Agilent Sample Prep, LC Columns, GC Columns, and Supplies Applications Compendium

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Below is a listing of Agilent Sample Preparation, Columns, and Supplies that support the Criminalistics applications. Click on the product name to get more information.

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### ORAL FLUIDS

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- **GC Columns:**
  - DB-1ms
  - DB-17ms
- **Agilent Inert Flow Path Solutions:**
  - Ultra Inert liners
  - Inert Inlet weldments
  - Ultra Inert gold seals
  - Inert MS source
  - Capillary Flow Technology purged union
  - UtiMetal Plus Flexible Metal ferrules
  - Gas Clean purifier
- **LC Columns:**
  - Poroshell 120
  - ZORBAX RRHD
  - ZORBAX RRHT
  - ZORBAX Eclipse Plus
- **LC Column Guards:**
  - Fast Guards for UHPLC
- **Sample Preparation:**
  - Bond Etut Plexa (SPE)
  - Bond Etut Plexa PCX (SPE)
  - Bond Etut Certify
- **Sample Filtration:**
  - Captiva Filter Cartridges
- **LC Supplies:**
  - 1290 LC
  - 1220 LC

### DESIGNER DRUGS

Below is a listing of Agilent Sample Preparation, Columns and Supplies that support the Designer Drugs applications. Click on the product name to get more information.

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- **GC Columns:**
  - HP-5ms UI
- **Agilent Inert Flow Path Solutions:**
  - Ultra Inert liners
  - Inert Inlet weldments
  - Ultra Inert gold seals
  - Inert MS source
  - Capillary Flow Technology purged union
  - UtiMetal Plus Flexible Metal ferrules
  - Gas Clean purifier
- **LC Columns:**
  - Poroshell 120
  - ZORBAX RRHD
  - ZORBAX RRHT
  - ZORBAX Eclipse Plus
- **LC Column Guards:**
  - Fast Guards for UHPLC
- **Sample Preparation:**
  - Bond Etut Certify (SPE)
  - Bond Etut Plexa Family (SPE)
  - Chem Etut (SLE)
- **Filtration:**
  - Captiva Filter Cartridges
- **LC Supplies:**
  - 1290 LC
  - 1220 LC

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### DRUG SCREENING & CONFIRMATION

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DRUG SCREENING AND CONFIRMATION (CON’T.)

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- **GC Columns:**
  - DB-1ms UI
  - DB-5ms UI
  - DB-35ms UI
  - DB-624 UI
  - DB-FFAP

- **Agilent Inert Flow Path Solutions**
  - Ultra Inert liners
  - Inert Inlet weldments
  - Ultra Inert gold seals
  - Inert MS source
  - Capillary Flow Technology purged union
  - UltiMetal Plus Flexible Metal ferrules
  - 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:**
  - 1290 LC
  - 1220 LC

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Effective detection, measurement, and analysis play a key part in crime prevention, criminal investigations, and law enforcement. The role of criminalistics will continue to grow as the nature of crime and evidence usage increases in complexity.

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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

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.
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 with SPE/LC products and procedures.

Experimental

Analytes

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (6-acetylmorphine) and 100 µg/mL (6-acetylmorphine-D6) 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 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 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% NH4OH 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.

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<tr>
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<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
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<td>6-AM</td>
<td>328.2</td>
<td>165.1</td>
<td>140</td>
<td>40</td>
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<tr>
<td>6-AM</td>
<td>328.2</td>
<td>211.1</td>
<td>140</td>
<td>25</td>
</tr>
<tr>
<td>6-AM</td>
<td>328.2</td>
<td>193.1</td>
<td>140</td>
<td>25</td>
</tr>
<tr>
<td>6-AM-D₆</td>
<td>334.2</td>
<td>165.1</td>
<td>140</td>
<td>40</td>
</tr>
<tr>
<td>6-AM-D₆</td>
<td>334.2</td>
<td>211.1</td>
<td>140</td>
<td>25</td>
</tr>
</tbody>
</table>

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-acetylmorphone. Calibration standards were prepared by spiking negative urine at 1.0, 10, 50, 200, and 400 ng/mL. Deuterated internal standard 6-AM-D₆ was added at 20 ng/mL. The excellent linear fit with R² > 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).

| %  |
| 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


For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.
SAMHSA-Compliant LC/MS/MS Analysis of Amphetamines in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

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.

Authors

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

Amphetamine  
Log P 1.79 pKa 9.8

Methamphetamine  
Log P 1.94 pKa 9.5

MDA  
Log P 1.67 pKa 9.7

Amphetamine-D6

Methamphetamine-D9

MDMA  
Log P 2.05 pKa 9.9

MDEA  
Log P 2.34 pKa 9.9

MDMA-D6

MDEA-D6

Ephedrine  
Log P 1.13 pKa 9.6

Pseudoephedrine  
Log P 0.89/1.1 pKa 9.9

Phentermine  
Log P 2.16 pKa 10.1

Phenylpropanolamine  
Log P 0.81 pKa 9.4

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-D₆, methamphetamine-D₉, MDA-D₅, MDMA-D₅, and MDEA-D₆) 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 (G 13 79B microdegasser, 13 12B binary pump in low delay volume configuration, G 13 67E autosampler, and G 13 30B 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_MS_DiverterValveToWaste()
  {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% \( \text{NH}_4 \text{OH} \) 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.

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.

### Table 1. MRM transitions.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine</td>
<td>136.1</td>
<td>119.1</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Amphetamine-D₆</td>
<td>142.1</td>
<td>125.1</td>
<td>66</td>
<td>5</td>
</tr>
<tr>
<td>MDA</td>
<td>180.1</td>
<td>163.1</td>
<td>92</td>
<td>5</td>
</tr>
<tr>
<td>MDA-D₅</td>
<td>185.1</td>
<td>168.1</td>
<td>68</td>
<td>5</td>
</tr>
<tr>
<td>MDEA</td>
<td>208.1</td>
<td>163.1</td>
<td>88</td>
<td>8</td>
</tr>
<tr>
<td>MDEA-D₅</td>
<td>208.1</td>
<td>133.1</td>
<td>88</td>
<td>17</td>
</tr>
<tr>
<td>MDEA-D₆</td>
<td>214.2</td>
<td>166.1</td>
<td>90</td>
<td>8</td>
</tr>
<tr>
<td>MDMA</td>
<td>214.2</td>
<td>163.1</td>
<td>84</td>
<td>5</td>
</tr>
<tr>
<td>MDMA-D₅</td>
<td>194.1</td>
<td>135.1</td>
<td>84</td>
<td>17</td>
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<tr>
<td>MDMA-D₆</td>
<td>194.1</td>
<td>105.1</td>
<td>84</td>
<td>21</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>159.1</td>
<td>125.2</td>
<td>77</td>
<td>5</td>
</tr>
<tr>
<td>Methamphetamine-D₆</td>
<td>159.2</td>
<td>93.1</td>
<td>77</td>
<td>13</td>
</tr>
<tr>
<td>Ephedrine-pseudoephedrine</td>
<td>166.1</td>
<td>133.1</td>
<td>80</td>
<td>21</td>
</tr>
<tr>
<td>Phenetermine</td>
<td>150.1</td>
<td>133.1</td>
<td>80</td>
<td>6</td>
</tr>
<tr>
<td>Phenylpropanolamine</td>
<td>152.1</td>
<td>117.1</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

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-D₆, 2’. methamphetamine-D₆, 3’. MDA-D₅, 4’. MDMA-D₅, 5’. MDEA-D₆. 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.

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

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


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

Application Note

Forensic Toxicology

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 benzoylcegonine 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.
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].

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

Experimental

Analytes
Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (benzoylecgonine) and 100 µg/mL (benzoylecgonine-D₈) solutions in methanol.
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% NH₄OH 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>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE</td>
<td>290.1</td>
<td>168.1</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>BE</td>
<td>290.1</td>
<td>105.1</td>
<td>90</td>
<td>30</td>
</tr>
<tr>
<td>BE-D₈</td>
<td>298.2</td>
<td>171.1</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>BE-D₉</td>
<td>298.2</td>
<td>110.1</td>
<td>90</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 1. MRM transitions.

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-D₈ 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-D₈ (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 2](image)

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

![Figure 3](image)
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.

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.

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

<table>
<thead>
<tr>
<th></th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process efficiency*</td>
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<tr>
<td>Extraction recovery*</td>
<td>86</td>
</tr>
<tr>
<td>Matrix effect*</td>
<td>99</td>
</tr>
<tr>
<td>Accuracy**</td>
<td>102</td>
</tr>
<tr>
<td>Precision** (CV)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

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


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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

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.
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-glucuronide) and 100 µg/mL (morphine-D₆ and codeine-D₆) 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
  - 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

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$_4$OH 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>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>300.2</td>
<td>215.1</td>
<td>130</td>
<td>23</td>
</tr>
<tr>
<td>Codeine</td>
<td>300.2</td>
<td>165.1</td>
<td>130</td>
<td>46</td>
</tr>
<tr>
<td>Codeine-D$_6$</td>
<td>306.2</td>
<td>165.1</td>
<td>130</td>
<td>44</td>
</tr>
<tr>
<td>Codeine-D$_6$</td>
<td>306.2</td>
<td>218.1</td>
<td>130</td>
<td>23</td>
</tr>
<tr>
<td>Morphine</td>
<td>286.1</td>
<td>201.1</td>
<td>130</td>
<td>23</td>
</tr>
<tr>
<td>Morphine</td>
<td>286.1</td>
<td>181.1</td>
<td>130</td>
<td>40</td>
</tr>
<tr>
<td>Morphine-D$_6$</td>
<td>292.1</td>
<td>181.1</td>
<td>130</td>
<td>43</td>
</tr>
<tr>
<td>Morphine-D$_6$</td>
<td>292.1</td>
<td>165.1</td>
<td>130</td>
<td>40</td>
</tr>
<tr>
<td>Morphine-3-glucuronide</td>
<td>462.2</td>
<td>286.1</td>
<td>162</td>
<td>45</td>
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<td>Oxycodone</td>
<td>316.2</td>
<td>298.1</td>
<td>130</td>
<td>15</td>
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<td>Oxydormorphine</td>
<td>302.2</td>
<td>284.1</td>
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<tr>
<td>Hydrocodone</td>
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<td>199.1</td>
<td>130</td>
<td>30</td>
</tr>
<tr>
<td>Norcodeine</td>
<td>286.1</td>
<td>225.1</td>
<td>130</td>
<td>20</td>
</tr>
<tr>
<td>Hydromorphine</td>
<td>286.1</td>
<td>185.1</td>
<td>130</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 1. MRM transitions.

![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](image)
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-D₆ and codeine-D₆ 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.

When processed according to the protocol, urine samples spiked with morphine-β-3-glucuronide at 10,000 ng/mL showed 97 to 99.2% conversion to morphine. MS parameters for the detection of morphine-β-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. Example calibration curves for morphine (upper panel) and codeine (lower panel) in urine extract. Concentration range 200 to 20,000 ng/mL. Linear fit, $R^2 \geq 0.998$. 

\[ y = 0.001292 * x + 0.225659 \]
\[ R^2 = 0.99932191 \]

\[ y = 0.001727 * x + 0.040572 \]
\[ R^2 = 0.9985013 \]
**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>
<thead>
<tr>
<th>Parameter</th>
<th>Morphine</th>
<th>Codeine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process efficiency (%)</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>Extraction recovery (%)</td>
<td>85</td>
<td>86</td>
</tr>
<tr>
<td>Matrix effect (%)</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>108</td>
<td>108</td>
</tr>
<tr>
<td>Precision (CV) (%)</td>
<td>0.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

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

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

Application Note

Forensic Toxicology

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.
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

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.

Figure 1. Phencyclidine analytes and their structures.
**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
  - 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 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% NH₄OH added shortly before sample elution.

The Poroshell 120 EC-C18 3 x 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.
Table 1. MRM Transitions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP</td>
<td>244.2</td>
<td>86.1</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>PCP</td>
<td>244.2</td>
<td>159.1</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>PCP</td>
<td>244.2</td>
<td>91.1</td>
<td>80</td>
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<tr>
<td>PCP-D₅</td>
<td>249.2</td>
<td>164.1</td>
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<tr>
<td>PCP-D₅</td>
<td>249.2</td>
<td>86.1</td>
<td>80</td>
<td>7</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process efficiency</td>
</tr>
<tr>
<td>Extraction recovery</td>
</tr>
<tr>
<td>Matrix effect</td>
</tr>
<tr>
<td>Accuracy</td>
</tr>
<tr>
<td>Precision (CV)</td>
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</tbody>
</table>

**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**


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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

Irina Dioumaeva, John M. Hughes
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.
**Introduction**

11-Nor-9-carboxy-$\Delta^9$-tetrahydrocannabinol (THCA, “THC-acid”, THCA-COOH) is a metabolite of tetrahydrocannabinol ($\Delta^9$-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-$\Delta^9$-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 x 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 $\Delta^9$-THC and 11-hydroxy-$\Delta^9$-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**

![Structures of 11-nor-9-carboxy-$\Delta^9$-THC and 11-nor-9-carboxy-$\Delta^9$-THC-D$_9$.](image)

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (11-nor-9-carboxy-$\Delta^9$-THC) and 100 µg/mL (11-nor-9-carboxy-$\Delta^9$-THC-D$_9$ and 11-nor-9-carboxy-$\Delta^9$-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 x 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.
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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-nor-9-carboxy-Δ⁹-THC</td>
<td>343.2</td>
<td>299.2</td>
<td>135</td>
<td>18</td>
</tr>
<tr>
<td>Δ⁹-THC</td>
<td>343.2</td>
<td>245.1</td>
<td>135</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>343.2</td>
<td>191.1</td>
<td>135</td>
<td>33</td>
</tr>
<tr>
<td>11-nor-9-carboxy-Δ⁹-THC-D₉</td>
<td>352.2</td>
<td>308.2</td>
<td>145</td>
<td>18</td>
</tr>
<tr>
<td>Δ⁹-THC-D₉</td>
<td>352.2</td>
<td>254.2</td>
<td>145</td>
<td>30</td>
</tr>
<tr>
<td>11-nor-9-carboxy-Δ⁹-THC-glucuronide</td>
<td>519.2</td>
<td>343.2</td>
<td>160</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>519.2</td>
<td>299.2</td>
<td>160</td>
<td>36</td>
</tr>
</tbody>
</table>

When processed according to the protocol, urine samples spiked with 11-nor-9-carboxy-Δ⁹-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-Δ⁹-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-D₉ (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-Δ9-tetrahydrocannabinol at the Cutoff Level, n = 5**

<table>
<thead>
<tr>
<th>%</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Process efficiency</td>
<td>73</td>
</tr>
<tr>
<td>Extraction recovery</td>
<td>65</td>
</tr>
<tr>
<td>Matrix effect</td>
<td>113</td>
</tr>
<tr>
<td>Accuracy</td>
<td>98.2</td>
</tr>
<tr>
<td>Precision (CV)</td>
<td>2.2</td>
</tr>
</tbody>
</table>

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


Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

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-Δ⁸-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 Δ⁸-tetrahydrocannabinol (Δ⁸-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.
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.

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

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

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).

---

**Table 2. Method Evaluation**

<table>
<thead>
<tr>
<th></th>
<th>JWH-018</th>
<th>JWH-073</th>
<th>JWH-200</th>
<th>JWH-250</th>
<th>CP 47497</th>
<th>CP 47497 C8</th>
<th>HU-210</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ (ng/mL)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Imprecision</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intra-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 ng/mL</td>
<td>3.9%</td>
<td>3.6%</td>
<td>5.0%</td>
<td>3.4%</td>
<td>4.9%</td>
<td>3.9%</td>
<td>8.6%</td>
</tr>
<tr>
<td>40 ng/mL</td>
<td>2.2%</td>
<td>2.1%</td>
<td>6.0%</td>
<td>2.0%</td>
<td>4.1%</td>
<td>4.3%</td>
<td>5.6%</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 ng/mL</td>
<td>8.8%</td>
<td>9.6%</td>
<td>6.2%</td>
<td>11%</td>
<td>7.7%</td>
<td>11%</td>
<td>10%</td>
</tr>
<tr>
<td>40 ng/mL</td>
<td>8.5%</td>
<td>7.9%</td>
<td>6.2%</td>
<td>11%</td>
<td>10%</td>
<td>11%</td>
<td>12%</td>
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<tr>
<td>Pad recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4 ng/mL</td>
<td>65.5%</td>
<td>67.4%</td>
<td>85.0%</td>
<td>66.5%</td>
<td>77.7%</td>
<td>76.0%</td>
<td>86.4%</td>
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<tr>
<td>40 ng/mL</td>
<td>70.6%</td>
<td>61.4%</td>
<td>81.4%</td>
<td>75.1%</td>
<td>71.3%</td>
<td>78.2%</td>
<td>75.7%</td>
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<tr>
<td>Matrix effect</td>
<td>-55%</td>
<td>-45%</td>
<td>-55%</td>
<td>-73%</td>
<td>-64%</td>
<td>-55%</td>
<td>-49%</td>
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<tr>
<td>Process efficiency</td>
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<td>51%</td>
<td>56%</td>
<td>24%</td>
<td>38%</td>
<td>45%</td>
<td>51%</td>
</tr>
</tbody>
</table>
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 ($R_{ES} / R_{PES} \times 100$).

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 ($R_{PES} / R_{NES} - 1 \times 100$). The overall efficiency of the process was calculated as ($R_{ES} / R_{NES} \times 100$).

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
cannabinol doxepin
cocaine fluoxetine
benzoylcegonine sertraline
norcoaine 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 chlor Diazepoxide
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 different 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.](image-url)
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".

**For More Information**

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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

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 R² 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.
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 (norbuprenorphine). 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

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (buprenorphine, norbuprenorphine) and 100 µg/mL (buprenorphine-D<sub>4</sub> and norbuprenorphine-D<sub>3</sub>) 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 NH₄OH eluent. Add NH₄OH 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
  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.
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% NH₄OH 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.
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 (C4H7) group with m/z 55.1. Its identification is confirmed by a fragment of buprenorphine-D4 with m/z 59.1. The most abundant product of norbuprenorphine fragmentation (m/z 83.1) probably comes from the branched side chain of the parent ion and includes the tert-butyl group (CH3)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 (R² > 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.
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.

References


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Rapid, Robust and Sensitive Detection of 11-nor-Δ⁹-
Tetrahydrocannabinol-9-Carboxylic Acid in Hair

Application Note
Forensic Toxicology/Doping Control

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 test-
ing 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-Δ⁹-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-Δ⁹-tetrahydrocannabinol 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 ≤ 0.2 pg/mg for THCA.
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>
<thead>
<tr>
<th>Table 1. Agilent 7890N/7000B Gas Chromatograph and Triple Quadrupole Mass Spectrometer Conditions</th>
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<tbody>
<tr>
<td><strong>GC Run Conditions</strong></td>
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<td>Pre-column</td>
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<td><strong>Analytical columns</strong></td>
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<td>Column 1</td>
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<td>Column 2</td>
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<tr>
<td><strong>Injection volume</strong></td>
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<td>Isothermal at 250 °C</td>
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<tr>
<td>Quench gas</td>
</tr>
<tr>
<td>Solvent delay</td>
</tr>
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<td>MS temperatures</td>
</tr>
</tbody>
</table>
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/acetone (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>
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<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>MRM</th>
<th>Dwell time (ms)</th>
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<td>THCA*</td>
<td>6.714</td>
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<td>620→383</td>
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*11-nor-Δ⁹-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 second 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.](image-url)
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.

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 R² 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.
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.
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


Rapid and Robust Detection of THC and Its Metabolites in Blood

Application Note

Forensic Toxicology/Doping Control

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).
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-hydroxy-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.

![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](image)
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>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>MRM</th>
<th>Dwell time (ms)</th>
<th>Collision energy (EV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC</td>
<td>3.5</td>
<td>386→293*</td>
<td>25</td>
<td>386→289</td>
</tr>
<tr>
<td>THC-d3</td>
<td>3.5</td>
<td>389→290*</td>
<td>20</td>
<td>389→289</td>
</tr>
<tr>
<td>11-OH-THC</td>
<td>4.5</td>
<td>371→289*</td>
<td>24</td>
<td>371→289</td>
</tr>
<tr>
<td>THCA (11-nor-Δ9-Tetrahydrocannabinol-9-Carboxylic Acid)</td>
<td>5.6</td>
<td>371→289*</td>
<td>23</td>
<td>371→289</td>
</tr>
<tr>
<td>THCA-d9</td>
<td>5.5</td>
<td>380→292*</td>
<td>15</td>
<td>380→380</td>
</tr>
</tbody>
</table>

*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 through 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.
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.
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.
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 4.8 to 4.9 min.
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


For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.
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
Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

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-Δ⁹-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 Δ⁹-tetrahydrocannabinol (Δ⁹-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.
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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition</th>
<th>Fragment voltage (V)</th>
<th>Collision energy (eV)</th>
<th>Polarity</th>
<th>Ratio of quantifying to qualifying transition (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d9-JWH-018</td>
<td>351.3 &gt; 223.4</td>
<td>140</td>
<td>20</td>
<td>Positive</td>
<td>n/a</td>
</tr>
<tr>
<td>JWH-018</td>
<td>342.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>16–24</td>
</tr>
<tr>
<td></td>
<td>342.2 &gt; 214.2</td>
<td>120</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JWH-250</td>
<td>336.3 &gt; 200.2</td>
<td>120</td>
<td>12</td>
<td>Positive</td>
<td>69–104</td>
</tr>
<tr>
<td></td>
<td>336.3 &gt; 188.2</td>
<td>120</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d7-JWH-073</td>
<td>335.3 &gt; 207.2</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>n/a</td>
</tr>
<tr>
<td>JWH-073</td>
<td>328.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>60–90</td>
</tr>
<tr>
<td></td>
<td>328.2 &gt; 127.1</td>
<td>120</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JWH-200</td>
<td>385.3 &gt; 155.1</td>
<td>140</td>
<td>20</td>
<td>Positive</td>
<td>54–81</td>
</tr>
<tr>
<td></td>
<td>385.3 &gt; 114.2</td>
<td>140</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP 47497 C8</td>
<td>331.3 &gt; 313.3</td>
<td>160</td>
<td>25</td>
<td>Negative</td>
<td>70–104</td>
</tr>
<tr>
<td></td>
<td>331.3 &gt; 259.3</td>
<td>160</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP 47497</td>
<td>317.3 &gt; 298.2</td>
<td>160</td>
<td>20</td>
<td>Negative</td>
<td>75–113</td>
</tr>
<tr>
<td></td>
<td>317.3 &gt; 245.2</td>
<td>160</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU-210</td>
<td>385.3 &gt; 367.4</td>
<td>120</td>
<td>30</td>
<td>Negative</td>
<td>13–20</td>
</tr>
<tr>
<td></td>
<td>385.3 &gt; 281.3</td>
<td>120</td>
<td>45</td>
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<td></td>
</tr>
</tbody>
</table>

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).

![Chromatogram for primary transitions at 10 ng/mL](image.png)

**Figure 1. Primary transition at 10 ng/mL.**

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC</td>
<td>amitriptyline</td>
</tr>
<tr>
<td>THC-COOH</td>
<td>cyclobenzaprine</td>
</tr>
<tr>
<td>11-OH-THC</td>
<td>imipramine</td>
</tr>
<tr>
<td>cannabinol</td>
<td>dothiepin</td>
</tr>
<tr>
<td>cannabinol</td>
<td>doxepin</td>
</tr>
<tr>
<td>cocaine</td>
<td>fluoxetine</td>
</tr>
<tr>
<td>benzoylecgonine</td>
<td>sertraline</td>
</tr>
<tr>
<td>norcocaine</td>
<td>trimipramine</td>
</tr>
<tr>
<td>cocaethylene</td>
<td>protriptyline</td>
</tr>
<tr>
<td>codeine</td>
<td>chlorpromazine</td>
</tr>
<tr>
<td>morphine</td>
<td>clomipramine</td>
</tr>
<tr>
<td>6-AM</td>
<td>nortriptyline</td>
</tr>
<tr>
<td>6-AC</td>
<td>paroxetine</td>
</tr>
<tr>
<td>oxycodone</td>
<td>desipramine</td>
</tr>
<tr>
<td>oxymorphone</td>
<td>bromazepam</td>
</tr>
<tr>
<td>hydrocodone</td>
<td>alprazolam</td>
</tr>
<tr>
<td>hydromorphone</td>
<td>clonazepam</td>
</tr>
<tr>
<td>amphetamine</td>
<td>lorazepam</td>
</tr>
<tr>
<td>methamphetamine</td>
<td>oxaepam</td>
</tr>
<tr>
<td>MDMA</td>
<td>diazepam</td>
</tr>
<tr>
<td>MDA</td>
<td>midazolam</td>
</tr>
<tr>
<td>MDEA</td>
<td>flurazepam</td>
</tr>
<tr>
<td>phentermine</td>
<td>flunitrazepam</td>
</tr>
<tr>
<td>fentanyl</td>
<td>nordiazepam</td>
</tr>
<tr>
<td>phenycyclidine</td>
<td>triazolam</td>
</tr>
<tr>
<td>tramadol</td>
<td>temazepam</td>
</tr>
<tr>
<td>carisoprodol</td>
<td>nitrazepam</td>
</tr>
<tr>
<td>meprobamate</td>
<td>chlor Diazepoxide</td>
</tr>
<tr>
<td>citalopram</td>
<td>methadone</td>
</tr>
<tr>
<td>venlafaxine</td>
<td></td>
</tr>
</tbody>
</table>

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 different 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.](image-url)
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 concentration: 11 ng/mL

Figure 4. Oral fluid from “Blueberry Posh” 40 minutes after smoking; JWH-018 concentration: 11 ng/mL.
DESIGNER DRUGS

Overcome obstacles to analyzing designer drugs

Designer drugs (such as Bath Salts, Ecstasy, Spice, and K2) are synthetic analogs of illegal drugs developed to circumvent drug laws. There is a growing demand for reliable detection and confirmation. Unfortunately, analyzing designer drugs can be difficult, both in bulk and in body fluids, due to matrix interferences.

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Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

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-Δ⁹-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 Δ⁹-tetrahydrocannabinol (Δ⁹-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.
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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Agilent ZORBAX RRHT Extend C18, (2.1 × 50 mm, 1.8 µm, p/n 727700-902)</td>
</tr>
<tr>
<td>Column temperature</td>
<td>60 °C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>5 µL</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Solvent A: 0.2% acetic acid and Solvent B: acetonitrile</td>
</tr>
<tr>
<td>Time 0:</td>
<td>95% A; 5% B; 5 min: 100% B; 7 min 5% B</td>
</tr>
<tr>
<td>Run time</td>
<td>9.2 min; Post-time 3 min</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.5 mL/min</td>
</tr>
<tr>
<td>Nitrogen gas</td>
<td>350 °C</td>
</tr>
<tr>
<td>Gas flow</td>
<td>10 L/min</td>
</tr>
<tr>
<td>Nebulizer pressure</td>
<td>55 psi</td>
</tr>
<tr>
<td>Capillary voltage</td>
<td>+4,000 V in positive mode; –4,000 V in negative mode</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Compound</th>
<th>Transition</th>
<th>Fragment voltage (V)</th>
<th>Collision energy (eV)</th>
<th>Polarity</th>
<th>Ratio of quantifying to qualifying transition (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d9-JWH-018</td>
<td>351.3 &gt; 223.4</td>
<td>140</td>
<td>20</td>
<td>Positive</td>
<td>n/a</td>
</tr>
<tr>
<td>JWH-018</td>
<td>342.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>16–24</td>
</tr>
<tr>
<td></td>
<td>342.2 &gt; 214.2</td>
<td>120</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JWH-250</td>
<td>336.3 &gt; 200.2</td>
<td>120</td>
<td>12</td>
<td>Positive</td>
<td>69–104</td>
</tr>
<tr>
<td></td>
<td>336.3 &gt; 188.2</td>
<td>120</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d7-JWH-073</td>
<td>335.3 &gt; 207.2</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>n/a</td>
</tr>
<tr>
<td>JWH-073</td>
<td>328.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>60–90</td>
</tr>
<tr>
<td></td>
<td>328.2 &gt;127.1</td>
<td>120</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JWH-200</td>
<td>385.3 &gt; 155.1</td>
<td>140</td>
<td>20</td>
<td>Positive</td>
<td>54–81</td>
</tr>
<tr>
<td></td>
<td>385.3 &gt; 114.2</td>
<td>140</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP 47497 C8</td>
<td>331.3 &gt; 313.3</td>
<td>160</td>
<td>25</td>
<td>Negative</td>
<td>70–104</td>
</tr>
<tr>
<td></td>
<td>331.3 &gt; 259.3</td>
<td>160</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP 47497</td>
<td>317.3 &gt; 299.2</td>
<td>160</td>
<td>20</td>
<td>Negative</td>
<td>75–113</td>
</tr>
<tr>
<td></td>
<td>317.3 &gt; 245.2</td>
<td>160</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU-210</td>
<td>385.3 &gt; 367.4</td>
<td>120</td>
<td>30</td>
<td>Negative</td>
<td>13–20</td>
</tr>
<tr>
<td></td>
<td>385.3 &gt; 281.3</td>
<td>120</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

**Table 2. Method Evaluation**

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

Figure 1. Primary transition at 10 ng/mL.
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

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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 ($R_{ES} / R_{PES} \times 100$).

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 ($R_{PES} / R_{NES} \times 100$. The overall efficiency of the process was calculated as ($R_{ES} / R_{NES} \times 100$).

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

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<td>imipramine</td>
</tr>
<tr>
<td>cannabinol</td>
<td>dothiepin</td>
</tr>
<tr>
<td>cannabidiol</td>
<td>doxepin</td>
</tr>
<tr>
<td>cocaine</td>
<td>fluoxetine</td>
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<tr>
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<td>sertraline</td>
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<tr>
<td>norcocaine</td>
<td>trimipramine</td>
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<tr>
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<td>protriptyline</td>
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<tr>
<td>codeine</td>
<td>chlorpromazine</td>
</tr>
<tr>
<td>morphine</td>
<td>clomipramine</td>
</tr>
<tr>
<td>6-AM</td>
<td>nortriptyline</td>
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<tr>
<td>6-AC</td>
<td>paroxetine</td>
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<td>desipramine</td>
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<td>chloridiazepoxide</td>
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<td>citalopram</td>
<td>methadone</td>
</tr>
<tr>
<td>venlafaxine</td>
<td></td>
</tr>
</tbody>
</table>

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 different 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.](image-url)
An extracted ion chromatogram showing the transitions and 
± 20% acceptability band around the intensity of the qualify-
ing 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” com-
ounds in oral fluid is reported for the first time. The proce-
dure 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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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

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.
**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**

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (6-acetylmorphine) and 100 µg/mL (6-acetylmorphine-D₆) solutions in acetonitrile.

**Materials and instrumentation**

**SPE**
- Bond Elut PLEXA 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 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 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% NH₄OH 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-AM</td>
<td>328.2</td>
<td>165.1</td>
<td>140</td>
<td>40</td>
</tr>
<tr>
<td>6-AM</td>
<td>328.2</td>
<td>211.1</td>
<td>140</td>
<td>25</td>
</tr>
<tr>
<td>6-AM-D₆</td>
<td>334.2</td>
<td>165.1</td>
<td>140</td>
<td>40</td>
</tr>
<tr>
<td>6-AM-D₆</td>
<td>334.2</td>
<td>211.1</td>
<td>140</td>
<td>25</td>
</tr>
</tbody>
</table>

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-acetylmophine. Calibration standards were prepared by spiking negative urine at 1.0, 10, 50, 200, and 400 ng/mL. Deuterated internal standard 6-AM-D₆ 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](image2.png)

![Figure 3](image3.png)

Figure 2. MRM extracted ion chromatograms for 6-AM (1 ng/mL) and 6-AM-D₆ (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.

<table>
<thead>
<tr>
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<th>%</th>
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<tr>
<td>Process efficiency*</td>
<td>83</td>
</tr>
<tr>
<td>Extraction recovery*</td>
<td>83</td>
</tr>
<tr>
<td>Matrix effect*</td>
<td>100</td>
</tr>
<tr>
<td>Accuracy**</td>
<td>106</td>
</tr>
<tr>
<td>Precision** (CV)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* 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


For More Information

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

Application Note

Forensic Toxicology

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.
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**

![Chemical structures of analytes](image)

- **Amphetamine**  
  Log P 1.79 pKa 9.8
- **Methamphetamine**  
  Log P 1.94 pKa 9.5
- **MDA**  
  Log P 1.67 pKa 9.7
- **Amphetamine-D₆**
- **Methamphetamine-D₉**
- **MDA-D₅**

- **MDMA**  
  Log P 2.05 pKa 9.9
- **MDEA**  
  Log P 2.34 pKa 9.9
- **MDMA-D₆**
- **MDEA-D₆**

- **Ephedrine**  
  Log P 1.13 pKa 9.6
- **Pseudoephedrine**  
  Log P 0.89/1.1 pKa 9.9
- **Phentermine**  
  Log P 2.16 pKa 10.1
- **Phenylpropanolamine**  
  Log P 0.81 pKa 9.4

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-\text{D}_6, methamphetamine-\text{D}_9, MDA-\text{D}_5, MDMA-\text{D}_5, and MDEA-\text{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 \( \leq 37 \) °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% NH₄OH 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.

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.

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.

Table 1. MRM transitions.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine</td>
<td>136.1</td>
<td>119.1</td>
<td>64</td>
<td>4</td>
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<td>92</td>
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<tr>
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<td>108.1</td>
<td>90</td>
<td>25</td>
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<td>163.1</td>
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<td>13</td>
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<td>152.1</td>
<td>117.1</td>
<td>80</td>
<td>20</td>
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</table>

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-D6, 2’. methamphetamine-D9, 3’. MDA-D6, 4’. MDMA-D5, 5’. MDEA-D6. 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.
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.

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

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


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These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.
SAMHSA-Compliant LC/MS/MS Analysis of Benzoylecgonine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

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.
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 ionization 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-D8) solutions in methanol.

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      | Flush port 75.25 methanol:water for 10 s |
| Needle wash                     | 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% NH₄OH 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
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<tr>
<td>BE</td>
<td>290.1</td>
<td>168.1</td>
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<td>15</td>
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<tr>
<td>BE</td>
<td>290.1</td>
<td>105.1</td>
<td>90</td>
<td>30</td>
</tr>
<tr>
<td>BE-D₆</td>
<td>298.2</td>
<td>171.1</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>BE-D₆</td>
<td>298.2</td>
<td>110.1</td>
<td>90</td>
<td>30</td>
</tr>
</tbody>
</table>

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-D₆ 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.
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.

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.

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

<table>
<thead>
<tr>
<th></th>
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<tr>
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<td>Matrix effect*</td>
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<td>Accuracy**</td>
<td>102</td>
</tr>
<tr>
<td>Precision ** (CV)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

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


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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.
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-glucuronide) and 100 µg/mL (morphine-D₆ and codeine-D₆) 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

**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: Flush port 75:25 methanol:water for 10 s
- 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()
- 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% NH₄OH 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 MassHunterQuantitative software calculates qualifier ion ratios, automatically highlighting those out of acceptable range.

Table 1. MRM transitions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>300.2</td>
<td>215.1</td>
<td>130</td>
<td>23</td>
</tr>
<tr>
<td>Codeine</td>
<td>300.2</td>
<td>165.1</td>
<td>130</td>
<td>46</td>
</tr>
<tr>
<td>Codeine</td>
<td>300.2</td>
<td>153.1</td>
<td>130</td>
<td>50</td>
</tr>
<tr>
<td>Codeine-D₆</td>
<td>306.2</td>
<td>165.1</td>
<td>130</td>
<td>44</td>
</tr>
<tr>
<td>Codeine-D₆</td>
<td>306.2</td>
<td>218.1</td>
<td>130</td>
<td>23</td>
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<tr>
<td>Morphine</td>
<td>286.1</td>
<td>201.1</td>
<td>130</td>
<td>23</td>
</tr>
<tr>
<td>Morphine</td>
<td>286.1</td>
<td>181.1</td>
<td>130</td>
<td>40</td>
</tr>
<tr>
<td>Morphine-D₆</td>
<td>292.1</td>
<td>181.1</td>
<td>130</td>
<td>40</td>
</tr>
<tr>
<td>Morphine-D₆</td>
<td>292.1</td>
<td>165.1</td>
<td>130</td>
<td>42</td>
</tr>
<tr>
<td>Morphine-3-glucuronide</td>
<td>462.2</td>
<td>286.1</td>
<td>162</td>
<td>45</td>
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<tr>
<td>Oxycodone</td>
<td>316.2</td>
<td>298.1</td>
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<tr>
<td>Oxymorphone</td>
<td>302.2</td>
<td>284.1</td>
<td>130</td>
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<td>Hydrocodone</td>
<td>300.2</td>
<td>199.1</td>
<td>130</td>
<td>30</td>
</tr>
<tr>
<td>Norcodeine</td>
<td>286.1</td>
<td>225.1</td>
<td>130</td>
<td>20</td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>286.1</td>
<td>185.1</td>
<td>130</td>
<td>28</td>
</tr>
</tbody>
</table>

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 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-D6 and codeine-D6 were added at 1,000 ng/mL. Excellent linear fit (R² ≥ 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 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.](image_url)

![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, R² ≥ 0.998.](image_url)
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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Morphine</th>
<th>Codeine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process efficiency (%)</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>Extraction recovery (%)</td>
<td>85</td>
<td>86</td>
</tr>
<tr>
<td>Matrix effect (%)</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>108</td>
<td>108</td>
</tr>
<tr>
<td>Precision (CV) (%)</td>
<td>0.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

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

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.
SAMHSA-Compliant LC/MS/MS Analysis of Phencyclidine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

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.

Authors

Irina Dioumaeva, John M. Hughes
Agilent Technologies, Inc.
**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 analytes and their structures.](image)

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (phencyclidine) and 100 µg/mL (phencyclidine-D₅) 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

<table>
<thead>
<tr>
<th>Mobile phase A</th>
<th>0.1% formic acid in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase B</td>
<td>0.1% formic acid in methanol</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8 mL/min</td>
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<td>Gradient</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>0.5 10</td>
</tr>
<tr>
<td></td>
<td>2.5 70</td>
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<td></td>
<td>2.51 90</td>
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<td></td>
<td>5.5 90</td>
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<td></td>
<td>5.51 10</td>
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<tr>
<td>Stop time</td>
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<tr>
<td>Post time</td>
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<td>Max pump pressure</td>
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<tr>
<td>Injection volume</td>
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</tr>
<tr>
<td>Injection with needle wash</td>
<td>Flush port 75.25 methanol:water for 10 s</td>
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<td>Disable overlapped injection</td>
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<tr>
<td>No automatic delay volume reduction</td>
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MS conditions

ES source parameters

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<th>Ionization mode</th>
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<td>Drying gas flow</td>
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<tr>
<td>Drying gas temperature</td>
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<td>Nebulizer gas</td>
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<tr>
<td>Sheath gas flow</td>
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<tr>
<td>Sheath gas temperature</td>
<td>400 °C</td>
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<tr>
<td>Nozzle voltage</td>
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</table>

MS parameters

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<th>Scan type</th>
<th>MRM</th>
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<td>Pre-run script</td>
<td>SCP_MS_DiverterValveToWaste()</td>
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<td></td>
<td>{MH_Acq_Scripts.exe}</td>
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<td>Time segments</td>
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<tr>
<td>Delta EMV (+)</td>
<td>200 V</td>
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</table>

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% NH₄OH added shortly before sample elution.

The Poroshell 120 EC-C18 3 x 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.
Table 1. MRM Transitions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP</td>
<td>244.2</td>
<td>86.1</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>PCP</td>
<td>244.2</td>
<td>159.1</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>PCP</td>
<td>244.2</td>
<td>91.1</td>
<td>80</td>
<td>34</td>
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<tr>
<td>PCP-D₅</td>
<td>249.2</td>
<td>164.1</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>PCP-D₅</td>
<td>249.2</td>
<td>86.1</td>
<td>80</td>
<td>7</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th></th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process efficiency</td>
<td>83</td>
</tr>
<tr>
<td>Extraction recovery</td>
<td>85</td>
</tr>
<tr>
<td>Matrix effect</td>
<td>98</td>
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<tr>
<td>Accuracy</td>
<td>93</td>
</tr>
<tr>
<td>Precision (CV)</td>
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</table>

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


SAMHSA-Compliant LC/MS/MS Analysis of 11-nor-9-carboxy-Δ⁹-Tetrahydrocannabinol in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensic Toxicology

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-Δ⁹-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.
Introduction

11-Nor-9-carboxy-\(\Delta^9\)-tetrahydrocannabinol (THCA, “THC-acid”, THCOOH) is a metabolite of tetrahydrocannabinol (\(\Delta^9\)-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-\(\Delta^9\)-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 x 50 mm, 2.7 \(\mu\)m column was chosen due to its high capacity and excellent separation properties. With superficially porous 2.7 \(\mu\)m particles, the Poroshell 120 provides similar efficiency to sub-2 \(\mu\)m 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 \(\Delta^9\)-THC and 11-hydroxy-\(\Delta^9\)-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 \(\mu\)L 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

![Figure 1. 11-nor-\(\Delta^9\)-tetrahydrocannabinol analytes and their structures.](image)

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (11-nor-9-carboxy-\(\Delta^9\)-THC) and 100 \(\mu\)g/mL (11-nor-9-carboxy-\(\Delta^9\)-THC-D\(_9\) and 11-nor-9-carboxy-\(\Delta^9\)-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 x 50 mm, 2.7 \(\mu\)m (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.
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>
<thead>
<tr>
<th>Compound</th>
<th>Parent</th>
<th>Product</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-nor-9-carboxy-D9-THC</td>
<td>343.2</td>
<td>299.2</td>
<td>135</td>
<td>18</td>
</tr>
<tr>
<td>Δ9-THC</td>
<td>343.2</td>
<td>245.1</td>
<td>135</td>
<td>30</td>
</tr>
<tr>
<td>Δ9-THC-D9</td>
<td>343.2</td>
<td>191.1</td>
<td>135</td>
<td>33</td>
</tr>
<tr>
<td>11-nor-9-carboxy-D9-THCA</td>
<td>352.2</td>
<td>308.2</td>
<td>145</td>
<td>18</td>
</tr>
<tr>
<td>11-nor-9-carboxy-Δ9-THC-glucuronide</td>
<td>519.2</td>
<td>343.2</td>
<td>160</td>
<td>22</td>
</tr>
<tr>
<td>11-nor-9-carboxy-D9-THCA-glucuronide</td>
<td>519.2</td>
<td>299.2</td>
<td>160</td>
<td>36</td>
</tr>
</tbody>
</table>

When processed according to the protocol, urine samples spiked with 11-nor-9-carboxy-Δ9-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-Δ9-THC-glucuronide are included in Table 1 for analysts interested in testing the hydrolysis efficiency.
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, THC A 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-D9 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>
<thead>
<tr>
<th>Table 2. Method Performance for 11-nor-carboxy-$\Delta^9$-tetrahydrocannabinol at the Cutoff Level, n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process efficiency</td>
</tr>
<tr>
<td>Extraction recovery</td>
</tr>
<tr>
<td>Matrix effect</td>
</tr>
<tr>
<td>Accuracy</td>
</tr>
<tr>
<td>Precision (CV)</td>
</tr>
</tbody>
</table>

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

$\Delta^9$ THC COOH · 5 Levels, 5 Levels used, 17 Points, 17 Points used, 13 QCs

$\gamma = 0.014212 \cdot x + 0.081876$

$R^2 = 0.99917930$
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


www.agilent.com/chem

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Synthetic Cannabinoids in Oral Fluid

Application Note

Forensic Toxicology

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-Δ⁹-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 Δ⁹-tetrahydrocannabinol (Δ⁹-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.
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>
<thead>
<tr>
<th>Compound</th>
<th>Transition</th>
<th>Fragment voltage (V)</th>
<th>Collision energy (eV)</th>
<th>Polarity</th>
<th>Ratio of quantifying to qualifying transition (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d9-JWH-018</td>
<td>351.3 &gt; 223.4</td>
<td>140</td>
<td>20</td>
<td>Positive</td>
<td>n/a</td>
</tr>
<tr>
<td>JWH-018</td>
<td>342.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>16–24</td>
</tr>
<tr>
<td></td>
<td>342.2 &gt; 214.2</td>
<td>120</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JWH-250</td>
<td>336.3 &gt; 200.2</td>
<td>120</td>
<td>12</td>
<td>Positive</td>
<td>69–104</td>
</tr>
<tr>
<td></td>
<td>336.3 &gt; 188.2</td>
<td>120</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d7-JWH-073</td>
<td>335.3 &gt; 207.2</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>n/a</td>
</tr>
<tr>
<td>JWH-073</td>
<td>328.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>Positive</td>
<td>60–90</td>
</tr>
<tr>
<td></td>
<td>328.2 &gt; 127.1</td>
<td>120</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JWH-200</td>
<td>385.3 &gt; 155.1</td>
<td>140</td>
<td>20</td>
<td>Positive</td>
<td>54–81</td>
</tr>
<tr>
<td></td>
<td>385.3 &gt; 114.2</td>
<td>140</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP 47497 C8</td>
<td>331.3 &gt; 313.3</td>
<td>160</td>
<td>25</td>
<td>Negative</td>
<td>70–104</td>
</tr>
<tr>
<td></td>
<td>331.3 &gt; 259.3</td>
<td>160</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP 47497</td>
<td>317.3 &gt; 299.2</td>
<td>160</td>
<td>20</td>
<td>Negative</td>
<td>75–113</td>
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<tr>
<td></td>
<td>317.3 &gt; 245.2</td>
<td>160</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU-210</td>
<td>385.3 &gt; 367.4</td>
<td>120</td>
<td>30</td>
<td>Negative</td>
<td>13–20</td>
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<tr>
<td></td>
<td>385.3 &gt; 281.3</td>
<td>120</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

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

Figure 1. Primary transition at 10 ng/mL.
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 ($R_{ES}/ R_{PES} \times 100$).

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 ($R_{PES}/ R_{NES} - 1 \times 100$). The overall efficiency of the process was calculated as ($R_{ES}/ R_{NES} \times 100$).

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.

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 different 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).

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![Figure 3. Stability of authentic specimens stored at 4 °C.](image-url)
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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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

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 R² 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.
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 (norbuprenorphine). 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

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (buprenorphine, norbuprenorphine) and 100 µg/mL (buprenorphine-D4 and norbuprenorphine-D3) 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 NH₄OH eluent. Add NH₄OH 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.
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% NH₄OH 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.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Precursor</th>
<th>MS1 Res</th>
<th>Product</th>
<th>MS2 Res</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine</td>
<td>468.3</td>
<td>Unit</td>
<td>55.1</td>
<td>Wide</td>
<td>200</td>
<td>62</td>
</tr>
<tr>
<td>Buprenorphine-D₄</td>
<td>472.3</td>
<td>Unit</td>
<td>59.1</td>
<td>Wide</td>
<td>200</td>
<td>62</td>
</tr>
<tr>
<td>Norbuprenorphine</td>
<td>414.3</td>
<td>Unit</td>
<td>83.1</td>
<td>Wide</td>
<td>188</td>
<td>60</td>
</tr>
<tr>
<td>Norbuprenorphine-D₃</td>
<td>417.3</td>
<td>Unit</td>
<td>83.1</td>
<td>Wide</td>
<td>188</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 1. MRM Transitions.

Figure 2. MRM extracted ion chromatograms:
A: buprenorphine, B: norbuprenorphine (both at 0.2 ng/mL), C: buprenorphine-D₄, and D: norbuprenorphine-D₃ (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 (C₄H₇) group with m/z 55.1. Its identification is confirmed by a fragment of buprenorphine-D₄ with m/z 59.1. The most abundant product of norbuprenorphine fragmentation (m/z 83.1) probably comes from the branched side chain of the parent ion and includes the tert-butyl group (CH₃)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 (R² > 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.**

<table>
<thead>
<tr>
<th></th>
<th>Accuracy (%)</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine</td>
<td>94.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Norbuprenorphine</td>
<td>103</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Figure 3. Typical calibration curves for buprenorphine and norbuprenorphine in whole blood extract. Concentration range 0.2 to 20 ng/mL. Linear fits R² > 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.

**References**


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Rapid, Robust and Sensitive Detection of 11-nor-Δ⁹-Tetrahydrocannabinol-9-Carboxylic Acid in Hair

Application Note
Forensic Toxicology/Doping Control

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, tetrahydrocannabininol (THC), is found in higher concentration in hair samples, but detection of the acid metabolite THCA (11-nor-Δ⁹-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-Δ³-tetrahydrocannabinol 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 ≤ 0.2 pg/mg for THCA.
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>
<thead>
<tr>
<th>GC Run Conditions</th>
<th>Pre-column</th>
<th>1 m × 0.15 mm × 1.2 µm DB-1 (p/n 12A-1015)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical columns</td>
<td>Column 1</td>
<td>15 m × 0.25 mm × 0.25 µm DB-1ms LTM Column Module (p/n 122-0112LTM)</td>
</tr>
<tr>
<td></td>
<td>Column 2</td>
<td>15 m × 0.25 mm × 0.25 µm DB-17ms LTM Column Module (p/n 122-4712LTM)</td>
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<tr>
<td>Injection volume</td>
<td>2 µL</td>
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<tr>
<td>Inlet temperature</td>
<td>Isothermal at 250 °C</td>
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</tr>
<tr>
<td>Injection mode</td>
<td>0.75 minute pulsed splitless at 35 psi</td>
<td></td>
</tr>
<tr>
<td>Oven temperatures</td>
<td>GC oven</td>
<td>7 minute hold at 250 °C (isothermal)</td>
</tr>
<tr>
<td></td>
<td>1st LTM module</td>
<td>50 sec hold at 100 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 °C to 210 °C at 200 °C/min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>210 °C to 267 °C at 10 °C/min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hold at 267 °C for 2 min</td>
</tr>
<tr>
<td></td>
<td>2nd LTM module</td>
<td>324 sec hold at 100 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 °C to 230 °C at 200 °C/min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>230 °C to 240 °C at 10 °C/min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hold at 240 °C for 2 min</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Helium in constant pressure mode.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-column: 1 psi; Column 1: 26.6 psi; Column 2: 19.6 psi</td>
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<tr>
<td>Transfer line temp</td>
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<td>MS conditions</td>
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<td>Autotune</td>
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<td></td>
<td>EMV Delta</td>
<td>1200 V</td>
</tr>
<tr>
<td></td>
<td>Acquisition parameters</td>
<td>NCI mode; multiple reaction monitoring (MRM)</td>
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<tr>
<td></td>
<td>Reagent gas</td>
<td>Ammonia, 35% flow</td>
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<td></td>
<td>Collision gas</td>
<td>Argon, constant flow, 0.9 mL/min</td>
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<tr>
<td></td>
<td>Quench gas</td>
<td>Helium, constant flow, 0.5 mL/min</td>
</tr>
<tr>
<td></td>
<td>Solvent delay</td>
<td>6.2 min</td>
</tr>
<tr>
<td></td>
<td>MS temperatures</td>
<td>Source 150 °C; Quadrupole 150 °C</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>MRM</th>
<th>Dwell time (ms)</th>
<th>Collision energy (EV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THCA*</td>
<td>6.714</td>
<td>620→492</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>620→383</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>THCA-d3</td>
<td>6.710</td>
<td>623→495</td>
<td>20</td>
<td>5</td>
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<tr>
<td></td>
<td></td>
<td>623→386</td>
<td>20</td>
<td>5</td>
</tr>
</tbody>
</table>

*11-nor-Δ^2-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.](image)
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.
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

![CALIBRATION CURVE](image)

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

![CALIBRATION CURVE](image)

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.
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


Rapid and Robust Detection of THC and Its Metabolites in Blood

Application Note

Forensic Toxicology/Doping Control

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)
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-hydroxy-Δ9-tetrahydrocannabinol) 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.

---

**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
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

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

*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 through 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 R² 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.
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.
Table: Quantitative Analysis Sample Report

<table>
<thead>
<tr>
<th>Compd</th>
<th>Signal</th>
<th>RT</th>
<th>Limits</th>
<th>Response</th>
<th>QRatio</th>
<th>Limits</th>
<th>FinalConc</th>
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</thead>
<tbody>
<tr>
<td>D3-THC</td>
<td>389.3 -&gt; 306.2</td>
<td>3.46</td>
<td></td>
<td>37956</td>
<td>175:1</td>
<td>3.6 - 3.9</td>
<td>0.010</td>
</tr>
<tr>
<td>THC</td>
<td>386.3 -&gt; 303.2</td>
<td>3.48</td>
<td>3.29 - 3.64</td>
<td>1554</td>
<td>64.3-104.3</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>THCC (Target)</td>
<td>386.3 -&gt; 330.2</td>
<td>1237</td>
<td>79.7</td>
<td>62.4-102.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3-THC (IntStd)</td>
<td>389.3 -&gt; 306.2</td>
<td>3.465</td>
<td></td>
<td>175:1</td>
<td>3.6 - 3.9</td>
<td>0.010</td>
<td></td>
</tr>
</tbody>
</table>

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.olex.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: 2011-05-05 13:57
Last Calib Update:

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<thead>
<tr>
<th>Cmpnd</th>
<th>Signal</th>
<th>RT</th>
<th>Limits</th>
<th>Response</th>
<th>QRatio</th>
<th>Limits</th>
<th>FinalConc</th>
</tr>
</thead>
<tbody>
<tr>
<td>d3-OH-THC</td>
<td>374.3 -&gt; 292.2</td>
<td>4.5</td>
<td></td>
<td>175705</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>374.3 -&gt; 308.2</td>
<td></td>
<td></td>
<td>157882</td>
<td>89.9</td>
<td>68.8-108.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>374.3 -&gt; 268.3</td>
<td></td>
<td></td>
<td>148545</td>
<td>84.5</td>
<td>59.4-99.4</td>
<td></td>
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<tr>
<td>OH-THC</td>
<td>371.3 -&gt; 289.2</td>
<td>4.51</td>
<td>4.27 - 4.72</td>
<td>4924</td>
<td>4.099</td>
<td>67.4-107.4</td>
<td>58.2-98.2</td>
</tr>
<tr>
<td></td>
<td>371.3 -&gt; 305.2</td>
<td></td>
<td></td>
<td>3707</td>
<td>75.3</td>
<td>67.4-107.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>371.3 -&gt; 265.2</td>
<td></td>
<td></td>
<td>4050</td>
<td>82.3</td>
<td>58.2-98.2</td>
<td></td>
</tr>
</tbody>
</table>

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 4.8 to 4.9 min.
Figure 5. Quantitative Analysis Sample Report for 1 ng/mL of THCA in blood. The RMS signal-to-noise is 38:1 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


For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.
Extraction of Benzodiazepines in Urine with Polymeric SPE Cation Exchange, Agilent Bond Elut Plexa PCX

Application Note

Forensic Toxicology

Authors

William Hudson
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.
Materials and Methods

Table 1. SPE reagents and solutions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Solution Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Formic acid</td>
<td>Add 2 mL of concentrated formic acid to 100 mL of DI water</td>
</tr>
<tr>
<td>Methanol</td>
<td>Reagent grade or better</td>
</tr>
<tr>
<td>50% Methanol</td>
<td>Add 50 mL of methanol to 50 mL of DI water</td>
</tr>
<tr>
<td>5% Ammonia in methanol</td>
<td>Add 5 mL of concentrated ammonia to 100 mL of methanol</td>
</tr>
</tbody>
</table>

Table 2. SPE method.

<table>
<thead>
<tr>
<th>Condition</th>
<th>1. 1 mL CH₃OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load:</td>
<td>Apply sample and extract under low or no vacuum</td>
</tr>
<tr>
<td>Wash 1:</td>
<td>2 mL 2% formic acid</td>
</tr>
<tr>
<td>Wash 2:</td>
<td>2 mL 50% CH₃OH in water</td>
</tr>
<tr>
<td>Elution:</td>
<td>1 mL 5% NH₃ in methanol</td>
</tr>
</tbody>
</table>

All samples are evaporated to dryness and reconstituted in 200 µL of 50:50 0.1% aqueous formic acid: CH₃OH.

Table 3. MS conditions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Q1</th>
<th>Q3</th>
<th>CE</th>
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<tr>
<td>Clonazepam</td>
<td>316.0</td>
<td>270.0</td>
<td>16.5 V</td>
</tr>
<tr>
<td>7-Aminoclonazepam</td>
<td>285.8</td>
<td>121.0</td>
<td>24.5 V</td>
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<tr>
<td>Flurazepam</td>
<td>388.0</td>
<td>315.0</td>
<td>18.0 V</td>
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<td>Desalkylflurazepam</td>
<td>288.9</td>
<td>140.0</td>
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<td>Midazolam</td>
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<td>Flunitrazepam</td>
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<td>284.1</td>
<td>135.0</td>
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<td>Chlordiazepoxide</td>
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<td>321.0</td>
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<td>Oxazepam</td>
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<td>23.0 V</td>
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<td>Temazepam</td>
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<td>255.0</td>
<td>17.0 V</td>
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Table 4. Analyte relative recoveries.

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<th>Analyte</th>
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<th>% RSD</th>
<th>% Rec (100 ng/mL)</th>
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<td>Clonazepam</td>
<td>116</td>
<td>13</td>
<td>103</td>
<td>7</td>
</tr>
<tr>
<td>7-Aminoclonazepam</td>
<td>102</td>
<td>10</td>
<td>99</td>
<td>2</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>117</td>
<td>14</td>
<td>106</td>
<td>8</td>
</tr>
<tr>
<td>Desalkylflurazepam</td>
<td>115</td>
<td>13</td>
<td>99</td>
<td>6</td>
</tr>
<tr>
<td>Midazolam</td>
<td>108</td>
<td>13</td>
<td>110</td>
<td>4</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>113</td>
<td>15</td>
<td>107</td>
<td>7</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>113</td>
<td>17</td>
<td>110</td>
<td>8</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>107</td>
<td>16</td>
<td>101</td>
<td>3</td>
</tr>
<tr>
<td>7-Aminoflunitrazepam</td>
<td>112</td>
<td>18</td>
<td>95</td>
<td>9</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>119</td>
<td>15</td>
<td>92</td>
<td>10</td>
</tr>
<tr>
<td>Diazepam</td>
<td>111</td>
<td>12</td>
<td>99</td>
<td>8</td>
</tr>
<tr>
<td>Temazepam</td>
<td>118</td>
<td>4</td>
<td>97</td>
<td>8</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>102</td>
<td>14</td>
<td>94</td>
<td>10</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>113</td>
<td>10</td>
<td>97</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 1a. Chromatograms of a 100 ng/mL urine extract (peaks 1-8).

Peak identification
1. Nordiazepam
2. 7-Aminoclonazepam
3. Desalkylflurazepam
4. Temazepam
5. Alprazolam
6. Clonazepam
7. Midazolam
8. Flurazepam

Figure 1b. Chromatograms of a 100 ng/mL urine extract (peaks 9-14).

Peak identification
9. 7-Aminoflunitrazepam
10. Diazepam
11. Oxazepam
12. Chlordiazepoxide
13. Flunitrazepam
14. Lorazepam
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.
Extraction of Basic Drugs from Plasma with Polymeric SPE

Application Note

Pharmaceuticals

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.
Materials and Methods

SPE reagents and solutions

2% ammonium hydroxide
Add 20 µL concentrated ammonium hydroxide to 1 mL DI H₂O

Methanol
Reagent grade or better

5% methanol
Add 5 mL methanol to 95 mL DI H₂O

Bond Elut Plexa 10 mg 96 well plate (p/n A4969010)

SPE method

Sample
100 µL human plasma

Pretreat
Dilute with 300 µL 2% NH₄OH

Condition
1. 500 µL CH₃OH
2. 500 µL H₂O

Wash
500 µL 5% CH₃OH in H₂O

Elute
500 µL CH₃OH

All samples evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% formic acid: CH₃OH 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

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00</td>
<td>40</td>
</tr>
<tr>
<td>0:15</td>
<td>40</td>
</tr>
<tr>
<td>1:00</td>
<td>80</td>
</tr>
<tr>
<td>3:00</td>
<td>80</td>
</tr>
<tr>
<td>4:30</td>
<td>40</td>
</tr>
</tbody>
</table>

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.
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 polarities and delivers reproducibly high recoveries. Bond Elut Plexa is therefore highly recommended for bioanalytical work, including contract research.

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com.

Table 1. High Recoveries of Basic Drugs with Bond Elut Plexa

<table>
<thead>
<tr>
<th>Drug</th>
<th>log P</th>
<th>pKa</th>
<th>0.5 µg/mL</th>
<th>1.0 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Recovery</td>
<td>%RSD</td>
<td>%Recovery</td>
<td>%RSD</td>
</tr>
<tr>
<td>Albuterol</td>
<td>1.3</td>
<td>10.3</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>4.6</td>
<td>9.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>3.9</td>
<td>6.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Propranolol</td>
<td>3.6</td>
<td>9.5</td>
<td>102</td>
<td>101</td>
</tr>
<tr>
<td>Atenolol</td>
<td>1.3</td>
<td>9.6</td>
<td>97</td>
<td>101</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>1.3</td>
<td>10.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Loratadine</td>
<td>5.2</td>
<td>4.9</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>1.8</td>
<td>9.2</td>
<td>103</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 1. Chromatograms of a 100 ng/mL human plasma extract.

Albuterol
Amitriptyline
Zolpidem
Propranolol
Atenolol
Metoprolol
Loratadine
Naltrexone
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.
Materials and Methods

Table 1. SPE Reagents and Solutions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Phosphoric Acid</td>
<td>Add 20 µL of concentrated H₃PO₄ to 1 mL of DI water</td>
</tr>
<tr>
<td>Methanol</td>
<td>Reagent grade or better</td>
</tr>
<tr>
<td>2% Formic Acid</td>
<td>Add 20 µL of concentrated formic acid to 1 mL of DI water</td>
</tr>
<tr>
<td>Methanol:acetonitrile</td>
<td>Add 1 mL of methanol to 1 mL of acetonitrile</td>
</tr>
<tr>
<td>(1:1, v/v)</td>
<td></td>
</tr>
<tr>
<td>5% NH₃</td>
<td>Add 50 µL of concentrated ammonia to 1 mL of methanol:acetonitrile</td>
</tr>
<tr>
<td>Methanol:acetonitrile</td>
<td></td>
</tr>
<tr>
<td>(1:1, v/v)</td>
<td></td>
</tr>
</tbody>
</table>

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% H₃PO₄                               |
| Condition                | 1. 500 µL CH₃OH 2. 500 µL DI H₂O                                           |
| 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% NH₃ methanol:acetonitrile                                        |

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% aqueous formic acid: CH₃OH.

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:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Q1</th>
<th>Q3</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albuterol</td>
<td>240.1</td>
<td>148.0</td>
<td>-23.5V</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>267.0</td>
<td>256.0</td>
<td>-34.0V</td>
</tr>
<tr>
<td>Metoprolol (IS)</td>
<td>296.1</td>
<td>201.1</td>
<td>-14.0V</td>
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</table>

Bond Elut Plexa 10 mg 96 well plate (part number A4968010)

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.

![Chromatograms of a 50 ng/mL extract](image)

Table 3. Recoveries of polar basic compounds from human plasma

<table>
<thead>
<tr>
<th>Analyte</th>
<th>log P</th>
<th>pKa</th>
<th>% Rec (500 ng/mL)</th>
<th>% RSD²</th>
<th>% Rec (1000 ng/mL)</th>
<th>% RSD²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sumatriptan</td>
<td>0.96</td>
<td>9.6</td>
<td>95</td>
<td>5</td>
<td>97</td>
<td>4</td>
</tr>
<tr>
<td>Atenolol</td>
<td>1.30</td>
<td>9.6</td>
<td>94</td>
<td>3</td>
<td>91</td>
<td>2</td>
</tr>
<tr>
<td>Albuterol</td>
<td>1.30</td>
<td>10.3</td>
<td>95</td>
<td>5</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>1.50</td>
<td>5.7</td>
<td>92</td>
<td>3</td>
<td>97</td>
<td>4</td>
</tr>
</tbody>
</table>

1Recoveries calculated as % of signal intensity of an extracted sample compared to that calibration curve.
2RSD = 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.
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.
Materials and Methods

Table 1. SPE Reagents and Solutions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Phosphoric Acid</td>
<td>Add 20 µL of concentrated H3PO4 to 1 mL of DI water</td>
</tr>
<tr>
<td>Methanol</td>
<td>Reagent grade or better</td>
</tr>
<tr>
<td>2% Formic Acid</td>
<td>Add 20 µL of concentrated formic acid to 1 mL of DI water</td>
</tr>
<tr>
<td>Methanol:acetonitrile</td>
<td>Add 1 mL of methanol to 1 mL of acetonitrile</td>
</tr>
<tr>
<td>5% NH3</td>
<td>Add 50 µL of concentrated ammonia to 1 mL of methanol:acetonitrile (1:1, v/v)</td>
</tr>
</tbody>
</table>

Bond Elut Plexa 10 mg 96 well plate (part number A4988010)

Table 2. SPE Method

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Pre-treatment</td>
<td>100 µL human plasma. Dilute 1:3 with 2% H3PO4</td>
</tr>
</tbody>
</table>
| Condition          | 1. 500 µL CH3OH  
2. 500 µL DI H2O                                                               |
| 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% NH3 methanol: acetonitrile                                         |

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% aqueous formic acid: CH3OH.

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:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Q1</th>
<th>Q3</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranitidine</td>
<td>315.0</td>
<td>176.0</td>
<td>-21.0V</td>
</tr>
<tr>
<td>Propranolol</td>
<td>260.1</td>
<td>116.0</td>
<td>-17.5V</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>278.1</td>
<td>233.0</td>
<td>-17.0V</td>
</tr>
<tr>
<td>Loratadine</td>
<td>383.1</td>
<td>337.0</td>
<td>-31.0V</td>
</tr>
</tbody>
</table>

Capillary = 25 V, Dry gas temp = 400 °C, 30 psi, CID = Argon  
Polarity: Positive

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.

\[ \text{Table 3. Recoveries of non-polar basic compounds from human plasma} \]

<table>
<thead>
<tr>
<th>Analyte</th>
<th>log P</th>
<th>pKa</th>
<th>% Rec (500 ng/mL)</th>
<th>% RSD\textsuperscript{2}</th>
<th>% Rec (1000 ng/mL)</th>
<th>% RSD\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranitidine</td>
<td>1.9</td>
<td>8.2</td>
<td>101</td>
<td>5</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>Propranolol</td>
<td>3.6</td>
<td>9.5</td>
<td>97</td>
<td>7</td>
<td>92</td>
<td>4</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>4.6</td>
<td>9.4</td>
<td>95</td>
<td>5</td>
<td>91</td>
<td>5</td>
</tr>
<tr>
<td>Loratadine</td>
<td>5.2</td>
<td>9.3</td>
<td>100</td>
<td>4</td>
<td>91</td>
<td>4</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Recoveries calculated as % of signal intensity of an extracted sample compared to that calibration curve.  
\textsuperscript{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.
Solid phase extraction of THC, THC-COOH and 11-OH-THC from whole blood

Application Note

Forensic Toxicology

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 $\Delta^9$-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-$\Delta^9$-tetrahydrocannabinol (11-OH-THC), which is then converted to 11-nor-$\Delta^9$-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 quaternary 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-OH-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-OH-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.
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.
**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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS number</th>
<th>Log P</th>
<th>pKa</th>
<th>Therapeutic use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lidocaine</td>
<td>137-58-6</td>
<td>2.4</td>
<td>8.01</td>
<td>Local anesthetic, antiarrhythmic</td>
</tr>
<tr>
<td>Tramadol</td>
<td>27203-92-5</td>
<td>2.5</td>
<td>9.41</td>
<td>Analgesic</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>50-48-6</td>
<td>4.92</td>
<td>9.4</td>
<td>Antidepressant</td>
</tr>
<tr>
<td>Biperidene</td>
<td>514-65-8</td>
<td>4.0</td>
<td>10.8</td>
<td>Anticholinergic</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>604-75-1</td>
<td>2.23</td>
<td>1.7, 11.3</td>
<td>Antianxiety</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>846-49-1</td>
<td>2.47</td>
<td>1.3, 11.5</td>
<td>Antidepressant</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>50-53-3</td>
<td>5.18</td>
<td>9.3</td>
<td>Antipsychotic</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>42399-41-7</td>
<td>3.63</td>
<td>7.7</td>
<td>Calcium channel blocker</td>
</tr>
<tr>
<td>Naloxone</td>
<td>465-65-6</td>
<td>1.45</td>
<td>7.9</td>
<td>Opioid receptor antagonist</td>
</tr>
<tr>
<td>Nortriptyline (IS)</td>
<td>72-69-5</td>
<td>5.65</td>
<td>9.7</td>
<td></td>
</tr>
</tbody>
</table>
**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\(_4\)OAc) pH 5 was made by dissolving 19.27 g NH\(_4\)OAc 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\(_2\)O (20:80) containing 5 mM NH\(_4\)OAc pH 5 was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL 1 M NH\(_4\)OAc, pH 5 stock solution. The 5 mM NH\(_4\)OAc in ACN was prepared by adding 5 mL 1 M NH\(_4\)OAc, 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\(_2\)O (0.1% FA). A 0.5 and 5.0 µg/mL standard solution in 1:1 ACN:H\(_2\)O (0.1% FA) was made for the preparation of calibration curves in the matrix blank extract with appropriate dilution.

**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\(_2\)O (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.
## 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.
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 MgSO₄ 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 2. Instrument Acquisition Data Used for the Analysis of Nine Drugs by LC/MS/MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM channels (m/z)</th>
<th>Fragmentor (V)</th>
<th>CE (V)</th>
<th>RT (min)</th>
<th>Delta RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lidocaine</td>
<td>1) 235.18 &gt; 86.1</td>
<td>97</td>
<td>11</td>
<td>1.37</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 235.18 &gt; 58.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tramadol</td>
<td>1) 264.2 &gt; 58.1</td>
<td>97</td>
<td>15</td>
<td>1.20</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 264.2 &gt; 246.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>1) 278.2 &gt; 117</td>
<td>112</td>
<td>19</td>
<td>4.25</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 278.2 &gt; 105</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biperidene</td>
<td>1) 312.23 &gt; 98.1</td>
<td>123</td>
<td>19</td>
<td>4.23</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>2) 312.23 &gt; 55.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazepam</td>
<td>1) 287.06 &gt; 240.9</td>
<td>112</td>
<td>19</td>
<td>3.99</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 287.06 &gt; 268.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lorazepam</td>
<td>1) 321.02 &gt; 274.9</td>
<td>113</td>
<td>15</td>
<td>4.09</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 321.02 &gt; 302.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>1) 319.11 &gt; 86.1</td>
<td>112</td>
<td>15</td>
<td>4.63</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 319.11 &gt; 58.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diltiazem</td>
<td>1) 415.17 &gt; 177.9</td>
<td>128</td>
<td>19</td>
<td>3.73</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 415.17 &gt; 149.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naloxone</td>
<td>1) 328.16 &gt; 310</td>
<td>123</td>
<td>15</td>
<td>0.82</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 328.16 &gt; 212</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nortriptyline (IS)</td>
<td>1) 264.18 &gt; 233</td>
<td>97</td>
<td>7</td>
<td>4.17</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2) 264.18 &gt; 91</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 MgSO₄ 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.

Table 3. Series of Experimental Conditions Investigated

<table>
<thead>
<tr>
<th>Sample (1 mL)</th>
<th>Extraction solvent</th>
<th>Extraction salts (mg)</th>
<th>d-SPE</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WB</td>
<td>ACN</td>
<td>none</td>
<td>none</td>
<td>Sample: solid mass</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 1% AA</td>
<td>none</td>
<td>none</td>
<td>Sample: solid mass</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>None</td>
<td>none</td>
<td>Sample: loose particles</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>Nonbuffered, 500</td>
<td>none</td>
<td>Dark extract</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>AOAC, 500</td>
<td>none</td>
<td>Clear extract</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>EN, 650</td>
<td>none</td>
<td>Dark extract</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>Nonbuffered, 500</td>
<td>50 mg PSA, 150 mg MgSO₄</td>
<td>Clear extract</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>AOAC, 500</td>
<td>50 mg PSA, 150 mg MgSO₄</td>
<td>Clear extract</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>EN, 650</td>
<td>25 mg PSA, 150 mg MgSO₄</td>
<td>Clear extract</td>
</tr>
<tr>
<td>WB</td>
<td>ACN, 0.4% FA</td>
<td>EN, 650</td>
<td>50 mg PSA, 150 mg MgSO₄</td>
<td>Clear extract</td>
</tr>
</tbody>
</table>

WB = whole blood; ACN = acetonitrile, AA = acetic acid; FA = formic acid, PSA = primary secondary amine, AOAC = MgSO₄ and NaAcetate, EN= MgSO₄ and citrate buffers, Nonbuffered = MgSO₄ and sodium chloride
Figure 1. Addition of ACN (A) or ACN (1% AA) (B) to the whole blood, common solvents used in the QuEChERS method.

Figure 2. Addition of ACN (0.4% FA) to the whole blood.

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 4. Extract after the addition of d-SPE clean-up containing 150 mg MgSO₄ 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 MgSO₄.
**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.

![Graph](https://via.placeholder.com/150)

**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>
<thead>
<tr>
<th>Compound</th>
<th>25 ng/mL Spiked</th>
<th>50 ng/mL Spiked</th>
<th>100 ng/mL Spiked</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery</td>
<td>RSD</td>
<td>Recovery</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>81.6</td>
<td>35.3</td>
<td>98.7</td>
</tr>
<tr>
<td>Tramadol</td>
<td>97.2</td>
<td>18.6</td>
<td>105</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>85</td>
<td>13.6</td>
<td>104</td>
</tr>
<tr>
<td>Biperidine</td>
<td>75.5</td>
<td>14.8</td>
<td>97</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>60.4</td>
<td>17.3</td>
<td>77.0</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>68.4</td>
<td>17.0</td>
<td>81.9</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>75</td>
<td>14.1</td>
<td>110</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>63.7</td>
<td>15.8</td>
<td>88.1</td>
</tr>
<tr>
<td>Naloxone</td>
<td>68</td>
<td>12.1</td>
<td>80.6</td>
</tr>
</tbody>
</table>
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.

References


For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.
Fractionation of Acidic, Basic, and Neutral Drugs from Urine with an SPE Mixed Mode Strong Anion Exchange Polymeric Resin (Agilent SampliQ-SAX)

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.

Authors

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Department of Chemistry
Rhodes University
South Africa

Application Note
Forensic Toxicology
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].

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 MΩ cm) 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.

Figure 1. Structures of the drugs used: ketoprofen and naproxen (acidic), secobarbital (neutral), and nortriptyline (basic).
**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 CH₃COONa (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.

<table>
<thead>
<tr>
<th>Condition: 1 mL CH₃OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrate: 1 mL H₂O</td>
</tr>
<tr>
<td>Load: 2 mL urine spiked or unspiked with drugs</td>
</tr>
<tr>
<td>Wash: 1 mL 5% NH₄OH</td>
</tr>
<tr>
<td>Elution 1: 1 mL MeOH (elutes basic and neutral drugs)</td>
</tr>
<tr>
<td>Elution 2: 1 mL 2% HCOOH in CH₃OH (acidic drugs)</td>
</tr>
</tbody>
</table>

Figure 2. SPE procedure for acidic, basic, and neutral drugs using Agilent SampliQ SAX.

**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).

**Separation and analysis**

The HPLC conditions are shown in Table 1.

**Table 1. HPLC conditions.**

<table>
<thead>
<tr>
<th>Column</th>
<th>Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 75 mm, 3.5 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>1.5 mL/min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>5 µL</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Isocratic elution</td>
</tr>
<tr>
<td>A: 55% CH₃OH</td>
<td></td>
</tr>
<tr>
<td>B: 45% 25 mM KH₂PO₄ pH 7</td>
<td></td>
</tr>
<tr>
<td>Run time</td>
<td>8 min</td>
</tr>
<tr>
<td>Post time</td>
<td>1 min</td>
</tr>
<tr>
<td>Detection:</td>
<td>DAD at 222 nm</td>
</tr>
</tbody>
</table>
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.

![Graphs of calibration curves for secobarbital, nortriptyline, ketoprofen, and naproxen.](image)

Figure 4. Calibration curves (at the \( \lambda_{max} \) of each) for secobarbital, nortriptyline, ketoprofen, and naproxen.

### Table 2. Chemical and physical characteristics of the studied drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Classification</th>
<th>Log P</th>
<th>pKα</th>
<th>( \lambda_{max} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secobarbital</td>
<td>Neutral</td>
<td>1.97</td>
<td>7.90</td>
<td>222</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>Basic</td>
<td>4.28</td>
<td>9.70</td>
<td>242</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>Acidic</td>
<td>0.97</td>
<td>5.94</td>
<td>258</td>
</tr>
<tr>
<td>Naproxen</td>
<td>Acidic</td>
<td>3.18</td>
<td>4.53</td>
<td>230</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Drug</th>
<th>SPE fraction</th>
<th>Spike level (µg/mL)</th>
<th>Recovery (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secobarbital</td>
<td>Neutral</td>
<td>5</td>
<td>79.63 1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>92.70 0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>86.47 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>91.20 1.04</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>Neutral</td>
<td>2.5</td>
<td>86.48 0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>85.32 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>109.34 0.54</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>Acidic</td>
<td>2.5</td>
<td>99.18 0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>85.88 0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>106.97 0.18</td>
</tr>
<tr>
<td>Naproxen</td>
<td>Acidic</td>
<td>1</td>
<td>87.66 0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>83.41 0.06</td>
</tr>
</tbody>
</table>

SPE procedure for drugs in urine

Agilent Sampler 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.

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.**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Concentration range (0 – 8 µg/mL)</th>
<th>Linear equation</th>
<th>Correlation coefficient ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secobarbital</td>
<td></td>
<td>$y = 1.3325x$</td>
<td>$R^2 = 0.9993$</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td></td>
<td>$y = 17.595x$</td>
<td>$R^2 = 0.9991$</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td></td>
<td>$y = -1.2748x^2 + 17.896x$</td>
<td>$R^2 = 0.9991$</td>
</tr>
<tr>
<td>Naproxen</td>
<td></td>
<td>$y = -1.9003x^2 + 33.527x$</td>
<td>$R^2 = 0.9993$</td>
</tr>
</tbody>
</table>

The LOD and LOQ results are shown in Table 5. Equations 1 and 2 were used to calculate LOD and LOQ, where $Syx = \text{standard error of the regression line}$ and $b = \text{gradient}$.

**Table 5. LOD and LOQ for the analytes.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secobarbital</td>
<td>0.21</td>
<td>0.81</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>0.04</td>
<td>0.12</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>0.03</td>
<td>1.04</td>
</tr>
<tr>
<td>Naproxen</td>
<td>0.03</td>
<td>2.74</td>
</tr>
</tbody>
</table>

**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.
References

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
Bellah O. Pule, Lesego C. Mmualefe, and Nelson Torto
Department of Chemistry, Rhodes
South Africa

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.
**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), 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 MΩcm) 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.**

<table>
<thead>
<tr>
<th>Condition: 1 mL CH₃OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrate: 1 mL H₂O</td>
</tr>
<tr>
<td>Load: 1 mL spiked plasma</td>
</tr>
<tr>
<td>Wash: 1 mL 2% HCOOH</td>
</tr>
<tr>
<td>Elute 1: 1 mL CH₃OH (acidic and neutral)</td>
</tr>
<tr>
<td>Elute 2: 1 mL 5% NH₄OH/CH₃OH (basic analytes)</td>
</tr>
<tr>
<td>Add 50 µL of IS</td>
</tr>
<tr>
<td>Elution 1</td>
</tr>
<tr>
<td>Add 50 µL of IS</td>
</tr>
<tr>
<td>Elution 2</td>
</tr>
<tr>
<td>Analyze Fraction using LC-UV</td>
</tr>
</tbody>
</table>

*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.

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.
**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.
Elution 1: acidic and neutral drugs

[Graph showing chromatogram of drugs with peaks labeled: Acetaminophen, Ranitidine (IS), p-Toluamide, and Phenobarbital.]

Figure 6A. Chromatograms of drugs in plasma, elution 1: 1. acetaminophen, 2. ranitidine (IS), 3. p-toluamide, and 4. phenobarbital.

Elution 2: basic drugs

[Graph showing chromatogram of drugs with peaks labeled: Ranitidine (IS), Amphetamine, m-Toluidine.]

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>
<thead>
<tr>
<th>Drugs</th>
<th>Concentration range (0–8 µg/mL)</th>
<th>Linear equation (y)</th>
<th>Correlation coefficient ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>0.099x</td>
<td></td>
<td>0.9999</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>0.0853x</td>
<td></td>
<td>0.9991</td>
</tr>
<tr>
<td>p-Toluidine</td>
<td>0.138x</td>
<td></td>
<td>0.9990</td>
</tr>
<tr>
<td>m-Toluidine</td>
<td>0.1804x</td>
<td></td>
<td>0.9991</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.1526x</td>
<td></td>
<td>0.9991</td>
</tr>
</tbody>
</table>
Equations 1 and 2 were used to calculate LOD and LOQ, where $S_y$ is the standard error of the regression line and $b$ is the gradient.

**Equation 1**

$$\text{LOD} = \frac{3.3 \times S_y}{b} \quad (1)$$

**Equation 2**

$$\text{LOQ} = \frac{10.0 \times S_y}{b} \quad (2)$$

Table 3 shows the LOD and LOQ determined for each analyte.

**Table 3. LOD and LOQ for the analytes.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>0.39</td>
<td>0.85</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>0.71</td>
<td>1.87</td>
</tr>
<tr>
<td>p-Toluamide</td>
<td>0.66</td>
<td>0.70</td>
</tr>
<tr>
<td>m-Toluidine</td>
<td>0.35</td>
<td>1.06</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.82</td>
<td>1.89</td>
</tr>
</tbody>
</table>

**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.**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Class</th>
<th>Spike level (µg/mL)</th>
<th>Recovery</th>
<th>%RSD</th>
<th>Recovery</th>
<th>%RSD</th>
<th>Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td></td>
<td>2.5</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Neutral</td>
<td>81.45</td>
<td>0.41</td>
<td></td>
<td>95.08</td>
<td>0.48</td>
<td>92.62</td>
<td>0.22</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Basic</td>
<td>83.16</td>
<td>0.62</td>
<td></td>
<td>88.83</td>
<td>1.09</td>
<td>86.64</td>
<td>0.28</td>
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<tr>
<td>p-Toluamide</td>
<td>Acidic</td>
<td>85.88</td>
<td>0.65</td>
<td></td>
<td>96.86</td>
<td>0.18</td>
<td>94.40</td>
<td>0.07</td>
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<tr>
<td>m-Toluidine</td>
<td>Basic</td>
<td>81.97</td>
<td>0.51</td>
<td></td>
<td>89.16</td>
<td>0.33</td>
<td>94.86</td>
<td>0.10</td>
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<tr>
<td>Phenobarbital</td>
<td>Acidic</td>
<td>85.49</td>
<td>0.39</td>
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<td>90.13</td>
<td>0.25</td>
<td>80.48</td>
<td>0.21</td>
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References


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