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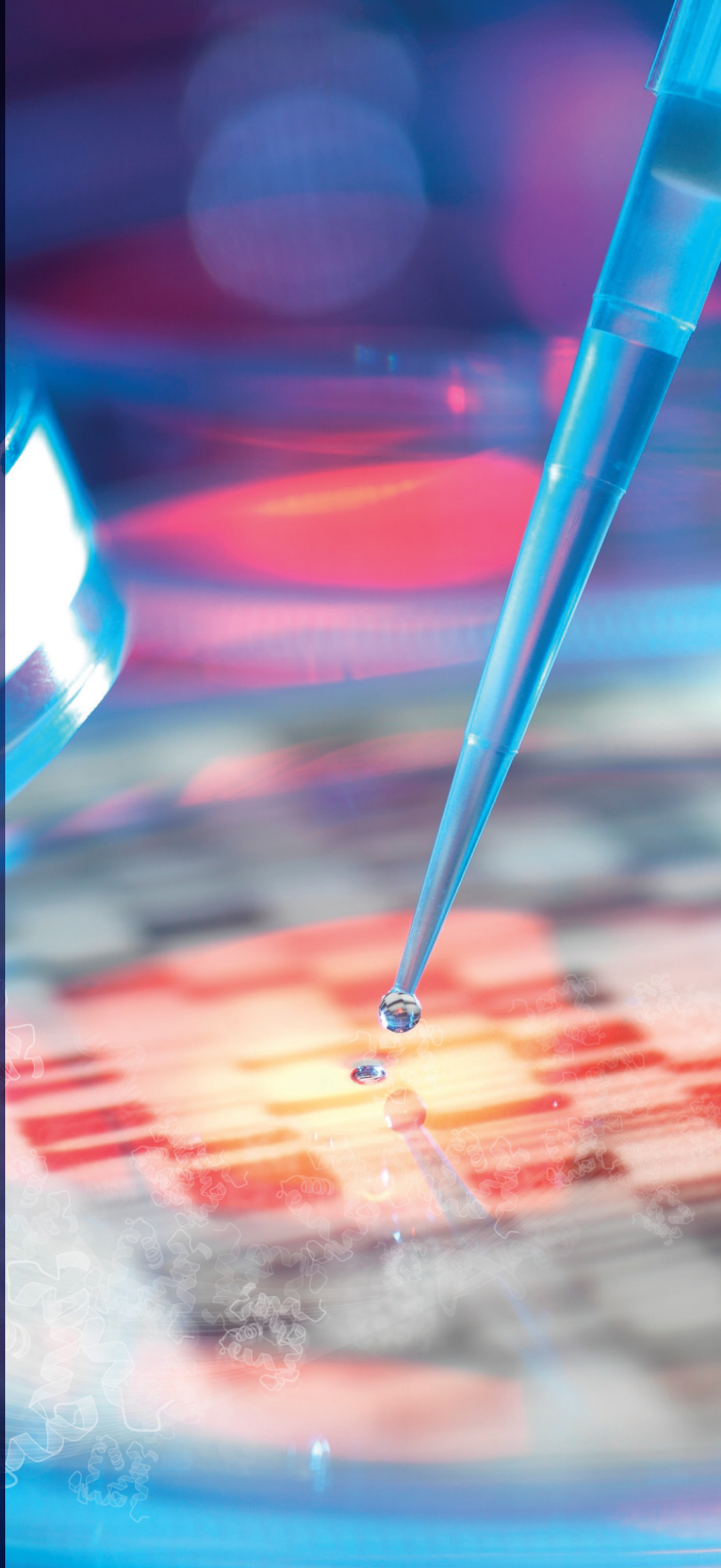

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- **Sample Preparation:**
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- **LC Column Guards:**
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- **Sample Preparation:**
  - Bond Elut Plexa (SPE)
  - Bond Elut Plexa PCX (SPE)
  - Bond Elut Certify
- **Sample Filtration:**
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- **LC Supplies:**
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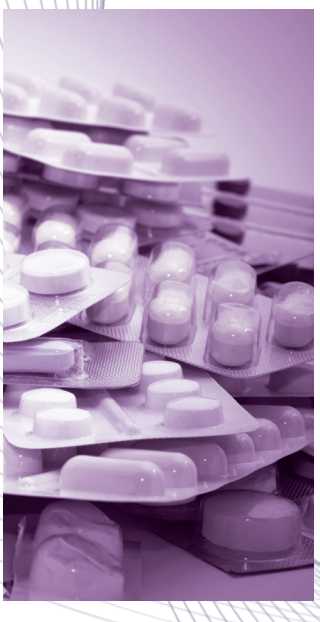
DESIGNER DRUGS



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  - Gas Clean purifier
- **LC Columns:**
  - Poroshell 120
  - ZORBAX RRHD
  - ZORBAX RRHT
  - ZORBAX Eclipse Plus
- **LC Column Guards:**
  - Fast Guards for UHPLC
- **Sample Preparation:**
  - Bond Elut Certify (SPE)
  - Bond Elut Plexa Family (SPE)
  - Chem Elut (SLE)
- **Filtration:**
  - Captiva Filter Cartridges
- **LC Supplies:**
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DRUG SCREENING & CONFIRMATION

Click on the title to view:

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DRUG SCREENING AND CONFIRMATION *(CON'T.)*

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SAMHSA-Compliant LC/MS/MS Analysis of 6-Acetylmorphine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they typically do not require a derivatization step. We present a method for analysis of 6-acetylmorphine that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy, and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products such as Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

A metabolite, 6-Acetylmorphine, or 6-monoacetylmorphine (6-AM) is unique to heroin. Heroin (or diacetylmorphine) is an opioid drug synthesized from morphine. In the body, heroin is rapidly metabolized through deacetylation to 6-AM and then to morphine at a somewhat slower rate [2]. The updated SAMHSA confirmation cutoff concentration for 6-AM is 10 ng/mL, and a LOD at 10% of the cutoff would be 1 ng/mL.

The simple extraction method described here provides reproducible high recoveries of 6-AM due to the unique properties of Bond Elut Plexa. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

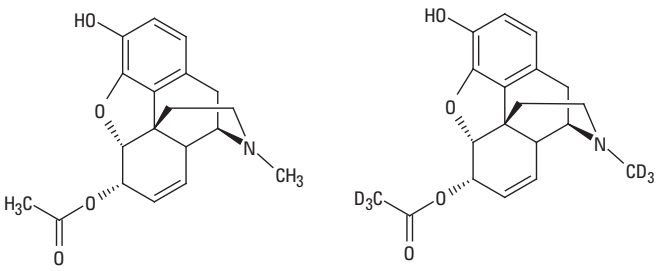
A Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with approximately 40% less back pressure, thereby allowing users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

With a low sample injection volume of 10 µL and no sample preconcentration, the presented method demonstrates excellent signal-to-noise ratios (> 190:1 at 1 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of an Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent [3,4] used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures.

Experimental

Analytes



6-Acetylmorphine
Log P 1.55
pK_{a1} 8.2

6-Acetylmorphine-D_6

Figure 1. 6-Acetylmorphine analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (6-acetylmorphine) and 100 µg/mL (6-acetylmorphine-D_6) solutions in acetonitrile.

Materials and instrumentation

SPE

- Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2-mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Pretreatment

Spike 1 mL of urine with ISTD at 20 ng/mL; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL. methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL methanol: ammonium hydroxide (100:10), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under stream of nitrogen to dryness.
8. Reconstitute in 1 mL initial mobile phase (10% methanol, 90% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	10
	1.5	25
	2.0	60
	2.1	90
	5.0	90
	5.1	10
Stop time	5.2 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	10 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES Source Parameters	
Ionization mode	Positive
Capillary voltage	2,800 V
Drying gas flow	13 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V
MS parameters	
Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.2 min - diverter valve to MS
Delta EMV (+)	400 V

Results and Discussion

At acidic pH, the tertiary amine of 6-acetylmorphine was protonated, and the analyte was efficiently retained on Bond Elute Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without 6-AM loss from the SPE column. A strong base was added to organic eluent to break ionic interaction between the analyte and strong cation exchange sorbent. 6-AM recovery was optimized with 10% NH_4OH added to methanol shortly before sample elution.

The Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column provided fast separation of 6-AM in urine extract and good peak shape (Figure 2). The LC separation started with a low fraction of organic solvent (10%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting a first portion of flow (0 to 1.2 minutes) to waste to minimize source contamination. Data collection started at 1.2 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed for short retention and re-equilibration times.

SAMHSA guidelines require one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for each target analyte (Table 1) was provided for additional confidence. Agilent MassHunter Quantitative software automatically calculated qualifier ion ratios, highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
6-AM	328.2	165.1	140	40
6-AM	328.2	211.1	140	25
6-AM	328.2	193.1	140	25
6-AM-D_6	334.2	165.1	140	40
6-AM-D_6	334.2	211.1	140	25

Normal, rather than dynamic, MRM scan type can be used with this method, because dynamic MRM has no advantages for detection of a single compound.

A signal-to-noise ratio of > 190:1 for the 1 ng/mL peak (Figure 2, upper panel) illustrated a state-of-the-art performance of the Agilent 6460 Triple Quadrupole LC/MS capable of reliably detecting 6-AM at a small fraction (10%) of the SAMHSA cutoff concentration.

Figure 3 is an example calibration curve for extracted urine standards at five concentration levels of 6-acetylmorphine. Calibration standards were prepared by spiking negative urine at 1.0, 10, 50, 200, and 400 ng/mL. Deuterated internal standard 6-AM-D_6 was added at 20 ng/mL. The excellent linear fit with $R^2 > 0.999$ demonstrates linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al* [5] and widely accepted as an industry standard approach for LC/MS/MS methods. Extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 10 ng/mL with 6-AM (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 10 ng/mL in urine (spiked mobile phase).

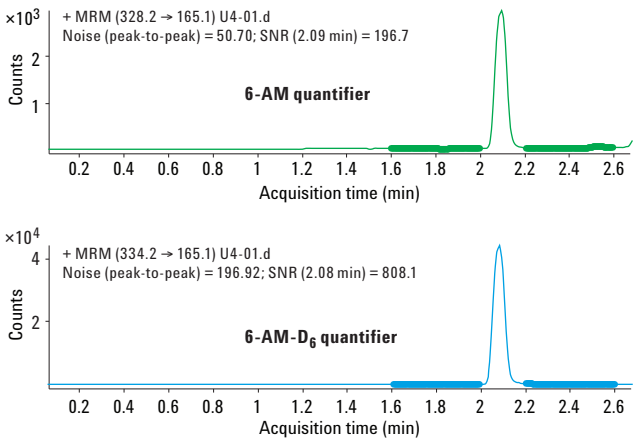


Figure 2. MRM extracted ion chromatograms for 6-AM (1 ng/mL) and 6-AM-D_6 (20 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

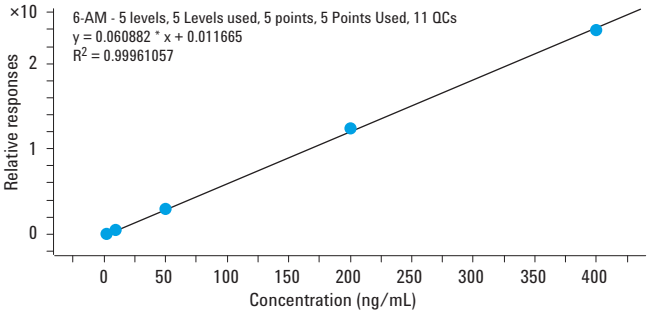


Figure 3. Example calibration curve for 6-AM in urine extract. Calibration range 1.0 to 400 ng/mL. Linear fit, $R^2 > 0.999$.

Table 2. Method performance for 6-Acetylmorphine, n = 5.

	%
Process efficiency*	83
Extraction recovery*	83
Matrix effect*	100
Accuracy**	106
Precision** (CV)	0.6

*determined at cutoff level **determined at 40% cutoff

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows high extraction recovery for 6-acetylmorphine (83%) together with very good accuracy (106%) and precision (0.6 %). Matrix effect of 100% indicated no suppression or enhancement of a signal due to matrix interferences, thus confirming an exceptional cleanliness of Plexa-processed extracts.

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described here is SAMHSA-compliant and provides accurate, precise, and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in the other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and 1200 Series LCs because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of the Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

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2. R. Baselt (2008) *Disposition of Toxic Drugs and Chemicals in Man.* 8th edition. Atlas Books, Ashland, OH, USA.

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Agilent Technologies

SAMHSA-Compliant LC/MS/MS Analysis of Amphetamines in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of five amphetamines that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy, and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

Amphetamines are psychostimulant drugs included in a group of sympathomimetic amines that mimic the effects of the endogenous neurotransmitters, such as epinephrine (adrenaline), norepinephrine (noradrenaline), and dopamine. Amphetamines are found in the leaves of *Ephedra sinica* (for example ephedrine) and were first produced synthetically at the end of the 19th century. Their chemical structure features a phenethylamine backbone with a methyl group attached to the alpha carbon, along with other substitutions (Figure 1). A significant portion of amphetamines is excreted intact in urine. By demethylation, more complicated amphetamine derivatives are metabolized into simpler structures, for example methamphetamine to amphetamine, and MDMA to MDA [2]. The 2011 SAMHSA guidelines require screening for and confirmation of five amphetamines – amphetamine, methamphetamine, MDA, MDMA, and MDEA. The confirmation method should demonstrate the ability to distinguish these drugs from structurally similar compounds that are potential interferences, including ephedrine, pseudoephedrine, phentermine, and phenylpropanolamine (PPA, or norephedrine).

In GC/MS methods traditionally employed for detection of amphetamines, it was common to apply periodate pretreatment to oxidize the hydroxyphenethylamines ephedrine and pseudoephedrine and, thus, exclude a chance of interference by these compounds. We eliminated this step, offering instead a reliable chromatographic separation of all analytes of interest required by the latest SAMHSA guidelines.

The new SAMHSA confirmation cutoff concentration for all amphetamines is 250 ng/mL and a limit of detection at 10% of the cutoff concentration is 25 ng/mL [1]. Because high concentrations of amphetamines can be expected in some urine samples, we chose to use a higher capacity 3 mm id Agilent Poroshell 120 column instead of a 2 mm id column for all Agilent SAMHSA methods. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure. Therefore, it allows users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

The simple extraction method described here provides reproducible high recoveries of amphetamines due to the unique properties of Agilent Bond Elut Plexa. Unlike other polymeric sorbents, Plexa possesses amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

With a low sample injection volume of 2 µL and no sample preconcentration, the presented method demonstrates excellent signal-to-noise (S/N) ratios (> 400:1 at 25 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system system and other SPE/LC products and procedures [3,4].

Experimental

Analytes

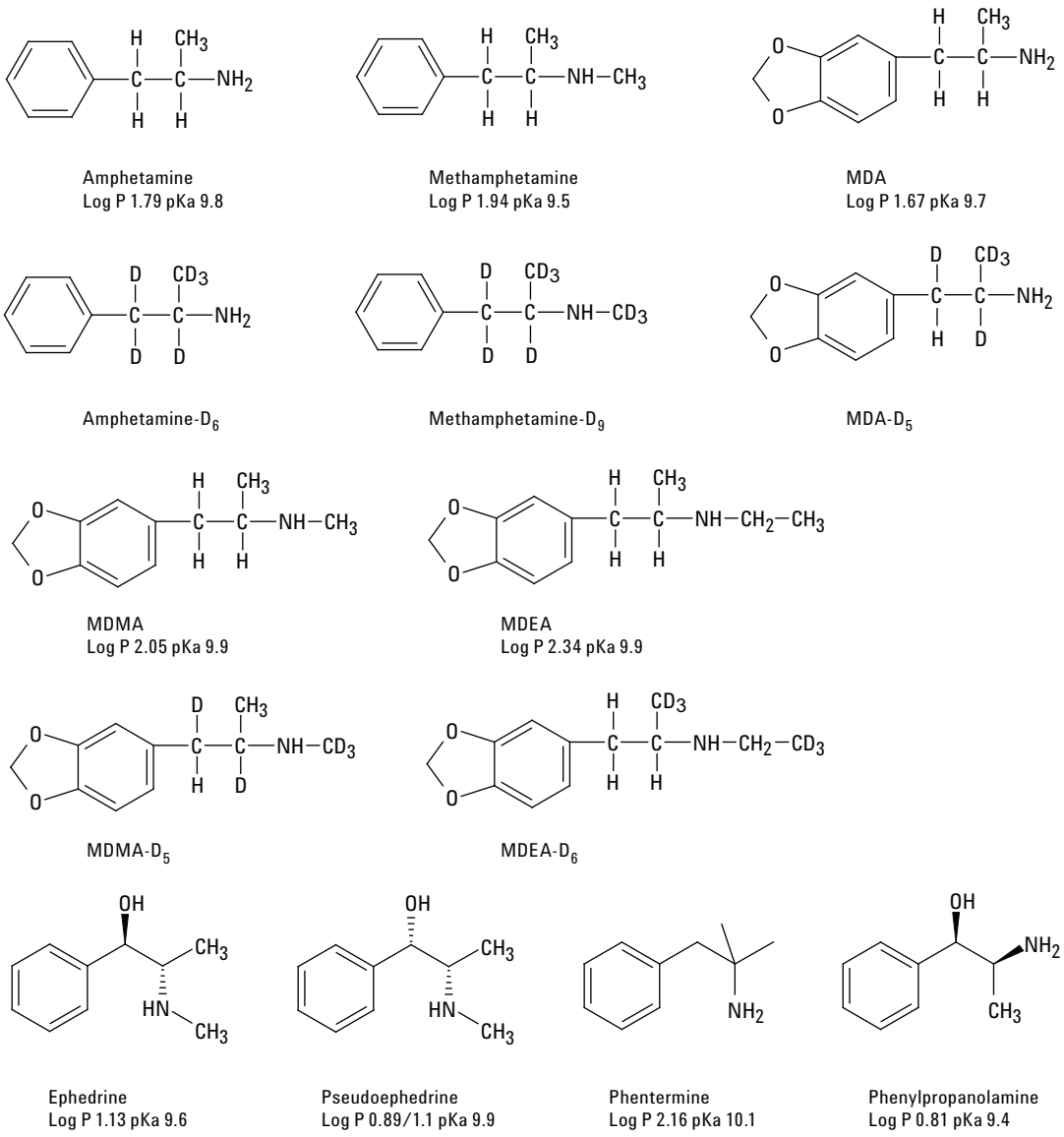

Figure 1. Amphetamines and interferences - analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (amphetamine, methamphetamine, MDA, MDMA, MDEA, ephedrine, pseudoephedrine, phentermine, and phenylpropanolamine) and 100 µg/mL (amphetamine-D_6, methamphetamine-D_9, MDA-D_5, MDMA-D_5, and MDEA-D_6) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2 mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Pretreatment

Spike 0.5 mL of urine with ISTDs at 500 ng/mL each; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL ethyl acetate: methanol: ammonium hydroxide (50:50:20), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under stream of nitrogen to 0.2 mL at ≤ 37 °C.
8. Add 100 µL of 0.025 N hydrochloric acid in methanol, vortex.
9. Evaporate to dryness.
10. Reconstitute in 0.5 mL initial mobile phase (15% methanol, 85% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	15
	1.5	15
	3.5	30
	3.6	90
	6.6	90
	6.7	15
Stop time	6.8 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES Source Parameters

Ionization mode	Positive
Capillary voltage	4,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V

MS parameters

Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 0.6 min (for interferences separation) or 1.2 min (for five amphetamines only) - diverter valve to MS
Delta EMV (+)	200 V

Results and Discussion

At acidic pH, the amine group of amphetamines was protonated, and the analytes were efficiently retained on Bond Elut Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without the loss of analytes from the sorbent. A strong base was added to organic eluent to break ionic interaction between the amphetamines and strong cation exchange sorbent. The recovery was optimized with two-component organic eluent consisting of 50% ethyl acetate and 50% methanol, with 20% NH_4OH added shortly before sample elution.

Amphetamines are rather volatile and could evaporate at the solvent evaporation step of sample preparation unless precipitated as salts by addition of the hydrochloric acid. It is best to add HCl toward the end of evaporation to avoid the formation of ammonium chloride salts which will cause ion suppression.

Figure 2 shows excellent separation of five amphetamines and potential interferences specified by SAMHSA on the Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column, which was completed within 3.2 minutes. LC separation started with a low fraction of organic solvent (15%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting the first portion of flow to waste to minimize source contamination. Data collection started immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed short separation and re-equilibration times.

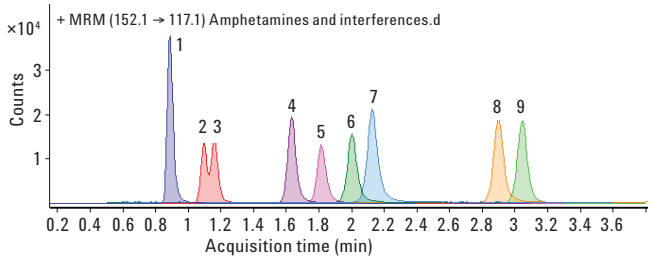

Figure 2. Separation of amphetamines and potential interferences on Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column – overlaid MRM extracted ion chromatograms. Concentration of each analyte corresponds to 50 ng/mL. Peaks in order of their elution are: 1. phenylpropanolamine, 2. ephedrine, 3. pseudoephedrine, 4. amphetamine, 5. methamphetamine, 6. MDA, 7. MDMA, 8. MDEA, 9. phentermine.

A dynamic MRM method using retention time and delta RT (time window) for a certain transition is recommended for the analysis of several compounds. When good separation from interferences is ensured, and data collection is focused on five amphetamines only, the valve can be switched from waste to mass spectrometer at 1.2 minutes instead of 0.6 minutes (time segment no. 1 in the MS method).

SAMHSA guidelines require the use of one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analytes (Table 1) was provided where possible for additional confidence. Agilent MassHunter Quantitative software calculated qualifier ion ratios, automatically highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound name	Precursor	Product	Fragmentor	Collision energy
Amphetamine	136.1	119.1	64	4
Amphetamine	136.1	91.1	64	14
Amphetamine-D_6	142.1	125.1	66	5
Amphetamine-D_6	142.1	93.1	66	13
MDA	180.1	163.1	92	5
MDA	180.1	105.1	92	17
MDA-D_5	185.1	168.1	68	5
MDA-D_5	185.1	110.1	68	21
MDEA	208.1	163.1	88	8
MDEA	208.1	133.1	88	17
MDEA	208.1	105.1	88	21
MDEA-D_6	214.2	166.1	90	8
MDEA-D_6	214.2	108.1	90	25
MDMA	194.1	163.1	84	5
MDMA	194.1	135.1	84	17
MDMA	194.1	105.1	84	21
MDMA-D_5	199.1	165.1	82	4
MDMA-D_5	199.1	107.1	82	25
Methamphetamine	150.1	119.1	80	4
Methamphetamine	150.1	91.1	80	16
Methamphetamine-D_9	159.2	125.2	77	5
Methamphetamine-D_9	159.2	93.1	77	13
Ephedrine-pseudoephedrine	166.1	133.1	80	21
Phentermine	150.1	133.1	80	6
Phenylpropanolamine	152.1	117.1	80	20

S/N ratios exceeding 400:1 were obtained for quantifier peaks of all five amphetamines at 25 ng/mL (Figure 3, upper panel: S/N is shown for the MDEA quantifier peak). This illustrated the state-of-the-art performance of the Agilent 6460 Triple Quadrupole LC/MS/MS capable of reliably detecting all five amphetamines at a small fraction of the SAMHSA cutoff.

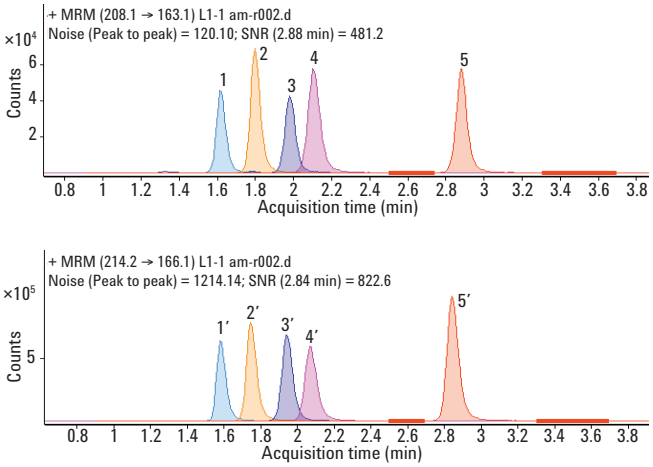

Figure 3. Overlaid MRM extracted ion chromatograms for amphetamines quantifiers (25 ng/mL) and ISTDs quantifiers (500 ng/mL) in urine extract on an Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Peaks in order of their elution are: upper panel - 1. amphetamine, 2. methamphetamine, 3. MDA, 4. MDMA, 5. MDEA, lower panel - 1′. amphetamine-D_6, 2′. methamphetamine-D_9 , 3′. MDA-D_5 , 4′. MDMA-D_5 , 5′. MDEA-D_6 . Noise regions are shown in bold.

Figure 4 gives examples of calibration curves for extracted urine standards at five concentration levels. Calibration standards were prepared by spiking negative urine at 25, 250, 1,000, 5,000, and 10,000 ng/mL with each of the five members of the amphetamines class. Deuterated internal standards for each analyte were added at 500 ng/mL. The excellent linear fits to all curves with $R^2 > 0.999$ demonstrated linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

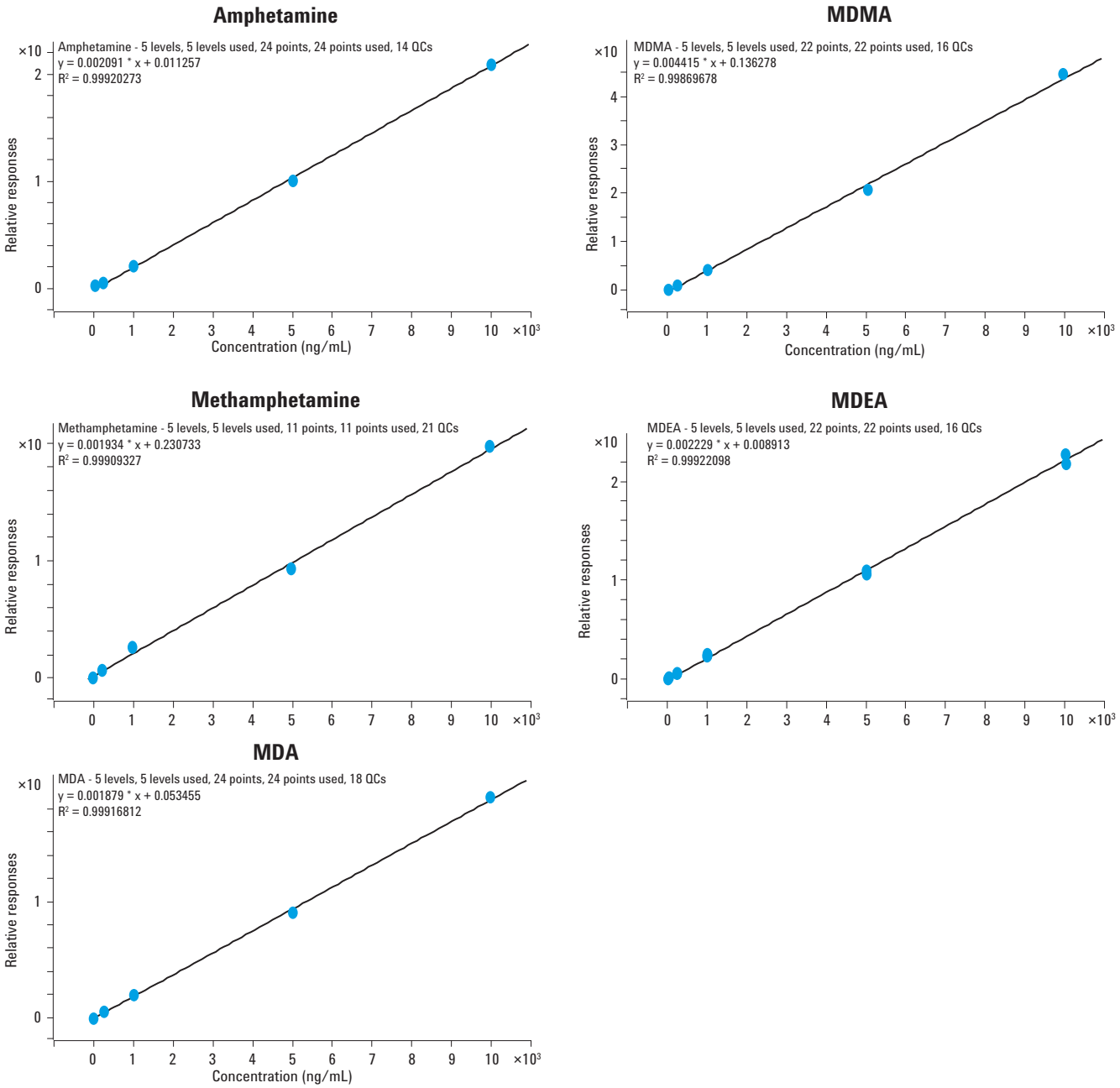

Figure 4. Example calibration curves for five amphetamines in urine extracts. Calibration range 25 to 10,000 ng/mL. All fits are linear, with $R^2 > 0.999$.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al* and widely accepted as an industry standard approach for LC/MS/MS methods [5]. The extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction with each of the five members of the amphetamines class at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase, and then fortified at 250 ng/mL (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 250 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows that the extraction recovery for all five amphetamines was ≥ 94%, with overall process efficiency higher than 90% in four out of five analytes; for amphetamine, process efficiency was 86%. The matrix effect of 91 to 99% means only a 1 to 9% signal reduction due to ion suppression, thus, confirming the exceptional cleanliness of Plexa PCX-processed extracts. High accuracy (within 10% of the target) and excellent precision (CV < 1.1%) is typical for this method.

Conclusions

The solid phase extraction procedure coupled with the LC/MS/MS detection method described here is SAMHSA-compliant and provides accurate, precise, and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. A hardware setup is the same as in other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 Series LC because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

Table 2. Method evaluations, n = 5.

Parameter	Amphetamine	Methamphetamine	MDA	MDMA	MDEA
Process efficiency* (%)	86	93	91	93	95
Extraction recovery* (%)	94	94	95	97	96
Matrix effect* (%)	91	99	95	96	98
Accuracy** (%)	107	105	92	101	106
Precision (CV)**(%)	0.6	0.5	1.1	0.5	0.3

*determined at cutoff level

**determined at 40% cutoff level for amphetamine, MDA, MDMA, MDEA, and at the cutoff level for methamphetamine

References

1. SAMHSA (2010) Manual for Urine Laboratories, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.
2. R. Baselt, (2008) *Disposition of Toxic Drugs and Chemicals in Man.* 8th edition. Atlas Books, Ashland, OH, USA.
3. P. Moorman and J. Hughes, (2010) "Amphetamines (expanded) in Urine by LC/Triple Quadrupole Mass Spectrometry (LC/MS/MS)". SOP, Agilent Technologies, Inc. Publication Number 5990-5865EN.
4. J. Hughes, and P. Moorman, (2011) "Confirmation by Triple Quadrupole LC/MS/MS for HHs-compliant Workplace Urine Drug Testing". Agilent Technologies, Inc. Seminar available from www.agilent.com/chem.
5. B. K. Matuszewski, M. L.Constanzer, and C. M. Chavez-Eng, (2003) "Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS". *Analytical Chemistry*, 75: 3019-3030.

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Agilent Technologies

SAMHSA-Compliant LC/MS/MS Analysis of Benzoylecgonine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of benzoylecgonine that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy, and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

Benzoylecgonine (BE) is a major urinary metabolite of cocaine. Cocaine hydrolysis to benzoylecgonine occurs enzymatically (in the liver), as well as without catalysts at alkaline pH [2]. The SAMHSA-established confirmation cutoff concentration for benzoylecgonine is 100 ng/mL, and a LOD at 10% of the cutoff would be 10 ng/mL [1].

The extraction method described in this application note provides reproducible high recoveries of benzoylecgonine due to unique properties of the Agilent Bond Elut Plexa polymer. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

A Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure, thereby allowing users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

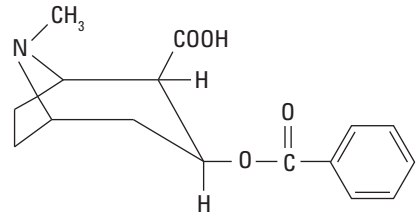
With a low sample injection volume of 2 µL and no sample preconcentration, the presented method demonstrates excellent signal-to-noise (S/N) ratios (> 400:1 at 10 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures [3,4].

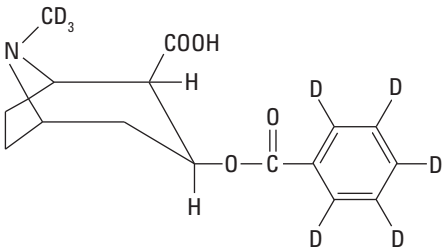
Experimental

Analytes

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (benzoylecgonine) and 100 µg/mL (benzoylecgonine-D_8) solutions in methanol.



Benzoylecgonine
Log P (predicted) -0.73–2.26
pK_{a1} 10.1



Benzoylecgonine –D_8

Figure 1. Benzoylecgonine analytes and their structures. Predicted log P values from DrugBank, ChemSpider, PubChem.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2-mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Pretreatment

Spike 1 mL of urine with ISTD at 200 ng/mL; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL methanol: ammonium hydroxide (100:20), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under a stream of nitrogen to dryness.
8. Reconstitute in 1 mL initial mobile phase (10% methanol, 90% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	10
	0.5	10
	2.5	70
	2.51	90
	5.5	90
	5.51	10
Stop time	5.6 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES source parameters	
Ionization mode	Positive
Capillary voltage	3,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V
MS parameters	
Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.2 min - diverter valve to MS
Delta EMV(+)	200 V

Results and Discussion

At acidic pH, the tertiary amine of benzoylecgonine was protonated, and the analyte was efficiently retained on Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without BE loss from the SPE column. A strong base was added to the organic eluent to break the ionic interaction between the analyte and the strong cation exchange sorbent. Benzoylecgonine recovery was optimized with 20% NH_4OH added to methanol shortly before sample elution.

The Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column provided fast separation of benzoylecgonine in urine extract and good peak shape (Figure 2). The LC separation started with a low fraction of the organic solvent (10%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting a first portion of flow (0 to 1.2 minutes) to waste to minimize source contamination. Data collection started at 1.2 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed for short analysis and re-equilibration times.

A S/N ratio >400:1 for the 10 ng/mL peak (Figure 2), upper panel) illustrates a state-of-the-art performance of the Agilent 6460 Triple Quadrupole capable of reliably detecting benzoylecgonine at a small fraction (10%) of the SAMHSA cutoff concentration.

SAMHSA guidelines require one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analyte (Table 1) was provided for additional confidence. The Agilent MassHunter Quantitative software automatically calculated qualifier ion ratios, highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
BE	290.1	168.1	90	15
BE	290.1	105.1	90	30
BE	290.1	82.1	90	32
BE-D_5	298.2	171.1	90	15
BE-D_5	298.2	110.1	90	30

Figure 3 is an example calibration curve for extracted urine standards at five concentration levels of benzoylecgonine. Calibration standards were prepared by spiking negative urine at 10, 100, 500, 1,000, and 4,000 ng/mL. Deuterated internal standard BE-D_8 was added at 200 ng/mL. The excellent linear fit with R^2 = 0.998 demonstrated linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

Normal, rather than dynamic, MRM scan type can be used with this method, because dynamic MRM has no advantages for detection of a single compound.

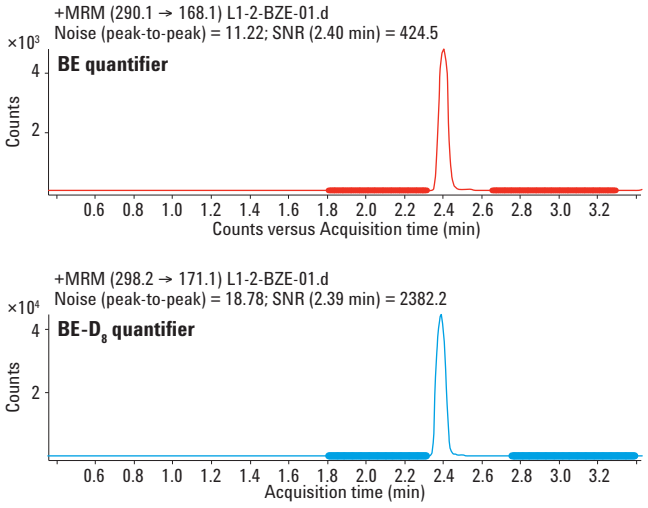


Figure 2. MRM extracted ion chromatograms for BE (10 ng/mL) and BE-D_8 (200 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

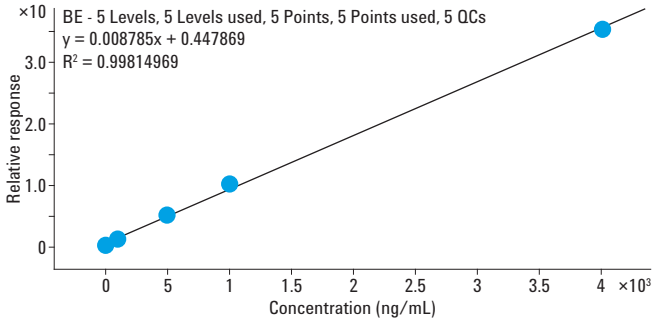


Figure 3. Example calibration curve for benzoylecgonine in urine extract. Calibration range 10 to 4,000 ng/mL. Linear fit, R^2 = 0.998.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al* and widely accepted as an industry standard approach for LC/MS/MS methods [5]. The extraction procedure and the LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 100 ng/mL with benzoylecgonine (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 100 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase.

Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows high extraction recovery for benzoylecgonine (86%) together with excellent accuracy (102%) and precision (0.7%). Matrix effect of 99% indicates minor ion suppression of a signal due to matrix interferences (1%), thus, confirming an exceptional cleanliness of Plexa PCX-processed extracts.

Table 2. Method evaluation at the cutoff level, n = 5.

	%
Process efficiency*	85
Extraction recovery*	86
Matrix effect*	99
Accuracy**	102
Precision** (CV)	0.7

*determined at cutoff level
**determined at 40% cutoff

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described in this application note is SAMHSA-compliant and provides accurate, precise, and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in the other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 Series LCs because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS/MS instruments. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

References

1. SAMHSA (2010) “Manual for Urine Laboratories”, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.
2. R. Baselt (2008) *Disposition of Toxic Drugs and Chemicals in Man*. 8th edition. Atlas Books, Ashland, OH, USA.
3. P. Moorman and J. Hughes (2010) “Benzoylecgonine in Urine by LC/Triple Quadrupole Mass Spectrometry (LC/MS/MS)”. SOP, Agilent Technologies, Inc. Publication number 5990-5866EN.
4. J. Hughes and P. Moorman (2011) “Confirmation by Triple Quadrupole LC/MS/MS for HHs-compliant Workplace Urine Drug Testing”. Agilent Technologies, Inc. Seminar available from www.agilent.com/chem.
5. B. K. Matuszewski, M. L. Constanzer, and C.M. Chavez-Eng (2003) “Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS”. *Analytical Chemistry*, 75: 3019-3030.

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SAMHSA-Compliant LC/MS/MS Analysis of Opiates (Morphine and Codeine) in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of opiates that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE, Agilent Poroshell 120 EC-C18, 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

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Introduction

Opiates (morphine and codeine) are natural alkaloids found in the resin of the opium poppy. In addition to detection of morphine and codeine, guidelines from SAMHSA require the confirmation method to demonstrate the ability to distinguish these drugs from structurally related compounds, such as the semisynthetic opioids: hydromorphone, oxymorphone, hydrocodone, oxycodone, and the codeine metabolite norcodeine [2].

Both morphine and codeine are extensively metabolized in the body. Morphine is metabolized primarily into morphine-3-glucuronide and morphine-6-glucuronide. Codeine's major metabolites are morphine, codeine-6-glucuronide, and norcodeine. Because both morphine and codeine are found in urine largely in the form of glucuronide conjugates, SAMHSA requires measurement of the total concentration of each compound. A full conversion of glucuronides back to parent species must be performed prior to analysis. The most reliable conversion method ensuring complete recovery of free opiates is acid hydrolysis. Frequently used enzymatic hydrolysis often leads to incomplete recovery of parent compounds which could lead to false negative results [3].

The SAMHSA-established confirmation cutoff concentration for morphine and codeine is 2,000 ng/mL [1]. Because high concentrations of opiates can be expected in some urine samples, we chose to use a higher capacity 3 mm id Poroshell 120 column instead of a 2 mm id column for all Agilent SAMHSA methods. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure. It, therefore, allows users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

The extraction method described in this application note provides reproducible high recoveries of morphine and codeine due to the unique properties of the Agilent Bond Elut Plexa polymer. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

With a low sample injection volume of 2 µL and no sample preconcentration, the method demonstrates excellent signal-to-noise (S/N) ratios for both morphine and codeine (>150:1 at 200 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures [4,5].

Experimental

Analytes

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (morphine, codeine, hydromorphone, norcodeine, hydrocodone, oxycodone, oxymorphone, and morphine-3-glucucronide) and 100 µg/mL (morphine-D_6 and codeine-D_6) solutions in methanol.

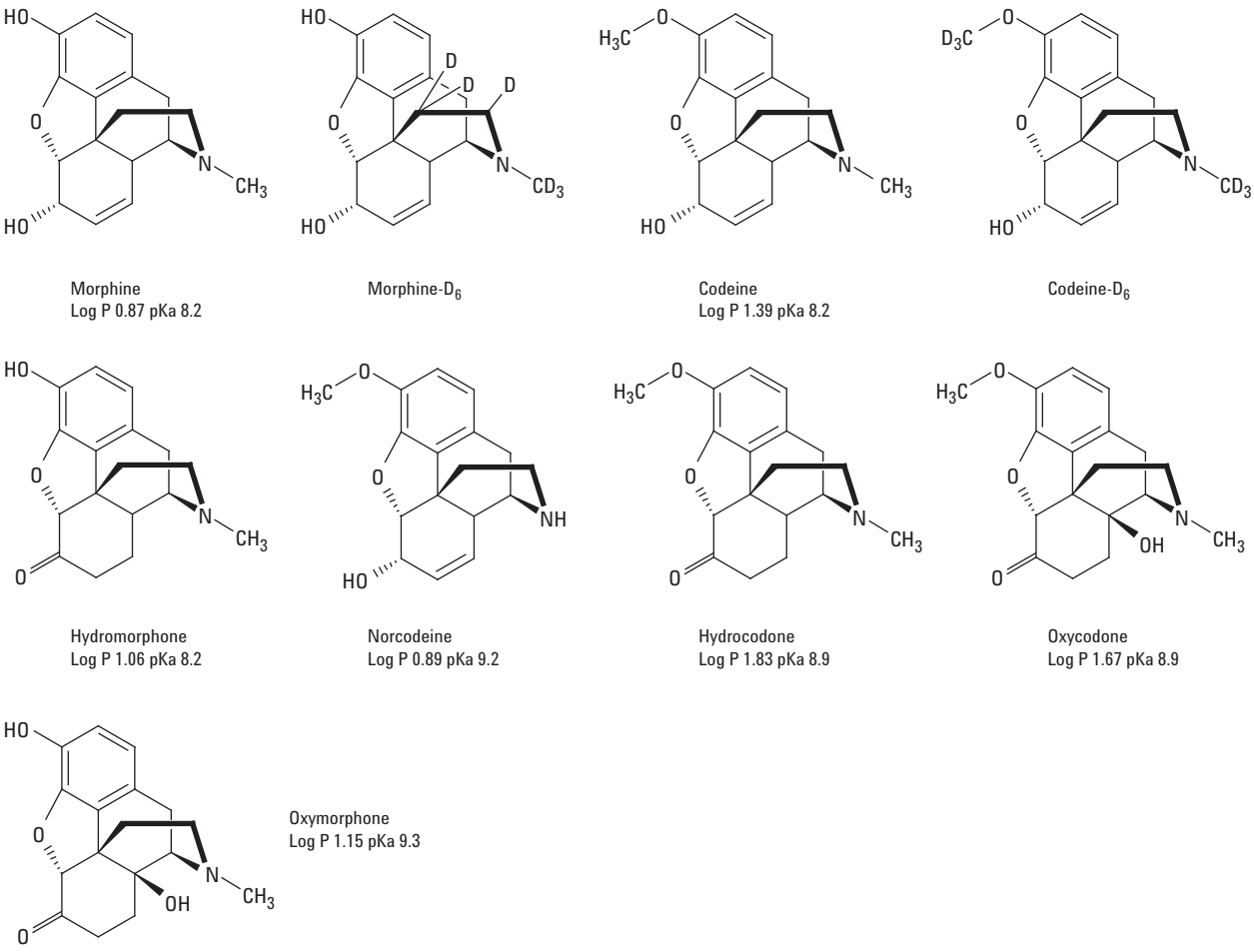

Figure 1. Opiate analytes and their structures.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2-mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Hydrolysis and sample pretreatment

1. Spike 0.5 mL of urine with ISTD at 1000 ng/mL; use of 12 × 75 mm glass tubes is recommended.
2. Add 125 µL concentration HCl.
3. Incubate in the heating block at 95 ±5 °C for 90 minutes.
4. Cool. Add 2 mL 0.1 M sodium acetate buffer (pH 4.5).
5. Neutralize with 250 µL 7 N KOH, vortex, and test pH; it should be <6.
6. Centrifuge 20 minutes at 6,000 rpm.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 2 mL methanol: ammonium hydroxide (100:20), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate to dryness at 40 °C.
8. Reconstitute in 0.5 mL initial mobile phase (5% methanol, 95% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	5
	0.5	5
	1.5	25
	2.5	55
	2.6	90
	5.6	90
	5.7	5
Stop time	5.8 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES source parameters

Ionization mode	Positive
Capillary voltage	3,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V

MS parameters

Scan type	Dynamic MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.0 min - diverter valve to MS
Delta EMV (+)	0 V

Results and Discussion

At low pH, morphine, codeine, and their derivatives were protonated at the tertiary amine group and were strongly retained on Plexa PCX polymeric sorbent by a combination of hydrophobic retention and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without loss of opiates from the SPE column. A strong base was added to the organic eluent to break ionic interaction between the analytes and the strong cation exchange sorbent. The opiates recovery was optimized with 20% NH_4OH added to methanol shortly before sample elution.

The Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column provided excellent separation and peak shapes for opiates and potentially interfering compounds, with the analysis completed within 2.5 minutes (Figure 2). LC separation started with a low fraction of organic solvent (5%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting a first portion of flow (0 to 1 minutes) to waste to minimize source contamination. Data collection started at 1.0 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed for short analysis and re-equilibration times.

The only partially unresolved pair in the chromatogram in Figure 2 were codeine and norcodeine (peaks 4 and 5), but because these compounds have different precursor ions and mass transitions, any possibility of interference of norcodeine signals with codeine quantitation was excluded.

In a separate experiment, Plexa PCX was tested for the possibility of norcodeine methylation and conversion to codeine. Test results were negative; no codeine was detected in negative urine samples that were spiked with norcodeine and then extracted using the method described in this application note.

When testing for interferences, a dynamic MRM method using retention time and delta RT (time window) for a certain transition is recommended. However, when good separation from interferences is ensured, data collection for morphine and codeine and their ISTDs can be performed with normal MRM.

SAMHSA guidelines require the use of one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for the target analyte is provided (Table 1) for additional confidence. Agilent MassHunter Quantitative software calculates qualifier ion ratios, automatically highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
Codeine	300.2	215.1	130	23
Codeine	300.2	165.1	130	46
Codeine	300.2	153.1	130	50
Codeine-D_6	306.2	165.1	130	44
Codeine-D_6	306.2	218.1	130	23
Morphine	286.1	201.1	130	23
Morphine	286.1	181.1	130	40
Morphine	286.1	165.1	130	43
Morphine-D_6	292.1	181.1	130	40
Morphine-D_6	292.1	165.1	130	42
Morphine-3-glucuronide	462.2	286.1	162	45
Oxycodone	316.2	298.1	130	15
Oxymorphone	302.2	284.1	130	17
Hydrocodone	300.2	199.1	130	30
Norcodeine	286.1	225.1	130	20
Hydromorphone	286.1	185.1	130	28

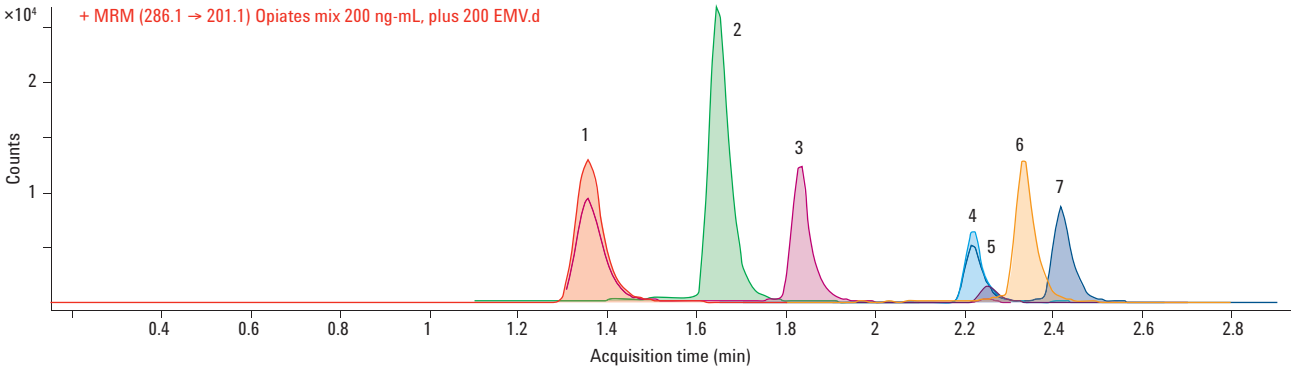

Figure 2. Separation of opiates and potential interferences on Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column - overlaid MRM extracted ion

When processed according to the protocol, urine samples spiked with morphine-ß-3-glucuronide at 10,000 ng/mL showed 97 to 99.2% conversion to morphine. MS parameters for the detection of morphine-ß-3-glucuronide are included in Table 1 for analysts interested in testing the hydrolysis efficiency.

S/N ratios exceeding 150:1 were obtained for quantifier peaks of morphine and codeine at 200 ng/mL (Figure 3, panel 1 and 2 from the top). This illustrates the state-of-the-art performance of the Agilent 6460 Triple Quadrupole LC/MS system, capable of reliably detecting opiates at a small fraction of the SAMHSA cutoff.

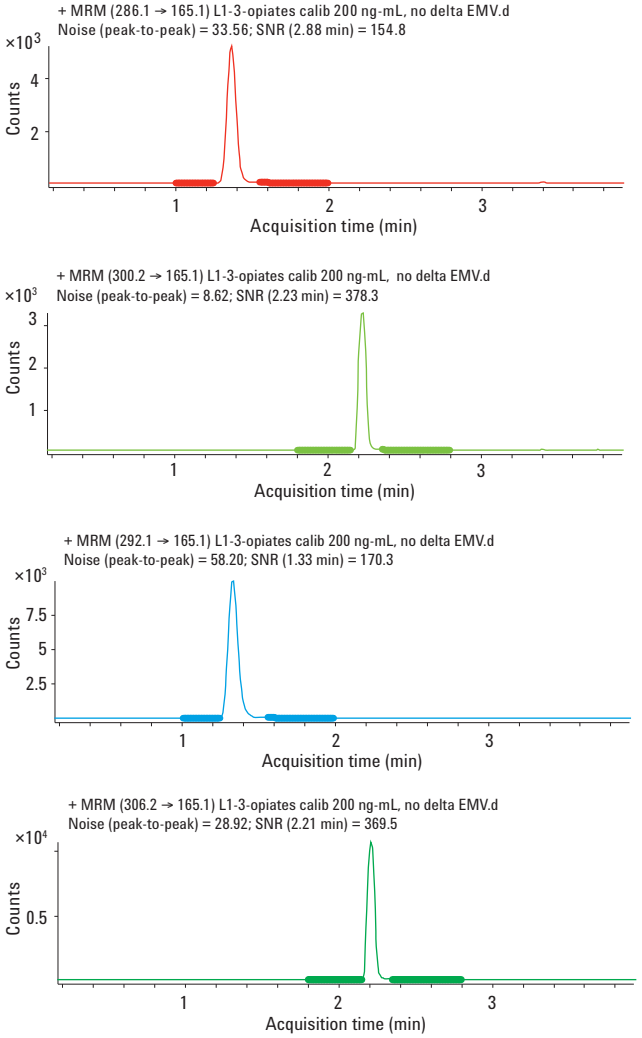

Figure 3. MRM extracted ion chromatograms for morphine and codeine quantifiers (200 ng/mL) and ISTD quantifiers (1,000 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

Figure 4 gives examples of calibration curves for extracted urine standards at five concentration levels. Calibration standards were prepared by spiking negative urine at 200, 1,000, 2,000, 10,000, and 20,000 ng/mL with morphine and codeine. Internal deuterated standard morphine-D_6 and codeine-D_6 were added at 1,000 ng/mL. Excellent linear fit ($R^2 \geq 0.998$) to each of the curves demonstrates linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

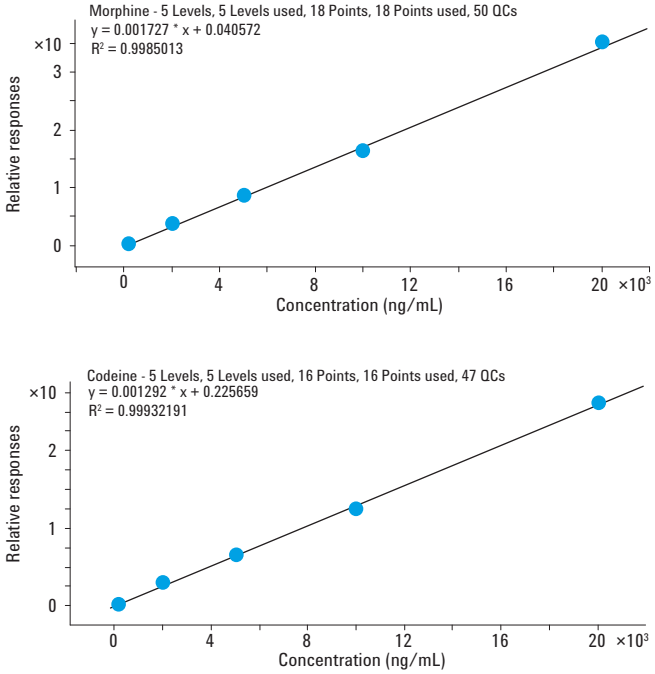

Figure 4. Example calibration curves for morphine (upper panel) and codeine (lower panel) in urine extract. Concentration range 200 to 20,000 ng/mL. Linear fit, $R^2 \geq 0.998$.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al*. and widely accepted as an industry standard approach for LC/MS/MS methods [6]. The extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction with morphine and codeine at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 2,000 ng/mL (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 2,000 ng/mL in urine (spiked mobile phase).

Table 2. Method evaluation of opiates at the cutoff level, n = 5.

Parameter	Morphine	Codeine
Process efficiency (%)	83	85
Extraction recovery (%)	85	86
Matrix effect (%)	98	99
Accuracy (%)	108	108
Precision (CV) (%)	0.6	0.7

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements

Table 2 shows high extraction recovery and process efficiency for morphine and codeine (approximately 85%). The high matrix effect value (98–99%) means only 1 to 2% signal reduction is due to ion suppression, thus, confirming the exceptional cleanliness of Plexa PCX-processed extracts. High accuracy (within 10% of the target) and excellent precision (CV<1%) are typical for the method.

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described in this application note is SAMHSA-compliant and provides reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 Series LCs because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

References

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2. R. Baselt (2008) Disposition of Toxic Drugs and Chemicals in Man. 8th edition. Atlas Books, Ashland, OH, USA.

3. P. Wang, J. A. Stone, K. H. Chen, S. F. Gross, C. A. Haller, and A. H. Wu (2006) Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites. *Journal of Analytical Toxicology*, 30: 570-575.

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5. J. Hughes and P. Moorman (2011) "Confirmation by Triple Quadrupole LC/MS/MS for HHs-compliant Workplace Urine Drug Testing". Agilent Technologies, Inc. Seminar available from www.agilent.com/chem.

6. B. K. Matuszewski, M. L. Constanzer, and C.M. Chavez-Eng (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, 75: 3019-3030.

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SAMHSA-Compliant LC/MS/MS Analysis of Phencyclidine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

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Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of phencyclidine that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18, 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

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Introduction

Phencyclidine (PCP) is a synthetic drug, a member of the family of dissociative anesthetics. Five to 20 % of administered PCP is excreted unchanged in urine [2]. Therefore, the drug can be detected in its original form and neither hydrolysis nor metabolite measurement are needed. PCP is stable in biological samples. In frozen urine samples, it is preserved for a year, and refrigeration at 4 °C is sufficient for short-term storage.

Phencyclidine has a three-ring structure, with one aryl, one cyclohexane, and one piperidine ring (Figure 1). It is a weak organic base, essentially nonpolar, with a high log P of 4.69. The new SAMHSA confirmation cutoff concentration for phencyclidine is 25 ng/mL, and a LOD at 10% of the cutoff is 2.5 ng/mL [1].

The simple extraction method described in this application note provides reproducible high recoveries of PCP due to the unique properties of the Agilent Bond Elut Plexa polymer. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface which excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

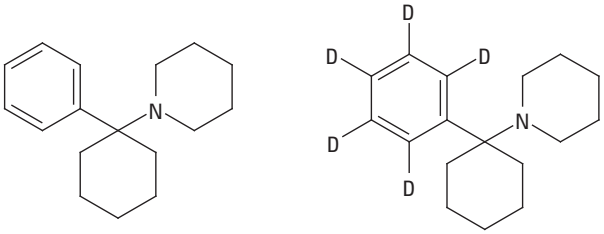
A Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7-µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure, thereby allowing users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

With a low sample injection volume of 2 µL and no sample preconcentration, the method demonstrates excellent signal-to-noise (S/N) ratios (>200:1 at 2.5 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures [3,4].

Experimental

Analytes



Phencyclidine
Log P 4.69
pK_a 8.5

Phencyclidine-D_5

Figure 1. Phencyclidine analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (phencyclidine) and 100 µg/mL (phencyclidine-D_5) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2 mL autosampler vials (p/n 5182-0716) or silanized vials (p/n 5183-2072)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Pretreatment

Spike 1 mL of urine with ISTD at 50 ng/mL; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL ethyl acetate: methanol: ammonium hydroxide (80:20:5), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under stream of nitrogen to dryness.
8. Reconstitute in 1 mL initial mobile phase (10% methanol, 90% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	10
	0.5	10
	2.5	70
	2.51	90
	5.5	90
	5.51	10
Stop time	5.6 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES source parameters

Ionization mode	Positive
Capillary voltage	3,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V

MS parameters

Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.2 min - diverter valve to MS
Delta EMV (+)	200 V

Results and Discussion

At acidic pH, the tertiary amine of phencyclidine was protonated, and the analyte was efficiently retained on Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without PCP loss from the SPE column. A strong base was added to the organic eluent to break the ionic interaction between the analyte and the strong cation exchange sorbent. PCP recovery is optimized with a two-component organic eluent consisting of 80% ethyl acetate and 20% methanol, with 5% NH_4OH added shortly before sample elution.

The Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column provided fast separation of phencyclidine in urine extract and good peak shape (Figure 2). The LC separation started with a low fraction of organic solvent (10%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting the first portion of flow to waste to minimize source contamination. Data collection started at 1.2 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed short retention and re-equilibration times.

A S/N ratio >200:1 for the 2.5 ng/mL peak (Figure 2, upper panel) illustrates state-of-the-art performance of the 6460 Triple Quadrupole LC/MS system, capable of reliably detecting PCP at a small fraction (10%) of the SAMHSA cutoff concentration. Being very hydrophobic, phencyclidine has the potential to adhere to any active surfaces. To avoid carryover, we recommend using the external needle wash flush port option of the high performance autosampler, and running a mobile phase blank after samples, which appear from screening results to have a high concentration. If needed, the needle wash can be increased from 10 to 20 seconds.

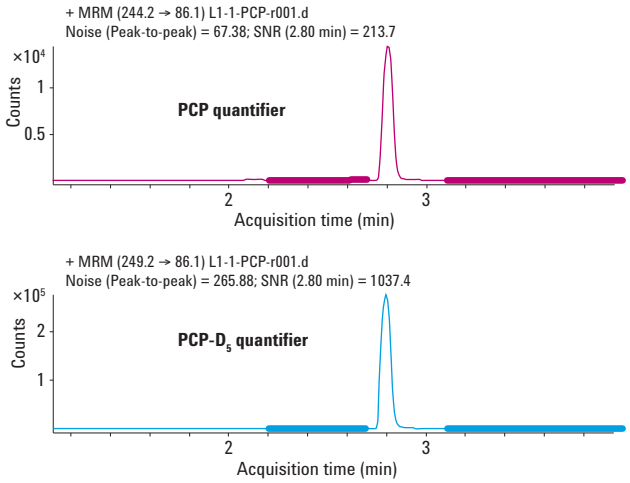


Figure 2. MRM extracted ion chromatograms for PCP (2.5 ng/mL) and PCP-D_5 (50 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm, column. Noise regions are shown in bold.

SAMHSA guidelines require one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analyte (Table 1) is provided for additional confidence. Agilent MassHunter Quantitative software automatically calculates qualifier ion ratios, highlighting those out of acceptable range.

Figure 3 shows an example calibration curve for extracted urine standards at five concentration levels of phencyclidine. Calibration standards were prepared by spiking negative urine at 2.5, 25, 100, 250, and 1,000 ng/mL. Deuterated internal standard PCP-D_5 was added at 50 ng/mL. Excellent linear fit with $R^2 > 0.999$ demonstrates the linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

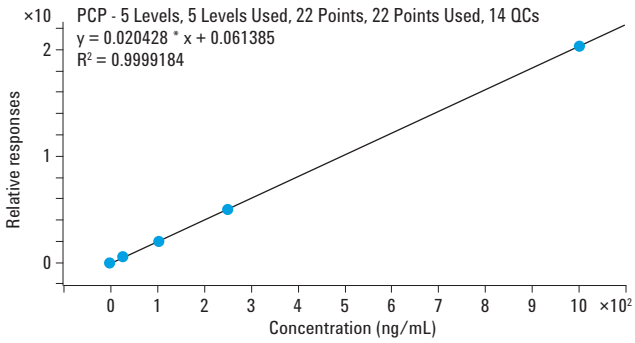


Figure 3. Example calibration curve for phencyclidine in urine extract. Calibration range 2.5 to 1000 ng/mL. Linear fit, R^2>0.999.

Table 1. MRM Transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
PCP	244.2	86.1	80	7
PCP	244.2	159.1	80	7
PCP	244.2	91.1	80	34
PCP-D_5	249.2	164.1	80	7
PCP-D_5	249.2	86.1	80	7

Normal, rather than dynamic, MRM scan type can be used with this method, since dynamic MRM has no advantages for detection of a single compound.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles proposed by Matuszewski *et al.* and widely accepted as an industry standard approach for LC/MS/MS methods [5]. Extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 25 ng/mL with PCP (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 25 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine extract spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows high extraction recovery for phencyclidine (85%) together with very good accuracy (93%) and precision (0.5 %). Matrix effect of 98% indicates only minor ion suppression of the signal due to matrix interferences (2%), thus confirming an exceptional cleanliness of Plexa PCX-processed extracts.

Table 2. Method performance for phencyclidine, n = 5.

	%
Process efficiency	83
Extraction recovery	85
Matrix effect	98
Accuracy	93
Precision (CV)	0.5

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described in this application note is SAMHSA-compliant and provides accurate, precise and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in the other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 LC series since the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

References

1. SAMHSA (2010) Manual for Urine Laboratories, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.

2. R. Baselt (2008) Disposition of Toxic Drugs and Chemicals in Man. 8th edition. Atlas Books, Ashland, OH, USA.

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SAMHSA-Compliant LC/MS/MS Analysis of 11-nor-9-carboxy-Δ^9-Tetrahydrocannabinol in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

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Agilent Technologies, Inc.

Abstract

Guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA) effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. This application note presents a method for analysis of 11-nor-9-carboxy-Δ^9– tetrahydrocannabinol that meets SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy and precision, as well as measurement of matrix effects, extraction recovery and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products such as Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

11-Nor-9-carboxy-Δ^9– tetrahydrocannabinol (THCA, "THC-acid", THC-COOH) is a metabolite of tetrahydrocannabinol (Δ^9–THC), an active constituent of marijuana. In the form of its glucuronide conjugates, THCA is excreted in urine for several weeks [2]. The SAMHSA confirmation cutoff concentration for THCA is 15 ng/mL and a LOD at 10% of the cutoff would be 1.5 ng/mL.

Sample preparation for 11-nor-9-carboxy-Δ^9–THC analysis requires base hydrolysis of urine to convert glucuronides back to THCA. Although THCA is a carboxylic acid, for the sake of a single method setup for all SAMHSA-regulated drugs, the Agilent sorbent chosen for extraction is Agilent Bond Elut Plexa PCX, a mixed-mode cation-exchange polymer. It efficiently retains THCA by hydrophobic interaction.

The extraction method provides reproducible high recoveries of THCA due to the unique properties of the Plexa sorbent. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

An Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7 µm particles, the Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns, with approximately 40% less back pressure, thereby allowing the users of even 400 bar LC systems to increase resolution and shorten analysis and re-equilibration times by applying a higher flow rate.

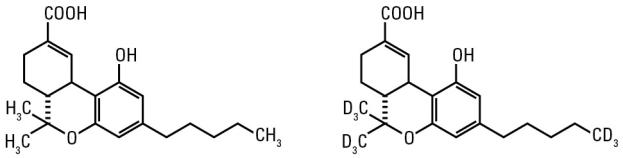
Being essentially nonpolar (log P>6), cannabinoids are not ideally suited for electrospray ionization and are often analyzed using APCI. However, due to its carboxylic moiety, THCA is much more efficiently ionized in negative ion mode than Δ^9–THC and 11-hydroxy- Δ^9–THC. A choice of electrospray source for THCA detection is warranted by the convenience of a single mass spectrometer configuration for all SAMHSA drugs.

With a low sample injection volume of 10 µL and no sample preconcentration, the method demonstrates excellent signal-to-noise ratios for cutoff and 10% of the cutoff concentrations (approximately 100:1 and 10:1, respectively) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the Jet Stream electrospray source.

Previous methods from Agilent [3,4] used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures.

Experimental

Analytes



11-nor-9-carboxy-Δ^9-THC
Log P (predicted) 6.06-6.36, pKa 4.5

11-nor-9-carboxy-Δ^9-THC-D_9

Figure 1. 11-nor- carboxy-Δ^9- tetrahydrocannabinol analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (11-nor-9-carboxy-Δ^9-THC) and 100 µg/mL (11-nor-9-carboxy-Δ^9-THC-D_9 and 11-nor-9-carboxy-Δ^9-THC-glucuronide) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent silanized 2 mL autosampler vials (p/n 5183-2072)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC system (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Hydrolysis and sample pretreatment

1. Spike 0.5 mL of urine with ISTD at 50 ng/mL; use of methanol-rinsed and 12 × 75 mm dried glass tubes is recommended.
2. Add 100 µL 7 N KOH, vortex.
3. Incubate in the heating block at 60 ±5 °C for 30 minutes.
4. Cool. Add 125 µL methanol, vortex.
5. Add 1.5 mL of 0.2 M sodium acetate buffer (pH 4).
6. Neutralize with 100 µL glacial acetic acid, vortex.
7. Centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol–soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 2 × 2 mL 10:90 ACN: 2% acetic acid.
4. Wash 2: 2 mL 30:70 ACN: 2% acetic acid.
5. Dry 5–10 minutes under high vacuum (10–15 in Hg).
6. Wash with 200 µL hexane, pull through with low vacuum (2–3 in Hg).
7. Dry under high vacuum, 3 to 4 minutes.
8. Elute with 0.5 mL 80:20 ethyl acetate:isopropanol. Soak, let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
9. Add 1 mL more of the same eluent, repeat soaking-elution procedure.
10. Evaporate to dryness at 40 °C.
11. Reconstitute in 0.5 mL initial mobile phase (30% methanol, 70% 5 mM ammonium formate).

LC/MS/MS

LC conditions

Mobile phase A	5 mM ammonium formate in water
Mobile phase B	methanol
Flow rate	0.8 mL/min

Gradient	Time (min)	% B
	0.0	30
	1	95
	5	95
	5.1	30

Stop time	5.2 minutes
Post time	2 minutes
Max pump pressure	400 bar
Injection volume	10 µL
Needle wash	Flush port 75:25 methanol:water for 10 seconds

Disable overlapped injection

No automatic delay volume reduction

MS conditions

ES Source Parameters

Ionization mode	negative
Capillary voltage	4,000 V
Drying gas flow	11 L/min
Drying gas temperature	320 °C
Nebulizer gas	18 psi
Sheath gas flow	12 L/min
Sheath gas temperature	320 °C
Nozzle voltage	0 V

MS parameters

Scan type	MRM

Pre-run script
SCP_MSDiverterValveToWaste(){MH_Acq_Scripts.exe}

Time segments	#1: 1.4 minutes - diverter valve to MS
Delta EMV (-)	800 V

Results and Discussion

The cannabinoids are notorious for their adsorption to glass and plastic. To minimize losses and to ensure method reproducibility, we strongly recommend the use of only freshly prepared stock solutions and calibrators, silanized or thoroughly washed, methanol-rinsed and dried glassware, and analyze final extracts immediately after reconstitution.

THCA is retained on a cation-exchange mixed mode Plexa PCX by hydrophobic interactions. The 100% methanol wash, commonly employed in ion-exchange SPE, is not practical for THCA extraction as high organic will elute the compound from the sorbent.

To minimize matrix interferences, 10 to 30% acetonitrile is added to wash one and two, respectively. The hexane wash serves the same purpose. When used alone and in a small amount (200 µL), hexane elutes most lipids but does not lead to analyte desorption, because THCA is very hydrophobic (log P>6) and is retained at the hydrophobic core of the Plexa particles very strongly. A soaking procedure is recommended at the elution step to enhance the solvent-analyte interaction and improve analyte recoveries.

The Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column provides fast separation of THCA in urine extract and good peak shape (Figure 2). The LC separation intentionally begins with a relatively low fraction of organic solvent (30%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Due to a steep gradient, the remaining hydrophobic interferences largely elute before the analyte, thus reducing matrix effect at the time of peak elution (1.96 minutes). A flow rate of 0.8 mL/min allows for a short retention and re-equilibration time. Each sample run begins with diverting a first portion of flow (0 to 1.4 minutes) to waste to minimize source contamination. Data collection begins at 1.4 minutes, immediately after the diverter valve switch.

SAMHSA guidelines require the use of one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analyte is provided for additional confidence (Table 1). Agilent MassHunter Quantitative software automatically calculates qualifier ion ratios, highlighting those out of acceptable range.

Table 1. MRM Transitions

Compound	Parent	Product	Fragmentor	Collision energy
11-nor-9-carboxy-Δ^9-THC	343.2	299.2	135	18
	343.2	245.1	135	30
	343.2	191.1	135	33
11-nor-9-carboxy-Δ^9-THC-D_9	352.2	308.2	145	18
	352.2	254.2	145	30
11-nor-9-carboxy-Δ^9-THC glucuronide	519.2	343.2	160	22
	519.2	299.2	160	36

When processed according to the protocol, urine samples spiked with 11-nor-9-carboxy-Δ^9-THC-glucuronide at 1,000 ng/mL tested negative for this compound. Instead, they displayed a very large THCA peak, far beyond the upper calibration level of 600 ng/mL. This is proof of full conversion of glucuronides to THCA by the base hydrolysis step. MS parameters for the detection of 11-nor-9-carboxy-Δ^9-THC-glucuronide are included in Table 1 for analysts interested in testing the hydrolysis efficiency.

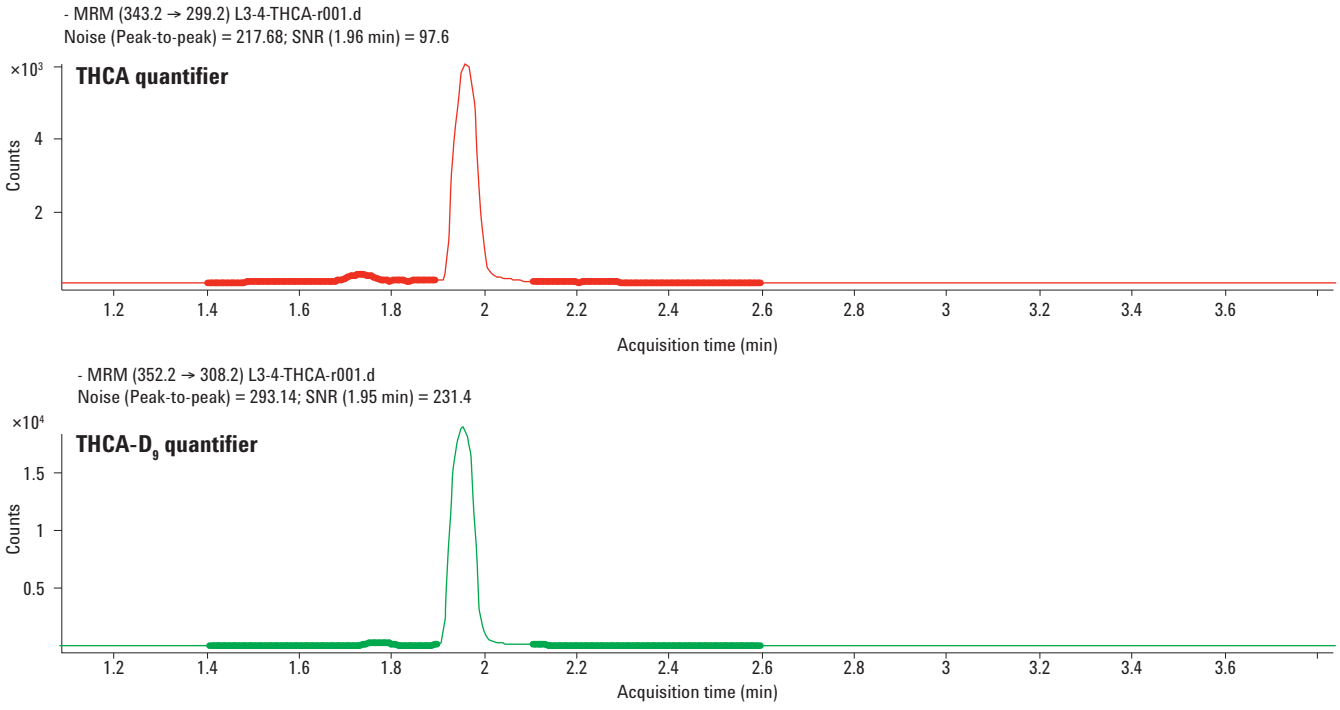


Figure 2. MRM extracted ion chromatograms for THCA (15 ng/mL) THCA-D_9 (50 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

Normal, rather than dynamic, MRM acquisition mode can be used with this method, since dynamic MRM has no advantages for detection of a single peak.

Due to its extreme hydrophobicity, THCA can adhere not only to glassware but also to injector parts and tubing. To avoid carryover, we recommend running a mobile phase blank after samples with high concentration, and to use the Injector Flush Pump option of the autosampler. If needed, the needle wash can be increased from 10 to 20 seconds.

A signal-to-noise ratio approximately 100:1 for the cutoff concentration of 15 ng/mL for THCA (Figure 2, upper panel) illustrates excellent performance of the 6460 Triple Quadrupole LC/MS system, capable of reliably detecting THCA at a small fraction (10%) of the SAMHSA cutoff concentration.

Figure 3 shows a calibration curve for extracted urine standards at five concentration levels. Calibration standards were prepared by spiking negative urine at 1.5, 15, 75, 300, and 600 ng/mL with THCA. Deuterated internal standard THCA-D_9 was added at 50 ng/mL. Excellent linear fit ($R^2 > 0.999$) demonstrates linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al*. [5] and widely accepted as an industry standard approach for LC/MS/MS methods. Extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 15 ng/mL with THCA (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 15 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine extract spiked post-SPE to its peak area in spiked mobile phase.

Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

The method is characterized by good recoveries together with very high accuracy (98%) and precision (2.2%) of the data (Table 2). Matrix effect in excess of 100% indicates ionization enhancement as opposed to ionization suppression. Signal enhancement of only 13% confirms cleanliness of Plexa PCX extracts. Overall process efficiency of 73% is rather high due to analytical challenge associated with the cannabinoid family.

Table 2. Method Performance for 11-nor- carboxy-Δ^9- tetrahydrocannabinol at the Cutoff Level, n = 5

	%
Process efficiency	73
Extraction recovery	65
Matrix effect	113
Accuracy	98.2
Precision (CV)	2.2

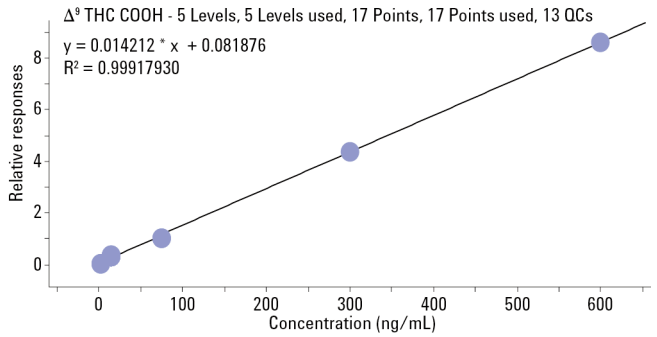


Figure 3. Example calibration curve for THCA in urine extract. Calibration range 1.5 to 600 ng/mL. Linear fit, R^2>0.999.

Conclusions

The solid phase extraction procedure coupled with the LC/MS/MS detection method described is SAMHSA-compliant and provides reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The THCA method uses the same hardware setup as the other Agilent SAMHSA methods. These methods are usable with all models of Agilent 1100 and Agilent 1200 LC series, since the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

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1. SAMHSA (2010) Manual for Urine Laboratories, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.
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Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

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Introduction

In 2011, five members of the "synthetic cannabinoids" group or 'Spice' compounds were banned in the USA. The substances were:

- 1-pentyl-3-(1-naphthoyl)-indole (JWH-018)
- 1-butyl-3-(1-naphthoyl)-indole (JWH-073)
- 1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)-indole (JWH-200)
- 5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (CP-47,497)
- 5-(1,1-dimethyloctyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (cannabicyclohexanol; CP-47,497 C8 homologue)

The drugs have been described as having cannabis-like effects, and some of these compounds show strong binding to cannabinoid receptors. The (–)-1,1-dimethylheptyl analog of 11-hydroxy-Δ^8-tetrahydrocannabinol, (1,1-dimethylheptyl-11-hydroxytetrahydrocannabinol) is known as HU-210 and has been reportedly found in seizures of "Spice Gold", "Spice Silver" and "Spice Diamond" made by the US Customs and Border Protection in 2009. HU-210 is considered to be more potent than Δ^9-tetrahydrocannabinol (Δ^9-THC), and was already classified as a controlled substance as an analog of marijuana. JWH-250 is also commonly encountered so was also included in the research.

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Oral fluid is becoming increasingly popular as a specimen for the detection of drugs at the roadside, and in workplace testing. It is easy to collect, non-invasive and can give information on recent drug intake. In the work described here, the Quantisal device was used for oral fluid collection, and the detection of "Spice" components is described.

Collection devices, reagents and standards

Quantisal devices for the collection of oral fluid specimens contain a cotton collection pad which is placed in the mouth. The incorporated volume adequacy indicator turns blue when 1 mL of oral fluid (± 10%) has been collected, then the pad is placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). Drug concentrations detected are adjusted accordingly.

Solid phase extraction columns (Bond Elut Plexa) and liquid chromatographic columns (ZORBAX RRHT) were obtained from Agilent Technologies. The standard compounds JWH-018, JWH-073, JWH-200, JWH-250, HU-210, CP-47,497 and CP-47,497 C8 homologue as well as deuterated *d9*-JWH-018 and *d7*-JWH-073 were purchased from Cayman Chemicals.

Calibrators and controls

The deuterated internal standards (*d9*-JWH-018 and *d7*-JWH-073) and unlabelled drug standards were prepared in methanol at a concentration of 100 µg/mL. The working solutions were diluted from stock to a concentration of 10 µg/mL in methanol. The solutions were stored at –20 °C when not in use. Controls were prepared by fortifying drug-free synthetic oral fluid with various concentrations of compounds. Drug free negative specimens, positive controls at 4 ng/mL and 40 ng/mL were included in every batch.

Sample preparation

Seven calibration standards were prepared in oral fluid at concentrations of 0.5, 2, 5, 10, 20, 50, and 100 ng/mL for all analytes; deuterated internal standards were added (10 ng/mL).

Agilent Bond Elut Plexa (30 mg/1 mL; p/n 12109301) solid phase extraction cartridges were used.

1. Condition: methanol (0.5 mL); 0.1 M acetic acid (0.1 mL)
2. To each 1mL aliquot of calibrator, control or specimen, add acetic acid (0.1 M; pH 4, 1 mL)
3. Load samples
4. Wash columns: DI water: glacial acetic acid (80:20; 1 mL); DI water: methanol (40:60; 1 mL)
5. Dry columns (5 minutes)
6. Elute acidic/neutral compounds: hexane: glacial acetic acid (98:2; 2 mL)
7. Evaporate extracts to dryness while allowing columns to dry (7 minutes)
8. Elute bases into corresponding tubes: ethyl acetate: ammonium hydroxide (98:2; 2 mL)
9. Evaporate to dryness under nitrogen at 40 °C
10. Reconstitute in methanol (50 µL); transfer to autosampler vials; cap
11. Analyze using LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

An Agilent Technologies 1200 Series liquid chromatography pump coupled to an Agilent 6430 Triple Quadrupole LC/MS System, operating in electrospray ionization mode (ESI) with either positive or negative polarity depending on the compound.

Column	Agilent ZORBAX RRHT Extend C18, (2.1 × 50 mm, 1.8 µm, p/n 727700-902)	
Column temperature	60 °C	
Injection volume	5 µL	
Mobile phase	Solvent A: 0.2% acetic acid and Solvent B: acetonitrile	
	Time 0:	95% A; 5% B; 5 min: 100% B; 7 min 5% B
	Run time	9.2 min; Post-time 3 min
	Flow rate:	0.5 mL/min
Nitrogen gas temperature	350 °C	
Gas flow	10 L/min	
Nebulizer pressure	55 psi.	
Capillary voltage	+4,000 V in positive mode; –4,000 V in negative mode	

Two transitions were selected and optimized for each drug. Table 1 shows the transitions, the optimized fragment voltages for the parent ion (M +1; M-1) as well as the collision energy for fragmentation of the product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ± 20% in order to meet the criterion for a positive result.

Table 1. Multiple Reaction Monitoring (MRM) Transitions; Optimized Fragmentation Voltages; Allowable Transition Ranges Determined at 10 µg/mL for "Spice" Compounds

Compound	Transition	Fragment voltage (V)	Collision energy (eV)	Polarity	Ratio of quantifying to qualifying transition (range)
d9-JWH-018	351.3 > 223.4	140	20	Positive	n/a
JWH-018	342.2 > 155.1	120	20	Positive	16–24
	342.2 > 214.2	120	20		
JWH-250	336.3 > 200.2	120	12	Positive	69–104
	336.3 > 188.2	120	20		
d7-JWH-073	335.3 > 207.2	120	20	Positive	n/a
JWH-073	328.2 > 155.1	120	20	Positive	60–90
	328.2 >127.1	120	35		
JWH-200	385.3 > 155.1	140	20	Positive	54–81
	385.3 > 114.2	140	25		
CP 47497 C8	331.3 > 313.3	160	25	Negative	70–104
	331.3 > 259.3	160	35		
CP 47497	317.3 > 299.2	160	20	Negative	75–113
	317.3 > 245.2	160	30		
HU-210	385.3 > 367.4	120	30	Negative	13–20
	385.3 > 281.3	120	45		

Underlined transitions used for quantitation; n/a = not applicable for internal standard

Figure 1 shows a chromatogram for the primary transitions of the compound at a concentration of 10 ng/mL; the ratio of primary to secondary transition for each compound was also determined at 10 ng/mL.

Recovery from the collection pad

Six synthetic oral fluid specimens fortified with the compounds at concentrations of 4 and 40 ng/mL were prepared. The collection pad was placed into the samples until 1 mL (±10%) had been collected, as evidenced by the blue volume adequacy indicator incorporated into the stem of the collector, then the pad was transferred to the Quantisal buffer, capped and stored overnight to simulate transportation to the laboratory. The following day an aliquot of the specimen was analyzed. The amount recovered from the pad was compared to an absolute concentration (100%) where drug was added to the buffer and left overnight at room temperature without the pad, then subjected to extraction and analysis.

The percentage recovery from the pad for the compounds at concentrations of 4 and 40 ng/mL (n = 6) were > 60% for all at both levels. The highest recovery was 86% for HU-210 at 4 ng/mL; the lowest was 61% for JWH-073 at 40 ng/mL. The recoveries were essentially equivalent at both levels (Table 2).

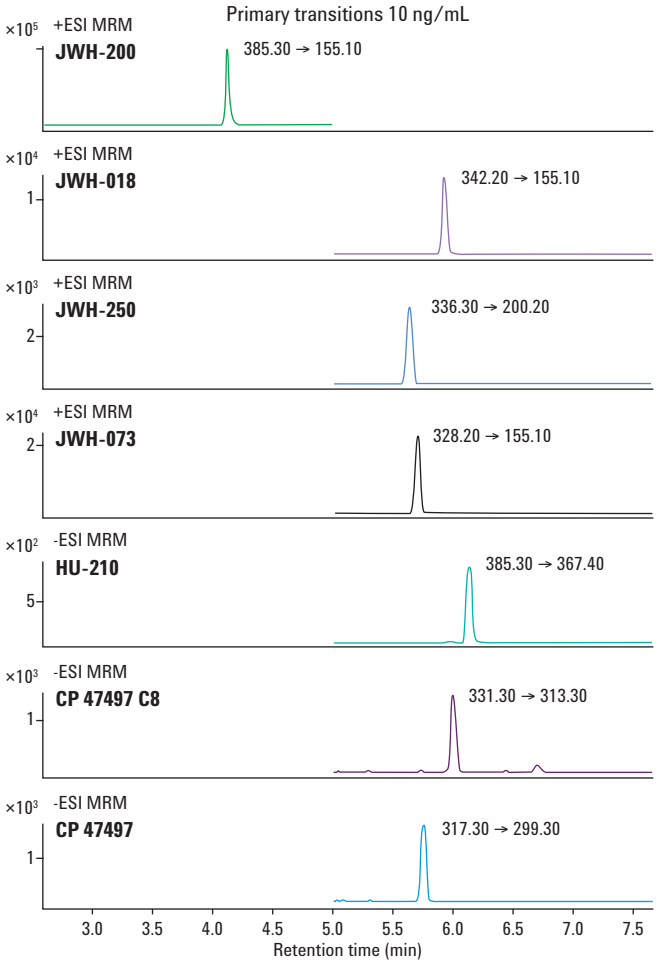

Figure 1. Primary transition at 10 ng/mL.

Table 2. Method Evaluation

	JWH-018	JWH-073	JWH-200	JWH-250	CP 47497	CP 47497 C8	HU-210
LOQ (ng/mL)	0.5	0.5	0.5	2	0.5	2	5
Imprecision intra-day							
4 ng/mL	3.9%	3.6%	5.0%	3.4%	4.9%	3.9%	8.6%
40 ng/mL	2.2%	2.1%	6.0%	2.0%	4.1%	4.3%	5.6%
Inter-day							
4 ng/mL	8.8%	9.6%	6.2%	11%	7.7%	11%	10%
40 ng/mL	8.5%	7.9%	6.2%	11%	10%	11%	12%
Pad recovery							
4 ng/mL	65.5%	67.4%	85.0%	66.5%	77.7%	76.0%	86.4%
40 ng/mL	70.6%	61.4%	81.4%	75.1%	71.3%	78.2%	75.7%
Matrix effect	-55%	-45%	-55%	-73%	-64%	-55%	-49%
Process efficiency	40%	51%	56%	24%	38%	45%	51%

Data Analysis

Calibration was carried out using linear regression analysis over a concentration range of 0.5–100 ng/mL. Peak area ratios of target analytes and the internal standard were calculated for each concentration using Agilent MSD software. The data were fit to a linear least squares regression curve, not forced through the origin, and with equal weighting. For confirmation, two transitions were monitored for each of the compounds; one for the internal standard. The ratio of the qualifying transition was required to be within 20% of that established using the known calibration standard to be acceptable.

Linearity and sensitivity

The limit of quantitation (LOQ) of the method was determined using serial dilutions to the lowest point where the acceptable criteria for the quantitation of a compound were met, that is, the chromatographic peak shape, retention time (within 2% of calibration standard), and qualifier transition ratio (± 20%) compared to the 10 ng/mL calibration standard were acceptable. The quantitative value of the LOQ had to be within ± 20% of the target concentration. The limit of quantitation was 0.5 ng/mL for JWH-018, JWH-073, JWH-200, and CP 47497; 2 ng/mL for CP 47497 C8 and JWH-250; 5 ng/mL for HU-210 (Figure 2). Linearity was acceptable from the LOQ to 100 ng/mL ($R^2 > 0.99$; n = 5) for all compounds.

Matrix effects

A nonextracted drug standard at a concentration of 10 ng/mL was prepared as well as drug free matrix extracts and negative controls (extracts containing only internal standard). The recovery of the compounds from the oral fluid was determined by first assessing the response of the extracted samples (n = 3) at a concentration of 10 ng/mL {R_{ES}}. Then, oral fluid was extracted and drug was added postextraction at a concentration of 10 ng/mL (n = 3) {R_{PES}}. The percentage recovery was then calculated from the equation (R_{ES}/ R_{PES}) × 100.

The reduction in response due to matrix effects (ion suppression) was determined by assessing the peak area response of a nonextracted neat drug standard (n = 3) at a concentration of 10 ng/mL {R_{NES}}. The nonextracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The % matrix effect was then calculated using the equation (R_{PES} / R_{NES}) -1 × 100. The overall efficiency of the process was calculated as (R_{ES} / R_{NES}) × 100.

Ion suppression effects were significant, but were limited by the use of solid-phase extraction and deuterated internal standards.

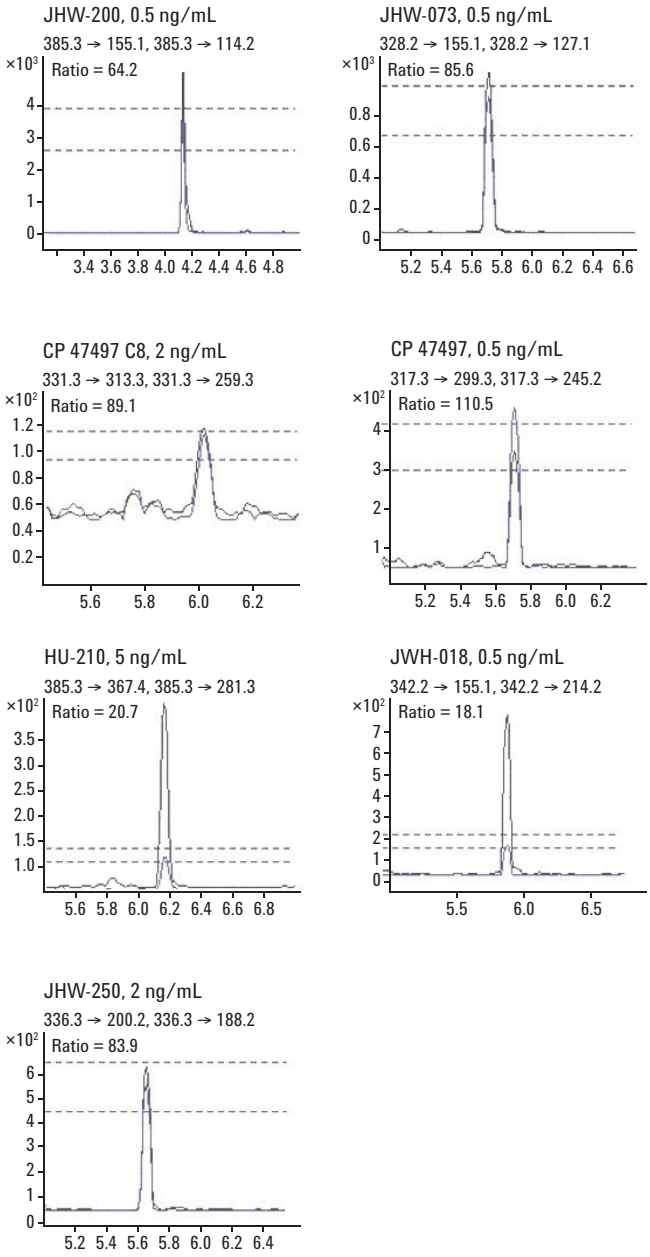

Figure 2. LOQ concentrations showing ± 20% ratio.

Selectivity

Five drug free oral fluid specimens were collected using the Quantisal device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer.

In addition, common drugs of abuse were added at concentrations of 2,000 ng/mL to other aliquots of the drug-free fluid, extracted, and analyzed as described.

THC	amitriptyline
THC-COOH	cyclobenzaprine
11-OH-THC	imipramine
cannabinol	dothiepin
cannabidiol	doxepin
cocaine	fluoxetine
benzoylecgonine	sertraline
norcocaine	trimipramine
cocaethylene	protriptyline
codeine	chlorpromazine
morphine	clomipramine
6-AM	nortriptyline
6-AC	paroxetine
oxycodone	desipramine
oxymorphone	bromazepam
hydrocodone	alprazolam
hydromorphone	clonazepam
amphetamine	lorazepam
methamphetamine	oxazepam
MDMA	diazepam
MDA	midazolam
MDEA	flurazepam
phentermine	flunitrazepam
fentanyl	nordiazepam
phencyclidine	triazolam
tramadol	temazepam
carisoprodol	nitrazepam
meprobamate	chlordiazepoxide
citalopram	methadone
venlafaxine	

No endogenous interference was noted from drug free extracts; or for exogenous interference from any of the commonly encountered drugs, including THC and its main metabolites, which were analyzed at high concentration.

Imprecision

Specimens were fortified with all the compounds simultaneously at concentrations of 4 ng/mL and 40 ng/mL. Each concentration was analyzed according to the described procedure (n = 6; intra-day imprecision) for 5 consecutive days (n = 30; inter-day imprecision). The intra-day imprecision of the assays for all drugs was < 9% at both concentrations; inter-day < 12% at both concentrations (Table 2).

Authentic samples

Specimens were collected from two volunteers, who had purchased the compounds while still legally available in the USA. Subject number 1 smoked "Blueberry Posh" and subject number 2 smoked "Black Mamba". Using Quantisal oral fluid collection devices, specimens were collected prior to the start of smoking, then at the various time points after smoking. Subject 1 gave specimens after 20 minutes, 40 minutes, 1 hour, 2 hours, and 12 hours; Subject 2 gave samples after 20 minutes, 40 minutes, 1 hour, 5 hours, and 12 hours. The specimens were analyzed the day after collection, then were stored at 4 °C for one month and re-analyzed with a dif-ferent method. A year later, they were re-analyzed using this procedure. It was not possible to procure authentic specimens at this time since the compounds are no longer available legally.

The main active compound in the two preparations was determined to be JWH-018. After storage at 4 °C for one month the samples were reanalyzed and found to be extremely stable with almost identical concentrations detected. When the specimens which had been stored at 4 °C for a year were re-analyzed, the concentrations in Subject number 1 were essentially the same as the previous year; the levels in Subject number 2, which were much lower originally, had generally declined (Figure 3).

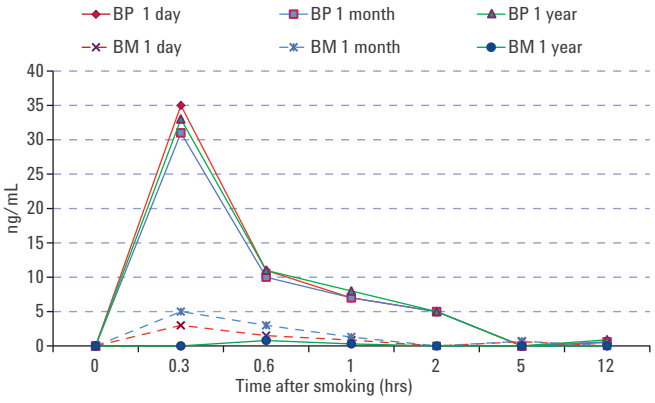

Figure 3. Stability of authentic specimens stored at 4 °C.

An extracted ion chromatogram showing the transitions and ± 20% acceptability band around the intensity of the qualifying transition from the sample collected 40 minutes after smoking (Subject number 1) is presented in Figure 4; the concentration of JWH-018 was 11 ng/mL.

Summary

The simultaneous determination of several "Spice" compounds in oral fluid is reported for the first time. The procedure is applicable to the analysis of specimens collected using the Quantisal device for the presence of synthetic cannabinoids, which were recovered from the pad > 60% at two concentrations. Following a single smoking session of two different herbal product brands, JWH-018 was detected in oral fluid with the highest concentrations appearing 20 minutes after a single smoking session. Even after a year, JWH-018 was detectable in the oral fluid 12 hours after a single smoking session of "Blueberry Posh".

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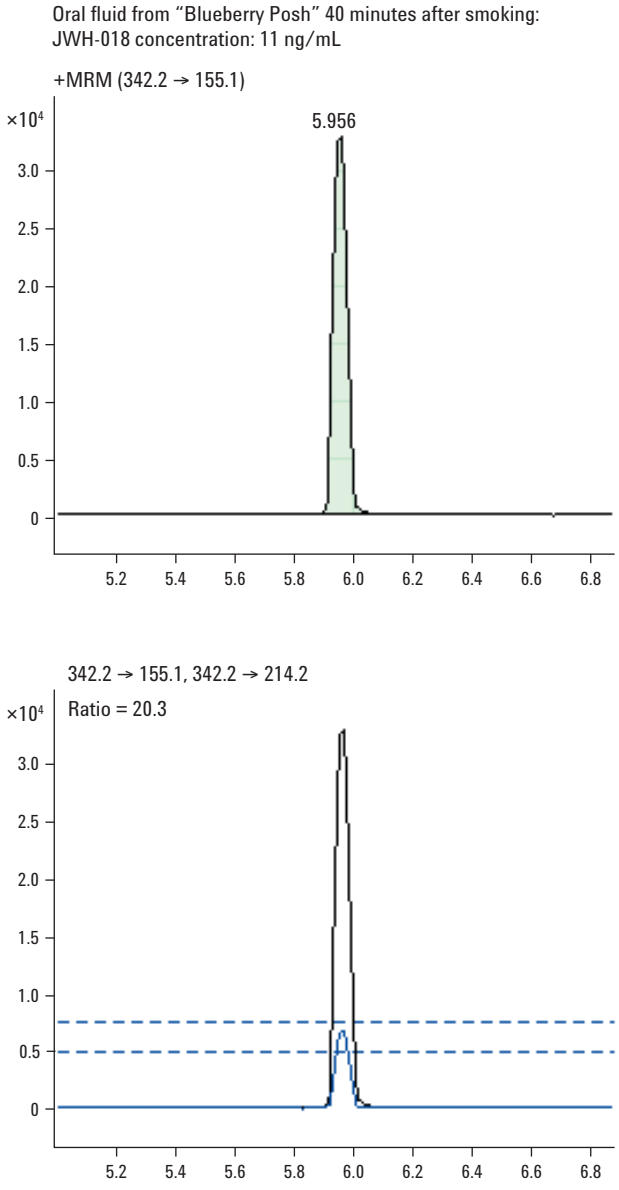

Figure 4. Oral fluid from Subject #1 40 minutes after smoking; JWH-018 = 11ng/mL.

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LC/MS/MS of Buprenorphine and Norbuprenorphine in Whole Blood Using Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 Column

Application Note

Forensic Toxicology

Author

Irina Dioumaeva
Agilent Technologies, Inc.

Abstract

Determination of buprenorphine and norbuprenorphine in whole blood by forensic toxicology laboratories requires an analytical method capable of reliable detection of these compounds at concentrations below 1 ng/mL. A simple sample cleanup procedure coupled with an LC/MS/MS method using mass transitions 468.2 → 55.1 and 414.2 → 83.1 allows for a limit of detection (LOD) below 0.1 ng/mL for both analytes. Typical calibration curves are linear in the range of 0.2 to 20 ng/mL for each analyte, with R^2 values equal or higher than 0.999. High sensitivity is achieved by using Agilent products, including an Agilent Bond Elut Plexa PCX mixed mode polymeric SPE sorbent, an Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, an Agilent 1200 Infinity LC system, and an Agilent 6460 Triple Quadrupole LC/MS System with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

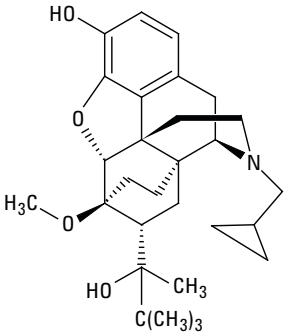
Buprenorphine is a semisynthetic opioid with a structure similar to morphine, although buprenorphine is much more hydrophobic (Figure 1). Buprenorphine is converted to norbuprenorphine, its major active metabolite [1,4]. Concentrations of buprenorphine and norbuprenorphine in blood are very similar, and in more than 50% cases, are below 1 ng/mL [9], presenting a challenge for an analyst. In addition, MS/MS detection of these compounds is complicated by the rigidity of the molecular structures of the analytes, resulting in very low amounts of collision-induced fragments. To achieve sensitivity below 1 ng/mL, analytical methods for determination of these compounds need not only excellent MS performance, but also an efficient sample cleanup procedure providing high recoveries and low ion suppression. We used an extraction method that delivered detection limits below 0.1 ng/mL, easily achieved due to the cleanliness of SPE-processed whole blood extracts. Unlike other polymeric sorbents, all members of the Agilent Bond Elut Plexa family possess an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

Good separation of analytes and excellent peak shapes achieved with this method are distinctive features of the Agilent Poroshell 120 column family. With superficially porous 2.7 µm particles, these columns provide similar efficiency to sub-2 µm UHPLC columns, but with approximately 40% less backpressure. This allows users of even 400 bar LC systems to increase resolution and to shorten analysis and re-equilibration times by applying a higher flow rate.

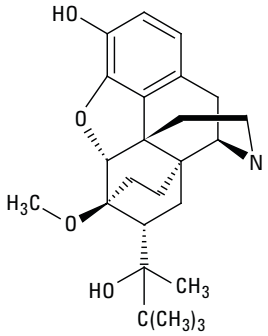
New ion transitions identified as the most abundant and used in this work for quantitation are 468.2 > 55.1 (buprenorphine) and 414.2 > 83.1 (norbupenorphine). With only 0.5 mL of blood, a low sample injection volume of 10 µL and preconcentration of only 5× at the extraction step, the method demonstrates excellent signal-to-noise ratios at 0.2 ng/mL:84:1 for buprenorphine and 20:1 for norbuprenorphine (Figure 2).

Experimental

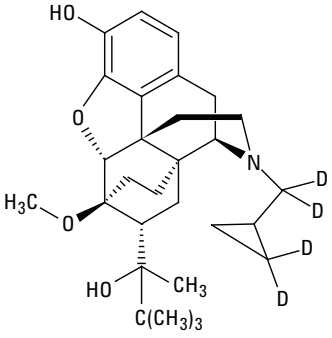
Analytes



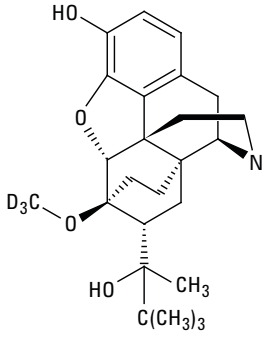
Buprenorphine
$C_{29}H_{41}NO_4$ M.W. 467.65
Log P 4.9-5.0
pKa 8.3



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



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



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

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

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (buprenorphine, norbuprenorphine) and 100 µg/mL (buprenorphine-D_4 and norbuprenorphine-D_3) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent silanized autosampler vials 2 mL (p/n 5183-2072)
- Agilent vial inserts, 250 µL, deactivated glass, with polymer feet (p/n 5181-8872)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column (p/n 699975-302)
- Agilent 1260 Infinity LC system (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Pretreatment

1. Spike 0.5 mL of blood with ISTD at 10 ng/mL, or prepare 10 ng/mL solution of ISTD in 0.1 M phosphate buffer (pH 6.0) and add 0.5 mL of this buffer to each blood sample. Use of methanol-rinsed and air-dried glass tubes 12 × 75 mm is recommended.
2. After adding ISTD, add 2 to 2.5 mL phosphate buffer (so that blood is diluted at least 1:5).
3. Vortex and centrifuge to obtain a good pellet.

Extraction

1. Condition Bond Elut Plexa PCX cartridge with 0.5 mL methanol, soak, then let drip.
2. Load sample/supernatants with a Pasteur glass pipette.
3. Wash 1: 2 × 2 mL 2% formic acid.
4. Wash 2: 3 mL of 70 MeOH:30 of 2% formic acid.
5. Dry 5-10 minutes under vacuum (10-15 in Hg).
6. Elute with 1.5 mL of 80 ethyl acetate:20 isopropanol: 5 NH_4OH eluent. Add NH_4OH shortly before elution. Apply eluent in 2 aliquots and soak the sorbent bed with each aliquot. Soak for approximately 0.5 minute with the stopcock valves closed, then let the eluate drip into the collection vials under gravity. When the dripping stops, apply low vacuum to extract eluate from the smallest pores.
7. Evaporate to dryness under a stream of nitrogen at 45 °C.
8. Reconstitute in 0.1 mL initial mobile phase (15% methanol, 85% water, 0.1% formic acid), vortex, and transfer into vial inserts with polymer feet.

LC/MS/MS

LC conditions

Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in methanol
Flow rate:	0.8 mL/min

Gradient:

Time (min)	% B
0.0	15
2.0	70
2.1	95
5.5	95
5.51	15

Stop time:	5.6 min
Post time:	2 min
Max pump pressure:	400 bar
Injection volume:	10 µL

Injection with needle wash

Needle wash:	Flush port 95 methanol:5 water for 10 s
Disable overlapped injection:	
No automatic delay volume reduction:	

MS conditions

ES source parameters

Ionization mode:	positive
Capillary voltage:	2,800 V
Drying gas flow:	10 L/min
Drying gas temperature:	350 °C
Nebulizer gas:	35 psi
Sheath gas flow:	12 L/min
Sheath gas temperature:	350 °C
Nozzle voltage:	0 V

MS parameters

Scan type:	MRM
Prerun script:	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments:	#1: 1.8 min - diverter valve to MS
Delta EMV (+):	400 V

Table 1 shows the MRM transitions for one quantifier and one qualifier product ion for the target compounds, and their deuterated internal standards.

Table 1. MRM Transitions.

Compound name	Precursor	MS1 Res	Product	MS2 Res	Fragmentor	Collision energy
Buprenorphine	468.3	Unit	55.1	Wide	200	62
Buprenorphine	468.3	Unit	396.2	Wide	200	45
Buprenorphine-D_4	472.3	Unit	59.1	Wide	200	62
Buprenorphine-D_4	472.3	Unit	400.2	Wide	200	45
Norbuprenorphine	414.3	Unit	83.1	Wide	188	60
Norbuprenorphine	414.3	Unit	57.1	Wide	188	50
Norbuprenorphine-D_3	417.3	Unit	83.1	Wide	188	60
Norbuprenorphine-D_3	417.3	Unit	57.1	Wide	188	50

Results and Discussion

At low pH, buprenorphine and norbuprenorphine are protonated at the tertiary amine group and strongly retained on Agilent Bond Elut Plexa PCX polymeric sorbent by a combination of hydrophobic retention and strong cation exchange.

A 100% methanol wash led to partial loss of analytes from the SPE column. The optimum wash that efficiently removed most matrix interferences without loss of analytes proved to be 70 MeOH:30 2% formic acid. A strong base is added to the organic eluent to break the ionic interaction between the analytes and the strong cation-exchange sorbent. The recovery of buprenorphine and norbuprenorphine was optimized with 5% NH_4OH added to the combination eluent (80 ethyl acetate: 20 isopropanol) shortly before sample elution. Two-step elution with a soaking procedure is recommended to enhance the solvent-analyte interaction and improve analyte recoveries.

Due to high hydrophobicity, buprenorphine and norbuprenorphine can adhere to glassware, LC tubing, and injector parts, which is why we recommend a 95% MeOH column rinse in the LC method and 95 MeOH:5 water flushing solution for the flushport needle rinse. Deactivated vials/inserts and MeOH-rinsed/air-dried glassware (both tubes and bottles for STD/ISTD dilutions) also ensure reproducible results.

The LC separation intentionally begins with a relatively low fraction of organic solvent (15%) to allow salts and other polar components of blood to elute at the beginning of the sample run. A flow rate of 0.8 mL/min allows for a short retention and re-equilibration time. Each sample run begins with diverting a first portion of flow (0 to 1.8 minutes) to waste to minimize source contamination. Data collection begins at 1.8 minutes, immediately after the diverter valve switch.

Chromatograms for buprenorphine and norbuprenorphine at the LOQ of 0.2 ng/mL and corresponding deuterated internal standards at 10 ng/mL are shown in Figure 2.

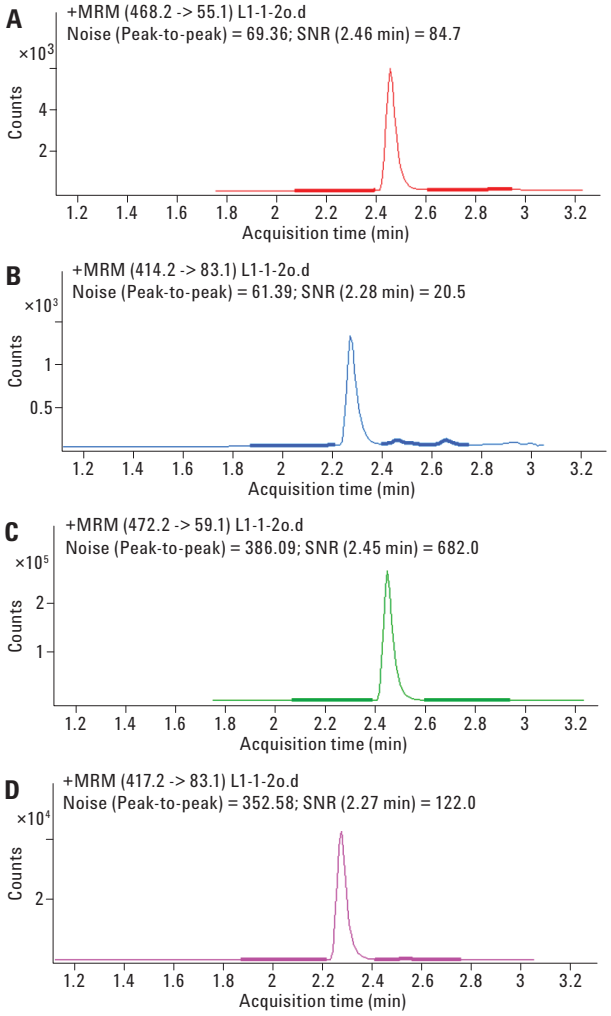


Figure 2. MRM extracted ion chromatograms: A: buprenorphine, B: norbuprenorphine (both at 0.2 ng/mL), C: buprenorphine-D_4, and D: norbuprenorphine-D_3 (both at 10 ng/mL) in whole blood extract processed on Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

The high stability of molecular ions of both buprenorphine and norbuprenorphine presents a challenge for MS/MS detection [3,9]. It led many researchers to quantitation in SIM mode [2,8], or in SRM mode by monitoring a molecular ion > molecular ion transition at relatively high collision energy without fragmentation [3,9]. Compared to a more selective quantitation by a parent-product transition, this approach is less reliable. It results in a much higher signal-to-noise (S/N) ratio and, therefore, in a higher lower limit of quantification (LLOQ). MS-MS transitions most commonly used for buprenorphine/norbuprenorphine quantification were 468 to 414, 396 *m/z* for buprenorphine, and 414 to 396, 340 and 101 *m/z* for norbuprenorphine [2, 3, 4, 5, 6, 7]. A new stable fragmentation pattern achieved with an Agilent 6460 Triple Quadrupole LC/MS System (Table 1) at high collision energy levels allows for a reliable quantitation with an LLOQ of 0.2 ng/mL for both analytes. The most abundant fragment of buprenorphine is the methylocyclopropyl (C_4H_7) group with *m/z* 55.1. Its identification is confirmed by a fragment of buprenorphine-D_4 with *m/z* 59.1. The most abundant product of norbupenorphine fragmentation (*m/z* 83.1) probably comes from the branched side chain of the parent ion and includes the tert-butyl group $(CH_3)_3C$. Compared to most commonly used fragmentation products obtained at their optimum collision energies, *m/z* 55.1 is a 8× more abundant product of buprenorphine than *m/z* 396.2, while *m/z* 83.1 is a 2× more abundant product of norbuprenorphine than *m/z* 101.1.

MRM transitions listed in Table 1 are for one quantifier and one qualifier product ion for both target compounds and their deuterated ISTDs. Agilent MassHunter software automatically calculates qualifier ion ratios, highlighting those out of the acceptable range. Either normal or dynamic MRM acquisition modes can be used with this method.

S/N ratios at the LLOQ level of 0.2 ng/mL were 84:1 for buprenorphine and 20:1 for norbuprenorphine Figures 2, A and B). This illustrates the efficiency of a sample cleanup procedure and the excellent sensitivity of the 6460 Triple Quadrupole, capable of detecting these analytes with LODs way below 0.1 ng/mL.

Figure 3 shows typical calibration curves for buprenorphine and norbuprenorphine in extracted whole blood standards at five concentration levels. Calibration standards were prepared by spiking whole blood with analytes at 0.2, 1, 5, 10, and 20 ng/mL. Deuterated internal standards were added at 10 ng/mL. Excellent linear fit (R^2 > 0.999) to each of the curves demonstrates linearity of the method. No weighting was applied, and the origin was included in the curve fit.

Table 2 shows recovery (accuracy) and precision (CV, or RSD) data collected for five samples of whole blood fortified with 1 ng/mL of each analyte. Quantitation was performed against calibration curves obtained from the spiked matrix standards (Figure 3).

Conclusions

A simple, solid phase extraction procedure coupled with an LC/MS/MS detection method allows determination of buprenorphine and norbuprenorphine in whole blood at concentrations below 0.2 ng/mL. This method is intended for users of Agilent 1100 and 1200 LC series since the backpressure in the LC system does not exceed 400 bar.

Table 2. Method Evaluation at 1 ng/mL of Each Analyte, n = 5.

	Accuracy (%)	CV %
Buprenorphine	94.2	0.6
Norbuprenorphine	103	0.2

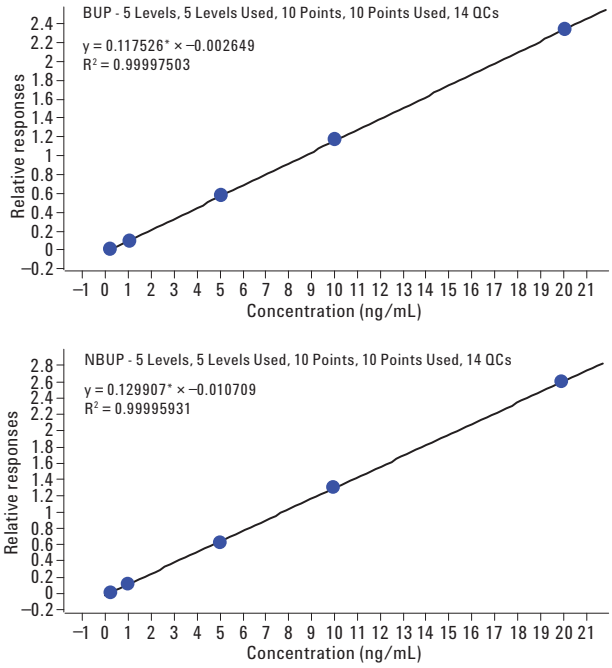


Figure 3. Typical calibration curves for buprenorphine and norbuprenorphine in whole blood extract. Concentration range 0.2 to 20 ng/mL. Linear fits R^2 > 0.999.

Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS System instruments. Low detection limits are achieved due to cleanliness of sample extracts and robust MS detection using newly identified ion transitions with abundant fragmentation products.

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Agilent Technologies

Rapid, Robust and Sensitive Detection of 11-nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid in Hair

Application Note

Forensic Toxicology/Doping Control

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Abstract

A robust method for the detection of the THCA marijuana metabolite in hair was developed with a run time of 7 min and a cycle time of 9 minutes using column switching and backflushing. The method LOD is 0.002 pg/mg and the LOQ is 0.01 pg/mg.

Introduction

Testing hair for drugs of abuse has been practiced for over 50 years, due in large part to the ability to detect drug use over a longer period of time, as compared to other biological matrices, because many drugs are well-preserved in hair. Hair testing is widely used in criminal investigations. Workplace programs include hair testing due to the ease of collection, difficulty of adulteration and longer detection times.

Marijuana is one of the drugs tested most often in forensic and drug screening applications. The parent compound, tetrahydrocannabinol (THC), is found in higher concentration in hair samples, but detection of the acid metabolite THCA (11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid) is preferred, in order to eliminate the possibility of potential environmental contamination from marijuana smoke. While guidelines for workplace hair testing have not yet been adopted by the Substance Abuse Mental Health Services Administration (SAMHSA) in the United States, a cutoff concentration for nor-9-carboxy-Δ^9-tetrahydrocannbinol as low as 0.05 pg/mg hair has been suggested, and such guidelines are a topic of additional study and analysis by this regulatory body. The Society of Hair Testing recommends a limit of quantification (LOQ) of $\leq$ 0.2 pg/mg for THCA.

Agilent Technologies

This application note describes a method developed on the Agilent 7890A GC System coupled with an Agilent 7000B Triple Quadrupole GC/MS System that provides rapid and sensitive detection of a THC metabolite in hair, using 2-D GC and negative ion chemical ionization (CI) MS/MS in the multiple reaction monitoring (MRM) mode (also called SRM, Selected Reaction Monitoring). The method is modified from a previous GC/MSD method [1] to take advantage of the lower chemical background and higher sensitivity provided by triple quadrupole MS/MS analysis. Backflush is used to increase robustness, and low thermal mass (LTM) column modules speed the chromatography process, enabling a run time of 7 min and a cycle time of 9 min. MRM MS/MS analysis on the Triple Quadrupole GC/MS System delivers excellent sensitivity, with an LOD of 0.002 pg/mg and an LOQ of 0.01 pg/mg.

Experimental

Standards and Reagents

Tri-deuterated THCA, which was used as the internal standard (100 µg/mL in methanol), and unlabelled THCA (100 µg/mL in methanol) were obtained from Cerilliant, (Round Rock, TX). The internal standard concentration in the method was 0.05 pg/mg of hair.

Methanol, acetonitrile, toluene, ethyl acetate, hexane, glacial acetic acid, and methylene chloride were obtained from Spectrum Chemicals (Gardena, CA). All solvents were high-performance liquid chromatography (HPLC) grade or better, and all chemicals were ACS grade. Bond Elut Certify I solid-phase extraction columns (130 mg) from Agilent, Inc. (Walnut Creek, CA), or Clean Screen ZSTHC020 extraction columns (200 mg) from United Chemical Technologies, Inc. (Bristol, PA) were interchangeable for the assay. The derivatizing agents, pentafluoropropionic anhydride (PFPA) and 1,1,1, 3, 3, 3-hexafluoro-2-propanol (HFIP), were purchased from Sigma –Aldrich (St. Louis, MO) and Campbell Science (Rockton, IL), respectively.

Instruments

The experiments were performed on an Agilent 7890N GC System equipped with a multimode inlet (MMI) and an LTM System, coupled to an Agilent 7000B Triple Quadrupole GC/MS System. Two dimensional chromatography was performed using a pre-column for backflushing, two Low Thermal Mass (LTM) columns connected by a Deans Switch, and a Purged Ultimate Union (Figure 1). The instrument conditions are listed in Table 1.

Table 1. Agilent 7890N/7000B Gas Chromatograph and Triple Quadrupole Mass Spectrometer Conditions

GC Run Conditions	
Pre-column	1 m × 0.15 mm × 1.2 µm DB-1 (p/n 12A-1015)
Analytical columns	
Column 1	15 m × 0.25 mm × 0.25 µm DB-1ms LTM Column Module (p/n 122-0112LTM)
Column 2	15 m × 0.25 mm × 0.25 µm DB-17ms LTM Column Module (p/n 122-4712LTM)
Injection volume	2 µL
Inlet temperature	Isothermal at 250 °C
Injection mode	0.75 minute pulsed splitless at 35 psi
Oven temperatures	
GC oven	7 minute hold at 250 °C (isothermal)
1st LTM module	50 sec hold at 100 °C
	100 °C to 210 °C at 200 °C/min
	210 °C to 267 °C at 10 °C/min
	Hold at 267 °C for 2 min
2nd LTM module	324 sec hold at 100 °C
	100 °C to 230 °C at 200 °C/min
	230 °C to 240 °C at 10 °C/min
	Hold at 240 °C for 2 min
Carrier gas	Helium in constant pressure mode. Pre-column: 1 psi; Column 1: 26.6 psi; Column 2: 19.6 psi
Transfer line temp	300 °C
MS conditions	
Tune	Autotune
EMV Delta	1200 V
Acquisition parameters	NCI mode; multiple reaction monitoring (MRM)
Reagent gas	Ammonia, 35% flow
Collision gas	Argon, constant flow, 0.9 mL/min
Quench gas	Helium, constant flow, 0.5 mL/min
Solvent delay	6.2 min
MS temperatures	Source 150 °C; Quadrupole 150 °C

Sample Preparation

Samples were prepared as previously described [2]. Calibrators, controls or hair specimens (20 mg) were weighed into silanized glass tubes and washed with methylene chloride (1.5 mL). The solvent was decanted and the hair samples were allowed to dry. The internal standard, THCA-d3 (0.05 pg/mg), was added to each hair specimen. For the calibration curve, unlabelled THCA was added to the hair at concentrations of 0.002, 0.01, 0.02, 0.05, 0.1, and 0.5 pg/mg of hair.

Deionized water (0.5 mL) and 2N sodium hydroxide (0.5 mL) were added, and the hair was heated at 75 °C for 15 min. The sample was allowed to cool and then centrifuged (2500 rpm, 15 min). The supernatant was poured into glass tubes already containing acetic acid (1 mL), 1 M acetic acid (3 mL), and 0.1 M sodium acetate buffer (pH 4, 2 mL). The tubes were capped and mixed.

SPE columns were conditioned with hexane/ethyl acetate (75:25, v/v; 2 mL), methanol (3 mL), deionized water (3 mL), and 0.1 M hydrochloric acid (1 mL). The acidified samples were loaded onto the SPE columns and allowed to dry. The SPE columns were washed with deionized water (2 to 3 mL) and allowed to dry for 5 min. The SPE columns were washed with 0.1 M hydrochloric acid/acetonitrile (70:30 v/v; 3 mL) and allowed to dry at 30 psi for 10 min. The SPE columns were finally rinsed with hexane/ethyl acetate (75:25 v/v; 3 mL) in order to elute the THCA into silanized glass tubes.

The eluent was evaporated to dryness under nitrogen at 40 °C and reconstituted in PFPA (70 µL) and HFIP (30 µL) for derivatization. The mixture was transferred to autosampler vials with glass inserts and capped. The vials were heated at 80 °C for 20 min, then left at room temperature for 10 min. The extracts were evaporated to dryness in a vacuum oven. The samples were finally reconstituted in toluene (50 µL), for injection into the GC–MS system.

Analysis Parameters

The Agilent Triple Quadrupole GC/MS System parameters used are shown in Table 2.

Table 2. Agilent 7000B Triple Quadrupole GC/MS System Analysis Parameters

Compound	RT (min)	MRM	Dwell time (ms)	Collision energy (EV)
THCA*	6.714	620→492	50	5
		620→383	50	5
THCA-d3	6.710	623→495	20	5
		623→386	20	5

*11-nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid

Results

Two Dimensional Gas Chromatography with Heart-Cutting

The use of two serial GC columns to separate background from the required peak is a well-established technology that is widely used to provide excellent separation of the analyte from matrix interferences. Once the analyte retention time on the first column has been determined, the pneumatic switch (Deans Switch) is turned on at that time to divert the flow to the second column, and turned off a short time later. This diverts a narrow, heart-cut "window" of the effluent from the first column that contains the analyte and minimal background, for further separation on the second column (Figure 1). The two columns function optimally when the stationary phases are as different as possible.

Exceptional Robustness and Speed

The unique combination of backflushing and low thermal mass (LTM) column modules make this a very robust and rapid method, compared to the traditional single column approach. Three independently programmed pressure zones are used in conjunction with three independently heated zones (Figure 1). The pre-column and the first LTM column are coated with relatively non-polar DB-1ms phase, and the second LTM column is coated with a more polar DB-17ms phase. The heart-cut window is only 0.2 min (5.5 to 5.7 min) wide.

A unique system for rapid and robust detection of THCA in hair

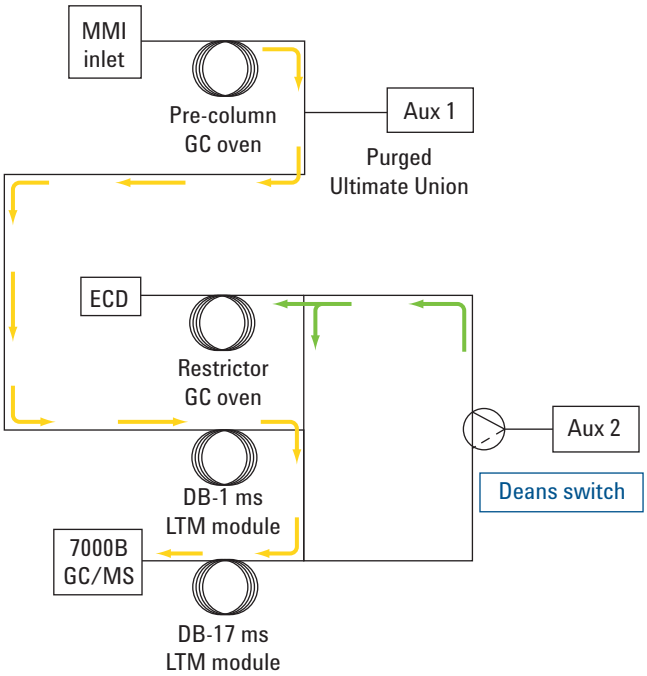

Figure 1. Schematic representation of the system used to develop the THCA method.

The precolumn and auxiliary pressure control module (AUX EPC) provides backflushing capability to protect and preserve the LTM analytical columns. The precolumn was in backflush mode with a constant pressure of 1 psi during the run. The inlet pressure pulse overrides the backflush for the initial 0.75 min. The use of backflushing prevents build-up of high-boiling compounds on the column, thus reducing retention time shifts, peak distortion, and chemical noise, while improving quantification. Contamination of the MS source and the resultant need for cleaning are also reduced, while the run time is shortened.

This method also employs LTM column modules external to the GC oven that enable independent and optimal temperature control of the two analytical columns (Figure 2). The unique design of these modules makes it possible to employ very fast temperature ramping and rapid cooling. The LTM column modules can be added to an Agilent GC without requiring any changes in the injectors, autosamplers, or detectors, and they can be controlled from the GC software.

The end result of this unique backflushing and LTM approach is a robust method that provides excellent quantification and sensitivity (see next section) with 7 min run times and 9 min cycle times.

Unique LTM Column Modules enable rapid temperature ramping and cooling

Figure 2. Low thermal mass (LTM) column modules interfaced with the Agilent 7890A GC.

Sensitivity and Quantification

This method has a limit of detection (LOD) of 0.002 pg/mg, demonstrating excellent sensitivity that is far below the suggested cutoff of 0.05 pg/mg (Figure 3). The accuracy of quantification is also quite good, with an R^2 of 0.995, from 0.002 to 0.5 pg/mg of hair (Figure 4). The limit of quantification (LOQ) is 0.01 pg/mg, which again is more than an order of magnitude below the 0.2 pg/mg LOQ suggested guideline established by the Society of Hair Testing (Figure 5). This method also provides a compliant quantitative analysis report that includes the retention times (with limits), response level, qualifier ion ratio (with limits), and the calculated concentration. The total ion current (TIC) trace and the quantifier and qualifier MRM traces are also displayed on the report, for both the sample and the THCA-d3 internal standard (Figure 6).

LOD of 0.002 pg/mg

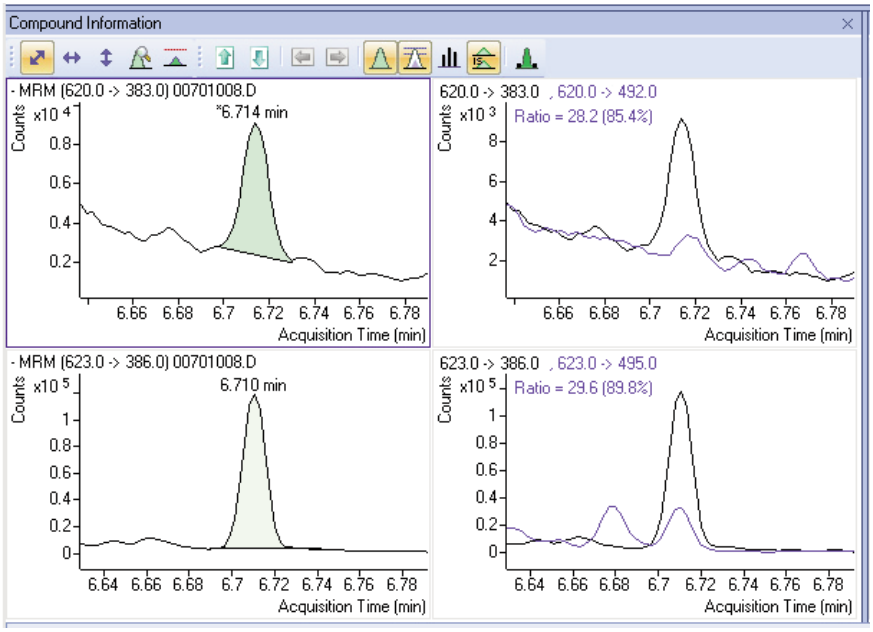

Figure 3. MRM traces for the quantifying transition (left) and both the quantifying and qualifying transitions (right) for the 0.002 pg/mg LOD of THCA (upper panel) and the deuterated standard (lower panel) spiked into a hair sample.

Reliable calibration

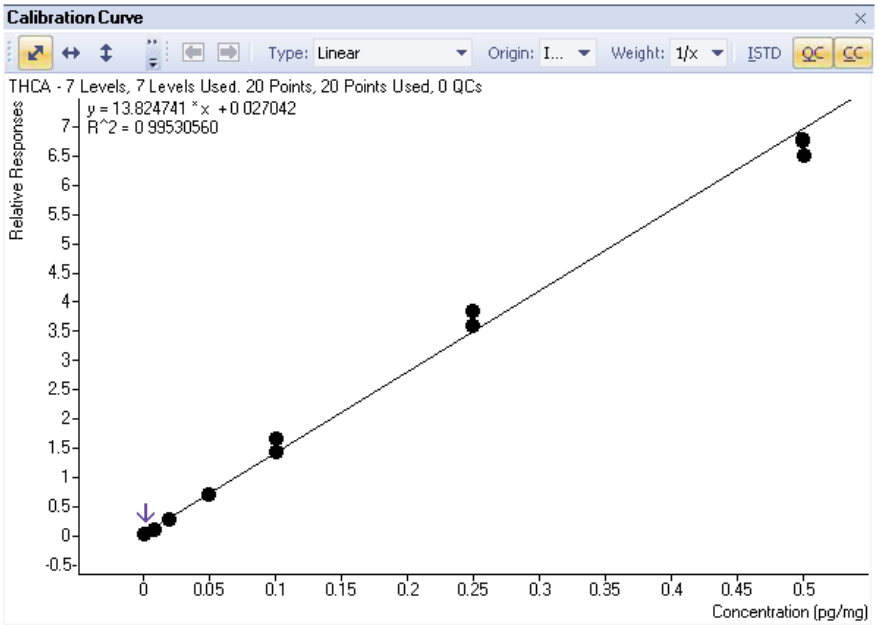

Figure 4. Calibration curve for THCA spiked into hair samples at 0.002, 0.01, 0.02, 0.05, 0.1, and 0.5 pg/mg of hair.

0.01 pg/mg LOQ

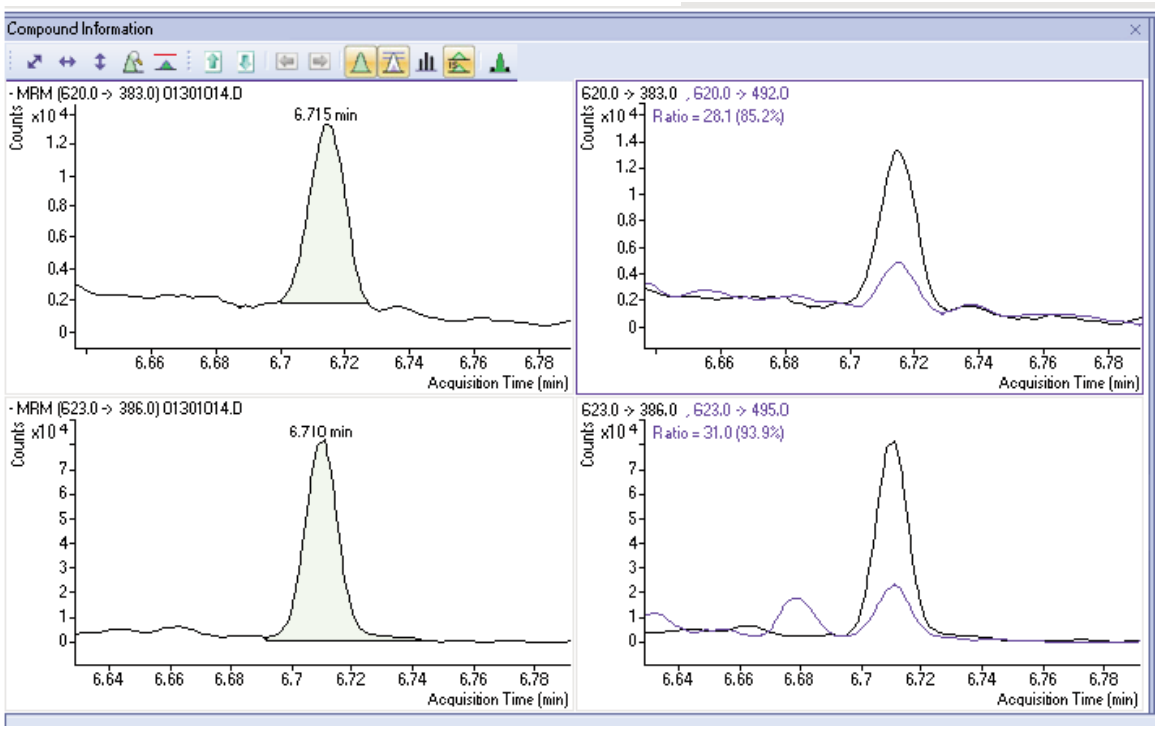

Figure 5. MRM traces for the quantifying transition (left) and both the quantifying and qualifying transitions (right) for the 0.01 pg/mg LOQ of THCA (upper panel) and the deuterated standard (lower panel) spiked into a hair sample.

Data File	01401015.D	Vial	14
Operator	DATASYSTEM01/Admin	Dillution	0.0
Acq method name		Sample information	
Acquisition date	2010-10-08 16:24	Last calib update	2010-11-28 09:34
Sample name and path	0.01 pg/mg, D:/MassHunter/GCMS/1/data/PFAA Curve Extracted/		

Compound	Signal	RT	Limits	Response	QRatio	Limits	Final conc
THCA-d3	623.0 -> 386.0	6.71		82558		35770 - 143081	
	623.0 -> 495.0			24962	30.2	23.1 - 42.9	
THCA	620.0 -> 383.0	6.71	6.38 - 7.05	10999			0.008
	620.0 -> 492.0			3908	35.5	23.1 - 42.9	

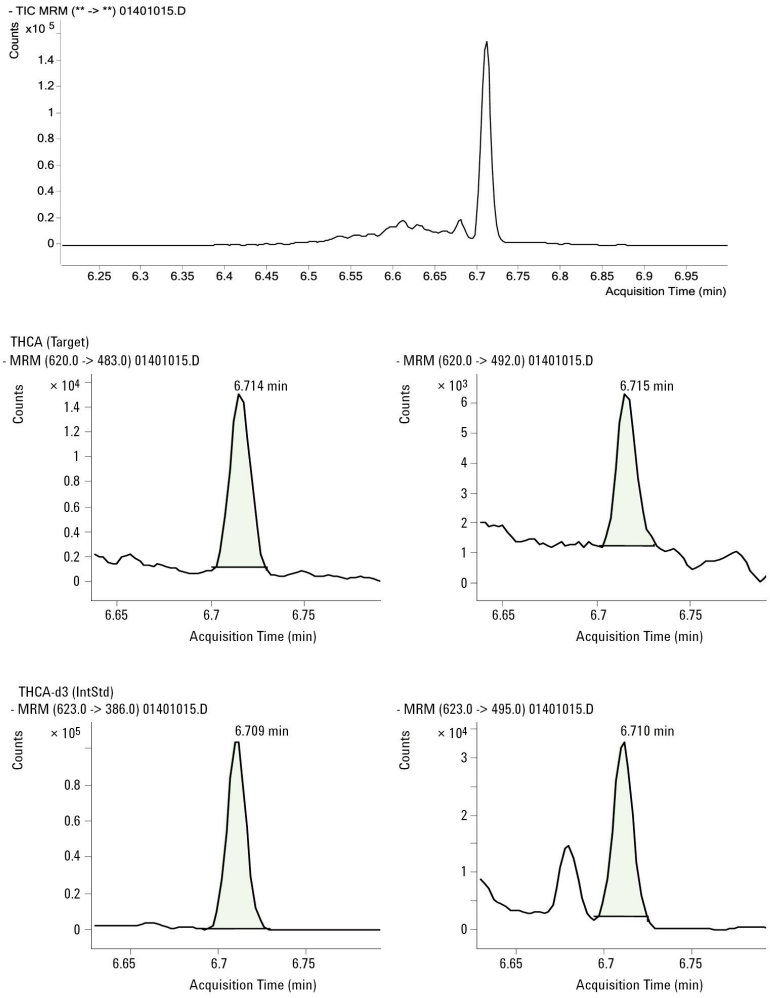

Figure 6. Quantitative Analysis Sample Report for a 0.01 pg/mg (the LOQ) sample spiked into hair.

Conclusion

The time-proven technique of heart-cutting to improve chromatographic separation is given new life in this unique method which utilizes state-of-the-art microfluidics-aided backflushing and low thermal mass column temperature ramping modules to deliver sensitive and robust detection and quantification of THCA in hair (LOD 0.002 pg/mg; LOQ 0.01 pg/mg) with run times of only 7 minutes, and cycle times of 9 minutes.

References

1. F. Feyerherm, R. Lowe, J. Stuff, D. Singer, "Rapid Multidimensional GC Analysis of Trace Drugs in Complex Matrices", Gerstel publication AN-2007-8.
2. C. Moore, S. Rana, C. Coulter, F. Feyerherm, H. Prest, "Application of Two-dimensional Gas Chromatography with Electron Capture Chemical Ionization Mass Spectrometry to the Detection of 11-nor-D9-Tetrahydrocannabinol-9-carboxylic acid (THCA) in Hair", J. Anal. Toxicol. 30, 171–177 (2006).

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Rapid and Robust Detection of THC and Its Metabolites in Blood

Application Note

Forensic Toxicology/Doping Control

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Abstract

A robust method for detection of THC and its metabolites in blood has been developed using SPE extraction and GC/MS/MS with backflushing. The dynamic range of quantification was 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA, with a run time of 6 minutes and a cycle time of 8 minutes.

Introduction

In the past decade, a great deal of research concerning the impact of cannabis use on road safety has been conducted. More specifically, studies on effects of cannabis smoking on driving performance, as well as epidemiological studies and cannabis-detection techniques have been published. As a result, several countries have adopted driving under the influence of drugs (DUID) legislation, with varying approaches worldwide. While a wide variety of bodily fluids have been used to determine the presence of cannabis, blood testing is considered the most reliable indicator of impairment. Blood testing for active tetrahydrocannabinol (THC) may also be considered by employers who wish to identify employees whose performance may be impaired by their cannabis use. Gas chromatography/mass spectrometry (GC/MS) is a standard method for detection and quantification of THC and its metabolites in blood.

One key to reliable THC testing in blood is an efficient extraction method. The use of tandem MS (MS/MS) also increases the sensitivity and reliability of quantification of THC and its metabolites in blood, due to the elimination of interferences. This application note describes a method using the High Flow Bond Elut Certify II SPE cartridge to rapidly and efficiently extract THC and its metabolites from blood. The extracts were derivatized to improve volatility and analyzed on the Agilent 7890A Triple Quadrupole GC/MS system equipped with a Low Thermal Mass Module (LTM)

Agilent Technologies

oven and backflushing. It was in turn coupled with an Agilent 7000B Triple Quadrupole GC/MS system, using MS/MS in the multiple reaction monitoring (MRM) mode to provide rapid and sensitive detection of THC and its metabolites, 11-OH-THC (11-hydoxy-Δ9-tetrahydrocannbinol) and THCA (11-nor-Δ9-Tetrahydrocannabinol-9-Carboxylic Acid). Backflushing was used to increase robustness and speed, enabling a run time of 6 minutes and a cycle time of 8 minutes. MRM MS/MS analysis on the Triple Quadrupole GC/MS system delivers excellent results, with a dynamic range of 0.1 to 50 ng/mL.

Experimental

Standards and Reagents

Tri-deuterated THC, 11-OH-THC and THCA, which were used as internal standards (100 µg/mL in methanol), and unlabelled THC, 11-OH-THC and THCA (100 µg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). The internal standard concentrations in the method were both 10 µg/mL.

Methanol, acetonitrile, toluene, ethyl acetate, hexanes, glacial acetic acid, and methylene chloride were obtained from Sigma Aldrich (St. Louis, MO). All solvents were high-performance liquid chromatography (HPLC) grade or better, and all chemicals were ACS grade. Agilent High Flow Bond Elut Certify II solid-phase extraction columns were used for the method. The derivatizing agents, BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) were purchased from Cerilliant. Normal human whole blood stabilized with potassium oxalate and sodium fluoride was obtained from Bioreclamation (Hicksville, NY). Standards were prepared in this drug-free matrix to construct the calibration curves.

Instruments

The experiments were performed on an Agilent 7890N gas chromatograph equipped with a multimode inlet (MMI) and an LTM oven, coupled to a 7000B Triple Quadrupole GC/MS. Chromatography was performed using a pre-column for backflushing, and a Low Thermal Mass (LTM) column connected by a Purged Ultimate Union (Figure 1). The instrument conditions are listed in Table 1.

a.

Loading the sample on the pre-column

b.

Backflushing the pre-column and separation of THC and its metabolites on the primary column

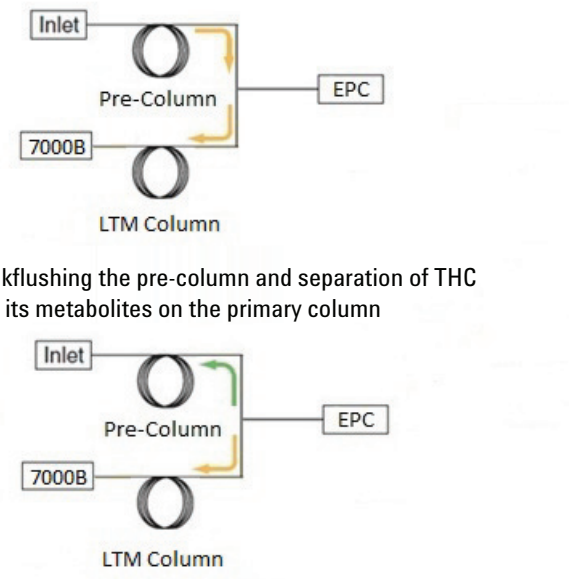


Figure 1. Schematic representation of the backflush system used to develop the method. EPC: Electronic Pneumatic Control module; 7000B: Agilent Triple Quadrupole GC/MS system

Table 1. Agilent 7890N/7000B Gas Chromatograph and Triple Quadrupole Mass Spectrometer Conditions

GC Run Conditions	
Pre-column	1 m section from a 15 m × 0.25 mm, 0.25 µm HP-5 ms Ultra Inert column (p/n 19091S-431UI)
Analytical column	15 m × 0.25 mm, 0.25 µm DB-17 ms LTM Column Module (p/n 122-4712LTM)
Injection volume	1 µL
Inlet temperature	Isothermal at 280 °C
Injection mode	0.5 min pulsed splitless at 35 psi
Oven temperatures	GC oven: 6 min hold at 280 °C (isothermal)
LTM module:	50 second hold at 100 °C 100 °C to 230 °C at 200 °C/min 230 °C to 280 °C at 10 °C/min Hold at 280 °C for 1 min
Carrier gas	Helium in constant pressure mode. Pre-column: 1 psi; Column 1: 5 psi; Column 2: 9.6 psi
Transfer line temp	300 °C
MS Conditions	
Tune	Autotune
Gain	20
Acquisition parameters	EI mode; multiple reaction monitoring (MRM)
Collision gas	Nitrogen constant flow, 1.5 mL/min
Quench gas	Helium, constant flow, 2.25 mL/min
Solvent delay	3.0 min
MS temperatures	Source 230 °C; Quadrupole 150 °C

Sample Preparation

A 2 mL blood sample containing 10 µg/mL of each internal standard (ISTD) and spiked with THC, 11-OH-THC and THCA was pipetted into a clean tube, and 4 mL of acetonitrile was added. After centrifugation at 2500 rpm for 5 minutes, the supernatant was transferred and evaporated to about 3 mL with nitrogen at 35-40 °C, and 7 mL of 0.1 M sodium acetate (pH 6.0) was added.

High Flow Bond Elut Certify II SPE columns were conditioned with 2 mL of methanol, then 2 mL 0.1 M sodium acetate buffer, pH 6.0 with 5% methanol. Cartridges were not be allowed to go to dryness prior to sample addition. The sample was drawn through the column slowly, at 1 to 2 mL/min. The column was then washed 2 mL sodium acetate buffer, pH 6.0, dried under maximum vacuum for approximately 5 minutes, then washed with 1 mL hexanes. THC was eluted under neutral conditions with 2 mL of 95:5 hexane: ethyl acetate. This was followed by a 5 mL 1:1 methanol:deionized water wash. The column was again dried under maximum vacuum for approximately 5 minutes and washed again with 1 mL hexanes. Elution of 11-OH-THC and THCA was performed with 2 mL 1% acetic acid in 75:25 hexane:ethyl acetate. The THC and the metabolite fractions were combined and dried before derivatization.

The eluent was evaporated under nitrogen at a temperature no higher than 40 °C, then reconstituted in 60 µL of toluene and 40 µL of BSTFA, 1% TMCS for derivatization. The sample tubes were capped and heated 20 minutes at 70 °C before injection into the tandem quadrupole GC/MS system.

Analysis Parameters

The Agilent Triple Quadrupole GC/MS system parameters used are shown in Table 2.

Table 2. Agilent 7000B Triple Quadrupole GC/MS System Analysis Parameters

Compound	RT (min)	MRM	Dwell time (ms)	Collision energy (EV)
THC (Δ9-Tetrahydrocannabinol)	3.5	386→303*	25	20
		386→330	27	10
		386→289	30	25
THC-d3	3.5	389→306*	10	20
		389→330	11	10
		389→292	15	25
11-OH-THC (11-hydoxy-Δ9-tetrahydrocannabinol)	4.5	371→289*	24	20
		371→305	26	15
		371→265	27	15
11-OH-THC-d3	4.5	374→292*	10	20
		374→308	12	15
		374→268	12	15
THCA (11-nor-Δ9-Tetrahydrocannabinol-9-Carboxylic Acid)	5.6	371→289*	23	15
		488→297	44	20
		488→371	29	20
THCA-d9	5.5	380→292*	15	15
		497→306	30	20
		497→380	22	20

*Target transition. All other transitions are qualifier transitions.

Results

SPE Sample Preparation with High Flow Bond Elut Certify II Columns

Screening for drugs of abuse in biological fluids requires rugged methods that provide high purification and recovery. The Bond Elut Certify was developed specifically for the rapid and effective extraction of compounds that possess both non-polar and anionic characteristics from urine and other biological matrices [1]. The mixed mode (non-polar C8 and strong anion exchange) sorbent takes advantage of non-polar, polar, and ion exchange properties to ensure rapid, reproducible, simple, and clean extraction of many drug classes. These columns enable the rapid and high recovery of THC, 11-OH-THC and THCA from whole blood.

Backflushing

Backflushing makes this a very robust and rapid method, preventing build-up of high-boiling compounds on the column and thus reducing retention time shifts, peak distortion, and chemical noise, while improving quantification. Contamination of the MS source and the resultant need for cleaning are also reduced, while the run time is shortened. The end result is a robust method that provides excellent dynamic range with 6 minute run times (not including sample prep) and 8 minute cycle times.

The suite of Agilent Capillary Flow Technology modules enables easy and rapid backflushing with minimal dead volumes for maintaining chromatographic resolution. During injection, the inlet Pneumatic Control Module (PCM) is held at an elevated pressure long enough to transfer the target analytes from the pre-column to the analytical column (Figure 1a). When backflushing, the inlet pressure is dropped to 1 psi, forcing the flow to reverse through the pre-column and out the split vent (Figure 1b). In this way, THC, 11-OH-THC and THCA are passed on to the primary column for further separation, while high-boiling compounds are swept back though the split vent.

Low Thermal Mass Modules

This method also employs a Low Thermal Mass (LTM) column module external to the GC oven that enables independent and optimal temperature control of the analytical column (Figure 1). The unique design of these modules makes it possible to employ very fast temperature ramping and rapid cooling. The LTM column modules can be added to an Agilent GC without requiring any changes in the injectors, autosamplers, or detectors.

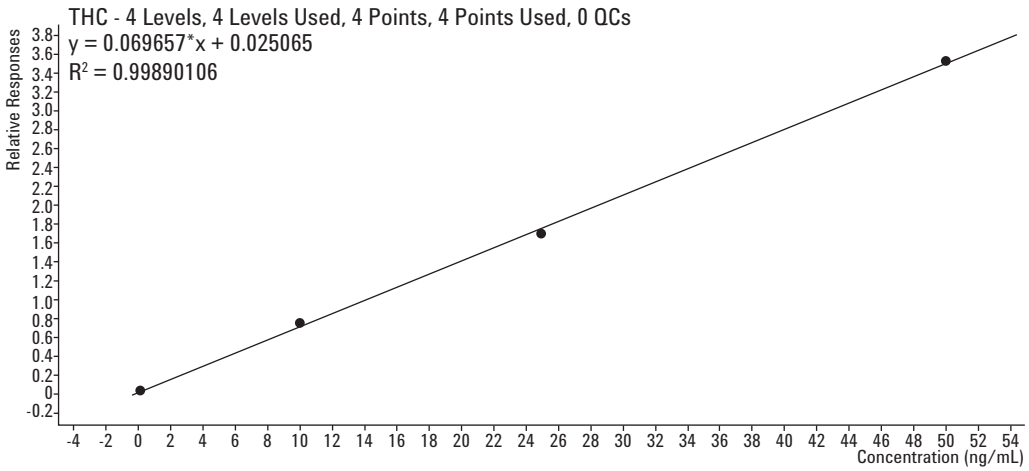
Dynamic Range

This method has a dynamic range of 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA (Figure 2), which match industry norms. The accuracy of quantification is also quite good, with an R^2 of 0.999 for all three analytes.

MRM Results

Using a MassHunter forensic report template, Quantitative Analysis Sample Reports were quickly and easily prepared for THC and its two analytes (Figures 3-5), featuring a Total Ion Current (TIC) chromatogram and spectra for all of the transitions, including the internal standard. Note the lack of interference in all of the transitions, even at the lowest end of the dynamic range for each analyte.

a.



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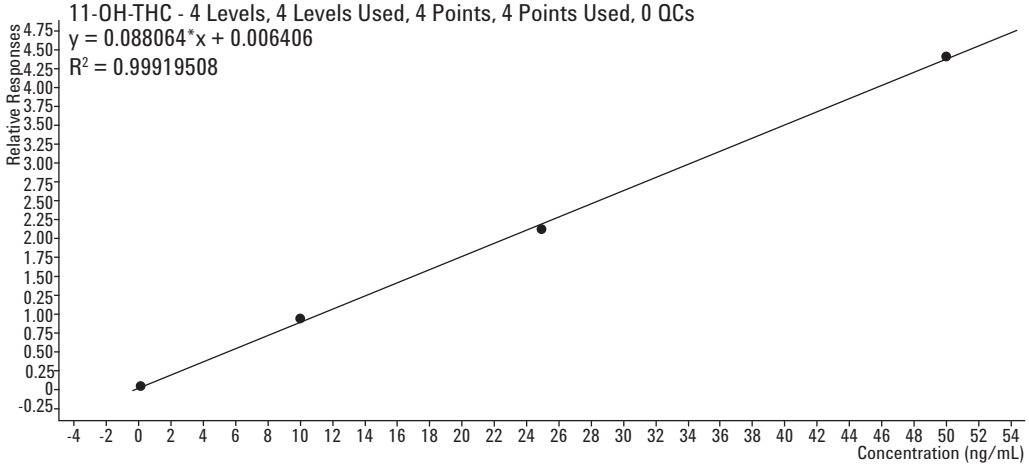

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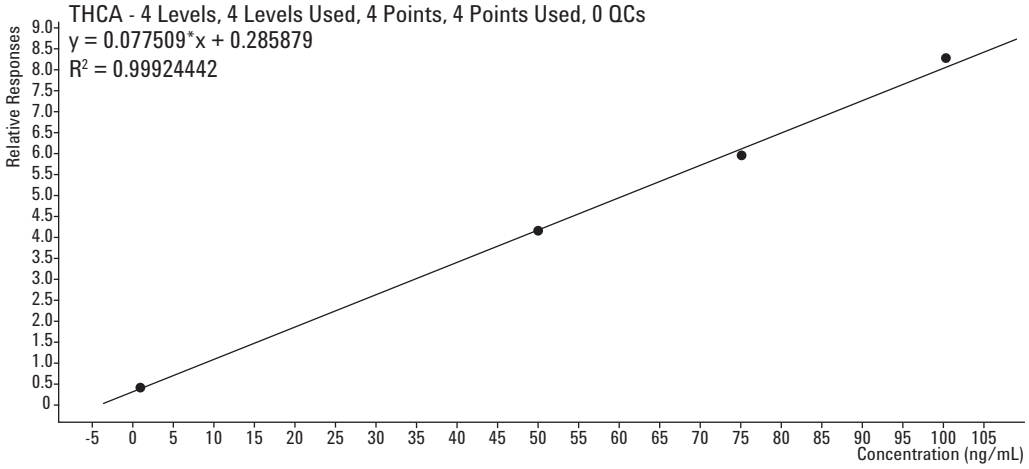
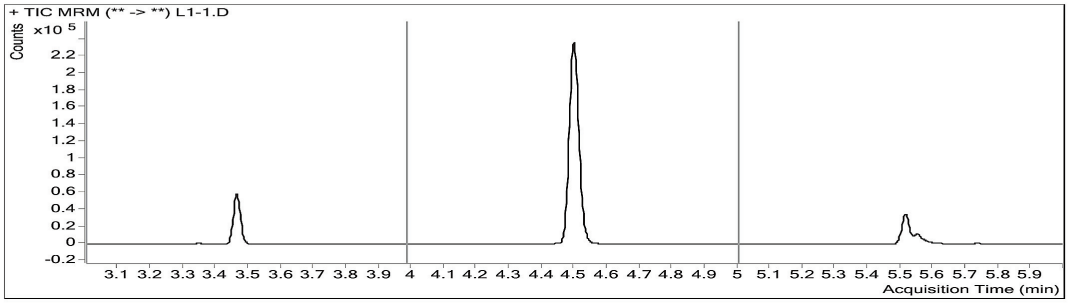


Figure 2. Calibration curves for THC (a), 11-OH-THC (b) and THCA (c) in blood. Data points were taken at 0.1, 10, 25, and 50 ng/mL for THC and 11-OH-THC, and at 1, 50, 75, and 100 ng/mL for THCA.

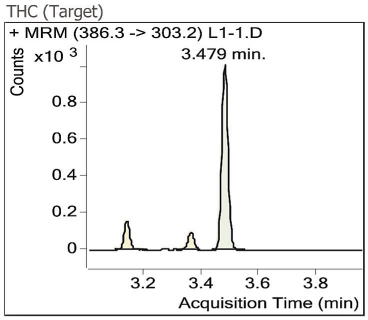
Quantitative Analysis Sample Report

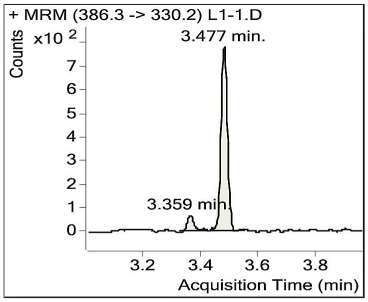
Data File	:	L1-1.D
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

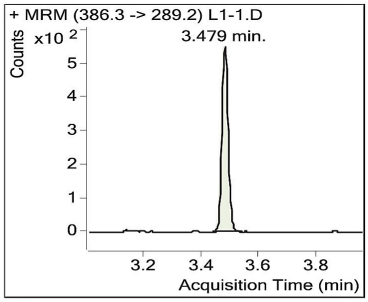
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
D3-THC	389.3 -> 306.2	3.46		37956			
	389.3 -> 330.2			32853	86.6	64.3-104.3	
	389.3 -> 292.2			18032	47.5	22.8-62.8	
THC	386.3 -> 303.2	3.48	3.29 - 3.64	1554			0.100
	386.3 -> 330.2			1237	79.7	62.4-102.4	
	386.3 -> 289.2			848	54.6	23.6-63.6	



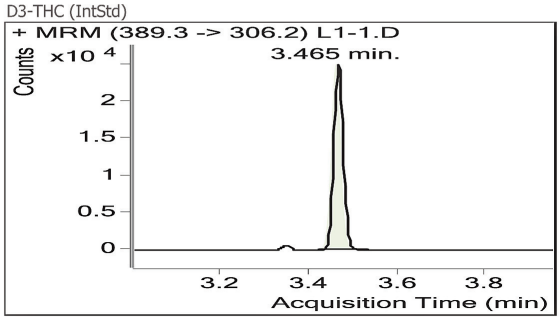
THC (Target)







D3-THC (IntStd)



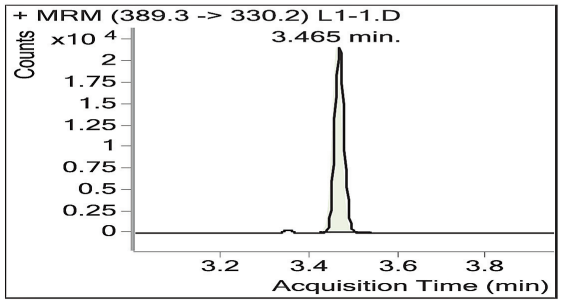
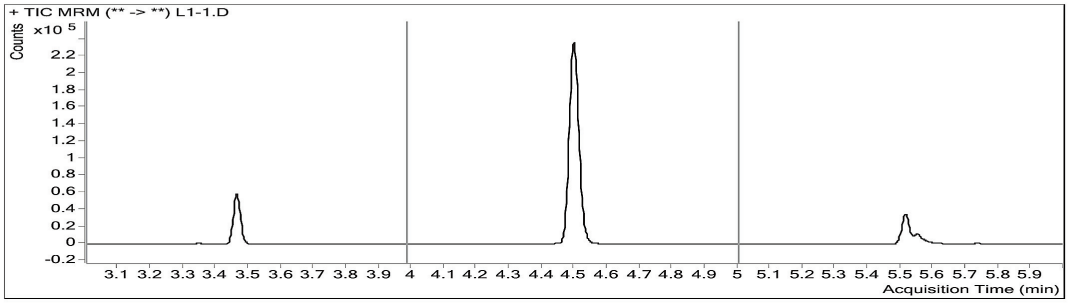


Figure 3. Quantitative Analysis Sample Report for 0.1 ng/mL of THC in blood. The RMS signal-to-noise is 175:1 with a noise region of 3.6 to 3.9 min.

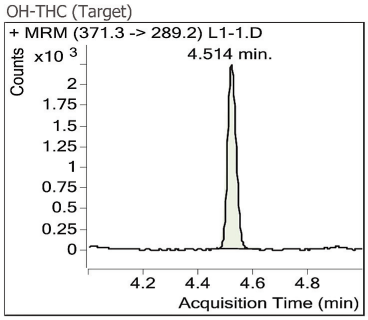
Quantitative Analysis Sample Report

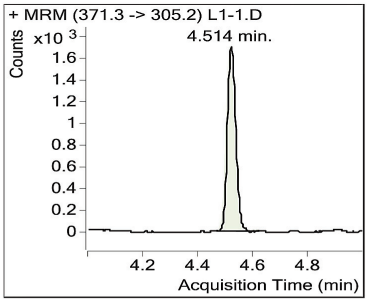
Data File	:	L1-1.D
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

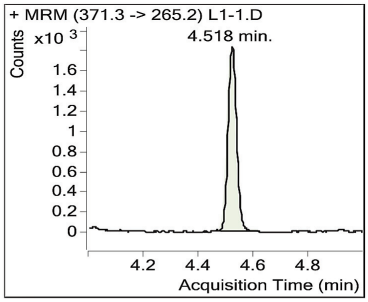
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
d3-OH-THC	374.3 -> 292.2	4.5		175705			
	374.3 -> 308.2			157882	89.9	68.8-108.8	
	374.3 -> 268.3			148545	84.5	59.4-99.4	
OH-THC	371.3 -> 289.2	4.51	4.27 - 4.72	4924			0.099
	371.3 -> 305.2			3707	75.3	67.4-107.4	
	371.3 -> 265.2			4050	82.3	58.2-98.2	



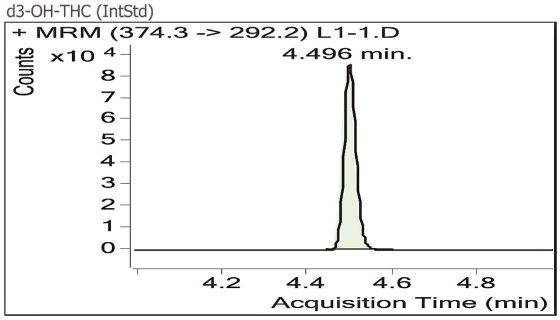
OH-THC (Target)







d3-OH-THC (IntStd)



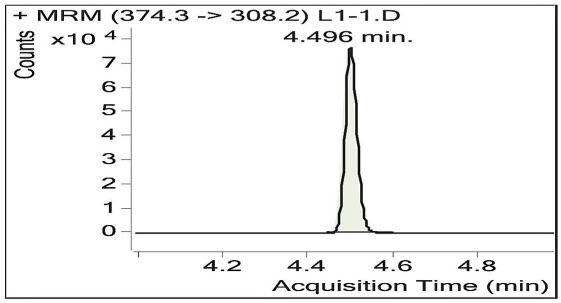
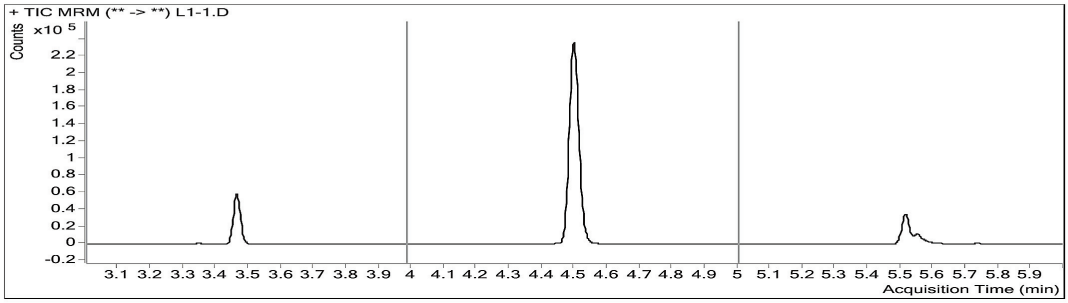


Figure 4. Quantitative Analysis Sample Report for 0.1 ng/mL of 11-OH-THC in blood. The RMS signal-to-noise is 46:1 with a noise region of 4.6 to 4.9 min.

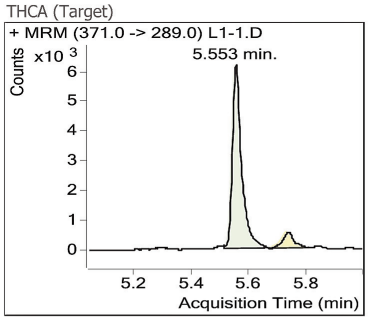
Quantitative Analysis Sample Report

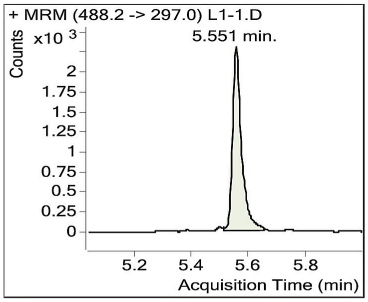
Data File	:	L1-1.D
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

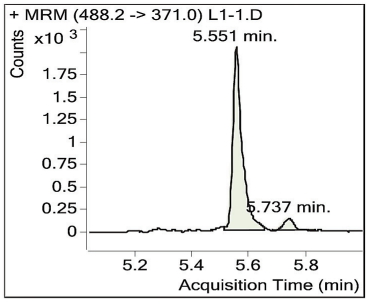
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
d9-THCA	380.0 -> 292.2	5.51		38358			
	497.0 -> 306.1			9800	25.5	5.6-45.6	
	497.0 -> 380.1			11765	30.7	12.5-52.5	
THCA	371.0 -> 289.0	5.55	5.26 - 5.82	13985			1.015
	488.2 -> 297.0			5124	36.6	9.1-49.1	
	488.2 -> 371.0			4758	34	14.4-54.4	



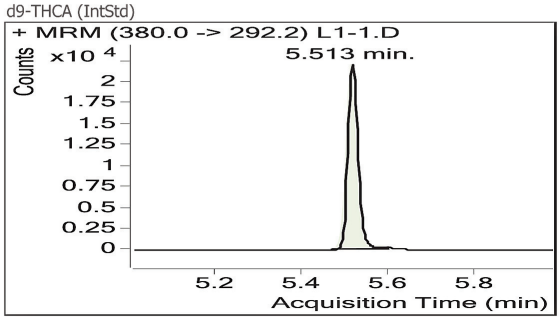
THCA (Target)







d9-THCA (IntStd)



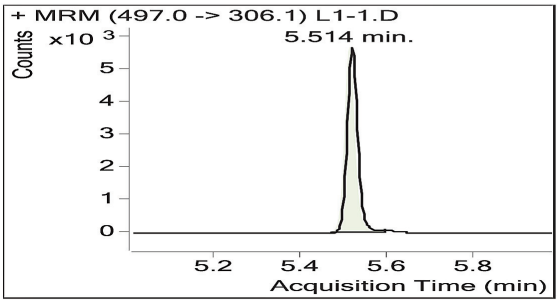


Figure 5. Quantitative Analysis Sample Report for 1 ng/mL of THCA in blood. The RMS signal-to-noise is 39:1 with a noise region of 5.1 to 5.3 min.

Conclusion

Coupling the Agilent 7890N gas chromatograph utilizing an LTM system with the Agilent 7000B Triple Quadrupole GC/MS system enables a rapid and robust method for the analysis of THC and its metabolites in blood. Using the High Flow Bond Elut Certify II SPE cartridge , backflushing of the GC column, and MRM eliminate all interferences, with a resulting dynamic range of quantification of 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA. The LTM module and backflushing facilitate rapid analysis, with a run time of 6 minutes and a cycle time of 8 minutes.

References

1. R.M Sears, Solid Phase Extraction of THD, THC-COOH and 11-OH-THC from Whole Blood, Agilent Technologies Application Note 00315.

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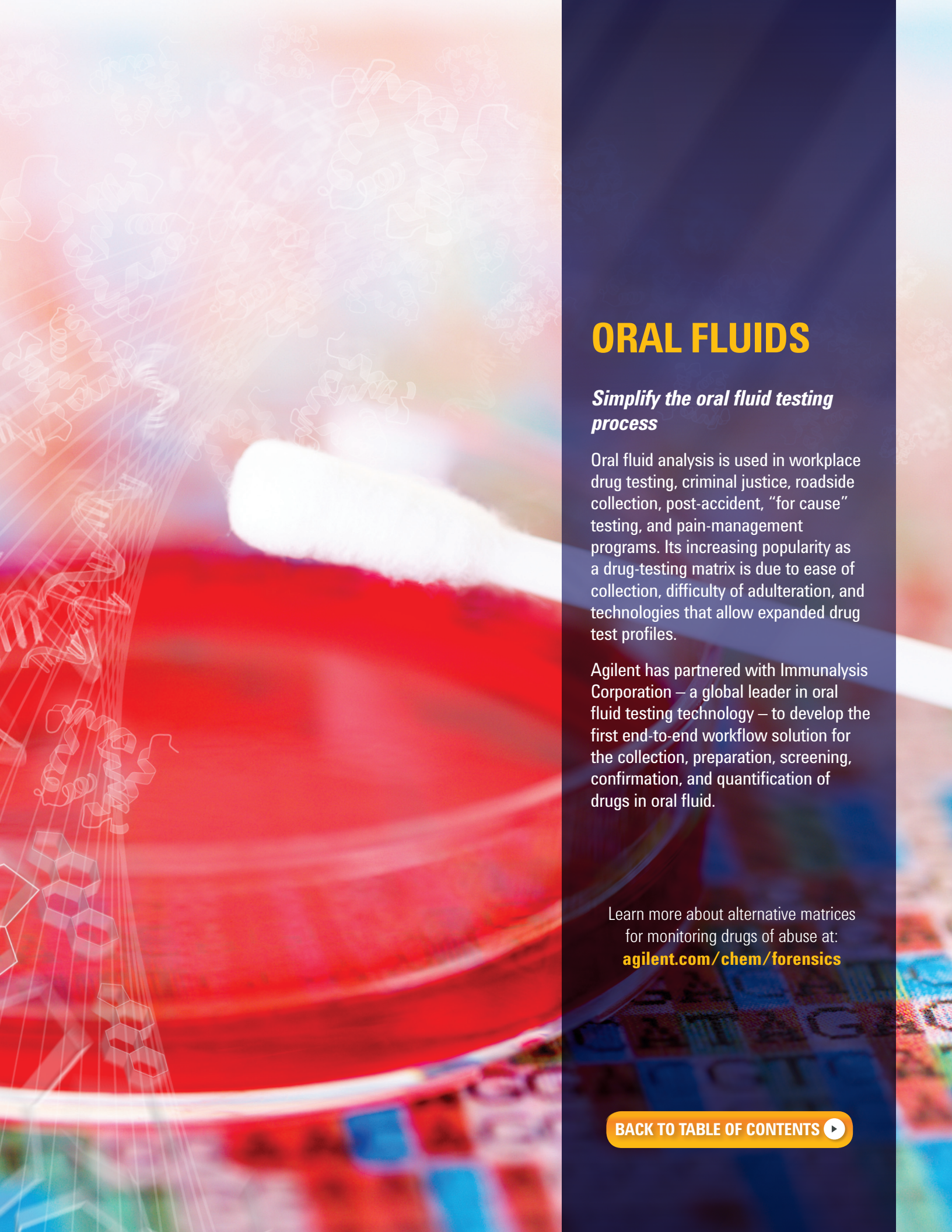

ORAL FLUIDS

Simplify the oral fluid testing process

Oral fluid analysis is used in workplace drug testing, criminal justice, roadside collection, post-accident, "for cause" testing, and pain-management programs. Its increasing popularity as a drug-testing matrix is due to ease of collection, difficulty of adulteration, and technologies that allow expanded drug test profiles.

Agilent has partnered with Immunalysis Corporation – a global leader in oral fluid testing technology – to develop the first end-to-end workflow solution for the collection, preparation, screening, confirmation, and quantification of drugs in oral fluid.

Learn more about alternative matrices for monitoring drugs of abuse at:
agilent.com/chem/forensics

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Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

Authors

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and Christine Moore
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Pomona, California 91767
USA

Introduction

In 2011, five members of the "synthetic cannabinoids" group or 'Spice' compounds were banned in the USA. The substances were:

- 1-pentyl-3-(1-naphthoyl)-indole (JWH-018)
- 1-butyl-3-(1-naphthoyl)-indole (JWH-073)
- 1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)-indole (JWH-200)
- 5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (CP-47,497)
- 5-(1,1-dimethyloctyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (cannabicyclohexanol; CP-47,497 C8 homologue)

The drugs have been described by users as having cannabis-like effects, and some of these compounds show strong binding to cannabinoid receptors. The (–)-1,1-dimethylheptyl analog of 11-hydroxy-Δ^8-tetrahydrocannabinol, (1,1-dimethylheptyl-11-hydroxytetrahydrocannabinol) is known as HU-210 and has been reportedly found in seizures of "Spice Gold", "Spice Silver" and "Spice Diamond" made by the US Customs and Border Protection in 2009. HU-210 is considered to be over 100 times more potent than Δ^9-tetrahydrocannabinol (Δ^9-THC), and was already classified as a controlled substance as an analog of marijuana. JWH-250 is also commonly encountered so was also included in the research.

Agilent Technologies

Oral fluid is becoming increasingly popular as a specimen for the detection of drugs at the roadside, and in workplace testing. It is easy to collect, non-invasive and can give information on recent drug intake. In the work described here, the Quantisal device was used for oral fluid collection, and the detection of "Spice" components is described.

Collection devices, reagents and standards

Quantisal devices for the collection of oral fluid specimens contain a cotton collection pad which is placed in the mouth. The incorporated volume adequacy indicator turns blue when 1 mL of oral fluid (± 10%) has been collected, then the pad is placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). Drug concentrations detected are adjusted accordingly.

Solid phase extraction columns (Bond Elut Plexa) and liquid chromatographic columns (ZORBAX RRHT) were obtained from Agilent Technologies. The standard compounds JWH-018, JWH-073, JWH-200, JWH-250, HU-210, CP-47,497 and CP-47,497 C8 homologue as well as deuterated *d9*-JWH-018 and *d7*-JWH-073 were purchased from Cayman Chemicals.

Calibrators and controls

The deuterated internal standards (*d9*-JWH-018 and *d7*-JWH-073) and unlabelled drug standards were prepared in methanol at a concentration of 100 µg/mL. The working solutions were diluted from stock to a concentration of 10 µg/mL in methanol. The solutions were stored at –20 °C when not in use. Controls were prepared by fortifying drug-free synthetic oral fluid with various concentrations of compounds. Drug free negative specimens, positive controls at 4 ng/mL and 40 ng/mL were included in every batch.

Sample preparation

Seven calibration standards were prepared in oral fluid at concentrations of 0.5, 2, 5, 10, 20, 50, and 100 ng/mL for all analytes; deuterated internal standards were added (10 ng/mL).

Agilent Bond Elut Plexa (30 mg/1 mL; p/n 12109301) solid phase extraction cartridges were used.

1. Condition: methanol (0.5 mL); 0.1 M acetic acid (0.1 mL)
2. To each 1mL aliquot of calibrator, control or specimen, add acetic acid (0.1 M; pH 4, 1 mL)
3. Load samples
4. Wash columns: DI water: glacial acetic acid (80:20; 1 mL); DI water: methanol (40:60; 1 mL)
5. Dry columns (5 minutes)
6. Elute acidic/neutral compounds: hexane: glacial acetic acid (98:2; 2 mL)
7. Evaporate extracts to dryness while allowing columns to dry (7 minutes)
8. Elute bases into corresponding tubes: ethyl acetate: ammonium hydroxide (98:2; 2 mL)
9. Evaporate to dryness under nitrogen at 40 °C
10. Reconstitute in methanol (50 µL); transfer to autosampler vials; cap
11. Analyze using LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

An Agilent Technologies 1200 Series liquid chromatography pump coupled to an Agilent 6430 Triple Quadrupole LC/MS System, operating in electrospray ionization mode (ESI) with either positive or negative polarity depending on the compound.

Column	Agilent ZORBAX RRHT Extend C18, (2.1 × 50 mm, 1.8 µm, p/n 727700-902)	
Column temperature	60 °C	
Injection volume	5 µL	
Mobile phase	Solvent A: 0.2% acetic acid and Solvent B: acetonitrile	
	Time 0:	95% A; 5% B; 5 min: 100% B; 7 min 5% B
	Run time	9.2 min; Post-time 3 min
	Flow rate:	0.5 mL/min
Nitrogen gas temperature	350 °C	
Gas flow	10 L/min	
Nebulizer pressure	55 psi.	
Capillary voltage	+4,000 V in positive mode; –4,000 V in negative mode	

Two transitions were selected and optimized for each drug. Table 1 shows the transitions, the optimized fragment voltages for the parent ion (M +1; M-1) as well as the collision energy for fragmentation of the product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ± 20% in order to meet the criterion for a positive result.

Table 1. Multiple Reaction Monitoring (MRM) Transitions; Optimized Fragmentation Voltages; Allowable Transition Ranges Determined at 10 µg/mL for "Spice" Compounds

Compound	Transition	Fragment voltage (V)	Collision energy (eV)	Polarity	Ratio of quantifying to qualifying transition (range)
d9-JWH-018	351.3 > 223.4	140	20	Positive	n/a
JWH-018	342.2 > 155.1	120	20	Positive	16–24
	342.2 > 214.2	120	20		
JWH-250	336.3 > 200.2	120	12	Positive	69–104
	336.3 > 188.2	120	20		
d7-JWH-073	335.3 > 207.2	120	20	Positive	n/a
JWH-073	328.2 > 155.1	120	20	Positive	60–90
	328.2 >127.1	120	35		
JWH-200	385.3 > 155.1	140	20	Positive	54–81
	385.3 > 114.2	140	25		
CP 47497 C8	331.3 > 313.3	160	25	Negative	70–104
	331.3 > 259.3	160	35		
CP 47497	317.3 > 299.2	160	20	Negative	75–113
	317.3 > 245.2	160	30		
HU-210	385.3 > 367.4	120	30	Negative	13–20
	385.3 > 281.3	120	45		

Underlined transitions used for quantitation; n/a = not applicable for internal standard

Figure 1 shows a chromatogram for the primary transitions of the compound at a concentration of 10 ng/mL; the ratio of primary to secondary transition for each compound was also determined at 10 ng/mL.

Recovery from the collection pad

Six synthetic oral fluid specimens fortified with the compounds at concentrations of 4 and 40 ng/mL were prepared. The collection pad was placed into the samples until 1 mL (±10%) had been collected, as evidenced by the blue volume adequacy indicator incorporated into the stem of the collector, then the pad was transferred to the Quantisal buffer, capped and stored overnight to simulate transportation to the laboratory. The following day an aliquot of the specimen was analyzed. The amount recovered from the pad was compared to an absolute concentration (100%) where drug was added to the buffer and left overnight at room temperature without the pad, then subjected to extraction and analysis.

The percentage recovery from the pad for the compounds at concentrations of 4 and 40 ng/mL (n = 6) were > 60% for all at both levels. The highest recovery was 86% for HU-210 at 4 ng/mL; the lowest was 61% for JWH-073 at 40 ng/mL. The recoveries were essentially equivalent at both levels (Table 2).

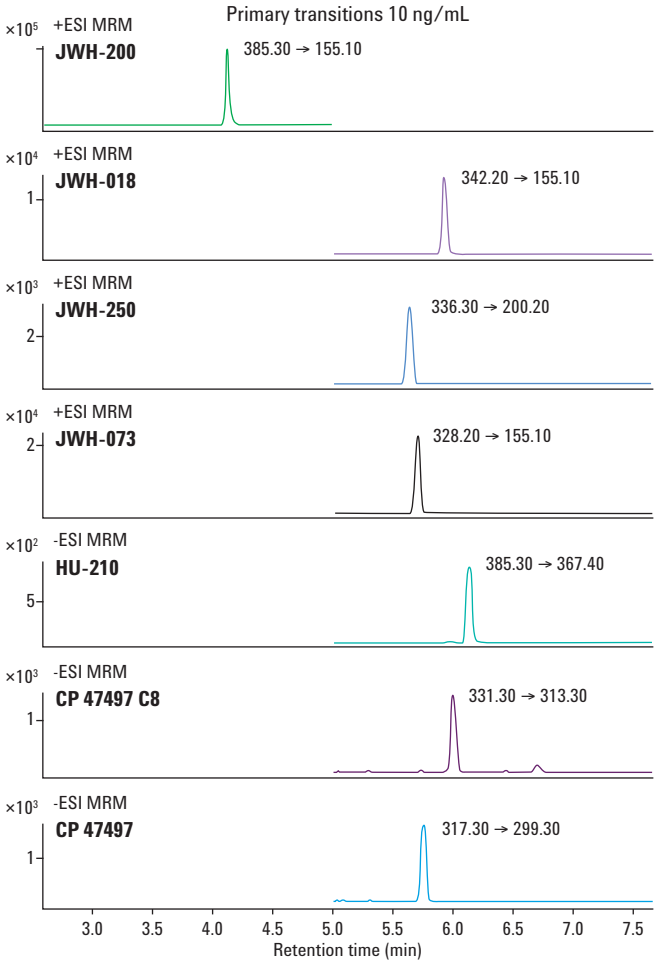

Figure 1. Primary transition at 10 ng/mL.

Table 2. Method Evaluation

	JWH-018	JWH-073	JWH-200	JWH-250	CP 47497	CP 47497 C8	HU-210
LOQ (ng/mL)	0.5	0.5	0.5	2	0.5	2	5
Imprecision intra-day							
4 ng/mL	3.9%	3.6%	5.0%	3.4%	4.9%	3.9%	8.6%
40 ng/mL	2.2%	2.1%	6.0%	2.0%	4.1%	4.3%	5.6%
Inter-day							
4 ng/mL	8.8%	9.6%	6.2%	11%	7.7%	11%	10%
40 ng/mL	8.5%	7.9%	6.2%	11%	10%	11%	12%
Pad recovery							
4 ng/mL	65.5%	67.4%	85.0%	66.5%	77.7%	76.0%	86.4%
40 ng/mL	70.6%	61.4%	81.4%	75.1%	71.3%	78.2%	75.7%
Matrix effect	-55%	-45%	-55%	-73%	-64%	-55%	-49%
Process efficiency	40%	51%	56%	24%	38%	45%	51%

Data Analysis

Calibration was carried out using linear regression analysis over a concentration range of 0.5–100 ng/mL. Peak area ratios of target analytes and the internal standard were calculated for each concentration using Agilent MSD software. The data were fit to a linear least squares regression curve, not forced through the origin, and with equal weighting. For confirmation, two transitions were monitored for each of the compounds; one for the internal standard. The ratio of the qualifying transition was required to be within 20% of that established using the known calibration standard to be acceptable.

Linearity and sensitivity

The limit of quantitation (LOQ) of the method was determined using serial dilutions to the lowest point where the acceptable criteria for the quantitation of a compound were met, that is, the chromatographic peak shape, retention time (within 2% of calibration standard), and qualifier transition ratio (± 20%) compared to the 10 ng/mL calibration standard were acceptable. The quantitative value of the LOQ had to be within ± 20% of the target concentration. The limit of quantitation was 0.5 ng/mL for JWH-018, JWH-073, JWH-200, and CP 47497; 2 ng/mL for CP 47497 C8 and JWH-250; 5 ng/mL for HU-210 (Figure 2). Linearity was acceptable from the LOQ to 100 ng/mL ($R^2 > 0.99$; n = 5) for all compounds.

Matrix effects

A nonextracted drug standard at a concentration of 10 ng/mL was prepared as well as drug free matrix extracts and negative controls (extracts containing only internal standard). The recovery of the compounds from the oral fluid was determined by first assessing the response of the extracted samples (n = 3) at a concentration of 10 ng/mL {R_{ES}}. Then, oral fluid was extracted and drug was added postextraction at a concentration of 10 ng/mL (n = 3) {R_{PES}}. The percentage recovery was then calculated from the equation (R_{ES}/ R_{PES}) × 100.

The reduction in response due to matrix effects (ion suppression) was determined by assessing the peak area response of a nonextracted neat drug standard (n = 3) at a concentration of 10 ng/mL {R_{NES}}. The nonextracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The % matrix effect was then calculated using the equation (R_{PES} / R_{NES}) -1 × 100. The overall efficiency of the process was calculated as (R_{ES} / R_{NES}) × 100.

Ion suppression effects were significant, but were limited by the use of solid-phase extraction and deuterated internal standards.

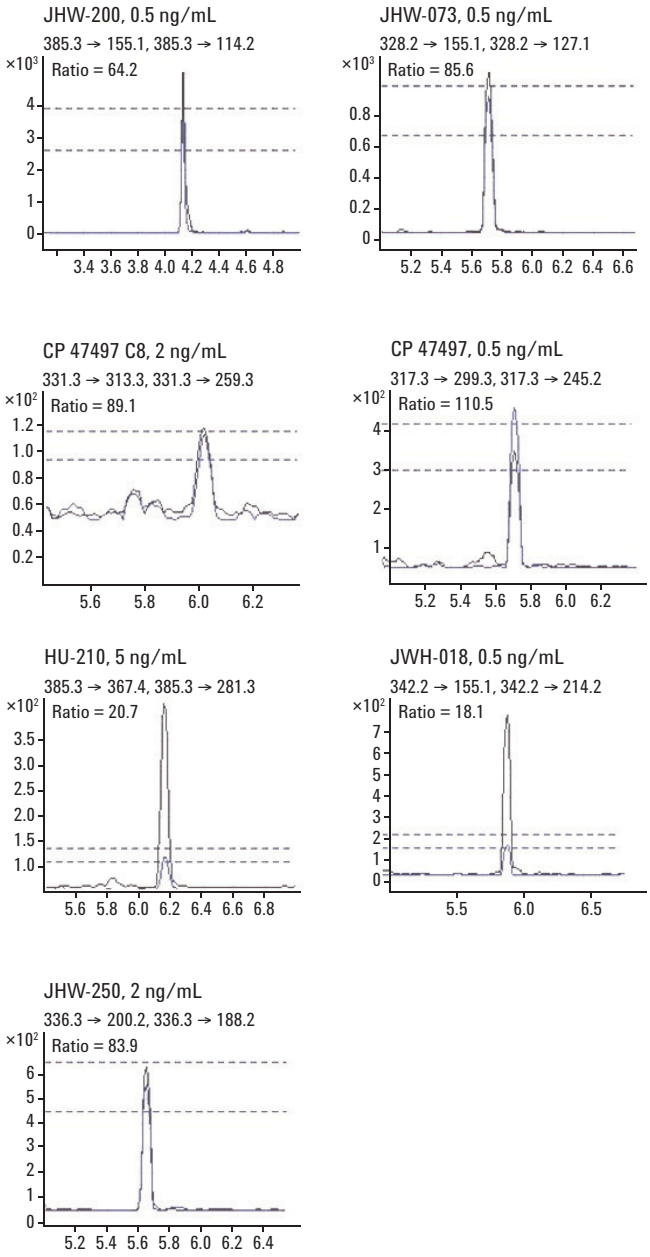

Figure 2. LOQ concentrations showing ± 20% ratio.

Selectivity

Five drug free oral fluid specimens were collected using the Quantisal device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer.

In addition, common drugs of abuse were added at concentrations of 2,000 ng/mL to other aliquots of the drug-free fluid, extracted, and analyzed as described.

THC	amitriptyline
THC-COOH	cyclobenzaprine
11-OH-THC	imipramine
cannabinol	dothiepin
cannabidiol	doxepin
cocaine	fluoxetine
benzoylecgonine	sertraline
norcocaine	trimipramine
cocaethylene	protriptyline
codeine	chlorpromazine
morphine	clomipramine
6-AM	nortriptyline
6-AC	paroxetine
oxycodone	desipramine
oxymorphone	bromazepam
hydrocodone	alprazolam
hydromorphone	clonazepam
amphetamine	lorazepam
methamphetamine	oxazepam
MDMA	diazepam
MDA	midazolam
MDEA	flurazepam
phentermine	flunitrazepam
fentanyl	nordiazepam
phencyclidine	triazolam
tramadol	temazepam
carisoprodol	nitrazepam
meprobamate	chlordiazepoxide
citalopram	methadone
venlafaxine	

No endogenous interference was noted from drug free extracts; or for exogenous interference from any of the commonly encountered drugs, including THC and its main metabolites, which were analyzed at high concentration.

Imprecision

Specimens were fortified with all the compounds simultaneously at concentrations of 4 ng/mL and 40 ng/mL. Each concentration was analyzed according to the described procedure (n = 6; intra-day imprecision) for 5 consecutive days (n = 30; inter-day imprecision). The intra-day imprecision of the assays for all drugs was < 9% at both concentrations; inter-day < 12% at both concentrations (Table 2).

Authentic samples

Specimens were collected from two volunteers, who had purchased the compounds while still legally available in the USA. Subject number 1 smoked "Blueberry Posh" and subject number 2 smoked "Black Mamba". Using Quantisal oral fluid collection devices, specimens were collected prior to the start of smoking, then at the various time points after smoking. Subject 1 gave specimens after 20 minutes, 40 minutes, 1 hour, 2 hours, and 12 hours; Subject 2 gave samples after 20 minutes, 40 minutes, 1 hour, 5 hours, and 12 hours. The specimens were analyzed the day after collection, then were stored at 4 °C for one month and re-analyzed with a dif-ferent method. A year later, they were re-analyzed using this procedure. It was not possible to procure authentic specimens at this time since the compounds are no longer available legally.

The main active compound in the two preparations was determined to be JWH-018. After storage at 4 °C for one month the samples were reanalyzed and found to be extremely stable with almost identical concentrations detected. When the specimens which had been stored at 4 °C for a year were re-analyzed, the concentrations in Subject number 1 were essentially the same as the previous year; the levels in Subject number 2, which were much lower originally, had generally declined (Figure 3).

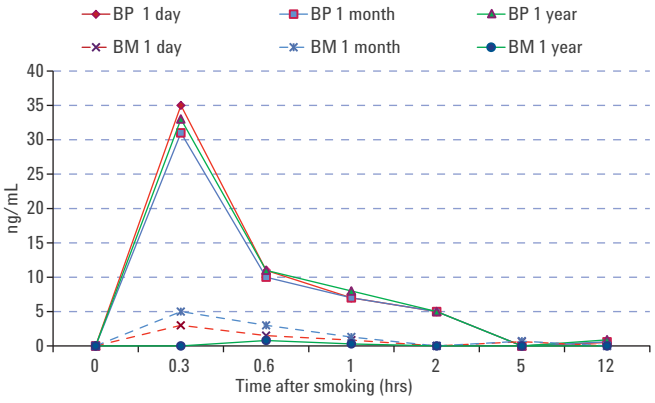

Figure 3. Stability of authentic specimens stored at 4 °C.

An extracted ion chromatogram showing the transitions and ± 20% acceptability band around the intensity of the qualifying transition from the sample collected 40 minutes after smoking (Subject number 1) is presented in Figure 4; the concentration of JWH-018 was 11 ng/mL.

Summary

The simultaneous determination of several "Spice" compounds in oral fluid is reported for the first time. The procedure is applicable to the analysis of specimens collected using the Quantisal device for the presence of synthetic cannabinoids, which were recovered from the pad > 60% at two concentrations. Following a single smoking session of two different herbal product brands, JWH-018 was detected in oral fluid with the highest concentrations appearing 20 minutes after a single smoking session. Even after a year, JWH-018 was detectable in the oral fluid 12 hours after a single smoking session of "Blueberry Posh".

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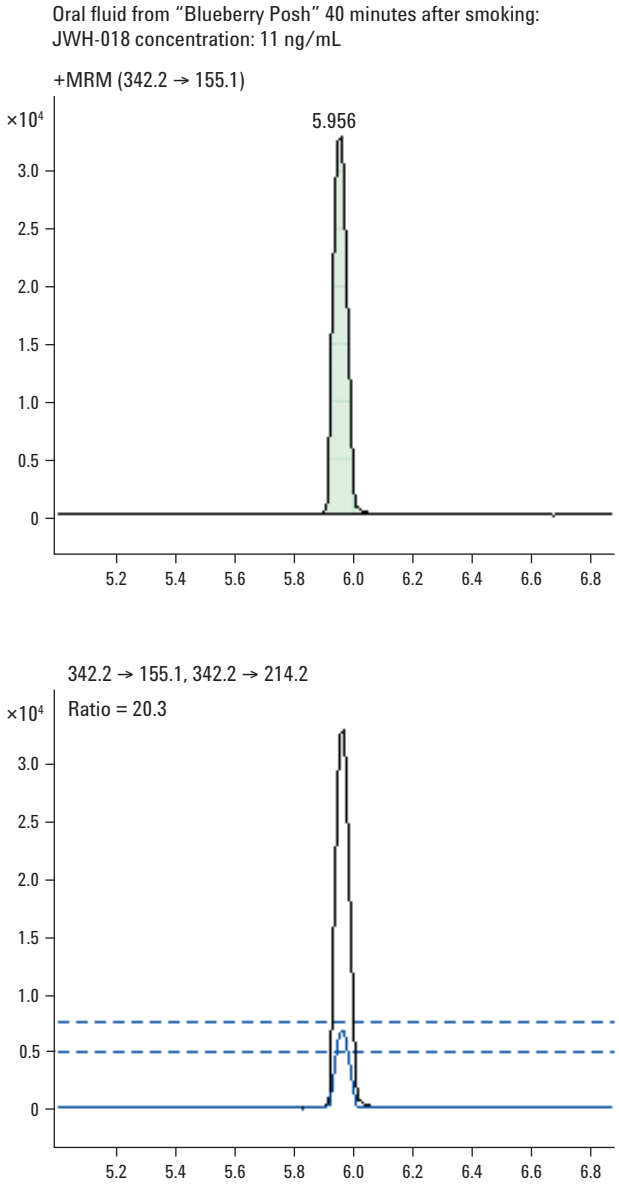

Figure 4. Oral fluid from Subject #1 40 minutes after smoking; JWH-018 = 11ng/mL.

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Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

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Introduction

In 2011, five members of the "synthetic cannabinoids" group or 'Spice' compounds were banned in the USA. The substances were:

- 1-pentyl-3-(1-naphthoyl)-indole (JWH-018)
- 1-butyl-3-(1-naphthoyl)-indole (JWH-073)
- 1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)-indole (JWH-200)
- 5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (CP-47,497)
- 5-(1,1-dimethyloctyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (cannabicyclohexanol; CP-47,497 C8 homologue)

The drugs have been described by users as having cannabis-like effects, and some of these compounds show strong binding to cannabinoid receptors. The (–)-1,1-dimethylheptyl analog of 11-hydroxy-Δ^8-tetrahydrocannabinol, (1,1-dimethylheptyl-11-hydroxytetrahydrocannabinol) is known as HU-210 and has been reportedly found in seizures of "Spice Gold", "Spice Silver" and "Spice Diamond" made by the US Customs and Border Protection in 2009. HU-210 is considered to be more potent than Δ^9-tetrahydrocannabinol (Δ^9-THC), and was already classified as a controlled substance as an analog of marijuana. JWH-250 is also commonly encountered so was also included in the research.

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Oral fluid is becoming increasingly popular as a specimen for the detection of drugs at the roadside, and in workplace testing. It is easy to collect, non-invasive and can give information on recent drug intake. In the work described here, the Quantisal device was used for oral fluid collection, and the detection of "Spice" components is described.

Collection devices, reagents and standards

Quantisal devices for the collection of oral fluid specimens contain a cotton collection pad which is placed in the mouth. The incorporated volume adequacy indicator turns blue when 1 mL of oral fluid (± 10%) has been collected, then the pad is placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). Drug concentrations detected are adjusted accordingly.

Solid phase extraction columns (Bond Elut Plexa) and liquid chromatographic columns (ZORBAX RRHT) were obtained from Agilent Technologies. The standard compounds JWH-018, JWH-073, JWH-200, JWH-250, HU-210, CP-47,497 and CP-47,497 C8 homologue as well as deuterated *d9*-JWH-018 and *d7*-JWH-073 were purchased from Cayman Chemicals.

Calibrators and controls

The deuterated internal standards (*d9*-JWH-018 and *d7*-JWH-073) and unlabelled drug standards were prepared in methanol at a concentration of 100 µg/mL. The working solutions were diluted from stock to a concentration of 10 µg/mL in methanol. The solutions were stored at –20 °C when not in use. Controls were prepared by fortifying drug-free synthetic oral fluid with various concentrations of compounds. Drug free negative specimens, positive controls at 4 ng/mL and 40 ng/mL were included in every batch.

Sample preparation

Seven calibration standards were prepared in oral fluid at concentrations of 0.5, 2, 5, 10, 20, 50, and 100 ng/mL for all analytes; deuterated internal standards were added (10 ng/mL).

Agilent Bond Elut Plexa (30 mg/1 mL; p/n 12109301) solid phase extraction cartridges were used.

1. Condition: methanol (0.5 mL); 0.1 M acetic acid (0.1 mL)
2. To each 1mL aliquot of calibrator, control or specimen, add acetic acid (0.1 M; pH 4, 1 mL)
3. Load samples
4. Wash columns: DI water: glacial acetic acid (80:20; 1 mL); DI water: methanol (40:60; 1 mL)
5. Dry columns (5 minutes)
6. Elute acidic/neutral compounds: hexane: glacial acetic acid (98:2; 2 mL)
7. Evaporate extracts to dryness while allowing columns to dry (7 minutes)
8. Elute bases into corresponding tubes: ethyl acetate: ammonium hydroxide (98:2; 2 mL)
9. Evaporate to dryness under nitrogen at 40 °C
10. Reconstitute in methanol (50 µL); transfer to autosampler vials; cap
11. Analyze using LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

An Agilent Technologies 1200 Series liquid chromatography pump coupled to an Agilent 6430 Triple Quadrupole LC/MS System, operating in electrospray ionization mode (ESI) with either positive or negative polarity depending on the compound.

Column	Agilent ZORBAX RRHT Extend C18, (2.1 × 50 mm, 1.8 µm, p/n 727700-902)	
Column temperature	60 °C	
Injection volume	5 µL	
Mobile phase	Solvent A: 0.2% acetic acid and Solvent B: acetonitrile	
	Time 0:	95% A; 5% B; 5 min: 100% B; 7 min 5% B
	Run time	9.2 min; Post-time 3 min
	Flow rate:	0.5 mL/min
Nitrogen gas temperature	350 °C	
Gas flow	10 L/min	
Nebulizer pressure	55 psi.	
Capillary voltage	+4,000 V in positive mode; –4,000 V in negative mode	

Two transitions were selected and optimized for each drug. Table 1 shows the transitions, the optimized fragment voltages for the parent ion (M +1; M-1) as well as the collision energy for fragmentation of the product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ± 20% in order to meet the criterion for a positive result.

Table 1. Multiple Reaction Monitoring (MRM) Transitions; Optimized Fragmentation Voltages; Allowable Transition Ranges Determined at 10 µg/mL for "Spice" Compounds

Compound	Transition	Fragment voltage (V)	Collision energy (eV)	Polarity	Ratio of quantifying to qualifying transition (range)
d9-JWH-018	351.3 > 223.4	140	20	Positive	n/a
JWH-018	342.2 > 155.1	120	20	Positive	16–24
	342.2 > 214.2	120	20		
JWH-250	336.3 > 200.2	120	12	Positive	69–104
	336.3 > 188.2	120	20		
d7-JWH-073	335.3 > 207.2	120	20	Positive	n/a
JWH-073	328.2 > 155.1	120	20	Positive	60–90
	328.2 >127.1	120	35		
JWH-200	385.3 > 155.1	140	20	Positive	54–81
	385.3 > 114.2	140	25		
CP 47497 C8	331.3 > 313.3	160	25	Negative	70–104
	331.3 > 259.3	160	35		
CP 47497	317.3 > 299.2	160	20	Negative	75–113
	317.3 > 245.2	160	30		
HU-210	385.3 > 367.4	120	30	Negative	13–20
	385.3 > 281.3	120	45		

Underlined transitions used for quantitation; n/a = not applicable for internal standard

Figure 1 shows a chromatogram for the primary transitions of the compound at a concentration of 10 ng/mL; the ratio of primary to secondary transition for each compound was also determined at 10 ng/mL.

Recovery from the collection pad

Six synthetic oral fluid specimens fortified with the compounds at concentrations of 4 and 40 ng/mL were prepared. The collection pad was placed into the samples until 1 mL (±10%) had been collected, as evidenced by the blue volume adequacy indicator incorporated into the stem of the collector, then the pad was transferred to the Quantisal buffer, capped and stored overnight to simulate transportation to the laboratory. The following day an aliquot of the specimen was analyzed. The amount recovered from the pad was compared to an absolute concentration (100%) where drug was added to the buffer and left overnight at room temperature without the pad, then subjected to extraction and analysis.

The percentage recovery from the pad for the compounds at concentrations of 4 and 40 ng/mL (n = 6) were > 60% for all at both levels. The highest recovery was 86% for HU-210 at 4 ng/mL; the lowest was 61% for JWH-073 at 40 ng/mL. The recoveries were essentially equivalent at both levels (Table 2).

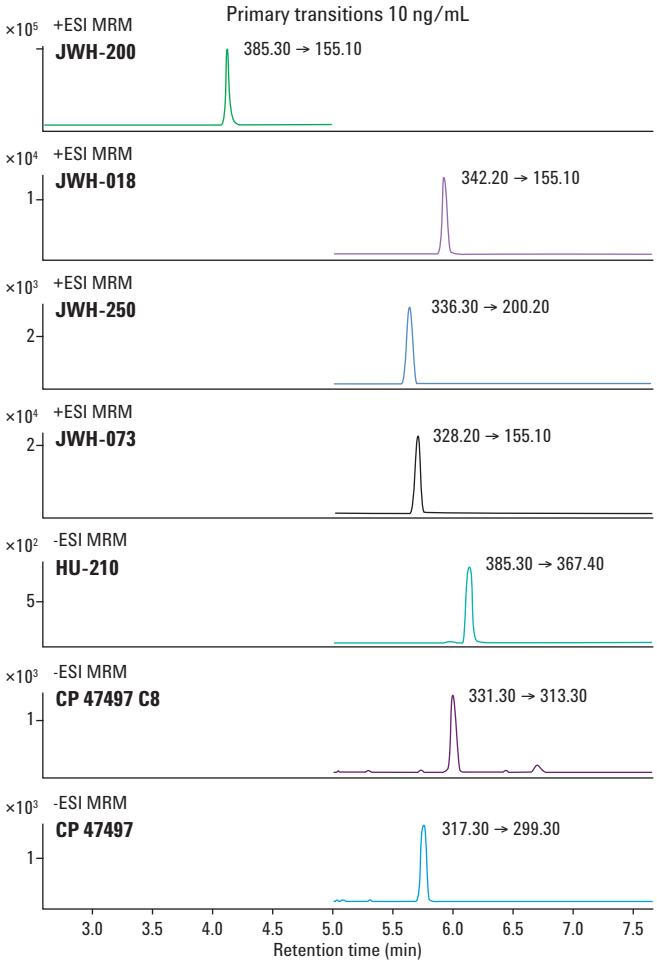

Figure 1. Primary transition at 10 ng/mL.

Table 2. Method Evaluation

	JWH-018	JWH-073	JWH-200	JWH-250	CP 47497	CP 47497 C8	HU-210
LOQ (ng/mL)	0.5	0.5	0.5	2	0.5	2	5
Imprecision intra-day							
4 ng/mL	3.9%	3.6%	5.0%	3.4%	4.9%	3.9%	8.6%
40 ng/mL	2.2%	2.1%	6.0%	2.0%	4.1%	4.3%	5.6%
Inter-day							
4 ng/mL	8.8%	9.6%	6.2%	11%	7.7%	11%	10%
40 ng/mL	8.5%	7.9%	6.2%	11%	10%	11%	12%
Pad recovery							
4 ng/mL	65.5%	67.4%	85.0%	66.5%	77.7%	76.0%	86.4%
40 ng/mL	70.6%	61.4%	81.4%	75.1%	71.3%	78.2%	75.7%
Matrix effect	-55%	-45%	-55%	-73%	-64%	-55%	-49%
Process efficiency	40%	51%	56%	24%	38%	45%	51%

Data Analysis

Calibration was carried out using linear regression analysis over a concentration range of 0.5–100 ng/mL. Peak area ratios of target analytes and the internal standard were calculated for each concentration using Agilent MSD software. The data were fit to a linear least squares regression curve, not forced through the origin, and with equal weighting. For confirmation, two transitions were monitored for each of the compounds; one for the internal standard. The ratio of the qualifying transition was required to be within 20% of that established using the known calibration standard to be acceptable.

Linearity and sensitivity

The limit of quantitation (LOQ) of the method was determined using serial dilutions to the lowest point where the acceptable criteria for the quantitation of a compound were met, that is, the chromatographic peak shape, retention time (within 2% of calibration standard), and qualifier transition ratio (± 20%) compared to the 10 ng/mL calibration standard were acceptable. The quantitative value of the LOQ had to be within ± 20% of the target concentration. The limit of quantitation was 0.5 ng/mL for JWH-018, JWH-073, JWH-200, and CP 47497; 2 ng/mL for CP 47497 C8 and JWH-250; 5 ng/mL for HU-210 (Figure 2). Linearity was acceptable from the LOQ to 100 ng/mL ($R^2 > 0.99$; n = 5) for all compounds.

Matrix effects

A nonextracted drug standard at a concentration of 10 ng/mL was prepared as well as drug free matrix extracts and negative controls (extracts containing only internal standard). The recovery of the compounds from the oral fluid was determined by first assessing the response of the extracted samples (n = 3) at a concentration of 10 ng/mL {R_{ES}}. Then, oral fluid was extracted and drug was added postextraction at a concentration of 10 ng/mL (n = 3) {R_{PES}}. The percentage recovery was then calculated from the equation (R_{ES}/ R_{PES}) × 100.

The reduction in response due to matrix effects (ion suppression) was determined by assessing the peak area response of a nonextracted neat drug standard (n = 3) at a concentration of 10 ng/mL {R_{NES}}. The nonextracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The % matrix effect was then calculated using the equation (R_{PES} / R_{NES}) -1 × 100. The overall efficiency of the process was calculated as (R_{ES} / R_{NES}) × 100.

Ion suppression effects were significant, but were limited by the use of solid-phase extraction and deuterated internal standards.

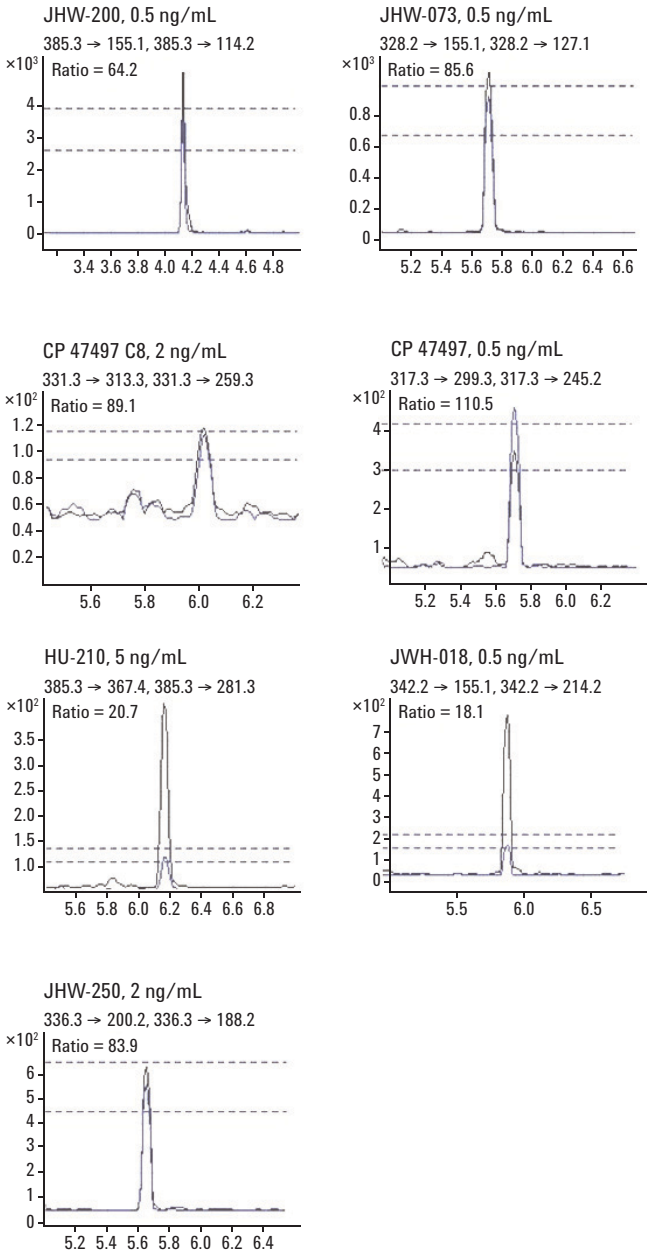

Figure 2. LOQ concentrations showing ± 20% ratio.

Selectivity

Five drug free oral fluid specimens were collected using the Quantisal device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer.

In addition, common drugs of abuse were added at concentrations of 2,000 ng/mL to other aliquots of the drug-free fluid, extracted, and analyzed as described.

THC	amitriptyline
THC-COOH	cyclobenzaprine
11-OH-THC	imipramine
cannabinol	dothiepin
cannabidiol	doxepin
cocaine	fluoxetine
benzoylecgonine	sertraline
norcocaine	trimipramine
cocaethylene	protriptyline
codeine	chlorpromazine
morphine	clomipramine
6-AM	nortriptyline
6-AC	paroxetine
oxycodone	desipramine
oxymorphone	bromazepam
hydrocodone	alprazolam
hydromorphone	clonazepam
amphetamine	lorazepam
methamphetamine	oxazepam
MDMA	diazepam
MDA	midazolam
MDEA	flurazepam
phentermine	flunitrazepam
fentanyl	nordiazepam
phencyclidine	triazolam
tramadol	temazepam
carisoprodol	nitrazepam
meprobamate	chlordiazepoxide
citalopram	methadone
venlafaxine	

No endogenous interference was noted from drug free extracts; or for exogenous interference from any of the commonly encountered drugs, including THC and its main metabolites, which were analyzed at high concentration.

Imprecision

Specimens were fortified with all the compounds simultaneously at concentrations of 4 ng/mL and 40 ng/mL. Each concentration was analyzed according to the described procedure (n = 6; intra-day imprecision) for 5 consecutive days (n = 30; inter-day imprecision). The intra-day imprecision of the assays for all drugs was < 9% at both concentrations; inter-day < 12% at both concentrations (Table 2).

Authentic samples

Specimens were collected from two volunteers, who had purchased the compounds while still legally available in the USA. Subject number 1 smoked "Blueberry Posh" and subject number 2 smoked "Black Mamba". Using Quantisal oral fluid collection devices, specimens were collected prior to the start of smoking, then at the various time points after smoking. Subject 1 gave specimens after 20 minutes, 40 minutes, 1 hour, 2 hours, and 12 hours; Subject 2 gave samples after 20 minutes, 40 minutes, 1 hour, 5 hours, and 12 hours. The specimens were analyzed the day after collection, then were stored at 4 °C for one month and re-analyzed with a dif-ferent method. A year later, they were re-analyzed using this procedure. It was not possible to procure authentic specimens at this time since the compounds are no longer available legally.

The main active compound in the two preparations was determined to be JWH-018. After storage at 4 °C for one month the samples were reanalyzed and found to be extremely stable with almost identical concentrations detected. When the specimens which had been stored at 4 °C for a year were re-analyzed, the concentrations in Subject number 1 were essentially the same as the previous year; the levels in Subject number 2, which were much lower originally, had generally declined (Figure 3).

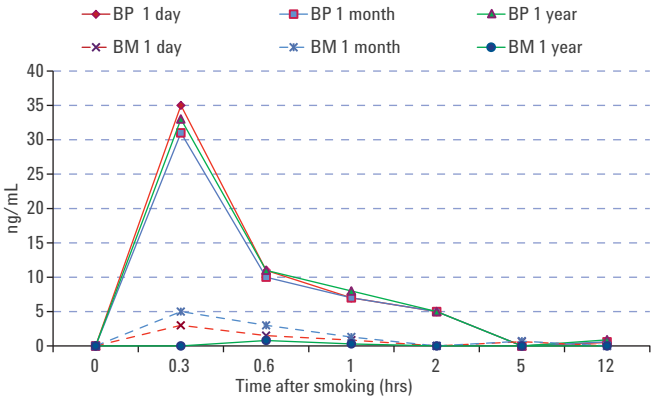

Figure 3. Stability of authentic specimens stored at 4 °C.

An extracted ion chromatogram showing the transitions and ± 20% acceptability band around the intensity of the qualifying transition from the sample collected 40 minutes after smoking (Subject number 1) is presented in Figure 4; the concentration of JWH-018 was 11 ng/mL.

Summary

The simultaneous determination of several "Spice" compounds in oral fluid is reported for the first time. The procedure is applicable to the analysis of specimens collected using the Quantisal device for the presence of synthetic cannabinoids, which were recovered from the pad > 60% at two concentrations. Following a single smoking session of two different herbal product brands, JWH-018 was detected in oral fluid with the highest concentrations appearing 20 minutes after a single smoking session. Even after a year, JWH-018 was detectable in the oral fluid 12 hours after a single smoking session of "Blueberry Posh".

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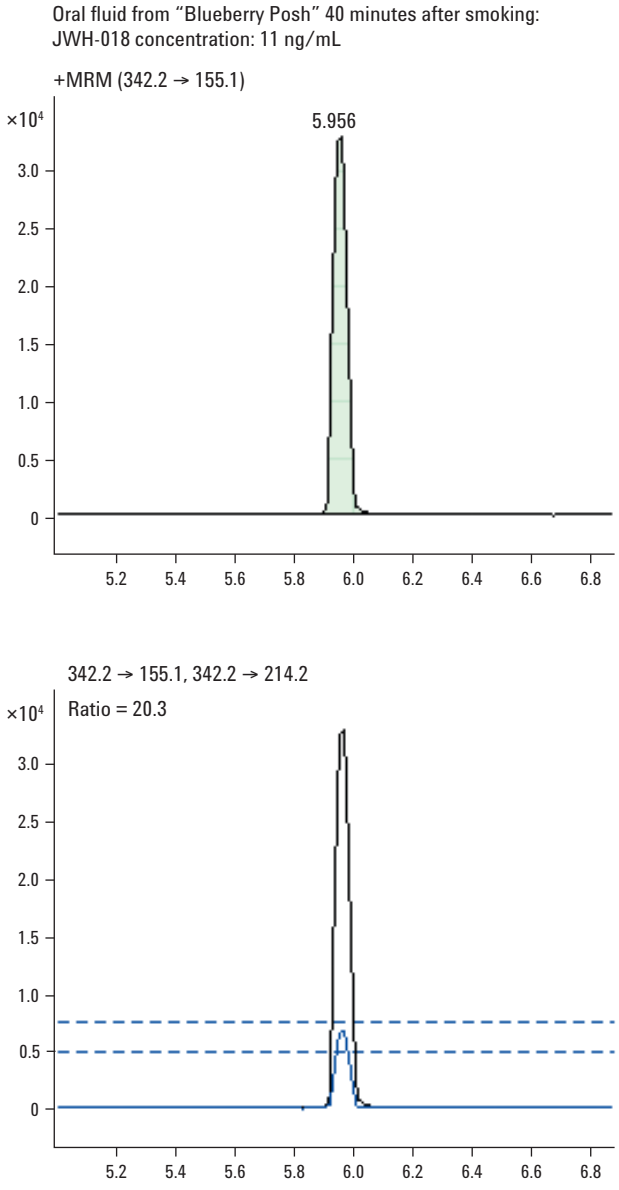

Figure 4. Oral fluid from Subject #1 40 minutes after smoking; JWH-018 = 11ng/mL.

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SAMHSA-Compliant LC/MS/MS Analysis of 6-Acetylmorphine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they typically do not require a derivatization step. We present a method for analysis of 6-acetylmorphine that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy, and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products such as Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

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Introduction

A metabolite, 6-Acetylmorphine, or 6-monoacetylmorphine (6-AM) is unique to heroin. Heroin (or diacetylmorphine) is an opioid drug synthesized from morphine. In the body, heroin is rapidly metabolized through deacetylation to 6-AM and then to morphine at a somewhat slower rate [2]. The updated SAMHSA confirmation cutoff concentration for 6-AM is 10 ng/mL, and a LOD at 10% of the cutoff would be 1 ng/mL.

The simple extraction method described here provides reproducible high recoveries of 6-AM due to the unique properties of Bond Elut Plexa. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

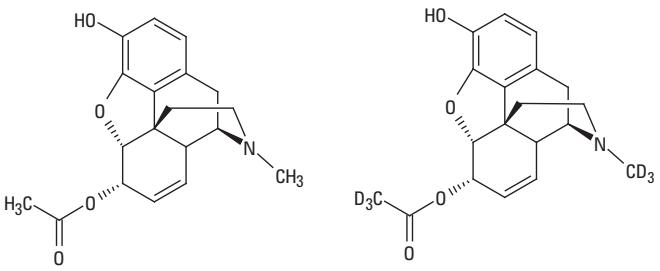
A Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with approximately 40% less back pressure, thereby allowing users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

With a low sample injection volume of 10 µL and no sample preconcentration, the presented method demonstrates excellent signal-to-noise ratios (> 190:1 at 1 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of an Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent [3,4] used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures.

Experimental

Analytes



6-Acetylmorphine
Log P 1.55
pK_{a1} 8.2

6-Acetylmorphine-D_6

Figure 1. 6-Acetylmorphine analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (6-acetylmorphine) and 100 µg/mL (6-acetylmorphine-D_6) solutions in acetonitrile.

Materials and instrumentation

SPE

- Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2-mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Pretreatment

Spike 1 mL of urine with ISTD at 20 ng/mL; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL. methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL methanol: ammonium hydroxide (100:10), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under stream of nitrogen to dryness.
8. Reconstitute in 1 mL initial mobile phase (10% methanol, 90% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	10
	1.5	25
	2.0	60
	2.1	90
	5.0	90
	5.1	10
Stop time	5.2 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	10 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES Source Parameters	
Ionization mode	Positive
Capillary voltage	2,800 V
Drying gas flow	13 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V
MS parameters	
Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.2 min - diverter valve to MS
Delta EMV (+)	400 V

Results and Discussion

At acidic pH, the tertiary amine of 6-acetylmorphine was protonated, and the analyte was efficiently retained on Bond Elute Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without 6-AM loss from the SPE column. A strong base was added to organic eluent to break ionic interaction between the analyte and strong cation exchange sorbent. 6-AM recovery was optimized with 10% NH_4OH added to methanol shortly before sample elution.

The Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column provided fast separation of 6-AM in urine extract and good peak shape (Figure 2). The LC separation started with a low fraction of organic solvent (10%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting a first portion of flow (0 to 1.2 minutes) to waste to minimize source contamination. Data collection started at 1.2 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed for short retention and re-equilibration times.

SAMHSA guidelines require one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for each target analyte (Table 1) was provided for additional confidence. Agilent MassHunter Quantitative software automatically calculated qualifier ion ratios, highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
6-AM	328.2	165.1	140	40
6-AM	328.2	211.1	140	25
6-AM	328.2	193.1	140	25
6-AM-D_6	334.2	165.1	140	40
6-AM-D_6	334.2	211.1	140	25

Normal, rather than dynamic, MRM scan type can be used with this method, because dynamic MRM has no advantages for detection of a single compound.

A signal-to-noise ratio of > 190:1 for the 1 ng/mL peak (Figure 2, upper panel) illustrated a state-of-the-art performance of the Agilent 6460 Triple Quadrupole LC/MS capable of reliably detecting 6-AM at a small fraction (10%) of the SAMHSA cutoff concentration.

Figure 3 is an example calibration curve for extracted urine standards at five concentration levels of 6-acetylmorphine. Calibration standards were prepared by spiking negative urine at 1.0, 10, 50, 200, and 400 ng/mL. Deuterated internal standard 6-AM-D_6 was added at 20 ng/mL. The excellent linear fit with $R^2 > 0.999$ demonstrates linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al* [5] and widely accepted as an industry standard approach for LC/MS/MS methods. Extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 10 ng/mL with 6-AM (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 10 ng/mL in urine (spiked mobile phase).

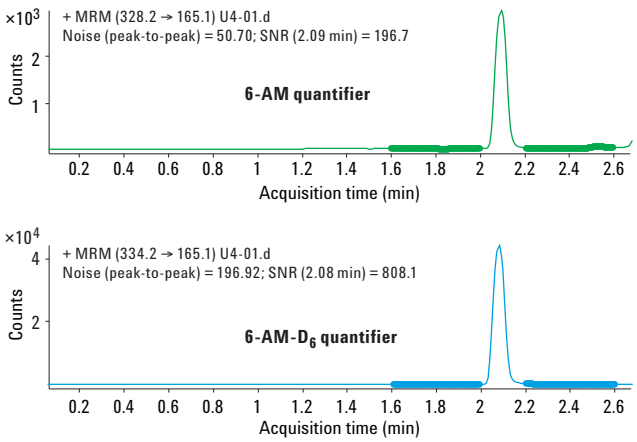


Figure 2. MRM extracted ion chromatograms for 6-AM (1 ng/mL) and 6-AM-D_6 (20 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

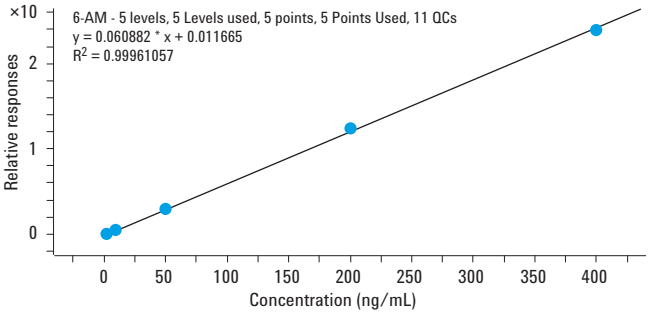


Figure 3. Example calibration curve for 6-AM in urine extract. Calibration range 1.0 to 400 ng/mL. Linear fit, $R^2 > 0.999$.

Table 2. Method performance for 6-Acetylmorphine, n = 5.

	%
Process efficiency*	83
Extraction recovery*	83
Matrix effect*	100
Accuracy**	106
Precision** (CV)	0.6

*determined at cutoff level **determined at 40% cutoff

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows high extraction recovery for 6-acetylmorphine (83%) together with very good accuracy (106%) and precision (0.6 %). Matrix effect of 100% indicated no suppression or enhancement of a signal due to matrix interferences, thus confirming an exceptional cleanliness of Plexa-processed extracts.

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described here is SAMHSA-compliant and provides accurate, precise, and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in the other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and 1200 Series LCs because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of the Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

References

1. SAMHSA (2010) Manual for Urine Laboratories, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.

2. R. Baselt (2008) *Disposition of Toxic Drugs and Chemicals in Man.* 8th edition. Atlas Books, Ashland, OH, USA.

3. P. Moorman and J. Hughes (2010) "6-Acetylmorphine in Urine by LC/Triple Quadrupole Mass Spectrometry (LC/MS/MS)". SOP, Agilent Technologies, Inc. Publication Number 5990-5857EN.

4. J. Hughes and P. Moorman (2011) "Confirmation by Triple Quadrupole LC/MS/MS for HHs-compliant Workplace Urine Drug Testing". Agilent Technologies, Inc. Seminar available from www.agilent.com/chem.

5. B. K. Matuszewski, M. L. Constanzer, and C. M. Chavez-Eng (2003) "Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS". *Analytical Chemistry*, 75: 3019-3030.

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Agilent Technologies

SAMHSA-Compliant LC/MS/MS Analysis of Amphetamines in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of five amphetamines that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy, and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

Amphetamines are psychostimulant drugs included in a group of sympathomimetic amines that mimic the effects of the endogenous neurotransmitters, such as epinephrine (adrenaline), norepinephrine (noradrenaline), and dopamine. Amphetamines are found in the leaves of *Ephedra sinica* (for example ephedrine) and were first produced synthetically at the end of the 19th century. Their chemical structure features a phenethylamine backbone with a methyl group attached to the alpha carbon, along with other substitutions (Figure 1). A significant portion of amphetamines is excreted intact in urine. By demethylation, more complicated amphetamine derivatives are metabolized into simpler structures, for example methamphetamine to amphetamine, and MDMA to MDA [2]. The 2011 SAMHSA guidelines require screening for and confirmation of five amphetamines – amphetamine, methamphetamine, MDA, MDMA, and MDEA. The confirmation method should demonstrate the ability to distinguish these drugs from structurally similar compounds that are potential interferences, including ephedrine, pseudoephedrine, phentermine, and phenylpropanolamine (PPA, or norephedrine).

In GC/MS methods traditionally employed for detection of amphetamines, it was common to apply periodate pretreatment to oxidize the hydroxyphenethylamines ephedrine and pseudoephedrine and, thus, exclude a chance of interference by these compounds. We eliminated this step, offering instead a reliable chromatographic separation of all analytes of interest required by the latest SAMHSA guidelines.

The new SAMHSA confirmation cutoff concentration for all amphetamines is 250 ng/mL and a limit of detection at 10% of the cutoff concentration is 25 ng/mL [1]. Because high concentrations of amphetamines can be expected in some urine samples, we chose to use a higher capacity 3 mm id Agilent Poroshell 120 column instead of a 2 mm id column for all Agilent SAMHSA methods. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure. Therefore, it allows users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

The simple extraction method described here provides reproducible high recoveries of amphetamines due to the unique properties of Agilent Bond Elut Plexa. Unlike other polymeric sorbents, Plexa possesses amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

With a low sample injection volume of 2 µL and no sample preconcentration, the presented method demonstrates excellent signal-to-noise (S/N) ratios (> 400:1 at 25 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system system and other SPE/LC products and procedures [3,4].

Experimental

Analytes

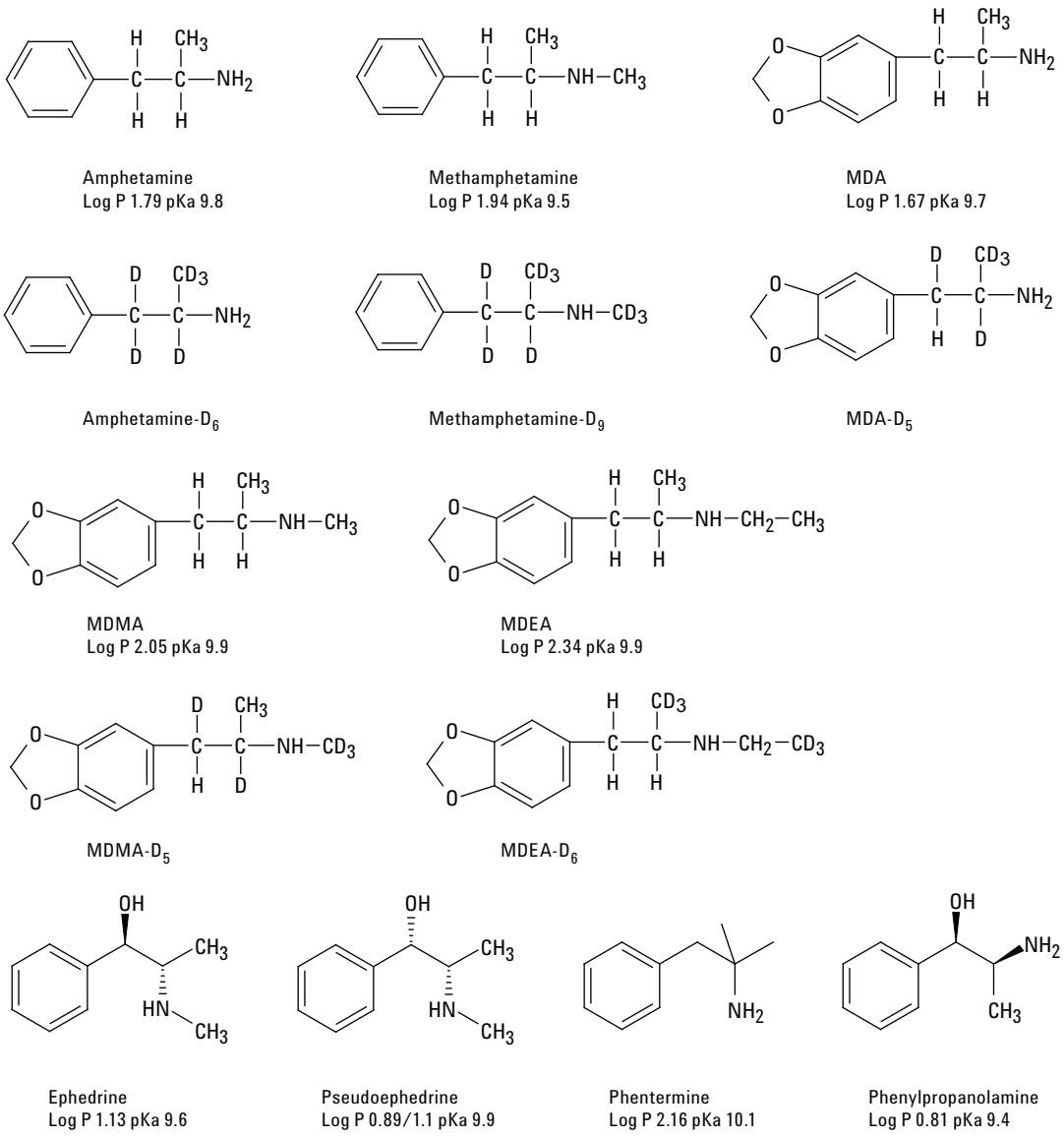

Figure 1. Amphetamines and interferences - analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (amphetamine, methamphetamine, MDA, MDMA, MDEA, ephedrine, pseudoephedrine, phentermine, and phenylpropanolamine) and 100 µg/mL (amphetamine-D_6, methamphetamine-D_9, MDA-D_5, MDMA-D_5, and MDEA-D_6) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2 mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Pretreatment

Spike 0.5 mL of urine with ISTDs at 500 ng/mL each; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL ethyl acetate: methanol: ammonium hydroxide (50:50:20), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under stream of nitrogen to 0.2 mL at ≤ 37 °C.
8. Add 100 µL of 0.025 N hydrochloric acid in methanol, vortex.
9. Evaporate to dryness.
10. Reconstitute in 0.5 mL initial mobile phase (15% methanol, 85% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	15
	1.5	15
	3.5	30
	3.6	90
	6.6	90
	6.7	15
Stop time	6.8 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES Source Parameters

Ionization mode	Positive
Capillary voltage	4,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V

MS parameters

Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 0.6 min (for interferences separation) or 1.2 min (for five amphetamines only) - diverter valve to MS
Delta EMV (+)	200 V

Results and Discussion

At acidic pH, the amine group of amphetamines was protonated, and the analytes were efficiently retained on Bond Elut Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without the loss of analytes from the sorbent. A strong base was added to organic eluent to break ionic interaction between the amphetamines and strong cation exchange sorbent. The recovery was optimized with two-component organic eluent consisting of 50% ethyl acetate and 50% methanol, with 20% NH_4OH added shortly before sample elution.

Amphetamines are rather volatile and could evaporate at the solvent evaporation step of sample preparation unless precipitated as salts by addition of the hydrochloric acid. It is best to add HCl toward the end of evaporation to avoid the formation of ammonium chloride salts which will cause ion suppression.

Figure 2 shows excellent separation of five amphetamines and potential interferences specified by SAMHSA on the Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column, which was completed within 3.2 minutes. LC separation started with a low fraction of organic solvent (15%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting the first portion of flow to waste to minimize source contamination. Data collection started immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed short separation and re-equilibration times.

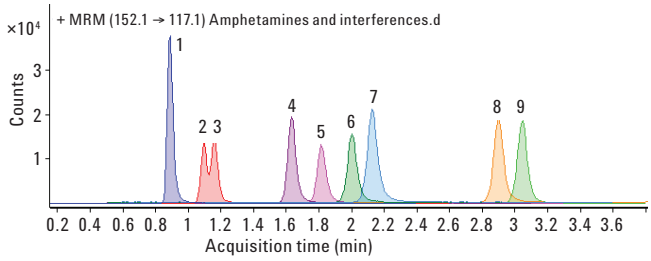

Figure 2. Separation of amphetamines and potential interferences on Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column – overlaid MRM extracted ion chromatograms. Concentration of each analyte corresponds to 50 ng/mL. Peaks in order of their elution are: 1. phenylpropanolamine, 2. ephedrine, 3. pseudoephedrine, 4. amphetamine, 5. methamphetamine, 6. MDA, 7. MDMA, 8. MDEA, 9. phentermine.

A dynamic MRM method using retention time and delta RT (time window) for a certain transition is recommended for the analysis of several compounds. When good separation from interferences is ensured, and data collection is focused on five amphetamines only, the valve can be switched from waste to mass spectrometer at 1.2 minutes instead of 0.6 minutes (time segment no. 1 in the MS method).

SAMHSA guidelines require the use of one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analytes (Table 1) was provided where possible for additional confidence. Agilent MassHunter Quantitative software calculated qualifier ion ratios, automatically highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound name	Precursor	Product	Fragmentor	Collision energy
Amphetamine	136.1	119.1	64	4
Amphetamine	136.1	91.1	64	14
Amphetamine-D_6	142.1	125.1	66	5
Amphetamine-D_6	142.1	93.1	66	13
MDA	180.1	163.1	92	5
MDA	180.1	105.1	92	17
MDA-D_5	185.1	168.1	68	5
MDA-D_5	185.1	110.1	68	21
MDEA	208.1	163.1	88	8
MDEA	208.1	133.1	88	17
MDEA	208.1	105.1	88	21
MDEA-D_6	214.2	166.1	90	8
MDEA-D_6	214.2	108.1	90	25
MDMA	194.1	163.1	84	5
MDMA	194.1	135.1	84	17
MDMA	194.1	105.1	84	21
MDMA-D_5	199.1	165.1	82	4
MDMA-D_5	199.1	107.1	82	25
Methamphetamine	150.1	119.1	80	4
Methamphetamine	150.1	91.1	80	16
Methamphetamine-D_9	159.2	125.2	77	5
Methamphetamine-D_9	159.2	93.1	77	13
Ephedrine-pseudoephedrine	166.1	133.1	80	21
Phentermine	150.1	133.1	80	6
Phenylpropanolamine	152.1	117.1	80	20

S/N ratios exceeding 400:1 were obtained for quantifier peaks of all five amphetamines at 25 ng/mL (Figure 3, upper panel: S/N is shown for the MDEA quantifier peak). This illustrated the state-of-the-art performance of the Agilent 6460 Triple Quadrupole LC/MS/MS capable of reliably detecting all five amphetamines at a small fraction of the SAMHSA cutoff.

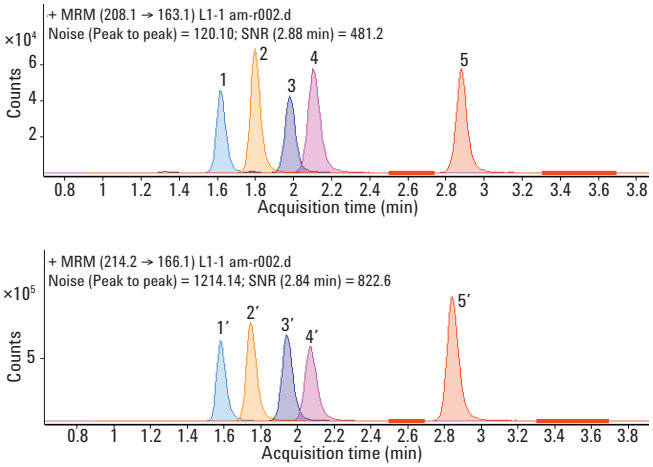

Figure 3. Overlaid MRM extracted ion chromatograms for amphetamines quantifiers (25 ng/mL) and ISTDs quantifiers (500 ng/mL) in urine extract on an Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Peaks in order of their elution are: upper panel - 1. amphetamine, 2. methamphetamine, 3. MDA, 4. MDMA, 5. MDEA, lower panel - 1′. amphetamine-D_6, 2′. methamphetamine-D_9 , 3′. MDA-D_5 , 4′. MDMA-D_5 , 5′. MDEA-D_6 . Noise regions are shown in bold.

Figure 4 gives examples of calibration curves for extracted urine standards at five concentration levels. Calibration standards were prepared by spiking negative urine at 25, 250, 1,000, 5,000, and 10,000 ng/mL with each of the five members of the amphetamines class. Deuterated internal standards for each analyte were added at 500 ng/mL. The excellent linear fits to all curves with $R^2 > 0.999$ demonstrated linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

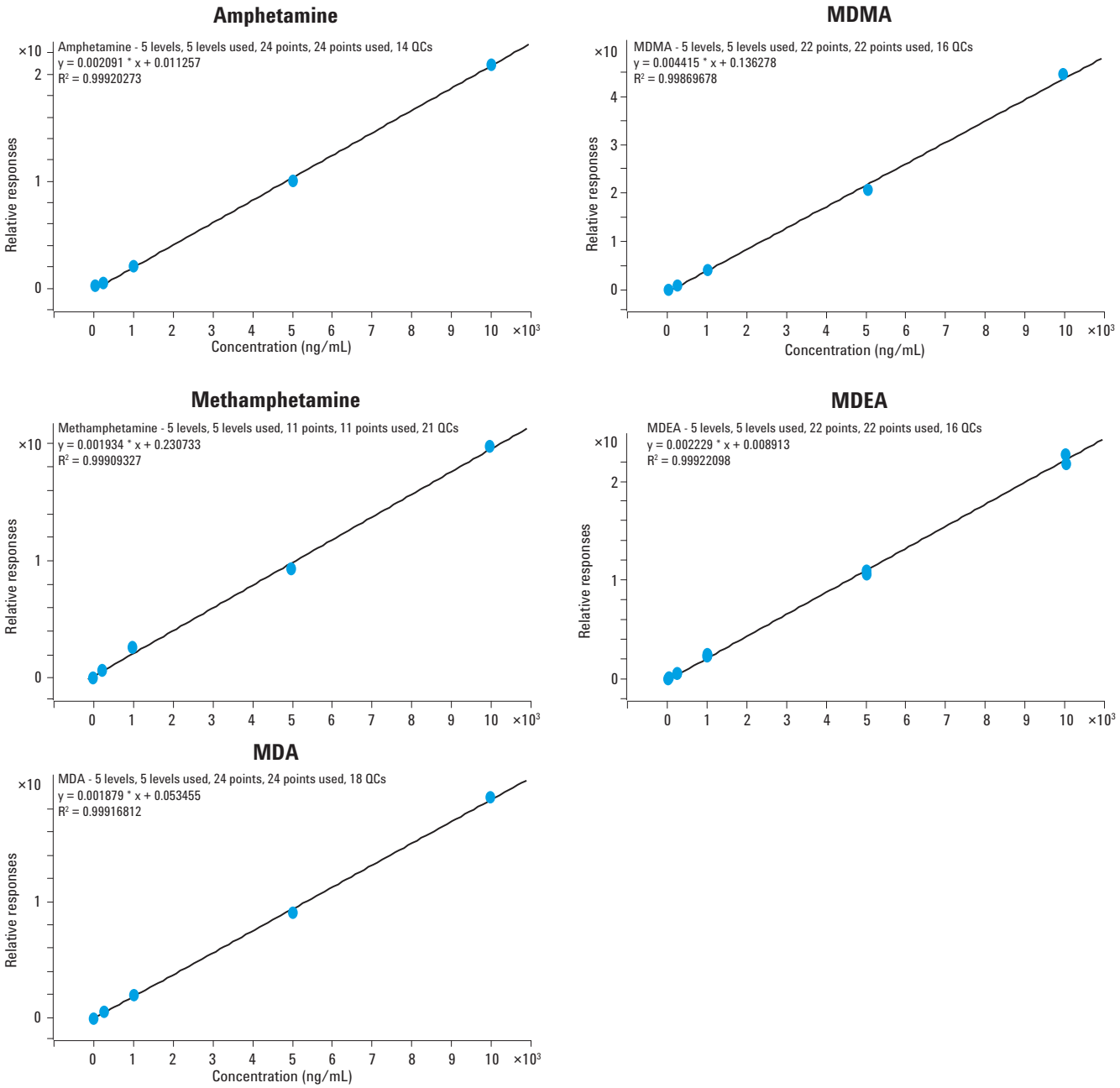

Figure 4. Example calibration curves for five amphetamines in urine extracts. Calibration range 25 to 10,000 ng/mL. All fits are linear, with $R^2 > 0.999$.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al* and widely accepted as an industry standard approach for LC/MS/MS methods [5]. The extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction with each of the five members of the amphetamines class at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase, and then fortified at 250 ng/mL (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 250 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows that the extraction recovery for all five amphetamines was ≥ 94%, with overall process efficiency higher than 90% in four out of five analytes; for amphetamine, process efficiency was 86%. The matrix effect of 91 to 99% means only a 1 to 9% signal reduction due to ion suppression, thus, confirming the exceptional cleanliness of Plexa PCX-processed extracts. High accuracy (within 10% of the target) and excellent precision (CV < 1.1%) is typical for this method.

Conclusions

The solid phase extraction procedure coupled with the LC/MS/MS detection method described here is SAMHSA-compliant and provides accurate, precise, and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. A hardware setup is the same as in other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 Series LC because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

Table 2. Method evaluations, n = 5.

Parameter	Amphetamine	Methamphetamine	MDA	MDMA	MDEA
Process efficiency* (%)	86	93	91	93	95
Extraction recovery* (%)	94	94	95	97	96
Matrix effect* (%)	91	99	95	96	98
Accuracy** (%)	107	105	92	101	106
Precision (CV)**(%)	0.6	0.5	1.1	0.5	0.3

*determined at cutoff level

**determined at 40% cutoff level for amphetamine, MDA, MDMA, MDEA, and at the cutoff level for methamphetamine

References

1. SAMHSA (2010) Manual for Urine Laboratories, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.
2. R. Baselt, (2008) *Disposition of Toxic Drugs and Chemicals in Man.* 8th edition. Atlas Books, Ashland, OH, USA.
3. P. Moorman and J. Hughes, (2010) "Amphetamines (expanded) in Urine by LC/Triple Quadrupole Mass Spectrometry (LC/MS/MS)". SOP, Agilent Technologies, Inc. Publication Number 5990-5865EN.
4. J. Hughes, and P. Moorman, (2011) "Confirmation by Triple Quadrupole LC/MS/MS for HHs-compliant Workplace Urine Drug Testing". Agilent Technologies, Inc. Seminar available from www.agilent.com/chem.
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SAMHSA-Compliant LC/MS/MS Analysis of Benzoylecgonine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of benzoylecgonine that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy, and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

Benzoylecgonine (BE) is a major urinary metabolite of cocaine. Cocaine hydrolysis to benzoylecgonine occurs enzymatically (in the liver), as well as without catalysts at alkaline pH [2]. The SAMHSA-established confirmation cutoff concentration for benzoylecgonine is 100 ng/mL, and a LOD at 10% of the cutoff would be 10 ng/mL [1].

The extraction method described in this application note provides reproducible high recoveries of benzoylecgonine due to unique properties of the Agilent Bond Elut Plexa polymer. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

A Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure, thereby allowing users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

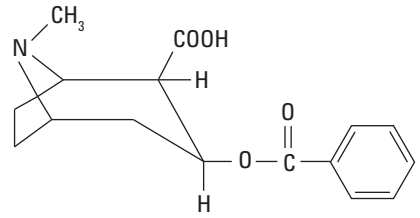
With a low sample injection volume of 2 µL and no sample preconcentration, the presented method demonstrates excellent signal-to-noise (S/N) ratios (> 400:1 at 10 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures [3,4].

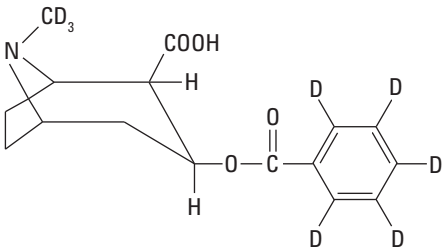
Experimental

Analytes

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (benzoylecgonine) and 100 µg/mL (benzoylecgonine-D_8) solutions in methanol.



Benzoylecgonine
Log P (predicted) -0.73–2.26
pK_{a1} 10.1



Benzoylecgonine –D_8

Figure 1. Benzoylecgonine analytes and their structures. Predicted log P values from DrugBank, ChemSpider, PubChem.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2-mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Pretreatment

Spike 1 mL of urine with ISTD at 200 ng/mL; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL methanol: ammonium hydroxide (100:20), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under a stream of nitrogen to dryness.
8. Reconstitute in 1 mL initial mobile phase (10% methanol, 90% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	10
	0.5	10
	2.5	70
	2.51	90
	5.5	90
	5.51	10
Stop time	5.6 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES source parameters	
Ionization mode	Positive
Capillary voltage	3,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V
MS parameters	
Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.2 min - diverter valve to MS
Delta EMV(+)	200 V

Results and Discussion

At acidic pH, the tertiary amine of benzoylecgonine was protonated, and the analyte was efficiently retained on Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without BE loss from the SPE column. A strong base was added to the organic eluent to break the ionic interaction between the analyte and the strong cation exchange sorbent. Benzoylecgonine recovery was optimized with 20% NH_4OH added to methanol shortly before sample elution.

The Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column provided fast separation of benzoylecgonine in urine extract and good peak shape (Figure 2). The LC separation started with a low fraction of the organic solvent (10%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting a first portion of flow (0 to 1.2 minutes) to waste to minimize source contamination. Data collection started at 1.2 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed for short analysis and re-equilibration times.

A S/N ratio >400:1 for the 10 ng/mL peak (Figure 2), upper panel) illustrates a state-of-the-art performance of the Agilent 6460 Triple Quadrupole capable of reliably detecting benzoylecgonine at a small fraction (10%) of the SAMHSA cutoff concentration.

SAMHSA guidelines require one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analyte (Table 1) was provided for additional confidence. The Agilent MassHunter Quantitative software automatically calculated qualifier ion ratios, highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
BE	290.1	168.1	90	15
BE	290.1	105.1	90	30
BE	290.1	82.1	90	32
BE-D_5	298.2	171.1	90	15
BE-D_5	298.2	110.1	90	30

Figure 3 is an example calibration curve for extracted urine standards at five concentration levels of benzoylecgonine. Calibration standards were prepared by spiking negative urine at 10, 100, 500, 1,000, and 4,000 ng/mL. Deuterated internal standard BE-D_8 was added at 200 ng/mL. The excellent linear fit with R^2 = 0.998 demonstrated linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

Normal, rather than dynamic, MRM scan type can be used with this method, because dynamic MRM has no advantages for detection of a single compound.

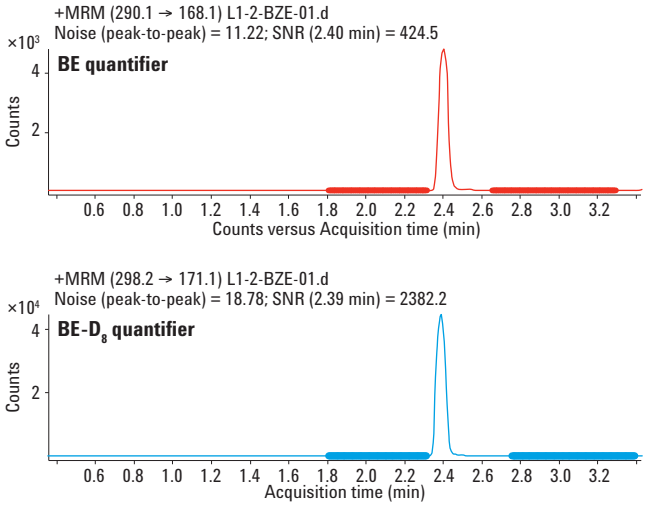


Figure 2. MRM extracted ion chromatograms for BE (10 ng/mL) and BE-D_8 (200 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

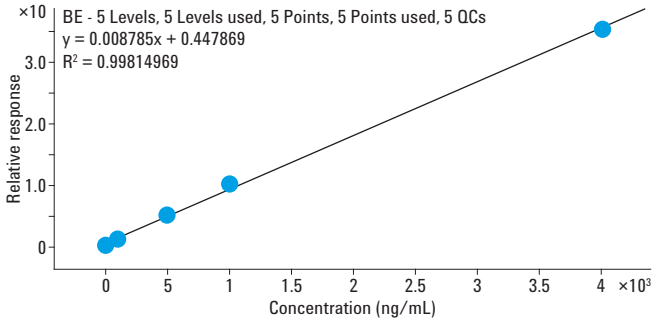


Figure 3. Example calibration curve for benzoylecgonine in urine extract. Calibration range 10 to 4,000 ng/mL. Linear fit, R^2 = 0.998.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al* and widely accepted as an industry standard approach for LC/MS/MS methods [5]. The extraction procedure and the LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 100 ng/mL with benzoylecgonine (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 100 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase.

Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows high extraction recovery for benzoylecgonine (86%) together with excellent accuracy (102%) and precision (0.7%). Matrix effect of 99% indicates minor ion suppression of a signal due to matrix interferences (1%), thus, confirming an exceptional cleanliness of Plexa PCX-processed extracts.

Table 2. Method evaluation at the cutoff level, n = 5.

	%
Process efficiency*	85
Extraction recovery*	86
Matrix effect*	99
Accuracy**	102
Precision** (CV)	0.7

*determined at cutoff level
**determined at 40% cutoff

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described in this application note is SAMHSA-compliant and provides accurate, precise, and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in the other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 Series LCs because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS/MS instruments. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

References

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2. R. Baselt (2008) *Disposition of Toxic Drugs and Chemicals in Man*. 8th edition. Atlas Books, Ashland, OH, USA.
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SAMHSA-Compliant LC/MS/MS Analysis of Opiates (Morphine and Codeine) in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of opiates that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE, Agilent Poroshell 120 EC-C18, 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

Opiates (morphine and codeine) are natural alkaloids found in the resin of the opium poppy. Codeine is currently the most widely used opiate in the world. In addition to detection of morphine and codeine, guidelines from SAMHSA require the confirmation method to demonstrate the ability to distinguish these drugs from structurally related compounds, such as the semisynthetic opioids: hydromorphone, oxymorphone, hydrocodone, oxycodone, and the codeine metabolite norcodeine [2].

Both morphine and codeine are extensively metabolized in the body. Morphine is metabolized primarily into morphine-3-glucuronide and morphine-6-glucuronide. Codeine's major metabolites are morphine, codeine-6-glucuronide, and norcodeine. Because both morphine and codeine are found in urine largely in the form of glucuronide conjugates, SAMHSA requires measurement of the total concentration of each compound. A full conversion of glucuronides back to parent species must be performed prior to analysis. The most reliable conversion method ensuring complete recovery of free opiates is acid hydrolysis. Frequently used enzymatic hydrolysis often leads to incomplete recovery of parent compounds which could lead to false negative results [3].

The SAMHSA-established confirmation cutoff concentration for morphine and codeine is 2,000 ng/mL [1]. Because high concentrations of opiates can be expected in some urine samples, we chose to use a higher capacity 3 mm id Poroshell 120 column instead of a 2 mm id column for all Agilent SAMHSA methods. With superficially porous 2.7 µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure. It, therefore, allows users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

The extraction method described in this application note provides reproducible high recoveries of morphine and codeine due to the unique properties of the Agilent Bond Elut Plexa polymer. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

With a low sample injection volume of 2 µL and no sample preconcentration, the method demonstrates excellent signal-to-noise (S/N) ratios for both morphine and codeine (>150:1 at 200 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures [4,5].

Experimental

Analytes

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (morphine, codeine, hydromorphone, norcodeine, hydrocodone, oxycodone, oxymorphone, and morphine-3-glucucronide) and 100 µg/mL (morphine-D_6 and codeine-D_6) solutions in methanol.

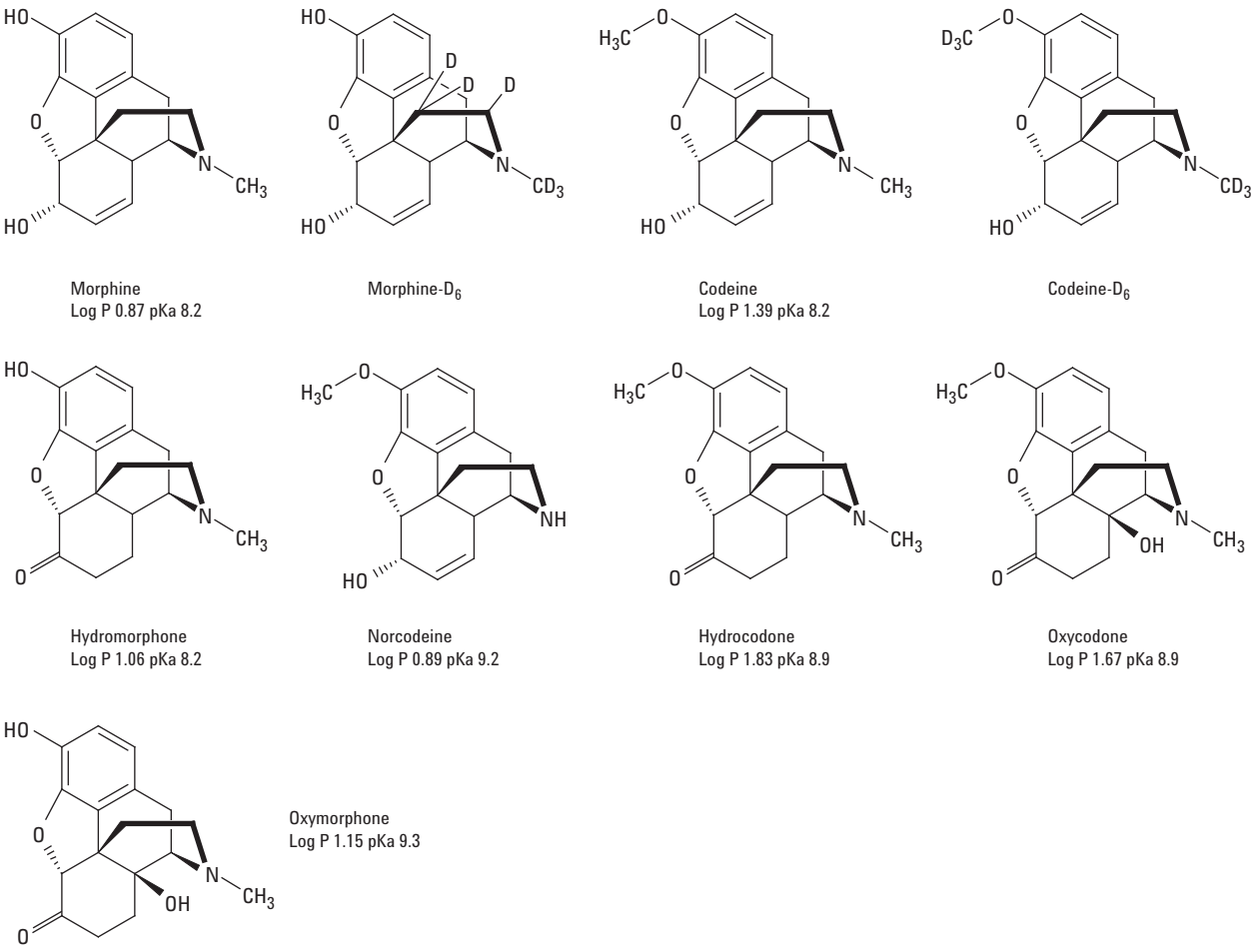

Figure 1. Opiate analytes and their structures.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2-mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Hydrolysis and sample pretreatment

1. Spike 0.5 mL of urine with ISTD at 1000 ng/mL; use of 12 × 75 mm glass tubes is recommended.
2. Add 125 µL concentration HCl.
3. Incubate in the heating block at 95 ±5 °C for 90 minutes.
4. Cool. Add 2 mL 0.1 M sodium acetate buffer (pH 4.5).
5. Neutralize with 250 µL 7 N KOH, vortex, and test pH; it should be <6.
6. Centrifuge 20 minutes at 6,000 rpm.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 2 mL methanol: ammonium hydroxide (100:20), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate to dryness at 40 °C.
8. Reconstitute in 0.5 mL initial mobile phase (5% methanol, 95% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	5
	0.5	5
	1.5	25
	2.5	55
	2.6	90
	5.6	90
	5.7	5
Stop time	5.8 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES source parameters

Ionization mode	Positive
Capillary voltage	3,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V

MS parameters

Scan type	Dynamic MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.0 min - diverter valve to MS
Delta EMV (+)	0 V

Results and Discussion

At low pH, morphine, codeine, and their derivatives were protonated at the tertiary amine group and were strongly retained on Plexa PCX polymeric sorbent by a combination of hydrophobic retention and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without loss of opiates from the SPE column. A strong base was added to the organic eluent to break ionic interaction between the analytes and the strong cation exchange sorbent. The opiates recovery was optimized with 20% NH_4OH added to methanol shortly before sample elution.

The Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column provided excellent separation and peak shapes for opiates and potentially interfering compounds, with the analysis completed within 2.5 minutes (Figure 2). LC separation started with a low fraction of organic solvent (5%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting a first portion of flow (0 to 1 minutes) to waste to minimize source contamination. Data collection started at 1.0 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed for short analysis and re-equilibration times.

The only partially unresolved pair in the chromatogram in Figure 2 were codeine and norcodeine (peaks 4 and 5), but because these compounds have different precursor ions and mass transitions, any possibility of interference of norcodeine signals with codeine quantitation was excluded.

In a separate experiment, Plexa PCX was tested for the possibility of norcodeine methylation and conversion to codeine. Test results were negative; no codeine was detected in negative urine samples that were spiked with norcodeine and then extracted using the method described in this application note.

When testing for interferences, a dynamic MRM method using retention time and delta RT (time window) for a certain transition is recommended. However, when good separation from interferences is ensured, data collection for morphine and codeine and their ISTDs can be performed with normal MRM.

SAMHSA guidelines require the use of one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for the target analyte is provided (Table 1) for additional confidence. Agilent MassHunter Quantitative software calculates qualifier ion ratios, automatically highlighting those out of acceptable range.

Table 1. MRM transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
Codeine	300.2	215.1	130	23
Codeine	300.2	165.1	130	46
Codeine	300.2	153.1	130	50
Codeine-D_6	306.2	165.1	130	44
Codeine-D_6	306.2	218.1	130	23
Morphine	286.1	201.1	130	23
Morphine	286.1	181.1	130	40
Morphine	286.1	165.1	130	43
Morphine-D_6	292.1	181.1	130	40
Morphine-D_6	292.1	165.1	130	42
Morphine-3-glucuronide	462.2	286.1	162	45
Oxycodone	316.2	298.1	130	15
Oxymorphone	302.2	284.1	130	17
Hydrocodone	300.2	199.1	130	30
Norcodeine	286.1	225.1	130	20
Hydromorphone	286.1	185.1	130	28

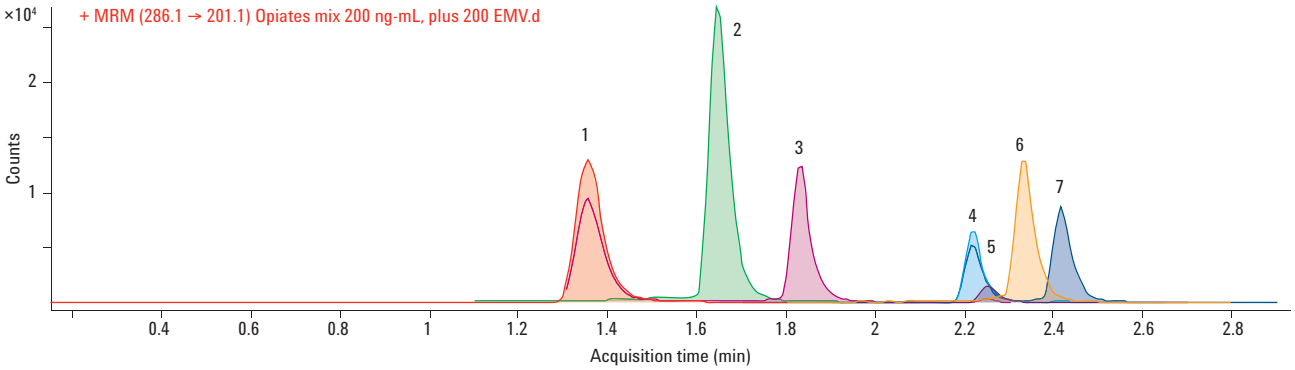

Figure 2. Separation of opiates and potential interferences on Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column - overlaid MRM extracted ion

When processed according to the protocol, urine samples spiked with morphine-ß-3-glucuronide at 10,000 ng/mL showed 97 to 99.2% conversion to morphine. MS parameters for the detection of morphine-ß-3-glucuronide are included in Table 1 for analysts interested in testing the hydrolysis efficiency.

S/N ratios exceeding 150:1 were obtained for quantifier peaks of morphine and codeine at 200 ng/mL (Figure 3, panel 1 and 2 from the top). This illustrates the state-of-the-art performance of the Agilent 6460 Triple Quadrupole LC/MS system, capable of reliably detecting opiates at a small fraction of the SAMHSA cutoff.

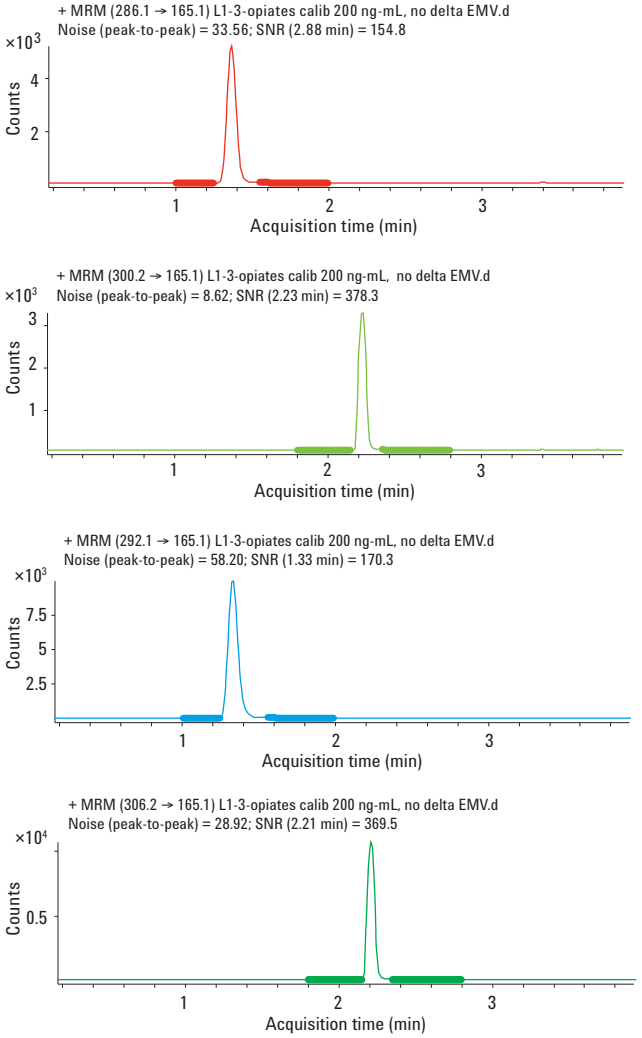

Figure 3. MRM extracted ion chromatograms for morphine and codeine quantifiers (200 ng/mL) and ISTD quantifiers (1,000 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

Figure 4 gives examples of calibration curves for extracted urine standards at five concentration levels. Calibration standards were prepared by spiking negative urine at 200, 1,000, 2,000, 10,000, and 20,000 ng/mL with morphine and codeine. Internal deuterated standard morphine-D_6 and codeine-D_6 were added at 1,000 ng/mL. Excellent linear fit ($R^2 \geq 0.998$) to each of the curves demonstrates linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

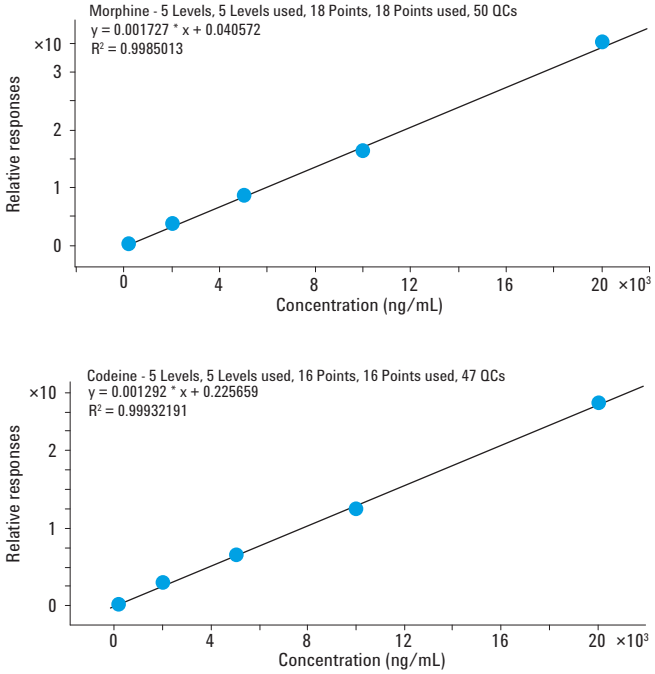

Figure 4. Example calibration curves for morphine (upper panel) and codeine (lower panel) in urine extract. Concentration range 200 to 20,000 ng/mL. Linear fit, $R^2 \geq 0.998$.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al*. and widely accepted as an industry standard approach for LC/MS/MS methods [6]. The extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction with morphine and codeine at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 2,000 ng/mL (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 2,000 ng/mL in urine (spiked mobile phase).

Table 2. Method evaluation of opiates at the cutoff level, n = 5.

Parameter	Morphine	Codeine
Process efficiency (%)	83	85
Extraction recovery (%)	85	86
Matrix effect (%)	98	99
Accuracy (%)	108	108
Precision (CV) (%)	0.6	0.7

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements

Table 2 shows high extraction recovery and process efficiency for morphine and codeine (approximately 85%). The high matrix effect value (98–99%) means only 1 to 2% signal reduction is due to ion suppression, thus, confirming the exceptional cleanliness of Plexa PCX-processed extracts. High accuracy (within 10% of the target) and excellent precision (CV<1%) are typical for the method.

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described in this application note is SAMHSA-compliant and provides reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 Series LCs because the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

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SAMHSA-Compliant LC/MS/MS Analysis of Phencyclidine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.

Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. We present a method for analysis of phencyclidine that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy and precision, as well as measurement of matrix effects, extraction recovery, and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18, 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

Phencyclidine (PCP) is a synthetic drug, a member of the family of dissociative anesthetics. Five to 20 % of administered PCP is excreted unchanged in urine [2]. Therefore, the drug can be detected in its original form and neither hydrolysis nor metabolite measurement are needed. PCP is stable in biological samples. In frozen urine samples, it is preserved for a year, and refrigeration at 4 °C is sufficient for short-term storage.

Phencyclidine has a three-ring structure, with one aryl, one cyclohexane, and one piperidine ring (Figure 1). It is a weak organic base, essentially nonpolar, with a high log P of 4.69. The new SAMHSA confirmation cutoff concentration for phencyclidine is 25 ng/mL, and a LOD at 10% of the cutoff is 2.5 ng/mL [1].

The simple extraction method described in this application note provides reproducible high recoveries of PCP due to the unique properties of the Agilent Bond Elut Plexa polymer. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface which excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

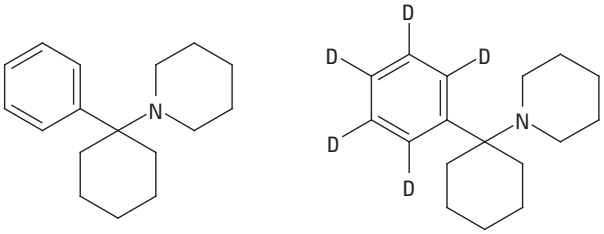
A Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7-µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns but with about 40% less back pressure, thereby allowing users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

With a low sample injection volume of 2 µL and no sample preconcentration, the method demonstrates excellent signal-to-noise (S/N) ratios (>200:1 at 2.5 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the AJST electrospray source.

Previous methods from Agilent used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures [3,4].

Experimental

Analytes



Phencyclidine
Log P 4.69
pK_a 8.5

Phencyclidine-D_5

Figure 1. Phencyclidine analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (phencyclidine) and 100 µg/mL (phencyclidine-D_5) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges, 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2 mL autosampler vials (p/n 5182-0716) or silanized vials (p/n 5183-2072)
- Agilent screw caps for autosampler vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, and G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Pretreatment

Spike 1 mL of urine with ISTD at 50 ng/mL; use of 12 × 75 mm glass tubes is recommended. Add 1 mL of 2% formic acid, vortex; centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 1 mL ethyl acetate: methanol: ammonium hydroxide (80:20:5), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate under stream of nitrogen to dryness.
8. Reconstitute in 1 mL initial mobile phase (10% methanol, 90% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	10
	0.5	10
	2.5	70
	2.51	90
	5.5	90
	5.51	10
Stop time	5.6 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	
Disable overlapped injection		
No automatic delay volume reduction		

MS conditions

ES source parameters

Ionization mode	Positive
Capillary voltage	3,000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V

MS parameters

Scan type	MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.2 min - diverter valve to MS
Delta EMV (+)	200 V

Results and Discussion

At acidic pH, the tertiary amine of phencyclidine was protonated, and the analyte was efficiently retained on Plexa PCX polymeric sorbent by a combination of hydrophobic interaction and a strong cation exchange.

A 100% methanol wash eliminated most matrix interferences without PCP loss from the SPE column. A strong base was added to the organic eluent to break the ionic interaction between the analyte and the strong cation exchange sorbent. PCP recovery is optimized with a two-component organic eluent consisting of 80% ethyl acetate and 20% methanol, with 5% NH_4OH added shortly before sample elution.

The Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column provided fast separation of phencyclidine in urine extract and good peak shape (Figure 2). The LC separation started with a low fraction of organic solvent (10%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run started with diverting the first portion of flow to waste to minimize source contamination. Data collection started at 1.2 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allowed short retention and re-equilibration times.

A S/N ratio >200:1 for the 2.5 ng/mL peak (Figure 2, upper panel) illustrates state-of-the-art performance of the 6460 Triple Quadrupole LC/MS system, capable of reliably detecting PCP at a small fraction (10%) of the SAMHSA cutoff concentration. Being very hydrophobic, phencyclidine has the potential to adhere to any active surfaces. To avoid carryover, we recommend using the external needle wash flush port option of the high performance autosampler, and running a mobile phase blank after samples, which appear from screening results to have a high concentration. If needed, the needle wash can be increased from 10 to 20 seconds.

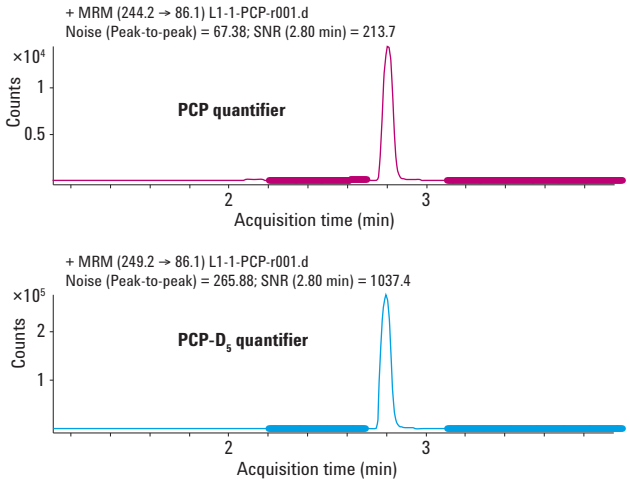


Figure 2. MRM extracted ion chromatograms for PCP (2.5 ng/mL) and PCP-D_5 (50 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm, column. Noise regions are shown in bold.

SAMHSA guidelines require one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analyte (Table 1) is provided for additional confidence. Agilent MassHunter Quantitative software automatically calculates qualifier ion ratios, highlighting those out of acceptable range.

Figure 3 shows an example calibration curve for extracted urine standards at five concentration levels of phencyclidine. Calibration standards were prepared by spiking negative urine at 2.5, 25, 100, 250, and 1,000 ng/mL. Deuterated internal standard PCP-D_5 was added at 50 ng/mL. Excellent linear fit with $R^2 > 0.999$ demonstrates the linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

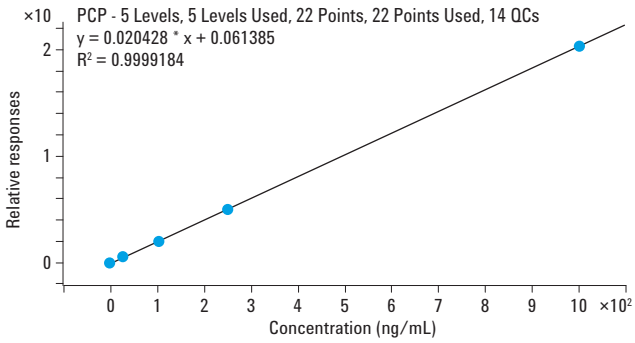


Figure 3. Example calibration curve for phencyclidine in urine extract. Calibration range 2.5 to 1000 ng/mL. Linear fit, R^2>0.999.

Table 1. MRM Transitions.

Compound	Precursor	Product	Fragmentor	Collision energy
PCP	244.2	86.1	80	7
PCP	244.2	159.1	80	7
PCP	244.2	91.1	80	34
PCP-D_5	249.2	164.1	80	7
PCP-D_5	249.2	86.1	80	7

Normal, rather than dynamic, MRM scan type can be used with this method, since dynamic MRM has no advantages for detection of a single compound.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles proposed by Matuszewski *et al.* and widely accepted as an industry standard approach for LC/MS/MS methods [5]. Extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 25 ng/mL with PCP (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 25 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine extract spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

Table 2 shows high extraction recovery for phencyclidine (85%) together with very good accuracy (93%) and precision (0.5 %). Matrix effect of 98% indicates only minor ion suppression of the signal due to matrix interferences (2%), thus confirming an exceptional cleanliness of Plexa PCX-processed extracts.

Table 2. Method performance for phencyclidine, n = 5.

	%
Process efficiency	83
Extraction recovery	85
Matrix effect	98
Accuracy	93
Precision (CV)	0.5

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described in this application note is SAMHSA-compliant and provides accurate, precise and reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in the other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 LC series since the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

References

1. SAMHSA (2010) Manual for Urine Laboratories, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.

2. R. Baselt (2008) Disposition of Toxic Drugs and Chemicals in Man. 8th edition. Atlas Books, Ashland, OH, USA.

3. P. Moorman and J. Hughes (2010) "Phencyclidine in Urine by LC/Triple Quadrupole Mass Spectrometry (LC/MS/MS)". SOP, Agilent Technologies, Inc. Publication number 5990-5864EN.

4 J. Hughes and P. Moorman (2011) "Confirmation by Triple Quadrupole LC/MS/MS for HHs-compliant Workplace Urine Drug Testing". Agilent Technologies, Inc. Seminar available from www.agilent.com/chem.

5. B. K. Matuszewski, M. L. Constanzer, and C.M. Chavez-Eng (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, 75: 3019-3030.

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SAMHSA-Compliant LC/MS/MS Analysis of 11-nor-9-carboxy-Δ^9-Tetrahydrocannabinol in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

Authors

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Agilent Technologies, Inc.

Abstract

Guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA) effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests [1]. LC/MS/MS methods are often less complicated than previously employed GC/MS methods because they do not typically require a derivatization step. This application note presents a method for analysis of 11-nor-9-carboxy-Δ^9– tetrahydrocannabinol that meets SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy and precision, as well as measurement of matrix effects, extraction recovery and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products such as Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE sorbent, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system, and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

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Introduction

11-Nor-9-carboxy-Δ^9– tetrahydrocannabinol (THCA, "THC-acid", THC-COOH) is a metabolite of tetrahydrocannabinol (Δ^9–THC), an active constituent of marijuana. In the form of its glucuronide conjugates, THCA is excreted in urine for several weeks [2]. The SAMHSA confirmation cutoff concentration for THCA is 15 ng/mL and a LOD at 10% of the cutoff would be 1.5 ng/mL.

Sample preparation for 11-nor-9-carboxy-Δ^9–THC analysis requires base hydrolysis of urine to convert glucuronides back to THCA. Although THCA is a carboxylic acid, for the sake of a single method setup for all SAMHSA-regulated drugs, the Agilent sorbent chosen for extraction is Agilent Bond Elut Plexa PCX, a mixed-mode cation-exchange polymer. It efficiently retains THCA by hydrophobic interaction.

The extraction method provides reproducible high recoveries of THCA due to the unique properties of the Plexa sorbent. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

An Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7 µm particles, the Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns, with approximately 40% less back pressure, thereby allowing the users of even 400 bar LC systems to increase resolution and shorten analysis and re-equilibration times by applying a higher flow rate.

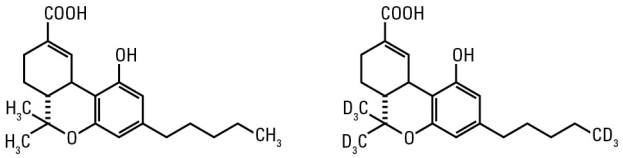
Being essentially nonpolar (log P>6), cannabinoids are not ideally suited for electrospray ionization and are often analyzed using APCI. However, due to its carboxylic moiety, THCA is much more efficiently ionized in negative ion mode than Δ^9–THC and 11-hydroxy- Δ^9–THC. A choice of electrospray source for THCA detection is warranted by the convenience of a single mass spectrometer configuration for all SAMHSA drugs.

With a low sample injection volume of 10 µL and no sample preconcentration, the method demonstrates excellent signal-to-noise ratios for cutoff and 10% of the cutoff concentrations (approximately 100:1 and 10:1, respectively) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS system with the Jet Stream electrospray source.

Previous methods from Agilent [3,4] used the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures.

Experimental

Analytes



11-nor-9-carboxy-Δ^9-THC
Log P (predicted) 6.06-6.36, pKa 4.5

11-nor-9-carboxy-Δ^9-THC-D_9

Figure 1. 11-nor- carboxy-Δ^9- tetrahydrocannabinol analytes and their structures.

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (11-nor-9-carboxy-Δ^9-THC) and 100 µg/mL (11-nor-9-carboxy-Δ^9-THC-D_9 and 11-nor-9-carboxy-Δ^9-THC-glucuronide) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent silanized 2 mL autosampler vials (p/n 5183-2072)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC system (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source

Sample preparation

Hydrolysis and sample pretreatment

1. Spike 0.5 mL of urine with ISTD at 50 ng/mL; use of methanol-rinsed and 12 × 75 mm dried glass tubes is recommended.
2. Add 100 µL 7 N KOH, vortex.
3. Incubate in the heating block at 60 ±5 °C for 30 minutes.
4. Cool. Add 125 µL methanol, vortex.
5. Add 1.5 mL of 0.2 M sodium acetate buffer (pH 4).
6. Neutralize with 100 µL glacial acetic acid, vortex.
7. Centrifuge if cloudy.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol–soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 2 × 2 mL 10:90 ACN: 2% acetic acid.
4. Wash 2: 2 mL 30:70 ACN: 2% acetic acid.
5. Dry 5–10 minutes under high vacuum (10–15 in Hg).
6. Wash with 200 µL hexane, pull through with low vacuum (2–3 in Hg).
7. Dry under high vacuum, 3 to 4 minutes.
8. Elute with 0.5 mL 80:20 ethyl acetate:isopropanol. Soak, let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
9. Add 1 mL more of the same eluent, repeat soaking-elution procedure.
10. Evaporate to dryness at 40 °C.
11. Reconstitute in 0.5 mL initial mobile phase (30% methanol, 70% 5 mM ammonium formate).

LC/MS/MS

LC conditions

Mobile phase A	5 mM ammonium formate in water
Mobile phase B	methanol
Flow rate	0.8 mL/min

Gradient	Time (min)	% B
	0.0	30
	1	95
	5	95
	5.1	30

Stop time	5.2 minutes
Post time	2 minutes
Max pump pressure	400 bar
Injection volume	10 µL
Needle wash	Flush port 75:25 methanol:water for 10 seconds

Disable overlapped injection

No automatic delay volume reduction

MS conditions

ES Source Parameters

Ionization mode	negative
Capillary voltage	4,000 V
Drying gas flow	11 L/min
Drying gas temperature	320 °C
Nebulizer gas	18 psi
Sheath gas flow	12 L/min
Sheath gas temperature	320 °C
Nozzle voltage	0 V

MS parameters

Scan type	MRM

Pre-run script
SCP_MSDiverterValveToWaste(){MH_Acq_Scripts.exe}

Time segments	#1: 1.4 minutes - diverter valve to MS
Delta EMV (-)	800 V

Results and Discussion

The cannabinoids are notorious for their adsorption to glass and plastic. To minimize losses and to ensure method reproducibility, we strongly recommend the use of only freshly prepared stock solutions and calibrators, silanized or thoroughly washed, methanol-rinsed and dried glassware, and analyze final extracts immediately after reconstitution.

THCA is retained on a cation-exchange mixed mode Plexa PCX by hydrophobic interactions. The 100% methanol wash, commonly employed in ion-exchange SPE, is not practical for THCA extraction as high organic will elute the compound from the sorbent.

To minimize matrix interferences, 10 to 30% acetonitrile is added to wash one and two, respectively. The hexane wash serves the same purpose. When used alone and in a small amount (200 µL), hexane elutes most lipids but does not lead to analyte desorption, because THCA is very hydrophobic (log P>6) and is retained at the hydrophobic core of the Plexa particles very strongly. A soaking procedure is recommended at the elution step to enhance the solvent-analyte interaction and improve analyte recoveries.

The Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column provides fast separation of THCA in urine extract and good peak shape (Figure 2). The LC separation intentionally begins with a relatively low fraction of organic solvent (30%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Due to a steep gradient, the remaining hydrophobic interferences largely elute before the analyte, thus reducing matrix effect at the time of peak elution (1.96 minutes). A flow rate of 0.8 mL/min allows for a short retention and re-equilibration time. Each sample run begins with diverting a first portion of flow (0 to 1.4 minutes) to waste to minimize source contamination. Data collection begins at 1.4 minutes, immediately after the diverter valve switch.

SAMHSA guidelines require the use of one quantifier and at least one qualifier ion for both target compound and ISTD. A third transition for target analyte is provided for additional confidence (Table 1). Agilent MassHunter Quantitative software automatically calculates qualifier ion ratios, highlighting those out of acceptable range.

Table 1. MRM Transitions

Compound	Parent	Product	Fragmentor	Collision energy
11-nor-9-carboxy-Δ^9-THC	343.2	299.2	135	18
	343.2	245.1	135	30
	343.2	191.1	135	33
11-nor-9-carboxy-Δ^9-THC-D_9	352.2	308.2	145	18
	352.2	254.2	145	30
11-nor-9-carboxy-Δ^9-THC glucuronide	519.2	343.2	160	22
	519.2	299.2	160	36

When processed according to the protocol, urine samples spiked with 11-nor-9-carboxy-Δ^9-THC-glucuronide at 1,000 ng/mL tested negative for this compound. Instead, they displayed a very large THCA peak, far beyond the upper calibration level of 600 ng/mL. This is proof of full conversion of glucuronides to THCA by the base hydrolysis step. MS parameters for the detection of 11-nor-9-carboxy-Δ^9-THC-glucuronide are included in Table 1 for analysts interested in testing the hydrolysis efficiency.

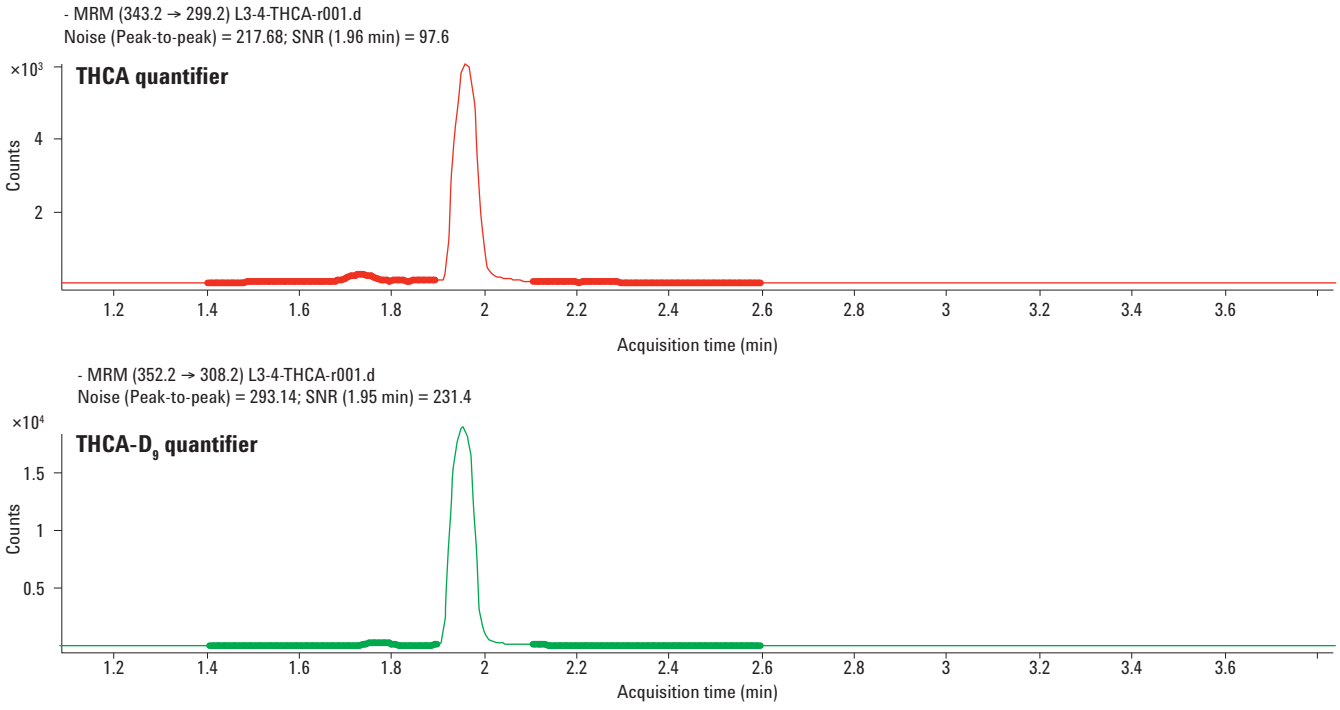


Figure 2. MRM extracted ion chromatograms for THCA (15 ng/mL) THCA-D_9 (50 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

Normal, rather than dynamic, MRM acquisition mode can be used with this method, since dynamic MRM has no advantages for detection of a single peak.

Due to its extreme hydrophobicity, THCA can adhere not only to glassware but also to injector parts and tubing. To avoid carryover, we recommend running a mobile phase blank after samples with high concentration, and to use the Injector Flush Pump option of the autosampler. If needed, the needle wash can be increased from 10 to 20 seconds.

A signal-to-noise ratio approximately 100:1 for the cutoff concentration of 15 ng/mL for THCA (Figure 2, upper panel) illustrates excellent performance of the 6460 Triple Quadrupole LC/MS system, capable of reliably detecting THCA at a small fraction (10%) of the SAMHSA cutoff concentration.

Figure 3 shows a calibration curve for extracted urine standards at five concentration levels. Calibration standards were prepared by spiking negative urine at 1.5, 15, 75, 300, and 600 ng/mL with THCA. Deuterated internal standard THCA-D_9 was added at 50 ng/mL. Excellent linear fit ($R^2 > 0.999$) demonstrates linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski *et al*. [5] and widely accepted as an industry standard approach for LC/MS/MS methods. Extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 15 ng/mL with THCA (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 15 ng/mL in urine (spiked mobile phase).

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine extract spiked post-SPE to its peak area in spiked mobile phase.

Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements.

The method is characterized by good recoveries together with very high accuracy (98%) and precision (2.2%) of the data (Table 2). Matrix effect in excess of 100% indicates ionization enhancement as opposed to ionization suppression. Signal enhancement of only 13% confirms cleanliness of Plexa PCX extracts. Overall process efficiency of 73% is rather high due to analytical challenge associated with the cannabinoid family.

Table 2. Method Performance for 11-nor- carboxy-Δ^9- tetrahydrocannabinol at the Cutoff Level, n = 5

	%
Process efficiency	73
Extraction recovery	65
Matrix effect	113
Accuracy	98.2
Precision (CV)	2.2

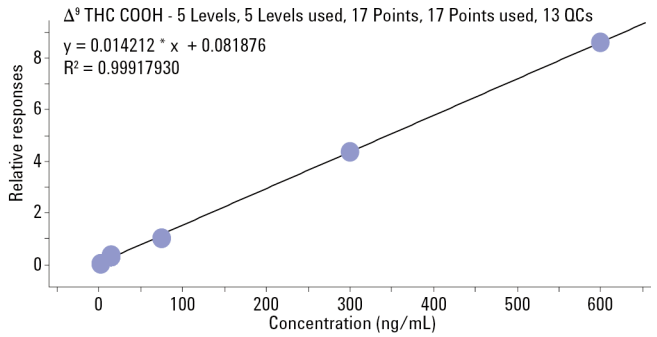


Figure 3. Example calibration curve for THCA in urine extract. Calibration range 1.5 to 600 ng/mL. Linear fit, R^2>0.999.

Conclusions

The solid phase extraction procedure coupled with the LC/MS/MS detection method described is SAMHSA-compliant and provides reproducible results for forensic toxicology or other analytical environments with similar requirements for legally defensible data. The THCA method uses the same hardware setup as the other Agilent SAMHSA methods. These methods are usable with all models of Agilent 1100 and Agilent 1200 LC series, since the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

References

1. SAMHSA (2010) Manual for Urine Laboratories, National Laboratory Certification Program, 1 October 2010. U. S. Department of Health and Human Services.
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Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

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Introduction

In 2011, five members of the "synthetic cannabinoids" group or 'Spice' compounds were banned in the USA. The substances were:

- 1-pentyl-3-(1-naphthoyl)-indole (JWH-018)
- 1-butyl-3-(1-naphthoyl)-indole (JWH-073)
- 1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)-indole (JWH-200)
- 5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (CP-47,497)
- 5-(1,1-dimethyloctyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (cannabicyclohexanol; CP-47,497 C8 homologue)

The drugs have been described by users as having cannabis-like effects, and some of these compounds show strong binding to cannabinoid receptors. The (–)-1,1-dimethylheptyl analog of 11-hydroxy-Δ^8-tetrahydrocannabinol, (1,1-dimethylheptyl-11-hydroxytetrahydrocannabinol) is known as HU-210 and has been reportedly found in seizures of "Spice Gold", "Spice Silver" and "Spice Diamond" made by the US Customs and Border Protection in 2009. HU-210 is considered to be more potent than Δ^9-tetrahydrocannabinol (Δ^9-THC), and was already classified as a controlled substance as an analog of marijuana. JWH-250 is also commonly encountered so was also included in the research.

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Oral fluid is becoming increasingly popular as a specimen for the detection of drugs at the roadside, and in workplace testing. It is easy to collect, non-invasive and can give information on recent drug intake. In the work described here, the Quantisal device was used for oral fluid collection, and the detection of "Spice" components is described.

Collection devices, reagents and standards

Quantisal devices for the collection of oral fluid specimens contain a cotton collection pad which is placed in the mouth. The incorporated volume adequacy indicator turns blue when 1 mL of oral fluid (± 10%) has been collected, then the pad is placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). Drug concentrations detected are adjusted accordingly.

Solid phase extraction columns (Bond Elut Plexa) and liquid chromatographic columns (ZORBAX RRHT) were obtained from Agilent Technologies. The standard compounds JWH-018, JWH-073, JWH-200, JWH-250, HU-210, CP-47,497 and CP-47,497 C8 homologue as well as deuterated *d9*-JWH-018 and *d7*-JWH-073 were purchased from Cayman Chemicals.

Calibrators and controls

The deuterated internal standards (*d9*-JWH-018 and *d7*-JWH-073) and unlabelled drug standards were prepared in methanol at a concentration of 100 µg/mL. The working solutions were diluted from stock to a concentration of 10 µg/mL in methanol. The solutions were stored at –20 °C when not in use. Controls were prepared by fortifying drug-free synthetic oral fluid with various concentrations of compounds. Drug free negative specimens, positive controls at 4 ng/mL and 40 ng/mL were included in every batch.

Sample preparation

Seven calibration standards were prepared in oral fluid at concentrations of 0.5, 2, 5, 10, 20, 50, and 100 ng/mL for all analytes; deuterated internal standards were added (10 ng/mL).

Agilent Bond Elut Plexa (30 mg/1 mL; p/n 12109301) solid phase extraction cartridges were used.

1. Condition: methanol (0.5 mL); 0.1 M acetic acid (0.1 mL)
2. To each 1mL aliquot of calibrator, control or specimen, add acetic acid (0.1 M; pH 4, 1 mL)
3. Load samples
4. Wash columns: DI water: glacial acetic acid (80:20; 1 mL); DI water: methanol (40:60; 1 mL)
5. Dry columns (5 minutes)
6. Elute acidic/neutral compounds: hexane: glacial acetic acid (98:2; 2 mL)
7. Evaporate extracts to dryness while allowing columns to dry (7 minutes)
8. Elute bases into corresponding tubes: ethyl acetate: ammonium hydroxide (98:2; 2 mL)
9. Evaporate to dryness under nitrogen at 40 °C
10. Reconstitute in methanol (50 µL); transfer to autosampler vials; cap
11. Analyze using LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

An Agilent Technologies 1200 Series liquid chromatography pump coupled to an Agilent 6430 Triple Quadrupole LC/MS System, operating in electrospray ionization mode (ESI) with either positive or negative polarity depending on the compound.

Column	Agilent ZORBAX RRHT Extend C18, (2.1 × 50 mm, 1.8 µm, p/n 727700-902)	
Column temperature	60 °C	
Injection volume	5 µL	
Mobile phase	Solvent A: 0.2% acetic acid and Solvent B: acetonitrile	
	Time 0:	95% A; 5% B; 5 min: 100% B; 7 min 5% B
	Run time	9.2 min; Post-time 3 min
	Flow rate:	0.5 mL/min
Nitrogen gas temperature	350 °C	
Gas flow	10 L/min	
Nebulizer pressure	55 psi.	
Capillary voltage	+4,000 V in positive mode; –4,000 V in negative mode	

Two transitions were selected and optimized for each drug. Table 1 shows the transitions, the optimized fragment voltages for the parent ion (M +1; M-1) as well as the collision energy for fragmentation of the product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ± 20% in order to meet the criterion for a positive result.

Table 1. Multiple Reaction Monitoring (MRM) Transitions; Optimized Fragmentation Voltages; Allowable Transition Ranges Determined at 10 µg/mL for "Spice" Compounds

Compound	Transition	Fragment voltage (V)	Collision energy (eV)	Polarity	Ratio of quantifying to qualifying transition (range)
d9-JWH-018	351.3 > 223.4	140	20	Positive	n/a
JWH-018	342.2 > 155.1	120	20	Positive	16–24
	342.2 > 214.2	120	20		
JWH-250	336.3 > 200.2	120	12	Positive	69–104
	336.3 > 188.2	120	20		
d7-JWH-073	335.3 > 207.2	120	20	Positive	n/a
JWH-073	328.2 > 155.1	120	20	Positive	60–90
	328.2 >127.1	120	35		
JWH-200	385.3 > 155.1	140	20	Positive	54–81
	385.3 > 114.2	140	25		
CP 47497 C8	331.3 > 313.3	160	25	Negative	70–104
	331.3 > 259.3	160	35		
CP 47497	317.3 > 299.2	160	20	Negative	75–113
	317.3 > 245.2	160	30		
HU-210	385.3 > 367.4	120	30	Negative	13–20
	385.3 > 281.3	120	45		

Underlined transitions used for quantitation; n/a = not applicable for internal standard

Figure 1 shows a chromatogram for the primary transitions of the compound at a concentration of 10 ng/mL; the ratio of primary to secondary transition for each compound was also determined at 10 ng/mL.

Recovery from the collection pad

Six synthetic oral fluid specimens fortified with the compounds at concentrations of 4 and 40 ng/mL were prepared. The collection pad was placed into the samples until 1 mL (±10%) had been collected, as evidenced by the blue volume adequacy indicator incorporated into the stem of the collector, then the pad was transferred to the Quantisal buffer, capped and stored overnight to simulate transportation to the laboratory. The following day an aliquot of the specimen was analyzed. The amount recovered from the pad was compared to an absolute concentration (100%) where drug was added to the buffer and left overnight at room temperature without the pad, then subjected to extraction and analysis.

The percentage recovery from the pad for the compounds at concentrations of 4 and 40 ng/mL (n = 6) were > 60% for all at both levels. The highest recovery was 86% for HU-210 at 4 ng/mL; the lowest was 61% for JWH-073 at 40 ng/mL. The recoveries were essentially equivalent at both levels (Table 2).

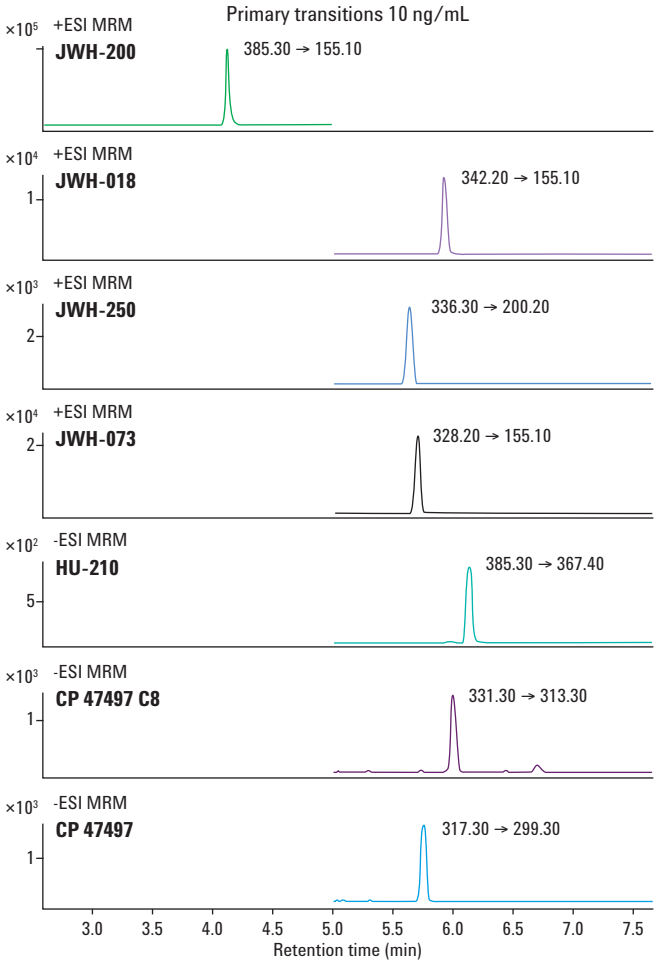

Figure 1. Primary transition at 10 ng/mL.

Table 2. Method Evaluation

	JWH-018	JWH-073	JWH-200	JWH-250	CP 47497	CP 47497 C8	HU-210
LOQ (ng/mL)	0.5	0.5	0.5	2	0.5	2	5
Imprecision intra-day							
4 ng/mL	3.9%	3.6%	5.0%	3.4%	4.9%	3.9%	8.6%
40 ng/mL	2.2%	2.1%	6.0%	2.0%	4.1%	4.3%	5.6%
Inter-day							
4 ng/mL	8.8%	9.6%	6.2%	11%	7.7%	11%	10%
40 ng/mL	8.5%	7.9%	6.2%	11%	10%	11%	12%
Pad recovery							
4 ng/mL	65.5%	67.4%	85.0%	66.5%	77.7%	76.0%	86.4%
40 ng/mL	70.6%	61.4%	81.4%	75.1%	71.3%	78.2%	75.7%
Matrix effect	-55%	-45%	-55%	-73%	-64%	-55%	-49%
Process efficiency	40%	51%	56%	24%	38%	45%	51%

Data Analysis

Calibration was carried out using linear regression analysis over a concentration range of 0.5–100 ng/mL. Peak area ratios of target analytes and the internal standard were calculated for each concentration using Agilent MSD software. The data were fit to a linear least squares regression curve, not forced through the origin, and with equal weighting. For confirmation, two transitions were monitored for each of the compounds; one for the internal standard. The ratio of the qualifying transition was required to be within 20% of that established using the known calibration standard to be acceptable.

Linearity and sensitivity

The limit of quantitation (LOQ) of the method was determined using serial dilutions to the lowest point where the acceptable criteria for the quantitation of a compound were met, that is, the chromatographic peak shape, retention time (within 2% of calibration standard), and qualifier transition ratio (± 20%) compared to the 10 ng/mL calibration standard were acceptable. The quantitative value of the LOQ had to be within ± 20% of the target concentration. The limit of quantitation was 0.5 ng/mL for JWH-018, JWH-073, JWH-200, and CP 47497; 2 ng/mL for CP 47497 C8 and JWH-250; 5 ng/mL for HU-210 (Figure 2). Linearity was acceptable from the LOQ to 100 ng/mL ($R^2 > 0.99$; n = 5) for all compounds.

Matrix effects

A nonextracted drug standard at a concentration of 10 ng/mL was prepared as well as drug free matrix extracts and negative controls (extracts containing only internal standard). The recovery of the compounds from the oral fluid was determined by first assessing the response of the extracted samples (n = 3) at a concentration of 10 ng/mL {R_{ES}}. Then, oral fluid was extracted and drug was added postextraction at a concentration of 10 ng/mL (n = 3) {R_{PES}}. The percentage recovery was then calculated from the equation (R_{ES}/ R_{PES}) × 100.

The reduction in response due to matrix effects (ion suppression) was determined by assessing the peak area response of a nonextracted neat drug standard (n = 3) at a concentration of 10 ng/mL {R_{NES}}. The nonextracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The % matrix effect was then calculated using the equation (R_{PES} / R_{NES}) -1 × 100. The overall efficiency of the process was calculated as (R_{ES} / R_{NES}) × 100.

Ion suppression effects were significant, but were limited by the use of solid-phase extraction and deuterated internal standards.

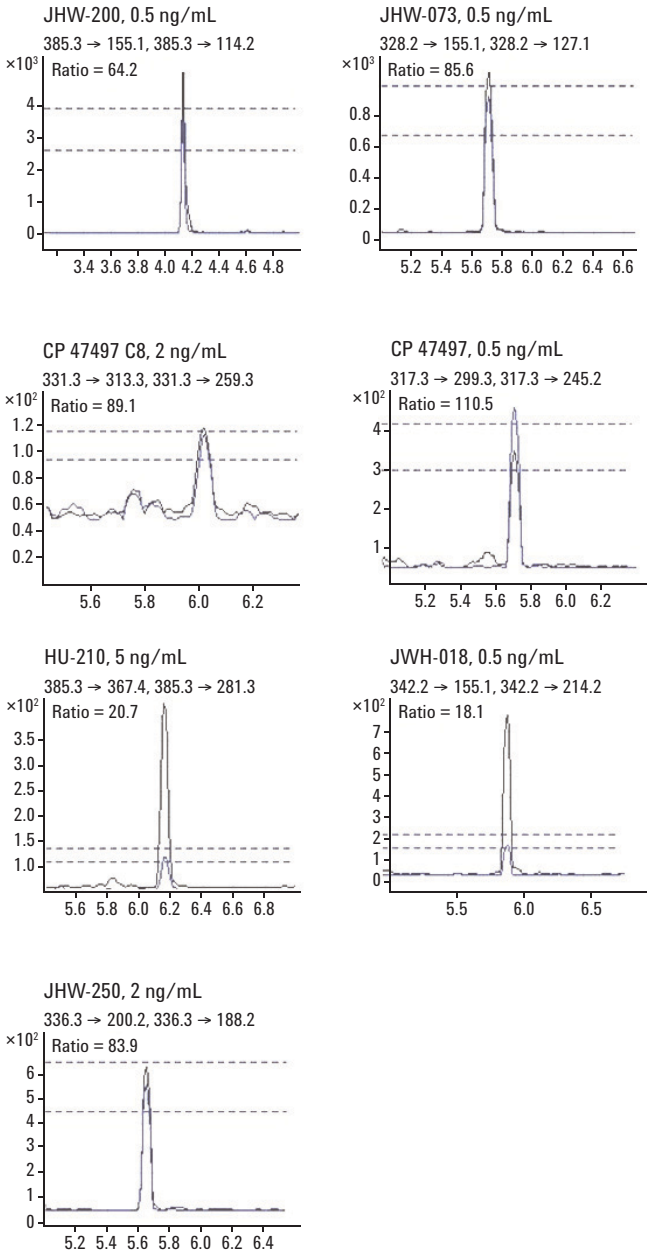

Figure 2. LOQ concentrations showing ± 20% ratio.

Selectivity

Five drug free oral fluid specimens were collected using the Quantisal device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer.

In addition, common drugs of abuse were added at concentrations of 2,000 ng/mL to other aliquots of the drug-free fluid, extracted, and analyzed as described.

THC	amitriptyline
THC-COOH	cyclobenzaprine
11-OH-THC	imipramine
cannabinol	dothiepin
cannabidiol	doxepin
cocaine	fluoxetine
benzoylecgonine	sertraline
norcocaine	trimipramine
cocaethylene	protriptyline
codeine	chlorpromazine
morphine	clomipramine
6-AM	nortriptyline
6-AC	paroxetine
oxycodone	desipramine
oxymorphone	bromazepam
hydrocodone	alprazolam
hydromorphone	clonazepam
amphetamine	lorazepam
methamphetamine	oxazepam
MDMA	diazepam
MDA	midazolam
MDEA	flurazepam
phentermine	flunitrazepam
fentanyl	nordiazepam
phencyclidine	triazolam
tramadol	temazepam
carisoprodol	nitrazepam
meprobamate	chlordiazepoxide
citalopram	methadone
venlafaxine	

No endogenous interference was noted from drug free extracts; or for exogenous interference from any of the commonly encountered drugs, including THC and its main metabolites, which were analyzed at high concentration.

Imprecision

Specimens were fortified with all the compounds simultaneously at concentrations of 4 ng/mL and 40 ng/mL. Each concentration was analyzed according to the described procedure (n = 6; intra-day imprecision) for 5 consecutive days (n = 30; inter-day imprecision). The intra-day imprecision of the assays for all drugs was < 9% at both concentrations; inter-day < 12% at both concentrations (Table 2).

Authentic samples

Specimens were collected from two volunteers, who had purchased the compounds while still legally available in the USA. Subject number 1 smoked "Blueberry Posh" and subject number 2 smoked "Black Mamba". Using Quantisal oral fluid collection devices, specimens were collected prior to the start of smoking, then at the various time points after smoking. Subject 1 gave specimens after 20 minutes, 40 minutes, 1 hour, 2 hours, and 12 hours; Subject 2 gave samples after 20 minutes, 40 minutes, 1 hour, 5 hours, and 12 hours. The specimens were analyzed the day after collection, then were stored at 4 °C for one month and re-analyzed with a dif-ferent method. A year later, they were re-analyzed using this procedure. It was not possible to procure authentic specimens at this time since the compounds are no longer available legally.

The main active compound in the two preparations was determined to be JWH-018. After storage at 4 °C for one month the samples were reanalyzed and found to be extremely stable with almost identical concentrations detected. When the specimens which had been stored at 4 °C for a year were re-analyzed, the concentrations in Subject number 1 were essentially the same as the previous year; the levels in Subject number 2, which were much lower originally, had generally declined (Figure 3).

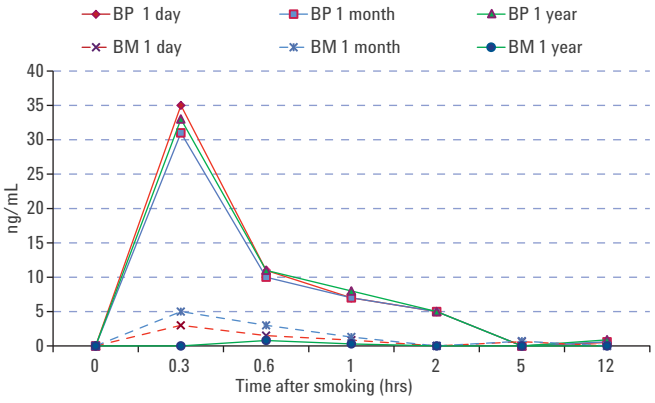

Figure 3. Stability of authentic specimens stored at 4 °C.

An extracted ion chromatogram showing the transitions and ± 20% acceptability band around the intensity of the qualifying transition from the sample collected 40 minutes after smoking (Subject number 1) is presented in Figure 4; the concentration of JWH-018 was 11 ng/mL.

Summary

The simultaneous determination of several "Spice" compounds in oral fluid is reported for the first time. The procedure is applicable to the analysis of specimens collected using the Quantisal device for the presence of synthetic cannabinoids, which were recovered from the pad > 60% at two concentrations. Following a single smoking session of two different herbal product brands, JWH-018 was detected in oral fluid with the highest concentrations appearing 20 minutes after a single smoking session. Even after a year, JWH-018 was detectable in the oral fluid 12 hours after a single smoking session of "Blueberry Posh".

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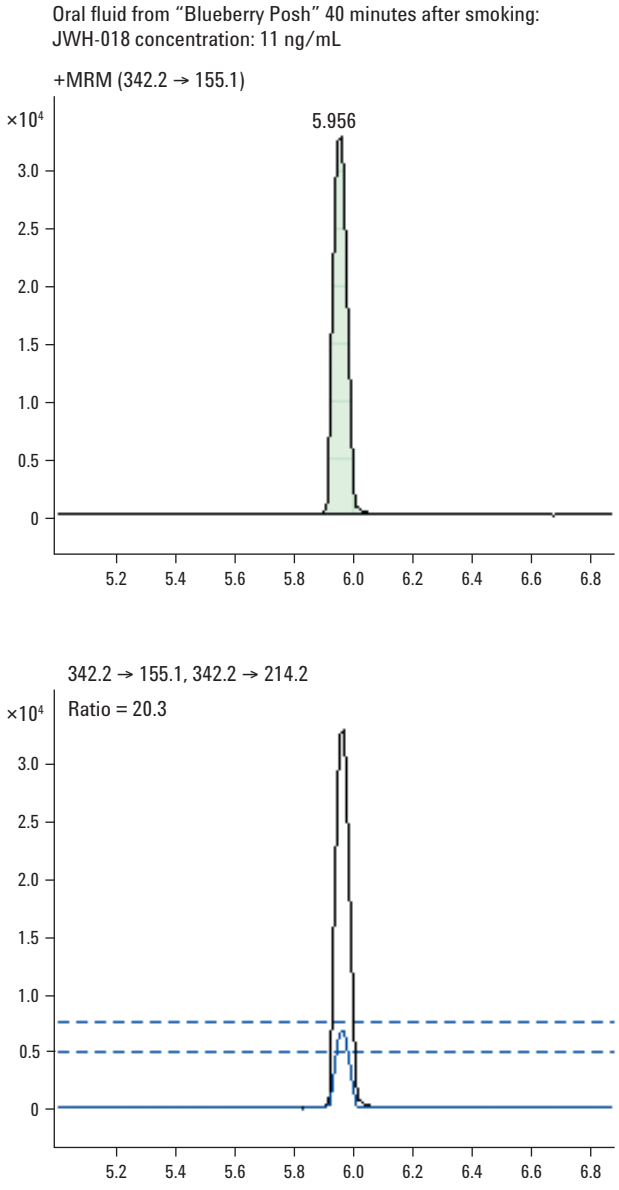

Figure 4. Oral fluid from Subject #1 40 minutes after smoking; JWH-018 = 11ng/mL.

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Agilent Technologies

LC/MS/MS of Buprenorphine and Norbuprenorphine in Whole Blood Using Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 Column

Application Note

Forensic Toxicology

Author

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Abstract

Determination of buprenorphine and norbuprenorphine in whole blood by forensic toxicology laboratories requires an analytical method capable of reliable detection of these compounds at concentrations below 1 ng/mL. A simple sample cleanup procedure coupled with an LC/MS/MS method using mass transitions 468.2 → 55.1 and 414.2 → 83.1 allows for a limit of detection (LOD) below 0.1 ng/mL for both analytes. Typical calibration curves are linear in the range of 0.2 to 20 ng/mL for each analyte, with R^2 values equal or higher than 0.999. High sensitivity is achieved by using Agilent products, including an Agilent Bond Elut Plexa PCX mixed mode polymeric SPE sorbent, an Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, an Agilent 1200 Infinity LC system, and an Agilent 6460 Triple Quadrupole LC/MS System with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

Agilent Technologies

Introduction

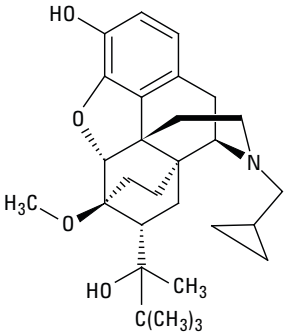
Buprenorphine is a semisynthetic opioid with a structure similar to morphine, although buprenorphine is much more hydrophobic (Figure 1). Buprenorphine is converted to norbuprenorphine, its major active metabolite [1,4]. Concentrations of buprenorphine and norbuprenorphine in blood are very similar, and in more than 50% cases, are below 1 ng/mL [9], presenting a challenge for an analyst. In addition, MS/MS detection of these compounds is complicated by the rigidity of the molecular structures of the analytes, resulting in very low amounts of collision-induced fragments. To achieve sensitivity below 1 ng/mL, analytical methods for determination of these compounds need not only excellent MS performance, but also an efficient sample cleanup procedure providing high recoveries and low ion suppression. We used an extraction method that delivered detection limits below 0.1 ng/mL, easily achieved due to the cleanliness of SPE-processed whole blood extracts. Unlike other polymeric sorbents, all members of the Agilent Bond Elut Plexa family possess an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

Good separation of analytes and excellent peak shapes achieved with this method are distinctive features of the Agilent Poroshell 120 column family. With superficially porous 2.7 µm particles, these columns provide similar efficiency to sub-2 µm UHPLC columns, but with approximately 40% less backpressure. This allows users of even 400 bar LC systems to increase resolution and to shorten analysis and re-equilibration times by applying a higher flow rate.

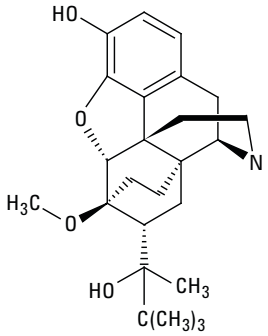
New ion transitions identified as the most abundant and used in this work for quantitation are 468.2 > 55.1 (buprenorphine) and 414.2 > 83.1 (norbupenorphine). With only 0.5 mL of blood, a low sample injection volume of 10 µL and preconcentration of only 5× at the extraction step, the method demonstrates excellent signal-to-noise ratios at 0.2 ng/mL:84:1 for buprenorphine and 20:1 for norbuprenorphine (Figure 2).

Experimental

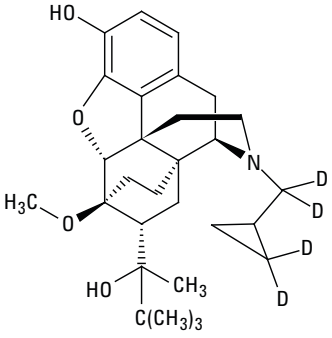
Analytes



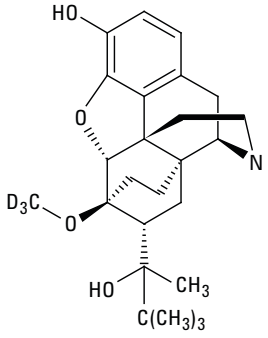
Buprenorphine
$C_{29}H_{41}NO_4$ M.W. 467.65
Log P 4.9-5.0
pKa 8.3



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



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



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

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

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (buprenorphine, norbuprenorphine) and 100 µg/mL (buprenorphine-D_4 and norbuprenorphine-D_3) solutions in methanol.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent silanized autosampler vials 2 mL (p/n 5183-2072)
- Agilent vial inserts, 250 µL, deactivated glass, with polymer feet (p/n 5181-8872)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm column (p/n 699975-302)
- Agilent 1260 Infinity LC system (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Pretreatment

1. Spike 0.5 mL of blood with ISTD at 10 ng/mL, or prepare 10 ng/mL solution of ISTD in 0.1 M phosphate buffer (pH 6.0) and add 0.5 mL of this buffer to each blood sample. Use of methanol-rinsed and air-dried glass tubes 12 × 75 mm is recommended.
2. After adding ISTD, add 2 to 2.5 mL phosphate buffer (so that blood is diluted at least 1:5).
3. Vortex and centrifuge to obtain a good pellet.

Extraction

1. Condition Bond Elut Plexa PCX cartridge with 0.5 mL methanol, soak, then let drip.
2. Load sample/supernatants with a Pasteur glass pipette.
3. Wash 1: 2 × 2 mL 2% formic acid.
4. Wash 2: 3 mL of 70 MeOH:30 of 2% formic acid.
5. Dry 5-10 minutes under vacuum (10-15 in Hg).
6. Elute with 1.5 mL of 80 ethyl acetate:20 isopropanol: 5 NH_4OH eluent. Add NH_4OH shortly before elution. Apply eluent in 2 aliquots and soak the sorbent bed with each aliquot. Soak for approximately 0.5 minute with the stopcock valves closed, then let the eluate drip into the collection vials under gravity. When the dripping stops, apply low vacuum to extract eluate from the smallest pores.
7. Evaporate to dryness under a stream of nitrogen at 45 °C.
8. Reconstitute in 0.1 mL initial mobile phase (15% methanol, 85% water, 0.1% formic acid), vortex, and transfer into vial inserts with polymer feet.

LC/MS/MS

LC conditions

Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in methanol
Flow rate:	0.8 mL/min

Gradient:

Time (min)	% B
0.0	15
2.0	70
2.1	95
5.5	95
5.51	15

Stop time:	5.6 min
Post time:	2 min
Max pump pressure:	400 bar
Injection volume:	10 µL

Injection with needle wash

Needle wash:	Flush port 95 methanol:5 water for 10 s
Disable overlapped injection:	
No automatic delay volume reduction:	

MS conditions

ES source parameters

Ionization mode:	positive
Capillary voltage:	2,800 V
Drying gas flow:	10 L/min
Drying gas temperature:	350 °C
Nebulizer gas:	35 psi
Sheath gas flow:	12 L/min
Sheath gas temperature:	350 °C
Nozzle voltage:	0 V

MS parameters

Scan type:	MRM
Prerun script:	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments:	#1: 1.8 min - diverter valve to MS
Delta EMV (+):	400 V

Table 1 shows the MRM transitions for one quantifier and one qualifier product ion for the target compounds, and their deuterated internal standards.

Table 1. MRM Transitions.

Compound name	Precursor	MS1 Res	Product	MS2 Res	Fragmentor	Collision energy
Buprenorphine	468.3	Unit	55.1	Wide	200	62
Buprenorphine	468.3	Unit	396.2	Wide	200	45
Buprenorphine-D_4	472.3	Unit	59.1	Wide	200	62
Buprenorphine-D_4	472.3	Unit	400.2	Wide	200	45
Norbuprenorphine	414.3	Unit	83.1	Wide	188	60
Norbuprenorphine	414.3	Unit	57.1	Wide	188	50
Norbuprenorphine-D_3	417.3	Unit	83.1	Wide	188	60
Norbuprenorphine-D_3	417.3	Unit	57.1	Wide	188	50

Results and Discussion

At low pH, buprenorphine and norbuprenorphine are protonated at the tertiary amine group and strongly retained on Agilent Bond Elut Plexa PCX polymeric sorbent by a combination of hydrophobic retention and strong cation exchange.

A 100% methanol wash led to partial loss of analytes from the SPE column. The optimum wash that efficiently removed most matrix interferences without loss of analytes proved to be 70 MeOH:30 2% formic acid. A strong base is added to the organic eluent to break the ionic interaction between the analytes and the strong cation-exchange sorbent. The recovery of buprenorphine and norbuprenorphine was optimized with 5% NH_4OH added to the combination eluent (80 ethyl acetate: 20 isopropanol) shortly before sample elution. Two-step elution with a soaking procedure is recommended to enhance the solvent-analyte interaction and improve analyte recoveries.

Due to high hydrophobicity, buprenorphine and norbuprenorphine can adhere to glassware, LC tubing, and injector parts, which is why we recommend a 95% MeOH column rinse in the LC method and 95 MeOH:5 water flushing solution for the flushport needle rinse. Deactivated vials/inserts and MeOH-rinsed/air-dried glassware (both tubes and bottles for STD/ISTD dilutions) also ensure reproducible results.

The LC separation intentionally begins with a relatively low fraction of organic solvent (15%) to allow salts and other polar components of blood to elute at the beginning of the sample run. A flow rate of 0.8 mL/min allows for a short retention and re-equilibration time. Each sample run begins with diverting a first portion of flow (0 to 1.8 minutes) to waste to minimize source contamination. Data collection begins at 1.8 minutes, immediately after the diverter valve switch.

Chromatograms for buprenorphine and norbuprenorphine at the LOQ of 0.2 ng/mL and corresponding deuterated internal standards at 10 ng/mL are shown in Figure 2.

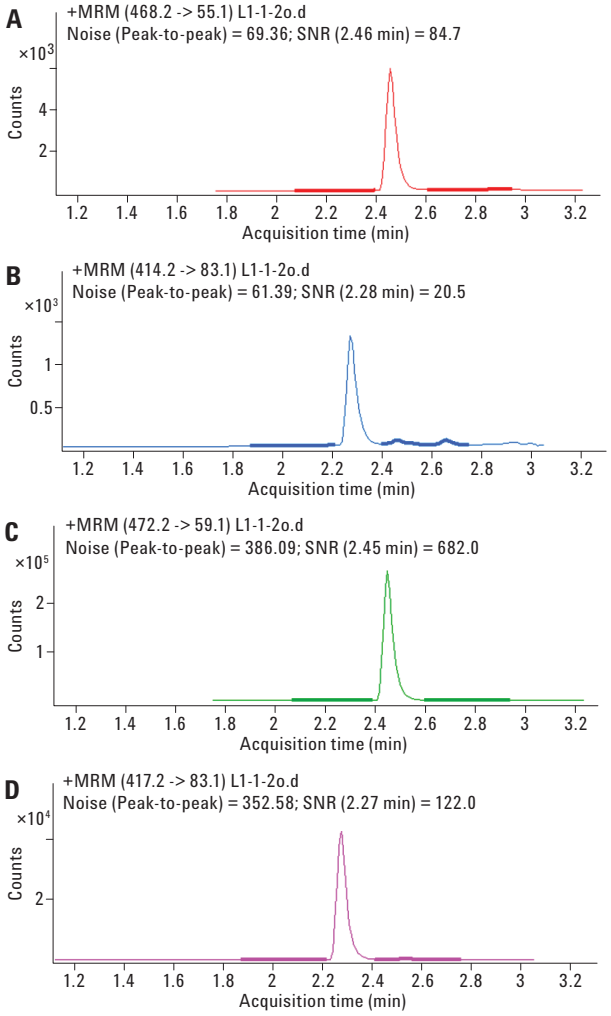


Figure 2. MRM extracted ion chromatograms: A: buprenorphine, B: norbuprenorphine (both at 0.2 ng/mL), C: buprenorphine-D_4, and D: norbuprenorphine-D_3 (both at 10 ng/mL) in whole blood extract processed on Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

The high stability of molecular ions of both buprenorphine and norbuprenorphine presents a challenge for MS/MS detection [3,9]. It led many researchers to quantitation in SIM mode [2,8], or in SRM mode by monitoring a molecular ion > molecular ion transition at relatively high collision energy without fragmentation [3,9]. Compared to a more selective quantitation by a parent-product transition, this approach is less reliable. It results in a much higher signal-to-noise (S/N) ratio and, therefore, in a higher lower limit of quantification (LLOQ). MS-MS transitions most commonly used for buprenorphine/norbuprenorphine quantification were 468 to 414, 396 *m/z* for buprenorphine, and 414 to 396, 340 and 101 *m/z* for norbuprenorphine [2, 3, 4, 5, 6, 7]. A new stable fragmentation pattern achieved with an Agilent 6460 Triple Quadrupole LC/MS System (Table 1) at high collision energy levels allows for a reliable quantitation with an LLOQ of 0.2 ng/mL for both analytes. The most abundant fragment of buprenorphine is the methylocyclopropyl (C_4H_7) group with *m/z* 55.1. Its identification is confirmed by a fragment of buprenorphine-D_4 with *m/z* 59.1. The most abundant product of norbupenorphine fragmentation (*m/z* 83.1) probably comes from the branched side chain of the parent ion and includes the tert-butyl group $(CH_3)_3C$. Compared to most commonly used fragmentation products obtained at their optimum collision energies, *m/z* 55.1 is a 8× more abundant product of buprenorphine than *m/z* 396.2, while *m/z* 83.1 is a 2× more abundant product of norbuprenorphine than *m/z* 101.1.

MRM transitions listed in Table 1 are for one quantifier and one qualifier product ion for both target compounds and their deuterated ISTDs. Agilent MassHunter software automatically calculates qualifier ion ratios, highlighting those out of the acceptable range. Either normal or dynamic MRM acquisition modes can be used with this method.

S/N ratios at the LLOQ level of 0.2 ng/mL were 84:1 for buprenorphine and 20:1 for norbuprenorphine Figures 2, A and B). This illustrates the efficiency of a sample cleanup procedure and the excellent sensitivity of the 6460 Triple Quadrupole, capable of detecting these analytes with LODs way below 0.1 ng/mL.

Figure 3 shows typical calibration curves for buprenorphine and norbuprenorphine in extracted whole blood standards at five concentration levels. Calibration standards were prepared by spiking whole blood with analytes at 0.2, 1, 5, 10, and 20 ng/mL. Deuterated internal standards were added at 10 ng/mL. Excellent linear fit (R^2 > 0.999) to each of the curves demonstrates linearity of the method. No weighting was applied, and the origin was included in the curve fit.

Table 2 shows recovery (accuracy) and precision (CV, or RSD) data collected for five samples of whole blood fortified with 1 ng/mL of each analyte. Quantitation was performed against calibration curves obtained from the spiked matrix standards (Figure 3).

Conclusions

A simple, solid phase extraction procedure coupled with an LC/MS/MS detection method allows determination of buprenorphine and norbuprenorphine in whole blood at concentrations below 0.2 ng/mL. This method is intended for users of Agilent 1100 and 1200 LC series since the backpressure in the LC system does not exceed 400 bar.

Table 2. Method Evaluation at 1 ng/mL of Each Analyte, n = 5.

	Accuracy (%)	CV %
Buprenorphine	94.2	0.6
Norbuprenorphine	103	0.2

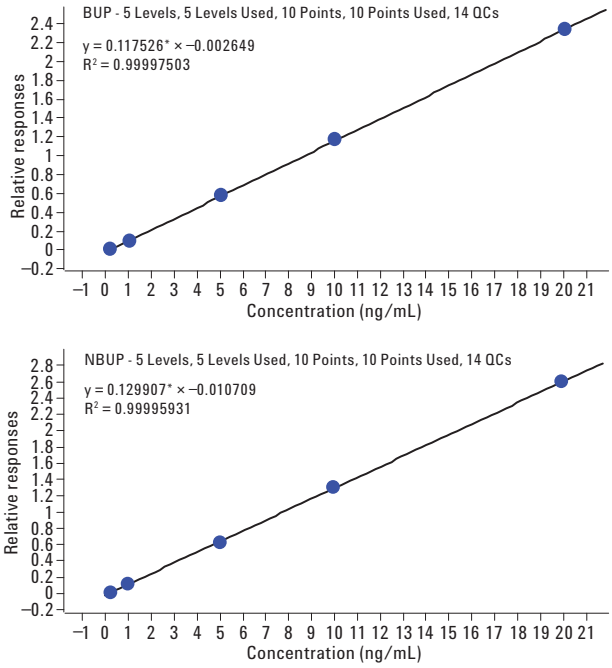


Figure 3. Typical calibration curves for buprenorphine and norbuprenorphine in whole blood extract. Concentration range 0.2 to 20 ng/mL. Linear fits R^2 > 0.999.

Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS System instruments. Low detection limits are achieved due to cleanliness of sample extracts and robust MS detection using newly identified ion transitions with abundant fragmentation products.

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Rapid, Robust and Sensitive Detection of 11-nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid in Hair

Application Note

Forensic Toxicology/Doping Control

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Abstract

A robust method for the detection of the THCA marijuana metabolite in hair was developed with a run time of 7 min and a cycle time of 9 minutes using column switching and backflushing. The method LOD is 0.002 pg/mg and the LOQ is 0.01 pg/mg.

Introduction

Testing hair for drugs of abuse has been practiced for over 50 years, due in large part to the ability to detect drug use over a longer period of time, as compared to other biological matrices, because many drugs are well-preserved in hair. Hair testing is widely used in criminal investigations. Workplace programs include hair testing due to the ease of collection, difficulty of adulteration and longer detection times.

Marijuana is one of the drugs tested most often in forensic and drug screening applications. The parent compound, tetrahydrocannabinol (THC), is found in higher concentration in hair samples, but detection of the acid metabolite THCA (11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid) is preferred, in order to eliminate the possibility of potential environmental contamination from marijuana smoke. While guidelines for workplace hair testing have not yet been adopted by the Substance Abuse Mental Health Services Administration (SAMHSA) in the United States, a cutoff concentration for nor-9-carboxy-Δ^9-tetrahydrocannbinol as low as 0.05 pg/mg hair has been suggested, and such guidelines are a topic of additional study and analysis by this regulatory body. The Society of Hair Testing recommends a limit of quantification (LOQ) of $\leq$ 0.2 pg/mg for THCA.

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This application note describes a method developed on the Agilent 7890A GC System coupled with an Agilent 7000B Triple Quadrupole GC/MS System that provides rapid and sensitive detection of a THC metabolite in hair, using 2-D GC and negative ion chemical ionization (CI) MS/MS in the multiple reaction monitoring (MRM) mode (also called SRM, Selected Reaction Monitoring). The method is modified from a previous GC/MSD method [1] to take advantage of the lower chemical background and higher sensitivity provided by triple quadrupole MS/MS analysis. Backflush is used to increase robustness, and low thermal mass (LTM) column modules speed the chromatography process, enabling a run time of 7 min and a cycle time of 9 min. MRM MS/MS analysis on the Triple Quadrupole GC/MS System delivers excellent sensitivity, with an LOD of 0.002 pg/mg and an LOQ of 0.01 pg/mg.

Experimental

Standards and Reagents

Tri-deuterated THCA, which was used as the internal standard (100 µg/mL in methanol), and unlabelled THCA (100 µg/mL in methanol) were obtained from Cerilliant, (Round Rock, TX). The internal standard concentration in the method was 0.05 pg/mg of hair.

Methanol, acetonitrile, toluene, ethyl acetate, hexane, glacial acetic acid, and methylene chloride were obtained from Spectrum Chemicals (Gardena, CA). All solvents were high-performance liquid chromatography (HPLC) grade or better, and all chemicals were ACS grade. Bond Elut Certify I solid-phase extraction columns (130 mg) from Agilent, Inc. (Walnut Creek, CA), or Clean Screen ZSTHC020 extraction columns (200 mg) from United Chemical Technologies, Inc. (Bristol, PA) were interchangeable for the assay. The derivatizing agents, pentafluoropropionic anhydride (PFPA) and 1,1,1, 3, 3, 3-hexafluoro-2-propanol (HFIP), were purchased from Sigma –Aldrich (St. Louis, MO) and Campbell Science (Rockton, IL), respectively.

Instruments

The experiments were performed on an Agilent 7890N GC System equipped with a multimode inlet (MMI) and an LTM System, coupled to an Agilent 7000B Triple Quadrupole GC/MS System. Two dimensional chromatography was performed using a pre-column for backflushing, two Low Thermal Mass (LTM) columns connected by a Deans Switch, and a Purged Ultimate Union (Figure 1). The instrument conditions are listed in Table 1.

Table 1. Agilent 7890N/7000B Gas Chromatograph and Triple Quadrupole Mass Spectrometer Conditions

GC Run Conditions	
Pre-column	1 m × 0.15 mm × 1.2 µm DB-1 (p/n 12A-1015)
Analytical columns	
Column 1	15 m × 0.25 mm × 0.25 µm DB-1ms LTM Column Module (p/n 122-0112LTM)
Column 2	15 m × 0.25 mm × 0.25 µm DB-17ms LTM Column Module (p/n 122-4712LTM)
Injection volume	2 µL
Inlet temperature	Isothermal at 250 °C
Injection mode	0.75 minute pulsed splitless at 35 psi
Oven temperatures	
GC oven	7 minute hold at 250 °C (isothermal)
1st LTM module	50 sec hold at 100 °C
	100 °C to 210 °C at 200 °C/min
	210 °C to 267 °C at 10 °C/min
	Hold at 267 °C for 2 min
2nd LTM module	324 sec hold at 100 °C
	100 °C to 230 °C at 200 °C/min
	230 °C to 240 °C at 10 °C/min
	Hold at 240 °C for 2 min
Carrier gas	Helium in constant pressure mode. Pre-column: 1 psi; Column 1: 26.6 psi; Column 2: 19.6 psi
Transfer line temp	300 °C
MS conditions	
Tune	Autotune
EMV Delta	1200 V
Acquisition parameters	NCI mode; multiple reaction monitoring (MRM)
Reagent gas	Ammonia, 35% flow
Collision gas	Argon, constant flow, 0.9 mL/min
Quench gas	Helium, constant flow, 0.5 mL/min
Solvent delay	6.2 min
MS temperatures	Source 150 °C; Quadrupole 150 °C

Sample Preparation

Samples were prepared as previously described [2]. Calibrators, controls or hair specimens (20 mg) were weighed into silanized glass tubes and washed with methylene chloride (1.5 mL). The solvent was decanted and the hair samples were allowed to dry. The internal standard, THCA-d3 (0.05 pg/mg), was added to each hair specimen. For the calibration curve, unlabelled THCA was added to the hair at concentrations of 0.002, 0.01, 0.02, 0.05, 0.1, and 0.5 pg/mg of hair.

Deionized water (0.5 mL) and 2N sodium hydroxide (0.5 mL) were added, and the hair was heated at 75 °C for 15 min. The sample was allowed to cool and then centrifuged (2500 rpm, 15 min). The supernatant was poured into glass tubes already containing acetic acid (1 mL), 1 M acetic acid (3 mL), and 0.1 M sodium acetate buffer (pH 4, 2 mL). The tubes were capped and mixed.

SPE columns were conditioned with hexane/ethyl acetate (75:25, v/v; 2 mL), methanol (3 mL), deionized water (3 mL), and 0.1 M hydrochloric acid (1 mL). The acidified samples were loaded onto the SPE columns and allowed to dry. The SPE columns were washed with deionized water (2 to 3 mL) and allowed to dry for 5 min. The SPE columns were washed with 0.1 M hydrochloric acid/acetonitrile (70:30 v/v; 3 mL) and allowed to dry at 30 psi for 10 min. The SPE columns were finally rinsed with hexane/ethyl acetate (75:25 v/v; 3 mL) in order to elute the THCA into silanized glass tubes.

The eluent was evaporated to dryness under nitrogen at 40 °C and reconstituted in PFPA (70 µL) and HFIP (30 µL) for derivatization. The mixture was transferred to autosampler vials with glass inserts and capped. The vials were heated at 80 °C for 20 min, then left at room temperature for 10 min. The extracts were evaporated to dryness in a vacuum oven. The samples were finally reconstituted in toluene (50 µL), for injection into the GC–MS system.

Analysis Parameters

The Agilent Triple Quadrupole GC/MS System parameters used are shown in Table 2.

Table 2. Agilent 7000B Triple Quadrupole GC/MS System Analysis Parameters

Compound	RT (min)	MRM	Dwell time (ms)	Collision energy (EV)
THCA*	6.714	620→492	50	5
		620→383	50	5
THCA-d3	6.710	623→495	20	5
		623→386	20	5

*11-nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid

Results

Two Dimensional Gas Chromatography with Heart-Cutting

The use of two serial GC columns to separate background from the required peak is a well-established technology that is widely used to provide excellent separation of the analyte from matrix interferences. Once the analyte retention time on the first column has been determined, the pneumatic switch (Deans Switch) is turned on at that time to divert the flow to the second column, and turned off a short time later. This diverts a narrow, heart-cut "window" of the effluent from the first column that contains the analyte and minimal background, for further separation on the second column (Figure 1). The two columns function optimally when the stationary phases are as different as possible.

Exceptional Robustness and Speed

The unique combination of backflushing and low thermal mass (LTM) column modules make this a very robust and rapid method, compared to the traditional single column approach. Three independently programmed pressure zones are used in conjunction with three independently heated zones (Figure 1). The pre-column and the first LTM column are coated with relatively non-polar DB-1ms phase, and the second LTM column is coated with a more polar DB-17ms phase. The heart-cut window is only 0.2 min (5.5 to 5.7 min) wide.

A unique system for rapid and robust detection of THCA in hair

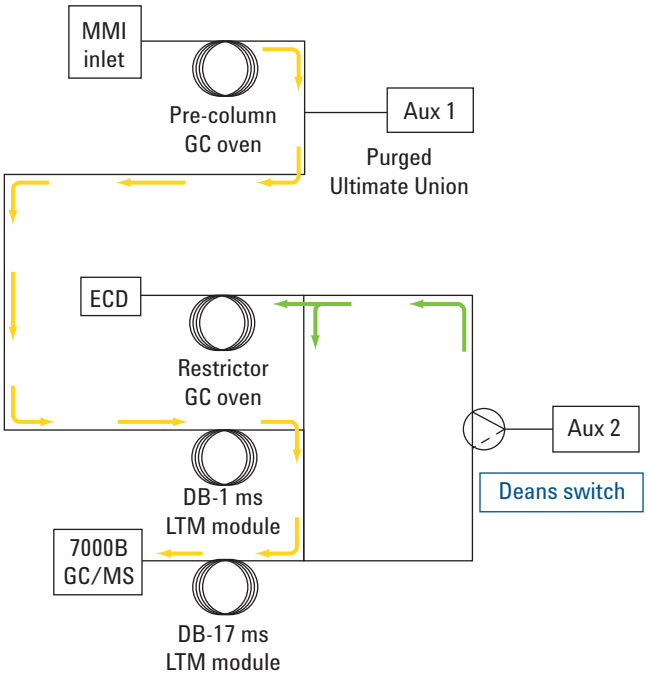

Figure 1. Schematic representation of the system used to develop the THCA method.

The precolumn and auxiliary pressure control module (AUX EPC) provides backflushing capability to protect and preserve the LTM analytical columns. The precolumn was in backflush mode with a constant pressure of 1 psi during the run. The inlet pressure pulse overrides the backflush for the initial 0.75 min. The use of backflushing prevents build-up of high-boiling compounds on the column, thus reducing retention time shifts, peak distortion, and chemical noise, while improving quantification. Contamination of the MS source and the resultant need for cleaning are also reduced, while the run time is shortened.

This method also employs LTM column modules external to the GC oven that enable independent and optimal temperature control of the two analytical columns (Figure 2). The unique design of these modules makes it possible to employ very fast temperature ramping and rapid cooling. The LTM column modules can be added to an Agilent GC without requiring any changes in the injectors, autosamplers, or detectors, and they can be controlled from the GC software.

The end result of this unique backflushing and LTM approach is a robust method that provides excellent quantification and sensitivity (see next section) with 7 min run times and 9 min cycle times.

Unique LTM Column Modules enable rapid temperature ramping and cooling

Figure 2. Low thermal mass (LTM) column modules interfaced with the Agilent 7890A GC.

Sensitivity and Quantification

This method has a limit of detection (LOD) of 0.002 pg/mg, demonstrating excellent sensitivity that is far below the suggested cutoff of 0.05 pg/mg (Figure 3). The accuracy of quantification is also quite good, with an R^2 of 0.995, from 0.002 to 0.5 pg/mg of hair (Figure 4). The limit of quantification (LOQ) is 0.01 pg/mg, which again is more than an order of magnitude below the 0.2 pg/mg LOQ suggested guideline established by the Society of Hair Testing (Figure 5). This method also provides a compliant quantitative analysis report that includes the retention times (with limits), response level, qualifier ion ratio (with limits), and the calculated concentration. The total ion current (TIC) trace and the quantifier and qualifier MRM traces are also displayed on the report, for both the sample and the THCA-d3 internal standard (Figure 6).

LOD of 0.002 pg/mg

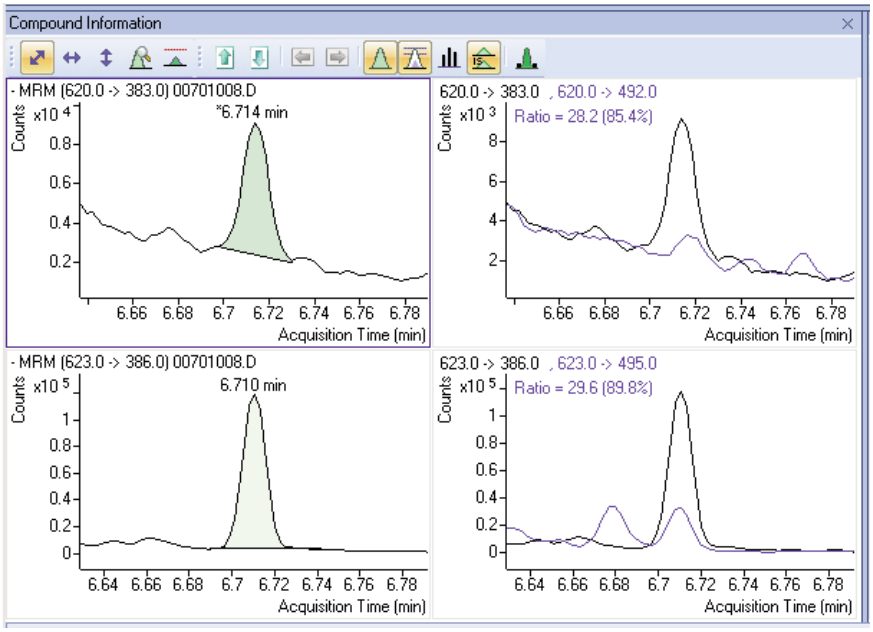

Figure 3. MRM traces for the quantifying transition (left) and both the quantifying and qualifying transitions (right) for the 0.002 pg/mg LOD of THCA (upper panel) and the deuterated standard (lower panel) spiked into a hair sample.

Reliable calibration

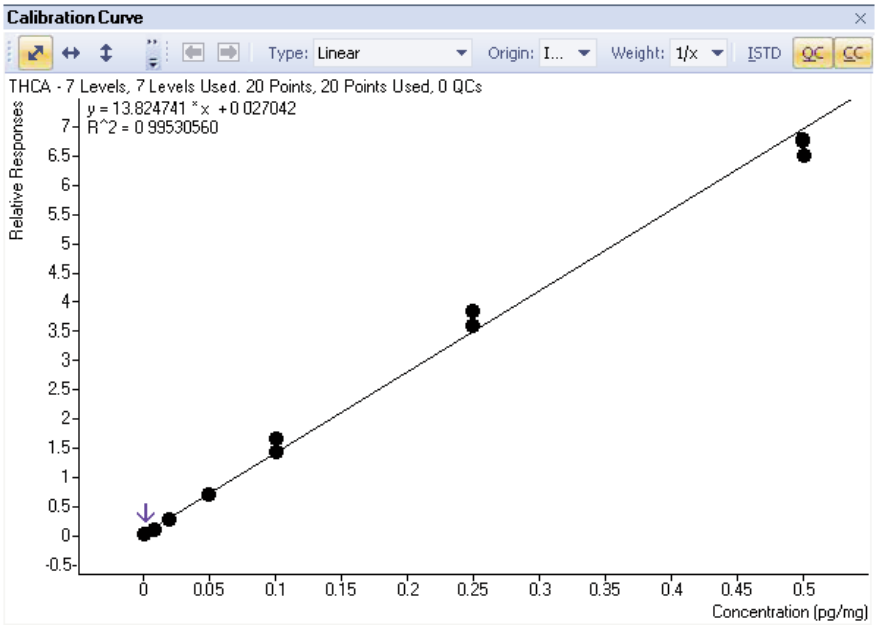

Figure 4. Calibration curve for THCA spiked into hair samples at 0.002, 0.01, 0.02, 0.05, 0.1, and 0.5 pg/mg of hair.

0.01 pg/mg LOQ

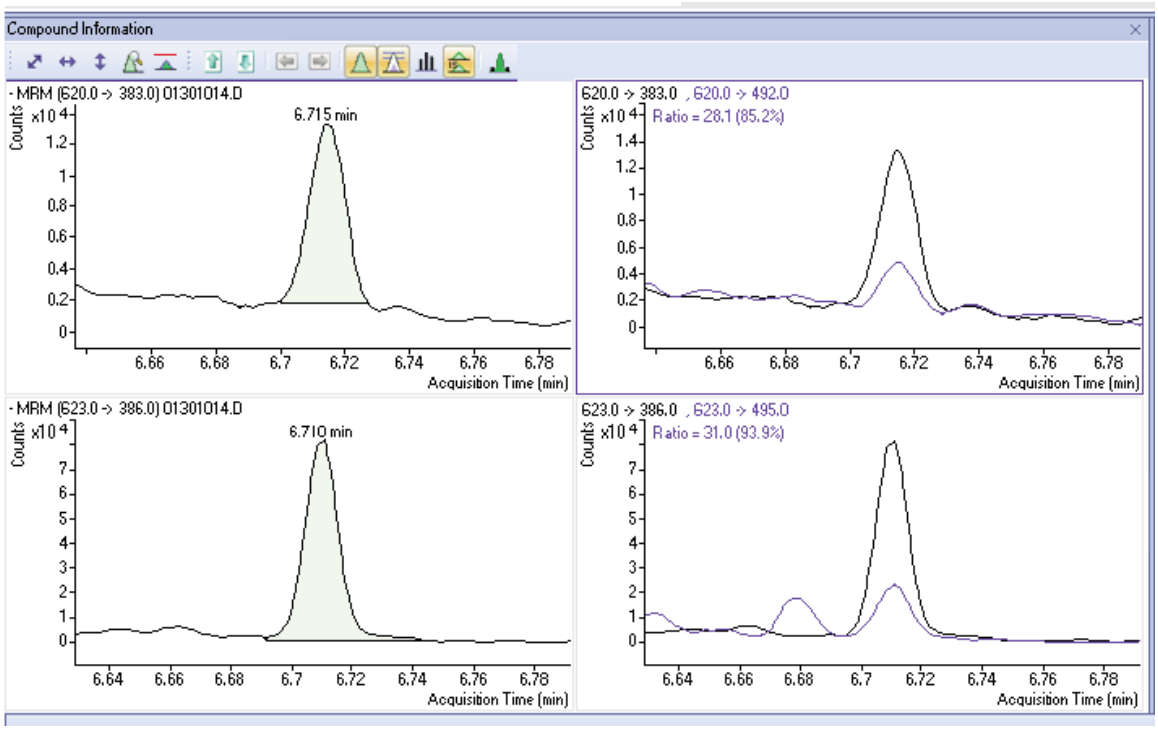

Figure 5. MRM traces for the quantifying transition (left) and both the quantifying and qualifying transitions (right) for the 0.01 pg/mg LOQ of THCA (upper panel) and the deuterated standard (lower panel) spiked into a hair sample.

Data File	01401015.D	Vial	14
Operator	DATASYSTEM01/Admin	Dillution	0.0
Acq method name		Sample information	
Acquisition date	2010-10-08 16:24	Last calib update	2010-11-28 09:34
Sample name and path	0.01 pg/mg, D:/MassHunter/GCMS/1/data/PFAA Curve Extracted/		

Compound	Signal	RT	Limits	Response	QRatio	Limits	Final conc
THCA-d3	623.0 -> 386.0	6.71		82558		35770 - 143081	
	623.0 -> 495.0			24962	30.2	23.1 - 42.9	
THCA	620.0 -> 383.0	6.71	6.38 - 7.05	10999			0.008
	620.0 -> 492.0			3908	35.5	23.1 - 42.9	

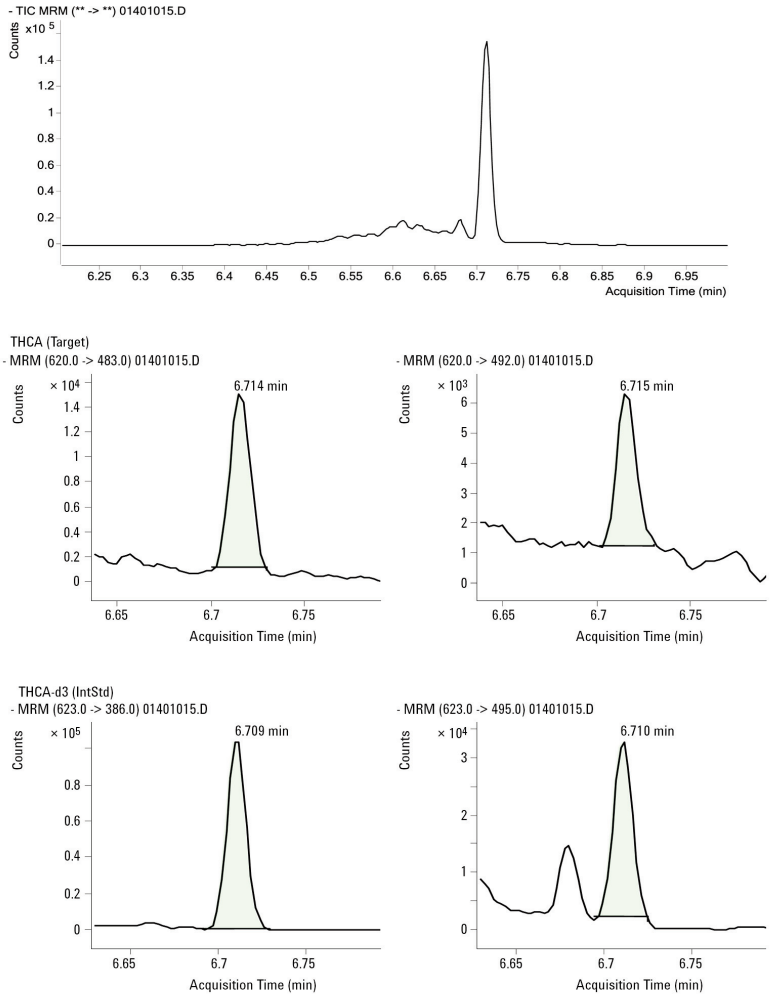

Figure 6. Quantitative Analysis Sample Report for a 0.01 pg/mg (the LOQ) sample spiked into hair.

Conclusion

The time-proven technique of heart-cutting to improve chromatographic separation is given new life in this unique method which utilizes state-of-the-art microfluidics-aided backflushing and low thermal mass column temperature ramping modules to deliver sensitive and robust detection and quantification of THCA in hair (LOD 0.002 pg/mg; LOQ 0.01 pg/mg) with run times of only 7 minutes, and cycle times of 9 minutes.

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Rapid and Robust Detection of THC and Its Metabolites in Blood

Application Note

Forensic Toxicology/Doping Control

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Abstract

A robust method for detection of THC and its metabolites in blood has been developed using SPE extraction and GC/MS/MS with backflushing. The dynamic range of quantification was 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA, with a run time of 6 minutes and a cycle time of 8 minutes.

Introduction

In the past decade, a great deal of research concerning the impact of cannabis use on road safety has been conducted. More specifically, studies on effects of cannabis smoking on driving performance, as well as epidemiological studies and cannabis-detection techniques have been published. As a result, several countries have adopted driving under the influence of drugs (DUID) legislation, with varying approaches worldwide. While a wide variety of bodily fluids have been used to determine the presence of cannabis, blood testing is considered the most reliable indicator of impairment. Blood testing for active tetrahydrocannabinol (THC) may also be considered by employers who wish to identify employees whose performance may be impaired by their cannabis use. Gas chromatography/mass spectrometry (GC/MS) is a standard method for detection and quantification of THC and its metabolites in blood.

One key to reliable THC testing in blood is an efficient extraction method. The use of tandem MS (MS/MS) also increases the sensitivity and reliability of quantification of THC and its metabolites in blood, due to the elimination of interferences. This application note describes a method using the High Flow Bond Elut Certify II SPE cartridge to rapidly and efficiently extract THC and its metabolites from blood. The extracts were derivatized to improve volatility and analyzed on the Agilent 7890A Triple Quadrupole GC/MS system equipped with a Low Thermal Mass Module (LTM)

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oven and backflushing. It was in turn coupled with an Agilent 7000B Triple Quadrupole GC/MS system, using MS/MS in the multiple reaction monitoring (MRM) mode to provide rapid and sensitive detection of THC and its metabolites, 11-OH-THC (11-hydoxy-Δ9-tetrahydrocannbinol) and THCA (11-nor-Δ9-Tetrahydrocannabinol-9-Carboxylic Acid). Backflushing was used to increase robustness and speed, enabling a run time of 6 minutes and a cycle time of 8 minutes. MRM MS/MS analysis on the Triple Quadrupole GC/MS system delivers excellent results, with a dynamic range of 0.1 to 50 ng/mL.

Experimental

Standards and Reagents

Tri-deuterated THC, 11-OH-THC and THCA, which were used as internal standards (100 µg/mL in methanol), and unlabelled THC, 11-OH-THC and THCA (100 µg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). The internal standard concentrations in the method were both 10 µg/mL.

Methanol, acetonitrile, toluene, ethyl acetate, hexanes, glacial acetic acid, and methylene chloride were obtained from Sigma Aldrich (St. Louis, MO). All solvents were high-performance liquid chromatography (HPLC) grade or better, and all chemicals were ACS grade. Agilent High Flow Bond Elut Certify II solid-phase extraction columns were used for the method. The derivatizing agents, BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) were purchased from Cerilliant. Normal human whole blood stabilized with potassium oxalate and sodium fluoride was obtained from Bioreclamation (Hicksville, NY). Standards were prepared in this drug-free matrix to construct the calibration curves.

Instruments

The experiments were performed on an Agilent 7890N gas chromatograph equipped with a multimode inlet (MMI) and an LTM oven, coupled to a 7000B Triple Quadrupole GC/MS. Chromatography was performed using a pre-column for backflushing, and a Low Thermal Mass (LTM) column connected by a Purged Ultimate Union (Figure 1). The instrument conditions are listed in Table 1.

a.

Loading the sample on the pre-column

b.

Backflushing the pre-column and separation of THC and its metabolites on the primary column

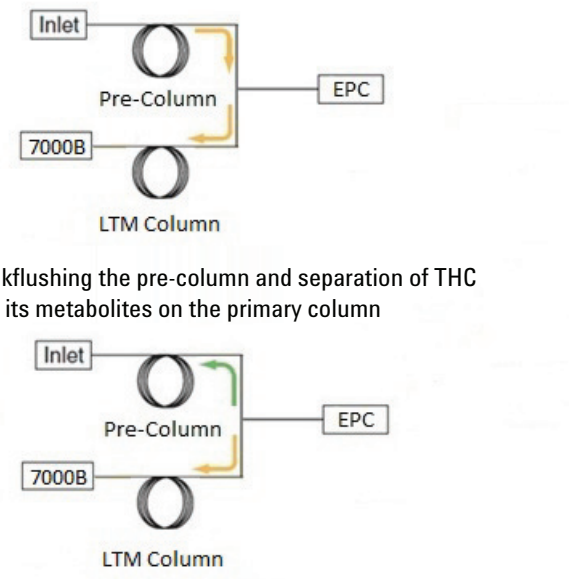


Figure 1. Schematic representation of the backflush system used to develop the method. EPC: Electronic Pneumatic Control module; 7000B: Agilent Triple Quadrupole GC/MS system

Table 1. Agilent 7890N/7000B Gas Chromatograph and Triple Quadrupole Mass Spectrometer Conditions

GC Run Conditions	
Pre-column	1 m section from a 15 m × 0.25 mm, 0.25 µm HP-5 ms Ultra Inert column (p/n 19091S-431UI)
Analytical column	15 m × 0.25 mm, 0.25 µm DB-17 ms LTM Column Module (p/n 122-4712LTM)
Injection volume	1 µL
Inlet temperature	Isothermal at 280 °C
Injection mode	0.5 min pulsed splitless at 35 psi
Oven temperatures	GC oven: 6 min hold at 280 °C (isothermal)
LTM module:	50 second hold at 100 °C 100 °C to 230 °C at 200 °C/min 230 °C to 280 °C at 10 °C/min Hold at 280 °C for 1 min
Carrier gas	Helium in constant pressure mode. Pre-column: 1 psi; Column 1: 5 psi; Column 2: 9.6 psi
Transfer line temp	300 °C
MS Conditions	
Tune	Autotune
Gain	20
Acquisition parameters	EI mode; multiple reaction monitoring (MRM)
Collision gas	Nitrogen constant flow, 1.5 mL/min
Quench gas	Helium, constant flow, 2.25 mL/min
Solvent delay	3.0 min
MS temperatures	Source 230 °C; Quadrupole 150 °C

Sample Preparation

A 2 mL blood sample containing 10 µg/mL of each internal standard (ISTD) and spiked with THC, 11-OH-THC and THCA was pipetted into a clean tube, and 4 mL of acetonitrile was added. After centrifugation at 2500 rpm for 5 minutes, the supernatant was transferred and evaporated to about 3 mL with nitrogen at 35-40 °C, and 7 mL of 0.1 M sodium acetate (pH 6.0) was added.

High Flow Bond Elut Certify II SPE columns were conditioned with 2 mL of methanol, then 2 mL 0.1 M sodium acetate buffer, pH 6.0 with 5% methanol. Cartridges were not be allowed to go to dryness prior to sample addition. The sample was drawn through the column slowly, at 1 to 2 mL/min. The column was then washed 2 mL sodium acetate buffer, pH 6.0, dried under maximum vacuum for approximately 5 minutes, then washed with 1 mL hexanes. THC was eluted under neutral conditions with 2 mL of 95:5 hexane: ethyl acetate. This was followed by a 5 mL 1:1 methanol:deionized water wash. The column was again dried under maximum vacuum for approximately 5 minutes and washed again with 1 mL hexanes. Elution of 11-OH-THC and THCA was performed with 2 mL 1% acetic acid in 75:25 hexane:ethyl acetate. The THC and the metabolite fractions were combined and dried before derivatization.

The eluent was evaporated under nitrogen at a temperature no higher than 40 °C, then reconstituted in 60 µL of toluene and 40 µL of BSTFA, 1% TMCS for derivatization. The sample tubes were capped and heated 20 minutes at 70 °C before injection into the tandem quadrupole GC/MS system.

Analysis Parameters

The Agilent Triple Quadrupole GC/MS system parameters used are shown in Table 2.

Table 2. Agilent 7000B Triple Quadrupole GC/MS System Analysis Parameters

Compound	RT (min)	MRM	Dwell time (ms)	Collision energy (EV)
THC (Δ9-Tetrahydrocannabinol)	3.5	386→303*	25	20
		386→330	27	10
		386→289	30	25
THC-d3	3.5	389→306*	10	20
		389→330	11	10
		389→292	15	25
11-OH-THC (11-hydoxy-Δ9-tetrahydrocannabinol)	4.5	371→289*	24	20
		371→305	26	15
		371→265	27	15
11-OH-THC-d3	4.5	374→292*	10	20
		374→308	12	15
		374→268	12	15
THCA (11-nor-Δ9-Tetrahydrocannabinol-9-Carboxylic Acid)	5.6	371→289*	23	15
		488→297	44	20
		488→371	29	20
THCA-d9	5.5	380→292*	15	15
		497→306	30	20
		497→380	22	20

*Target transition. All other transitions are qualifier transitions.

Results

SPE Sample Preparation with High Flow Bond Elut Certify II Columns

Screening for drugs of abuse in biological fluids requires rugged methods that provide high purification and recovery. The Bond Elut Certify was developed specifically for the rapid and effective extraction of compounds that possess both non-polar and anionic characteristics from urine and other biological matrices [1]. The mixed mode (non-polar C8 and strong anion exchange) sorbent takes advantage of non-polar, polar, and ion exchange properties to ensure rapid, reproducible, simple, and clean extraction of many drug classes. These columns enable the rapid and high recovery of THC, 11-OH-THC and THCA from whole blood.

Backflushing

Backflushing makes this a very robust and rapid method, preventing build-up of high-boiling compounds on the column and thus reducing retention time shifts, peak distortion, and chemical noise, while improving quantification. Contamination of the MS source and the resultant need for cleaning are also reduced, while the run time is shortened. The end result is a robust method that provides excellent dynamic range with 6 minute run times (not including sample prep) and 8 minute cycle times.

The suite of Agilent Capillary Flow Technology modules enables easy and rapid backflushing with minimal dead volumes for maintaining chromatographic resolution. During injection, the inlet Pneumatic Control Module (PCM) is held at an elevated pressure long enough to transfer the target analytes from the pre-column to the analytical column (Figure 1a). When backflushing, the inlet pressure is dropped to 1 psi, forcing the flow to reverse through the pre-column and out the split vent (Figure 1b). In this way, THC, 11-OH-THC and THCA are passed on to the primary column for further separation, while high-boiling compounds are swept back though the split vent.

Low Thermal Mass Modules

This method also employs a Low Thermal Mass (LTM) column module external to the GC oven that enables independent and optimal temperature control of the analytical column (Figure 1). The unique design of these modules makes it possible to employ very fast temperature ramping and rapid cooling. The LTM column modules can be added to an Agilent GC without requiring any changes in the injectors, autosamplers, or detectors.

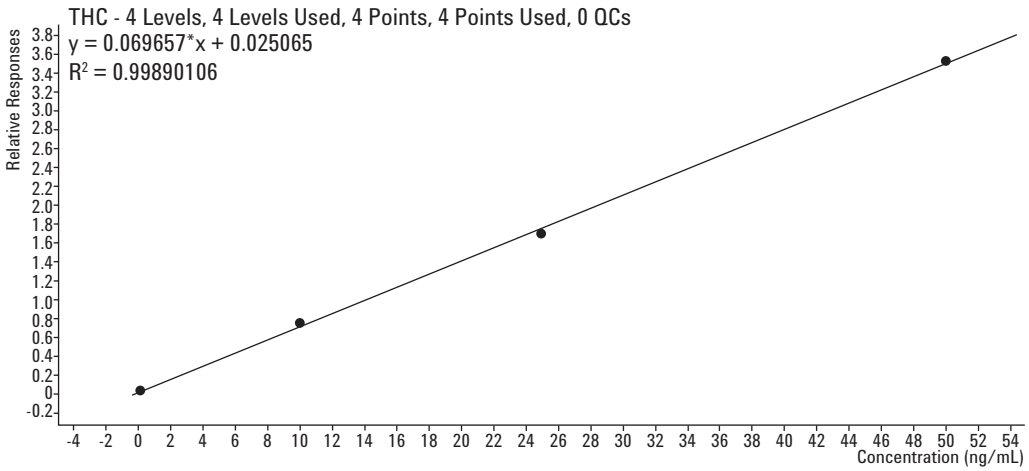
Dynamic Range

This method has a dynamic range of 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA (Figure 2), which match industry norms. The accuracy of quantification is also quite good, with an R^2 of 0.999 for all three analytes.

MRM Results

Using a MassHunter forensic report template, Quantitative Analysis Sample Reports were quickly and easily prepared for THC and its two analytes (Figures 3-5), featuring a Total Ion Current (TIC) chromatogram and spectra for all of the transitions, including the internal standard. Note the lack of interference in all of the transitions, even at the lowest end of the dynamic range for each analyte.

a.



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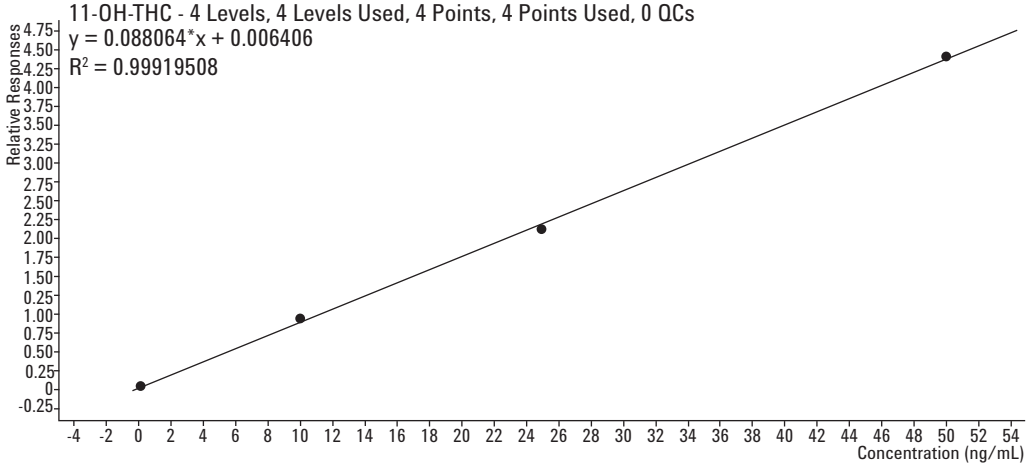

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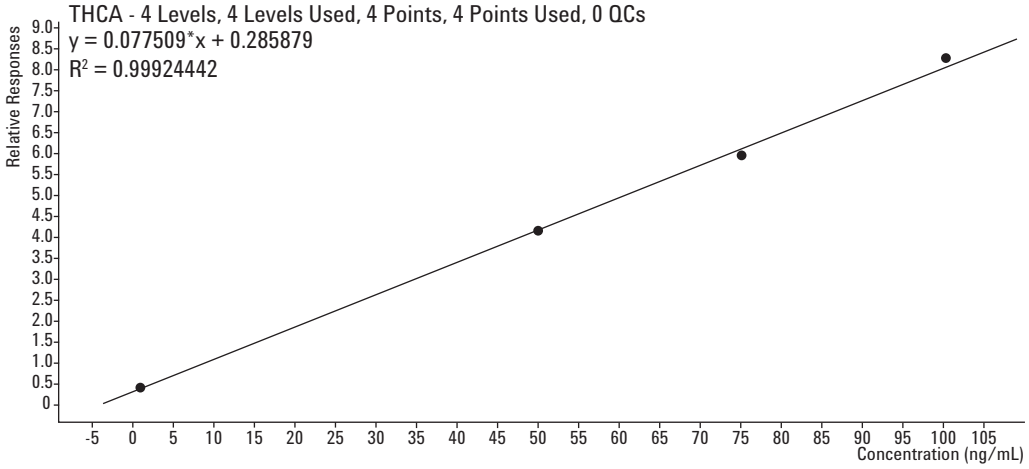
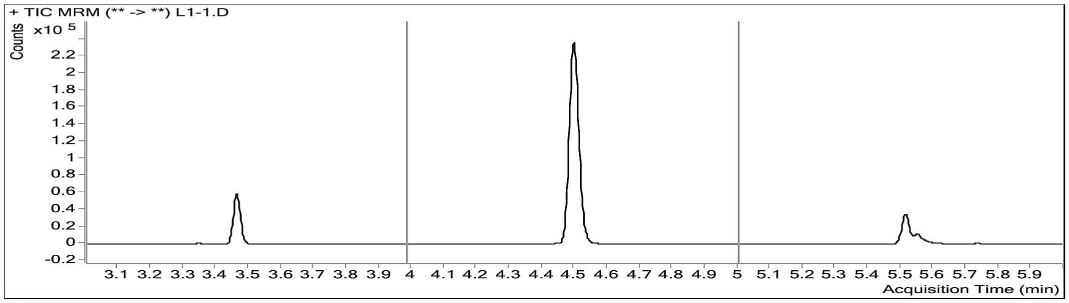


Figure 2. Calibration curves for THC (a), 11-OH-THC (b) and THCA (c) in blood. Data points were taken at 0.1, 10, 25, and 50 ng/mL for THC and 11-OH-THC, and at 1, 50, 75, and 100 ng/mL for THCA.

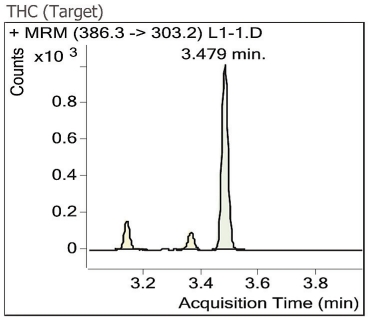
Quantitative Analysis Sample Report

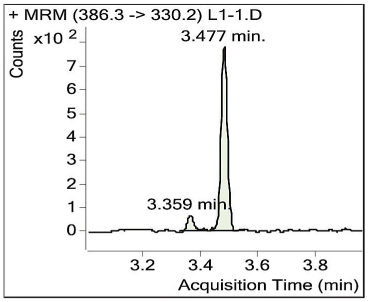
Data File	:	L1-1.D
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

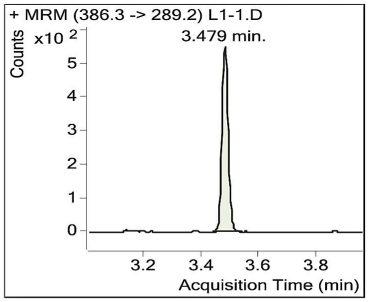
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
D3-THC	389.3 -> 306.2	3.46		37956			
	389.3 -> 330.2			32853	86.6	64.3-104.3	
	389.3 -> 292.2			18032	47.5	22.8-62.8	
THC	386.3 -> 303.2	3.48	3.29 - 3.64	1554			0.100
	386.3 -> 330.2			1237	79.7	62.4-102.4	
	386.3 -> 289.2			848	54.6	23.6-63.6	



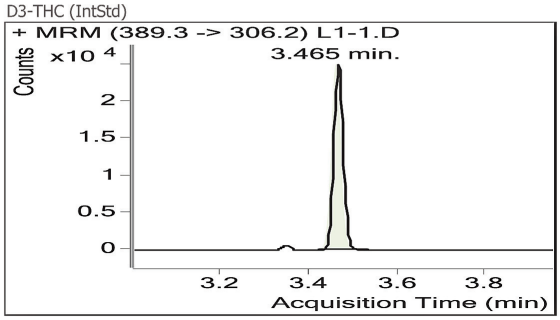
THC (Target)







D3-THC (IntStd)



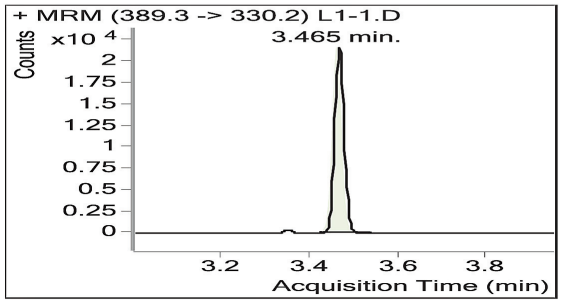
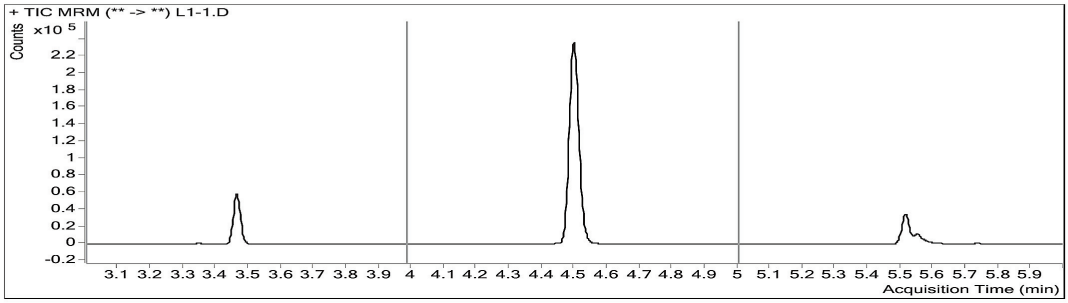


Figure 3. Quantitative Analysis Sample Report for 0.1 ng/mL of THC in blood. The RMS signal-to-noise is 175:1 with a noise region of 3.6 to 3.9 min.

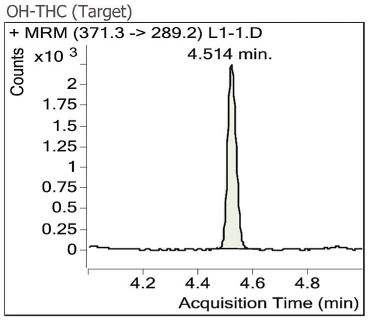
Quantitative Analysis Sample Report

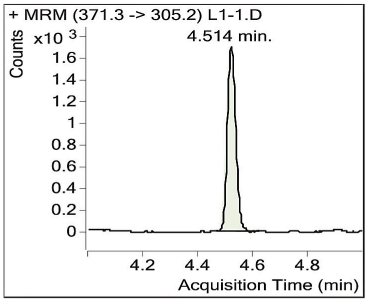
Data File	:	L1-1.D
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

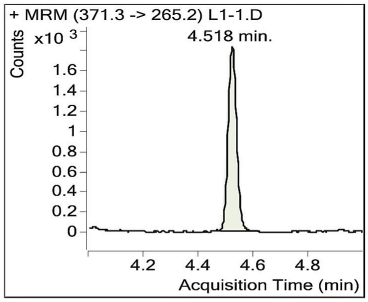
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
d3-OH-THC	374.3 -> 292.2	4.5		175705			
	374.3 -> 308.2			157882	89.9	68.8-108.8	
	374.3 -> 268.3			148545	84.5	59.4-99.4	
OH-THC	371.3 -> 289.2	4.51	4.27 - 4.72	4924			0.099
	371.3 -> 305.2			3707	75.3	67.4-107.4	
	371.3 -> 265.2			4050	82.3	58.2-98.2	



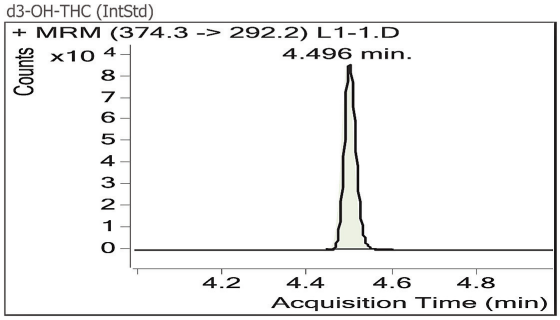
OH-THC (Target)







d3-OH-THC (IntStd)



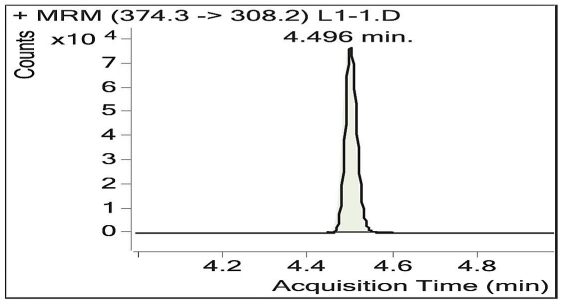
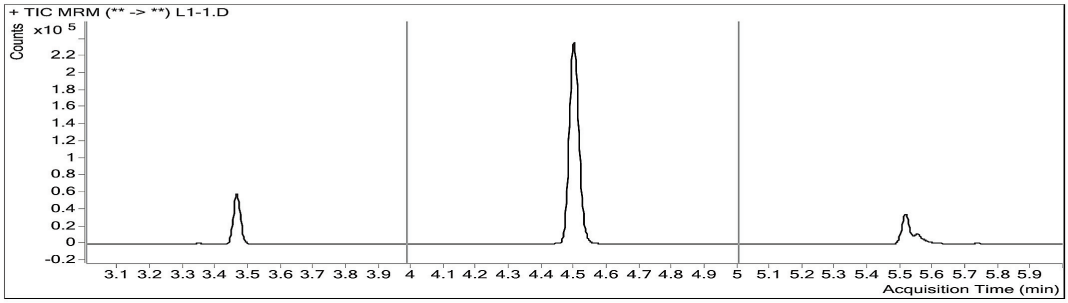


Figure 4. Quantitative Analysis Sample Report for 0.1 ng/mL of 11-OH-THC in blood. The RMS signal-to-noise is 46:1 with a noise region of 4.6 to 4.9 min.

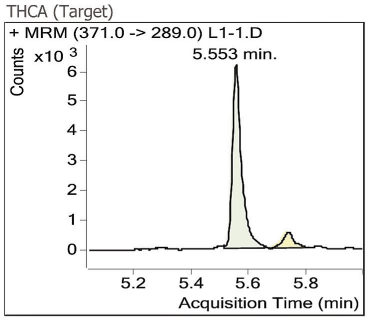
Quantitative Analysis Sample Report

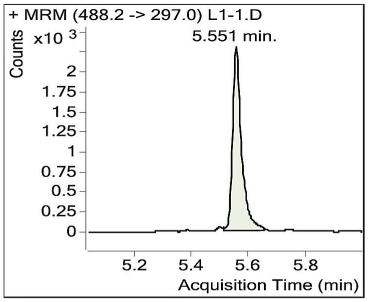
Data File	:	L1-1.D
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

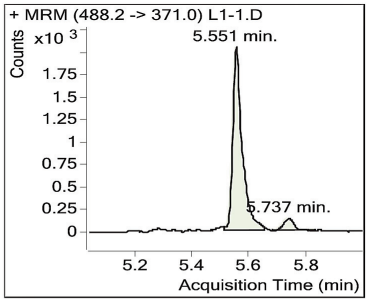
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
d9-THCA	380.0 -> 292.2	5.51		38358			
	497.0 -> 306.1			9800	25.5	5.6-45.6	
	497.0 -> 380.1			11765	30.7	12.5-52.5	
THCA	371.0 -> 289.0	5.55	5.26 - 5.82	13985			1.015
	488.2 -> 297.0			5124	36.6	9.1-49.1	
	488.2 -> 371.0			4758	34	14.4-54.4	



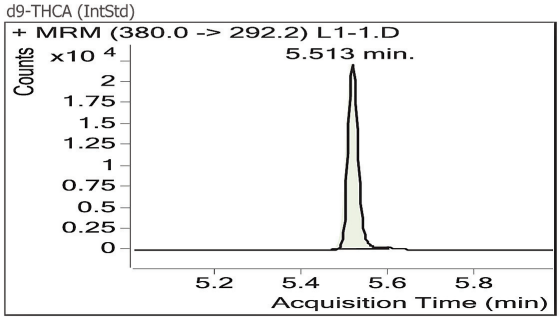
THCA (Target)







d9-THCA (IntStd)



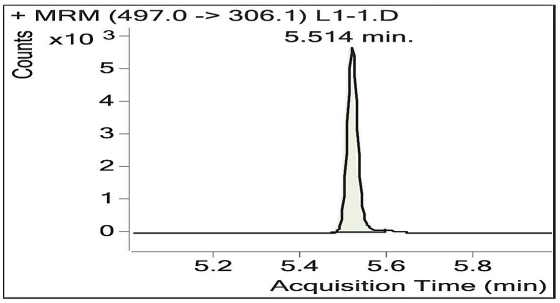


Figure 5. Quantitative Analysis Sample Report for 1 ng/mL of THCA in blood. The RMS signal-to-noise is 39:1 with a noise region of 5.1 to 5.3 min.

Conclusion

Coupling the Agilent 7890N gas chromatograph utilizing an LTM system with the Agilent 7000B Triple Quadrupole GC/MS system enables a rapid and robust method for the analysis of THC and its metabolites in blood. Using the High Flow Bond Elut Certify II SPE cartridge , backflushing of the GC column, and MRM eliminate all interferences, with a resulting dynamic range of quantification of 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA. The LTM module and backflushing facilitate rapid analysis, with a run time of 6 minutes and a cycle time of 8 minutes.

References

1. R.M Sears, Solid Phase Extraction of THD, THC-COOH and 11-OH-THC from Whole Blood, Agilent Technologies Application Note 00315.

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Agilent Technologies

Extraction of Benzodiazepines in Urine with Polymeric SPE Cation Exchange, Agilent Bond Elut Plexa PCX

Application Note

Forensic Toxicology

Authors

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Agilent Technologies, Inc.

Introduction

Benzodiazepines are a large class of drugs and include compounds such as diazepam (Valium), chlordiazepoxide (Librium), oxazepam (Serax), lorazepam (Ativan), alprazolam (Xanax), clonazepam (Clonopin), and others. 1,4-Benzodiazepines, such as diazepam, nordiazepam, and temazepam, are metabolized and excreted as oxazepam and oxazepam glucuronide. The nitrobenzodiazepines, such as clonazepam and flunitrazepam, are metabolized to a 7-amino metabolite in urine. Flurazepam is rapidly desalkylated.

Quantitative analysis of benzodiazepines in urine by LC/MS can be difficult due to the high level of matrix components. Organic salts as well as pigments and proteins cause ion suppression and the loss of signal intensity. Agilent Bond Elut Plexa PCX SPE products are a member of the Plexa family based on a polymeric cation exchanger. Plexa PCX products use a generic and simplified method to remove neutral and acidic interferences from the matrix and concentrate basic analytes, resulting in improved analytical performance and sensitivity in the quantification of basic compounds.

In addition, Bond Elut Plexa PCX SPE products offer faster and highly reproducible flow rates, resulting in excellent tube-to-tube and well-to-well performance. Bond Elut Plexa PCX SPE products exhibit significantly reduced ion suppression because their highly polar, hydroxylated surfaces are entirely amide free. Therefore, the particle exterior minimizes strong binding of proteins and phospholipids. An LC/MS/MS method is presented for the quantitative determination of benzodiazepines and their target metabolites in human urine specimens with Bond Elut Plexa PCX SPE tubes. Hydrolysis may also be necessary by adding 5,000 units of β-glucuronidase to a 1 M acetic acid (pH = 3.8) buffered urine sample. The sample was vortexed and incubated for 2 hours at 60 °C prior to extraction.

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Materials and Methods

Table 1. SPE reagents and solutions.

2% Formic acid	Add 2 mL of concentrated formic acid to 100 mL of DI water
Methanol	Reagent grade or better
50% Methanol	Add 50 mL of methanol to 50 mL of DI water
5% Ammonia in methanol	Add 5 mL of concentrated ammonia to 100 mL of methanol

Table 2. SPE method.

Column:	Agilent Bond Elut Plexa PCX 30 mg 3 mL tube (p/n 12108303)
Sample pretreatment:	1 mL human urine. Dilute 1:2 with 2% formic acid.
Condition:	1. 1 mL CH_3OH 2. 1 mL H_2O
Load:	Apply sample and extract under low or no vacuum
Wash 1:	2 mL 2% formic acid
Wash 2:	2 mL 50% CH_3OH in water
Elution:	1 mL 5% NH_3 in methanol

All samples are evaporated to dryness and reconstituted in 200 µL of 50:50 0.1% aqueous formic acid: CH_3OH.

Table 3. MS conditions.

Compound	Q1	Q3	CE
Clonazepam	316.0	270.0	16.5 V
7-Aminoclonazepam	285.8	121.0	24.5 V
Flurazepam	388.0	315.0	18.0 V
Desalkylflurazepam	288.9	140.0	24.0 V
Midazolam	326.4	290.9	21.5 V
Alprazolam	309.0	204.9	37.0 V
Flunitrazepam	314.0	268.0	21.0 V
7-Aminoflunitrazepam	284.1	135.0	22.0 V
Chlordiazepoxide	300.3	227.0	19.5 V
Diazepam	285.0	222.0	20.5 V
Lorazepam	321.0	274.9	18.0 V
Oxazepam	286.8	241.0	16.5 V
Nordiazepam	271.0	165.0	23.0 V
Temazepam	301.0	255.0	17.0 V

LC conditions

Mobile phase:	A: 0.1% Formic acid	
	B: Methanol	
Gradient:	t = 0-1 minutes	40% A : 60% B
	t = 2.0-4.30 minutes	20% A : 80% B
	t = 4.31-5.30 minutes	40% A : 60% B
Flow rate:	0.2 mL/min	
Column:	Agilent Pursuit XRs$^{Ultra\ 2.8}$ C18, 2.0 × 100 mm (p/n A7511100X020)	
Capillary:	70 V	
Dry gas temperature:	350 °C, 30 psi	
CID:	Argon	
Polarity:	Negative	

Results and Discussion

The procedure describes a method for extracting and determining 14 different benzodiazepines in human urine. The limit of detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a first order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to three orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.995. To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). Table 4 shows that the extractions produced very reproducibly high recoveries.

Table 4. Analyte relative recoveries.

Analyte	% Rec (1 ng/mL)	% RSD	% Rec (100 ng/mL)	% RSD
Clonazepam	116	13	103	7
7-Aminoclonazepam	102	10	99	2
Flurazepam	117	14	106	8
Desalkylflurazepam	115	13	99	6
Midazolam	108	13	110	4
Nordiazepam	113	15	107	7
Alprazolam	113	17	110	8
Flunitrazepam	107	16	101	3
7-Aminoflunitrazepam	112	18	95	9
Chordiazepoxide	119	15	92	10
Diazepam	111	12	99	8
Temazepam	118	4	97	8
Lorazepam	102	14	94	10
Oxazepam	113	10	97	5

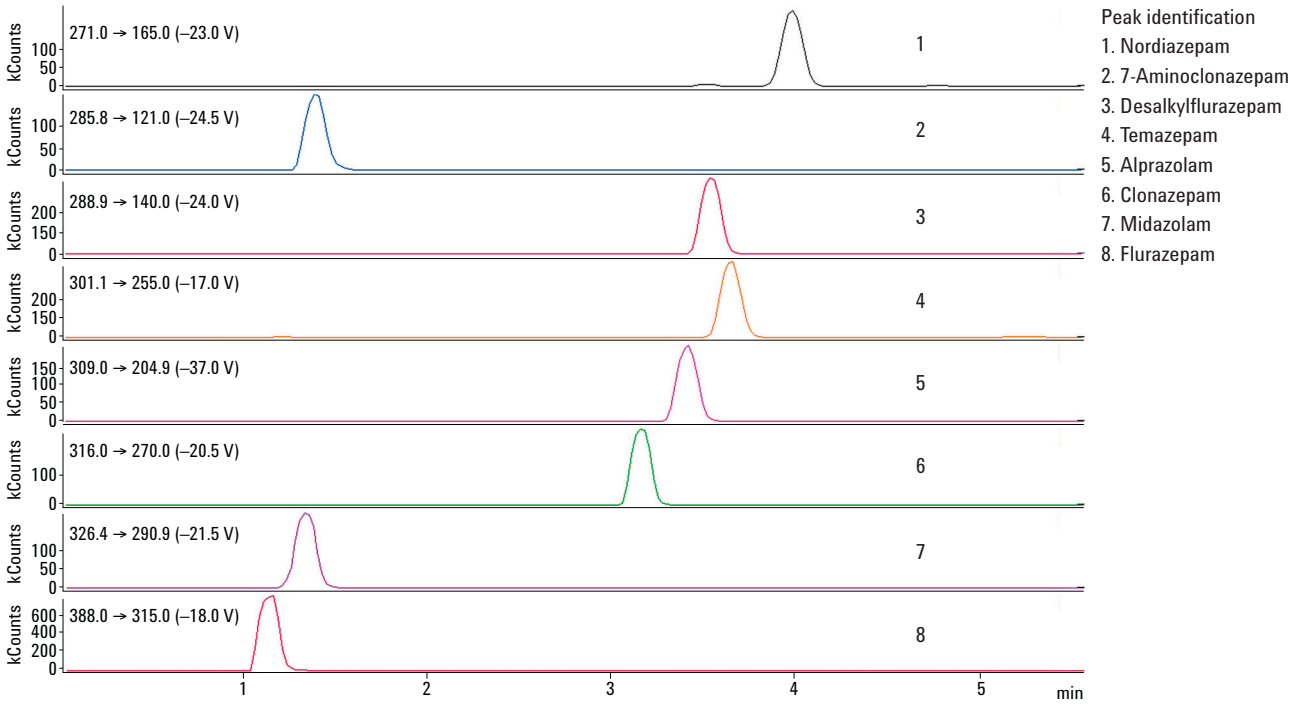

Figure 1a. Chromatograms of a 100 ng/mL urine extract (peaks 1-8).

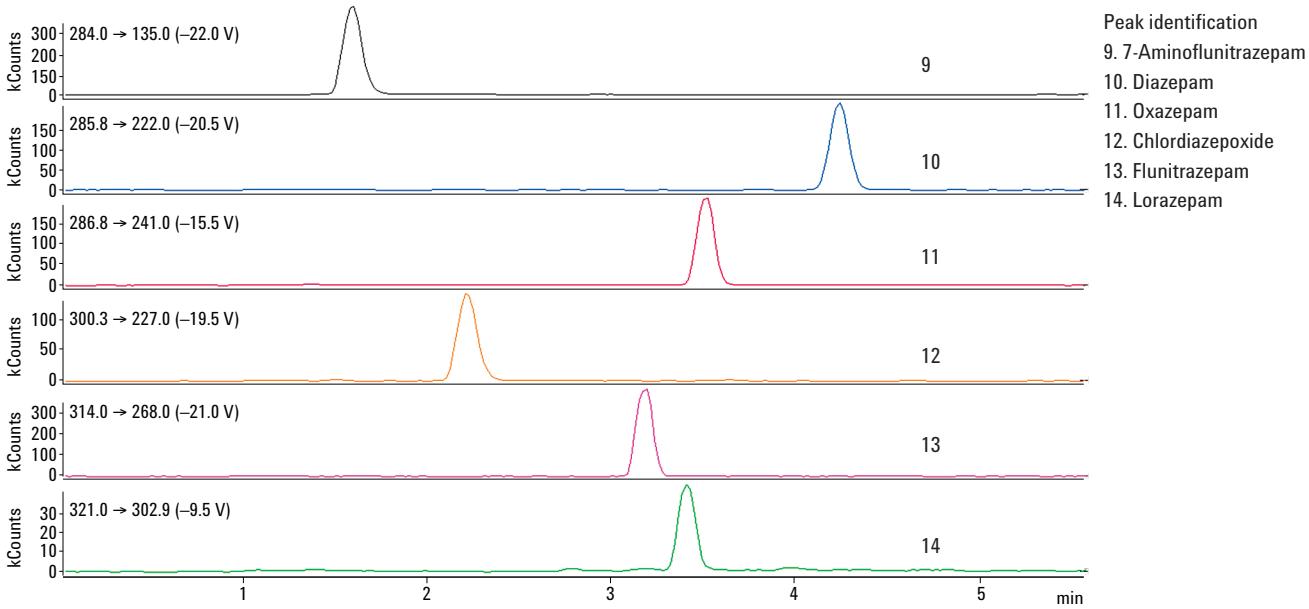

Figure 1b. Chromatograms of a 100 ng/mL urine extract (peaks 9-14).

Conclusions

Agilent Bond Elut Plexa PCX is a useful tool for high throughput SPE applications, which require analysis at low analyte levels, need validated reproducibility, and must be quickly implemented with minimal method development. Bond Elut Plexa PCX products meet these requirements.

With Bond Elut Plexa PCX, a generic drug extraction protocol can be applied to polar analytes with basic amino functional groups. Under acidic conditions, the charged analyte binds to the cation exchange groups of the sorbent. Polar interferences and proteins are washed away with an acidic, aqueous solution. A wash with 50% aqueous methanol is possible without a significant loss of analytes. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, ammoniated methanol was used to disrupt the cation exchange interaction, resulting in the elution of the benzodiazepines.

Flow rate is fast because Bond Elut Plexa PCX particles have much narrower particle size distribution with no fines to cause blockages, thus resulting in excellent tube-to-tube reproducibility. Bond Elut Plexa PCX tubes are, therefore, a useful tool for high throughput SPE applications, which require analysis at low analyte levels, validated reproducibility and quick implementation, with minimal method development.

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Agilent Technologies

Extraction of Basic Drugs from Plasma with Polymeric SPE

Application Note

Pharmaceuticals

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Introduction

Bioanalytical solid phase extraction (SPE) has been dominated by polymeric sorbents in recent years. The ease-of-use, good flow, and resistance to effects of drying relative to silica-based sorbents make polymeric sorbents an obvious choice for high volume, high throughput assays requiring quick validation and minimal method development.

Because the method validation process is time consuming and requires high quality data, SPE methods that are fast, yet produce good recoveries with high reproducibility, are desirable. To the extent that the SPE process is streamlined without compromising data integrity, method validation can be simplified and shortened. Bond Elut Plexa minimizes method development with simple and effective methods and improves analytical sensitivity and reproducibility with an advanced polymeric structure that minimizes binding of large biomolecules to the surface, with the end result of simplifying and streamlining the SPE process.

Agilent Technologies

Materials and Methods

SPE reagents and solutions

2% ammonium hydroxide	Add 20 µL concentrated ammonium hydroxide to 1 mL DI H_2O
Methanol	Reagent grade or better
5% methanol	Add 5 mL methanol to 95 mL DI H_2O
Bond Elut Plexa	10 mg 96 well plate (p/n A4969010)

SPE method

Sample	100 µL human plasma
Pretreat	Dilute with 300 µL 2% NH_4OH
Condition	1. 500 µL CH_3OH 2. 500 µL H_2O
Wash	500 µL 5% CH_3OH in H_2O
Elute	500 µL CH_3OH

All samples evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% formic acid: CH_3OH aq.

LC/MS performed – ESI, drying gas @ 400 °C, 30 psi

LC conditions

Mobile phase

A	0.1% Formic acid
B	Methanol

LC gradient program

Time (min)	%B
0:00	40
0:15	40
1:00	80
3:00	80
4:30	40

Column

Type	Pursuit XRs C18 3 µm, 50 × 2.0 mm (p/n A3001050X020)
Flow rate	0.2 mL/min

Results and Discussion

The procedure described provides a simple and effective SPE method for the extraction of basic or neutral drugs from human plasma. The Limit of Quantitation (LOQ) of the combined SPE and LC/MS/MS analysis was 1.0 ng/mL. The internal standard for the application was 50 ng/mL quetiapine.

Recoveries were calculated from a second order regression with RSD values based on a sampling of n = 6. Excellent recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to three orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.995 (n = 6). To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). Figure 1 shows the chromatograms of the extractions at 100 ng/mL. As shown in Table 1, the extractions produced reproducibly high recoveries.

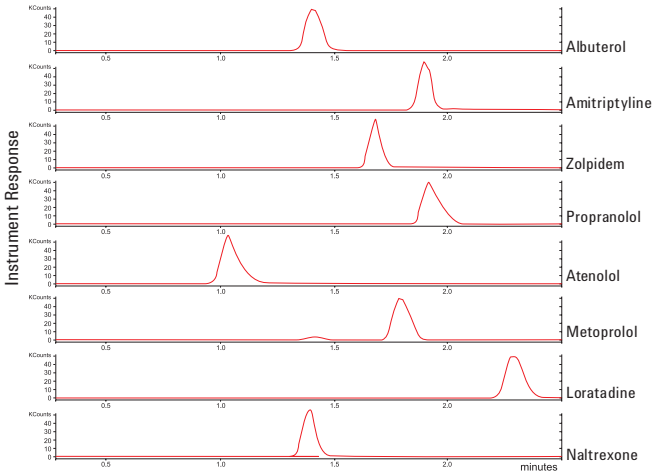

Figure 1. Chromatograms of a 100 ng/mL human plasma extract.

Table 1. High Recoveries of Basic Drugs with Bond Elut Plexa

Drug	log P	pKa	0.5 µg/mL %Recovery	%RSD	1.0 µg/mL %Recovery	%RSD
Albuterol	1.3	10.3	95	5	100	2
Amitriptyline	4.6	9.4	100	10	100	4
Zolpidem	3.9	6.2	100	8	103	2
Propranolol	3.6	9.5	102	6	101	6
Atenolol	1.3	9.6	97	4	101	4
Metoprolol	1.3	10.8	100	5	100	5
Loratadine	5.2	4.9	97	5	95	3
Naltrexone	1.8	9.2	103	11	100	4

Conclusions

Bond Elut Plexa is a useful tool for high-throughput SPE applications that require analysis at low analyte levels, need validated reproducibility, and must be quickly implemented with minimal method development. A single method for basic analytes covers a broad range of analyte polarites and delivers reproducibly high recoveries. Bond Elut Plexa is therefore highly recommended for bioanalytical work, including contract research.

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Agilent Technologies

Extraction of Polar Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

BioPharma

Authors

William Hudson and
Andrea Junker-Buchheit
Agilent Technologies, Inc.

Introduction

Basic pharmaceutical drugs are ideal for a cation exchange sorbent. Analytes are easily charged in an acidic solution and readily interact with the ion exchange function of the sorbent. Polar basic compounds can be problematic for reversed phase sorbents due to their poor hydrophobic interaction and water solubility.

Agilent Bond Elut Plexa PCX is a new addition to the Plexa family and uses a polymeric cation exchange technique. Plexa PCX uses a generic and simplified method to remove neutral and acidic interferences from the matrix and concentrate basic analytes, resulting in improved analytical performance and sensitivity in the quantification of basic compounds.

In addition, Plexa PCX offers faster and highly reproducible flow rates, resulting in excellent tube-to-tube and well-to-well performance. Plexa PCX significantly reduces ion suppression because its highly polar, hydroxylated surface is entirely amide-free. The particle exterior minimizes strong binding of proteins and phospholipids. Efficient removal of phospholipids from plasma is ensured. A simple generic method was developed for the extraction of polar basic drugs in human plasma.

Agilent Technologies

Materials and Methods

Table 1. SPE Reagents and Solutions

2% Phosphoric Acid	Add 20 µL of concentrated H_3PO_4 to 1 mL of DI water
Methanol	Reagent grade or better
2% Formic Acid	Add 20 µL of concentrated formic acid to 1 mL of DI water
Methanol:acetonitrile (1:1, v/v)	Add 1 mL of methanol to 1 mL of acetonitrile
5% NH_3 Methanol:acetonitrile (1:1, v/v)	Add 50 µL of concentrated ammonia to 1 mL of methanol:acetonitrile (1:1, v/v)
Bond Elut Plexa 10 mg 96 well plate (part number A4968010)	

Table 2. SPE Method

Sample Pre-treatment	100 µL human plasma. Dilute 1:3 with 2% H_3PO_4.
Condition	1. 500 µL CH_3OH 2. 500 µL DI H_2O
Load	Sample with the drug mixture at the flow rate of 1 mL/min
Wash 1	500 µL 2% formic acid
Wash 2	500 µL acetonitrile:methanol (1:1, v/v)
Elution	500 µL 5% NH_3 methanol:acetonitrile

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% aqueous formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 0.1% Formic acid
B: Methanol

Gradient:
t = 0 min 80% A : 0% B
t = 0-2 min 20% A : 80% B
t = 3.5-5 min 80% A : 20% B

Column: Agilent Pursuit C18 3 µm, 2.0 × 50 mm (part number A3051050X020)

MS Conditions

Transition ions and collision energy were:

Compound	Q1	Q3	CE
Albuterol	240.1	148.0	-23.5V
Lamotrignine	256.0	256.0	-5.0V
Atenolol	267.0	145.0	-34.0V
Sumatriptan	296.1	201.1	-14.0V

Capillary = 25 V, Dry gas temp = 400 °C, 30 psi, CID = Argon

Polarity: Positive

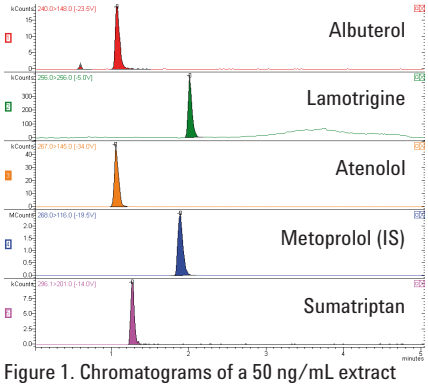

Figure 1. Chromatograms of a 50 ng/mL extract

This LC/MS method describes the quantitative determination of polar basic compounds in human plasma using Bond Elut Plexa PCX for SPE (Figure 1). The limit of detection (LOD) of the solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 2nd order regression with RSD values based on a sampling of n = 6.

Excellent recoveries were achieved, which demonstrated good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.999. To demonstrate reproducibility, samples were analyzed at two different concentrations (n = 6). As shown in Table 3, reproducibly high recoveries were obtained according to the generic standard protocol.

Table 3. Recoveries of polar basic compounds from human plasma

Analyte	log P	pKa	% Rec (500 ng/mL)	% RSD[2]	% Rec (1000 ng/mL)	% RSD[2]
Sumatriptan	0.96	9.6	95	5	97	4
Atenolol	1.30	9.6	94	3	91	2
Albuterol	1.30	10.3	95	5	100	7
Lamotrigine	1.50	5.7	92	3	97	4

[1]Recoveries calculated as % of signal intensity of an extracted sample compared to that calibration curve.
[2]RSD = standard deviation/average recovery x 100; n = 6.

Conclusions

With Agilent Bond Elut Plexa PCX, a generic drug extraction protocol from plasma can be applied to polar analytes with basic amino functional groups. Under acidic conditions, the charged analyte binds to the cation exchange groups of the sorbent (see Table 3 for pKa). Polar interferences and proteins are washed away with an acidic, aqueous solution. A neutral wash with relatively strong solvents, such as 50% methanol:acetonitrile, is possible without any loss of analyte. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, a mixture of organic solvents with ammonia is used to disrupt the cation exchange interaction, resulting in the elution of the basic drugs.

Flow rate all over the 96-well plate is fast because Plexa PCX particles have a much narrower particle size distribution with no fines to cause blockages, thus resulting in excellent well-to-well reproducibility. Automated 96-well technology is easily possible, which opens up new opportunities to maximize efficiency. Bond Elut Plexa PCX is therefore a useful tool for high throughput SPE applications, which require analysis at low analyte levels, validated reproducibility and quick implementation, with minimal method development. It is therefore highly recommended for bioanalytical work, including contract research.

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Agilent Technologies

Extraction of Non-Polar Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

BioPharma

Authors

William Hudson and
Andrea Junker-Buchheit
Agilent Technologies, Inc.

Introduction

Bioanalytical methods for pharmaceutical analysis require quick and easy method development and validation to reduce bottlenecks in drug development. Biological samples can be complicated to analyze due to proteins, peptides, salts, phospholipids and other endogenous compounds. Sample clean-up is necessary to remove these inferences without significant loss of the target analytes. Solid phase extraction utilizing simplified methodologies for routine analysis is the technique of choice.

Agilent Bond Elut Plexa PCX is a new addition to the Plexa family and uses a polymer cation exchange technique. Plexa PCX utilizes a generic and simplified method to remove neutral and acidic interferences from the matrix and concentrate basic analytes, resulting in improved analytical performance and sensitivity in the quantitation of basic compounds. In addition, faster and highly reproducible flow rates are the norm, resulting in excellent tube-to-tube and well-to-well performance. Plexa PCX significantly reduces ion suppression because its highly polar, hydroxylated surface is entirely amide-free. The particle exterior excludes proteins and avoids strong binding of phospholipids. Thus, efficient removal of phospholipids from plasma is ensured. A simple generic method was developed for the extraction and analysis of non-polar basic compounds in human plasma.

Agilent Technologies

Materials and Methods

Table 1. SPE Reagents and Solutions

2% Phosphoric Acid	Add 20 µL of concentrated H_3PO_4 to 1 mL of DI water
Methanol	Reagent grade or better
2% Formic Acid	Add 20 µL of concentrated formic acid to 1 mL of DI water
Methanol:acetonitrile (1:1, v/v)	Add 1 mL of methanol to 1 mL of acetonitrile
5% NH_3 Methanol:acetonitrile (1:1, v/v)	Add 50 µL of concentrated ammonia to 1 mL of methanol:acetonitrile (1:1, v/v)
Bond Elut Plexa 10 mg 96 well plate (part number A4968010)	

Table 2. SPE Method

Sample Pre-treatment	100 µL human plasma. Dilute 1:3 with 2% H_3PO_4.
Condition	1. 500 µL CH_3OH 2. 500 µL DI H_2O
Load	Sample with the drug mixture at the flow rate of 1 mL/min
Wash 1	500 µL 2% formic acid
Wash 2	500 µL acetonitrile:methanol (1:1, v/v)
Elution	500 µL 5% NH_3 methanol:acetonitrile

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% aqueous formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 0.1% Formic acid
B: Methanol

Gradient: t = 0 min 80% A : 20% B
t = 0-2 min 20% A : 80% B
t = 3.5-5 min 80% A : 20% B

Column: Agilent Pursuit C18, 2.0 × 50 mm, 3 µm (p/n A3051050X020)

MS Conditions

Transition ions and collision energy were:

Compound	Q1	Q3	CE
Ranitidine	315.0	176.0	-21.0V
Propranolol	260.1	116.0	-17.5V
Amitriptyline	278.1	233.0	-17.0V
Loratadine	383.1	337.0	-31.0V

Capillary = 25 V, Dry gas temp = 400 °C, 30 psi, CID = Argon

Polarity: Positive

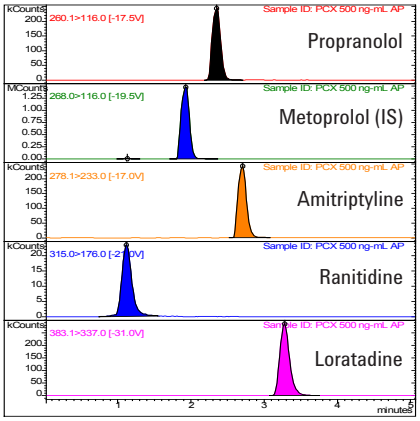

Figure 1. Chromatograms of a 50 ng/mL extract

This LC/MS method describes the quantitative determination of non-polar basic compounds in human plasma using Bond Elut Plexa PCX for SPE (Figure 1). The limit of detection (LOD) of the solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 2nd order regression with RSD values based on a sampling of n = 6. Excellent recoveries were achieved, demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.999.

To demonstrate reproducibility, samples were analyzed at two different concentrations (n = 6). As shown in Table 3, reproducibly high recoveries were obtained according to the generic standard protocol.

Table 3. Recoveries of non-polar basic compounds from human plasma

Analyte	log P	pKa	% Rec (500 ng/mL)	% RSD[2]	% Rec (1000 ng/mL)	% RSD[2]
Ranitidine	1.9	8.2	101	5	94	6
Propranolol	3.6	9.5	97	7	92	4
Amitriptyline	4.6	9.4	95	5	91	5
Loratadine	5.2	9.3	100	4	91	4

[1]Recoveries calculated as % of signal intensity of an extracted sample compared to that calibration curve.
[2]RSD = standard deviation/average recovery x 100; n = 6.

Conclusions

With Agilent Bond Elut Plexa PCX, it is possible to use a single method for the extraction of non-polar basic analytes from plasma that delivers reproducibly high recoveries. Under acidic conditions, the charged analyte binds to the cation-exchange groups of the sorbent (see Table 3 for pKa). Polar interferences and proteins are washed away with an acidic, aqueous solution. A neutral wash with relatively strong solvents, such as 50% methanol:acetonitrile, is possible without loss of analyte. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, a mixture of organic solvents with ammonia is used to disrupt the cation exchange interaction, resulting in the elution of the basic drugs.

Flow rate over the 96-well plate is fast because Plexa PCX particles have much smaller interstitial paths with no fines to cause blockages, resulting in high well-to-well reproducibility. Automated 96-well technology is convenient which opens new opportunities to maximize efficiency. Bond Elut Plexa PCX is therefore a useful tool for high-throughput SPE applications which require analysis at low analyte levels, need validated reproducibility, and that must be quickly implemented with minimal method development. It is highly recommended for bioanalytical work, including contract research.

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Agilent Technologies

Solid phase extraction of THC, THC-COOH and 11-OH-THC from whole blood

Application Note

Forensic Toxicology

Authors

Robert M. Sears, DFTCB

Forensic Toxicologist

South Carolina Law Enforcement Division

Introduction

Marijuana, one of the most widely abused drugs, after alcohol, is derived from *Cannabis Sativa*. There are more than 400 chemicals in the cannabis plant. The Δ^9-tetrahydrocannabinol (THC) is the most psychoactive of the various forms of THC. Marijuana is most often used in cigarette form, the user inhaling the marijuana smoke. THC and other forms of cannabinoids are lipid soluble and can enter body tissues rapidly. THC is rapidly metabolized to 11-hydroxy-Δ^9-tetrahydocannabinol (11-0H-THC), which is then converted to 11-nor-Δ^9 -THC-9-carboxylic acid (THC-COOH).

Detection of THC metabolites in urine, primarily THC-COOH, can indicate prior THC exposure but provides no indication of impairment. Testing for THC and its metabolites in blood can give a better indication of recent drug usage and can be of merit when testing for impairment.

This application note shows an effective SPE method for the extraction of THC and key metabolites from human blood and the GC/MS analysis of these compounds.

Instrumentation

GC with single quad mass spectrometer

Materials and Reagents

High flow Agilent Bond Elut Certify II SPE cartridge 200 mg (p/n 14113051). Bond Elut Certify II is a mix of C8 and a quanternary amine, a strong anion-exchange bonded silica. The two functionalities are effective in retaining the polar and non - polar functionalities of the THC compounds

5% phenyl substituted, low bleed GC/MS column 30 m x 0.25 mm x 0.25 µm

d3-THC, d3-11-0H-THC and d9-carboxy-THC from Cerilliant

Sample Preparation

Pipette 2 mL blood into a clean tube with ISTD equivalent to 10 - 11g/L (ng/mL)

Add 4 mL cold acetonitrile drop-wise while vortexing

Centrifuge sample 5 min minimum 2500 rpm

Transfer supernatant to a clean labeled tube.

Evaporate sample to about 3 mL with nitrogen at 35 - 40 °C

Add 7 mL 0.1 M sodium acetate buffer, pH 6.0 to each sample

SPE Method

Conditioning

Condition Certify cartridge with 2 mL MeOH. (All steps, except where noted, utilize low vacuum of approximately 2 - 5 in Hg).

Condition cartridge next with 2 mL 0.1 M sodium acetate buffer, pH 6.0 with 5% MeOH.

Cartridges should not be allowed to dry prior to sample addition.

Pour sample into column reservoir and draw sample through the column slowly, 1-2 mL/min.

Washes

2 mL sodium acetate buffer, pH 6.0.

Dry column under maximum vacuum for approximately 5 minutes.

Wash with 1 mL hexane

Elution

Elute THC with 2 mL 95:5 hexane:ethyl acetate.

Wash column with 5 mL 1:1 MeOH:DI water.

Dry column under maximum vacuum for approximately 5 minutes.

Wash with 1 mL hexanes.

Elute (in a separate tube) THC-COOH and 11-0H-THC with 2 mL 1% acetic acid in 75:25 hexane:ethyl acetate.

For best results, do not combine fractions. Run as two samples. Evaporate elution fractions under nitrogen no higher than 40 °C.

Derivitization

Add 500 µL elution solvent to sample, vortex and transfer to a clean, high recovery GC vial. Evaporate to dryness with nitrogen no higher than 40 °C

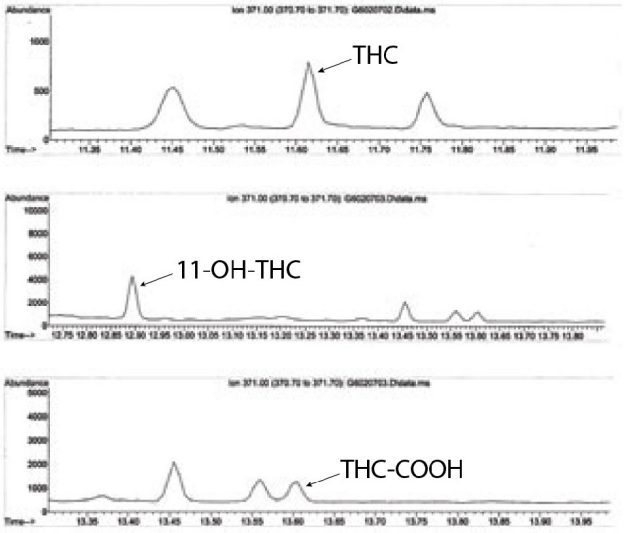
Add 35 µL BSTFA with 1% TMCS and 35 µL ethyl acetate. Overlay samples with nitrogen, cap and heat 20 minutes at 70 °C

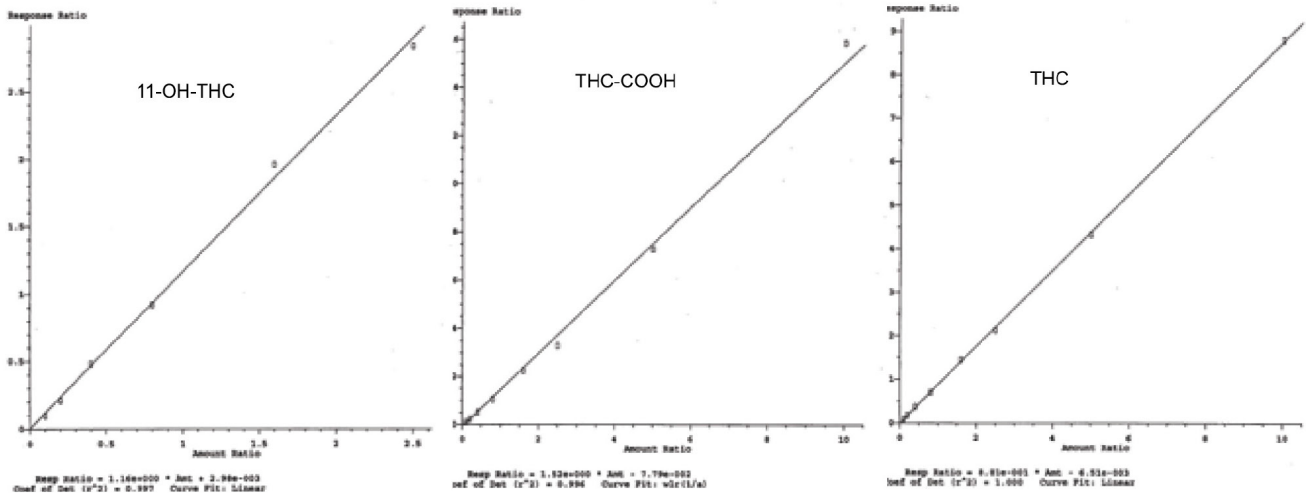
Conditions

Inlet temperature:	250 °C
Mode:	Pulsed pressure injection
Injection volume:	2 µL
Initial oven temperature:	120 °C Hold 1 min 15 °C/min to 300 °C Hold 0 30 °C/min to 310 °C Hold 5.57 min

Target Ions

d3-THC	374, 389, 346 (dwell time 50 ms)
THC	371, 386, 343
Linear range	1 - 50 µg/L
d3-11-0H-THC	374, 462, 477
11-0H-THC	371, 459, 474
Linear Range	1 - 50 µg/L
d9-THC-COOH	380, 479, 497
THC-COOH	371, 473, 488
Linear Range	1 - 100 µg/L





Conclusion

The above data shows the effective use of mixed-mode SPE with GC/MS detection for the extraction and quantification of THC and key metabolites from whole blood at low levels.

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The Measure of Confidence

Agilent Technologies

Pharmaceuticals in Whole Blood Using Mini-Extraction Sample Prep and Poroshell 120

Application Note

Small Molecule Pharmaceuticals and Generics

Author

Joan Stevens
Agilent Technologies, Inc.

Abstract

A convenient analytical method for determination of pharmaceuticals in various therapeutic categories in whole blood involves the addition of acetonitrile and salts to a small amount of blood. The mixture is shaken and centrifuged for extraction/partitioning, which removes water and proteins from the sample. An aliquot of the organic layer is cleaned by dispersive solid-phase extraction (SPE) employing SPE sorbent and salts, to remove endogenous matrix components. Analytes are then isolated from spiked samples with recoveries above 80% on average, and RSDs typically below 10% for a wide range of substances. This mini-extraction approach in whole blood delivers successful separation for a variety of pharmaceuticals, with limits of detection below 10 ng/mL. The method is quick, easy, inexpensive, and effective.

Agilent Technologies

Introduction

Determination of pharmaceuticals in biological matrixes is commonly employed in ADME (DMPK), clinical research and forensic analysis. The main techniques used for analysis are immunoassays, LC, and GC. Mass spectral chromatographic methods are the first choice for many applications, based on their flexibility, selectivity, sensitivity, qualitative, and quantitative capabilities. Analysis of pharmaceuticals in biological samples requires sample preparation that can range from simple protein precipitation (PPT) to more complex solid- phase extraction (SPE). There is a need in classic sample preparation for a method to determine multi-classes of pharmaceuticals in biological samples. Polymeric or mixed-mode SPE sorbents that can isolate acidic, neutral and basic drugs by hydrophobic and, or ion-exchange interactions address this need, but there is always room for sample preparation techniques that are rapid and inexpensive to implement.

Previously reported methods provide analysis of multi-residue pesticides in foods. They are known as QuEChERS (a quick, easy, cheap, effective, rugged, and safe sample preparation approach) [1]. The authors reported outstanding recoveries for a wide range of pesticide classes. Since its inception, there have been many reported articles employing QuEChERS for the analysis of a wide range of compounds including, but not specific to, antibiotics [2], toxins [3], contaminants [4], and pharmaceuticals [5].

In this note we describe an extension of the work presented by Plössl *et al*. [5] for the determination of pharmaceuticals in whole blood employing a modified mini-extraction procedure with LC/MS/MS analysis. The experiments presented in this application note used human whole blood containing either EDTA or citrate as an anticoagulant and were evaluated with both nonbuffered and buffered extraction salts used in the QuEChERS methodology, namely nonbuffered, AOAC 2007.01 and EN 15662. Modifications to the acetonitrile (extraction solvent) used in the first step (extraction/partitioning) were also evaluated. The experiments were performed using nine different pharmaceuticals (lidocaine, tramadol, amitriptyline, biperidene, oxazepam, lorazepam, chlorpromazine, diltiazem, and naloxone), with a broad range of hydrophobicity and dissociation constants (Table 1). Agilent Poroshell 120 is a good column for this analysis, in part because it has standard 2-µm frits and is more forgiving for more complex samples relative to a sub-2-µm column. Poroshell 120 has mass transfer such that it acts very much like a sub-2-µm particle LC column, without the high back pressure associated with a sub-2-µm column. The efficient mass transfer equates with faster analysis time and higher throughput with optimum resolution.

Table1. Characteristics of Pharmaceuticals Under Investigation

Compound	CAS number	Log P	pKa	Therapeutic use
Lidocaine	*137-58-6*	*2.4*	*8.01*	*Local anesthetic, antiarrhythmic*
Tramadol	*27203-92-5*	*2.5*	*9.41*	*Analgesic*
Amitriptyline	*50-48-6*	*4.92*	*9.4*	*Antidepressant*
Biperidene	*514-65-8*	*4.0*	*10.8*	*Anticholinergic*
Oxazepam	*604-75-1*	*2.23*	*1.7, 11.3*	*Antianxiety*
Lorazepam	*846-49-1*	*2.47*	*1.3, 11.5*	*Antidepressant*
Chlorpromazine	*50-53-3*	*5.18*	*9.3*	*Antipsychotic*
Diltiazem	*42399-41-7*	*3.63*	*7.7*	*Calcium channel blocker*
Naloxone	*465-65-6*	*1.45*	*7.9*	*Opioid receptor antagonist*
Nortriptyline (IS)	*72-69-5*	*5.65*	*9.7*	

Experimental

All reagents and solvents were HPLC analytical grade. The compounds were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

A stock solution of 1 M ammonium acetate (NH_4OAc) pH 5 was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The pH was adjusted to 5 with acetic acid monitored with a pH meter. The solution was stored at 4 °C. MeOH:H_2O (20:80) containing 5 mM NH_4OAc pH 5 was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL 1 M NH_4OAc, pH 5 stock solution. The 5 mM NH_4OAc in ACN was prepared by adding 5 mL 1 M NH_4OAc, pH 5 stock solution to 1 L ACN, sonicating well.

Standard and internal standard solutions (2.0 mg/mL) were made in MeOH and stored at –20 °C. A QC spiking solution of 5.0 µg/mL was made fresh daily in 1:1 ACN:H_2O (0.1% FA). A 0.5 and 5.0 µg/mL standard solution in 1:1 ACN:H_2O (0.1% FA) was made for the preparation of calibration curves in the matrix blank extract with appropriate dilution. Five µg/mL nortriptyline in 1:1 ACN:H_2O (0.1% FA) was used as the internal standard (IS).

Equipment

- Agilent 1260 Infinity LC with Diode Array
- Agilent 6460 Triple Quadrupole LC/MS system with Electrospray Ionization
- Agilent Bond Elut QuEChERS AOAC Extraction kit (p/n 5982-6755)
- Bond Elut QuEChERS EN Extraction kit (p/n 5982-6650)
- Bond Elut QuEChERS Non-Buffered Extraction kit (p/n 5982-6550)
- Bond Elut QuEChERS AOAC Dispersive SPE kit for General Fruits and Vegetables (p/n 5982-5022)
- Bond Elut QuEChERS EN Dispersive SPE kit for General Fruits and Vegetables (p/n 5982-5021)
- Bond Elut Ceramic Homogenizers (p/n 5982-9312)
- Sorvall ST 16R Centrifuge (Thermo IEC, MA, USA)
- Micro centrifuge 5415D Eppendorf (Brinkman Instruments, Westbury, NY, USA)
- Geno Grinder 2010 (SPEX CertiPrep, Inc., Metuchen, NJ, USA)
- DVX 2500 Multi-Tube Vortexer (VWR International, West Chester, PA, USA)

HPLC conditions

Column	Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
Flow rate	0.4 mL/min
Column temperature	30 °C
Injection	10 µL
Mobile phase	A. 5 mM Ammonium acetate, pH 5 in 20:80 MeOH:water
	B. 5 mM Ammonium acetate, pH 5 in ACN
Needle wash	1:1:1:1 ACN:MeOH:IPA:H_2O (0.2% FA)
Gradient	20 to 75% B over 5.5 min

MS conditions

ESI	Positive mode
GT	300 °C
GF	7 L/min
Nebulizer	40 psi
SGT	400 °C
SFG	12 L/min
Capillary	3500 V
NV	500 V

Other MS conditions relating to the analytes are listed in Table 2.

Table 2. Instrument Acquisition Data Used for the Analysis of Nine Drugs by LC/MS/MS

Compound	MRM channels (m/z)	Fragmentor (V)	CE (V)	RT (min)	Delta RT
Lidocaine	1) 235.18 > 86.1 2) 235.18 > 58.1	97	11 35	1.37	0.4
Tramadol	1) 264.2 > 58.1 2) 264.2 > 246.1	97	15 3	1.20	0.4
Amitriptyline	1) 278.2 > 117 2) 278.2 > 105	112	19 19	4.25	0.4
Biperidene	1) 312.23 > 98.1 2) 312.23 > 55.1	123	19 60	4.23	0.7
Oxazepam	1) 287.06 > 240.9 2) 287.06 > 268.9	112	19 7	3.99	0.4
Lorazepam	1) 321.02 > 274.9 2) 321.02 > 302.9	113	15 7	4.09	0.4
Chlorpromazine	1) 319.11 > 86.1 2) 319.11 > 58.1	112	15 43	4.63	0.4
Diltiazem	1) 415.17 > 177.9 2) 415.17 > 149.9	128	19 43	3.73	0.4
Naloxone	1) 328.16 > 310 2) 328.16 > 212	123	15 39	0.82	0.4
Nortriptyline (IS)	1) 264.18 > 233 2) 264.18 > 91	97	7 19	4.17	0.4

General procedure

1. Add 1 mL of whole blood to a centrifuge tube.
2. Spike with appropriate volume from a concentrated stock mixture to yield 25, 50, and 100 ng/mL of the component mix.
3. Add 20 µL of IS stock solution, yield 100 ng/mL (nortriptyline), and two ceramic homogenizers.
4. Vortex.
5. Add 2 mL acetonitrile solution (with or without acid), see Table 3.
6. Vortex.
7. Add a premixed amount (see Table 3) of the extraction salts and vigorously shake.
8. Centrifuge at 5,000 rpm for 5 minutes.
9. Transfer 1 mL of the extract into a d-SPE tube (2 mL centrifuge tube) containing 50 mg PSA and 150 mg $MgSO_4$ for matrix cleanup.
10. Vortex for 1 minute.
11. Centrifuge at 18,000 rpm for 3 minutes.
12. Transfer 200 µL aliquot of the extract into a LC vial containing 800 µL of water.
13. Vortex and analyze.

The entire series of experiments are in Table 3. A matrix-matched calibration curve from 10 to 250 ng/mL was employed to determine recovery.

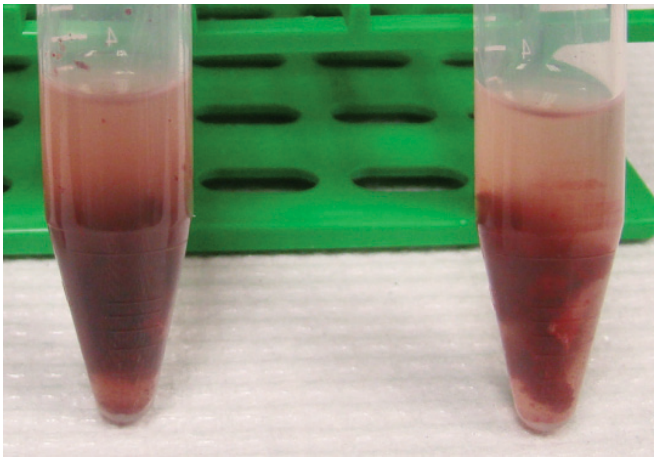
Table 3. Series of Experimental Conditions Investigated

Sample (1 mL)	Extraction solvent	Extraction salts (mg)	d-SPE	Observation
WB	ACN	none	none	Sample: solid mass
WB	ACN, 1% AA	none	none	Sample: solid mass
WB	ACN, 0.4% FA	none	none	Sample: loose particles
WB	ACN, 0.4% FA	Nonbuffered, 500	none	Dark extract
WB	ACN, 0.4% FA	AOAC, 500	none	Clear extract
WB	ACN, 0.4% FA	EN, 650	none	Dark extract
WB	ACN, 0.4% FA	Nonbuffered, 500	50 mg PSA, 150 mg $MgSO_4$	Clear extract
WB	ACN, 0.4% FA	AOAC, 500	50 mg PSA, 150 mg $MgSO_4$	Clear extract
WB	ACN, 0.4% FA	EN, 650	25 mg PSA, 150 mg $MgSO_4$	Clear extract
WB	ACN, 0.4% FA	EN, 650	50 mg PSA, 150 mg $MgSO_4$	Clear extract

WB = whole blood; ACN = acetonitrile, AA = acetic acid; FA = formic acid,
PSA = primary secondary amine, AOAC = $MgSO_4$ and NaAcetate, EN= $MgSO_4$ and citrate buffers,
Nonbuffered = $MgSO_4$ and sodium chloride

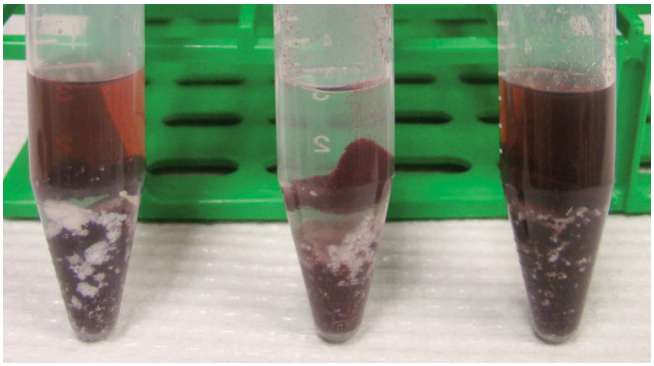
Results and Discussion

The experiments showed that the use of ACN (0.4% FA) as the extraction solvent offered a better lysed sample versus the other extraction solvents where the sample became a solid mass (see Figures 1 and 2). The AOAC-buffered salts yielded the cleanest extract, visually (Figure 3) and was chosen for use with the d-SPE containing 50 mg PSA, 150 mg $MgSO_4$ for the extraction of the pharmaceuticals in whole blood (Figure 4). It is worth noting that the d-SPE step does in fact offer substantial cleanup for all the extracted samples, especially from the EN and nonbuffered salt extracts, which initially showed a significant amount of red blood cells remaining in the extract.



A B

Figure 1. Addition of ACN (A) or ACN (1% AA) (B) to the whole blood, common solvents used in the QuEChERS method.



A B C

Figure 3. After the addition of ACN (0.4% FA), QuEChERS salts, shake and vortex.
A EN method citrate salts,
B AOAC method acetate salts
C Nonbuffered method chloride salts

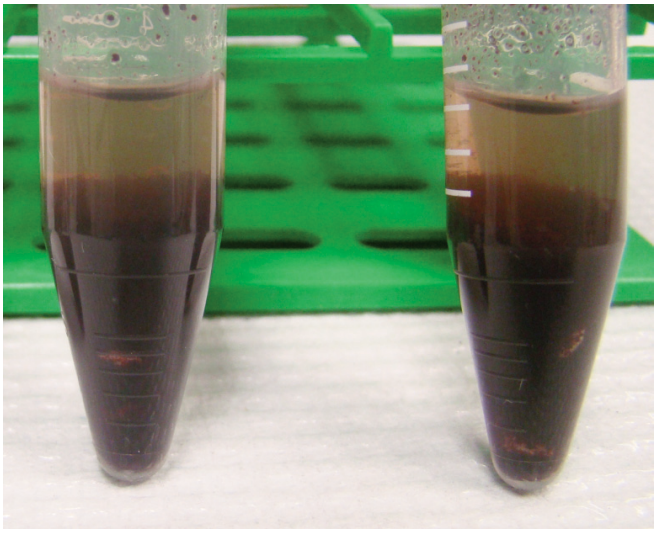
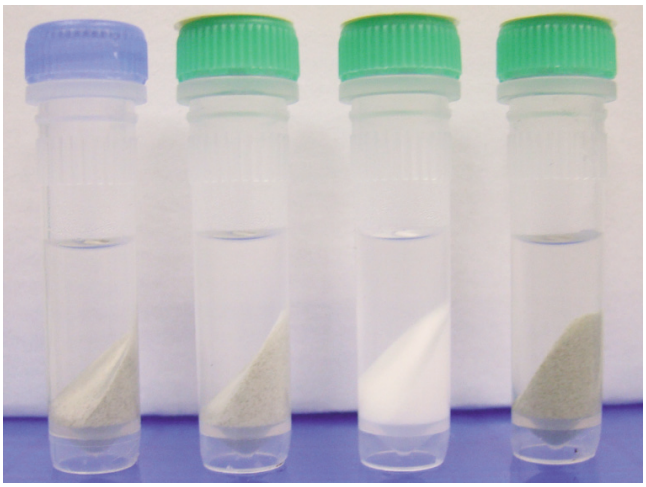


Figure 2. Addition of ACN (0.4% FA) to the whole blood.



A B C D

Figure 4. Extract after the addition of d-SPE clean-up containing 150 mg $MgSO_4$ and varying amounts of PSA.
A EN citrate salts and EN d-SPE 25 mg PSA
B EN citrate salts and AOAC d-SPE 50 mg PSA
C AOAC acetate salts and AOAC d-SPE 50 mg PSA
D Nonbuffered chloride salts and AOAC d-SPE 50 mg PSA

The mini-extraction procedure is based on the principles behind the QuEChERS methodology. It provides an alternative to more complicated techniques, offering a simplified sample preparation technique for complex matrixes such as whole blood. This type of sample preparation technique is extremely complementary to the powerful selectivity of LC/MS/MS multiple reaction monitoring (MRM) mode. The whole blood extract appeared to be clean and free of impurities, indicating that the blank whole blood extract did not contribute any interferences with target compounds. Figure 5 shows the chromatogram of a 10 ng/mL spiked whole blood sample after the mini-extraction procedure.

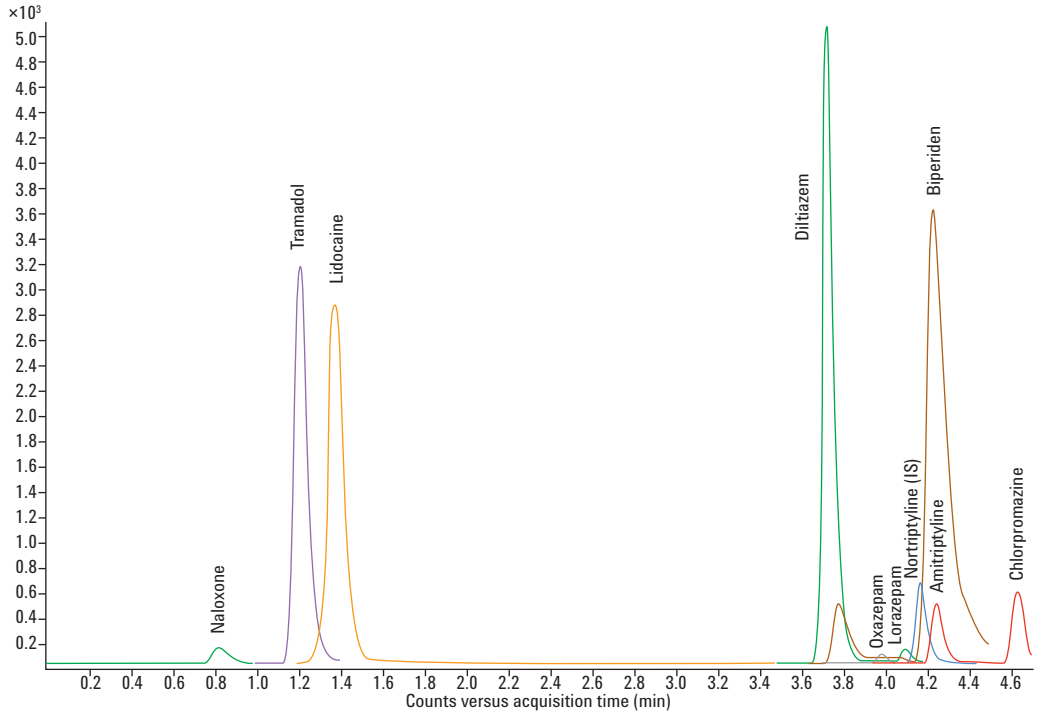


Figure 5. LC/MS/MS chromatograms of 10 ng/mL spiked whole blood sample after mini-extraction; AOAC acetate salts and AOAC d-SPE with 50 mg PSA and 150 mg $MgSO_4$.

Linearity and limit of quantification (LOQ)

The linear calibration range evaluated for all the pharmaceuticals was 10 to 250 ng/mL. Matrix blank extracts were prepared for the calibration curves. Calibration curves, spiked in the matrix blank extracts, were made at 10, 25, 50, 100, and 250 ng/mL. The nortriptyline (IS) was used at 100 ng/mL. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). Figure 6 is an example of the regression equation and correlation coefficient (R^2) observed for the nine pharmaceuticals from whole blood.

Recovery and reproducibility

The recovery and reproducibility were evaluated by spiking standards in the whole blood sample at 25, 50, and 100 ng/mL. These QC samples were quantitated against the matrix-spiked calibration curve. The analysis was performed in six replicates at each level. The recovery and reproducibility (RSD) data are shown in Table 4.

It can be seen from the results that all the pharmaceuticals give acceptable recoveries (average > 90%) and precision (average of 7% RSD). We have observed a small degree of matrix interference at low levels of concentration, < 25 ng/mL, with the pharmaceuticals investigated.

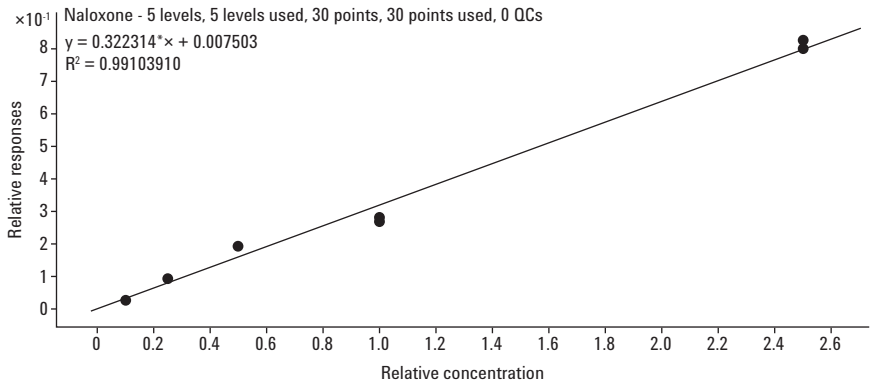


Figure 6. Example of the results from the mini-extraction, standard linear curve for naloxone from 10-250 ng/mL, R^2 = 0.991.

Table 4. Recovery and RSD for the Extracted Drug Compounds

	25 ng/mL Spiked		50 ng/mL Spiked		100 ng/mL Spiked	
Compound	**Recovery**	**RSD**	**Recovery**	**RSD**	**Recovery**	**RSD**
Lidocaine	81.6	35.3	98.7	15.7	100	11.8
Tramadol	97.2	18.6	105	3.0	104	8.2
Amitriptyline	85	13.6	104	2.1	104	8.2
Biperidene	75.5	14.8	97	4.5	99	8.2
Oxazepam	60.4	17.3	77.0	9.2	78	8.6
Lorazepam	68.4	17.0	81.9	6.8	81.8	8.6
Chlorpromazine	75	14.1	110	10.3	105	6.3
Diltiazem	63.7	15.8	88.1	2.7	91.7	8.3
Naloxone	68	12.1	80.6	9.0	75.5	7.7

Conclusion

Mini-extraction sample preparation is a simple, easy, and cost-effective approach, requiring minimal sample preparation expertise, solvent, or equipment. The mini-extraction approach for the extraction of pharmaceuticals from whole blood offers an alternative sample preparation technique that can be easily implemented by laboratories. Although matrix interference was observed at low-level concentrations for some of the pharmaceuticals, improvements in the method can include a dispersive SPE that contains additional solid phase extraction materials to facilitate matrix removal. The Poroshell 120 EC-C18 column offers different selectivity and exceptional peak shape across the wide range of pharmaceuticals used in this study.

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Fractionation of Acidic, Basic, and Neutral Drugs from Urine with an SPE Mixed Mode Strong Anion Exchange Polymeric Resin (Agilent SampliQ-SAX)

Application Note

Forensic Toxicology

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Abstract

A polymeric mixed mode strong anion exchange resin, Agilent SampliQ SAX, was evaluated in terms of its ability to extract acidic, basic, and neutral drugs from urine. A solid phase extraction (SPE) procedure was applied whereby acidic drugs were eluted in the acidic fraction while the neutral and basic drugs were eluted in the neutral fraction. High recoveries (79.6–109%) and high reproducibilities (RSDs ranged from 0.06–1.12%) were obtained. The calibration curves were linear for nortriptyline, ketoprofen, and naproxen ($R^2 > 0.999$) in the 0 to 10 µg/mL concentration range. Secobarbital was, however, linear from 0–25 µg/mL. The limits of detection were 0.21 µg/mL, 0.04 µg/mL, 0.03 µg/mL, and 0.02 µg/mL. Quantification values were 0.81 µg/mL, 0.12 µg/mL, 1.04 µg/mL, and 2.74 µg/mL for secobarbital, nortriptyline, ketoprofen, and naproxen, respectively.

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Introduction

Forensic toxicology laboratories commonly employ SPE prior to chromatographic analysis. In bioanalysis, urine and blood present a very complex matrix for the determination of drugs and their metabolites. Therefore, sample preparation for cleanup and preconcentration of analytes to improve their detection is very important.

The fractionation of different classes of drugs (acidic, basic, and neutral) in biological fluids has been reported in a number of studies [1-4]. Protein precipitation, liquid-liquid extraction (LLE), and SPE are among the most popular sample preparation techniques. The versatility of SPE allows for the preferential use of the technique, as it is not only employed for class fractionation but also for trace enrichment and purification. Commercial sorbents, such as chemically-modified silica gel and polymer and graphitized or porous carbon, are available [5]. These offer interactions based on normal phase, reversed phase, ion exchange, and mixed mode ion exchange (combination of reversed phase and ion exchange) mechanisms. The mixed mode sorbents have proven to give cleaner extracts and better separations than standard reversed phase or ion exchange sorbents because they take advantage of both the ion exchange and hydrophobic interactions [6].

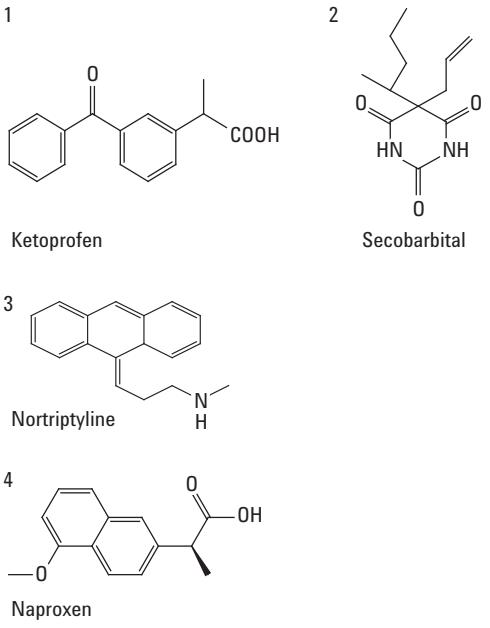

Figure 1. Structures of the drugs used: ketoprofen and naproxen (acidic), secobarbital (neutral), and nortriptyline (basic).

In this application note, a method based on SPE was developed for the fractionation of acidic, basic, and neutral drugs in urine with Agilent SampliQ-SAX, a mixed mode strong anion exchange polymer. The resin is a tertiary amine-modified divinylbenzene polymer that exhibits both anion exchange and reversed phase behavior. In addition, it provides excellent reproducibility and enables a simple extraction protocol. Specific drugs (Figure 1) were used as representatives of the three classes of drugs (acidic, basic, and neutral).

Experimental

Chemicals

Ketoprofen, secobarbital, nortriptyline, and naproxen were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Phosphoric acid, formic acid, and potassium hydroxide were purchased from Merck Chemicals (Gauteng, South Africa) while the HPLC-grade methanol (MeOH) was from Merck KGaA (Darmstadt, Germany) and potassium dihydrogen phosphate was purchased from Saarchem Analytical (Krugersdorp, South Africa).

The mobile phase was prepared with ultrapure water (18.2 MΩcm) from a MilliQ system by Millipore (Milford, Mass, USA) and filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size). The stock solutions (1,000 ppm) of the four analytes were prepared in methanol and kept at 4 °C while the working solutions were prepared daily by diluting the stock solutions, to appropriate concentrations, in methanol. The urine was from a donor who was not using or has not used the drugs investigated in this study.

Instrumental

The analysis was performed on an Agilent 1200 Series High Performance LC System (HPLC) equipped with a binary pump and a diode array detector (DAD) set at λ = 222 nm. Separation of the compounds was achieved on an Agilent ZORBAX Eclipse Plus C18 column 4.6 mm × 75 mm, 3.5 µm, (p/n 959933-902). The data was processed by Agilent ChemStation HPLC-2D software. The SPE cartridges were Agilent SampliQ SAX, 1 mL/30 mg containing a polymeric anion exchanger with 25–35 µm average particle size (p/n 5982-3313). A Jenway 3510 pH meter (London, UK) was employed for pH adjustments.

Sample pretreatment: SPE procedure

A 5 mL amount of urine was hydrolyzed with 1 M KOH at 60 °C for 15 minutes and diluted with 10 mM CH_3COONa (1:1 v/v). The pH was then adjusted to 2 with phosphoric acid. The urine sample, unspiked (blank) and spiked with drugs, was loaded onto the SampliQ SAX cartridges using the conditions shown in Figure 2. This SPE procedure was optimized for maximum recovery and reproducibility of experimental results.

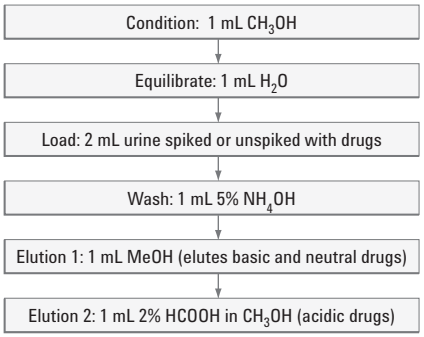

Figure 2. SPE procedure for acidic, basic, and neutral drugs using Agilent SampliQ SAX.

Separation and analysis

The HPLC conditions are shown in Table 1.

Table 1. HPLC conditions.

Column	Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 75 mm, 3.5 µm
Flow rate	1.5 mL/min
Column temperature	30 °C
Injection volume	5 µL
Mobile phase	Isocratic elution
	A: 55% CH_3OH
	B: 45% 25 mM KH_2PO_4, pH 7
Run time	8 min
Post time	1 min
Detection:	DAD at 222 nm

Results and Discussion

Separation

The chromatogram of a standard solution containing secobarbital, nortriptyline, naproxen, and ketoprofen is shown in Figure 3. A baseline separation of these standards was obtained. Under the conditions used in Table 1, all analytes were eluted within 9 minutes.

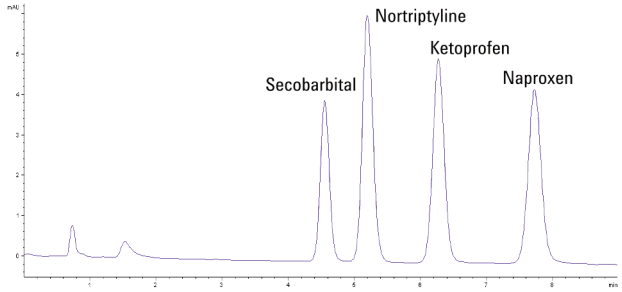

Figure 3. Chromatogram of a standard solution (5 µL) containing 1) secobarbital (10 µg/mL), 2) nortriptyline (5 µg/mL), 3) ketoprofen (5 µg/mL), and 4) naproxen (2 µg/mL).

Analysis of standard solutions

Calibration curves were constructed in the concentration range 0.0–8.0 µg/mL for nortriptyline and ketoprofen, 0–7 µg/mL for naproxen, and 0–35 µg/mL for secobarbital as shown in Figure 4. Good linearity was obtained with $R^2 > 0.999$. Due to the diverse polarities and pH characteristics of the compounds tested, each one was monitored at its maximum absorption wavelength (Table 2). It can be seen that secobarbital gave a weak response compared to the other drugs in the standard mix. Therefore, in later experiments, the concentration of this drug was adjusted upward to provide a stronger signal.

Table 2. Chemical and physical characteristics of the studied drugs.

Drug	Classification	Log P	pKa	λmax (nm)
Secobarbital	Neutral	1.97	7.90	222
Nortriptyline	Basic	4.28	9.70	242
Ketoprofen	Acidic	0.97	5.94	258
Naproxen	Acidic	3.18	4.53	230

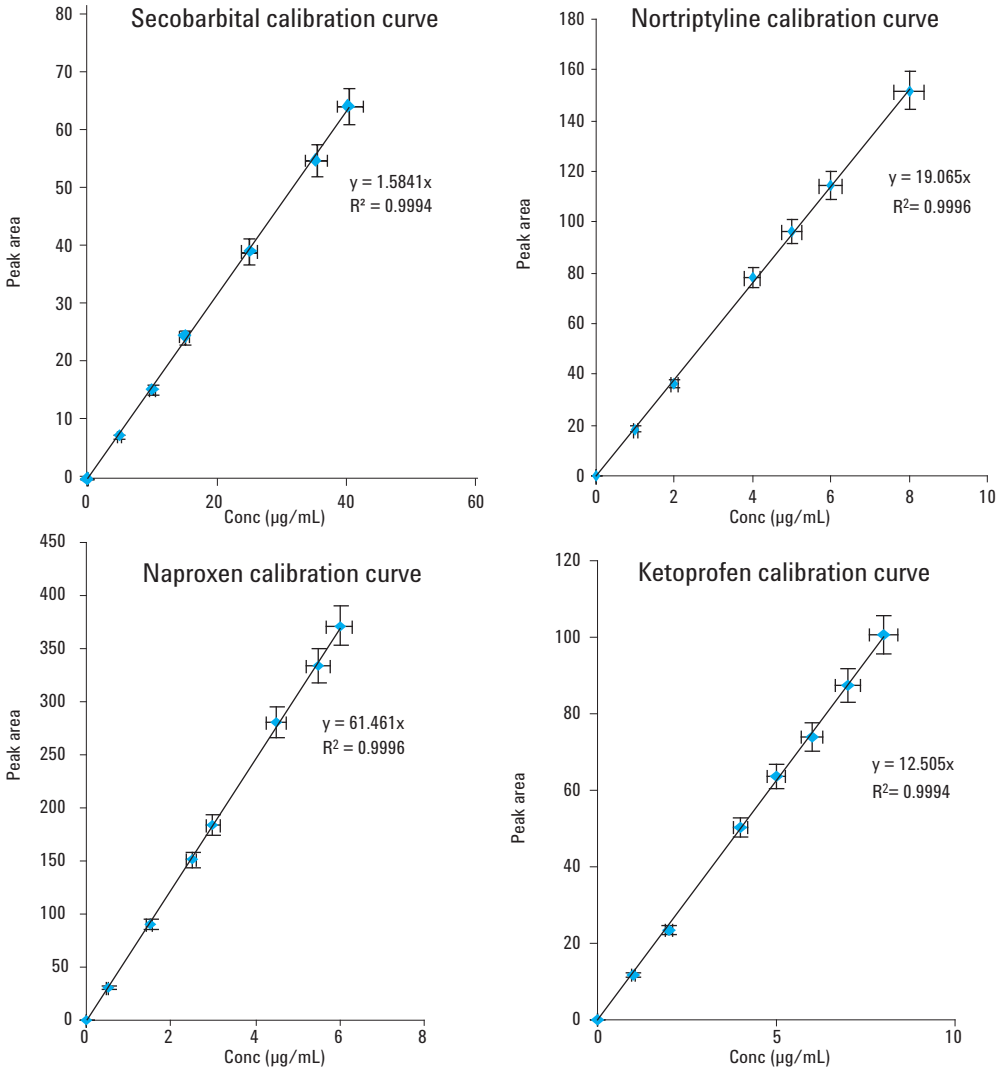

Figure 4. Calibration curves (at the λ max of each) for secobarbital, nortriptyline, ketoprofen, and naproxen.

SPE procedure for drugs in urine

Agilent SampliQ SAX, a polymeric mixed-mode, strong anion exchange SPE sorbent was successfully used to simultaneously extract acidic, basic, and neutral drugs from a spiked urine sample using the SPE procedure depicted in Figure 2. First, blank urine containing no drugs was treated using the SPE method. Figure 5A, for the basic and neutral elution conditions, showed nothing eluting in the region of the acidic and neutral drugs in the standards. Figure 5B, which depicts a blank urine using the acidic elution conditions, also showed nothing eluting in the region of the acidic drugs. For the spiked urine samples, the neutral (secobarbital) and basic (nortriptyline) drugs were eluted in the neutral fraction (Figure 6A) because they were retained through hydrophobic interactions. The acidic drugs (naproxen and ketoprofen), retained by the strong anion exchange functionalities of the sorbent, eluted separately in the acidic fraction as shown in Figure 6B. A small amount (< 10%) of the neutral/basic drugs were also found in the acidic fraction. A larger volume of methanol in the prior step could have been used to improve extraction efficiency.

Recovery and reproducibility

The recoveries were calculated by comparing the peak area of the analyte concentration in the spiked urine after SPE to that of the standard solution at the same concentration level. To demonstrate reproducibility, the samples were analyzed at three different concentration levels (n = 6). As indicated in Table 3, high recoveries (> 85%) were obtained except for the lowest level of secobarbital. The RSD values were excellent and ranged from 0.06 to 1.12 for n = 6 runs.

Table 3. Recoveries for secobarbital, nortriptyline, ketoprofen, and naproxen from urine.

Drug	SPE fraction	Spike level (µg/mL)	Recovery	%RSD` (n = 6)
Secobarbital	Neutral	5	79.63	1.12
		10	92.70	0.78
		15	86.47	0.31
		1	91.20	1.04
Nortriptyline	Neutral	2.5	86.48	0.47
		4	85.32	0.12
		1	109.34	0.54
Ketoprofen	Acidic	2.5	99.18	0.58
		4	85.88	0.16
		0.5	106.97	0.18
Naproxen	Acidic	1	87.66	0.63
		2.5	83.41	0.06

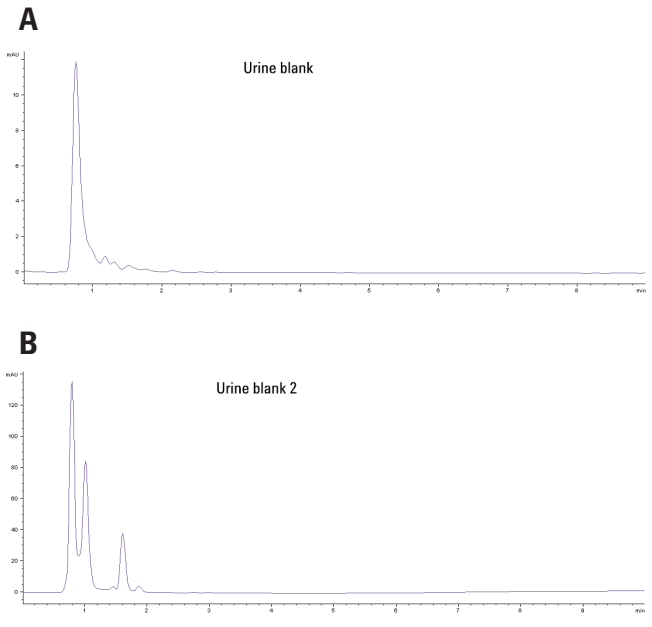

Figure 5A. Chromatograms of blank urine extract by SPE method using Elution 1 for neutral and basic compounds (see Figure 2).

Figure 5B. Chromatograms of blank urine extract by SPE method using Elution 2 for acidic compounds (see Figure 2).

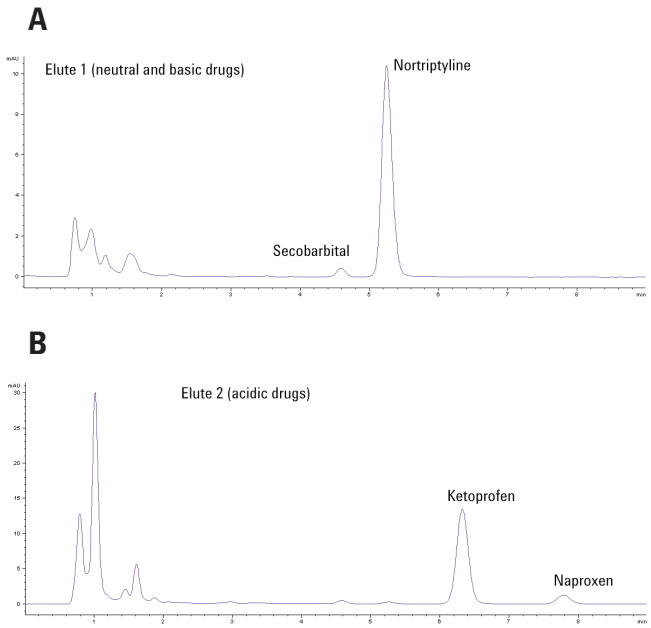

Figure 6A. Chromatograms of neutral and basic drugs (Elution 1) extracted from spiked urine: 1) secobarbital and 2) nortriptyline.

Figure 6B. Chromatograms of acidic drugs (Elution 2) extracted from spiked urine: 3) ketoprofen and 4) naproxen.

Linearity, limits of detection and limits of quantification

After SPE was performed, the method linearity as well as the limits of detection (LOD) and limits of quantification (LOQ) were determined. Linearity was determined in the concentration range 0–25 µg/mL for secobarbital and 0–10 µg/mL for nortriptyline, ketoprofen, and naproxen. Secobarbital and nortriptyline were linear in the chosen range while ketoprofen and naproxen showed linearity from 0–4.5 µg/mL. Table 4 shows the linearity equations and correlation coefficients.

Table 4. Linearity after SPE.

	Concentration range (0 – 8 µg/mL)	
Drugs	**Linear equation**	**Correlation coefficient (R^2)**
Secobarbital	$y = 1.3325x$	$R^2 = 0.9993$
Nortriptyline	$y = 17.595x$	$R^2 = 0.9991$
Ketoprofen	$y = -1.2748x^2 + 17.896x$	$R^2 = 0.9991$
Naproxen	$y = -1.9003x^2 + 33.527x$	$R^2 = 0.9993$

The LOD and LOQ results are shown in Table 5. Equations 1 and 2 were used to calculate LOD and LOQ, where Syx = standard error of the regression line and b = gradient.

Table 5. LOD and LOQ for the analytes.

Drug	LOD (µg/mL)	LOQ (µg/mL)
Secobarbital	0.21	0.81
Nortriptyline	0.04	0.12
Ketoprofen	0.03	1.04
Naproxen	0.03	2.74

$$LOD = \frac{3.3 \times Syx}{b} \qquad \text{Equation 1}$$

$$LOQ = \frac{10.0 \times Syx}{b} \qquad \text{Equation 2}$$

Conclusion

The SPE method employed is relatively simpler than other protocols reported in literature. With the strong anionic exchange polymer, Agilent SampliQ SAX, the simultaneous extractions of acidic drugs, a basic drug, and a neutral drug from a spiked urine matrix were obtained. High recoveries and good reproducibilities were achieved for extraction of all drugs from the urine.

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Fractionation of Acidic, Basic, and Neutral Drugs from Plasma with an SPE Mixed Mode Strong Cation Exchange Polymeric Resin (Agilent SampliQ SCX)

Application Note

Forensic Toxicology

Authors

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Abstract

A method for the simultaneous extraction of drugs (amphetamine, acetaminophen, p-toluamide, m-toluidine, and phenobarbital) from spiked human plasma sample was developed. This procedure employed solid phase extraction with a mixed mode strong cation exchange resin, Agilent SampliQ SCX. The chromatographic separation and analysis of solid phase extraction extracts were achieved using 30% methanol and 70% potassium dihydrogen phosphate as a mobile phase under isocratic conditions on an Agilent ZORBAX Eclipse Plus C18 4.6 mm × 75 mm, 3.5 µm column at 1 mL/min flow rate and a diode array detector (DAD) set at 210 nm. High and reproducible recoveries (> 80%) for all the analytes were obtained. The limits of detection (LOD) and quantification (LOQ) were 0.39 and 0.71 µg/mL for acetaminophen, 0.84 and 1.87 µg/mL for amphetamine, 0.36 and 1.06 µg/mL for m-toluidine, 0.66 and 0.70 µg/mL for p-toluamide, as well as 0.80 and 1.89 µg/mL for phenobarbital, respectively.

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Introduction

Sample preparation prior to chromatographic analysis presents a major challenge for the determination of drugs and their metabolites in complex matrices, such as biological fluids (for example, blood, plasma, urine, serum, saliva). Drugs normally exhibit a diverse polarity with acidic, basic, or neutral functionalities depending on the pH of the matrix. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) have traditionally been employed for the extraction of drugs, their metabolites, and endogenous compounds from plasma [1-2]. Their quantification at a low concentration has proven to be a difficulty in pharmaceutical and forensic toxicology analyses. SPE is, however, the preferred method as it is not only employed for class fractionation, but it is also for trace enrichment and purification. Available commercial sorbents, such as chemically modified silica gel, polymer, and graphitized or porous carbon, are used [3]. These offer separations based on normal phase, reversed phase, ion exchange, and mixed mode ion exchange (combination of reversed phase and ion exchange) sorbents. The mixed mode sorbents have proven to give cleaner extracts and better separations than standard reversed phase or ion exchange sorbents as they take advantage of both the ion exchange and hydrophobic interactions [4,5]. For the extraction of compounds with a wide variety of polarity, polymeric sorbents have proven to be superior to other sorbents (for example, alkylated silica) and are, therefore, the choice of sorbent for this study [6–8].

In this application note, a method employing solid phase extraction was developed for the fractionation of acidic, basic, and neutral drugs from plasma with Agilent SampliQ SCX, a mixed mode strong cation exchange polymer. The resin is a sulfonic acid modified divinyl benzene polymer that exhibits both cation exchange and reversed phase behavior. In addition, it provides excellent reproducibility and enables a simple extraction protocol. Specific drugs (Figure 1) have been used as representatives of the three classes of drugs, for example, p-toluamide (acidic), amphetamine (basic), and acetaminophen (neutral).

Experimental

Chemicals

Acetaminophen, phenobarbital, p-toluamide, amphetamine, m-toluidine, and ranitidine (IS) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Phosphoric acid, formic acid, and potassium hydroxide were purchased from Merck Chemicals (Gauteng, South Africa) while the HPLC grade methanol was from Merck KGaA (Darmstadt, Germany),

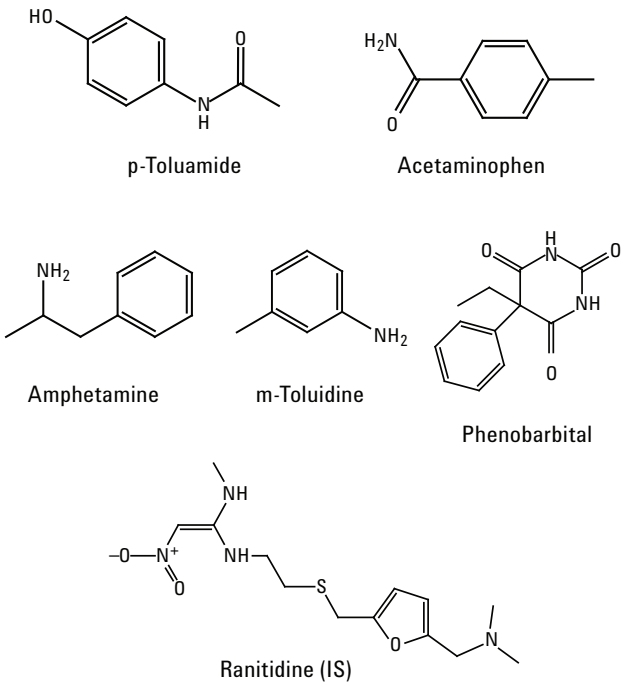

Figure 1. Structures of drugs used in present study.

dipotassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Saarchem Analytical (Krugersdorp, South Africa).

The mobile phase was prepared with ultrapure water (18.2 M Ωcm) from a MilliQ system by Millipore (Milford, Mass, USA) and filtered through a Whatman membrane filter (47 mm diameter and 0.2 µm pore size). The stock solutions of the drugs and the internal standard were prepared in methanol (1,000 µg/mL) and kept at 4 °C while the working solutions were prepared daily by diluting the stock solutions, to appropriate concentrations, also in methanol. The plasma (ECZ HQ Donation 5497780, O^+) was from SANBS (Port Elizabeth, South Africa).

Instrumental

The analysis was performed on an Agilent 1200 Series LC composed of a binary pump and a DAD set at λ = 210 nm. Separation of the compounds was achieved on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm, p/n 959933-902, Agilent Technologies, Santa Clara, CA). The data was processed by Agilent LC 2D ChemStation software. The SPE cartridges were Agilent SampliQ SCX, 1 mL/30 mg, p/n 5982-3213, a polymeric strong cation exchanger with 25–35 µm average particle sizes. A Jenway 3510 pH meter (London, UK) was employed for pH adjustments.

Sample pretreatment: SPE procedure

The plasma sample (1 mL) was hydrolyzed with 1% formic acid (3 mL) for 30 minutes. The sample, spiked with drugs, was then loaded onto the SampliQ SCX cartridges as described in Figure 2. An internal standard, 50 µL of the ranitidine stock solution, was added to each SPE fraction. A blank plasma sample (1 mL) was carried through the procedure also.

Separation and analysis

Table 1 shows the reversed-phase chromatographic conditions. All drugs were separated within 5 minutes.

Table 1. HPLC conditions.

Column:	Agilent ZORBAX Eclipse Plus C18 4.6 mm × 75 mm, 3.5 µm
Flow rate:	1.5 mL/min
Column temperature:	35 °C
Injection volume:	5 µL
Mobile phase:	Isocratic elution: 30/70 A/B A: CH_3OH B: 25 mM K_2HPO_4, pH 7

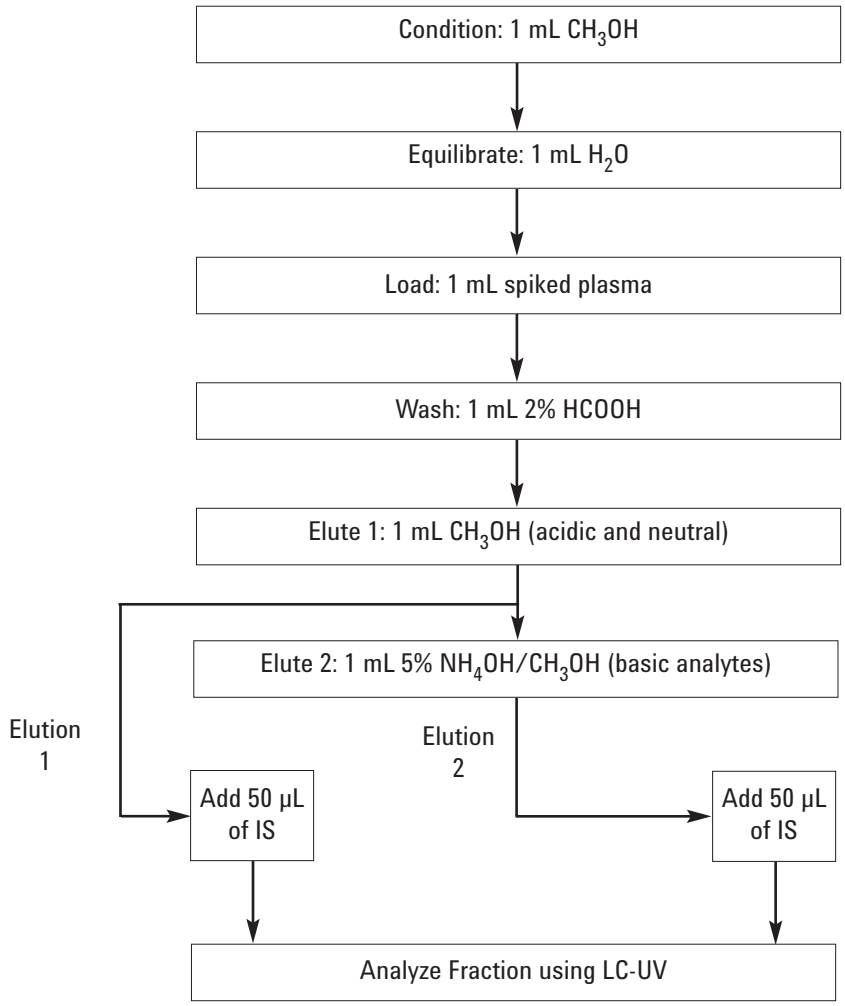

Figure 2. SPE procedure.

Results and Discussion

Separation

The standard mixture of the analytes was separated with the set chromatographic conditions (Table 1), and the chromatogram is reported in Figure 3.

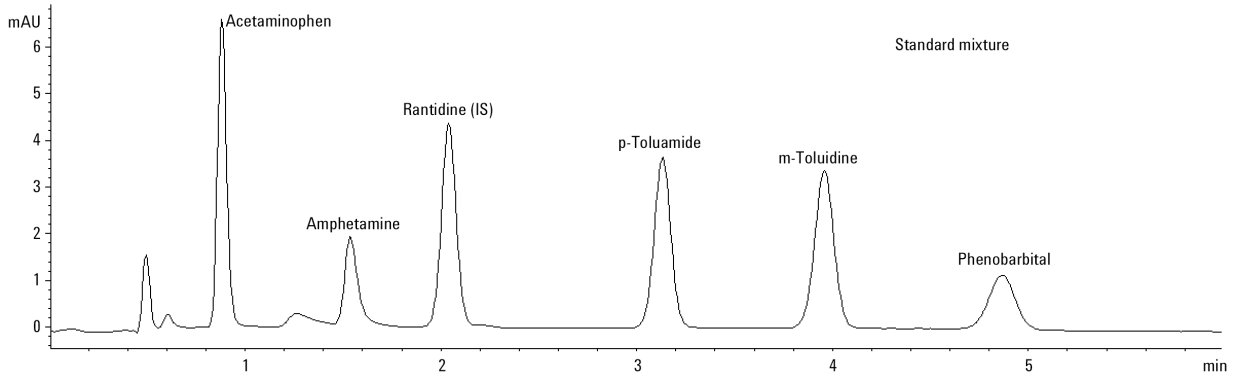

Figure 3. Chromatogram of a standard mixture (7 µg/mL): (1) acetaminophen, (2) amphetamine, (3) p-toluamide, (4) m-toluidine, and (5) phenobarbital ranitidine (IS).

Analysis of standard solutions

Calibration curves were processed at 0–10 µg/mL concentration ranges for all the analytes. They were linear with coefficient of regression (R^2) greater than 0.999 as shown in Figure 4.

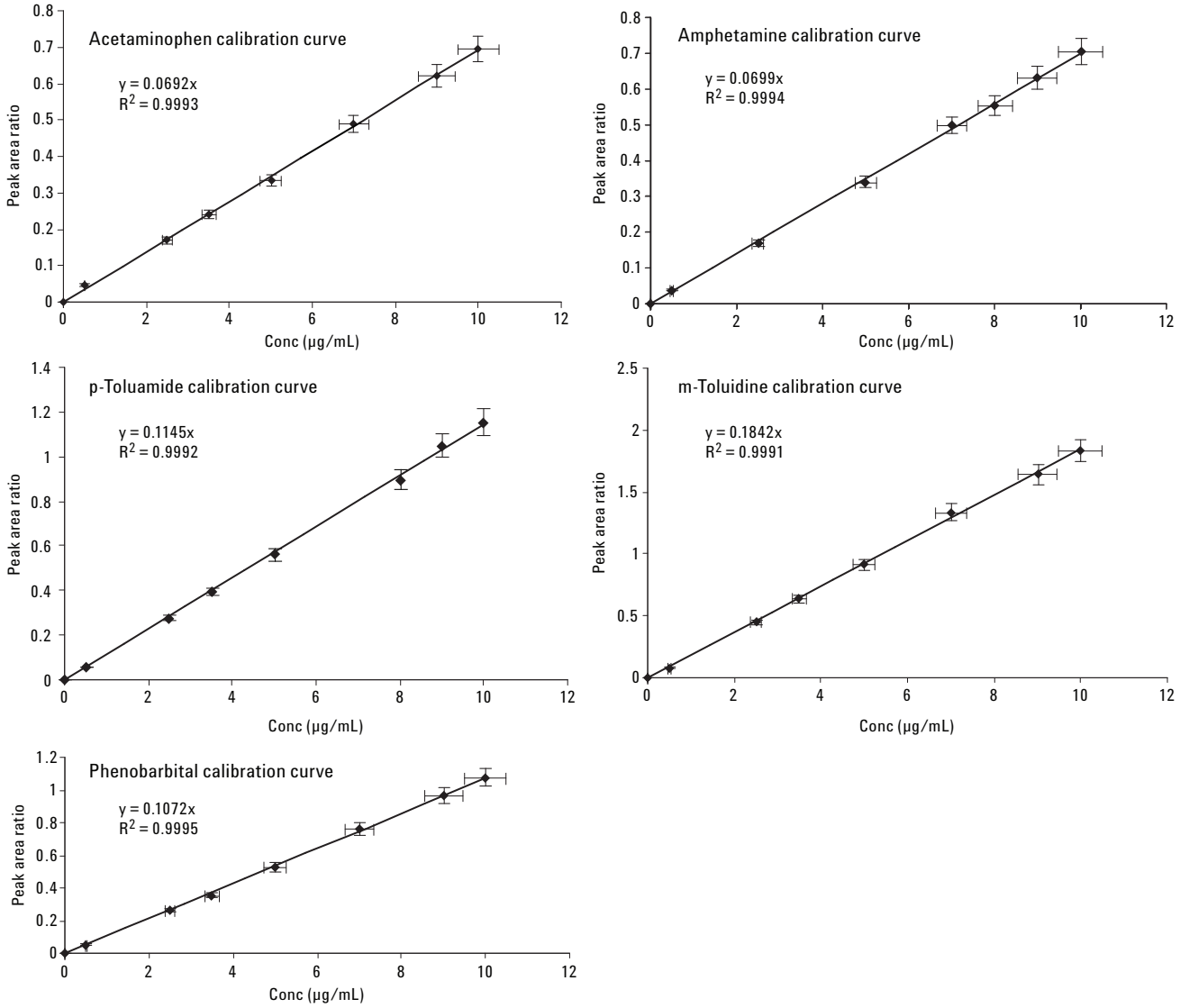

Figure 4. Calibration curves of the standards.

SPE procedure

A mixed mode strong cation exchange sorbent was used to extract basic drugs from the acidic and neutral drugs in plasma. The acidic and the neutral drugs, which exhibit a similar retention mechanism to that of the undissociated acidic compounds, were adsorbed in the hydrophobic portion of the sorbent and eluted in the neutral fraction while the basic drugs were retained by the cation exchange interactions with the sorbent and eluted in the ammoniated fraction.

First, blank urine containing no drugs was treated using the SPE method. Figure 5A, for the acidic and neutral elution conditions (Elution 1), showed nothing eluting in the region of the acidic and neutral drugs in the standards. Figure 5B, which depicts a blank urine using the basic elution conditions (Elution 2), also showed nothing eluting in the region of the acidic drugs. For the spiked urine samples, the neutral and acidic drugs were eluted in the neutral fraction (Figure 6A, Elution 1) because they were retained through hydrophobic interactions while the basic drugs, retained by the strong anion exchange functionalities of the sorbent, eluted separately in the basic fraction as shown in Figure 6B, Elution 2. A small amount (< 10%) of the neutral/acidic drugs were also found in the basic fraction. A larger volume of methanol in the prior step could have been used to improve extraction efficiency.

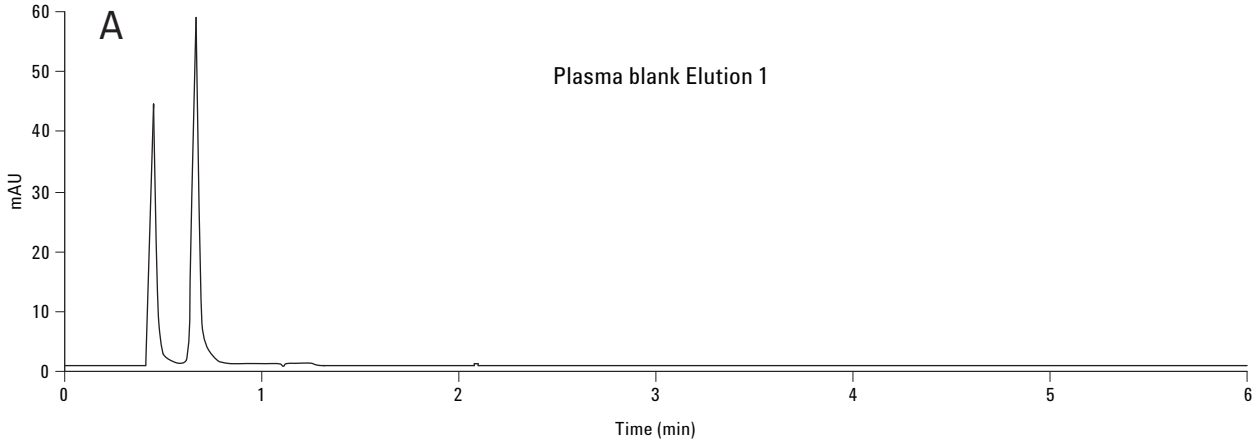

Figure 5A. Chromatograms of blank plasma sample from Elution 1.

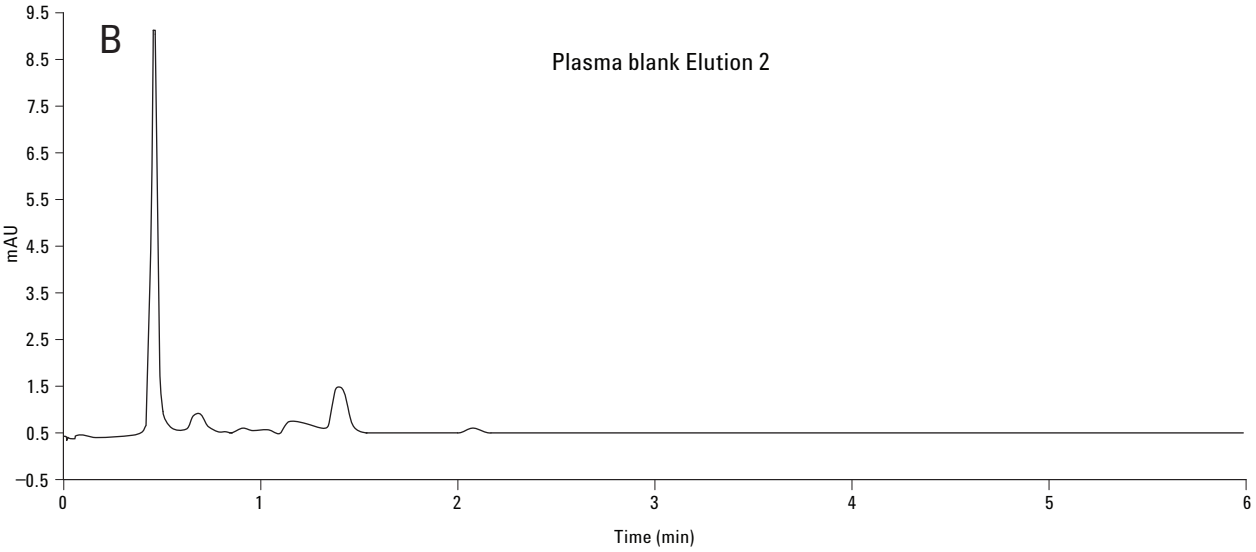

Figure 5B. Chromatograms of blank plasma sample from Elution 2.

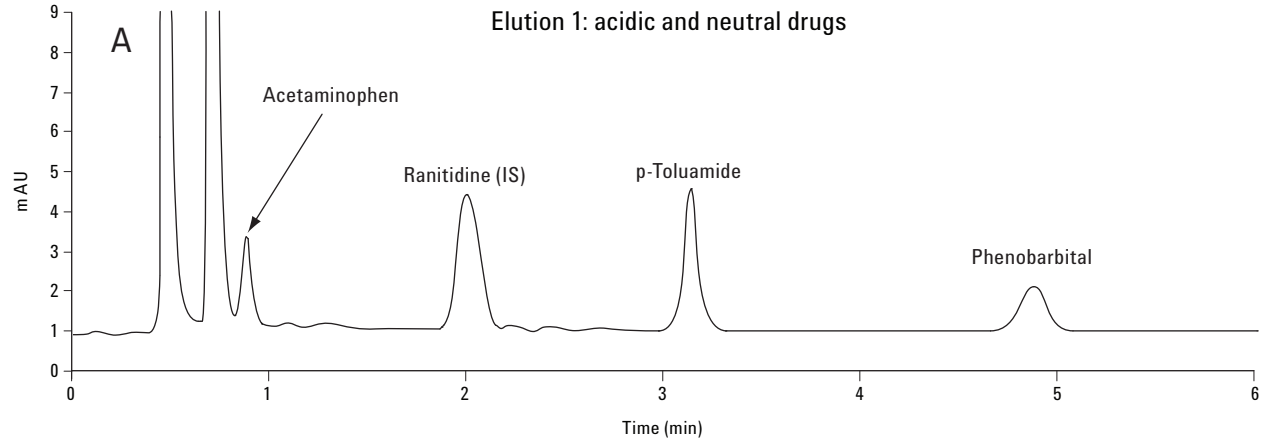

Figure 6A. Chromatograms of drugs in plasma, elution 1: 1. acetaminophen, 2. ranitidine (IS), 3. p-toluamide, and 4. phenobarbital.

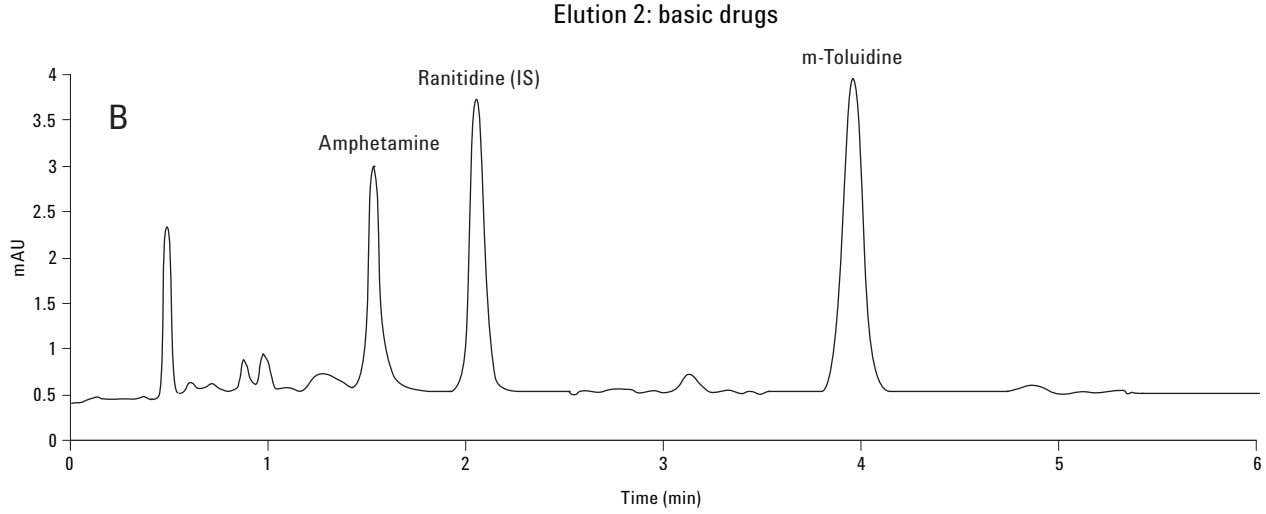

Figure 6B. Chromatograms of drugs in plasma, elution 2: 1. amphetamine, 2. ranitidine (IS), and 3. m-toluidine.

Linearity, limits of detection, and limits of quantification

The blank plasma was spiked with the analytes at five different concentrations and subjected to the SPE procedure in Figure 2. The internal standard, 50 µL, was added. Each spiked plasma sample was prepared in triplicate. The analyte/IS peak area ratios were plotted against the corresponding concentrations. All the analytes were linear in the chosen concentration range (0–8 µg/mL) with $R^2 > 0.999$ as shown in Table 2.

Table 2. Linearity of the method employing SPE.

	Concentration range (0–8 µg/mL)	
Drugs	**Linear equation equation (y)**	**Correlation coefficient (R^2)**
Acetaminophen	0.099x	0.9999
Amphetamine	0.0853x	0.9991
p-Toluidine	0.138x	0.9990
m-Toluamide	0.1804x	0.9991
Phenobarbital	0.1526x	0.9991

Equations 1 and 2 were used to calculate LOD and LOQ, where Syx is the standard error of the regression line and b is the gradient.

Equation 1

$$LOD = \frac{3.3 \times Syx}{b} \quad (1)$$

Equation 2

$$LOQ = \frac{10.0 \times Syx}{b} \quad (2)$$

Table 3 shows the LOD and LOQ determined for each analyte.

Table 3. LOD and LOQ for the analytes.

Drug	LOD (µg/mL)	LOQ (µg/mL)
Acetaminophen	0.39	0.85
Amphetamine	0.71	1.87
p-Toluamide	0.66	0.70
m-Toluidine	0.35	1.06
Phenobarbital	0.82	1.89

Recovery and reproducibility studies

Spiked plasma samples at three concentration levels: 0.5, 2.5, and 5 µg/mL, corresponding to the lower, middle, and upper limit of the linearity curve, were subjected to SPE cleanup. The analyte chromatographic peak areas obtained were compared to those obtained from standard solutions at the same concentration, and the percentage extraction yield was calculated. To demonstrate reproducibility, the samples were analyzed at the three mentioned concentration levels (n = 6). Good recoveries were obtained as indicated in Table 4.

Conclusions

The SPE procedure was successfully carried out on Agilent SampliQ SCX sorbents for the simultaneous extraction of acidic, basic, and neutral drugs from plasma with high and reproducible recoveries (> 80%). The LOD ranged from 0.39 to 0.84 µg/mL for the drugs studied. The LOQ ranged from 0.71 to 1.89 µg/mL. This method can be applied to compounds that exhibit a diverse polarity and acidic, basic, or neutral functionalities.

Table 4. Recoveries for the drugs in the study.

		Spike level (µg/mL)					
		0.5		2.5		5	
Drugs	Class	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
Acetaminophen	Neutral	81.45	0.41	95.08	0.48	92.62	0.22
Amphetamine	Basic	83.16	0.62	88.83	1.09	86.64	0.28
p-Toluamide	Acidic	85.88	0.65	96.86	0.18	94.40	0.07
m-Toluidine	Basic	81.97	0.51	89.16	0.33	94.86	0.10
Phenobarbital	Acidic	85.49	0.39	90.13	0.25	80.48	0.21

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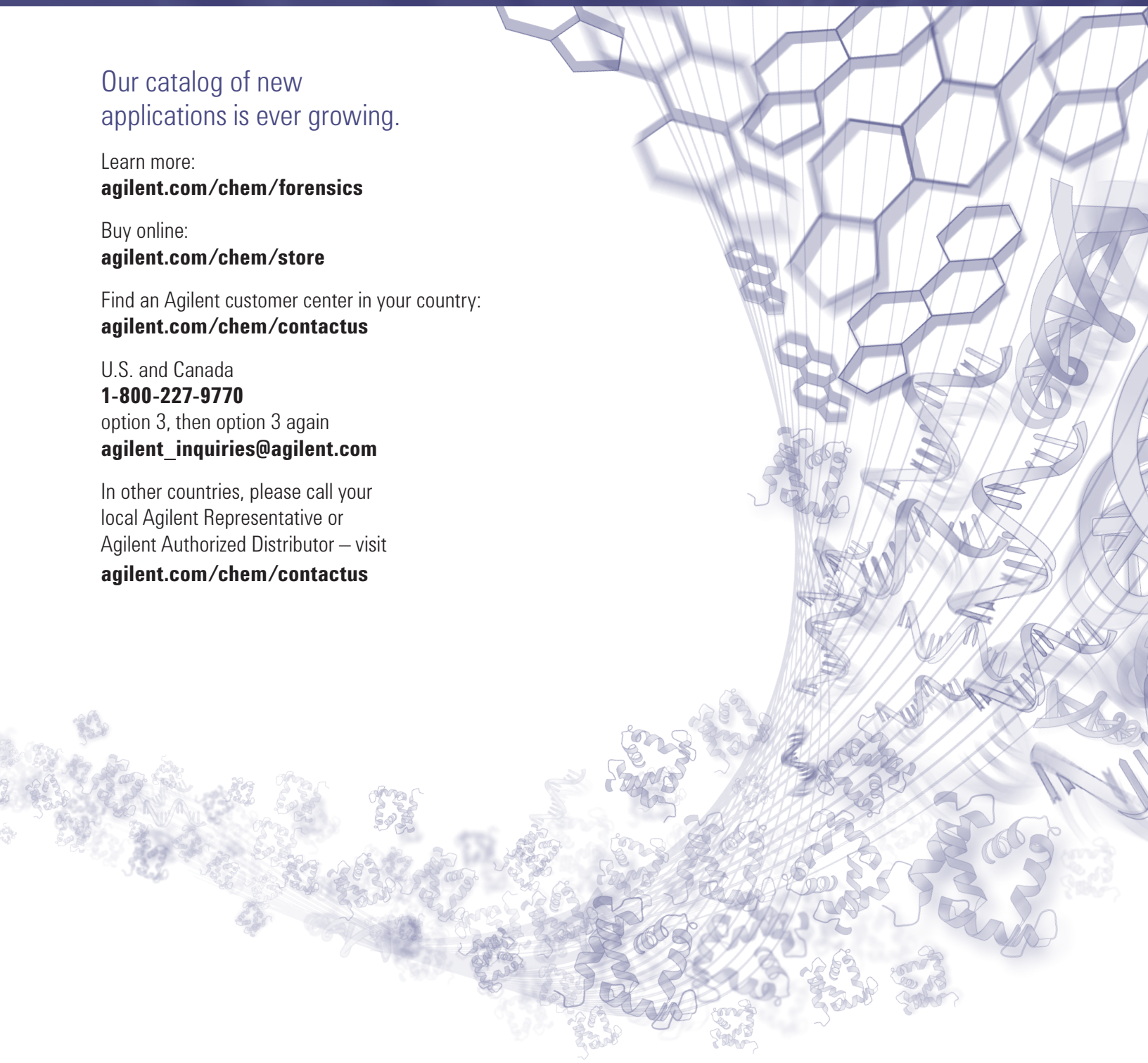

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