[APPLICATION NOTE]

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Quantitative Analysis of THC and Metabolites in Urine With a Simple, Fast, and Clean Oasis PRiME HLB µElution Plate

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APPLICATION BENEFITS

- Faster, simplified sample preparation workflow
- Consistent recovery and minimal matrix effects
- No evaporation or reconstitution necessary
- Linear, accurate, and precise results for all analytes

WATERS SOLUTIONS

Oasis PRiME HLB 96-well µElution Plate (p/n 186008052)

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

ACQUITY UPLC® BEH C_{18} Column, 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186002350)

ACQUITY UPLC I-Class System

Xevo® TQ-S Mass Spectrometer

KEY WORDS

Δ-9-tetrahydrocannabinol (THC) and its metabolites, THC-COOH, THC-OH, urine, Oasis PRiME HLB, µElution plate, sample preparation, LC-MS

INTRODUCTION

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, which is designed to have some key advantages over traditional SPE sorbents. These include the ability to eliminate sorbent preconditioning and equilibration, allowing a more rapid workflow compared to traditional SPE products, and the ability to remove more interferences, resulting in a cleaner extracts and reducing the risk of short column lifetimes or MS source fouling.

This application note details the extraction and UPLC-MS/MS analysis of Δ-9-tetrahydrocannabinol (THC) and its metabolites, 11-hydroxy- Δ-9-THC (THC-OH) and 11-nor-9-Carboxy-Δ-9-THC (THC-COOH) from urine using Oasis PRiME µElution Plates. Δ-9-tetrahydrocannabinol (THC) is the main psychoactive element present in the plant Cannabis sativa. Quantitative analysis of these compounds in urine is an indicator of cannabis consumption, with high levels indicating recent and/or chronic use.

The use of Oasis PRiME resulted in consistent and highly reproducible recoveries of all compounds with minimal matrix effects. The µElution format allowed for the concentration of the sample on the SPE column, eliminating the need to evaporate and reconstitute the sample, minimizing the risk of analyte loss due to nonspecific binding and streamlining the laboratory workflow. This resulted in a method that was linear, accurate and precise for all analytes, with limits of quantification of 0.1 ng/mL for THC and its metabolites.

EXPERIMENTAL

Methods

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 of 1 µg/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. 80 µL of each working calibrator or QC standard was added to 1920 µL of human urine to make calibration curves and QC samples.

β-Glucoronidase from *E. Coli* K 12 was purchased from Roche Life Science (Indianapolis, IN)

Sample preparation

Glucuronide hydrolysis: 40 µL internal standards was added to 2 mL spiked human urine sample in a glass vial, then 2.4 mL 0.1 M potassium phosphate buffer (pH 6.8) containing 10 µL β-Glucoronidase was added. Vials were capped, vortex mixed, and incubated at 37 °C water bath for 16 hours. After allowing samples to cool down to room temperature, 150 µL of 10 M NaOH was added, vortex mixed and hydrolyzed in a dry heating block for 30 min at 70 °C. Once the samples had cooled, 850 µL glacial acetic acid was added to the samples and vortex mixed.

Solid-Phase Extraction with Oasis PRiME µElution Plate: 500 µL pretreated sample (equivalent to 180 µL urine) was directly applied to the Oasis PRiME µElution Plate. All wells of the SPE plate were then washed with 2 x 300 µL aliquots of 25% methanol. The samples were then eluted with 2 x 25 µL aliquots of 60:40 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.

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:

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

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.

LC conditions

LC system:	ACQUITY I-Class UPLC System
Column:	ACQUITY UPLC BEH C_{18} Column, 130Å, 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 vol.:	5 µL

The gradient ramp is shown in Table 1.

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.

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™

RESULTS AND DISCUSSION

Chromatography

Figure 2 shows chromatography of the three cannabinoids from an extracted calibrator at 2 ng/mL. All compounds eluted within 3 minutes with all peak widths were under 3 seconds at 5% of baseline. All peaks were symmetrical with symmetries between 0.95–1.15.

Table 2 lists the 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).

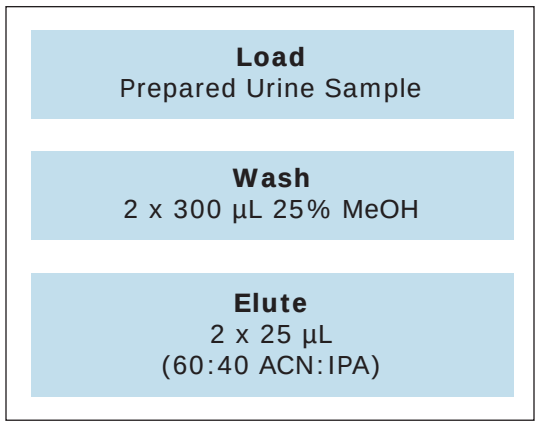

Figure 1. Oasis PRiME extraction methodology for urine THCs. With no conditioning and equilibration, sample extraction is simplified to just three steps.

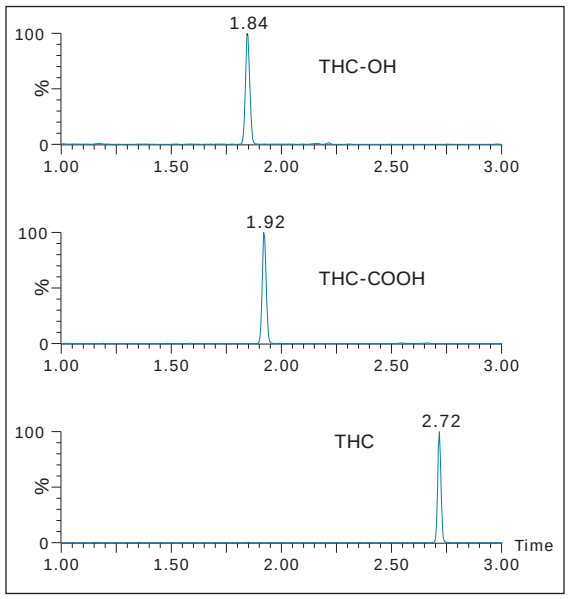

Figure 2. Chromatography of THC-OH, THC-COOH and THC from an extracted urine sample on the ACQUITY UPLC BEH C_{18} column, 1.8 µm; 2.1 x 50 mm. The concentrations are 4 ng/mL for all compounds.

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.

Recovery and matrix effects

Extraction recoveries were very consistent. As Figure 3 shows, recovery for THC-OH and THC-COOH was around 90% and THC was 60% with all %RSDs under 7.5%, demonstrating the reproducibility of Oasis PRiME. Matrix effects were minimal, at less than 15% for all compounds. Once again, the low standard deviations (7.5% or less) and high recoveries for THC-OH and THC-COOH 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 also provided better recovery, variability and matrix effects than LLE, with a more simplified procedure.[1]

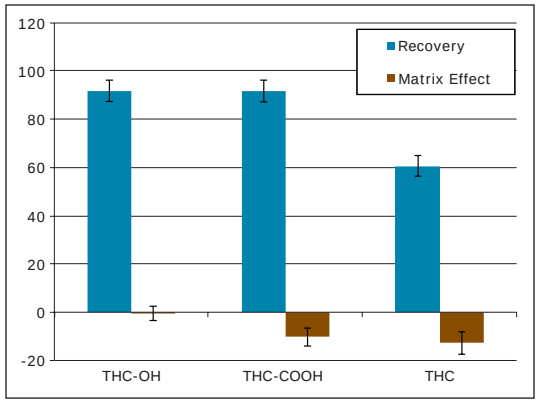

Figure 3. Recovery and matrix effects of THC-OH, THC-COOH, and THC after extraction using the Oasis PRiME µElution plate. %RSDs for extraction recovery were less than 5% for all compounds. Matrix effects were all within 20%.

Quantitative results

Calibration and quality control samples were prepared as previously described in the materials and method section. Calibration ranges were from 0.1–100.0 ng/mL for THC-COOH and THC-OH and 0.2–100.0 ng/mL for THC. Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges.

All compounds had linear responses over the entire calibration range with R^2 values of 0.99 or greater with 1/x weighting. Table 4 summarizes the data from the calibration curves. Lower limits of quantification (LLOQ) were 0.1 ng/mL for THC-COOH and THC-OH and 0.2 ng/mL for THC. In each case, all FDA recommendations for accuracy, precision, linearity and analytical sensitivity were met for validated methods.[2]

Quality control samples were accurate and precise. All results were within 15% of expected values and %RSDs were under 2% (N=6). This data can be seen in Table 5. The excellent accuracy and precision demonstrate the consistency and robustness of this sorbent.

	Recovery			Matrix effects	
	Mean	S.D.	%RSD	Mean	S.D.
THC-OH	91.9	4.5	4.9	-0.4	3.0
THC-COOH	91.5	4.5	4.9	-10.4	3.7
THC	60.6	4.5	7.4	-12.9	4.5

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

	R^2	Mean % dev.
THC-OH	0.997	2.8
THC-COOH	0.998	2.0
THC	0.998	1.2

Table 4. Calibration Curve Summary for THC and its metabolites with 1/x fit 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
0.75	0.66	88.6	1.7	0.76	100.8	1.4	0.72	96.3	0.4
7.5	6.70	89.3	1.3	7.37	98.3	1.3	7.15	95.3	1.2
75	73.4	97.9	1.8	73.6	98.2	0.7	75.5	100.7	0.8
Mean		91.7			99.7			97.4	

Table 5. Quality control results from extracted urine samples. (N=6 for each compound at all three levels).

CONCLUSIONS

Calibration and quality control samples were prepared as previously described in the materials and method section. Calibration ranges were from 0.1–100.0 ng/mL for THC-COOH and THC-OH and 0.2–100.0 ng/mL for THC. Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges.

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References

1. Lee, R., Traynor, A., LeCount, J., Wood, M., Quantitative analysis of 11-nor-carboxy Δ9-THC in urine using UPLC-MS/MS. Waters Application Note 720004808EN (2012).
2. Bansal, S., DeStefano, A., Key elements of bioanalytical method validation for small molecules. *The AAPS Journal* 9(1), E109-E114 (2007).

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[TECHNOLOGY BRIEF]

Oasis PRiME HLB Cartridge for Effective Cleanup of Meat Extracts Prior to Multi-Residue Veterinary Drug UPLC-MS Analysis

Michael S. Young and Kim Tran

GOAL

To demonstrate the effectiveness of the Oasis® PRiME HLB Cartridge for cleanup of meat extracts prior to UPLC®-MS analysis.

BACKGROUND

Waters has developed an optimized sample preparation and analysis protocol for multi-class, multi-residue LC-MS/MS screening of veterinary drug residues in meat. The major constituents of a typical meat sample are water (up to 70%), protein (15–25%), fat (5–25%) and phospholipid (1–3%). During the sample pre-extraction, the protein is removed from the extract by precipitation and centrifugation. However, significant amounts of fat and phospholipid are co-extracted along with the target veterinary drugs. The presence of these co-extracted substances can lead to interference in the LC-MS analysis, contamination of the analytical column and other components of the UPLC System, and contamination of the mass spectrometer itself. Fats have traditionally been removed from meat extracts using cumbersome hexane defatting steps or by the use of reversed-phase sorbents such as C_{18}- silica. Although these techniques may be effective for fat removal, neither of these procedures removes phospholipids.

Fats and phospholipids are significant potential instrument and column contaminants. Oasis PRiME HLB Cartridges provide a rapid cleanup to remove these substances from meat extracts prior to LC-MS analysis.

THE SOLUTION

Pass-through cleanup with the Oasis PRiME HLB Cartridge. This procedure is highly effective for removal of both fat and phospholipid from meat extracts. Just as important, the recoveries of the veterinary drugs are not compromised with Oasis PRiME HLB Cartridge cleanup. The recoveries are similar to those obtained using hexane defatting or C_{18}-silica cleanup but Oasis PRiME HLB Cartridge cleanup is more effective.

Experimental

Initial Extraction. Typical pork samples (5 g, 15% fat) were fortified with representative compounds chosen from major classes of veterinary drugs. The homogenized meat samples were extracted with 10 mL of 80:20 acetonitrile/water with 0.2% formic acid. The samples were vortexed for 30 seconds, shaken for 30 minutes, and then centrifuged at 12000 rpm for 5 minutes.

Oasis PRiME HLB Cartridge Cleanup. An Oasis PRiME HLB Cartridge (3 cc, 60 mg) was mounted on a pre-cleaned vacuum manifold. No cartridge conditioning is required or was performed. A 0.5 mL aliquot of the supernatant was passed-through the Oasis PRiME HLB Cartridge and collected. The collected sample was diluted three-fold with aqueous 10 mM ammonium formate buffer (pH 4.5) prior to UPLC-MS/MS analysis.

Results

Little or no recovery loss was observed in the pass-through cleanup step for any of the tested compounds. Absolute recoveries (measuring mostly the effectiveness of the initial liquid extraction) averaged over 80% for the tested compounds except for phenylbutazone (32%). These recoveries are consistent with C_{18}-silica cleanup but no phospholipids are removed with C_{18}-silica. UPLC-MS/MS conditions and chromatograms are presented in Figure 1. Figure 2 shows chromatograms that illustrate the effectiveness of the Oasis PRiME HLB Cartridge for phospholipid removal; greater than 90% more phospholipid is removed compared with C_{18}-silica cleanup. Using gravimetric analysis it was also determined that the Oasis PRiME HLB Cartridge removed more than 90% of the co-extracted fat from the pork extract.

UPLC Conditions

Instrument:	ACQUITY UPLC® I-Class
Column:	1.7 µm CSH C_{18}, 2.1 x 100 mm (p/n 186005297)
Column temp.:	30 °C
Sample temp.:	10 °C
Detection:	MS:TQ-S
Injection vol.:	5 µL for 3 cc and 6 cc
Run time (flow rate):	7 minutes (0.04 mL/min)
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	ACN with 0.1% formic acid
Gradient:	15% B initial to 40% B at 2.5 min, to 95% B at 3.9 min, hold to 4.9 min, back to 15% B at 5.0 min and hold to 7.0 min
Needle:	Standard needle for UPLC, 15
Weak wash (solvent 3 and 6 cc):	10/90 ACN:water
Strong wash (solvent 3 and 6 cc):	50/30/20 water:ACN:IPA
Seal wash:	10% MeOH

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Sulfamerazine	265.0	156.0	30	15
Enrofloxacin	360.2	316.1	50	25
Dexamethasone	393.2	355.3	30	15
Phenylbutazone	309.4	160.0	37	20
Penicillin	335.1	160.0	20	30
Chloramphenicol*	321.1	152.1	30	17
Oxytetracycline	461.2	426.2	30	21

*Chloramphenicol ESI-, others ESI+ ionization

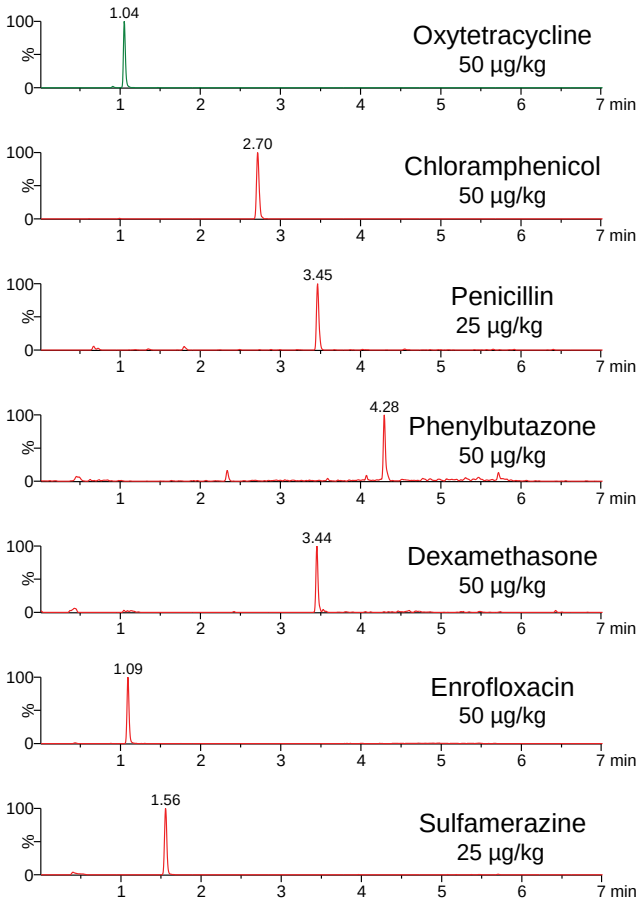

Figure 1. UPLC-MS/MS conditions and resulting chromatograms from a typical pork sample spiked at the levels indicated.

CONCLUSIONS

- Oasis PRiME HLB Cartridges did not require conditioning or equilibration prior to use; a simple one-step SPE cleanup was effective
- Oasis PRiME HLB Cartridges removed greater than 90% of fats and greater than 90% of phospholipids from acetonitrile based extracts of pork
- When used in the pass-through mode, Oasis PRiME HLB Cartridges did not affect the recovery of the test compounds but gave significant removal of fats and phospholipids from the extract

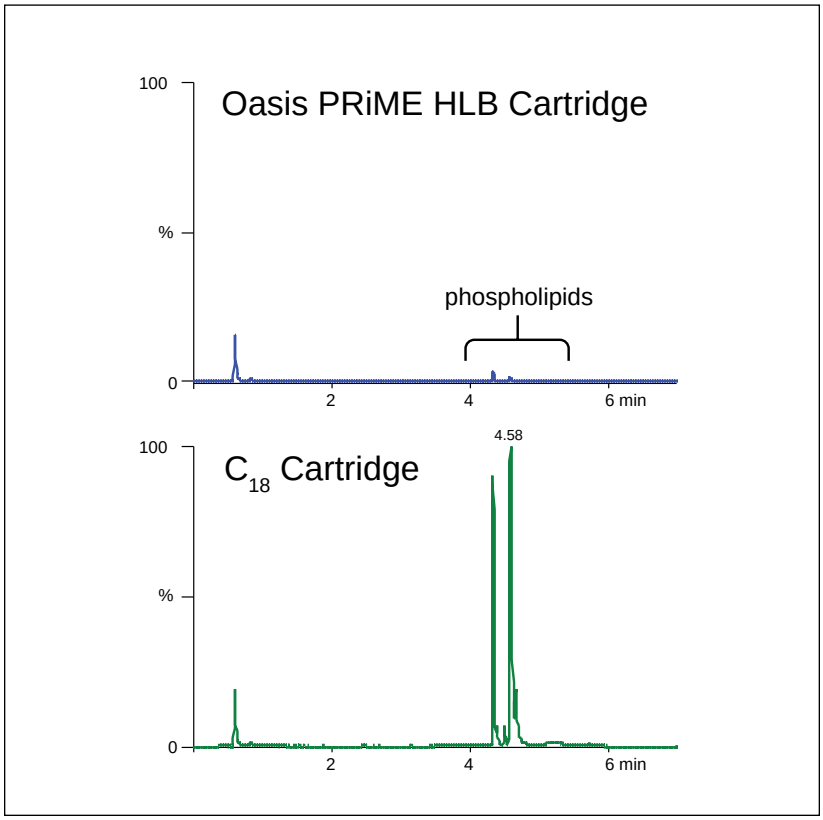

Figure 2. Removal of phospholipids from meat extracts comparing Oasis PRiME HLB cleanup (upper trace) with C_{18}-silica cleanup (lower trace).

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A Comprehensive Comparison of Solid Phase Extraction (SPE) vs. Solid Liquid Extraction (SLE) vs. Liquid Liquid Extraction (LLE) Sample Prep Techniques in Bioanalysis and Forensic Toxicology Analyses

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APPLICATION BENEFITS

- Provide a process and results comparison of different sample preparation techniques for bioanalysis and forensic toxicology
- Significantly faster extraction protocols compared to SLE and LLE
- Oasis® PRiME HLB shows higher analyte recoveries and improved matrix effects compared to SLE and LLE in plasma samples
- Oasis PRiME HLB resulted in higher analyte recoveries for polar bases than SLE and LLE in urine samples
- Flexible sample capacity options with Oasis PRiME HLB compared with SLE (rigid sample amount on specific SLE plate)

WATERS SOLUTIONS

Oasis PRiME HLB µElution plate

ACQUITY® UPLC® I-Class System

Xevo® TQ-S mass spectrometer

ACQUITY UPLC CORTECS® C_{18} Column

1 mL round collection plates

1 mL 96 well cap mat

TruView™ LCMS Certified Vial

KEYWORDS

Oasis PRiME HLB, SPE, Supported Liquid Extraction, SLE, liquid-liquid extraction, LLE, urine, plasma, bioanalysis, forensic toxicology

INTRODUCTION

Solid-phase extraction (SPE) is a sample preparation technique by which compounds that are dissolved or suspended in a liquid matrix are extracted according to their physical and chemical properties. Reversed phase SPE sorbents can be either polymeric or silica based. In both cases, compounds are retained on the sorbent mainly by hydrophobic interactions. A washing step helps to remove matrix interferences. The analyte(s) can be eluted with an organic solvent, which disrupts the interaction of the analyte and the sorbent.[1,2] Waters® Oasis PRiME HLB is a novel reversed phase SPE sorbent developed to enable simpler and faster SPE protocols, while at the same time generating cleaner extracts than other sample preparation methods with a simple, generic three step protocol.

Liquid-liquid extraction (LLE) employs water-immiscible solvents to extract analytes from aqueous solutions. This is usually accomplished by shaking and collecting the solvent layer containing the analytes of interest.

Supported liquid extraction (SLE, aka, solid supported liquid extraction SSLE) is analogous to traditional liquid-liquid extraction (LLE) and utilizes the same water-immiscible solvent systems for analyte extraction from aqueous solutions. Instead of shaking the two immiscible phases together as in LLE, in SLE, the aqueous sample is immobilized on an inert support, and the organic phase flows through the supported matrix to extract the targeted analytes.[3]

In this application note, a comparison was performed between Oasis PRiME HLB SPE, SLE, and LLE in both plasma and urine matrices for bioanalysis and forensic toxicology. In plasma, 22 commonly analyzed pharmaceuticals, steroids, and drugs of abuse were extracted using the three aforementioned methods and the results were compared. In urine, 23 drugs of abuse representing opioids, stimulants, benzodiazepines, and synthetic cannabinoid metabolites were tested for forensic toxicology analysis.

Key areas of comparison were: procedure simplicity, analyte recoveries, and matrix effects (ME). The mechanisms behind these three techniques and how they affect their respective performances are discussed as well. Oasis PRiME SPE shows very high and consistent recoveries and excellent matrix effects across all of the tested analytes in both matrices. For SLE and LLE, lower recoveries were observed for polar basic analytes in urine samples and acidic analytes in plasma samples. The LLE and SLE methods were then optimized for these specific compounds and improvements in the recoveries of problematic analytes were successfully achieved, but only at the expense of other analytes. Only Oasis PRiME HLB was able to successfully extract all analytes from plasma and urine samples with a single method.

MATERIALS

RCS-4 M10, RCS-4 M11, JWH-073 4-COOH, JWH-073 4-OH, and JWH-018 5-COOH were purchased from Cayman Chemical (Ann Arbor, MI). All other compounds and metabolites were purchased from Cerilliant (Round Rock, TX).

Individual stocks (1 mg/mL) were prepared in methanol, DMSO, or 50:50 DMSO:methanol. A combined stock solution of all compounds (5 µg/mL) was prepared in methanol, except naproxen, which was at 50 µg/mL. Working solutions were prepared daily by spiking standards into matrices (plasma and urine) and performing serial dilutions to achieve the desired concentrations.

In plasma, 22 drugs were analyzed including acids (naproxen), bases (most analytes), and neutrals (phenacetin, 17 α-OH progesterone) used in a variety of application areas. In urine, 23 drugs of abuse representing opioids, stimulants, benzodiazepines, and synthetic cannabinoid metabolites were tested.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC I-Class, (FL)
Column:	ACQUITY UPLC CORTECS C_{18} 2.1 × 100 mm, 1.6 µm
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Flow rate:	0.6 mL/min
Gradient:	See Table 1
Column temp.:	40 °C
Sample temp.:	10 °C
Strong needle wash:	70/30 ACN/water with 2% formic acid
Weak needle wash:	5/95 ACN/water with 1% formic acid
Injection mode:	Partial loop with needle overfill
Injection volume:	2–5 µL

Table 1. LC gradient.

Time (min)	Profile		Curve
	%A	%B	
0	95	5	
2.0	75	25	6
6.0	50	50	6
6.1	30	70	6
7.0	5	95	6
7.5	95	5	6
9.0	95	5	6

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI+
Capillary voltage:	3.0 kV
Desolvation temp.:	500 °C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	1000 L/Hr
MRM transition monitored:	See Table 2

Table 2. Drug functions, MRM transitions, cone voltages (Cone V), and collision energies (Coll. E) for test analytes.

Compound	Function	MRM	Cone V	Coll. E	Matrix
Phenacetin	Analgesic, fever reducer	180.1>110.1	26	20	Plasma
Propranolol	β-blocker	260.2>116.2	48	16	Plasma
Cortisol	Corticosteroid	363.2>121.1	42	22	Plasma
Protriptyline	Antidepressant	264.2>155.1	38	26	Plasma
Amirtriptyline	Antidepressant	278.2>91.0	44	22	Plasma
Naproxen	Analgesic, fever reducer	231.1>185.1	20	16	Plasma
17α-OH-progesterone	Steroid	331.2>97.1	58	26	Plasma
Cocaine	Stimulant	304.1>82.0	40	30	Plasma/urine
Fentanyl	Opiate/opioid	337.2>188.2	48	22	Plasma/urine
Oxazepam	Benzodiazepine	287.0>104.0	44	30	Plasma/urine
Benzoylecgonine (BZE)	Stimulant (Cocaine metabolite)	290.1>168.1	55	19	Plasma/urine
Clonazepam	Benzodiazepine	316.0>214.1	54	42	Plasma/urine
Lorazepam	Benzodiazepine	321.0>229.1	40	28	Plasma/urine
Alprazolam	Benzodiazepine	309.1>205.1	60	42	Plasma/urine
Flunitrazepam	Benzodiazepine	314.1>268.1	50	25	Plasma/urine
Temazepam	Benzodiazepine	301.1>177.1	36	46	Plasma/urine
Diazepam	Benzodiazepine	285.1>154.0	54	26	Plasma/urine
RCS-4, M10	Synthetic cannabinoid	324.2>121.0	40	36	Plasma/urine
RCS-4, M11	Synthetic cannabinoid	322.2>121.0	42	32	Plasma/urine
JWH-073, 4-COOH	Synthetic cannabinoid	358.2>155.1	52	32	Plasma/urine
JWH-073, 4-OH	Synthetic cannabinoid	344.2>155.1	52	32	Plasma/urine
JWH-018, 5-COOH	Synthetic cannabinoid	372.2>155.1	54	32	Plasma/urine
Amphetamine	Amine stimulant	136.0>119.0	22	8	Urine
MDEA	Amine stimulant	208.1>105.0	26	24	Urine
Methamphetamine	Amine stimulant	150.0>91.0	24	20	Urine
MDMA	Amine stimulant	194.1>163.0	26	12	Urine
MDA	Amine stimulant	180.1>163.0	22	11	Urine
Phentermine	Amine stimulant	150.0>91.0	24	20	Urine
Norfentanyl	Fentanyl metabolite	233.2>177.2	30	14	Urine
6-Acetylmorphine	Heroin metabolite	328.2>165.1	60	26	Urine

Sample preparation protocols

In this evaluation, the protocol used with Oasis PRiME HLB was the generic 3-step load-wash-elute protocol. Depending on the matrix, either 400 µL of plasma diluted 1:1 with 4% H_3PO_4 or 400 µL hydrolyzed urine diluted 1:1 with 4% H_3PO_4 was directly loaded onto an Oasis PRiME HLB µElution plate. No conditioning or equilibration was needed or performed for either matrix. The samples were then washed with 2 × 200 µL 5% MeOH and eluted with 2 × 25 µL 90:10 ACN:MeOH. The eluate was then diluted with 100 µL water, vortexed, and directly injected into the LC-MS system without evaporation or reconstitution.

For SLE, there are multiple dilution buffers (to dilute the biological sample for loading) and extraction solvents suggested depending on the analytes of interest. Since the aim of this work was to compare one single method targeting all compounds, we evaluated protocols with the highest likelihood of success. The protocols selected for this evaluation were designed for neutral and basic analytes as they are predominant in the mixture. For plasma samples, 400 µL diluted plasma (200 µL rat plasma + 200 µL water) was loaded into an SLE plate (obtained from a competitor, rigidly designed for 400 µL sample load). Loading was initiated by applying gentle vacuum (~ 3 psi) for 2–5 seconds and waiting 5 minutes for the sample to completely absorb onto the support matrix. To begin the extraction of the analytes, 800 µL of extraction solvent (MTBE: Methyl tert-butyl ether) was then applied and allowed to flow over the matrix for 5 minutes under gravity. Vacuum (10 psi) was applied again for 10–30 seconds to complete the elution. The extraction steps were then repeated by adding another 800 µL of MTBE. To ensure compatibility with LC-MS analysis and concentrate the analytes, the extract was evaporated to dryness under N_2 gas flow at 40 °C and then reconstituted in 200 µL of 30% acetonitrile (ACN). For urine samples, two similar pretreatment protocols were used. 200 µL hydrolyzed urine was diluted 1:1 with either water or 0.5 M NH_4OH. Samples were then loaded onto the SLE plate and processed as described above for plasma samples.

For LLE, the experiments were performed using single 2 mL centrifuge tubes. As LLE and SLE share a similar mechanism, similar protocols were applied. 1000 µL MTBE was added to either 400 diluted plasma or hydrolyzed urine for the LLE experiments. As with SLE, plasma samples were diluted with 200 µL water. 200 µL hydrolyzed urine samples were diluted with either 200 µL water or 200 µL of 0.5 M NH_4OH. The samples were then vortexed for 5 min and centrifuged for 5 min at 11000 rcf. The top layer was transferred to a collection plate and evaporated to dryness under N_2 gas flow at 40 °C and reconstituted in 200 µL of 30% acetonitrile (ACN).

Urine hydrolysis for all samples/techniques: 200 µL of spiked urine was mixed with 160 µL of water and 40 µL of β-glucuronidase enzyme (Roche, *E. coli*) at room temperature for 30 minutes to simulate enzymatic hydrolysis.

Recovery and matrix effect calculations

Analyte recovery was calculated according to the following equation:

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

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:

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

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 chromatogram of all compounds from a 20 ng/mL extracted plasma sample is shown in Figure 1. The urinary chromatography is shown in Figure 2. Using a CORTECS UPLC C_{18} Column (90Å, 1.6 µm, 2.1 x 100 mm), all analytes were analyzed within 6.5 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. All potentially interfering compounds such as methamphetamine and phentermine, which share an MRM transition (150>91) were baseline separated.

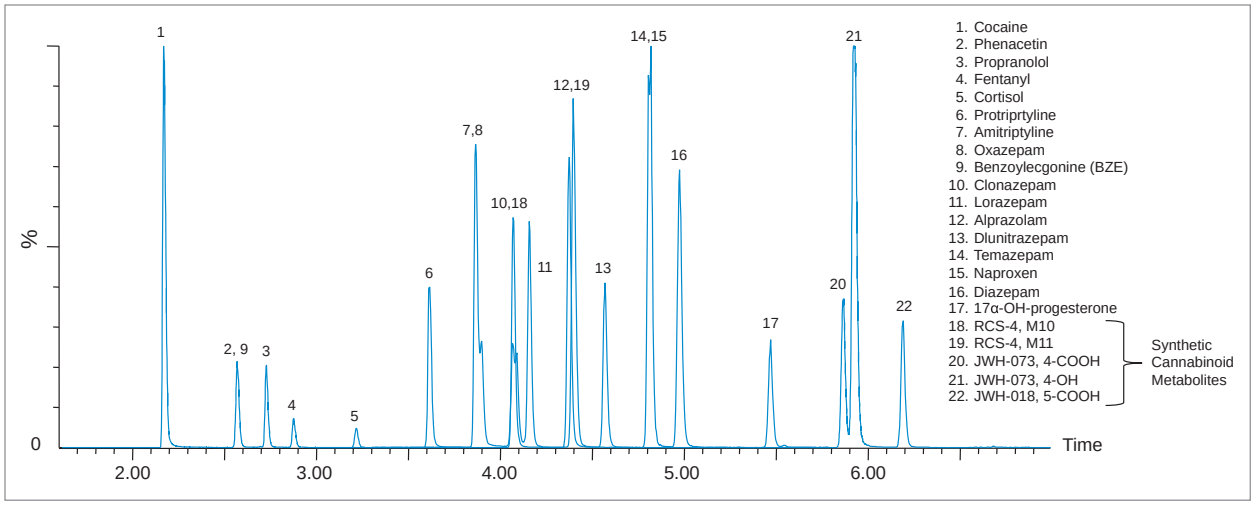

Figure 1. Chromatography of analytes in an extracted plasma sample. The LC gradient is shown in Table 1. MRM transitions for all compounds are listed in Table 2.

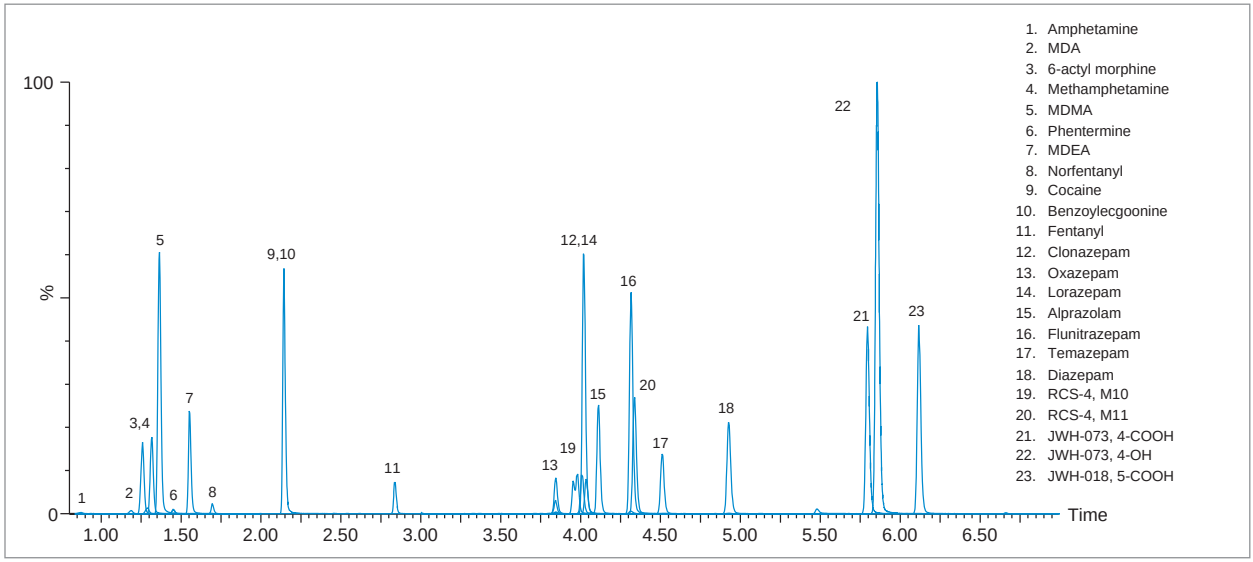

Figure 2. Chromatography of analytes in an extracted urine sample. The LC gradient is shown in Table 1. MRM transitions for all compounds are listed in Table 2.

Figure 3 details the extraction protocols and processing time for SPE, SLE, and LLE. The total time required to prepare 96 plasma samples is 15 minutes for Oasis PRiME HLB, 40 minutes for SLE, and 60 minutes for LLE. Oasis PRiME HLB uses a simple, generic three step SPE technique that removes salt, proteins, and phospholipids without the need for evaporation and reconstitution (in the µElution format), whereas SLE and LLE require method development with different sample pretreatment or extraction solvents for different classes of analytes. SLE requires a 5 minute waiting time after loading to allow the sample to fully adsorb onto the support matrix. In addition, an additional 5 minute waiting time is required after the extraction solvent is applied to allow the analytes to interact with the extraction solvent. Since a water-immiscible solvent is used in extraction step, evaporation and reconstitution are required for LC-MS analysis. In addition, the initiation of the flow in the SLE sample loading step, which is accomplished by applying very gentle vacuum (~3 psi) for 2–5 seconds, is very subtle and takes time and practice to perfect. If the initiation time is too short (shorter than 2–5 seconds) or the pressure is too low, the aqueous sample won't be able to successfully immobilize to the sorbent. If the time is too long or the pressure is too high, the plasma sample will directly elute and result in a cloudy elution solution and higher matrix factors. In SLE and LLE, the use of harsh water-immiscible extraction solvents may also extract impurities from the frits and plates, contaminating the extraction solution. Extraction solvents such as MTBE also have a negative impact on both operators' health and the environment.

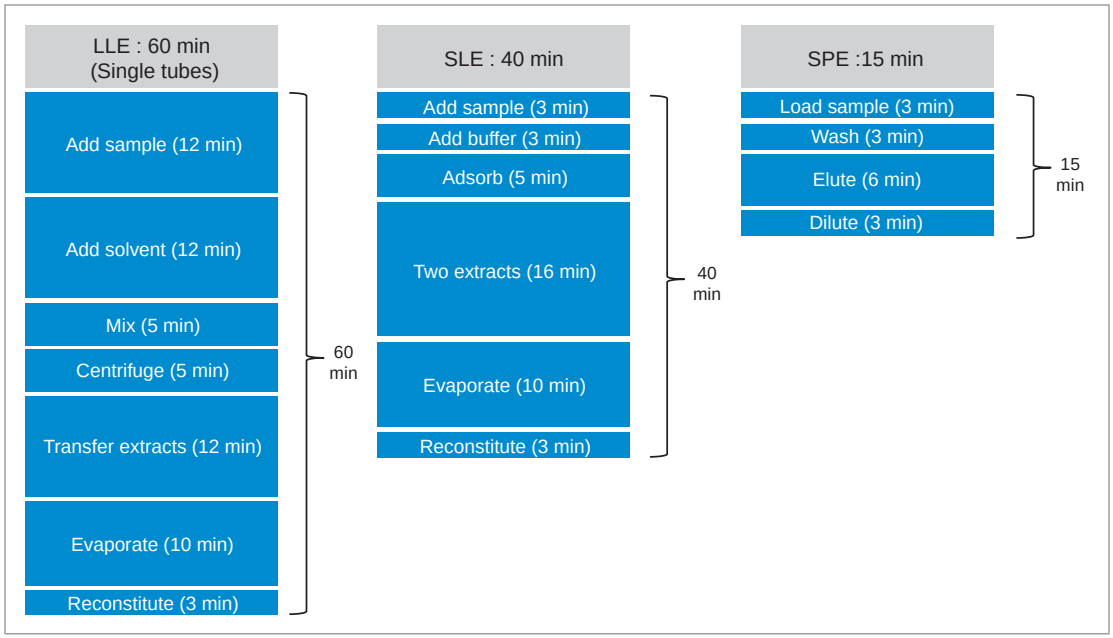

Figure 3. Protocols and processing times for LLE, SLE, and Oasis PRiME HLB extraction protocols.

PLASMA SAMPLES

With the simple three step SPE protocol, Oasis PRiME HLB demonstrates excellent and consistent recoveries across all the tested analytes (Figure 4A) with an average % recovery of 98±8%. All tested recoveries were within 75–110%. SLE showed good recoveries for neutral and basic drugs, but poor recoveries for acidic analytes such as naproxen and the COOH metabolite of the synthetic cannabinoid, JWH-073. Average recoveries were 89±7%. All analyte recoveries for LLE were lower than 80% with an average recovery at 70 ± 10%. Only one extraction was performed during the experiment, which may have resulted in decreased extraction efficiency. A second extraction may have increased recovery, but would also have increased processing time. Previous work has also indicated that additional extractions can contribute to increased matrix effects. For SLE and LLE, the extraction method was selected for neutral and basic analytes. Acidic analytes such as naproxen, JWH-073, 4-COOH, and JWH-018, 5-COOH were not recovered well at all (less than 30% recovery). Further method development or a separate protocol would be required for SLE or LLE to improve acidic analyte recovery such as different sample pretreatment or buffering. However, this could adversely affect the recovery of the basic drugs. Under these conditions, only Oasis PRiME HLB was able to extract the full complement of basic, neutral, and acidic compounds with a single protocol.

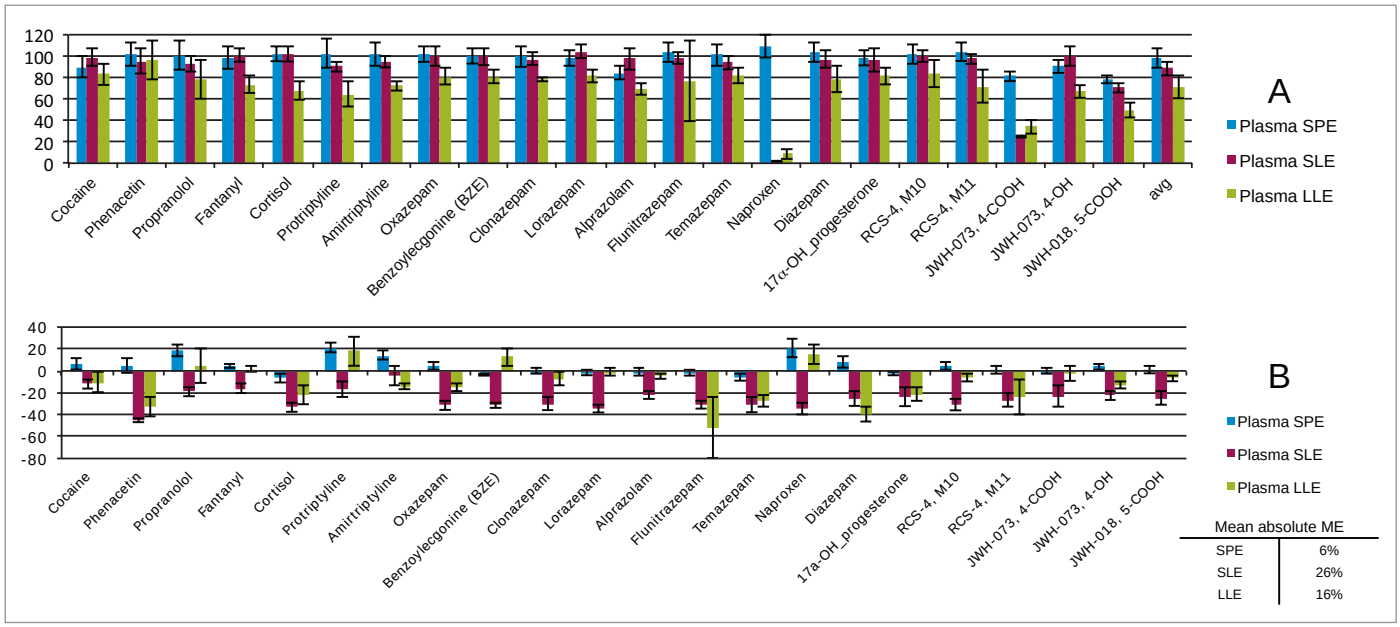

Figure 4A. Extraction recoveries for compounds in plasma samples (N=4). Blue, red, and green bars represent recoveries from Oasis PRiME HLB, SLE, and LLE, respectively. Error bars represent standard deviations. Figure 4B. Matrix effects for compounds extracted from plasma samples. Blue, red, and green bars represent mean matrix effects from Oasis PRiME HLB, SLE, and LLE, respectively. The means of the absolute values of matrix effects are listed on the lower right.

The overall matrix effects for Oasis PRiME HLB were lower than SLE or LLE (Figure 4B). All matrix effects for Oasis PRiME HLB were <20%, while 17/22 drugs from SLE and 7/22 drugs from LLE processing have MEs that are greater than 20%. The average magnitude of matrix effects for Oasis PRiME HLB was only 6%, while SLE was 26% and LLE was 16%. Furthermore, matrix effects for LLE were more variable. Matrix effect standard deviation values ranged from 1.4–8.8% for SPE, 1.9–10.3% for SLE and 2.6–28.3% for LLE. The three step protocol on Oasis PRiME HLB removed salts, proteins, and phospholipids and resulted in very clean final extracts with minimal matrix effects for all 22 different drugs, from several diverse classes. The higher matrix effects seen in SLE extracts may be a result of impurities extracted from the SLE sorbent as this wasn't seen in LLE extracts where the same sample and extraction solvent were used. Alternatively, it could also be simply a result of the more efficient extraction seen with SLE vs. LLE. Since LLE appears to be more effective at extracting the analytes from urine, it may also extract other components that could contribute to ion suppression.

Overall, Oasis PRiME HLB demonstrated superior recovery and minimal matrix effects when the sample matrix contains a wide variety of compounds. In this case, this included acids (naproxen and the synthetic cannabinoid metabolites), bases (most drugs), and neutral compounds of varying polarities. SLE yielded acceptable recoveries for neutral and basic analytes, but with much higher matrix effects. LLE, due to its limited extraction efficiency, had lower recoveries (10–20% lower in recoveries compared to SPE and SLE). LLE also demonstrated higher variability in matrix effects, particularly for compounds such as flunitrazepam and propranolol. Using an SLE or LLE extraction, acidic analytes can't be efficiently recovered with this single procedure, and additional method development would be required to improve acid recoveries.

URINE SAMPLES

A wide panel of 23 drugs of abuse which included stimulants, opioids, benzodiazepines, benzoylecgonine (BZE), and synthetic cannabinoid metabolites was hydrolyzed and extracted with SPE, SLE, and LLE. As shown in Figure 5, high and consistent recoveries were obtained using the Oasis PRiME HLB generic 3-step protocol. Recoveries were >75% for 21/23 tested drugs and the overall average recovery was 86% ± 6.6%. Two extraction protocols for SLE and LLE extractions were performed as described in the materials and methods section. When samples were diluted with water (Figure 5A) SLE showed good recoveries for many drugs with the exception of the hydrophilic bases such as most of the amine stimulants and norfentanyl (the recoveries were lower than 60%). LLE exhibited a similar trend to SLE with much lower recoveries. When SLE and LLE extractions were performed after adjusting the pH of the urine samples to 11 with 0.5 M ammonium hydroxide, recoveries of the polar amines improved significantly (Figure 5B). However, this was at the expense of the more acidic compounds such as the carboxy metabolites of JWH-073 and JWH-018. Unlike Oasis PRiME HLB, a single protocol for SLE or LLE was unable to extract all of the analytes from the samples with acceptable recovery.

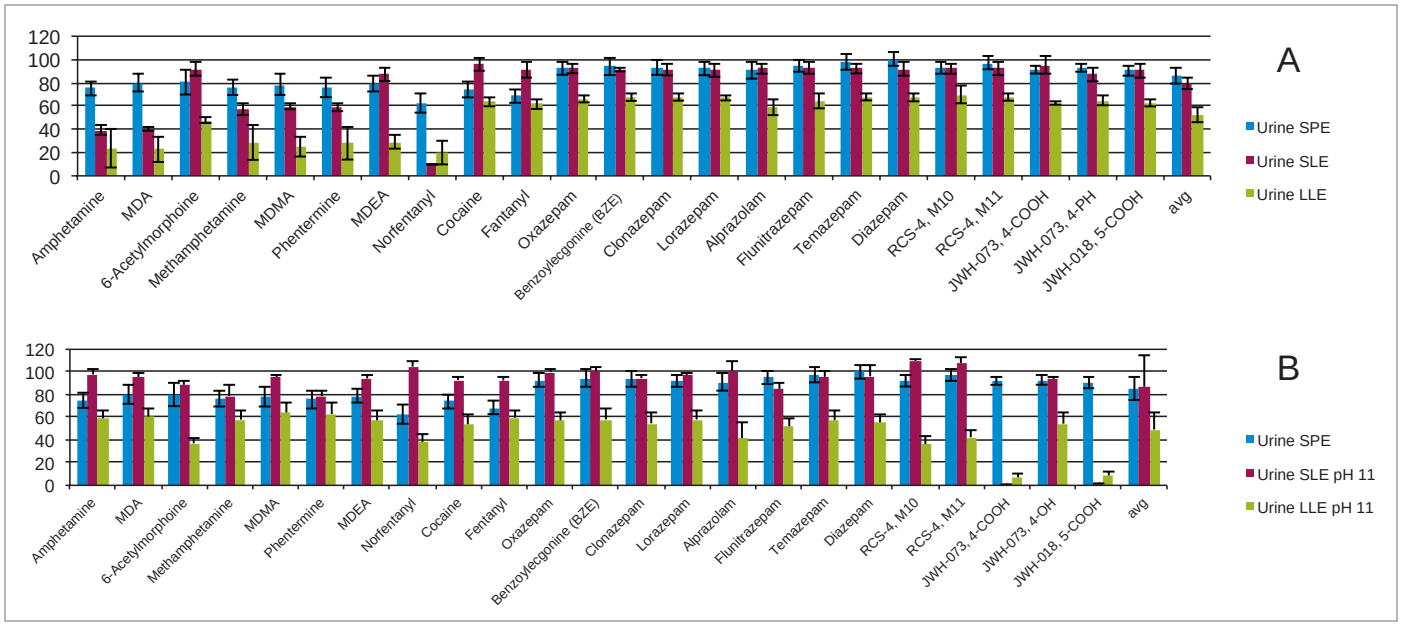

Figure 5A. Extraction recoveries for compounds in urine samples. SLE and LLE processed samples were diluted with water (N=4). Blue, red, and green bars represent recoveries from Oasis PRiME HLB, SLE, and LLE, respectively. Error bars represent standard deviations. Figure 5B. Extraction recoveries for samples extracted from urine. In this case, the LLE and SLE samples were diluted with 0.5 M NH_4OH.

The matrix effects for Oasis PRiME HLB, SLE, and LLE are roughly comparable (Figure 6A). The absolute average of matrix effects for SPE, SLE, and LLE were 12, 12, and 17 respectively, all of which are acceptable. Matrix effects were within 20% for the majority of the compounds using any of the three extraction techniques. When the urine pH was adjusted with ammonium hydroxide (Figure 6B), matrix effects for SLE increased to an average of 25%, while those for LLE remained relatively low, with a mean absolute value of 14%.

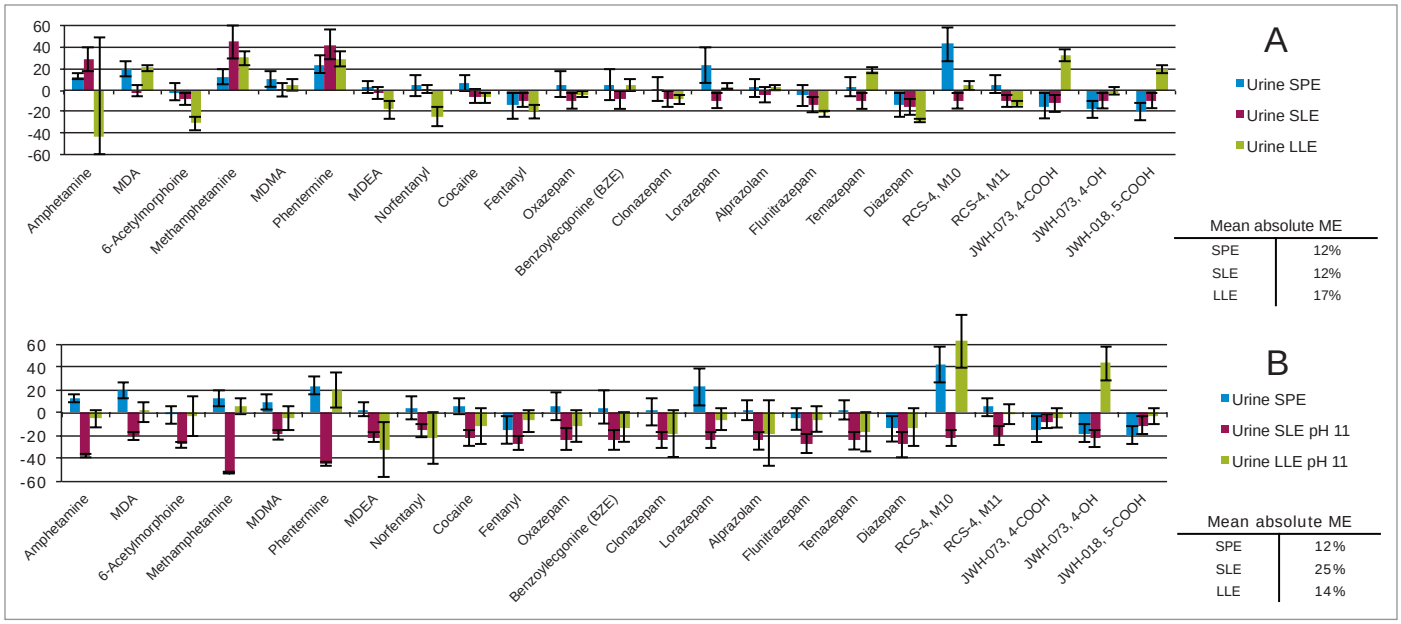

Figure 6A. Matrix effects from urine samples. Samples diluted with water and extracted by SLE or LLE. Figure 6B. Samples were diluted with 0.5 M NH_4OH to pH 11 and extracted by SLE or LLE. The means of the absolute values of matrix effects are listed on the right.

CONCLUSIONS

In this application, a comprehensive comparison of sample preparation techniques including SPE, SLE, and LLE was conducted in plasma and urine, using a wide variety of compounds found in bioanalysis and forensic toxicology. Oasis PRiME HLB employed a simple, three step protocol in which reduced extraction time by 60% and 75% compared to SLE, and LLE, respectively, for forensic toxicology. Oasis PRiME HLB also demonstrated superior recoveries and matrix effects for a variety of tested drugs without any additional method development. SLE and LLE required additional method development or multiple extraction protocols to achieve recoveries that were comparable to Oasis PRiME HLB for all of the tested analytes. The unique nature of Oasis PRiME HLB enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. The µElution format enabled the direct injection of extracts without evaporation or reconstitution.

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An Improved SPE-LC-MS/MS Method for the Quantification of Bradykinin in Human Plasma Using the ionKey/MS System

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- 2x reduction in sample and 10x increase in sensitivity facilitates multiple injections of samples for improved accuracy or to meet guidelines for ISR.
- 50x reduction in solvent consumption reduces cost of analysis.
- SPE using mixed-mode SPE reduces matrix interferences and enhances selectivity of the extraction for bradykinin in plasma.
- 96-well µElution™ plate format enables concentration of the sample while maintaining solubility and minimizes peptide losses due to adsorption to reach detection limits of 2.5 pg/mL for brakyinin in plasma.
- Selective, fast SPE extraction (<30 minutes) without time-consuming immunoaffinity purification.

WATERS SOLUTIONS

ionKey/MS™ System
ACQUITY UPLC® M-Class System
ionKey™ Source
Xevo® TQ-S
iKey™ Separation Device
MassLynx® 4.1 Software
Oasis® WCX 96-well µElution Plate
Waters Collection Plate
TargetLynx™ Application Manager

KEY WORDS

bioanalysis, Oasis, sample preparation, peptide quantification, bradykinin, UPLC, 2D Technology, plasma, ionKey/MS, iKey

INTRODUCTION

The need for robust and sensitive analysis of peptide species challenges both chromatographic separation and mass spectrometry. Peptides, in general, are often difficult to analyze by LC-MS/MS, as mass spectrometer (MS) sensitivity is low due to the formation of multiple precursors and poor or overly extensive fragmentation, making liquid chromatography (LC) and sample preparation even more critical. A previous application note (720004833EN) described in detail the development of a fast, flexible analytical scale, SPE-LC-MS/MS method for the quantification of the peptide bradykinin (Figure 1) in human plasma for use as a biomarker in the preclinical or discovery setting.[1] Accurate quantification of bradykinin in plasma is particularly challenging because it is present in low pg/mL levels, is rapidly metabolized, and is also artificially produced during blood sampling and sample preparation via proteolytic processes.[2]

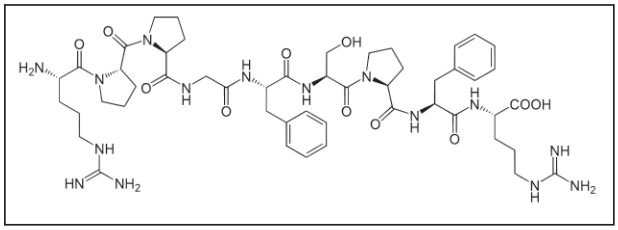

Figure 1. Representative structure and amino acid sequence of bradykinin.

In this work, the LC-MS platform was updated to incorporate the use of the ionKey/MS System which integrates the UPLC® analytical separation directly into the source of the MS (Figure 2). The iKey Separation Device (150 µm I.D.), shown in Figure 3, contains the fluidic channel, electronics, ESI interface, heater, eCord,™ and the chemistry to perform UPLC separations. Additionally, this technology offers significant increases in sensitivity compared to 2.1 mm I.D. chromatography, making it ideal for peptide analyses. Most bioanalytical LC-MS/MS assays often consume high volumes of both solvent and sample, thus increasing the cost of the assay and limiting the number of replicates that can be analyzed. In addition to the sensitivity increase the ionKey/MS System provides over the 2.1 mm diameter scale, it also reduces solvent and sample consumption and provides enough sample to perform multiple injections that may be required to meet incurred sample reanalysis (ISR) guidelines.

EXPERIMENTAL

Method conditions

UPLC conditions

LC system:	ACQUITY UPLC M-Class with 2D Technology configured with optional trap and back flush elution
Separation device:	Key Peptide BEH C_{18} Separation Device, 300Å, 1.7 µm, 150 µm x 50 mm (p/n 186006969)
Trap column:	ACQUITY UPLC M-Class Symmetry® C_{18}, 5 µm, 300 µm x 50 mm (p/n 186007498)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	99:1 mobile phase A:B, 25 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column)
Analytical gradient:	See Table 1
Elution flow rate:	2.5 µL/min
iKey temp.:	75 °C
Sample temp.:	15 °C
Injection vol.:	10 µL
Total run time:	12.0 minutes
Collection plates:	Waters 1 mL collection plates (p/n 186002481)

MS conditions

MS system:	Xevo TQ-S Mass Spectrometer with ionKey Source and iKey Seperation Device
Ionization mode:	ESI positive
Capillary voltage:	3.8 kV
Source temp.:	120 °C
Cone gas flow:	50 L/hr
Collision cell pressure:	3.83 x $10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 2
Cone voltage:	Optimized by component, see Table 2

Data management

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx

Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
0.00	2.5	98.0	2.0	Initial
0.50	2.5	98.0	2.0	6
5.00	2.5	50.0	50.0	6
6.00	2.5	5.0	95.0	6
7.00	2.5	5.0	95.0	6
8.00	2.5	98.0	2.0	6

Table 1. UPLC gradient conditions.

This study utilizes specifically designed blood collection techniques to inhibit bradykinin formation *ex vivo*, takes advantage of mixed mode solid-phase extraction (SPE) and use of the novel and highly efficient ionKey/MS System for selective, sensitive, and robust chromatographic separation, and quantification of the nonopeptide bradykinin. The sensitivity increase that ionKey/MS System provides over the 2.1 mm diameter scale method for bradykinin enables a 2x reduction in plasma and a 7–10x increase in signal-to-noise (S:N). As a result, we can accurately and precisely quantify 2.5 pg/mL of bradykinin above the basal level.

Sample preparation

Blood collection

Human plasma was obtained from one male donor whose blood was collected in BD™ P100, P700, P800, and blood collection tubes containing only K_2EDTA. The various BD P blood collection tubes contain various mixtures of proprietary stabilizers/inhibitors that immediately solubilize during blood collection, and enable preservation of human plasma proteins and peptides.

Sample pretreatment

10 µL of the internal standard (IS), [Lys-des-Arg9]-bradykinin (5 ng/mL) was added to 100 µL of human plasma and mixed. The samples were then diluted 1:1 with 5% NH_4OH in water and mixed.

Sample extraction

Pretreated plasma samples were extracted according to the protocol in Figure 4. All solutions are made up by volume. All extraction steps were applied to all wells of the Oasis WCX 96-well µElution Plate that contained samples.

Figure 2. ionKey/MS System: comprised of the Xevo TQ-S, the ACQUITY UPLC M-Class, the ionKey Source, and the iKey Separation Device.

Figure 3. iKey Separation Device.

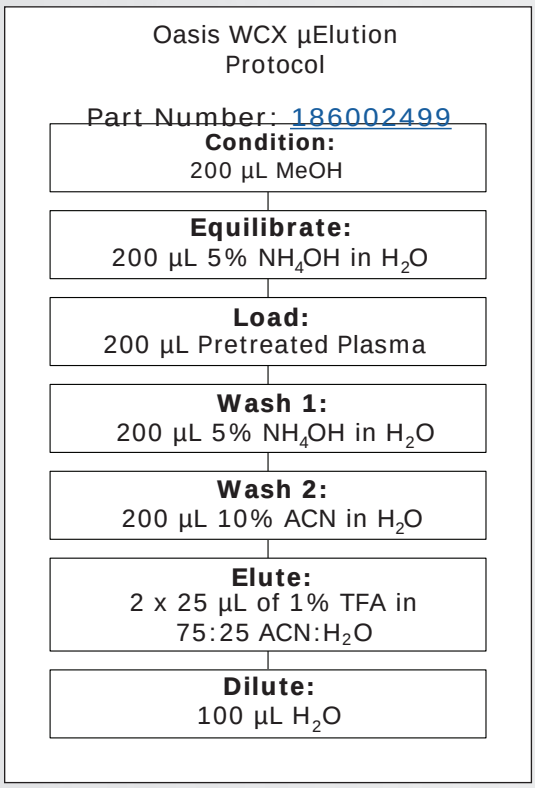

Figure 4. Oasis µElution WCX extraction protocol.

RESULTS AND DISCUSSION

Mass spectrometry

The 3+ precursors of bradykinin (*m/z* 354.18) and IS (344.94) were used for quantitation. The fragment at *m/z* 419.18 $y3^{1+}$ was chosen as the primary fragment for bradykinin quantitative analysis, while the *m/z* 408.18 $b4^{1+}$ fragment was used for confirmatory purposes. For the IS, the fragment at *m/z* 386.03 $b7^{2+}$ was chosen. Optimal MS conditions are shown in Table 2. Although many peptides produce intense fragments below *m/z* 200, these ions (often immonium ions) result in high background in extracted samples due to their lack of specificity. In this assay, the use of highly specific b or y ion fragments with *m/z* values higher than their precursors yielded significantly improved specificity, facilitating the use of simpler LC and SPE methodologies.

Compound	Precursor	MRM transition	Cone voltage (V)	Collision energy (eV)	Production type
Bradykinin	$[M+3H]3^+$	354.18 > 419.18	10	8	$[1H^+]$ 1/γ3
	$[M+3H]3^+$	354.18 > 408.18	10	10	$[1H^+]$ 1/b4
[Lys-des-arg9]-Bradykinin(IS)	$[M+3H]3^+$	344.94 > 386.03	10	10	$[2H^+]$ 1/b7

Table 2. MRM transitions, collision energies, and cone voltages for bradykinin and [Lys-des-Arg9] bradykinin, the internal standard (IS).

Chromatographic separation

Chromatographic separation of bradykinin and its IS was achieved using the novel microfluidic chromatographic iKey Separation Device. The iKey Separation Device has a channel with UPLC-grade, sub-2-µm particles that permits operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume are avoided. Use of the iKey Peptide BEH C18 Separation Device, 300Å, 1.7 µm, 150 µm x 50 mm (p/n 186006969) provided excellent peak shape, increased peak height, and improved S:N compared to the analytical scale (2.1 mm I.D.) LC-MS analysis. Representative chromatograms of bradykinin and the IS using the iKey Separation Device are shown in Figure 5. The use of multidimensional chromatography, specifically a trap and back-elute strategy, provided further sample cleanup and facilitated the loading of 10 µL of the high organic SPE eluate (required to maintain solubility of the peptides) without experiencing analyte break through. Additionally, the ability to inject the larger sample volumes typical for analytical scale LC analysis (e.g. 10 µL) on the iKey Separation Device can provide the substantial gains in sensitivity that are often required to accurately and reliably detect low pg/mL levels of peptide and protein in complex matrices.

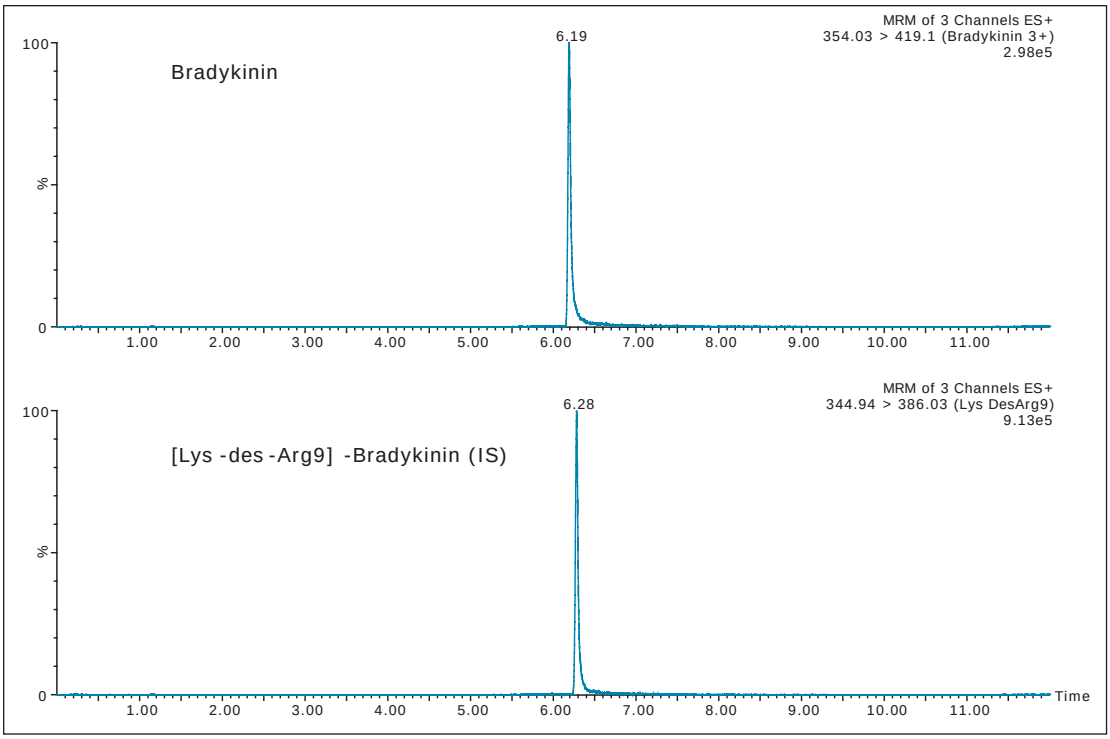

Figure 5. UPLC separation of bradykinin and internal standard, from extracted plasma, using the iKey Peptide BEH C_{18} Separation Device, 300Å, 1.7 µm, 150 µm x 50 mm (p/n 186006969)

Enhanced sensitivity with the use of ionKey/MS System

Use of the ionKey/MS System facilitated the development of a highly efficient LC separation of bradykinin in plasma with significant improvement in sensitivity and S:N over the analytical scale LC-MS using 2.1 mm I.D. chromatography. Initially, samples were extracted using the protocol described in the previous application note (720004833EN). Briefly, 200 µL of plasma was extracted followed by a 1:1 dilution of the eluate with water. A 3 µL injection of this sample on the iKey Separation Device provided a 5x improvement in S:N compared to a 10 µL injection of the same sample analyzed at the 2.1 mm scale, and is shown in Figure 6. The improvement in ionization efficiency and subsequent increase in sensitivity afforded by the iKey Separation Device facilitated this lower injection volume. The ability to obtain comparable or improved sensitivity with smaller injection volumes (1–3 µL) using the ionKey/MS System makes this technology ideal when sample is limited or when multiple injections are required to meet ISR guidelines.

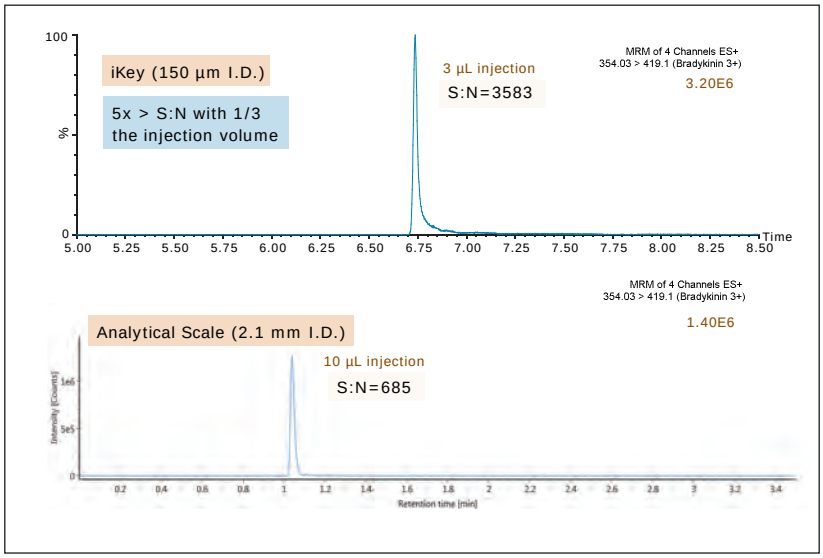

Figure 6. Comparison of 1 ng/mL over-spiked bradykinin extracted from human plasma (200 µL): iKey Separation Device (150 µm I.D.) vs. traditional analytical flow (2.1 mm I.D.).

Method optimization resulted in the reduction of the required plasma sample by half and an increase in eluate dilution to 1:2, both of which minimized matrix interferences. A comparison of a 10 µL injection of extracted plasma (using the optimized method) the ionKey/MS System and a traditional analytical flow system (ACQUITY UPLC and Xevo TQ-S with UNIFI®) resulted in a 10x increase in signal and 7x increase in S:N with the ionKey/MS System. This improvement is illustrated in Figure 7, with a comparison of endogenous levels of bradykinin. Ultimately, the use of the 150 µm iKey Separation Device enabled the development of a low flow quantitative MRM method for bradykinin that achieved a detection limit of 2.5 pg/mL with only 100 µL of plasma.

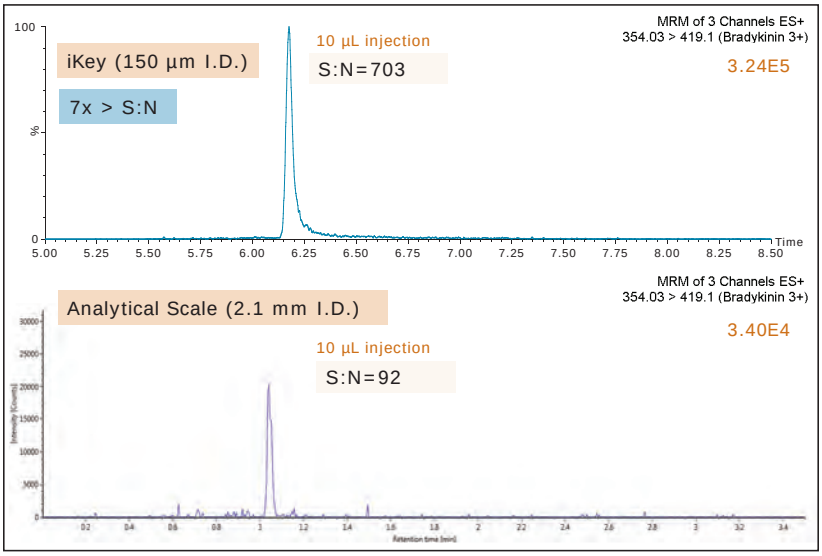

Figure 7. Comparison of endogenous levels of bradykinin extracted from human plasma (100 µL): iKey Separation Device (150 µm I.D.) vs. traditional analytical flow (2.1 mm I.D.).

Sample preparation

The development and optimization of the SPE method was described in detail in the previous application note (720004833EN) and was employed for this study. Use of the Oasis WCX SPE, provided both reversed-phase and ion-exchange modes of retention, enabling greater sample cleanup, selectivity, and ultimate sensitivity for this peptide. Additionally, the Oasis WCX 96-well µElution Plate (p/n 186002499) can be processed manually in under 30 minutes and is compatible with most liquid-handling robotic systems for automation to meet sample throughput requirements. This format also provides the ability to elute in very small sample volumes of only 50 µL, minimizing the potential for peptide losses that might occur during evaporation due to adsorption to the walls of collection plates and/or chemical instability.

Linearity, accuracy, and precision

To generate standard curves, human plasma (derived from blood collected in BD P100 tubes) was fortified with bradykinin at the following final concentrations: 2.5, 5, 10, 20, 40, 60, 100, 600, 1,000, 2,000, 4,000, and 8,000 pg/mL. Each standard level was prepared in duplicate. Quality control (QC) samples were prepared from the same plasma at 15, 30, 50, 150, 300, 800, and 6,000 pg/mL. QC samples at each level were prepared in triplicate. [Lys-des-Arg9]-Bradykinin (final concentration of 0.5 ng/mL) was used as the internal standard (IS). Peak area ratios (PARs) of the analyte peak area to the IS peak were calculated. The calibration curve was constructed using PARs of the calibration samples by applying a one/concentration (1/x) weighted linear regression model. All QC sample concentrations were then calculated from their PARS against the calibration curve. Due to the presence of endogenous bradykinin, standard addition was used. The mean basal level of bradykinin (0.19 ng/mL) in control plasma samples was determined by calculating the x-intercept. The calculated basal level was then added to the spiked concentration for all standard curve and QC samples to enable accurate quantification. Using 1/x regression, bradykinin was linear with an R^2 value of >0.99. A summary of standard curve performance is shown in is shown in Table 3. Results from QC analysis are shown in Table 4. At all levels, QC samples demonstrated very good accuracy and precision, with mean accuracies ranging from 92.7–104.0 and mean %CV's of 1.21–4.31. These results easily meet the recommended FDA acceptance criteria outlined in the white papers describing best practices in bioanalytical method validation for LC-MS/MS assays.[3,4]

Bradykinin overspiked concentration (ng/mL)	Final bradykinin concentration (ng/mL)	Area	IS area	Response	Calculated bradykinin concentration (pg/mL)	Mean accuracy
0.0025	0.1925	8595	20655	0.417	0.1891	98.2
0.0050	0.1950	8369	19474	0.430	0.1956	100.3
0.0100	0.2000	8493	19296	0.441	0.2008	100.4
0.0200	0.2100	8906	19386	0.460	0.2100	100.0
0.0400	0.2300	10287	19462	0.528	0.2432	105.8
0.0600	0.2500	10775	19588	0.551	0.2542	101.7
0.1000	0.2900	11441	19119	0.598	0.2771	95.5
0.3000	0.4900	20435	20694	0.988	0.4656	95.0
0.6000	0.7900	30256	18599	1.628	0.7753	98.2
1.0000	1.1900	54216	20792	2.608	1.2495	105.0
2.0000	2.1900	92974	19438	4.782	2.3018	105.1
4.0000	4.1900	181824	21490	8.454	4.0784	97.4
8.0000	8.1900	349881	20616	16.966	8.1969	0.1

Table 3. Standard curve summary and statistics for bradykinin extracted from human plasma.

Bradykinin overspiked concentration (ng/mL)	Bradykinin QC concentration (ng/mL)	Mean concentration (ng/mL)	SD	%CV	Mean accuracy
0.0000	–	0.1860	0.003	1.62	–
0.0150	0.2050	0.2078	0.003	1.47	101.4
0.0300	0.2200	0.2268	0.003	1.26	103.1
0.0500	0.2400	0.2360	0.010	4.11	98.3
0.1500	0.3400	0.3152	0.014	4.31	92.7
0.3000	0.4900	0.4854	0.010	2.16	99.1
0.8000	0.9900	1.0293	0.031	3.06	104.0
6.0000	6.1900	6.0504	0.073	1.21	97.8

Table 4. QC statistics from bradykinin extracted from human plasma.

Assessment of pre-analytical handling and endogenous bradykinin levels

Accurate quantification of bradykinin in plasma is particularly challenging because it is metabolized rapidly, with a half life of less than 30 seconds, and can be artificially produced during blood sampling and sample preparation, via proteolytic processes.[2,5,6] To assess the best preservation of bradykinin in blood, as well as to prevent the formation of bradykinin *ex vivo*, particular attention was paid to the protocol for blood collection which employed the use of commercially-available blood collection tubes containing proprietary additives that provide enhanced recovery plasma analytes. More specifically, the BD P100, P700, and P800 collection tubes provide a means of preservation of plasma to be used in peptide and protein analysis.[7] The original work presented (720004833EN) only assessed the preservation of bradykinin in P100 blood collection tubes. The BD P100 and P700 blood collection tubes contain proprietary mixtures of additives and inhibitors. The BD P100 collection tubes also contain a mechanical separator that allow for ease of collection and separation of the plasma after blood centrifugation. The P700 tubes contain the same inhibitors as the P100 tubes, with an additional inhibitor for stabilization of Glucagon-Like Peptide I (GLP-1) and contains no mechanical separator. P800 blood collection tubes, like the P100 and P700 blood cllection tubes, contain a proprietray cocktail of inhibitors that provide preservation of bioactive peptide in plasma, and contains no mechanical separator. The P800 blood collection tubes are marketed for assays that require quantitation and measurement of the GLP-1, Glucose-Dependent Insulinotropic Polypeptide (GIP), Glucagon, and Ghrelin.

Mean extracted endogenous plasma bradykinin concentrations, in which the blood was collected with (P100, P700), and without protease inhibitors (K2EDTA only, days 1 and 4) are shown in Table 5. Average CV's of the endogenous bradykinin levels ranged from 0.88–2.18%, indicating a very robust and reproducible method. Representative chromatograms for these results are shown in Figure 8 (panels A-D). Panel A is a representative chromatogram of endogenous plasma bradykinin obtained from blood collected in the P100 tubes, with a mean calculated concentration of 0.1860 ng/mL. P700 blood collection yielded a mean endogenous bradykinin plasma level of 0.0945 ng/mL, and is shown in Panel B. This concentration was approximately half of the concentration determined using the P100 tubes. The artifactual formation of bradykinin in plasma without inhibitor is demonstrated in panels C and D. In these cases, blood was collected in K2EDTA-only blood collection tubes, and the subsequent plasma was brought though 1 freeze/thaw (F/T) cycle. Panel C represents the bradykinin concentration on day 1, where the bradykinin plasma level increased to 0.8107 ng/mL. Panel D represents the bradykinin concentration after 4 days of storage at 10 °C, where bradykinin plasma levels increased to 5.4916 ng/mL. Endogenous levels of bradykinin using the P800 showed relative area counts similar to that of the P700 collection (data not shown), but due to a 10x signal loss of the IS in the P800 tube samples endogenous levels of bradykinin were not calculated for the P800 sample collection. It is assumed that the analogue IS was not protected from metabolism and/or degradation in the P800 tube due to differences in the cocktail of inhibitors. Further, the reduced endogenous bradykinin plasma levels using the P700 collection tube indicated that this cocktail of inhibitors may be more appropriate for stabilization and prevention of ex vivo bradykinin formation. However, another possibility that was not explored was that the presence of the plasma mechanical separator provided a mechanism of bradykinin formation prior or during blood collection and centrifugation. These results further emphasize the need for proper sample collection and storage to accurately quantify endogenous bradykinin plasma levels.

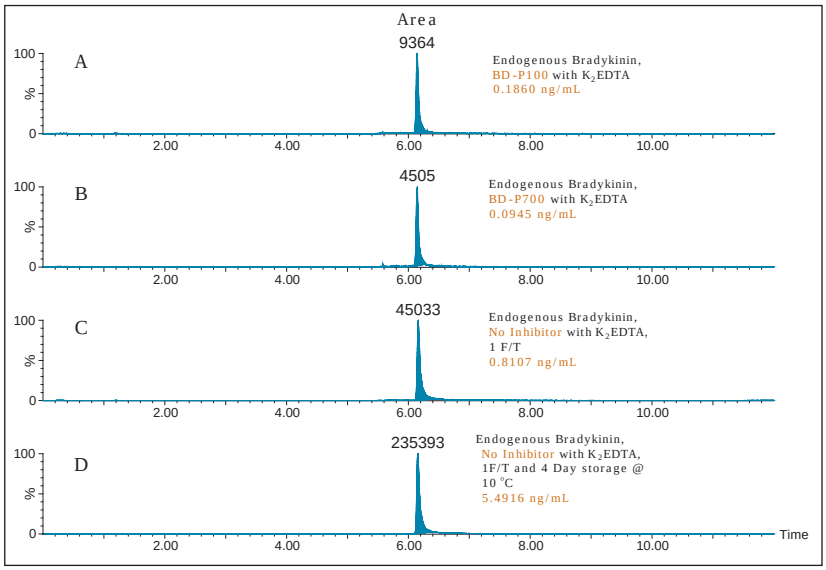

Figure 8. Representative chromatograms of extracted endogenous plasma bradykinin, in which the blood was collected with (P100, P700), and without protease inhibitors (K2EDTA only).

Plasma treatment	Mean concentration (ng/mL)	SD	%CV
BD-P100	0.1860	0.003	1.62
BD-P700	0.0945	0.002	2.18
BD-No inhibitor with K2EDTA, 1F/T	0.8107	0.007	0.88
BD-No inhibitor, K_2EDTA, 1 F/T, 4 days at 10 °C.	5.4916	0.110	2.01

Table 5. Mean extracted endogenous plasma bradykinin, in which the blood was collected with (P100, P700), and without protease inhibitors (K2 EDTA only, days 1 and 4).

CONCLUSIONS

Use of the ionKey/MS System, mixed-mode SPE and higher *m/z* b or y ion MS fragments provided the level of selectivity and sensitivity necessary to accurately quantify bradykinin and distinguish subtle differences in concentrations. The current analysis uses 100 µL of plasma and provides a significant improvement in sensitivity and S:N over the analytical scale LC-MS analysis which uses twice as much sample. The use of the 150 µm iKey Separation Device enabled the development of a highly sensitive, low flow quantitative MRM method for bradykinin that could distinguish a change of 2.5 pg/mL of bradykinin over the basal level. Standard curves were accurate and precise from 2.5–8,000 pg/mL. QC samples at all levels easily met recommended FDA regulatory criteria[4,5] with mean accuracies ranging from 92.7–104.0 and mean %CV's of 1.20–4.31, indicating an accurate, precise, and reproducible method. Furthermore, an injection of the same volume (10 µL) of sample corresponded to a >10x increase in on-column sensitivity as compared to the traditional analytical flow method for this peptide. In addition to the sensitivity increase the ionKey/MS System provides over the 2.1 mm I.D. scale, it also reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for ISR. This study also demonstrates the importance of proper sample collection with appropriate additives for the stabilization/preservation of bradykinin in plasma to accurately represent endogenous levels. This method shows great promise for high sensitivity quantification of bradykinin in patient samples from PK and clinical studies using the ionKey/MS System if further validation was performed.

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3-step protocol (catch and release SPE)

Load pre-treated sample

Wash: 5% MeOH

Elute: 90/10 acetonitrile/MeOH

2-step protocol (pass-through SPE)

Load pre-treated sample
Analytes pass through unretained

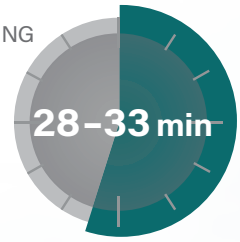
Rinse to collect hold up volume

DisQuE Reduces Sample Preparation Time and Steps with Oasis PRiME HLB Compared to SSLE

SSLE

1. Add sample (3 min)
2. Wait (5–10 min)
3. Add extraction solvent (2 min)
4. Wait (5–10 min)
5. Extract (1 min)
6. Evaporate (5 min)
7. Reconstitute (2 min)

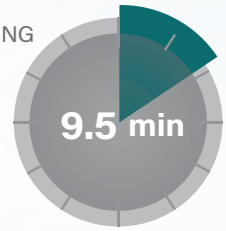
TOTAL PROCESSING TIME:



Oasis PRiME HLB

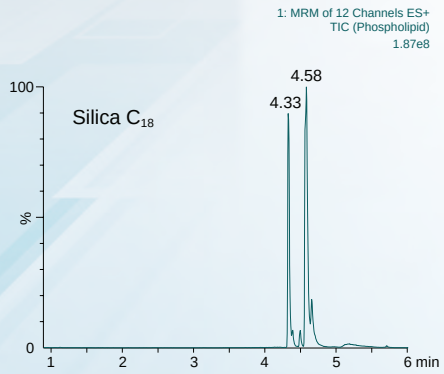
1. Load pre-treated sample (4 min)
2. Wash: 5% MeOH (3.5 min)
3. Elute: 90/10 acetonitrile/MeOH (2 min)

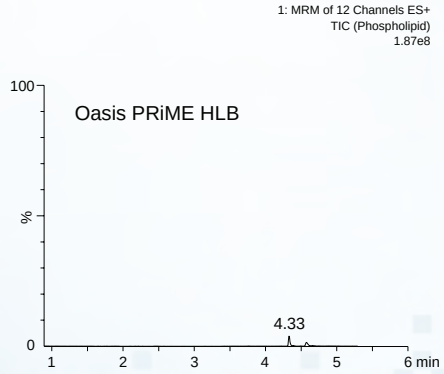
TOTAL PROCESSING TIME:



Oasis PRiME HLB reduces sample preparation time and complexity by providing efficient sample preparation. Compared to SSLE, samples can be processed in fewer steps and less time.

Phospholipids Remaining After Pass-through Sample Clean Up





Lipid removal from acetonitrile-based meat extract results: Oasis PRiME HLB removes more than 90% of hexane-extractable total lipids (determined gravimetrically). Oasis PRiME HLB successfully removes both phospholipids and fats in pass-through method. The silica C_{18} sorbent removes only fats, NOT phospholipids. Removal of both of these components results in fewer matrix effects and less column and/or instrument contamination.

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APPLICATION NOTES

A Simple Cleanup Protocol Using a Novel SPE Device for UPLC-MS/MS Analysis of Multi-Residue Veterinary Drugs in Milk

Oasis PRiME HLB Cartridge for Cleanup of Infant Formula Extracts Prior to UPLC-MS/MS Multiresidue Veterinary Drugs Analysis

Oasis PRiME HLB Cartridges and DisQuE QuEChERS Products for UPLC-MS/MS Mycotoxin Analysis in Cereal Grains

Oasis PRiME HLB Cartridge for Effective Cleanup of Meat Extracts Prior to Multi-Residue Veterinary Drug UPLC-MS Analysis

Oasis PRiME HLB Cartridge for Rapid and Effective Cleanup of Avocado, a High Fat Matrix, Prior to APGC-MS/MS Analysis

Rapid, Simple, and Effective Cleanup of Bovine Liver Samples Prior to UPLC-MS/MS Multiresidue Veterinary Drugs Analysis

Rapid, Simple, and Effective Cleanup of Seafood Extracts Prior to UPLC-MS/MS Multiresidue Veterinary Drug Analysis

Simple and Effective Cleanup for UPLC-MS/MS Determination of Veterinary Drug Residues in Egg

APPLICATION NOTEBOOK

Oasis PRiME HLB Food Applications

BROCHURE

Oasis Sample Extraction Products

CARE AND USE MANUAL

Oasis Cartridges and 96-Well Plates

ORDERING INFORMATION

Description	Format	Qty.	P/N
Oasis PRiME SPE			
Oasis PRiME HLB µElution Plate	3 mg	1/pk	186008052
Oasis PRiME HLB 96-Well Plate	10 mg	1/pk	186008053
Oasis PRiME HLB 96-Well Plate	30 mg	1/pk	186008054
Oasis PRiME HLB Cartridge	1 cc/30 mg	100/pk	186008055
Oasis PRiME HLB Cartridge	3 cc/60 mg	100/pk	186008056
Oasis PRiME HLB Cartridge	3 cc/150 mg	100/pk	186008717
Oasis PRiME HLB Cartridge	6 cc/200 mg	30/pk	186008057
Oasis PRiME HLB Cartridge	6 cc/500 mg	30/pk	186008718
Oasis PRiME HLB Plus Light Cartridge	100 mg	50/pk	186008886
Oasis PRiME HLB Plus Short Cartridge	335 mg	50/pk	186008887
Columns and Vials			
ACQUITY UPLC® CSH™ C_{18}, 2.1 x 100 mm, 1.7 µm			186005297
ACQUITY UPLC BEH C_{18}, 2.1 x 100 mm, 1.7 µm			186003555
Sample Vials			Vials Selector

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