5%	4.2%
8	Dihydrocodeine	0.984	99.8	11.4	7.0%	2.2%
9	Codeine	0.979	101.1	14.1	4.3%	2.2%
10	Oxycodone	0.986	99.0	12.4	4.4%	3.6%
11	6-Acetylmorphone (6-AM)	0.984	100.4	7.1	11.7%	3.4%
12	O-desmethyl Tramadol	0.990	100.3	6.7	5.2%	2.6%
13	Hydrocodone	0.990	100.5	6.8	5.5%	4.8%
14	Norbuprenorphine-glucuronide	0.989	101.0	7.4	11.1%	6.9%
15	Tramadol	0.988	100.5	10.2	3.4%	1.9%
16	Normeperedine	0.995	100.4	4.9	4.2%	2.6%
17	Meperidine	0.994	100.3	6.4	4.2%	2.3%
18	Norbuprenorphine	0.989	100.5	7.6	6.6%	4.4%
19	Fentanyl	0.992	99.6	5.8	5.2%	2.3%
20	Buprenorphine	0.994	100.3	5.5	5.5%	2.3%
21	Propoxyphene	0.990	100.5	7.5	4.2%	1.7%
22	Methadone	0.994	100.3	6.3	2.8%	0.4%

Table 2. Accuracy and coefficients of variation (%CV) from opiate calibration curves prepared using a simple sample dilution protocol. The concentrations of fentanyl and norfentanyl were ¼ that of the other compounds.

CONCLUSIONS

The method presented in this study demonstrates the use of Ostro Pass-through Sample Preparation Plates combined with UPLC-MS/MS for the analysis of 22 opioid compounds and metabolites of interest in whole blood samples. All compounds were analyzed in less than 5.5 minutes with complete resolution of all isobaric compound pairs. The use of Ostro Pass-through Sample Preparation Plates allowed for rapid, in-well cell lysis and protein precipitation, followed by elution into a 96-well collection plate. This procedure resulted in improved throughput compared to protein precipitation in individual tubes, with the added benefit of removing endogenous phospholipid compounds. All analytes demonstrated good linearity over the entire calibration range, and the method was sensitive for reliable detection at the lowest curve points.

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Direct Analysis of Opioids and Metabolites in Oral Fluid by Mixed-mode µElution SPE Combined with UPLC-MS/MS for Forensic Toxicology

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Analysis of all metabolites without enzymatic hydrolysis
- Comprehensive panel of 26 opiate and opioid analgesic compounds
- Rapid and simple sample preparation in oral fluid
- Linear, accurate and precise responses for all analytes and metabolites

WATERS SOLUTIONS

Oasis® MCX µElution Plate

ACQUITY® 96-Well Plate, 700 µL

ACQUITY UPLC® BEH
1.7 µm, 2.1 x 100 mm

ACQUITY UPLC I-Class

XEVO® TQD Mass Spectrometer

KEY WORDS

Opiates, opioids, oral fluid, UPLC, forensic toxicology, SPE, solid-phase extraction, sample preparation, oasis

INTRODUCTION

The analysis of natural and synthetic opioid drugs continues to be an important aspect of forensic toxicology. In the past, analyses were typically conducted by GC/MS after first subjecting the samples to acid or enzymatic hydrolysis to liberate glucuronide metabolites.[1] With the advent of LC-MS/MS techniques, glucuronide metabolites can now be analyzed directly.[2-5] Direct analyses of these metabolites can eliminate the risk of false negatives due to incomplete hydrolysis, as enzymatic efficiency can vary greatly depending upon the enzyme used and the drug substrate analyzed.[6]

One particular sample matrix that has become increasingly popular recently is oral fluid. Unlike urine, oral fluid can be more indicative of current impairment or intoxication. Collection can also be easily accomplished without the privacy issues and adulteration possibilities associated with urine collection. Oral fluid also has similar advantages over blood as a matrix. Once again, collection is much easier, since it is non-invasive and there is no need for specialized training. This application highlights a method for the analysis of 26 opioid drugs and metabolites by mixed-mode SPE followed by UPLC®-MS/MS. Glucuronide metabolites are directly analyzed, eliminating the need for enzymatic or chemical hydrolysis.

EXPERIMENTAL

Final method conditions

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC BEH C_{18} 1.7 µm; 2.1 x 100 mm (p/n 186002352)
Column temp.:	30 °C
Injection volume:	10 µL
Flow rate:	0.4 mL/min.
Mobile phase A:	0.1% formic acid in MilliQ water
Mobile phase B:	0.1% formic acid in ACN
Weak needle wash:	2% ACN in water
Strong needle wash:	10% ACN in water
Gradient:	Initial Conditions were 2% B. The %B was increased to 52.8% over 6 minutes and then returned to 2% over 0.5 min. The system was allowed to re-equilibrate for 1.5 min. The total cycle time was 8.0 min.

MS conditions

MS System:	XEVO TQD Mass Spectrometer
Ionization mode:	ESI Positive
Acquisition mode:	MRM (See Table 1 for transitions)
Capillary voltage:	1 kV
Collision energy (eV):	Optimized for individual compounds (See Table 1)
Cone voltage (V):	Optimized for individual compounds (See Table 1)
Data management:	All data was acquired and analyzed using Waters MassLynx® Software v.4.1

Materials

All compounds and internal standards (IS) were purchased from Cerilliant (Round Rock, TX). Complementary, deuterated internal standards were used for all compounds with the exception of hydromorphone-3-glucuronide, codeine-6-glucuronide, norbuprenorphine-glucuronide, norfentanyl, and buprenorphine-glucuronide. For these compounds, a deuterated IS with the most similar response was chosen as a surrogate.

A combined stock solution of all compounds (10 µg/mL; 2.5 µg/mL for fentanyl and norfentanyl) was prepared in methanol. Working solutions were prepared daily by preparing high standards and QCs in matrix (oral fluid) and performing serial dilutions to achieve the desired concentrations. Calibrator concentrations ranged from 5-500 ng/mL for all analytes with the exception of fentanyl and norfentanyl, which were prepared at 25% of the concentration of the other analytes (1.25-125 ng/mL). A combined internal standard stock solution (5 µg/mL; 1.25 µg/mL for fentanyl and norfentanyl) was prepared in methanol. Working IS solutions were prepared daily in MeOH at 500 ng/mL.

Sample preparation

Sample collection

Oral fluid samples were collected with the Quantisal collection device from Immunalysis according to the manufacturer's directions. The collection applicator was saturated with oral fluid, and then placed in the collection vial, which contained 3.0 mL of sample stabilization buffer. This was claimed to be the equivalent of collecting 1.0 mL ± 0.1 mL of sample. The collection kit was stored overnight to simulate the transit time of the sample and to allow for complete equilibration between the sample in the applicator and the stabilization buffer in the collection vial.

Solid-Phase Extraction

400 µL aliquots of buffer stabilized oral fluid samples (equivalent to 100 µL oral fluid) were pretreated by adding 200 µL 4% H_3PO_4 and 20 µL of the working IS mixture (500 ng/mL in MeOH). Wells in the 96-well Oasis MCX µElution plate (p/n 186001830BA) were conditioned with 200 µL MeOH followed by 200 µL MilliQ water. The entire pretreated sample was then added to each well. After loading, the wells were washed with 200 µL of 2% formic acid, followed by 200 µL of methanol and 200 µL of isopropanol (IPA). All samples were then eluted with 2 x 50 µL of 60:40 ACN:IPA containing 5% of a concentrated NH_4OH solution (Fisher, 20-22%). After elution, all samples were evaporated under N_2 to dryness at 37 °C (approximately 5 min.) and reconstituted with a solution of 98:2 water: ACN containing 0.1% formic acid and 0.1% (by volume) human plasma. 10 µL was injected onto the LC-MS/MS system.

Recovery calculation

Recovery was calculated according to the following equation:

$$\% \text{ Recovery} = \left(\frac{\text{Area A}}{\text{Area B}}\right) \times 100\%$$

Area A refers to the peak area of a sample spiked with analytes before extraction, and area B refers to the peak area of a sample in which the analytes were spiked into the final eluate after extraction.

RESULTS AND DISCUSSION

The 26 compounds and metabolites screened are listed in Table 1 and constitute a comprehensive panel of natural opiate drugs, semi-synthetic opioids, and synthetic narcotic analgesic compounds. Most of the compounds are weak bases, with pKa values of approximately 8-9. They have a wide range of polarities, with LogP values ranging from -3.48 for morphine-3β-d-glucuronide to 5.0 for methadone. MRM transitions, cone voltage and collision energies are also listed in Table 1.

Chromatography

A representative chromatogram of all compounds is shown in Figure 1. Peak assignments can be found in Table 1. Using an ACQUITY UPLC BEH C_{18} Column (1.7 µm, 2.1 x 100 mm), we were able to analyze all compounds in under 5.5 minutes with baseline separation between all critical pairs of isomers, such as morphine-3-glucuronide, morphine-6-glucuronide and hydromorphone-3-glucuronide (compounds 1, 3, and 4, respectively).

	Compound	RT	Formula	Molecular Mass	MRM Transitions	Cone Voltage	Coll. Energy
1	Morphine-3β-D-glucuronide	1.13	$C_{23}H_{27}NO_9$	461.17	462.2 > 286.1, 201.1	58	30, 52
2	Oxymorphone-3β-D-glucuronide	1.12	$C_{23}H_{27}NO_{10}$	477.16	478.2 > 284.1, 227.1	56	44, 68
3	Hydromorphone-3β-D-glucuronide	1.24	$C_{23}H_{27}NO_9$	461.17	462.2 > 286.1, 185.1	58	50, 70
4	Morphine-6β-D-glucuronide	1.37	$C_{23}H_{27}NO_9$	461.17	462.2 > 286.2, 113.0	66	50, 65
5	Morphine	1.40	$C_{17}H_{19}NO_3$	285.14	286.1 > 201.1, 165.1	54	28, 34
6	Oxymorphone	1.51	$C_{17}H_{19}NO_4$	301.13	302.2 > 284.2, 227.1	44	30, 37
7	Hydromorphone	1.65	$C_{17}H_{19}NO_3$	285.13	286.1 > 185.0, 157.0	65	46, 62
8	Codeine-6β-D-glucuronide	1.90	$C_{24}H_{29}NO_9$	475.18	476.2 > 300.2, 113.0	70	50, 60
9	Dihydrocodeine	1.97	$C_{18}H_{23}NO_3$	301.17	302.2 > 199.1, 128.1	60	45, 75
10	Codeine	2.04	$C_{18}H_{21}NO_3$	299.15	300.2 > 199.1, 165.1	58	42, 54
11	Oxycodone	2.26	$C_{18}H_{21}NO_4$	315.15	316.2 > 298.2, 241.1	44	25, 44
12	6-Acetylmorphone (6-AM)	2.30	$C_{19}H_{21}NO_4$	327.15	328.2 > 165.1, 211.1	60	58, 40
13	O-desmethyl Tramadol	2.35	$C_{15}H_{23}NO_2$	249.17	250.2 > 58.0	30	20
14	Hydrocodone	2.38	$C_{18}H_{21}NO_3$	299.15	300.2 > 199.1, 171.0	65	40, 58
15	Norbuprenorphine-glucuronide	2.74	$C_{31}H_{43}NO_{10}$	589.29	590.3 > 414.3, 101.0	74	62, 80
16	Norfentanyl	2.82	$C_{14}H_{20}N_2O$	232.16	233.2 > 177.2, 150.1	38	18, 24
17	Tramadol	3.10	$C_{16}H_{25}NO_2$	263.19	264.2 > 58.0	30	25
18	Normeperedine	3.45	$C_{14}H_{19}NO_2$	233.1	234.1 > 160.1, 131.1	40	20, 35
19	Meperidine	3.46	$C_{15}H_{21}NO_2$	247.16	248.2 > 220.2, 147.1	50	34, 28
20	Buprenorphine-glucuronide	3.52	$C_{35}H_{49}NO_{10}$	643.34	644.3 > 468.3, 396.4	75	60, 80
21	Norbuprenorphine	3.64	$C_{25}H_{35}NO_4$	413.26	414.3 > 101.0, 187.2	70	55, 55
22	Fentanyl	4.15	$C_{22}H_{28}N_2O$	336.22	337.2 > 188.2, 105.1	50	36, 56
23	Buprenorphine	4.41	$C_{29}H_{41}NO_4$	467.3	468.3 > 396.3, 101.0	82	55, 68
24	EDDP+	4.63	$C_{20}H_{24}N^+$	278.19	278.2 > 249.2, 234.2	60	33, 40
25	Propoxyphene	5.02	$C_{22}H_{29}NO_2$	339.3	340.3 > 266.2, 128.0	20	14, 60
26	Methadone	5.09	$C_{21}H_{27}NO$	309.2	310.3 > 265.2, 105.0	32	20, 38

Table 1. Chemical names, formulae, retention times, and MS conditions of test compounds.

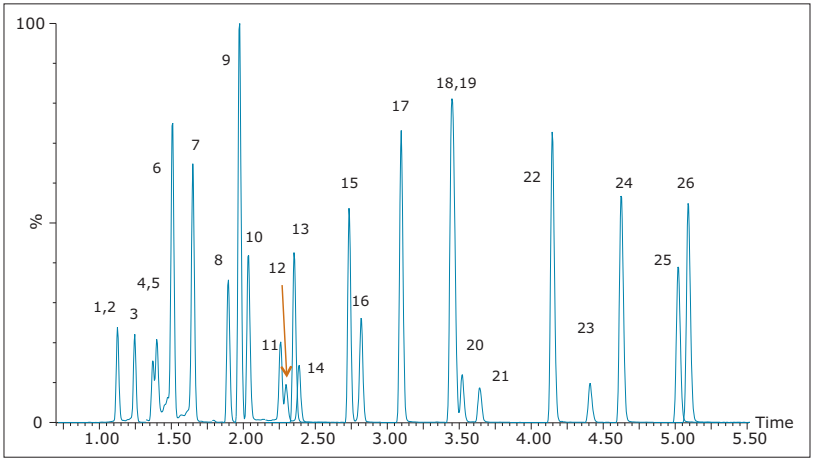

Figure 1. Chromatography of opiates and synthetic analgesic compounds. Peak assignments are listed in Table 1.

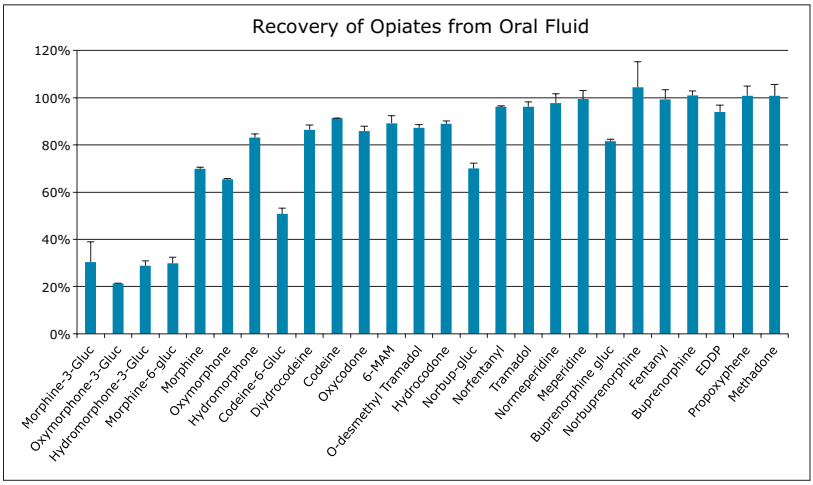

Figure 2. Recovery of opioid compounds from oral fluid extracted using Oasis MCX µElution Plates. Error bars indicate standard deviations (N=4).

Analyte recovery

Recovery was evaluated using both IPA and MeOH as a co-elution solvent with ACN. Both solvents resulted in similar recovery patterns for the 26 opiate compounds. When MeOH was used, recoveries were slightly better for the 4 earliest eluting glucuronide metabolites. However, the average recovery for all compounds was improved when using IPA. Eluting with 60:40 ACN:IPA resulted in an average recovery of 78.8% for all compounds vs. 74.2% using 60:40 ACN:MeOH. Figure 2 shows the average recovery for all compounds when eluted with 60:40 ACN:IPA.

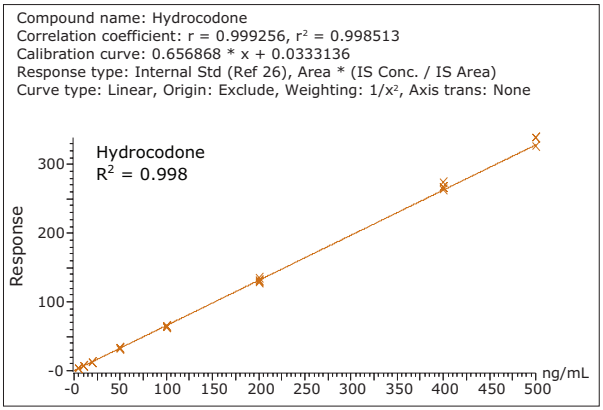

Figure 3. Calibration curve for hydrocodone. R^2 = 0.998. Fit – linear 1/x2 weighting.

For this application, evaporation of the organic eluate and reconstitution in a high aqueous solution (2% ACN) was necessary to prevent strong solvent effects that would otherwise affect the chromatography of the glucuronide metabolites by causing peak distortion that prevents proper retention and integration of the resulting peaks. However, use of the Oasis MCX Plate in the µElution Plate format results in only 100 µL of eluate that is easily evaporated in under 5 minutes. An additional benefit of using the µElution plate format is that only 100 µL of sample is needed for the assay. This can be a significant advantage for oral fluid analysis, since sample sizes are often quite small (1.0 mL). The ability to use minimal sample volumes allows for repeat analysis, or the use of additional aliquots for other analyses, if necessary.

Linearity, accuracy, and precision

Calibration and quality control (QC) results indicate that this method is linear, accurate and precise. Calibration standards were prepared in oral fluid at concentrations ranging from 5-500 ng/mL (1.25-125 ng/mL for fentanyl and norfentanyl). An example calibration curve is shown for hydrocodone in Figure 3. The mean accuracies and R^2 values for the calibration curves are listed in Table 2. All compounds had R^2 values of at least 0.989 and many were 0.995 or greater. Quality control samples (N=4) were prepared at 4 concentrations: 7.5, 25, 150, and 300 ng/mL. Analytical accuracy and precision were very good. With only 2 exceptions, all QC results were within 15% of their intended values and all but 2 points had % CVs that were under 15%.

		QC Concentration (ng/mL)							
		7.5		25		150		300	
Compound	R^2	%CV	Bias	%CV	Bias	%CV	Bias	%CV	Bias
Morphine-3-β-d-glucuronide	0.995	10.2%	14.4%	3.5%	9.0%	6.8%	5.3%	3.3%	2.0%
Oxymorphone-3-b-d-glucuronide	0.994	14.4%	14.9%	5.9%	-0.8%	3.8%	11.2%	1.9%	4.2%
Hydromorphone-3-b-d-glucuronide	0.992	8.2%	8.0%	5.4%	2.2%	7.2%	4.9%	3.9%	2.5%
Morphine-6-gluc	0.993	17.4%	0.8%	6.4%	2.4%	4.6%	3.6%	3.9%	3.3%
Morphine	0.989	15.3%	19.7%	2.7%	18.2%	12.2%	11.6%	6.9%	5.9%
Oxymorphone	0.997	9.2%	2.7%	6.4%	3.3%	2.6%	4.1%	2.7%	5.1%
Hydromorphone	0.997	7.7%	1.1%	3.6%	5.1%	3.2%	5.4%	3.8%	6.4%
Codeine-6-β-d-glucuronide	0.993	2.6%	-7.3%	5.2%	1.9%	3.9%	-3.8%	6.0%	5.7%
Dihydrocodeine	0.996	2.3%	6.7%	3.6%	11.4%	2.7%	4.4%	2.2%	1.5%
Codeine	0.994	8.7%	7.2%	3.7%	11.7%	3.8%	4.3%	3.9%	1.4%
Oxycodone	0.996	7.0%	5.3%	5.6%	10.6%	5.1%	7.5%	2.7%	2.2%
6-Acetylmorphone (6-AM)	0.996	5.3%	5.4%	3.6%	8.5%	3.6%	3.3%	7.1%	4.5%
O-desmethyl Tramadol	0.999	5.6%	6.1%	2.5%	7.7%	2.1%	5.8%	1.7%	5.4%
Hydrocodone	0.998	5.6%	6.4%	3.4%	4.6%	2.7%	4.7%	3.0%	6.6%
Norbuprenorphine-glucuronide	0.992	2.5%	-11.4%	2.8%	1.7%	7.1%	-4.9%	5.9%	8.8%
Norfentanyl	0.998	7.0%	0.8%	3.9%	8.3%	2.9%	2.6%	3.3%	4.9%
Tramadol	0.999	4.8%	6.4%	3.1%	8.8%	2.6%	6.7%	2.2%	4.8%
Normeperedine	0.999	4.8%	-0.7%	3.3%	3.5%	2.2%	3.1%	2.8%	2.4%
Meperidine	0.999	5.5%	5.2%	4.1%	4.9%	2.6%	6.6%	2.5%	6.2%
Buprenorphine-gluc	0.999	4.8%	-4.5%	7.0%	2.2%	3.9%	1.1%	3.7%	7.1%
Norbuprenorphine	0.996	5.9%	5.4%	3.6%	8.3%	2.3%	4.8%	1.5%	2.9%
Fentanyl	0.999	4.6%	4.8%	2.5%	7.4%	2.7%	6.8%	1.5%	6.4%
Buprenorphine	0.999	4.5%	6.5%	2.8%	8.1%	3.0%	7.9%	1.5%	7.5%
EDDP+	0.999	4.7%	4.8%	2.4%	5.8%	2.7%	6.8%	2.5%	7.3%
Propoxyphene	0.999	3.8%	6.8%	3.0%	8.6%	2.4%	7.0%	2.2%	7.0%
Methadone	0.999	5.3%	6.1%	3.2%	8.0%	3.0%	6.8%	2.4%	6.5%

Table 2. Regression coefficients (R^2) and quality control statistics for opioid compounds extracted from oral fluid using Oasis MCX µElution Plates. For each concentration, mean, %CV and % bias are listed (N=4).

CONCLUSIONS

The method presented here demonstrates the advantages of mixed-mode µElution SPE combined with UPLC-MS/MS for the analysis of 26 opioid compounds and metabolites of interest. All compounds are analyzed in under 5.5 minutes with complete resolution of all isobaric compound pairs. Linearity, analytical accuracy, and precision were excellent over the entire calibration range for all 26 compounds. The µElution format enabled the extraction of 100 µL aliquots of oral fluid, leaving the remaining sample for additional assays, or repeat analyses, if necessary. The ability to achieve LOQs of 5 ng/mL for nearly all analytes and the ability to measure glucuronide metabolites directly without hydrolysis make this method well suited for the analysis of these compounds in oral fluid.

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Advantages of CORTECS C_{18} 2.7 µm and XBridge BEH Phenyl *XP* 2.5 µm Columns for the Analysis of a Comprehensive Panel of Pain Management Drugs for Forensic Toxicology

Jonathan Danaceau, Erin Chambers, and Kenneth Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Rapid analysis of 35 forensic toxicology drugs
- Enhanced retention of polar compounds
- Improved resolution vs. competitive biphenyl columns
- Low backpressures compatible with traditional HPLC systems

WATERS SOLUTIONS

Xevo® TQD

ACQUITY UPLC® I-Class System

CORTECS® C_{18} 2.7 µm, 3.0 x 50 mm Column (p/n 186007370)

XBridge® BEH Phenyl ***XP***, 2.5 µm, 3.0 x 50 mm Column (p/n 186006069)

MassLynx® Software

KEY WORDS

Forensic toxicology, pain management, opiates, benzodiazepines, amphetamines, stimulants

INTRODUCTION

In forensic toxicology, drug screening panels often include such commonly used substances such as opiates, benzodiazepines and stimulants. These panels are often analyzed by LC-MS using traditional C_{18} column technologies. Key considerations include the ability to chromatographically resolve the various pairs of isobaric compounds included in these panels, while maintaining good peak shape for a variety of compounds. In addition, when using traditional HPLC systems, the ability to analyze samples as rapidly as possible without exceeding the pressure limitations of the system is very important. This application note highlights the capabilities of Waters' new CORTECS C_{18} 2.7 µm Columns and XBridge BEH Phenyl ***XP*** 2.5 µm Columns for this type of application. In the case of the CORTECS C_{18} Column, the high efficiency packing of solid core 2.7 µm particles yields excellent performance that equals or exceeds competitive columns at lower operating backpressures. If alternative selectivity is desired, the phenyl functionality of the BEH phenyl column enhances the retention of opiate compounds. This enhanced retention can potentially result in reducing ion suppression from urinary matrix components. Both columns achieve excellent baseline separation between isomers, and the entire panel of 35 compounds, including opioids, benzodiazepines, stimulants, and other drugs of abuse can be analyzed in under 4 minutes at backpressures compatible with any HPLC system.

EXPERIMENTAL

Stock solutions were obtained from Cerilliant Corporation, Round Rock, TX. Stock solutions were prepared in methanol. Working solutions were prepared in 5% acetonitrile containing 0.1% formic acid.

LC conditions

LC system:	ACQUITY UPLC I-Class, Fixed Loop (FL) with Column Manager (CMA)
Columns:	CORTECS C_{18} 2.7 µm, 3.0 x 50 mm (p/n 186007370)
	XBridge BEH Phenyl ***XP*** 2.5 µm, 3.0 x 50 mm (p/n 186006069)
Column temp.:	30 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	MilliQ water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid

The mobile phase gradient is listed in Table 1.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI Positive
Capillary voltage:	0.5 V
Collision energy:	Optimized for individual components
Cone voltage:	Optimized for individual components
Data management:	MassLynx v 4.1 scn 855 Software

RESULTS AND DISCUSSION

Waters CORTECS C_{18} 2.7 µm Column, an XBridge BEH Phenyl ***XP*** 2.5 µm Column, and a competitor's biphenyl core shell column (2.6 µm) were used to analyze a panel of 35 common pain management compounds (Figure 1), including opioids, benzodiazepines, stimulants, benzoylecgonine (BZE), and phencyclidine (PCP). All columns had the same dimensions (3.0 x 50 mm). The solvent gradient is listed in Table 1. The entire gradient cycle was 5 minutes.

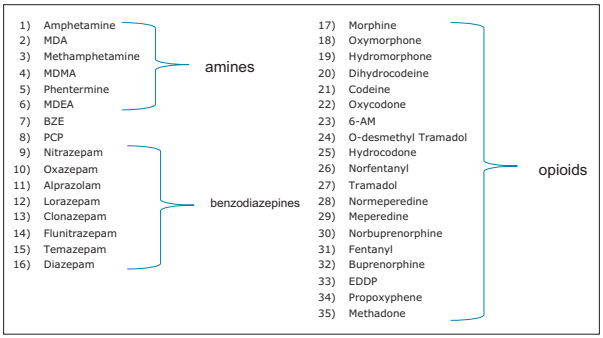


Figure 1. Compound key.

Time (min)	Flow (mL/min)	% MPA	% MPB
0.0	0.6	95	5
4.0	0.6	40	60
4.1	0.6	95	5
5.0	0.6	95	5

Table 1. LC Gradient.

All compounds eluted within 4 minutes and showed good, symmetrical peak shape. Average peak width and maximum backpressure for all columns are shown in Table 2.

Column	Particle size (µm)	Backpressure	Mean peaks width (s)
CORTECS C_{18}	2.7	2206	2.52
XBridge BEH Phenyl	2.5	3274	2.94
Competitor biphenyl	2.6	2492	2.71

Table 2. Performance summary.

The columns operated at backpressures well within the limit of traditional HPLC systems and, predictably, backpressure increased with decreasing particle size. Interestingly, the CORTECS C_{18} Column, despite its larger particle size, demonstrated the best resolution, as measured by average peak width (see Table 2). The chromatography of all opioid compounds is shown in Figure 2a and the separation of key isobaric opiates can be seen in Figure 2b. All opioid drugs elute within 3.5 minutes and demonstrate good peak shape. As Figure 2b shows, the isobaric pairs of morphine and hydromorphone (peaks 17 and 19), and codeine and hydrocodone (peaks 21 and 25) are well separated on all columns. This is an important feature as these compounds must be resolved from each other for accurate identification and quantification. While the BEH phenyl and biphenyl column both show increased retention of these compounds, which is most likely a result of their phenyl functionality, excellent resolution is easily achieved on the CORTECS C_{18} Column.

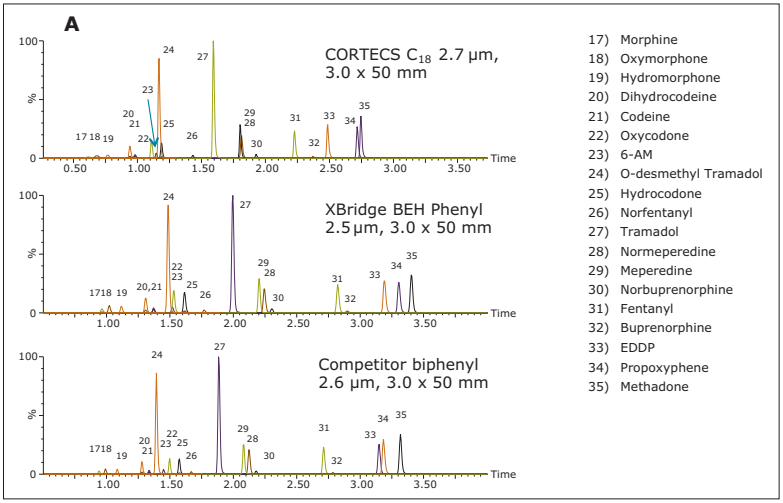
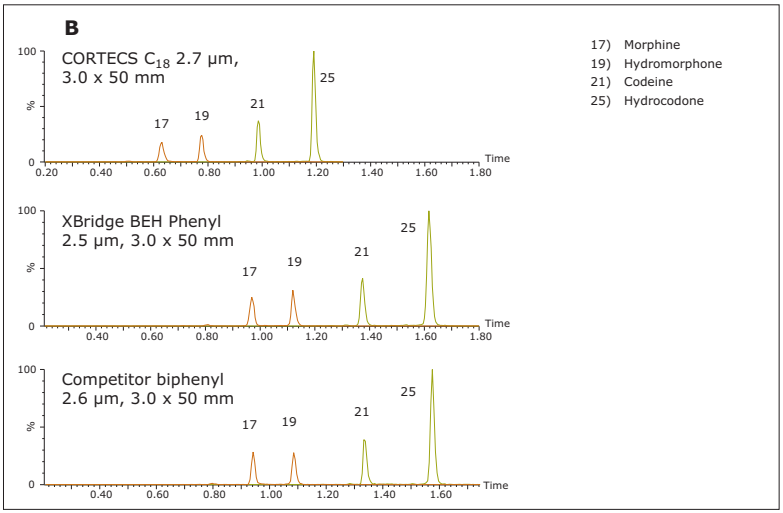


Figure 2a. Chromatographic separation of opioids.



2b. Chromatographic separation of key isobaric opiates.

Figure 3 shows the chromatography of the amines, PCP, and BZE. While all peaks demonstrate good peak shape, the CORTECS C_{18} Column and XBridge BEH Phenyl ***XP*** Column both show excellent separation of these compounds. Of particular note are methamphetamine and phentermine (peaks 3 and 5) which demonstrate baseline separation on these two columns, but co-elute on the biphenyl column. This is an important feature as these compounds have identical molecular formulas and both have a major fragment ion at *m/z* 91. The ability to separate these compounds eliminates the risk of cross talk between these two stimulants and can be crucial to unambiguous identification. Figure 3 also demonstrates that MDEA and benzoylecgonine (peaks 6 and 7), which coelute on the biphenyl column, are separated on both the C_{18} and BEH phenyl columns.

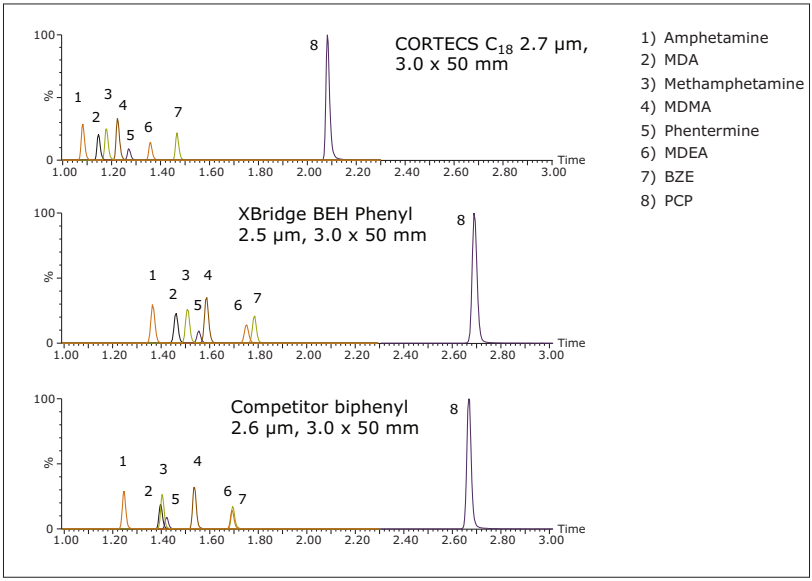


Figure 3. Chromatographic separation of amines, BZE & PCP.

The benzodiazepine chromatography is shown in Figure 4. Good peak shape can be achieved on all columns. Once again, the CORTECS C_{18} Column, despite its larger particle size, demonstrates the highest resolution for this group of compounds (average peak width of 2.89 s).

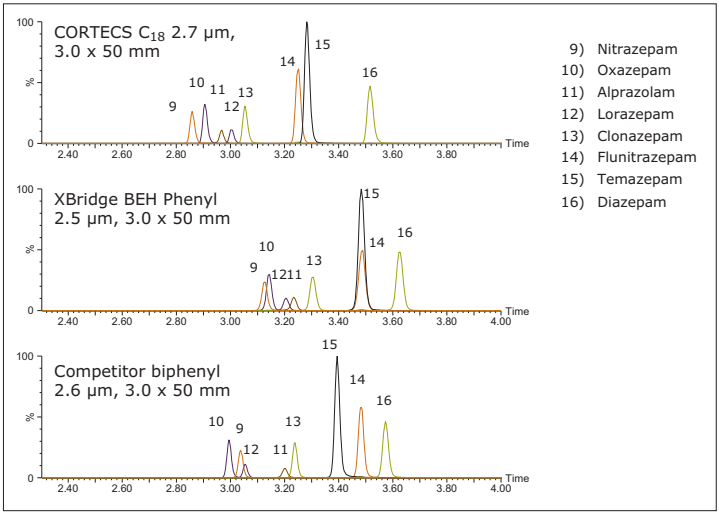


Figure 4. Chromatographic separation of benzodiazepines.

CONCLUSION

This application note highlights the analysis of a comprehensive panel of opiates, benzodiazepines, and other drugs of abuse. Using either Waters' 2.7 µm CORTECS C_{18} Column, or a 2.5 µm XBridge BEH Phenyl ***XP*** Column, all compounds were analyzed within 4 minutes with excellent peak shape and narrow peak widths. Maximum backpressures were were respectively 2206 and 3274 psi, enabling the use of these columns on traditional HPLC systems. Perhaps most importantly, baseline separation was achieved between isobaric compounds, allowing for their unambiguous identification and quantification. Whether laboratories prefer the performance and efficiency of the solid-core/superficially porous CORTECS C_{18} Column, or the unique selectivity of the XBridge BEH Phenyl ***XP*** Column, each can be used to rapidly analyze this important group of compounds.

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Quantitative Analysis of Cannabinoids in Whole Blood Using UPLC/MS/MS for Forensic Laboratories

Rob Lee,[1] Elodie Saussereau,[2] Christian Lacroix,[2] and Michelle Wood[1]
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APPLICATION BENEFITS

- Reduced sample volume
- Elimination of derivatization step prior to analysis
- Shorter analytical run times
- Improved sample throughput

WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ-S Mass Spectrometer

ACQUITY UPLC BEH C_{18} Column

Oasis® MAX SPE Cartridge

Total Recovery Vial

KEY WORDS

Cannabinoids, whole blood, UPLC®/MS/MS

INTRODUCTION

Cannabis is the most widely used illicit substance in the world, and long-term use can lead to dependency. Consequently, the cannabinoids are one of the most commonly detected classes of illegal drugs, and their analysis plays a key role in both forensic and roadside drug testing.

Δ-9 tetrahydrocannabinol (THC) is the main psychoactive element present in the plant *Cannabis sativa*.[1] THC produces a number of metabolites, including the active hydroxy-THC (THC-OH), and inactive carboxy-THC (THC-COOH), which can be detected circulating in blood after smoking or ingestion of cannabis.[2,3] Quantitative analysis of the psychoactive constituents in whole blood is an indicator of cannabis consumption and may provide information relating to the individual's state of impairment at the time of sample collection.

Previous publications have described the use of GC/MS, after solid-phase extraction (SPE),[4] or liquid-liquid extraction,[5] and pre-column derivatization for the determination of cannabinoids in whole blood. Recently a publication described the use of pre-column derivatization in conjunction with HPLC/MS/MS for this analysis.[6] This study reports a quantitative method based on SPE, following protein precipitation and UPLC/MS/MS. The method has been verified and its performance evaluated using authentic samples. Data were compared to results obtained with a GC/MS/MS method.

EXPERIMENTAL

Sample description

THC, THC-OH and THC-COOH (1 mg/mL), and their deuterated (d-3) analogues for use as internal standards (ISTD) at 0.1 mg/mL were purchased from LGC Standards (Teddington, UK). A mixture of pooled ISTDs at 50 ng/mL in methanol was prepared and stored at -20 °C.

Whole blood calibrators were prepared by spiking blank whole blood samples with known amounts of cannabinoids.

Forty-five anonymized samples containing pre-analyzed cannabinoids were obtained from J Monod Hospital, Le Havre, France. The samples were collected in the presence of either sodium fluoride or lithium heparin as anticoagulant.

Sample preparation

Twenty microlitres ISTD were added to 0.2 mL whole blood (either sample or calibrator), which was then precipitated by drop-wise addition of 0.4 mL acetonitrile while vortex-mixing. The sample was then centrifuged at 4000 g for 10 minutes at 4 °C. Supernatant (0.4 mL) was then added to 0.6 mL 1% ammonium hydroxide, and the resulting solution loaded onto the Oasis MAX SPE Cartridge (p/n 186000366).

Solid-phase extraction with Oasis MAX

Condition:	1 mL methanol followed by 1 mL 1% ammonium hydroxide
Load:	prepared 1 mL sample
Wash:	0.5 mL 50% acetonitrile
Dry:	10 minutes under full vacuum
Elute:	1.5 mL hexane/ethyl acetate/acetic acid (49:49:2 v/v/v)
Evaporate:	under nitrogen at 40 °C

The sample was reconstituted in 0.133 mL 70% aqueous methanol, vortex-mixed, then transferred to a Waters® Total Recovery Vial.

UPLC conditions

Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 100 mm, (p/n 186002352)
Column temp.:	30 °C
Sample temp.:	10 °C
Injection volume:	15 µL (PLNO)
Strong wash:	methanol/acetonitrile/ propan-2-ol (1:1:1 v/v/v)
Weak wash:	50% aqueous methanol
Flow rate:	400 µL/min
Mobile phase A:	0.1% formic acid
Mobile phase B:	acetonitrile
Gradient:	Linear from 60% B to 90% B over four minutes

MS conditions

Mass spectrometer:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	2.5 kV
Cone voltage:	25 V
Cone offset:	50 V
Desolvation temp.:	550 °C
Desolvation gas:	900 L/h
Cone gas:	150 L/h
Acquisition mode:	multiple reaction monitoring (MRM), see Table 1.

Cannabinoid	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
THC	4.1	**315.3**	**193.1**	**22**
		315.3	123.0	32
THC-d3	4.1	318.3	196.1	22
THC-OH	2.3	**331.3**	**193.1**	**24**
		331.3	313.2	14
THC-OH-d3	2.3	334.3	196.1	24
THC-COOH	2.4	**345.2**	**193.1**	**26**
		345.2	299.2	18
THC-COOH-d3	2.4	348.2	196.1	26

Table 1. Retention times and MRM transitions for analytes and ISTDs, the quantifier transitions are in bold.

Data management

MassLynx® Software incorporating TargetLynx™ Application Manager

RESULTS AND DISCUSSION

Method verification

The MRM transitions for all of the cannabinoids and ISTDs are shown in Table 1. The analytes were monitored using two transitions (quantifier and qualifier). The acceptance criteria for a positive identification of analytes include retention time within 0.2 minutes of predicted, and the quantifier/qualifier ion ratio within 20% of the predicted ratio, which was based on the average of the ratios across the entire calibrator range. The ISTD was monitored using a single transition. Figure 1 shows a chromatogram of a whole blood calibrator spiked at 0.5 ng/mL.

To investigate linearity for all cannabinoids, spiked whole blood calibrators were prepared at 0.0, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 ng/mL. ISTDs were added, then the samples were precipitated and extracted as previously described, and subsequently analyzed by UPLC/MS/MS.

Quantification was performed by integrating the area under the peak for each analyte MRM trace, and referencing to the appropriate ISTD peak area. Data were processed using the TargetLynx Application Manager, and calibration curves plotted with a 1/x weighting. Inter-day coefficient of determination (assessed over five days) was >0.995 for each cannabinoid.

The limit of detection (LOD) was defined as the lowest concentration with a signal- to-noise ratio >10:1 (for both transitions) in spiked whole blood. The lower limit of quantitation (LLOQ) was defined as the lowest concentration with a signal-to-noise ratio >10:1 (for both transitions), and demonstrated a mean concentration bias <20% of target and %RSD of <20% in spiked whole blood. The LOD and LLOQ for each cannabinoid are summarized in Table 2.

	LOD ng/mL	LLOQ ng/mL
THC-OH	0.15	0.25
THC-COOH	0.10	0.15
THC	0.05	0.15

Table 2. LOD and LLOQ for cannabinoids in whole blood.

Matrix effects and recovery (from six different sources of blank whole blood) were investigated at the following concentrations: 0.5 ng/mL (low), 5 ng/mL (medium), 25 ng/mL (high), and at 2.5 ng/mL for the ISTDs. Matrix effects were determined by comparing the peak areas obtained for blank whole blood spiked with cannabinoids after SPE, with peak areas obtained when spiked into 70% aqueous methanol. Recovery was determined by comparing cannabinoid peak areas from whole blood spiked pre-extraction with peak areas from whole blood spiked post-extraction. The results for each cannabinoid are shown in Table 3, whereby the values for matrix effects and recovery of the ISTDs matched those of the relevant cannabinoid.

	% Matrix effects			% Recovery		
	Low	Medium	High	Low	Medium	High
THC-OH	3.5 (3.0)	0.1 (6.5)	4.7 (2.5)	72.3 (3.9)	79.6 (5.2)	70.1 (4.8)
THC-COOH	-5.4 (5.4)	-11.9 (4.2)	-17.6 (3.2)	62.7 (2.6)	64.1 (3.5)	72.0 (4.6)
THC	-6.0 (3.3)	-9.4 (4.5)	-5.5 (1.9)	55.2 (6.6)	63.2 (3.0)	60.6 (3.0)
THC-OH-d3		-2.4 (3.3)			74.5 (2.2)	
THC-COOH-d3		4.8 (3.2)			68.1 (2.6)	
THC-d3		10.1 (3.5)			62.4 (3.0)	

Table 3. Mean % matrix effects and % recovery (n=6) for cannabinoid spiked whole blood at low, medium, and high concentrations. The figures in brackets are standard deviation.

Inter-day accuracy and precision were assessed by analyzing three quality control (QC) concentrations (3.33, 16.67, 33.30 ng/mL) over five different days. The mean achieved values for the quality control replicates over the five-day period at the three concentration levels were within 20% of target, and the %RSD was <20%, as shown in Table 4.

	Inter-day accuracy % Target			Inter-day precision %RSD		
	3.33 ng/mL	16.67 ng/mL	33.3 ng/mL	3.33 ng/mL	16.67 ng/mL	33.30 ng/mL
THC-OH	104.1	100.9	97.5	5.0	4.5	6.3
THC-COOH	102.7	99.2	96.5	6.7	4.4	3.8
THC	106.5	102.5	97.7	5.8	4.7	4.3

Table 4. Inter-day accuracy and precision (n=20) for cannabinoid spiked whole blood at three QC levels.

Extracted sample stability was assessed by extracting multiple replicates of whole blood spiked at 2.5 ng/mL, pooling the reconstituted extracts, and storing the sample in the ACQUITY® Autosampler at 10 °C. Injections were made every 60 minutes over the subsequent 24-hour period; no significant change in the peak area for the cannabinoids or ISTDs was observed.

Carryover of cannabinoids, following the injection of a 100 ng/mL spiked blood sample, was investigated and any cannabinoid observed was below the limits of detection.

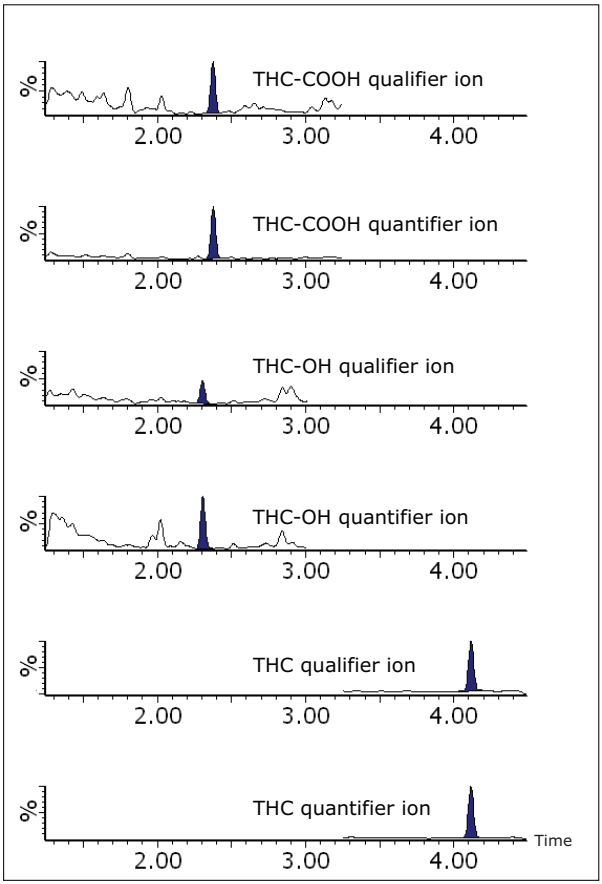

Figure 1. Chromatogram showing cannabinoids spiked into whole blood at 0.5 ng/mL (ISTDs not shown).

Analysis of authentic whole blood samples

A total of 45 authentic whole blood samples were prepared and analyzed by UPLC/MS/MS, and the concentrations of detected cannabinoids calculated. Cannabinoids were detected in 35 of the 45 samples, passing both acceptance criteria for detection. A cannabinoid positive whole blood sample, shown in Figure 2, includes a cannabinoid negative sample for comparison. THC-COOH was present in all cannabinoid positive samples, while THC and THC-OH were found at levels above the LLOQ in 35 and 29 positive samples, respectively. These values were compared to those obtained at a separate laboratory using a GC/MS/MS method. The (r^2) correlation values between the two data sets ranged from 0.9178 for THC-COOH to 0.9961 for THC-OH, as shown in Figure 3.

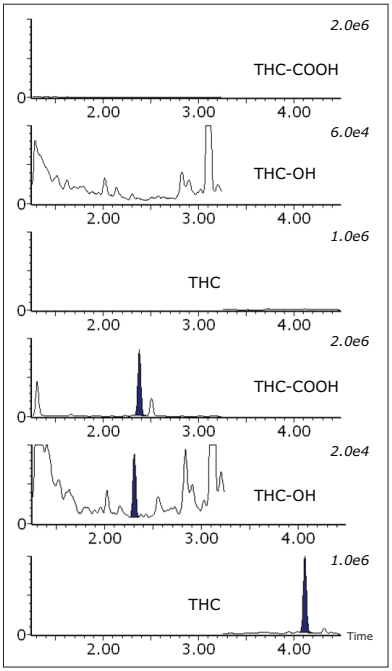

Figure 2. Positive whole blood sample (lower traces) containing THC at 1.22 ng/mL, THC-COOH at 8.25 ng/mL, and THC-OH at 0.44 ng/mL. A negative sample is shown for comparison (upper traces). The traces show the quantifier ions for the analytes only (ISTDs not shown).

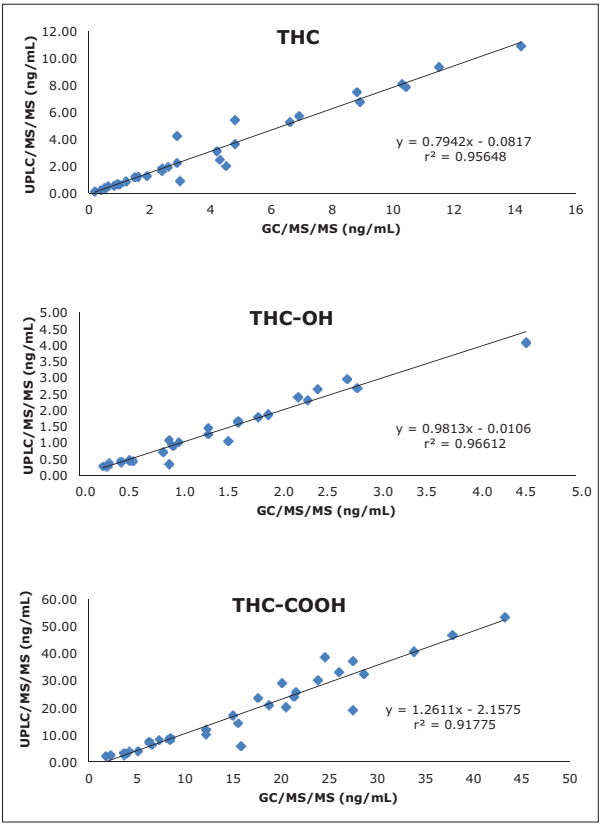

Figure 3. Comparison between UPLC/MS/MS and GC/MS/MS analysis of cannabinoids in whole blood.

CONCLUSIONS

Cannabinoids should be monitored in both forensic and roadside drug testing laboratories, thus requiring an accurate, reliable, and robust method to quantify these compounds in biological samples. The developed approach meets these requirements, and demonstrates excellent correlation with an alternative GC/MS/MS method for the analysis of cannabinoids in human whole blood samples.

The method offers a number of noteworthy benefits over the GC/MS/MS approach including the following: utilization of UPLC rather than GC separation means that the lengthy post-extraction derivatization, used by the latter technique, can be eliminated with the analytical run time reduced from 20 minutes to 6.5 minutes, a three-fold reduction. The combination of these factors allows for significantly higher sample throughput. Furthermore, the superior sensitivity of the Xevo TQ-S permits detection of the required low levels of cannabinoids even with much smaller blood sample volumes, for example 0.2 mL compared with 1 mL required for other reported methods, even without the need of a post-extraction concentration step.[4,5 & 7] This can be particularly advantageous as the volumes of whole blood available for testing can be small and must be sufficient for testing a number of drug classes.

A full validation by the user would be necessary prior to adoption in a laboratory.

Acknowledgement

J Monod Hospital, Le Havre, France for supplying the anonymized authentic whole blood samples.

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Quantitative Analysis of Barbiturates in Urine Using UPLC/MS/MS

Rob Lee,[1] Allan Traynor,[2] Jane LeCount,[2] and Michelle Wood[1]
[1]Waters Corporation, MS Technologies Centre, Manchester, UK
[2]Concateno plc, London, UK

APPLICATION BENEFITS

- Elimination of extraction step
- Elimination of derivatization step prior to analysis
- Improved sample throughput

WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ Mass Spectrometer

ACQUITY® BEH C_{18} Column

Waters® maximum recovery vial

KEY WORDS

Barbiturates, UPLC/MS/MS

INTRODUCTION

Barbiturates act as central nervous system depressants producing effects ranging from mild sedation to general anesthesia. They have largely been replaced by benzodiazepines as prescription medicines, owing to their relatively low therapeutic index and their high potential for dependence. However, it is known that the use of barbiturates is still common in certain regions of Eastern Europe;[1] consequently, their analysis is still of key importance in both forensic analysis and workplace drug testing.

Barbiturates have traditionally been measured by GC.[2,3] The arrival of newer technologies into the modern laboratory, such as UPLC®/MS/MS, often leads to an overall requirement to consolidate analytical methods and transfer existing methodologies to the newer platforms. Furthermore, UPLC/MS/MS permits the development of more sensitive techniques.

We report a quantitative method based on simple dilution and UPLC/MS/MS. The method has been verified, and its performance evaluated using authentic samples. Data were compared to results obtained with a traditional method that used liquid-liquid extraction followed by derivatization and analysis by GC/MS.

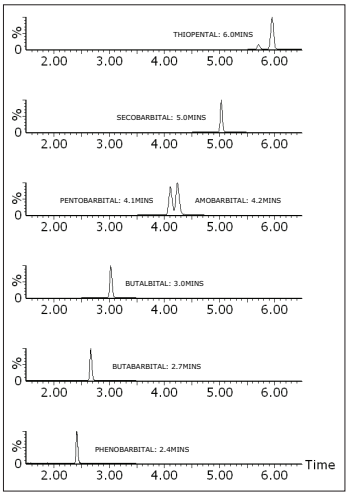

Figure 1. Chromatogram showing barbiturates spiked into urine at 500 ng/mL.

EXPERIMENTAL

Method Conditions

UPLC Conditions

System:	ACQUITY UPLC
Column:	ACQUITY BEH C_{18} 2.1 x 100 mm with BEH C_{18} 2.1 x 5 mm Vanguard pre-column
Column temp.:	50 °C
Sample temp.:	5 °C
Injection volume:	15 µL (PLNO)
Strong wash:	0.001% formic acid in acetonitrile
Weak wash:	0.001% formic acid in water
Flow rate:	400 µL/min
Mobile phase A:	0.001% formic acid in water
Mobile phase B:	0.001% formic acid in acetonitrile
Gradient:	Hold at 5% B for 0.5 min, then switch to 27.5% B, hold until 4 min, then switch to 35% B, hold until 5.25 min, then switch to 90% B, hold until 6.25 min, then switch to 5% B.

MS Conditions

Mass Spectrometer:	Xevo TQ
Ionization mode:	ESI negative
Capillary voltage:	2.75 kV
Cone voltage:	25 V
Collision energy:	12 eV
Desolvation temp.:	500 °C
Desolvation gas:	1000 L/h
Cone gas:	25 L/h
Acquisition mode:	Multiple reaction monitoring (MRM), as shown in Table 1.

Sample description

Phenobarbital and thiopental were purchased from Sigma Aldrich (Dorset, UK) and dissolved in methanol to 1 mg/mL. All other barbiturates (1 mg/mL) and deuterated internal standards (ISTDs) at 0.1 mg/mL were obtained as certified standard solutions from LGC Standards (Teddington, UK). Deuterated internal standards were not available for all of the barbiturates.

Quality control reference urine samples (Bio-Rad Liquichek Urine Toxicology Control: C2, C3, C4, S1, and S2) were obtained from Bio-Rad Laboratories Ltd (Hemel Hempstead, UK).

Urine samples for method development were obtained from donors at Waters Corporation.

Nineteen samples containing pre-analyzed barbiturates were obtained from Concateno, London, UK.

Sample preparation

Urine, either sample or calibrator, was centrifuged at 13,000 rpm for 5 min, then 50 µL was transferred to a Waters maximum recovery vial and diluted with 950 µL water containing 25 ng of each available ISTD.

Barbiturate	Precursor ion (*m/z*)	Product ion (*m/z*)	ISTD
Amobarbital	225.1	182.0	Pentobarbital-d5
Butabarbital	211.1	168.0	Phenobarbital-d5
Butalbital	223.0	180.0	Butalbital-d5
Pentobarbital	225.1	182.0	Pentobarbital-d5
Phenobarbital	231.1	188.0	Phenobarbital-d5
Secobarbital	237.1	194.1	Secobarbital-d5
Thiopental	241.1	57.9	Secobarbital-d5
Butalbital-d5	228.0	185.0	
Pentobarbital-d5	230.1	186.9	
Phenobarbital-d5	236.1	193.0	
Secobarbital-d5	242.1	199.1	

Table 1. MRM transitions for analytes and ISTDs.

Data management

MassLynx™ *v*4.1 incorporating TargetLynx™ Application Manager

RESULTS AND DISCUSSION

Method verification

The MRM transitions for all of the barbiturates and ISTDs are shown in Table 1. All were monitored using a single transition. Figure 1 shows a chromatogram of a 500 ng/mL barbiturate-spiked urine.

To investigate linearity for all barbiturates, spiked urine calibrators were prepared at 0, 25, 50, 100, 250, 500, 1000, 750, 1250, and 1500 ng/mL. Samples were diluted 20-fold with water, containing ISTDs as previously described, and subsequently analyzed by UPLC/MS/MS.

Quantification was performed by integrating the area under the peak for each analyte MRM trace, and referencing to the appropriate ISTD peak area. Data were processed using the TargetLynx Application Manager, and calibration curves plotted with a 1/x weighting. Interday coefficient of determination (assessed over five days) was >0.995.

The limit of detection (LOD) was defined as the lowest concentration, which produced a signal to noise ratio >5:1 in spiked urine. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a signal to noise ratio >10:1, and demonstrated a mean concentration bias <20% of target, and a %RSD of <20% in spiked urine. The LOD and LLOQ are summarized in Table 2.

Barbiturate matrix effects (from six different sources of blank urines) were investigated in triplicate at the following concentrations: 100 (low), 500 (medium), and 1000 ng/mL (high). Matrix effects were determined by comparing the response in spiked urine sample to the response in water. The results for each barbiturate are shown in Table 2. The %RSD for the six urines at each concentration was <20%.

Interday accuracy and precision were assessed by analyzing three quality control (QC) concentrations (150, 600, 1200 ng/mL) over five different days. The mean achieved values for the quality control replicates over the five-day period at the three concentration levels were within 10% of target and the %RSD was <15%, as shown in Table 3.

	LOD	LLOQ	% Matrix effects		
	ng/mL	ng/mL	100 ng/mL	500 ng/mL	1000 ng/mL
Amobarbital	5	20	102.7 (2.4)	101.8 (2.3)	101.4 (2.3)
Butabarbital	5	20	85.1 (12.9)	83.2 (14.1)	83.6 (14.0)
Butalbital	5	20	104.0 (4.6)	96.0 (4.4)	95.3 (4.7)
Pentobarbital	5	20	97.4 (1.8)	98.4 (3.1)	98.5 (2.5)
Phenobarbital	5	20	84.8 (14.8)	85.6 (12.9)	86.2 (12.1)
Secobarbital	5	20	105.7 (2.8)	102.9 (2.4)	102.7 (1.7)
Thiopental	5	20	93.5 (1.8)	91.4 (2.8)	91.0 (3.3)

Table 2. LOD, LLOQ, and mean % matrix effects (n=6) for barbiturate-spiked urine at low, medium, and high concentrations. The figures in brackets are %RSD.

	Interday accuracy % target			Interday precision %RSD		
	150 ng/mL	600 ng/mL	100 ng/mL	150 ng/mL	600 ng/mL	1200 ng/mL
Amobarbital	104.1	108.0	99.3	5.7	2.4	1.6
Butabarbital	103.3	106.0	101.0	5.6	2.1	2.6
Butalbital	103.4	108.3	99.9	5.8	2.1	3.3
Pentobarbital	102.5	108.4	101.9	4.9	1.7	1.6
Phenobarbital	103.3	106.8	98.4	5.3	2.3	3.2
Secobarbital	105.3	108.7	98.3	4.9	1.2	1.4
Thiopental	103.5	107.0	97.3	7.9	4.2	3.4

Table 3. Interday accuracy and precision (n=20) for barbiturate-spiked urine at three QC levels.

Analysis of authentic urine samples and quality control reference urine samples

A total of nineteen authentic urine samples, and five quality control reference urines were diluted and analyzed using UPLC/MS/MS, and the concentrations of detected barbiturates calculated. For positive identification of barbiturates in the UPLC/MS/MS method, the analyte retention time had to be within 0.2 min of the expected retention time. Phenobarbital was the only barbiturate present in the authentic urine samples and was detected in all nineteen samples of which seventeen results fell within the calibration range. A phenobarbital positive urine sample at 375 ng/mL is shown in Figure 2 with a negative control for comparison.

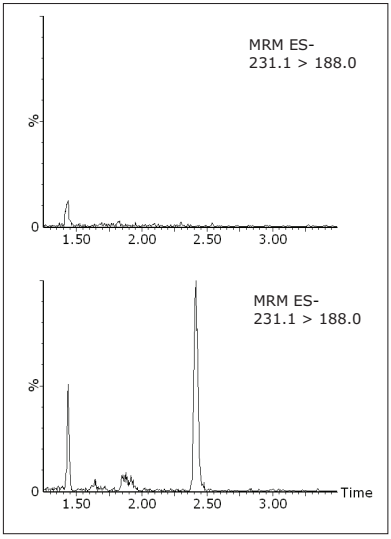

Figure 2. A positive result for phenobarbital at 375 ng/mL. The top trace is a blank urine calibrator and the bottom trace is the authentic sample.

These values were compared to those obtained at a separate laboratory using a liquid-liquid extraction, followed by derivatization and analysis by GC/MS. The correlation (r^2) between the two data sets was excellent, as shown in Figure 3. Amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital were found in commercial reference urines C2, C3, and C4; while secobarbital was the only barbiturate found in reference urines S1 and S2. The correlation between the UPLC/MS/MS data and the vendor's stated concentration by GC for the commercial reference urines was >0.9971.

Utilizing a simple sample dilution rather than a liquid-liquid extraction reduces the sample preparation time and utilizes smaller sample volumes, for example, 50 µL compared to the 1 mL required for the liquid-liquid extraction. Prior to injection, modern GC/MS methods require methylation of the barbiturates using trimethylanilinium hydroxide and ethyl acetate in the hot injection port of the GC. Derivatization of barbiturates is not needed for UPLC/MS/MS analysis, thus this step can be eliminated. The combination of these factors allows for higher sample throughput.

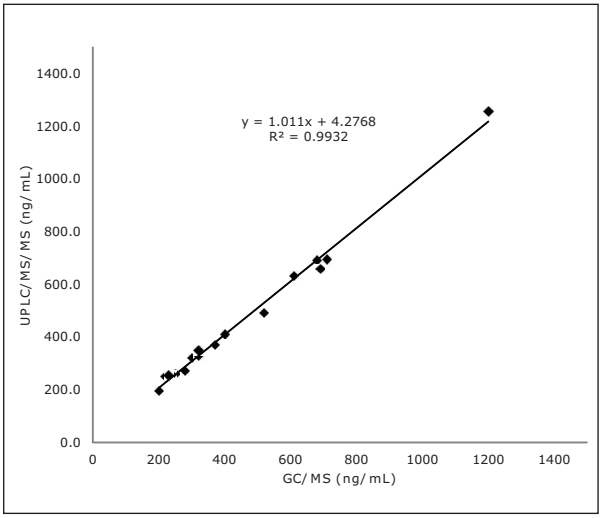

Figure 3. Comparison of GC/MS and UPLC/MS/MS analysis of phenobarbital.

CONCLUSIONS

Barbiturates need to be monitored in both forensic and workplace drug testing laboratories; therefore, an accurate, reliable, and robust method is needed to quantify these compounds in biological samples.

The Xevo TQ MS meets the sensitivity requirement for barbiturates in this particular matrix, without the need for a post-extraction concentration step. When analyzing barbiturates by UPLC/MS/MS, the use of a very simple sample dilution step eliminates both the liquid-liquid extraction and post-extraction derivatization steps that are required for GC/MS analysis. The elimination of the extraction step would reduce the time taken to prepare a typical batch of samples by more than 50%.

The 8.5-min ACQUITY UPLC System separation method run time is similar to the current GC methods for barbiturate analysis; therefore, when coupled with the simple sample dilution, it allows for high sample throughput.

UPLC/MS/MS showed excellent correlation with an alternative GC/MS method for the analysis of phenobarbital in nineteen human urine samples.

A full validation by the user would be necessary prior to adoption in a laboratory.

Acknowledgments

Concateno plc, London, UK for supplying the anonymized authentic urine samples.

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Quantitative Analysis of 11-nor-9-Carboxy Δ9-THC in Urine Using UPLC/MS/MS

Rob Lee[1], Allan Traynor[2], Jane LeCount[2] and Michelle Wood[1]
[1]Waters Corporation, MS Technologies Centre, Manchester, UK. [2]Concateno plc, London, UK.

APPLICATION BENEFITS

- Extended linear range
- Elimination of derivatisation step prior to analysis
- Shorter analytical run times, improved sample throughput

WATERS SOLUTIONS

ACQUITY UPLC®

Xevo® TQ MS

ACQUITY® BEH C_{18} Column

Waters Maximum Recovery Vials

KEY WORDS

Cannabinoids

UPLC®/MS/MS

INTRODUCTION

Cannabis is the most widely used illicit substance in the world and long-term use can lead to dependency. Cannabinoids are one of the most commonly detected classes of illegal drugs, consequently their analysis is of key importance in forensic testing.

Δ-9 tetrahydrocannabinol (THC) is the main psychoactive element present in the plant *Cannabis sativa* (1).

THC produces a number of metabolites, the most significant for urine drug testing is 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), which is the major metabolite eliminated in urine, as the free acid or the ester-linked β-glucuronide (2). Prior to analysis the glucuronide is usually hydrolyzed back to the free acid under alkaline conditions.

THC-COOH is monitored in urine as it allows for a longer window of detection and thus can be used as a marker of consumption due to previous Cannabis use.

Previous publications have described the use of GC/MS after solid phase extraction (SPE) and pre-column derivitisation for the determination of cannabinoids in urine (3, 4). We report a quantitative method based on liquid liquid extraction (LLE) and UPLC/MS/MS. The method has been verified and its performance evaluated using authentic samples. Data were compared to results obtained with a GC/MS method.

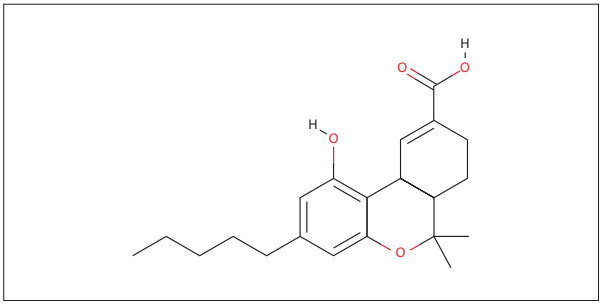

Figure 1. Structure of 11-nor-9-carboxy- Δ9 tetrahydrocannabinol (THC-COOH)

EXPERIMENTAL

ACQUITY UPLC conditions

Column:	ACQUITY BEH C_{18}, 1.0 x 50 mm
Column Temp.:	55°C
Sample Temp.:	10°C
Injection Volume:	10 µL (PLNO)
Strong Wash:	methanol/acetonitrile (1/1 v/v)
Weak Wash:	50% methanol
Flow Rate:	200 µL/min.
Mobile Phase A:	5 mM ammonium acetate + 0.05% formic acid
Mobile Phase B:	methanol
Gradient:	Linear, 50% B to 60% B over 2 min. to 80% B at 3 min to 95% B at 4 min. hold at 95% B to 5 min. to 50% B at 5.5 min.

MS Conditions

MS System:	Xevo TQ MS
Ionization Mode:	ESI positive
Capillary Voltage:	3.0 V
Desolvation Temp.:	550 °C
Desolvation Gas:	1000 L/Hr
Acquisition Mode:	multiple reaction monitoring (MRM)

MATERIALS

THC-COOH (1 mg/mL) and its deuterated (d-3) analogue for use as internal standard (ISTD) at 0.1 mg/mL were purchased from LGC Standards (Teddington, UK). The ISTD was diluted in methanol to 1.25 µg/mL.

Urine samples for method development were obtained from donors at Waters Corporation and the pH adjusted to 7.0 with 0.5 M potassium hydroxide.

One hundred and three anonymised samples containing pre-analysed THC-COOH were obtained from Concateno, London, UK.

Sample Preparation

Glucuronide hydrolysis: In a glass vial add 10 µL (12.5 ng) ISTD to 500 µL sample or calibrator along with 15 µL 10 M sodium hydroxide before vortex mixing and heating at 70°C for 30 minutes. Once the samples have cooled, add 85 µL glacial acetic acid to the sample and vortex mix.

Liquid liquid extraction (LLE): Add 3 volumes hexane/ethyl acetate (9/1 v/v) to the hydrolysed sample and vortex mix for 30 seconds. Centrifuge to separate the layers and then transfer the supernatant to a clean tube. Repeat extraction step and pool the extracts. Dry the pooled extracts at 40°C under a stream of oxygen-free nitrogen and reconstitute in 70 % methanol before transferring to a Waters maximum recovery vial. Analyse 10 µL by UPLC/MS/MS.

Table 1. MRM transitions and conditions.

MRM Transitions	Cone (V)	Collision (eV)
THC-COOH (ESI+)		
345.2 > 193.2	32	30
345.2 > 299.1	32	20
348.2 > 196.2 (d3-ISTD)	32	30

Data Management:

MassLynx v4.1 SCN714 incorporating TargetLynx™ application manager.

RESULTS AND DISCUSSION:

Method Verification

The MRM transitions and optimal MS conditions for THC-COOH and ISTD are shown in Table 1. THC-COOH was monitored using two transitions (quantifier and qualifier). For THC-COOH the ratio of the areas for the 2 transitions was to be within 20% of the predicted ratio, which was based on the average of the ratios across the entire calibrator range). The ISTD was monitored using a single transition.

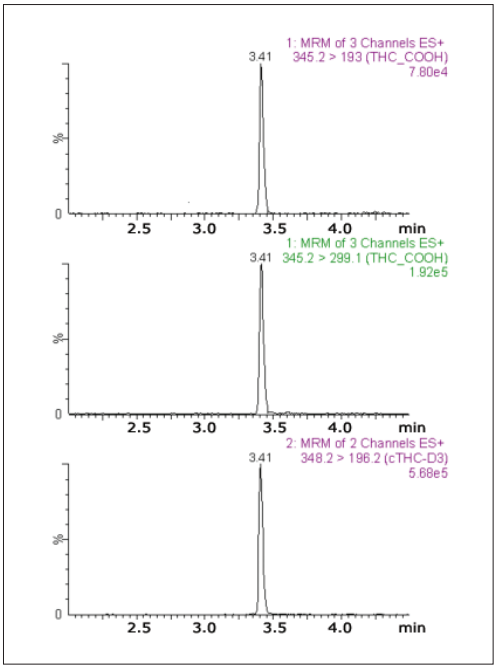

Figure 2. THC-COOH quantifier ion (top), qualifier ion (middle) and ISTD ion traces (bottom) from extracted spiked urine at 5 ng/mL, ISTD at 25 ng/mL.

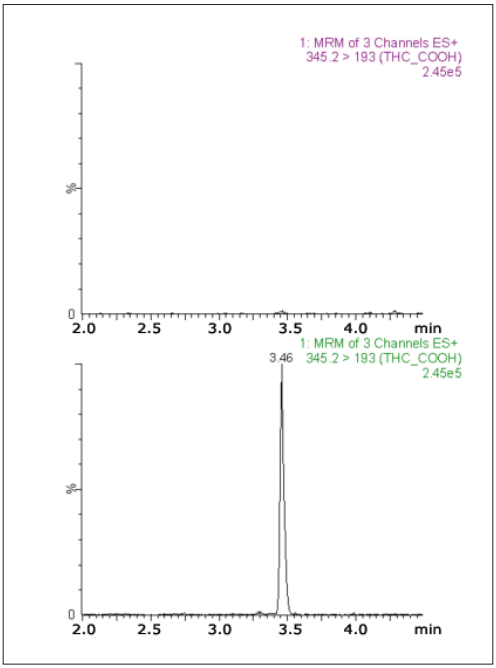

Figure 3. A positive result for THC-COOH at 16 ng/mL The top trace is the zero urine calibrator, the bottom trace is the authentic sample, both traces show quantifier ion only.

Figure 2 shows the quantifier and qualifier ion traces for THC-COOH spiked urine including the ISTD trace.

To investigate linearity of response for THC-COOH, urine calibrators were prepared in duplicate at 0, 1, 5, 10, 25, 50, 100, 250, 500 ng/mL, hydrolysed and extracted as described above and subsequently analysed using UPLC/MS/MS.

Data were processed using the TargetLynx application manager and calibration curves plotted with a 1/x weighting. Interday coefficient of determination (assessed over 5 days) was >0.995.

The limit of detection (LOD) was defined as the concentration which gave a signal to noise ratio >10:1 (for both quantifier and qualifier ions) in extracted urine and was determined to be 1 ng/mL.

THC-COOH and ISTD recovery (from three different sources of blank urines), and matrix effects (from six different sources of blank urines) were investigated at the following concentrations: 5, 50, 500 ng/mL (25 ng/mL for the ISTD). Recovery was determined by comparing urine spiked pre and post extraction.

Matrix effects were determined by comparing the response in urine spiked post extraction to the standard response in mobile phase. The results for THC-COOH are shown in Table 2, the values for recovery and matrix effects of the ISTD matched those for THC-COOH.

Table 2. Mean % recovery (n=3) and matrix effects (n=6) for THC-COOH spiked urine at low, medium and high concentrations.

Recovery (%)				Matrix effects (%)			
5 ng/mL	50 ng/mL	500 ng/mL	ISTD (25 ng/mL)	5 ng/mL	50 ng/mL	500 ng/mL	ISTD (25 ng/mL)
61	66	69	61	-22	-20	-18	-19

Intraday precision and accuracy were assessed by analysing three quality control (QC) concentrations (7.5, 75, 375 ng/mL). Interday precision was assessed by analysing the QC samples in quadruplicate on five different days. Intra and interday precision and accuracy were found to be within 10% of expected values and are shown in Table 3.

Table 3. Interday accuracy and precision (n=20) and intraday (n=4) precision for THC-COOH spiked urine at three QC levels.

QC level (ng/mL)	Interday accuracy (%)	Intraday precision CV (%)	Interday precision CV (%)
7.5	101.1	3.2	5.0
75	101.9	2.4	6.3
375	97.4	3.7	4.1

Extracted sample stability was assessed by performing multiple extractions of a 10 ng/mL spiked urine, pooling the reconstituted extracts and storing the sample in the ACQUITY autosampler at 10°C for 24 hours. Injections were made every 60 minutes. No significant change in either THC-COOH or ISTD peak areas was observed over the investigated period; the %RSD was less than 2% for both THC-COOH and ISTD.

Authentic Sample Analysis

A total of 103 urine samples were hydrolysed and processed by LLE followed by UPLC/MS/MS analysis and the concentrations of THC-COOH calculated.

The following criteria were used for a positive identification in the UPLC/MS/MS method: analyte peak within 0.2 min of the expected retention time and qualifier/quantifier ratio within 20% of predicted ratio.

A THC-COOH positive urine sample at 16 ng/mL is shown in Figure 3 with a negative control for comparison.

THC-COOH was detected in 101 of the 103 samples; 99 results fell within the calibration range. Values were compared to those obtained at a separate laboratory using a SPE GC/MS method. The correlation between the data sets ($r^2 > 0.945$) is shown in Figure 4.

Utilising ACQUITY UPLC® rather than GC means that the derivatisation step can be eliminated; furthermore the reference GC method has a run time in excess of 14 minutes compared to the 7 minute run time for the UPLC/MS/MS method described here. The reduction in both sample preparation time and analytical run time allows for higher sample throughput.

Future work is planned to extend the methodology to include for the measurement of THC and other metabolites in other matrices such as whole blood.

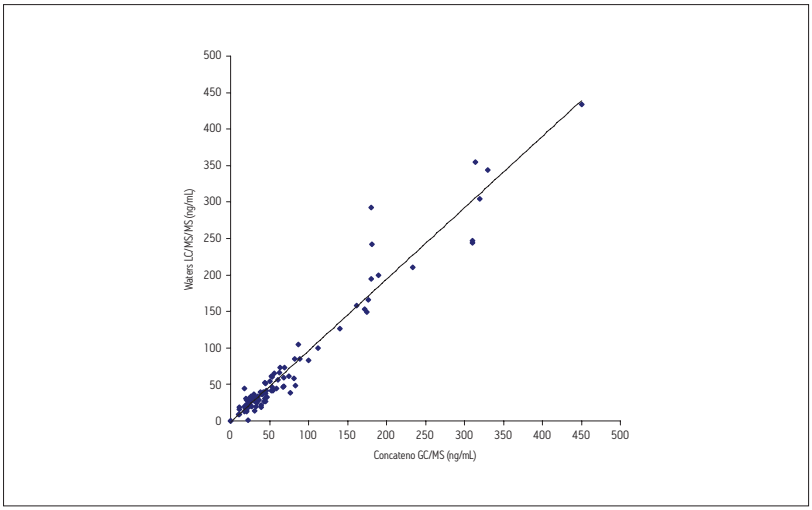

Figure 4. Comparison between UPLC/MS/MS and GC/MS for THC-COOH in urine.

CONCLUSIONS

Cannabinoids need to be monitored in forensic drug testing laboratories. Therefore an accurate, reliable and robust method is needed to quantify these compounds in biological samples.

The Xevo TQ MS meets the sensitivity requirement for THC-COOH in this particular matrix, without the need for a post extraction concentration step, and its extended linear range allows for samples with high levels of THC-COOH to be measured without dilution. Regardless of the type of sample preparation method used, the post-extraction derivatisation step that is required for GC/MS analysis is eliminated when analysing THC-COOH by UPLC/MS/MS.

The 7 minute ACQUITY UPLC separation method allows for high sample throughput, and is significantly shorter than current GC methods for THC-COOH analysis.

UPLC/MS/MS showed excellent correlation with an alternative GC/MS method for the analysis of THC-COOH in 103 human urine samples.

A full validation by the user would be necessary prior to adoption in a laboratory.

ACKNOWLEDGMENTS

Concateno plc, London. UK for supplying the anonymised, authentic urine samples.

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Quantitative Analysis of 21 Benzodiazepine Drugs, Zolpidem and Zopiclone in Serum Using UPLC/MS/MS

Mark Roberts and Michelle Wood. Waters Corporation, MS Technologies Centre, Manchester, UK.

OBJECTIVE

To develop a UPLC/MS/MS method for the quantitation of 21 benzodiazepines, Zolpidem and Zopiclone in human serum.

INTRODUCTION

Benzodiazepines (Figure 1) are the most frequently prescribed drugs in the western world. They are indicated for a variety of disorders including: anxiety; insomnia; agitation; muscle spasms and alcohol withdrawal. They work primarily due to their interaction with the $GABA_A$ receptor.

Many of the benzodiazepines are potentially addictive[1] and long-term use can lead to dependency. Consequently their analysis is of key importance in both clinical and forensic settings. Misuse of these medications by vulnerable populations such as the elderly[2] or the mentally-ill[3] is common. The elderly are at particular risk, as sensitivity to benzodiazepines tends to increase with age thus, these analytes are commonly reported in self-poisonings[4-7]. Recreational use of benzodiazepines has also been reported; they are often used in combination with other narcotics e.g., they can be used to augment the 'high' of heroin or cocaine or can be used to reduce the after-effects of LSD or amphetamine use[8]. Drug-facilitated crime often involves benzodiazepines due to their sedative properties and amnesia-producing effects[9].

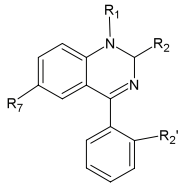

Figure 1. Core Structure of the Benzodiazepines

Traditional techniques used for the quantitation of benzodiazepines include gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography/mass spectrometry (HPLC/MS). The former typically requires inclusion of an additional derivatisation step and analysis can be problematic due to the thermo-labile nature of some of the analytes within this class. In contrast, HPLC/MS can separate a wide range of substances without the need for derivatisation and utilises 'softer' ionisation techniques e.g., electrospray ionisation (ESI) which allows the protonated molecular species to remain intact.

Since its introduction in 2004, UltraPerformance® LC (UPLC) has repeatedly demonstrated significant advantages compared to HPLC-based methods e.g., enhanced resolution, sensitivity and throughput. Thus our aim was to develop a method utilising this technique.

We describe a quantitative method based on liquid/liquid extraction (LLE) and UPLC with tandem mass spectrometry (MS/MS). The method's performance has been evaluated using authentic samples. Data were compared to results obtained with a validated method based on HPLC/MS/MS[10].

MATERIALS

Samples

Twenty-seven authentic serum and plasma samples were received which had previously been analysed by a published method involving conventional SPE and HPLC/MS/MS analysis[10].

Standards, Internal Standards and Blank Serum

Standards (1 mg/mL) and their deuterated internal standards (IS) at 0.1 mg/mL were purchased from LGC Standards (Teddington, UK). Internal standards were made into a mixed stock of 5 µg/mL in water.

Blank serum was obtained from Scipac (Sittingbourne, UK). Serum calibrators (0 to 1000 ng/mL) were prepared by adding mixtures of drug standards to the blank serum.

EXPERIMENTAL

Sample Preparation

Liquid/Liquid Extraction (LLE)

Spike:
Spike 300 µL sample/calibrator with 10 µL IS, add 150 µL borate buffer*, vortex to mix

↓

Extract:
Add 900 µL extraction mixture**, mix

↓

Centrifuge:
3000 rpm for 5 min

↓

Transfer and Evaporate:
Transfer the supernatant to a clean Eppendorf tube and dry on a 40 °C heating block under nitrogen gas.

↓

Reconstitute and Inject:
Reconstitute in 50 µL 80% water and 20% methanol, (6 x concentration step), mix and inject

**Borate buffer made using saturated solution of disodium tetraborate decahydrate.*
***Extraction mixture made from dichloromethane/ether/hexane (30/50/20) with 0.5% isoamyl alcohol.*

LC Conditions

LC System:	Waters® ACQUITY UPLC® System
Column:	ACQUITY UPLC BEH C_{18} Column 2.1 x 100 mm, 1.7 µm
Column Temp:	50 °C
Flow Rate:	400 µL/min.
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in methanol†

Gradient:

Time/min	%A	%B	Curve
0	70	30	
2.5	35	65	6
3.25	30	70	6
4.5	23	77	6
4.55	5	95	6
5.55	5	95	6
5.6	70	30	6
7.5	70	30	6

Injection Volume:	10 µL
Strong Wash:	Mobile Phase B (500 µL)
Weak Wash:	Mobile Phase A (1500 µL)

†The organic mobile phase chosen for this UPLC chromatography method was methanol, avoiding the use of acetonitrile which has been more difficult to obtain due to global shortages.

MS Conditions

MS System:	Waters® TQ Detector (TQD)
Ionization Mode:	ESI Positive
Capillary Voltage:	3 kV
Desolvation Temp:	400 °C
Desolvation Gas:	800 L/Hr
Source Temp:	120 °C
Acquisition Mode:	Multiple Reaction Monitoring (MRM)

RESULTS AND DISCUSSION

Method Validation

The MRM transitions and optimised conditions for all of the drugs and metabolites are shown in Table 1. Each analyte was monitored using two transitions i.e., a quantifier and qualifier. Internal standards were monitored using a single transition.

To investigate linearity for all of the analytes, spiked serum calibrators were prepared in triplicate at 0, 1, 5, 10, 100, and 1,000 ng/mL, and extracted using LLE as previously described.

Following analysis, calibration curves were plotted with a 1/x weighting. Average r^2 values were all >0.995 except for alpha-hydroxy triazolam which was 0.975 for 1-100 ng/mL.

Quantitation was performed by integrating the area under the peak for each analyte MRM trace and referencing to the appropriate deuterated internal standard peak area. Figure 2 shows the quantifier ion traces for all analytes at 1 ng/mL. The limit of detection (LOD) was defined as the concentration which gave a signal to noise (S:N)

Compound	Precursor Ion (m/z)	CV (V)	Product 1 (m/z)	CE (V)	Product 2 (m/z)	CE (V)	Internal Standard (IS)	LOD (ng/mL)	Matrix Effect		Recovery
									%	RSD	%
7-aminoclonazepam	286	53	121	31	222	24	7-aminoclonazepam d4	1	0	2	62
7-aminoflunitraz-epam	284	50	135	28	227	27	7-aminoflunitrazepam d7	1	1	4	73
alpha-hydroxy alprazolam	325	55	297	26	205	44	alpha-hydroxy alprazolam d5	1	1	6	66
alpha-hydroxy midazolam	342	35	324	20	203	25	Nitrazepam d5	1	-13	12	70
alpha-hydroxy triazolam	359	50	331	27	176	27	alpha-hydroxy triazolam d4	1	-4	7	69
Alprazolam	309	50	281	26	205	43	Alprazolam d5	1	-4	5	72
Bromazepam	316	43	182	32	209	26	Nitrazepam d5	1	-1	7	70
Chlordiazepoxide	300	35	283	15	227	25	Nitrazepam d5	1	-7	7	70
Clonazepam	316	55	270	24	214	39	Clonazepam d4	1	2	6	74
Diazepam	285	50	154	28	193	32	Diazepam d5	1	-21	27	89
Estazolam	295	50	267	25	205	40	Estazolam d5	1	-1	6	72
Flunitrazepam	314	50	268	24	239	34	Flunitrazepam d7	1	6	7	73
Lorazepam	321	40	275	25	229	25	Oxazepam d5	5	-1	5	74
Lormetazepam	335	35	289	20	177	40	Nordiazepam d5	1	5	7	71
Midazolam	326	50	291	26	244	26	7-aminoflunitrazepam d7	1	-28	30	83
Nitrazepam	282	45	236	25	180	35	Nitrazepam d5	1	0	4	69
Nordiazepam	271	45	140	25	165	27	Nordiazepam d5	5	-5	11	71
Oxazepam	287	40	241	19	269	15	Oxazepam d5	5	-5	7	69
Prazepam	325	40	271	25	140	27	Prazepam d5	1	-25	25	78
Temazepam	301	35	255	22	283	15	Nordiazepam d5	5	-2	4	73
Triazolam	343	56	308	26	239	44	Triazolam d4	1	-1	5	72
Zolpidem	308	57	235	32	263	26	Zolpidem d6	1	-11	12	87
Zopiclone	389	25	245	27	217	35	7-aminoclonazepam d4	1	-26	28	62

Table 1. MRM transitions with cone voltages (CV) and collision energies (CE) for 23 analytes, product 1 is the quantifier ion and product 2 is the qualifier ion.

ratio >7:1 (for both qualifier and quantifier). The data is summarised in Table 1.

Recoveries and matrix effects were assessed using six different sources of blank sera (Table 1). Recoveries were investigated using pre- and post-spiked serum at 50 ng/mL and ranged from 62% to 89%.

Matrix effects were determined by comparing the responses for analytes spiked into extracted blank sera to those spiked into mobile phase. Generally these were considered to be satisfactory and ranged from -28% suppression to +6% enhancement (0% indicates no matrix effect).

Extracted sample stability was assessed using 16 blank serum samples which were spiked at 50 ng/mL and extracted by LLE as described. The reconstituted extracts were pooled into one vial which was placed in the ACQUITY autosampler at 5 °C for 11 hours and injections made every 45 minutes. No significant loss in peak area, for either the standards or the IS, was observed over the period investigated.

Sample Analysis

A total of 27 authentic serum and plasma samples were anonymised, extracted by LLE and analysed by the described method. The following criteria were used for a positive identification: analytes must be within 0.2 min of the expected retention time, ion ratios within 15% of the predicted ratio. For these analyses, the predicted ratio was defined as an average of the calibrator ratios at 1, 10 and 100 ng/mL. The TargetLynx™ application manager was used to process the data for these samples, it was set to automatically flag any analytes with a retention time or ratio that fell outside the user-defined settings mentioned above. This minimises the amount of time required for the user to interrogate processed data.

The data were subsequently compared to those obtained previously at a separate laboratory using a published, validated method for a smaller panel i.e., 13 benzodiazepines. These data are shown in Figures 3 and 4.

Overall there was excellent correlation (r^2 values above 0.98) between the newly-developed method and the published method. An example of a positive authentic sample is shown in Figure 5; a negative control is also included for comparison.

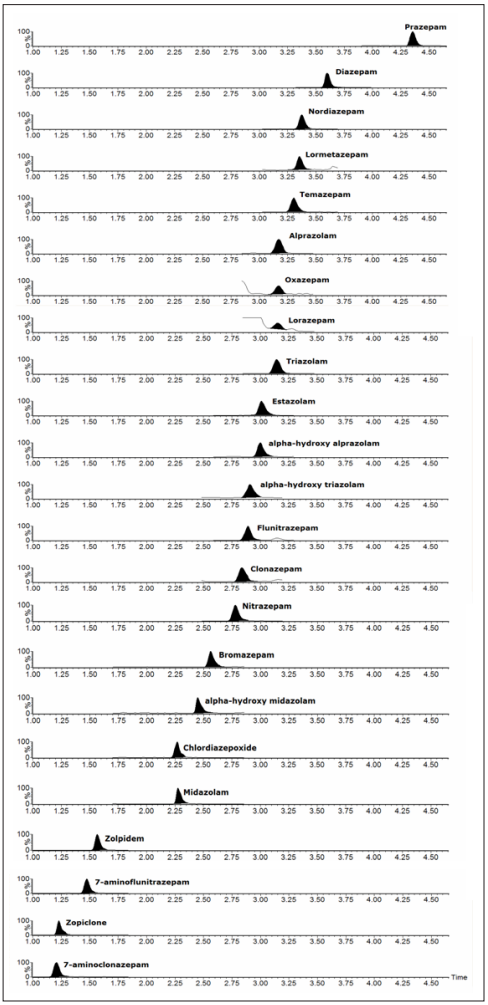

Figure 2. Quantifier ion traces for all analytes for the extracted 1 ng/mL serum calibrator. N.B. Lorazepam, Nordiazepam, Oxazepam and Temazepam are below LOD for this UPLC/MS/MS method.

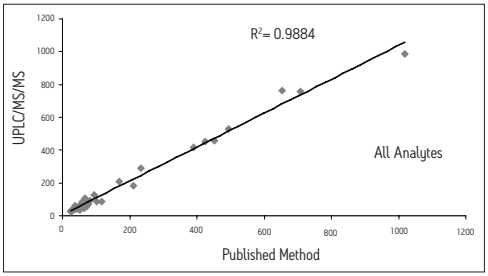

Figure 3. Comparative data for the newly-developed UPLC/MS/MS method versus a previously-published method. The 39 results plotted include all found analytes with concentrations in ng/mL.

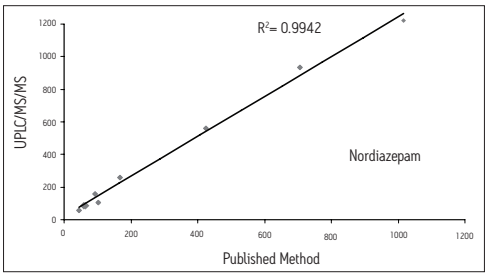

Figure 4. Comparative data for the newly-developed UPLC/MS/MS method versus a previously-published method. Nine results are plotted for nordiazepam only with concentrations in ng/mL.

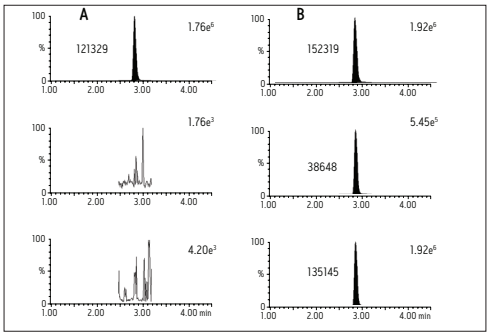

Figure 5. A positive result for clonazepam at 40 ng/mL. A is the zero serum calibrator and B is the authentic sample. The top trace is the internal standard, middle is 316>214 (qualifier) and bottom is 316>270 (quantifier). The numbers above the peaks show peak area and to the right of the peak is the peak intensity.

CONCLUSIONS

Benzodiazepines need to be monitored in both clinical and forensic laboratories and so an accurate, reliable and robust method is needed to quantitate these drugs in biological samples. To this end we have developed a fast, sensitive method for an extensive panel of commonly-prescribed benzodiazepines using UPLC/MS/MS.

Unlike GC/MS, this technique requires no derivatisation and due to softer ionisation it is possible to monitor specific fragmentations from the protonated molecular species of the analytes. In comparison to HPLC-based methods, those based on UPLC offer superior chromatographic resolution, enhanced sensitivity and shorter analytical run times.

The results for 27 authentic serum and plasma samples analysed using the described LLE-UPLC/MS/MS method were compared to those obtained previously with a published, validated method; the data showed excellent agreement.

This application is an example of an assay that can be performed using Waters systems. Complete method validation by the end user is required.

ACKNOWLEDGMENTS

Stephanie Marin (ARUP laboratories, Salt Lake City, Utah, USA) for supplying pre-analysed authentic samples containing benzodiazepines.

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This method is an example of an application using the instrumentation, software and consumables described in this Application Note. Waters has not verified that this method is transferable to different instrumentation, software or consumables. Application Notes are intended as a proof of concept and may serve as a reference as end users develop & validate their own laboratory developed tests (LDTs). The end user is responsible for completion of the method development and validation of any such LDT. The method has not been cleared by any regulatory entity for diagnostic purposes.

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Using UPLC-MS/MS for the Quantitation of Illicit or Prescription Drugs in Preserved Oral Fluid

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GOAL

To develop a quantitative analytical method for 14 illicit or prescription drugs in preserved oral fluid.

BACKGROUND

The analysis of illicit or prescription drugs in workplace or roadside drug-testing schemes has become an important aspect of forensic toxicology. The use of oral fluid as an alternate matrix for these tests has increased in popularity over the last decade due to a number of reasons. Oral fluid collection is a non-invasive technique and it can be achieved without the privacy and adulteration issues associated with urine collection. In contrast to blood samples, oral fluid does not require medically-trained staff to collect the sample. Unlike urine, oral fluid can be more indicative of current impairment or intoxication. The Quantisal™ Oral Fluid Collection Device (Immunalysis, USA) allows 1 mL of sample to be collected into a stabilizing buffer which promotes stability of the sample during transportation to the testing laboratory.

A simple, sensitive, and quantitative UPLC-MS/MS method for substances commonly measured in oral fluid drug-testing schemes.

THE SOLUTION

A simple solid-phase extraction is used to eliminate matrix effects that result from additives and stabilizers in oral fluid collection devices. Combining the Waters® ACQUITY UPLC® I-Class System with the Xevo® TQD allows these compounds to be detected at levels lower than the currently recommended maximum cut-offs for confirmation assays in workplace drug-testing schemes.[1] This UPLC-MS/MS combination also permits a compound-specific quantitative determination of the relevant analytes.

EXPERIMENTAL

Sample collection

Oral fluid samples were collected and preserved using the Quantisal Oral Fluid Collection Device according to the manufacturer's directions. It is generally understood that the collected oral fluid is diluted by a factor of four once it has been added to the buffer in the device, and the concentrations stated in this technical brief relate to those in neat oral fluid. Once collected, the samples were stored at 4 °C for at least 24 hours prior to analysis.

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Sample preparation

Ten microlitres (1.125 ng) of deuterated internal standard (ISTD) mixture was added to 350 µL preserved oral fluid (either calibrator or quality control samples) and the sample was diluted with 4% phosphoric acid (350 µL). The samples were extracted by solid-phase extraction (SPE) using a modified version of Danaceau et al.[2] the wells in a 96-well Waters Oasis® MCX µElution plate (P/N 186001830BA) were conditioned with 200 µL methanol followed by 200 µL 18.2 MΩ water. The entire diluted sample was added to each well. After loading, the wells were washed with 200 µL 2% formic acid followed by 200 µL 50% methanol. After drying under vacuum for 10 min, all samples were eluted with 200 µL acetonitrile/propan-2-ol (60:40, v/v) containing 5% ammonium hydroxide. The samples were evaporated to dryness under a stream of nitrogen at 50 °C (for a maximum of 15 min) and reconstituted in 87.5 µL water/acetonitrile (95:5, v/v). The collection plate was covered with a silicone/PTFE treated cap mat and vortex-mixed for 1 min. Five microlitres were analyzed by UPLC-MS/MS.

LC conditions

System:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC BEH C_{18}, 130Å, 1.7 µm, 2.1 mm x 100 mm (P/N 186002352)
Column temp.:	40 °C
Flow rate:	0.40 mL/min
Mobile phase A:	0.1% formic acid
Mobile phase B:	acetonitrile
Wash solvent:	acetonitrile/water (95:5, v/v)
Purge solvent:	0.1% formic acid

Gradient elution:

Time (min)	%B	Curve
0	2	initial
1.50	13	6
1.80	13	6
2.65	36	6
3.00	36	6
3.40	50	6
4.25	95	6
4.75	95	6
4.80	2	6

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI+
Capillary voltage:	1.0 KV
Source temp.:	150 °C
Desolvation temp.:	500 °C
Desolvation gas:	800 L/Hr
Cone gas:	20 L/Hr

Compound	RT (min)	Time window (min)	MRM transitions	Cone voltage (V)	Collision energy (eV)
Morphine	1.37	1.0–1.9	286.1 > 165.1	55	42
Codeine	2.11	1.9–2.3	300.1 > 215.1	50	25
Amphetamine	2.29	2.1–2.5	136.0 > 91.0	22	13
6-Monoacetylmorphine (6-MAM)	2.37	2.2–2.6	328.2 > 165.1	55	37
Methamphetamine	2.49	2.3–2.7	150.0 > 91.0	25	20
MDMA	2.55	2.4–2.8	194.0 > 163.0	25	10
Ketamine	2.88	2.7–3.1	238.1 > 124.9	30	20
Benzoylecgonine (BZE)	2.91	2.7–3.1	290.1 > 168.1	55	19
Cocaine	3.17	3.0–3.5	304.1 > 82.0	40	30
Methadone	3.90	3.5–4.1	310.1 > 105.1	30	30
Oxazepam	4.10	3.9–4.3	287.0 > 241.1	20	20
Nordiazepam	4.21	4.0–4.4	271.0 > 140.0	50	30
Temazepam	4.31	4.1–4.5	301.0 > 177.0	30	40
Diazepam	4.47	4.2–4.7	285.0 > 154.0	35	20

Table 1. Retention times and MRM conditions for the analytes (quantifier transition only listed). Compound-specific deuterated ISTDs were monitored using a single transition.

RESULTS

The acceptance criteria for a positive identification of analytes were: the retention time to be within 0.2 min of predicted and the quantifier/qualifier ion ratio to be within 20% of the predicted ratio, which was based on the average of the ratios across the entire calibrator range.

To investigate linearity for all analytes, spiked preserved oral fluid calibrators and quality control (QC) samples were prepared daily (the concentrations in neat oral fluid ranged from 0 ng/mL to 500 ng/mL) and analyzed on four different days. Peak areas for each MRM trace were generated automatically using the TargetLynx™ Application Manager and referenced to the appropriate ISTD peak area. Quantitative calibration curves were plotted using a 1/x weighting with a quadratic fit applied to all analytes. Interday coefficient of determination (assessed over four days) was >0.995 for each analyte.

The limit of detection (LOD) was defined as the lowest concentration which gave a signal-to-noise ratio >10:1 (for both transitions) in spiked preserved oral fluid. The lower limit of quantitation (LLOQ) was defined as the lowest concentration which gave a signal to noise ratio >10:1 (for both transitions) and ion ratios within 20% of expected and the achieved concentration was within 20% of target with a %RSD of <20% in preserved oral fluid over the four day period. The LOD and LLOQ for each analyte are summarised in Table 2 along with the concentration of the lowest QC sample assayed. Extraction recovery and matrix effects for each analyte were investigated in six different sources of preserved oral fluid at three concentrations (5, 25, and 100 ng/mL), with the ISTDs at 12.5 ng/mL. The mean % recovery for each analyte was matched by that of the appropriate deuterated internal standard and was acceptable for this assay. The matrix effects were matched by the appropriate deuterated internal standard and were shown to be less than 25% for the majority of analytes with the %RSD less than 15% for all analytes.

Interday accuracy and precision were assessed by analyzing three QC samples at low, medium, and high concentrations over four different days. The mean achieved values for the quality control replicates over the four day period at the three concentration levels were within 15% of target and the % RSD was <10%.

Figure 1 shows a chromatogram of a processed oral fluid QC sample at the lowest level assayed.

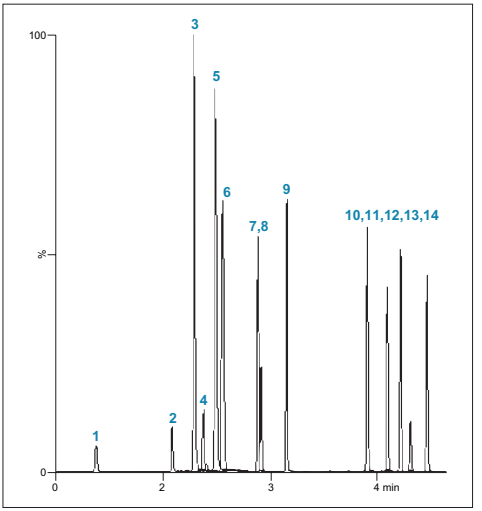

Figure 1. Chromatogram of the quantitation ion for all the analytes in spiked preserved oral fluid at the lowest QC level. Concentrations and peak assignments are listed in Table 2.

Peak #	Compound	LOD (ng/mL)	LLOQ (ng/mL)	Lowest QC (ng/mL)
1	Morphine	<0.1	2.0	7.5
2	Codeine	<0.1	4.0	7.5
3	Amphetamine	<0.1	1.0	3.75
4	6-MAM	<0.1	0.8	7.5
5	Methamphetamine	<0.1	1.0	3.75
6	MDMA	<0.1	1.0	3.75
7	Ketamine	<0.1	1.0	3.75
8	BZE	<0.1	1.0	3.75
9	Cocaine	<0.1	0.4	3.75
10	Methadone	<0.1	0.16	1.5
11	Oxazepam	<0.1	0.8	7.5
12	Nordiazepam	<0.1	2.0	7.5
13	Temazepam	<0.1	0.8	7.5
14	Diazepam	<0.1	2.0	7.5

Table 2. LODs, LLOQs, and the lowest QC sample assayed (ng/mL) based on the µ-Elution SPE protocol described above. Quoted concentrations refer to concentrations in the neat oral fluid and have been adjusted for the dilution effect associated with sample collection.

SUMMARY

The rise of workplace and roadside drug testing has highlighted the need for a quick, accurate, reliable, and robust method to quantify both illicit and prescribed drugs in various biological matrices. The use of preserved oral fluid allows for simple, supervised, and non-invasive collection of a matrix which contains analytes commonly measured in such testing schemes.

The use of the Waters ACQUITY UPLC I-Class System allows for a quick and robust analytical method that can detect the analytes in a single run. The demonstrated injection-to-injection time of 7 min, combined with the simple sample preparation method utilizing Oasis MCX plates, minimizes matrix effects from the stabilizers used in commercial collection devices. This allows for high sample throughput. Furthermore the superior sensitivity of the Xevo TQD permits detection of the analytes at levels lower than the currently recommended maximum cut-offs for confirmation assays in workplace drug testing.

References

1. European Workplace Drug Testing Society Guidelines. http://www.ewdts.org (accessed 29 May 2015).
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This is a proof of principle demonstration of an analytical method, which may include examples of typical results that can be achieved with the stated configuration. This method represents a basic starting point from which users should perform their own in-house validation.

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Improved Extraction of THC and its Metabolites from Oral Fluid Using Oasis PRiME HLB Solid Phase Extraction (SPE) and a UPLC CORTECS C_{18} Column

Xin Zhang, Jonathan P. Danaceau, and Erin E. Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Semi-validated method for a non-invasive, easy to collect matrix–oral fluid
- Faster, simplified sample preparation workflow compared to traditional SPE sorbents
- Excellent and consistent recoveries and minimal matrix effects
- No evaporation or reconstitution necessary with µElution plate format
- Linear, accurate, and precise results for all analytes

WATERS SOLUTIONS

Oasis® PRiME HLB µElution Plate (p/n 186008052)

ACQUITY® 96-well Sample Collection Plate, 700 µL Round Well (p/n 186005837)

CORTECS® UPLC® C_{18} Column 1.6 µm, 2.1 x 100 mm (p/n 186007095)

ACQUITY I-Class UPLC System

Xevo® TQ-S Mass Spectrometer

KEYWORDS

Quantification, THC and metabolites, Oasis PRiME HLB, Oral fluid, ACQUITY I-Class, Xevo TQ-S Mass Spectrometer

INTRODUCTION

Cannabis continues to be a highly abused recreational drug. The increasing number of states legalizing it for medical use combined with the trend towards legalization for recreational purposes, means that there is a growing need for analytical methods for the quantification of Δ-9-tetrahydrocannabinol (THC), its metabolites including the active metabolite 11-hydroxy Δ-9-THC (THC-OH) and non-active metabolite 11-nor-9-Carboxy-Δ-9-THC (THC-COOH).[1] While urine has traditionally been used to assess cannabis use, oral fluid has become increasingly popular as a matrix. Collection of oral fluid is relatively easy to perform, non-invasive and can be achieved under close supervision. Moreover, drug and metabolite concentrations in oral fluid provide better indications of current impairment than urine concentrations, so there is a higher probability that the subject is experiencing pharmacological effects at the time of sampling.[2,3] The cut off level for THC use was reported as 2 ng/mL in oral fluid,[5] which means any analytical method should be able to accurately quantify at this concentration.

This method details the extraction and analysis of THC and its major metabolites, 11-THC-OH and 11-THC-COOH from oral fluid using the Oasis PRiME HLB µElution Plate, followed by UPLC-MS/MS analysis. The SPE procedure is simple and very efficient, with elution in LC compatible solvents, allowing for direct injection, without evaporation and reconstitution of samples. Analysis is rapid with all analytes eluting in 3 minutes. Recoveries were excellent (all greater than 75% with %RSDs <6) and matrix effects were minimal (<10% ME) for all compounds. Quantitative results were highly reproducible. All calibration curves were linear and R^2 values were greater than 0.999. Quality control results were within 10% of expected concentrations with average %RSDs less than 3%.

EXPERIMENTAL

LC conditions

UPLC system:	ACQUITY I-Class UPLC System
Column:	CORTECS® UPLC® C_{18} Column 1.6 µm, 2.1 x 100 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	Water with 0.1% formic acid
Mobile phase B (MPB):	ACN with 0.1% formic acid
Strong wash solvent:	70:30 ACN:Water with 2% formic acid
Weak wash solvent:	10% ACN
Injection volume:	5 µL

The gradient ramp is shown in Table 1.

Mass spectrometry

MS system:	Xevo TQ-S Mass Spectrometer
Ionization mode:	ESI Positive
Capillary voltage:	2.0 kV
Cone voltage:	Optimized for each analyte
Cone gas:	150 L/hr
Desolvation temp.:	500 °C
Source temp:	150 °C

Time (min.)	Flow (mL/min.)	%A	%B
0	0.6	50	50
1.0	0.6	50	50
3.0	0.6	5	95
5	0.6	5	95
5.6	0.6	50	50
6.0	0.6	50	50

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Methods section.

Materials

All standards and stable isotope labelled internal standards were purchased from Cerilliant (Round Rock, TX, USA). Stock standards at 100 µg/mL were prepared in 40% methanol (THC, THC-OH and THC-COOH). A working internal standard solution, consisting of 100 ng/mL THC-D3, THC-OH-D3 and THC-COOH-D3 was also prepared in 40% methanol. Individual calibrators and quality control standards were prepared daily in 40% methanol. 200 µL of each working calibrator or QC standard was added to 1800 µL of oral fluid to make calibration curves and QC samples. Calibrator concentrations ranged from 0.05–100 ng/mL for all analytes. Quality control samples were prepared at 0.375, 1.75, 7.5 and 37.5 ng/mL, in oral fluid.

Sample preparation

Sample pre-treatment

Oral fluid samples were collected with Quantisal collection device from Immunalysis according to the manufacturer's directions. The collection applicator was saturated with oral fluid (spiked), and then placed in a collection vial, which contained 3.0 mL of sample stabilization buffer. Per Quantisal instruction, this was claimed to be the equivalent of collecting 1.0±0.1 mL of sample. 1 mL acetonitrile was then added to the collection vial to help improve extraction. The collection kit was stored in a refrigerator overnight to simulate the transit time of the sample and to allow for complete equilibration between the sample in the pad and the stabilization buffer mix in the collection vial.

SPE with an Oasis PRiME HLB µElution Plate

500 µL aliquots of buffer stabilized oral fluid samples (equivalent to 100 µL oral fluid) were pre-treated by adding 200 µL 4% H_3PO_4 and 10 µL of working IS mixture (100 ng/mL in 40% MeOH).

The entire pre-treated sample (total of 710 µL) was directly loaded on to the Oasis PRiME HLB µElution Plate without conditioning or equilibration, followed by washing with 2 x 250 µL 5% NH_4OH in 25:75 methanol:water. All the wells were then eluted with 2 x 25 µL 90:10 ACN:MeOH and diluted with 50 µL of water. 5 µL was injected onto the UPLC-MS/MS system. The SPE extraction procedure is summarized in Figure 1.

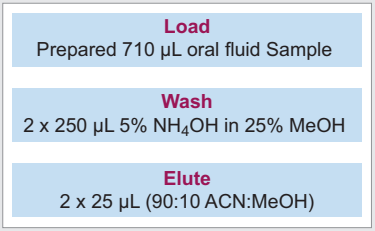

Figure 1. Oasis PRiME HLB extraction methodology for THC, COOH-THC, and OH-THC from oral fluid. With no conditioning and equilibration, sample extraction is simplified to just three steps.

Analyte recovery was calculated according to the following equation:

$$\% \text{ Recovery} = \left(\frac{\text{Area A}}{\text{Area B}}\right) \text{x } 100\%$$

Where A equals the peak area of an extracted sample and B equals the peak area of an extracted blank matrix sample in which the compounds were added post-extraction.

$$\text{Matrix Effects} = \left(\left(\frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}}\right)\right) \text{x } 100\%$$

The peak area in the presence of matrix refers to the peak area of an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

RESULTS AND DISCUSSION

CHROMATOGRAPHY

A representative UPLC chromatogram of the three cannabinoids from an extracted calibrator at 1 ng/mL is shown in Figure 2. Using a CORTECS UPLC C_{18} Column, all compounds eluted in 3 minutes. Peak shape was excellent for all compounds with all peak widths were under 1.8 seconds at 5% of baseline.

Table 2 lists the UPLC separation retention time and individualized MS parameters of the cannabinoids and their stable isotope labelled internal standards, including MRM transitions, cone voltage, and collision energy. Two MRM transitions were used for each compound, a primary (listed first) and a confirmatory transition (listed second).

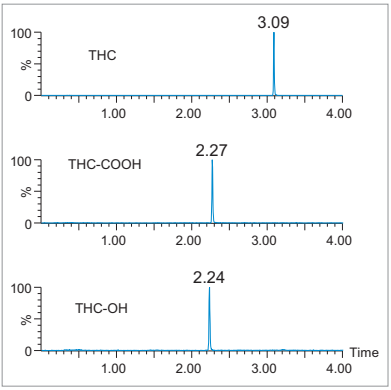

Figure 2. Chromatography of THC-OH, THC-COOH, and THC from extracted oral fluid samples at 1ng/mL of each analytes.

RECOVERY AND MATRIX EFFECTS

Extraction recoveries were high and consistent. As Figure 3 shows, recovery for all analytes was at least 75% with all %RSDs within 6% demonstrating the reproducibility of Oasis PRiME HLB. Matrix effects were minimal, at less than 10% for all compounds. Once again, the very low standard deviations (6% or less) demonstrate the consistency of extraction and cleanup seen with Oasis PRiME HLB. All recovery and matrix effect data are summarized in Table 3. The SPE wash step required optimization to eliminate suppression from the oral fluid matrix. The addition of 5% strong ammonia to the wash solution minimized the suppression, resulting in the near complete elimination of matrix effects.

Analyte	RT (min)	MRM transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)
THC-OH	2.24	331.3>313.1 331.3>193.1	40 40	18 30
THC-OH-d3	2.24	334.3>316.1	40	18
THC-COOH	2.27	345.3>327.3 345.3>299.3	50 50	20 25
THC_COOH-d3	2.27	348.3>330.3	50	20
THC	3.09	315.1>193.2 315.1>135.1	40 40	25 25
THC-d3	3.09	318.1>196.2	40	25

Table 2. Mass spectral parameters for all analytes and internal standards.

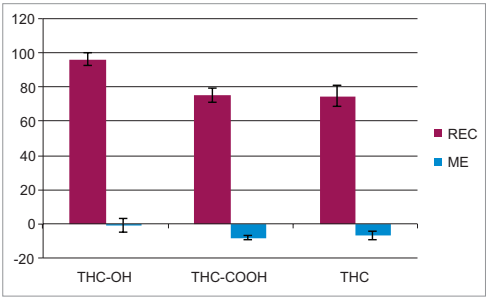

Figure 3. % Recovery and matrix effects of THC-OH, THC-COOH, and THC after extraction using the Oasis PRiME HLB µElution Plate. Error bars indicate standard deviations. %RSDs for extraction recovery were less than 8% for all compounds. Matrix effects were all under 10%.

	Recovery		Matrix Effects	
	Mean	%RSD	Mean	%RSD
THC-OH	96	4	-1	4
THC-COOH	75	4	-8	2
THC	75	6	-7	3

Table 3. Recovery and Matrix effects for THC and its metabolites (N=4 for all tests)

QUANTITATIVE RESULTS

Calibration and quality control samples were prepared as previously described in the materials and methods section. Calibration ranges were from 0.1–100 ng/mL for THC-OH and THC-COOH, and 0.05–100 ng/mL for THC. Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges.

Calibration and quality control (QC) results indicate that this method is linear, accurate and precise. All compounds had linear responses over the entire calibration range with R^2 values of 0.999 or greater using 1/x weighting. Figure 4 shows the calibration curves and Table 4 summarizes the data from these curves for all the compounds. Lower limits of quantification (LLOQ) were 0.1 ng/mL for THC-OH and THC-COOH and 0.05 ng/mL for THC. In each case, all FDA recommendations for accuracy, precision, linearity and analytical sensitivity were met for validated methods.[4]

Quality control samples prepared at 0.375, 1.75, 7.5, and 37.5 ng/mL were accurate and precise. All QC values were within 10% of their target values, and most were within 5%. This data can be seen in Table 5. This demonstrates that the method is linear, accurate and precise over a calibration range that includes the entire scope of expected values of samples. The method was also proved to be both selective and sensitive enough to routinely measure THC in oral fluid well below 2 ng/mL cut off level. This was exemplified by the excellent accuracy and precision at the 0.375 ng/mL QC sample level, where calculated concentrations of all six replicates were within an average of 6% of expected.

	R^2	Mean % dev.	Range (ng/mL)	Curve type
THC-OH	0.9992	3.7%	0.1–100	Linear
THC-COOH	0.9994	2.5%	0.1–100	Linear
THC	0.9995	1.8%	0.05–100	Linear

Table 4. Calibration Curve Summary for THC and its metabolites with 1/x fit weighting.

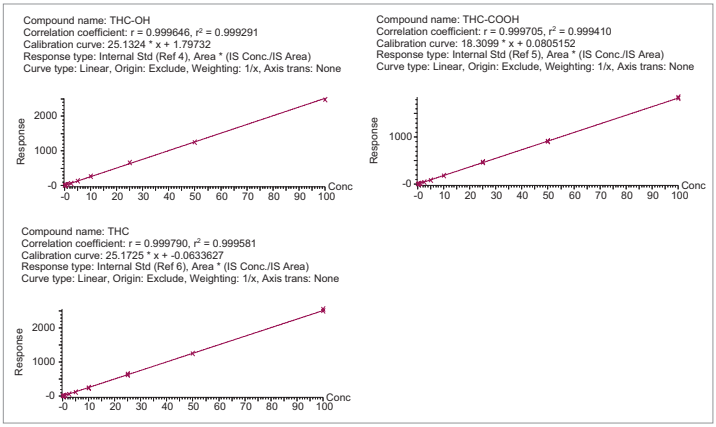

Figure 4. Calibration curves for THC and its metabolites, R^2> 0.999, fit – linear with 1/x weighting.

	Accuracy and precision								
N=6	THC-OH			THC-COOH			THC		
QC Level (ng/mL)	Mean (ng/mL)	% Acc.	%RSD	Mean (ng/mL)	% Acc.	%RSD	Mean (ng/mL)	% Acc.	%RSD
0.375	0.36	96.6	8.3%	0.35	93.8	7.1%	0.39	105.2	5.7%
1.75	1.77	101.1	3.4%	1.65	94.3	2.7%	1.69	96.6	3.2%
7.5	7.57	101.0	2.7%	6.94	92.5	3.9%	7.12	94.9	2.4%
37.5	36.88	98.3	1.9%	37.77	100.7	1.4%	36.34	96.9	0.8%
Mean		100	4%		96	3%		96.1	3%

Table 5. Quality control results from extracted oral fluid samples. (N=6 for each compound at all three levels). Mean values at the bottom indicate averages of all compounds at particular concentrations.

CONCLUSIONS

This application note details the extraction of THC-OH, THC-COOH, and THC from oral fluid samples using a novel SPE sorbent, Oasis PRiME HLB, in a µElution format for forensic toxicology applications. This sorbent enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. In addition, the µElution format enabled the direct injection of extracts without evaporation or reconstitution, minimizing the risk of nonspecific binding and sample losses. The unique nature of oral fluid resulted in some ion suppression that was not seen in other matrices. In order to overcome this signal suppression, a CORTECS C_{18} Column was utilized for high efficiency and the SPE wash step was optimized with the addition of 5% strong ammonia. These changes resulted in a method with negligible matrix effects (<10%) and calibration curves R^2 value all greater than 0.999.

Recoveries were very consistent, with recoveries >75%, with RSDs under 6%, and minimal matrix effects for all compounds. Linearity, accuracy, precision and analytical sensitivities were excellent for all compounds. All accuracies were within 10% of target concentrations with average %RSDs less than 3% for QC samples, demonstrating the high reproducibility arising from the combination of this sorbent and the UPLC-MS/MS method. In conclusion, Oasis PRiME HLB has been successfully used to achieve consistent recoveries with minimal matrix effects as well as accurate quantification 4 orders of magnitude from oral fluid samples.

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2. Kintz, P., Cirimele, V., Luders, B.: Detection of Cannabis in Oral Fluid (Saliva) and Forehead Wipes (Sweat) from Impaired Drivers. *Journal of Analytical Toxicology* 24, 557–561 (2000).
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Quantitative Analysis of THC and its Metabolites in Whole Blood Using LC-MS/MS for Toxicology and Forensic Laboratories

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Faster, simpler sample preparation workflow compared to traditional SPE sorbents
- Efficient and consistent recoveries and minimal matrix effects
- No evaporation or reconstitution necessary with µElution plate format
- Linear, accurate, and precise results for all analytes
- Cleaner eluates with removal of over 99% of phospholipids

WATERS SOLUTIONS

Oasis® PRiME HLB µElution Plate (p/n 186008052)

ACQUITY® 96-well Sample Collection Plate, 700 µL Round well (p/n 186005837)

ACQUITY UPLC® BEH C_{18} Column 1.7 µm, 2.1 x 50 mm (p/n 186002350)

ACQUITY UPLC I-Class System

Xevo® TQ-S Mass Spectrometer

KEYWORDS

THC and metabolites, Oasis PRiME HLB extraction, whole blood, Acquity UPLC I-Class, Xevo TQ-S, forensic toxicology

INTRODUCTION

Cannabis continues to be a highly abused recreational drug. The increasing number of states legalizing it for medical use combined with the trend towards legalization for recreational purposes, means that there is a growing need for analytical methods for the quantification of Δ-9-tetrahydrocannabinol (THC) and its metabolites. Furthermore, a growing number of laboratories are interested in the quantification of THC and its metabolites in whole blood for toxicology and forensic purposes. The complex nature of whole blood introduces many unique challenges that must be addressed to achieve consistent and reproducible results.

Sample preparation is an important consideration for any bioanalytical LC-MS/MS method designed for forensic toxicology. Waters has developed a novel sample preparation sorbent, Oasis PRiME HLB, which is designed to provide several key advantages over traditional SPE sorbents. These include the ability to eliminate sorbent preconditioning and equilibration, creating a faster workflow compared to traditional SPE products. It also has the ability to remove more matrix interferences, particularly phospholipids, resulting in a cleaner extracts and reducing the risk of short column lifetimes or MS source fouling.

This method details the extraction and analysis of THC and its major metabolites, 11-hydroxy Δ-9-THC (THC-OH) and 11-nor-9-Carboxy-Δ-9-THC (THC-COOH)[1] from whole blood using an Oasis PRiME HLB µElution Plate, followed by UPLC-MS/MS analysis. The SPE procedure is simple and very efficient, with elution in LC compatible solvents, allowing for direct injection without evaporation and reconstitution of samples. Analysis is rapid and highly consistent, with all analytes eluting in less than 3 minutes. Recoveries were excellent and matrix effects were minimal for all compounds. Quantitative results were highly reproducible. Quality control results were within 10% of expected concentrations and average %RSDs within 2–4%.

While this application was performed with UPLC-MS/MS system, an HPLC separation method for THC and its metabolites was developed to provide HPLC-MS/MS users guidance as well.

EXPERIMENTAL

MATERIALS

All standards and stable isotope labelled internal standards were purchased from Cerilliant (Round Rock, TX, USA). Stock standards at 100 µg/mL were prepared in 40% methanol (THC, THC-OH and THC-COOH). A working internal standard solution, consisting of 100 ng/mL THC-D3, THC-OH-D3 and THC-COOH-D3 was also prepared in 40% methanol. Individual calibrators and quality control standards were prepared daily in 40% methanol.

SPIKED WHOLE BLOOD SOLUTION

100 µL of each working calibrator or QC standard and 100µL internal standard (I.S.) were added to 1800 µL of rat whole blood to make calibration curves and QC samples. Calibrator concentrations ranged from 0.05–100 ng/mL for all analytes. Quality control samples were prepared at 0.375, 2, 7.5, 20 and 37.5 ng/mL, in whole blood.

SAMPLE PREPARATION

Sample pretreatment

Samples were extracted using Oasis PRiME HLB µElution Plates. 100 µL spiked whole blood was added to 25 µL of a solution of 0.1 M zinc sulfate/ammonium acetate, and the mixture was vortexed for 5 seconds to lyse the cells. All samples were then precipitated by adding 375 µL 0.1% formic acid in ACN. The entire sample was vortexed for 10 seconds and centrifuged for 5 min at 7000 rcf. The supernatant was then diluted with 800 µL water prior to loading.

SPE with Oasis PRiME HLB µElution Plate

The entire pretreated sample was directly loaded on to the Oasis PRiME HLB µElution Plate in 2 aliquots without conditioning or equilibration. All wells were then washed with 2 x 250 µL aliquots of 25:75 methanol:water. All the wells were then eluted with 2 x 25 µL aliquots of 90:10 ACN:IPA and diluted with 50 µL of water. 5 µL was injected onto the UPLC-MS/MS system. The SPE extraction procedure is summarized in Figure 1.

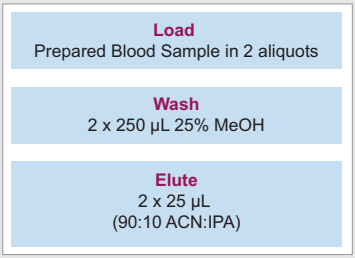

Figure 1. Oasis PRiME HLB extraction methodology for THC, COOH-THC, and OH-THC from whole blood. With no conditioning and equilibration, sample extraction is simplified to just three steps.

Analyte recovery was calculated according to the following equation:

$$\%\text{Recovery} = \left(\frac{\text{Area A}}{\text{Area B}}\right) \times 100\%$$

Where A equals the peak area of an extracted sample and B equals the peak area of an extracted blank matrix sample in which the compounds were added post-extraction.

Matrix effects were calculated according to the following equation:

$$\text{Matrix Effects} = \left(\left(\frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}}\right)\right) - 1 \times 100\%$$

The peak area in the presence of matrix refers to the peak area of an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

LC conditions

UPLC system:	ACQUITY I-Class UPLC System
Column:	ACQUITY UPLC BEH C_{18} Column, 1.7 µm, 2.1 x 50 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	Water with 0.1% formic acid
Mobile phase B (MPB):	ACN with 0.1% formic acid
Strong wash solvent:	70:30 ACN:Water with 2% formic acid
Weak wash solvent:	10% ACN
Injection volume:	5 µL

The gradient ramp is shown in Table 1.

Mass spectrometry

MS system:	Xevo TQ-S Mass Spectrometer
Ionization mode:	ESI Positive
Capillary voltage:	2.0 kV
Cone voltage:	Optimized for each analyte
Desolvation gas:	1000 L/hr
Cone gas:	150 L/hr
Desolvation temp.:	500 °C
Source temp.:	150 °C

Data were acquired and analyzed using MassLynx® Software (V4.1). Quantification was performed using TargetLynx.™

Time (min)	Flow (mL/min)	%A	%B
0	0.6	50	50
1.0	0.6	50	50
3.0	0.6	5	95
3.5	0.6	5	95
3.6	0.6	50	50
4.0	0.6	50	50

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Methods section.

RESULTS AND DISCUSSION

CHROMATOGRAPHY

Figure 2A shows UPLC chromatograms of the three cannabinoids from an extracted calibrator at 1 ng/mL. All compounds eluted within 3 minutes and all peak widths were between 2.2–2.6 seconds at 5% of baseline. All peaks were symmetrical with symmetries between 0.95–1.15. Figure 2B shows an HPLC chromatogram conducted with an XBridge BEH C_{18}, 2.5 µm; 2.1 x 50 mm Column with ACQUITY UPLC H-Class/Xevo TQD with a maximum system pressure of 4,000 psi. All peak widths were between 3.6–4.8 seconds at 5% baseline, an average of around 60% more than UPLC chromatogram. Similar symmetries were obtained. The slight difference in analyte retention time between UPLC and HPLC is due to the different system dwell volume (system delay volume, an ACQUITY UPLC I-Class dwell volume is around 100 µL and an H-Class is 300 µL).

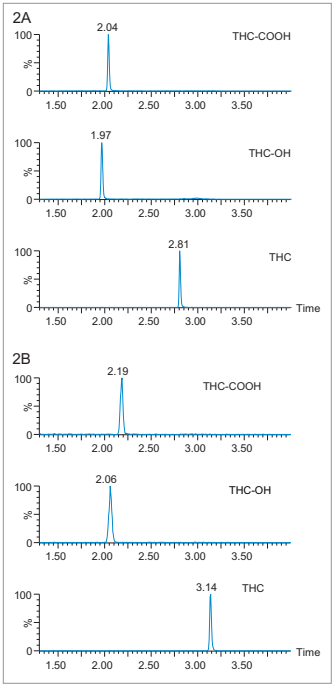

Figure 2. Chromatograms of THC-OH, THC-COOH and THC from an extracted whole blood samples.

Figure 2A is on the ACQUITY UPLC I-Class – Xevo TQ-S, BEH C_{18} Column, 1.7 µm; 2.1 x 50 mm with system back pressure around 6500 psi. The concentrations are 1 ng/mL for all compounds.

Figure 2B is on the ACQUITY UPLC H-Class System coupled to a Xevo TQD. The separation was achieved using an XBridge BEH C_{18}, 2.5 µm; 2.1 x 50mm with system back pressure around 4000 psi. The LC solvent gradient and flows were the same as the UPLC separation.

Table 2 lists the UPLC retention times and individualized MS parameters of the cannabinoids and their stable isotope labelled internal standards, including MRM transitions, cone voltage, and collision energy. Two MRM transitions were used for each compound, a primary (listed first) and a confirmatory transition (listed second).

Compared to HPLC, UPLC offers improved resolution and sensitivity, higher efficiency, a faster run time, and reduced solvent use. In this application, all recoveries, matrix effects, phospholipid removal and method validation were performed on the ACQUITY UPLC I-Class/Xevo-TQ-S System to maximize the aforementioned benefits.

RECOVERY AND MATRIX EFFECTS

Extraction recoveries were high and consistent. As Figure 3 shows, recovery for all analytes was greater than 85% with average RSDs within 5–7%, demonstrating the high reproducibility of Oasis PRiME HLB. Matrix effects were minimal, at less than 15% for all compounds. Once again, the low standard deviations (average at 5–7%) demonstrate the consistency of extraction and cleanup seen with Oasis PRiME HLB. All recovery and matrix effect data are summarized in Table 3. Oasis PRiME HLB provided comparable recovery, variability and matrix effects as mixed-mode SPE, with a more simplified procedure than previously published.[3]

Analyte	RT (min)	MRM transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)
THC-OH	1.84	331.3>313.1 331.3>193.1	40 40	18 30
THC-OH-d3	1.84	334.3>316.1	40	18
THC-COOH	1.92	345.3>327.3 345.3>299.3	50 50	20 25
THC-COOH-d3	1.92	348.3>330.3	50	20
THC	2.72	315.1>193.2 315.1>135.1	40 40	25 25
THC-d3	2.72	318.1>196.2	40	25

Table 2. Mass spectral parameters for all analytes and internal standards.

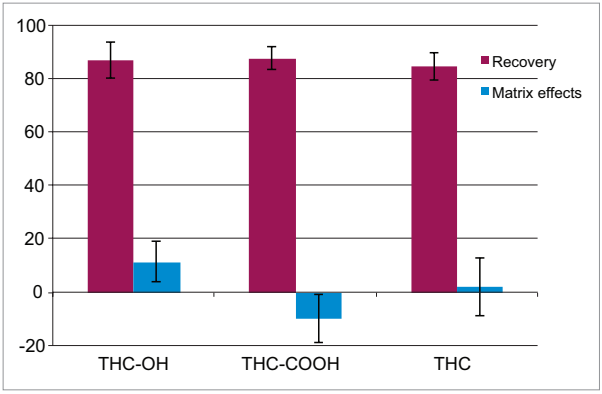

Figure 3. Recovery and matrix effects for THC-OH, THC-COOH, and THC after extraction using the Oasis PRiME HLB µElution Plate. Low average RSDs between 5–7% for all compounds. Matrix effects were all less than 15% with RSDs under 12%.

	% Recovery		% Matrix effects	
	Mean	%RSD	Mean	%RSD
THC-OH	87	7	12	7
THC-COOH	88	4	-10	9
THC	85	5	2	11

Table 3. Recovery and Matrix effects for THC and its metabolites (N=4 for all tests).

PHOSPHOLIPID REMOVAL

One of the key attributes of Oasis PRiME HLB is its ability to deliver cleaner extracts than other sample preparation methods. One way that this is achieved is by removing endogenous phospholipids. Figure 4 shows chromatograms of combined phospholipid traces from an Oasis PRiME HLB extract (B) and an identical sample subject to protein precipitation (C). Compared with protein precipitation (PPT), Oasis PRiME HLB removes over 99% phospholipids, resulting in a much cleaner extraction. This can translate to reduced matrix effects, longer column lifetimes, and less mass spectrometer source maintenance.

The chromatography of the three target compounds is also shown (A), demonstrating the potential interference with phospholipids if they were not removed during the extraction.

QUANTITATIVE RESULTS

The SPE method developed has been shown to deliver high and consistent extraction recoveries from whole blood. Research data shows that 2–3 ng/mL THCs are an indicator of recent marijuana exposure (cut off concentration).[1] This method detects THC and its metabolites down to 0.1 ng/mL, well below threshold for recent marijuana exposure.

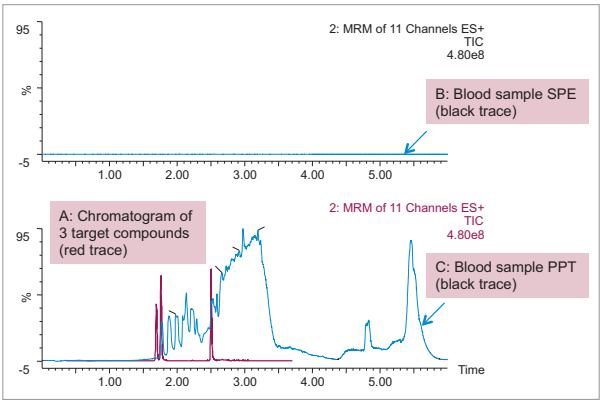

Figure 4. Chromatography of phospholipids remaining in Oasis PRiME HLB extraction vs. whole blood protein precipitation. Scales are linked. An overlaid chromatogram shows the retention times of THC-OH, THC-COOH, and THC in relation to the phospholipid traces. A: Chromatogram of 3 target compounds; B: blood sample SPE extract; C: blood sample PPT supernatant.

	R^2	Mean % dev.	Range (ng/mL)	Curve type
THC-OH	0.998	7.8	0.1–100	Linear
THC-COOH	0.999	4.0	0.05–100	Linear
THC	0.998	2.4	0.05–100	Linear

Table 4. Calibration Curve Summary for THC and its metabolites with 1/x fit weighting.

Calibration samples were prepared as previously described in the materials section. Calibration ranges were from 0.1–100 ng/mL for THC-OH and 0.05–100 ng/mL for THC and THC-COOH. All compounds had linear responses over the entire calibration range with R^2 values of 0.99 or greater with 1/x weighting. Figure 5 shows the calibration curves and Table 4 summarizes the data from these curves for all the compounds. Lower limits of quantification (LLOQ) were 0.1 ng/mL for THC-OH and 0.05 ng/mL for THC and THC-COOH, which are much lower than cut off concentration. In each case, all FDA recommendations for accuracy, precision, linearity and analytical sensitivity were met for validated methods.[2]

Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges. Quality control samples were accurate and precise. All results were within 10% of expected values with average RSDs between 2–4% (N=6). This data can be seen in Table 5. The excellent accuracy and precision demonstrate the consistency and robustness of this sorbent.

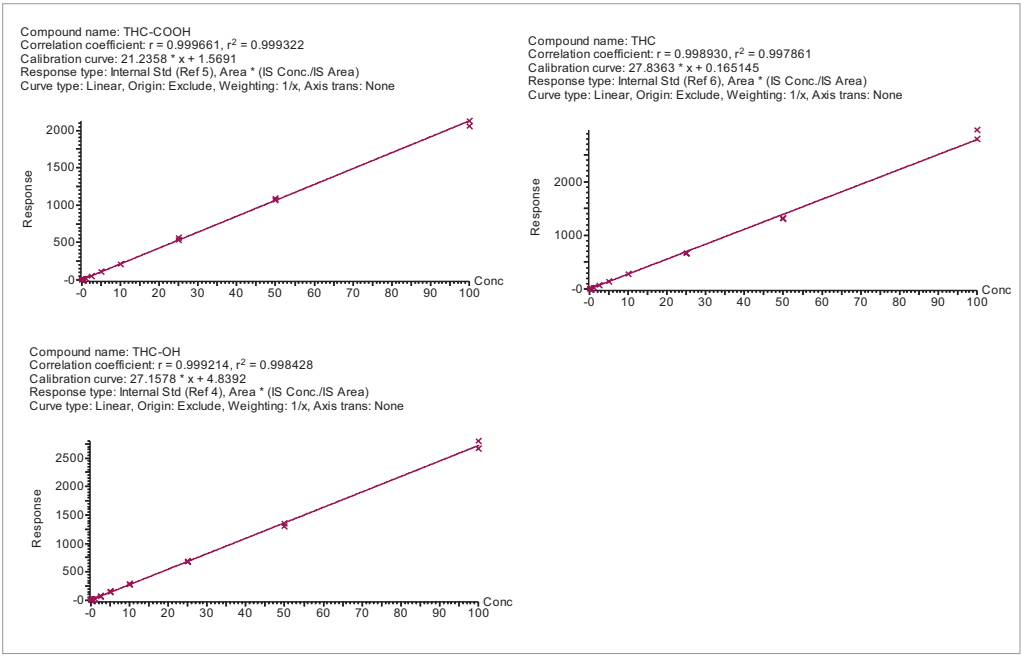

Figure 5. Calibration curves for THC and its metabolites, R^2> 0.99, fit – linear 1/x weighting.

CONCLUSIONS

This application note details the extraction of THC-OH, THC-COOH and THC from whole blood samples using a novel SPE sorbent, Oasis PRiME HLB, in a µElution format for forensic toxicology applications. The unique nature of this sorbent enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. In addition, the µElution format enabled the direct injection of extracts without evaporation or reconstitution, minimizing the risk of nonspecific binding. One key attribute of this sorbent is its ability to retain phospholipids. As mentioned previously and shown in Figure 4, >99% of residual phospholipids were eliminated from extracted samples, some of which would have co-eluted with the target analytes in this assay.

Recoveries were very consistent, with recoveries >85% and average RSDs at 5–7%. Matrix effects were less than 15% for all compounds. Linearity, accuracy, precision and analytical sensitivities were excellent for all compounds. All accuracies were within 10% of target concentrations with an average RSDs between 2–4% for QC samples, demonstrating the high reproducibility of the combination of this sorbent and the UPLC-MS/MS method. In conclusion, Oasis PRiME HLB has been successfully used to achieve consistent recoveries with minimal matrix effects as well as accurate quantification over 4 orders of magnitude from whole blood samples.

While this application was conducted using UPLC conditions, the chromatography illustrated in Figure 2B shows that this assay can also be run on an HPLC scale at reduced backpressures.

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Quantitative Determination of Urinary COOH-THC for Forensic Toxicology: Comparison to a Validated Reference Method

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APPLICATION BENEFITS

- Rapid extraction of COOH-THC from urine
- Elimination of evaporation and reconstitution steps, minimizing the risk of analyte loss from non-specific binding
- Direct sample concentration on the SPE device
- Excellent recovery and minimal matrix effects
- Excellent correlation with a fully validated quantification method

WATERS SOLUTIONS

Oasis® PRiME HLB µElution™ Plate

ACQUITY UPLC® BEH C_{18} Column

ACQUITY UPLC I-Class System

Xevo® TQ-S Mass Spectrometer

KEYWORDS

COOH THC, SPE, Oasis PRiME HLB, cannabis, tetrahydrocannabinol, forensic toxicology

INTRODUCTION

Cannabis continues to be the most widely abused recreational drug in the United States. In addition, the growing number of states legalizing cannabis for medical and/or recreational purposes means that there is a growing need for analytical methods for the quantification of THC and its metabolites. We have recently developed a simple, rapid, and clean SPE extraction method for these analytes using Oasis PRiME HLB.[1] While this method demonstrated excellent accuracy over a wide calibration range, a side by side comparison with a fully validated method from an external laboratory is a key component of method validation. This application note details an inter laboratory comparison of COOH-THC quantification using Oasis PRiME HLB.

MATERIALS AND METHODS

Authentic urine samples were obtained from an outside lab that conducts toxicology tests. Samples were prepared as follows: 25 µL of internal standard (100 ng/mL) was added to 350 µL of urine. 320 µL of 50% KOH was added and the samples were incubated for 2 hours at 65 °C to hydrolyze the COOH-THC glucuronide. 75 µL of the pretreated sample was added directly to the wells of an Oasis PRiME HLB µElution Plate (p/n 186008052). All samples were washed with 2 x 300 µL of 25:75 MeOH:H_2O and eluted with 2 x 25 µL of 60:40 ACN:IPA. The samples were then diluted with 100 µL of 40% ACN in water. 5 µL was injected onto the UPLC-MS/MS system. The SPE procedure is shown in Figure 1.

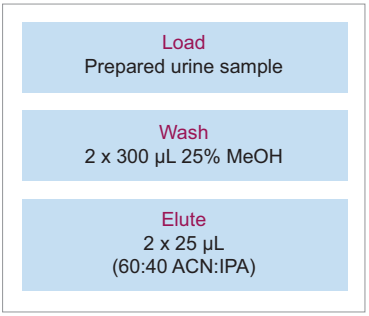

Figure 1. SPE procedure for the extraction of COOH-THC from urine samples using Oasis PRiME HLB µElution Plates.

Mobile phases A and B consisted of 0.1% formic acid in water (MPA) and ACN (MPB). The LC gradient is shown in Table 1. Separation was achieved on an ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 50 mm Column (p/n 186002350) at a temperature of 40 °C.

Detection was achieved with a Xevo TQ-S. MRM parameters are listed in Table 2.

RESULTS AND DISCUSSION

25 authentic urine samples were analyzed and compared to the validated comparison method. The samples ranged in concentration from 6.70–458 ng/mL, covering nearly the entire linear range of the reference method (5.00–500 ng/mL). A Deming regression (Figure 2) had a slope of 0.995 demonstrating parallelism between the two methods. The correlation (R) of 0.998 indicated an excellent correlation between the results obtained by the two laboratories. The Bland-Altman plot for the Oasis PRiME HLB method for the analysis of COOH-THC in authentic urine samples vs. reference method indicated that the two sets of data are similar at 95% limits of agreement (Figure 3). Table 3 details the results obtained by the two methods. 78% of the sample results are within 20% of each other, exceeding the FDA-GLP specification of 67% for incurred sample reanalysis.[2] Most results showed a slight negative bias not seen in the standards or QCs. Since the standards and QC samples were prepared in surrogate matrix (Surine), it is possible that the combination of different SPE methods and different chromatographic conditions differentially remove or chromatographically resolve an endogenous substance from the urine samples causing slight signal suppression during ionization. Despite the fact that the samples were subject to different extraction procedures as well as different LC-MS/MS conditions, the results show excellent agreement and indicate that the simplified SPE methodology, which eliminates conditioning and equilibration, gives equivalent results for authentic urine samples.

Time (min)	Flow (mL/min)	%A	%B
0	0.6	50	50
1.0	0.6	50	50
3.0	0.6	5	95
3.5	0.6	5	95
3.6	0.6	50	50
4.0	0.6	50	50

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Materials and Methods section.

Analyte	RT (min)	MRM transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)
THC-COOH	1.92	345.3>327.3	50	20
		345.3>299.3	50	25
THC-COOH-d3	1.92	348.3>330.3	50	20

Table 2. Mass spectral parameters for all analytes and internal standards.

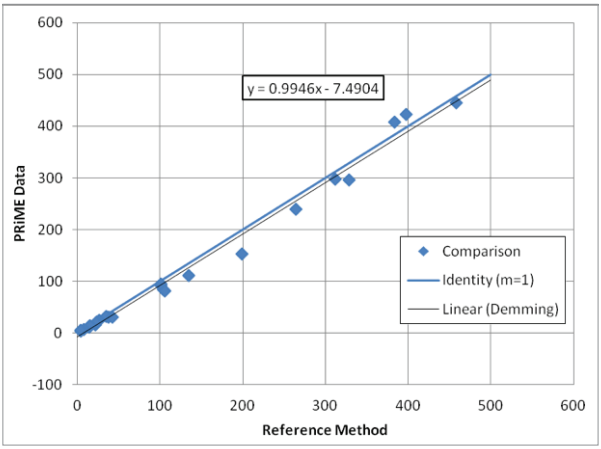

Figure 2. Correlation between the reference method and the Oasis PRiME HLB method for the analysis of COOH-THC in authentic urine samples. The blue line indicates the theoretical identity line of a perfect correlation. The black line is the plotted Deming regression with the equation listed on the chart.

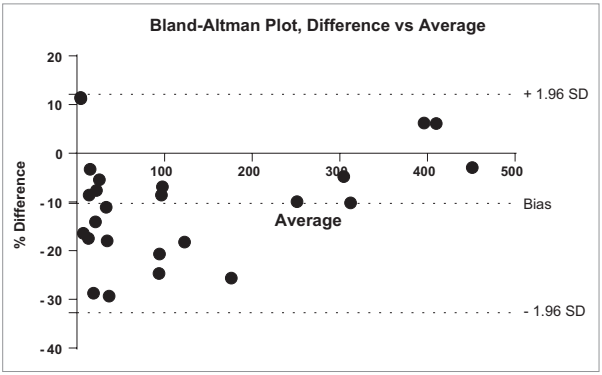

Figure 3. Bland-Altman plot at 95% limits of agreement for the Oasis PRiME HLB method for the analysis of COOH-THC in authentic urine samples vs. reference method.

Sample number	Urine THC concentration (ng/mL) original Oasis MAX reference method	Urine THC concentration (ng/mL) PRiME HLB method	Mean concentration (ng/mL)	%BIAS
1	BQL*	BQL	N/A	N/A
2	BQL	BQL	N/A	N/A
3	7.90	6.70	7.30	-16%
4	14.3	12.0	13.2	-17%
5	14.6	13.4	14.0	-9%
6	15.5	15.0	15.3	-3%
7	21.9	16.4	19.2	-29%
8	22.8	19.8	21.3	-14%
9	23.1	21.4	22.3	-8%
10	26.5	25.1	25.8	-5%
11	35.2	31.5	33.4	-11%
12	37.6	31.4	34.5	-18%
13	42.2	31.4	36.8	-29%
14	101	92.4	96.6	-9%
15	101	94.4	97.8	-7%
16	104	84.5	94.3	-21%
17	105	82.1	93.7	-25%
18	134	112	123	-18%
19	199	154	176	-26%
20	264	239	251	-10%
21	312	297	304	-5%
22	328	297	312	-10%
23	384	409	396	6%
24	398	423	410	6%
25	458	445	451	-3%
			AGREEMENT	78%

Table 3. COOH-THC concentrations from the reference method and the Oasis PRiME HLB method detailed in this application note.
**BQL: Below Quantifiable Limit.*

CONCLUSIONS

This work demonstrates the applicability of a simplified SPE method using Oasis PRiME HLB µElution Plates for the analysis of COOH-THC in authentic urine samples. Correlation was excellent with a validated method from Dominion Diagnostics that used a different SPE protocol and LC-MS/MS analysis procedure. Previous work had shown that Oasis PRiME HLB had excellent recovery with very low variability and could nearly eliminate matrix effects in urine samples. These data demonstrate that the differences between different samples do not affect the quantification of COOH–THC. The use of Oasis PRiME HLB enables a clean extraction without the need for conditioning or equilibration and gives quantitative results equivalent to a fully validated method.

For Forensic Toxicology Use Only.

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LC-MS/MS Analysis of Urinary Benzodiazepines and Z-drugs via a Simplified, Mixed-Mode Sample Preparation Strategy

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Rapid, simplified sample preparation of urinary benzodiazapines
- Significant savings in solvent usage and disposal vs. liquid-liquid extraction
- Consistent recovery for all compounds
- Excellent accuracy and reproducibility
- All sample pretreatment and extraction performed in-well, eliminating transfer steps
- Reduced matrix effects vs. reversed-phase SPE

WATERS SOLUTIONS

Xevo® TQ-S micro

ACQUITY UPLC® I-Class System (FL)

ACQUITY UPLC H-Class System

CORTECS UPLC® C_{18}+ Column

Oasis® MCX µElution Plate

MassLynx® Software

TargetLynx™ Application Manager

KEYWORDS

SPE, sample preparation, forensic toxicology, benzodiazepines, urine

INTRODUCTION

Benzodiazepines are frequently prescribed drugs used for their sedative, anxiolytic, and hypnotic properties.[1] They work by potentiating the inhibitory neurotransmitter gamma-amino butyric acid (GABA). Nationally, overdose deaths from benzodiazepines have risen 600% from under 1,600/year in 2001 to 8,000 in 2014, greater than any other drug class with the exception of heroin.[2] So-called "Z-drugs" (zolpidem and zopiclone) are commonly used sleep aids that act in a similar manner to benzodiazepines.[1] While the use of LC-MS/MS for benzodiazepine analysis has increased in recent years, many published techniques still rely on labor intensive liquid-liquid extraction techniques.[3-5] Some of the drawbacks of these techniques include the need to process individual samples one by one, the use of toxic solvents, and the need to evaporate and reconstitute samples after extraction.

This application note details a sample preparation and LC-MS/MS analysis strategy for a comprehensive panel of benzodiazepines, metabolites, and Z-drugs for forensic toxicology use. Using an abbreviated, modified solid phase extraction (SPE) method, Waters® Oasis® MCX µElution Plates were used to rapidly extract this panel of drugs and metabolites from urine samples. All sample preparation steps, including enzymatic hydrolysis, were performed within the wells of the Oasis MCX µElution Plates, and the extraction method was simplified by eliminating conditioning and equilibration steps. This enabled a streamlined workflow that minimized sample transfer steps while still achieving excellent and reproducible quantitative results. Chromatographic separation was achieved using a CORTECS UPLC® C_{18}+ Column while a Xevo® TQ-S micro Mass Spectrometer was used for detection. Extraction recovery was efficient, averaging 91%, and the use of the mixed-mode sorbent reduced matrix effects compared to reversed-phase SPE. The CORTECS UPLC C_{18}+ Column enabled the baseline separation of all target analytes from internal standards with identical nominal masses. This eliminated the risk of chromatographic interference between the labeled internal standards and the native compounds. All within and between batch quality control samples had mean accuracies within 5% of nominal values.

This method was also performed at HPLC scale using a CORTECS UPLC C_{18}+ 2.7 µm Column (3.0 x 100 mm) (p/n 186007372). The same efficient separation was seen as with the 1.6 µm column (p/n 186007402), with backpressures that remained under 4000 psi and a separation time that was increased by only 30%.

EXPERIMENTAL

All standards were obtained from Cerilliant (Round Rock, TX). Deuterated internal standards were used for all compounds with the exception of flurazepam. Stock solutions were prepared in methanol. Working standards were prepared daily by diluting stock standards in 80:20 water:methanol. Calibrators and QC samples were prepared in urine from working standards. All analytes are listed in Table 1, along with retention times and MS transitions and parameters.

Table 1. Analyte list, retention times, and MS parameters for benzodiazepines and metabolites analyzed in this application.

	Compound	RT	M+H+	MRM product ions	Cone voltage	Collision energy
1	N-desmethyl zopiclone	1.07	375.1	245.0 331.0	6 6	14 8
2	Zopiclone	1.13	389.1	245.0 111.9	8 8	12 58
3	Zolpidem	1.62	308.1	235.1 92.0	34 34	32 52
4	7-aminoclonazepam	1.92	286.1	121.0 222.1	50 50	26 30
5	Flurazepam	2.32	388.2	315.1 100.0	40 40	26 28
6	7-aminoflunitrazepam	2.36	284.1	135.0 226.9	34 34	26 22
7	Chlordiazepoxide	2.35	300.0	227.0 283.0	34 34	20 12
8	Midazolam	2.53	326.0	291.0 222.9	16 16	36 24
9	α-OH-midazolam	2.91	342.0	203.0 168.0	2 2	24 40
10	α-OH-triazolam	3.78	359.0	176.0 140.8	28 28	24 38
11	α-OH-alprazolam	3.77	325.1	297.1 243.1	50 50	25 30
12	Oxazepam[1]	3.84	289.0	103.9 243.0	50 50	30 20
13	Nitrazepam	3.87	282.1	180.1 236.0	50 50	36 20
14	Lorazepam	4.01	321.0	277.0 229.0	50 50	20 30
15	Clonazepam	4.10	316.0	214.1 241.1	54 54	42 40
16	Alprazolam	4.35	309.1	205.0 281.1	50 50	40 26
17	Nordiazepam	4.36	271.0	140.0 165.0	50 50	30 28
18	Flunitrazepam	4.41	314.1	239.2 268.1	50 50	30 25
19	Temazepam	4.45	301.1	177.0 255.1	36 50	46 20
20	Triazolam	4.47	343.0	308.0 239.0	28 28	24 38
21	Diazepam	5.14	285.1	154.0 193.1	50 50	26 30

[1] *Oxazepam's parent ion was set at 289 to avoid interference with Nitrazepam-d5 seen with m/z 287.*

Sample pretreatment: 200 µL of urine was added to individual wells of an Oasis MCX µElution Plate, along with 20 µL of internal standard solution (250 ng/mL), and 200 uL of 0.5 M ammonium acetate buffer (pH 5.0) containing 10 µL of β-glucuronidase enzyme/mL of buffer (Sigma Aldrich, *P. vulgate*, 85k units/mL). The entire plate was incubated at 50 °C for 1 hr. and then quenched with 200 µL of 4% H_3PO_4.

SPE extraction: Pretreated samples were drawn into the sorbent bed by vacuum. All samples were subsequently washed with 200 µL of 0.02 N HCl, followed by 200 µL of 20% MeOH. After washing, the plate was dried under high vacuum (~15 inch Hg) for 30 seconds. Samples were eluted with 2 x 25 µL of 60:40 ACN:MeOH containing 5% strong ammonia solution (Fisher, 28–30%). All samples were then diluted with 100 µL of sample diluent (2% ACN:1% formic acid in MilliQ water). A graphical workflow of the extraction procedure is shown in Figure 1.

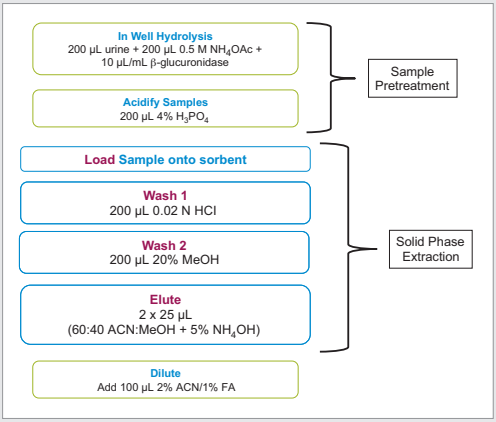

Figure 1. Details of the extraction method for the analysis of urinary benzodiazepines using Oasis MCX µElution Plates. Enzymatic hydrolysis and sample pretreatment are performed in the wells of the extraction plate, minimizing transfer steps. Conditioning and equilibration steps are eliminated, significantly simplifying the procedure.

Method conditions

LC conditions

System:	ACQUITY UPLC I-Class (FL)
Column:	CORTECS UPLC C_{18}+ 1.6 µm, 2.1 x 100 mm (p/n: 186007402)
Column temp.:	30 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.5 mL/min
Mobile phase A (MPA):	0.01% Formic acid in MilliQ water
Mobile phase B (MPB):	0.01% Formic acid in acetonitrile (ACN)
Gradient:	Initial conditions were 90:10 MPA:MPB. The percentage of MPB was increased to 50% over five minutes, ramped up to 95% by 5.25 minutes, held at 95% for 0.75 minutes and returned to 10% over 0.1 minute.

MS conditions

System:	Xevo TQ-S micro
Ionization mode:	ESI+
Detection:	MRM (transitions optimized for individual compounds, Table 1)
Capillary voltage:	0.5 kV
Collision energy:	Optimized for individual compounds (See Table 1)
Cone voltage:	Optimized for individual compounds (See Table 1)

Data management

MassLynx® Software with TargetLynx™ Application Manager

Analyte recovery was calculated according to the following equation:

$$\%Recovery = \left(\frac{Area\ A}{Area\ B} \right) x100\%$$

Where A = the peak area of an extracted sample and B = the peak area of an extracted matrix sample in which the compounds were added post-extraction.

RESULTS AND DISCUSSION

CHROMATOGRAPHY

All test compounds are listed in Table 1, and their chromatography is shown in Figure 2. Table 1 also lists the retention times and MS conditions of all compounds. Several columns were evaluated for this application, but the selectivity of the CORTECS UPLC C_{18}+ Column enables the baseline separation of all potentially interfering peaks. Two key pairs are shown in Figure 3. While clonazepam-d4 (R.T. 4.08) generates a slight contribution to the primary lorazepam MRM (323>277), the two peaks are baseline separated. Even at the LLOQ (0.5 ng/mL), the clonazepam IS does not interfere with lorazepam and does not affect quantification of the peak. Another critical pair is alprazolam-d5 and flunitrazepam. In this case, flunitrazepam makes a contribution that can be seen in the MRM trace of alprazolam-d5 (314.1>210.1). However, the baseline separation of these peaks ensures that even at the ULOQ (500 ng/mL) the baseline separation prevents flunitrazepam from affecting the integration and quantification if the alprazolam IS.

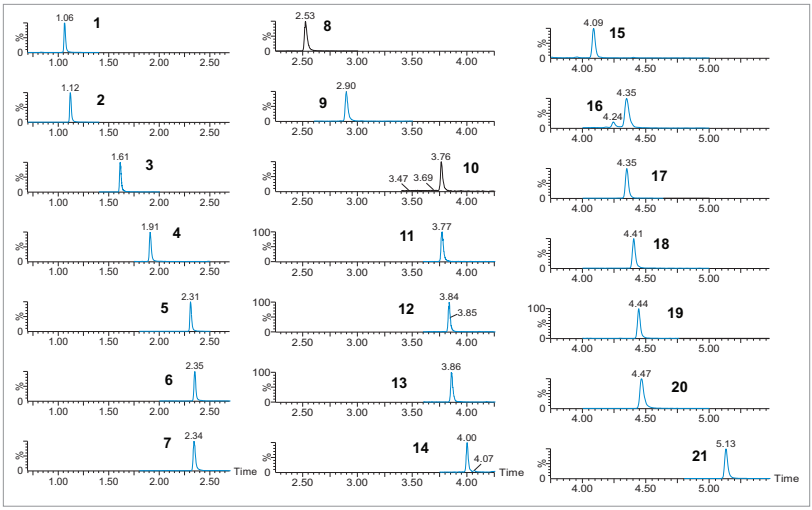

Figure 2. Chromatography of benzodiazepines analyzed in this application. See Table 1 for compound key. Column: CORTECS UPLC C_{18}+ 1.6 µm, 2.1 x 100 mm.

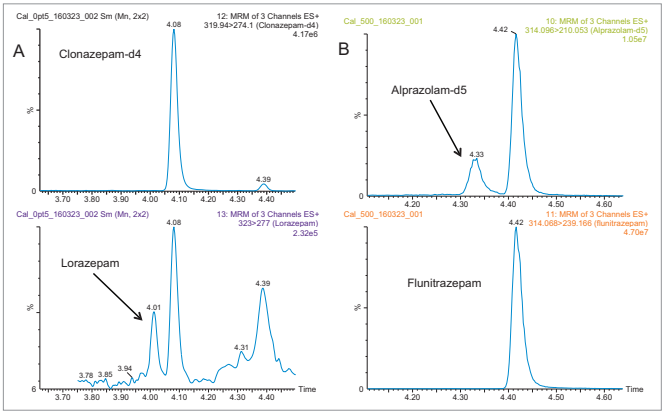

Figure 3. Chromatographic separation of key analyte pairs on the CORTECS UPLC C_{18}+ 1.6 µm Column. A. Clonazepam-d4 contributes to the lorazepam MRM but is baseline separated on this column. B. Alprazolam-d5 at 4.33 minutes is baseline separated from flunitrazepam at 4.42 minutes.

This panel was also analyzed on an HPLC scale using a CORTECS UPLC C_{18}+ 2.7 µm Column (3.0 x 100 mm) and an ACQUITY UPLC H-Class System. Table 2 compares the retention times of the UPLC and HPLC methods. All critical separations were maintained under HPLC conditions. The maximum system pressure stayed below 4000 psi. The retention time of diazepam, the latest eluting peak, only increased from 5.14 to 6.69, a 30% increase, and the solvent ramp duration increased from seven to nine minutes. The increase in retention time was likely due to the decreased linear velocity of the mobile phase resulting from the larger interior diameter of the HPLC column (3.0 mm vs 2.1 mm) and the decrease in the slope of the solvent ramp. If run on a traditional HPLC system, the increase in dwell volume would likely result in an increase in peak width. Nevertheless, the scalability of the CORTECS UPLC C_{18}+ Column should make this adjustment straightforward. While ACQUITY UPLC will provide the fastest and most efficient separation, this enables the method to be performed on HPLC instrumentation if necessary.

Table 2. UPLC and HPLC retention times for benzodiazepines and z-drugs.

	Compound	RT-UPLC	RT-HPLC
1	N-desmethyl zopiclone	1.07	1.98
2	Zopiclone	1.13	2.05
3	Zolpidem	1.62	2.58
4	7-aminoclonazepam	1.92	3.05
5	Flurazepam	2.32	3.37
6	7-aminoflunitrazepam	2.36	3.55
7	Chlordiazepoxide	2.35	3.39
8	Midazolam	2.53	3.57
9	α–OH-midazolam	2.91	3.98
10	α–OH-triazolam	3.78	4.95
11	α–OH-alprazolam	3.77	4.93
12	Oxazepam[1]	3.84	5.16
13	Nitrazepam	3.87	5.28
14	Lorazepam	4.01	5.32
15	Clonazepam	4.10	5.51
16	Alprazolam	4.35	5.52
17	Nordiazepam	4.36	5.78
18	Flunitrazepam	4.41	5.90
19	Temazepam	4.45	5.89
20	Triazolam	4.47	5.65
21	Diazepam	5.14	6.69

RECOVERY AND MATRIX EFFECTS

Figure 4 shows the composite extraction recoveries of the entire panel of compounds from four separate experiments. Recoveries ranged from 76 to 102% with an average of 91%, demonstrating excellent extraction efficiency. The recoveries were consistent as well, with coefficients of variation (%CVs) ranging from 5.2% to 15%, with a mean of 8.6%. The extraction method was modified from a traditional MCX method for basic compounds. The first wash step was modified from aqueous 2% formic acid to 0.02 N HCl to account for the low pKas of compounds such as clonazepam, flunitrazepam, and alprazolam and ensure ion-exchange retention on the MCX sorbent. A series of experiments performed during method development revealed that more than 20% methanol in the wash step resulted in loss of the acidic benzodiazapines, such as oxazepam, lorazepam, and temazepam. Thus, the second wash step consisted of 20% methanol, the strongest organic wash possible that did not result in analyte loss during the wash step. These modifications maximized reversed-phase and ion-exchange retention and enabled the highly efficient and most selective extraction of the entire panel of benzodiazepines.

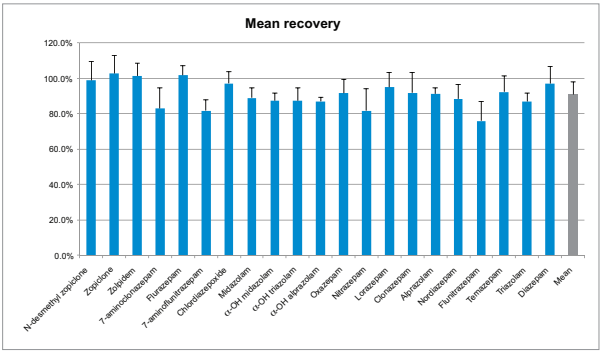

Figure 4. Extraction recovery for the compounds in this application. Values represent the mean of four individual extractions. Recoveries ranged from 76%–102.5% with an average recovery of 91%. Direct loading of the sorbent, without conditioning and equilibration had no impact on analyte recovery.

Two key benefits of this method take advantage of the water-wettable nature of the Oasis sorbent, the ability to directly load without conditioning and equilibration, and the ability to conduct all hydrolysis and pretreatment within the well of the SPE plate. The traditional six-step mixed-mode SPE method was simplified into just four steps. This was accomplished by eliminating the conditioning and equilibration steps. This simplification had no effect on the extraction efficiency of the method (data not shown), and is consistent with the water wettable nature of the Oasis sorbent. This also enables all sample hydrolysis and pretreatment to be performed within the wells of the 96-well plate, eliminating the need to transfer the sample from an incubation vessel to the SPE plate, a step that can be time consuming and error prone. After incubation within the wells of the Oasis MCX µElution Plate, the samples were simply mixed with 4% H_3PO_4 to quench the hydrolysis reaction and ionize the basic benzodiazepines, which were then drawn directly onto the sorbent. No leakage or well blockages were seen in any of the method development or validation experiments. Overall, this method reduces the number of post-incubation steps from nine to five by eliminating conditioning, equilibration, the transfer of samples to the SPE device, and sample evaporation compared to a traditional SPE workflow.

Matrix effects are shown in Figure 5. As with analyte recoveries, matrix effects were equivalent between the direct loaded samples and those in which the sorbent was conditioned and equilibrated. Matrix effects were also compared to traditional reversed phase extraction with Oasis PRiME HLB. Absolute matrix effects were 17.7% for Oasis MCX µElution plate prepared samples vs. 25.3% for Oasis PRiME HLB prepared samples (data not shown), demonstrating the superior cleanup of mixed-mode SPE for this group of analytes.

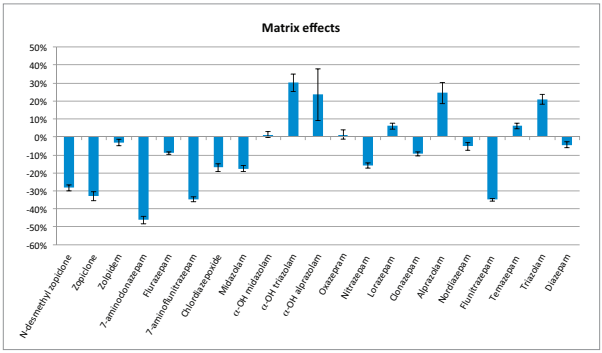

Figure 5. Matrix effects for benzodiazepines. Absolute matrix effects were reduced from 25.3% to 17.7% by using Oasis MCX mixed-mode SPE Plates vs. reversed-phase sorbent (Oasis HLB).

QUANTITATIVE RESULTS

Calibration curves ranged from 0.5 ng/mL through 500 ng/mL for all compounds. All compounds had LOQs of 0.5 ng/mL and ULOQs of 500 ng/mL. Quality control samples were prepared at 1.5, 7.5, 75, and 300 ng/mL. A calibration summary is shown in Table 3. Six of the curves were fitted with a 1/x weighted linear curve, while 15 were best fit with a 1/x weighted quadratic curve. Figure 6 shows examples of compounds best fit with a linear curve (nitrazepam, alprazolam), and a quadratic fit curve (diazepam, 7-aminoclonazepam). Regardless of the function used, fits were excellent and fit for purpose for the analytical needs of the method. Seventeen compounds had R^2 values of 0.999 or greater, and the remaining compounds had R^2 values of 0.997 or greater. Table 3 also shows that the mean % deviations for all compounds were less than 10%. Additionally, Tables 4 and 5 show the results of within-batch and between-batch QC results. The within-batch results show both excellent accuracy and precision. The mean accuracies for all compounds at the four QC levels were 107.8%, 98.5%, 97.5%, and 97.5%. For the highest three QC values (7.5, 75, and 300 ng/mL) all individual accuracies were within 10% of target values and all %CVs were less than 10%. The between-batch results shown in Table 5 were, if anything, even better. Mean accuracies were 102.1%, 99.3%, 98.2%, and 96.8% at the four QC levels. Individual CVs ranged from 1.1% to 9.0%. These high levels of accuracy and precision demonstrate the consistency and reliability of the Oasis MCX sorbent and extraction technique, and demonstrate that there is no compromise of result quality, even with the in-well hydrolysis and direct sorbent loading used in this assay. They also show that the quadratic curves used are fit for purpose and meet the needs of the assay.

Table 3. Calibration summary for all compounds in this application. The mean %deviation refers to the average of the absolute value of the deviations of all points in the curve.

Name	R^2	Lin/Quad	Mean %Dev
N-desmethyl zopiclone	0.999	L	5.4
Zopiclone	0.998	L	5.4
Zolpidem	0.999	Q	4.1
7-aminoclonazepam	1.000	Q	2.3
Flurazepam	0.998	Q	4.1
7-aminoflunitrazepam	0.997	L	6.2
Chlordiazepoxide	1.000	Q	3.4
Midazolam	1.000	Q	4.8
α-OH midazolam	0.999	Q	4.0
α-OH triazolam	1.000	Q	4.4
α-OH alprazolam	0.999	Q	9.0
Oxazepam	1.000	Q	6.2
Nitrazepam	0.999	L	4.6
Lorazepam	0.999	Q	4.4
Clonazepam	1.000	Q	6.2
Alprazolam	0.998	L	9.9
Nordiazepam	0.999	Q	6.6
Flunitrazepam	0.999	L	3.9
Temazepam	0.999	Q	5.3
Triazolam	0.999	Q	4.1
Diazepam	0.999	Q	3.7

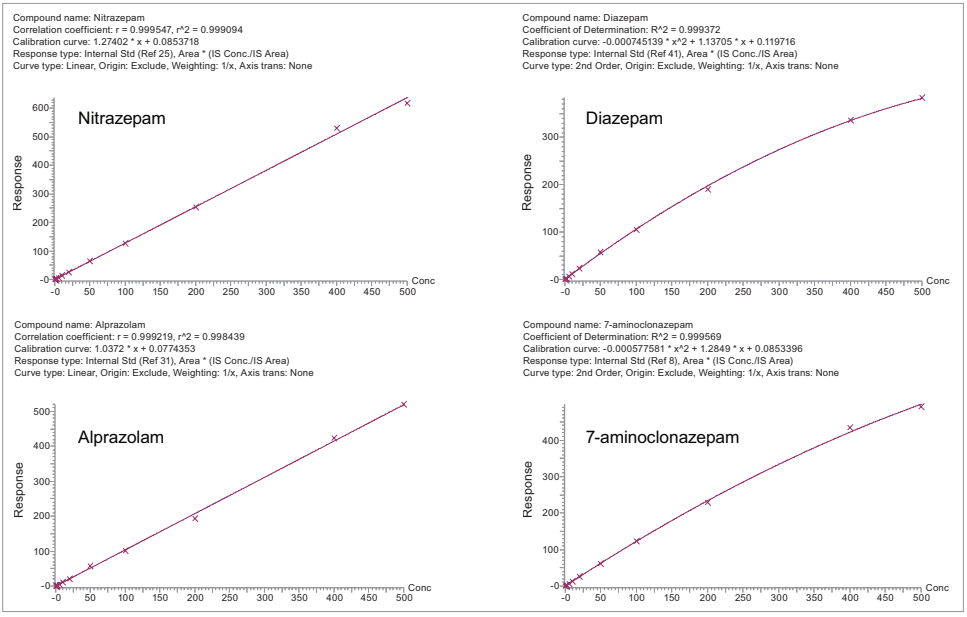

Figure 6. Representative calibration curves of benzodiazepines. Nitraepam and alprazolam were fit with a 1/x linear curve, while diazepam and 7-aminoclonazepam were best fit with a quadratic 1/x weighted curve.

Table 4. Within-batch QC results. N=6. Mean values show the average for each compound and the average for all compounds at each QC level.

	QC 1.5		QC 7.5		QC 75		QC 300		
Name	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**
N-desmethyl zopiclone	103.6%	7.3%	99.3%	3.1%	99.8%	2.0%	98.2%	3.9%	**100.2%**
Zopiclone	101.4%	7.4%	100.6%	3.2%	100.9%	2.3%	98.1%	4.0%	**100.3%**
Zolpidem	102.7%	6.7%	100.1%	2.5%	96.8%	0.9%	93.4%	4.2%	**98.2%**
7-aminoclonazepam	102.3%	8.1%	96.5%	2.6%	95.4%	1.0%	96.8%	3.5%	**97.8%**
Flurazepam	111.0%	9.0%	95.8%	4.5%	96.0%	2.1%	99.2%	4.7%	**100.5%**
7-aminoflunitrazepam	101.9%	10.9%	95.8%	4.5%	98.4%	1.7%	97.5%	3.6%	**98.4%**
Chlordiazepoxide	100.7%	9.5%	97.8%	4.2%	98.5%	1.0%	100.3%	6.1%	**99.3%**
Midazolam	107.0%	9.9%	98.3%	2.3%	98.6%	2.3%	99.4%	2.8%	**100.8%**
α-OH midazolam	107.4%	8.1%	99.5%	2.3%	99.0%	1.6%	101.1%	3.8%	**101.8%**
α-OH triazolam	109.9%	9.2%	95.1%	2.3%	93.1%	1.6%	94.5%	5.4%	**98.1%**
α-OH alprazolam	114.5%	12.6%	98.9%	5.2%	94.1%	4.5%	95.4%	8.3%	**100.7%**
Oxazepam	105.4%	6.3%	94.6%	3.2%	96.9%	1.4%	95.6%	3.1%	**98.1%**
Nitrazepam	108.8%	7.7%	96.8%	2.6%	97.0%	0.8%	98.2%	3.5%	**100.2%**
Lorazepam	107.0%	7.2%	95.5%	2.0%	96.1%	2.0%	97.4%	4.0%	**99.0%**
Clonazepam	106.7%	10.6%	97.2%	3.0%	95.4%	2.0%	94.6%	3.8%	**98.4%**
Alprazolam	116.8%	10.0%	99.3%	5.7%	98.7%	4.4%	101.3%	6.1%	**104.0%**
Nordiazepam	110.9%	10.1%	103.2%	2.4%	99.2%	1.6%	96.3%	3.0%	**102.4%**
Flunitrazepam	111.1%	8.2%	101.4%	2.4%	97.2%	1.9%	100.7%	4.3%	**102.6%**
Temazepam	110.6%	8.0%	102.8%	2.7%	98.5%	1.4%	95.4%	6.0%	**101.8%**
Triazolam	113.6%	8.4%	103.4%	2.5%	101.1%	2.4%	99.4%	1.8%	**104.4%**
Diazepam	110.3%	7.9%	101.5%	2.3%	97.3%	0.8%	95.3%	3.6%	**101.1%**
Mean	**107.8%**		**98.7%**		**97.5%**		**97.5%**		

Table 5. Between-batch QC results. Values represent the mean and %CV of four separate extraction batches. Mean values show the average for each compound and the average for all compounds at each QC level.

	QC 1.5		QC 7.5		QC 75		QC 300		
Name	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**
N-desmethyl zopiclone	99.2%	3.8%	96.7%	2.4%	96.6%	2.9%	97.1%	4.7%	**97.4%**
Zopiclone	97.7%	3.2%	96.7%	3.4%	98.0%	2.8%	96.2%	3.5%	**97.2%**
Zolpidem	99.4%	3.4%	98.8%	1.5%	95.8%	1.1%	91.7%	1.6%	**96.4%**
7-aminoclonazepam	100.4%	1.9%	95.6%	1.0%	93.8%	2.4%	95.1%	2.0%	**96.2%**
Flurazepam	103.6%	7.1%	97.6%	4.3%	99.3%	7.4%	97.6%	5.0%	**99.5%**
7-aminoflunitrazepam	99.3%	2.3%	93.7%	2.3%	96.1%	4.7%	97.0%	3.2%	**96.5%**
Chlordiazepoxide	100.5%	1.1%	100.3%	2.1%	99.3%	1.5%	98.4%	3.2%	**99.6%**
Midazolam	103.7%	4.4%	104.2%	5.4%	102.1%	3.1%	98.9%	2.0%	**102.2%**
α-OH midazolam	103.4%	4.3%	102.5%	4.7%	100.8%	5.0%	99.1%	2.5%	**101.4%**
α-OH triazolam	101.5%	8.4%	98.8%	4.9%	98.3%	4.9%	95.1%	2.6%	**98.4%**
α-OH alprazolam	104.4%	9.6%	101.4%	2.2%	99.1%	5.9%	97.7%	2.4%	**100.7%**
Oxazepam	100.4%	4.3%	98.5%	4.1%	98.2%	4.7%	97.6%	4.6%	**98.7%**
Nitrazepam	102.0%	6.2%	95.8%	1.3%	95.7%	2.4%	98.1%	1.8%	**97.9%**
Lorazepam	100.3%	6.9%	100.2%	4.2%	100.8%	5.4%	98.7%	4.9%	**100.0%**
Clonazepam	102.0%	4.9%	98.2%	3.0%	97.5%	3.3%	95.2%	4.5%	**98.2%**
Alprazolam	107.0%	8.7%	94.6%	4.7%	95.0%	4.6%	98.8%	4.5%	**98.9%**
Nordiazepam	106.1%	9.0%	106.7%	3.7%	101.7%	4.6%	95.4%	5.2%	**102.5%**
Flunitrazepam	101.8%	8.1%	98.2%	2.8%	96.3%	2.6%	96.3%	7.8%	**98.1%**
Temazepam	102.9%	7.3%	101.6%	1.2%	97.5%	2.8%	94.7%	1.8%	**99.2%**
Triazolam	104.4%	8.4%	102.4%	2.3%	99.9%	3.2%	98.2%	3.4%	**101.2%**
Diazepam	104.3%	6.5%	103.8%	2.1%	99.6%	4.1%	94.9%	7.6%	**100.6%**
Mean	**102.1%**		**99.3%**		**98.2%**		**96.8%**		

CONCLUSIONS

This application note describes a rapid and simplified solid phase extraction protocol and LC-MS/MS method for the analysis of urinary benzodiazepines and metabolites for forensic toxicology use. The unique water wettable nature of the Oasis MCX sorbent enables the elimination of the common conditioning and equilibration steps without any loss in recovery or reproducibility. This property of Oasis also enables the entire hydrolysis step to be conducted within the wells of the Oasis MCX µElution plate, eliminating time consuming and error-prone transfer steps, reducing the total number of post-incubation steps from nine to five. This extraction procedure combining the chromatography of the CORTECS UPLC C_{18}+ Column and the sensitive and reproducible quantification of the Xevo TQ-S micro results in a rapid and efficient analysis method that is also exceptionally accurate. This method is simpler, faster, and easier than liquid-liquid extraction. It is also cleaner than reversed-phase SPE while providing excellent sensitivity, accuracy, and precision for the analysis of this important class of compounds.

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Phosphatidylethanol (PEth) – A Direct Biomarker for Ethanol Abuse Analyzed with UPLC-MS/MS for Forensic Toxicology

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The ACQUITY UPLC I-Class/Xevo TQD System allows analytically sensitive detection of PEth in whole blood for forensic toxicology analysis.

GOAL

Develop an improved chromatographic method that enables the analytically sensitive measurement of PEth 16:0/18:1 in whole blood, using Waters® ACQUITY® UPLC® I-Class/Xevo® TQD and compare a new sample preparation method to a widely used liquid:liquid extraction.

BACKGROUND

As the legal consequences of alcohol abuse can have significant and far-reaching implications, specific and reliable biomarkers are needed. PEth comprises a group of abnormal phospholipids that are enzymatically formed in cell membranes only in the presence of ethanol. The determination of PEth in blood is attractive owing to high specificity, as formation is reported to correlate with ingested ethanol dose.[1] PEth can also be used to distinguish drinking patterns and behaviors, e.g., to identify moderate or excessive drinkers, or to identify episodes of binge drinking.[2]

A number of LC-MS/MS methods for PEth have been described, but some aspects of the procedure can be challenging, e.g., for the determination of PEth in blood,

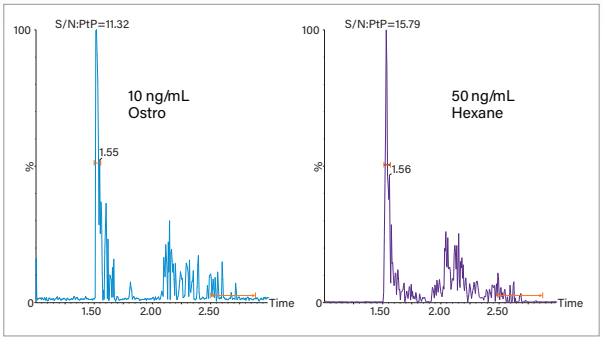

Figure 1. Signal:noise ratios (peak to peak) for PEth 16:0/18:1 whole blood calibrators, prepared by Ostro Protein Precipitation & Phospholipid Removal Plate and by LLE using hexane.

sample preparation procedures using liquid:liquid extraction (LLE) have been reported but recoveries can vary. Furthermore, the lipophilicity of the molecule can result in poor chromatographic peak shape and/or high background responses. All of these issues can affect overall sensitivity of the assay and robustness.

THE SOLUTION

Here we describe a procedure to measure the predominant species PEth 16:0/18:1 in human whole blood using an alternative sample preparation method with the Ostro™ Protein Precipitation & Phospholipid Removal Plate.

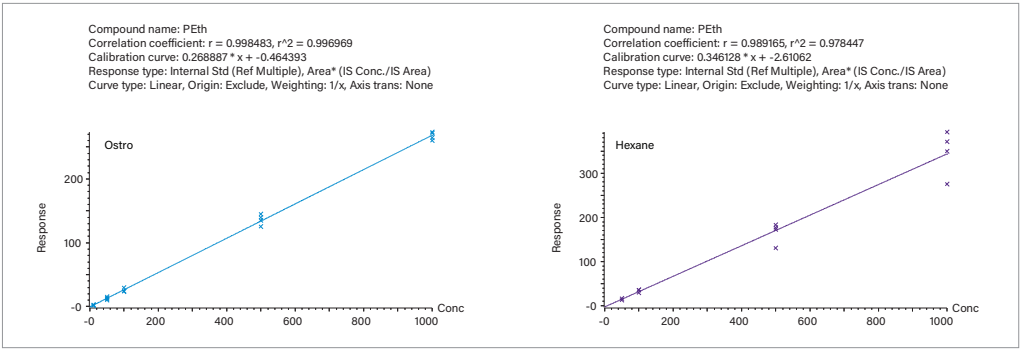

Figure 2. Calibration (ng/mL) lines for PEth 16:0/18:1 in whole blood following extraction using Ostro Plate (left) and by LLE using hexane (right). For each concentration, four different blank human blood samples were spiked and extracted. Peak-area responses for PEth were calculated relative to the response for the IStd. (d_5-PEth 16:0/18:1).

EXPERIMENTAL

Materials

PEth 16:0/18:1 was obtained from Sigma-Aldrich (UK). The deuterated analogue d_5-PEth 16:0/18:1, was used as an internal standard (IStd) and was from RedHot Diagnostics AB (Sweden). A working solution of IStd was prepared, at a concentration of 1 µg/mL, in acetonitrile.

Sample preparation

Thirty microlitres of IStd were added to a vial with 30 µL whole blood (calibrator or sample) and 600 µL of acetonitrile/1% formic acid, and extracted for 20 minutes at room temperature. The extract was applied to an Ostro Protein Precipitation & Phospholipid Removal Plate (P/N 186005518), washed with acetonitrile/1% formic acid, and eluted twice with dichloromethane/methanol (1/1, v/v). The pooled eluate was dried under a stream of nitrogen. Following reconstitution in 300 µL mobile phase A, samples were transferred to UPLC Total Recovery Vials (P/N 186000384C) before analysis of 2 µL using an ACQUITY UPLC I-Class (FTN)/Xevo TQD System.

UPLC-MS/MS analysis

Separation was achieved using a CORTECS® UPLC C_8 Column (P/N 186008399) maintained at 35 °C and eluted with a gradient comprising ammonium acetate buffer (mobile phase A) and acetonitrile (mobile phase B). An extended washing step was included at the end of the run to provide robustness and to avoid use of strong additives, such as tetrahydrofuran, which have been used to prevent carryover in methods with shorter run times. The following MRM transitions were monitored in electrospray negative (ES-): PEth 16:0/18:1, *m/z* 701.5 > 255.3 (quantifier) and 281.3 (qualifier), d_5-PEth 16:0/18:1, *m/z* 706.5 > 255.3.

RESULTS

Figure 1 shows a comparison of the responses for a calibrator prepared using the described Ostro Plate extraction protocol and a calibrator prepared by LLE (hexane). The lower limit of quantification (LLOQ) for PEth (based on a minimum signal:noise ratio of 5:1 for the qualifier transition) was estimated at 10 ng/mL for the Ostro Plate procedure and 50 ng/mL for LLE.

Figure 2 shows the calibration lines for four blood samples per concentration prepared by both techniques; even with this small sample set, better reproducibility was observed with the Ostro Plate.

Carryover was evaluated by monitoring the response obtained for blank samples injected immediately following the analysis of high concentrations (3000 ng/mL) of PEth – no carryover was observed for either extraction method.

Preparation time for a batch of 24 samples was significantly shorter (1 hour *vs.* 3 hours) with the Ostro Plate procedure owing to the requirement for additional transfer steps (vial to glass tubes) and larger volumes to dry down with the LLE method.

SUMMARY

The use of the Ostro Protein Precipitation & Phospholipid Removal Plate as trapping material provided cleaner extracts, a lower LLOQ, and improved reproducibility. Sample preparation time was significantly shorter compared with a commonly-used LLE procedure, the Ostro Plate format also offers potential for automation. The use of the ACQUITY UPLC I-Class/Xevo TQD System allows analytically sensitive detection of PEth in whole blood for forensic toxicology analysis. The total run time was 6.3 minutes and included an extended washing step to eliminate the use of strong additives to prevent carryover.

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Incorporation of Tetrahydrocannabinol into a Mixed Drug Substances Panel – Application to Three Commercially Available Oral Fluid Collection Devices

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GOAL

To develop a single sample preparation method, using Waters® Oasis® MCX 96-well µElution™ Plate, for the analysis of a mixed drug panel containing Tetrahydrocannabinol (THC) that can be applied to different oral fluid collection devices.

BACKGROUND

The requirement to analyze drugs at low levels in oral fluid has become an important requirement for many forensic laboratories around the world. Many different collection devices are commercially available which offer a simple way of collecting oral fluid samples in a non-invasive, yet supervised, manner. These devices provide the laboratory with either neat oral fluid or oral fluid that has been diluted in a variety of different preservative buffers. The lack of standardization for collection devices highlights the need for a sample preparation strategy suitable for all analytes that can be used with all the commonly available devices. The most commonly detected drug in these schemes is THC, however many other drug classes such as opiates, opioids, amphetamines, and benzodiazepines must also be measured, and as sample volume can be limited, this has to be performed using a single sample preparation method. Oasis MCX µElution offers the ability to extract all of these analytes from limited volumes of this complex matrix with sufficient efficiency to meet current guidelines such as those applied by the European Workplace Drug Testing Society (EWDTS).[1]

Inclusion of THC into an oral fluid mixed analyte panel using a single sample preparation method.

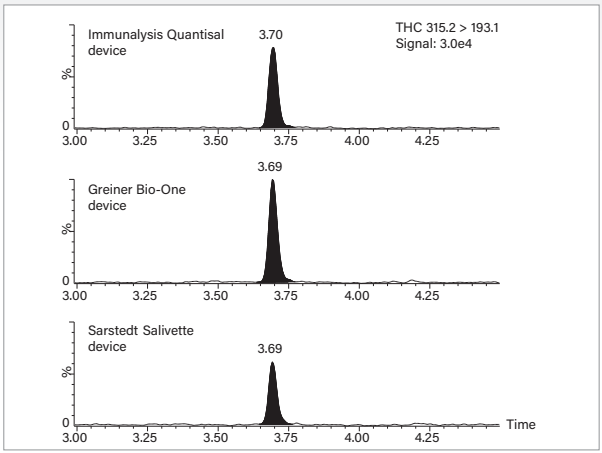

Figure 1. Chromatograms showing the quantifier MRM transition for THC spiked into oral fluid collected by the three different collection devices. The samples were prepared to give a concentration equivalent to 1 ng/mL in neat oral fluid.

THE SOLUTION

The oral fluid collection devices tested in this study were the Salivette® saliva collection device from Sarstedt, the Quantisal™ Oral Fluid Collection Device from Immunalysis, and the Saliva Collection System from Greiner Bio-One. Control oral fluid samples were collected as per the manufacturer's instructions and spiked with a mixture of 27 illicit or prescription substances commonly measured in oral fluid.

The analytes were extracted from the matrix using a simple Oasis MCX 96-well µElution Plate (P/N 186001830BA) protocol, based on previously reported methods.[2,3] The volume of sample loaded onto the plate for each collection device equated to the same volume (<75 µL) of neat oral fluid. The data was collected using a dual transition MRM method (quantifier and qualifier ions for each analyte) and processed using the TargetLynx™ Application Manager.

The chemical properties of THC are very different to the other analytes in the panel, and as such, THC requires a different chromatographic gradient to be able to meet the 2 ng/mL cut-off for confirmation tests recommended by the EWDTS guidelines for oral fluid analysis, as seen in Figure 1.

The eluant from the Oasis MCX µElution Plate was split into two equal aliquots in a 96-well Sample Collection Plate; 700 µL Round well (P/N 186005837). Following evaporation under nitrogen the separate aliquots were reconstituted in one of two alternative solvents. One aliquot was reconstituted in 50% acetonitrile and analyzed specifically for THC; the other aliquot was reconstituted in 5% acetonitrile containing 0.1% blank human plasma and used to determine all the other compounds. Figure 2 shows the chromatographic separation of all the analytes (except THC) scaled to the most intense.

To avoid the need for column switching, the two analytical methods were run on the same ACQUITY® UPLC® BEH C_{18} Column (P/N 186002352) using the same mobile phases, allowing for the methods to be run consecutively.

The high sensitivity Xevo® TQ-S micro mass spectrometer in conjunction with the ACQUITY UPLC I-Class System (FTN) is ideally suited to this application as the analyte concentrations in oral fluid can be relatively low in comparison to other biological matrices.

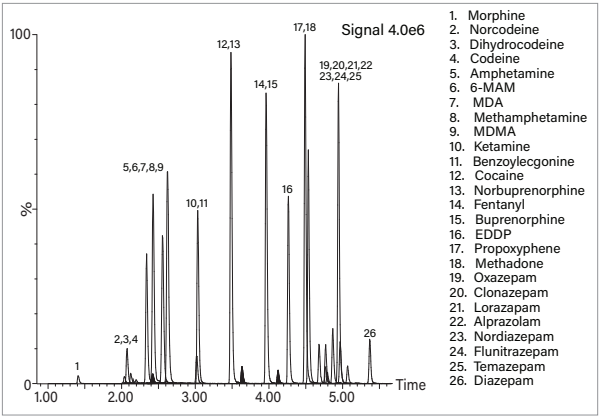

Figure 2. Chromatogram showing the quantifier MRM transitions for all analytes (except THC) spiked into oral fluid collected using the Immunalysis Quantisal Collection Device. The sample was prepared to give a concentration equivalent to 2 ng/mL in neat oral fluid. 6-MAM (peak #6), norbuprenorphine (peak #13), and buprenorphine (peak #15) are highlighted as compounds with EWDTS cut-off concentrations (≤2 ng/mL).

SUMMARY

The presence of multiple chemical classes with very different chemical properties in a drug panel, in combination with the limited sample volume that is often provided by oral fluid collection devices, creates a very challenging application. The use of a single Oasis MCX µElution sample preparation method and two UPLC-MS/MS methods allow for the analysis of a mixed drug panel, which includes THC, in less than 15 minutes and at concentrations which meet current guidelines such as those applied by the EWDTS. This sample preparation method can be easily automated to increase sample throughput.

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