Determination of Bath Salts (Pyrovalerone Analogs) in Biological Samples

Application Note

Forensic Toxicology

Abstract

A method has been developed on the Agilent 220 Quadrupole Ion Trap using EI-MS/MS for the identification and quantification of Pyrovalerone Analogs in biological samples. A working range of 50–1,000 µg/mL shows the method linearity of the Pyrovalerone Analogs

Introduction

Pyrrolidinopentiphenone (PVP), Methyleneoxydpyrovalerone (MDPV) and Naphylypyrovalerone (Naphyrone) are designer drugs marketed as bath salts. These drugs are analogs of Pyrovalerone. The drugs are marketed as legal substitutes for drugs such as Cocaine and MDMA.

This application note describes a method for the analysis of serum whole blood, vitreous fluid, urine, or tissue homogenate specimens. A minimum of 3 mL of sample is required for analysis.

Pyrrolidinopentiphenone (PVP), Pyrovalerone, MDPV, and Naphyrone and the internal standard Ropivacaine are extracted from alkalinized samples into an organic solvent using a liquid-liquid method of extraction. The extracts are reconstituted with ethyl acetate and injected into the Quadrupole Ion Trap GC/MS/MS for analysis.
Experimental

Standards and reagents

Reagents

- N-Chlorobutane (Nanograde), ethyl acetate, concentrated ammonia (NH₄OH), sodium carbonate/bicarbonate buffer pH = 9.8 (mix 100 g Na₂CO₃ and 50 g NaHCO₃ in 1,000 mL de-ionized water, adjust the pH to 9.8). Stable for 1 year at room temperature.
- Concentrated HCl, methanol (HPLC Grade), 0.1% HCl in methanol (dilute 0.1 mL of concentrated HCl in 100 mL of methanol. Stable for 1 year at room temperature).
- MDPV, naphyrone, and pyrovalerone stock standards (1 mg/mL in methanol) purchased from Cerilliant (Store in freezer until outdated).
- PVP stock standard purchased from Cayman Chemical (10-mg bottle), transfer contents to a 10-mL flask and fill to mark with methanol. Stable for 2 years in a freezer.
- Ropivacaine stock standard purchased from Sigma (R0283), 11.9 mg diluted to 10 mL with methanol. Stable for 2 years in a freezer.
- MDPV QC stock standard purchased from Cayman Chemical, 5 mg in 50 µL of Methyl Acetate (dilute to 5 mL in methanol). Stable for 1 year stored in a freezer.
- Naphyrone QC stock standard (1 mg/mL in methanol) purchased from Cerilliant. Store in a freezer until outdated.
- Pyrovalerone QC stock standard purchased from Cayman Chemical (5 mg in 100 µL of methanol, transfer contents to a 5-mL flask and dilute to mark with methanol. Stable for 2 years in freezer.
- PVP QC stock standard was purchased from Cayman Chemical as 10-mg bottle (transfer contents to a 10-mL flask and dilute to mark with methanol. Stable for 2 years in a freezer.

Working standards

- MDPV, naphyrone, pyrovalerone, PVP calibration intermediate standard 15 µg/mL (add 150 µL of each stock standard to a 10-mL flask and fill to 10 mL with methanol).
- MDPV, naphyrone, pyrovalerone, PVP QC intermediate standard 15 µg/mL (add 150 µL of each stock standard to a 10-mL flask and fill to 10 mL with methanol).
- Working internal standard ropivacaine 15 µg/mL (add 150 µL of stock solution to a 10-mL flask and fill to 10 mL with methanol). Store at 2–8 °C. Stable for 1 year.

Controls and Calibration Standards

Negative Control- drug free whole blood obtained from American Red Cross or pooled blank urine. The matrix must be tested prior to use and found to be drug free. When stored at –20 °C, it is stable for 1 year.

Low Control (125 ng/mL) - Prepared in blood or urine fresh as needed, 25 µL of the QC Intermediate Standard to 3 mL blank blood or urine.

High Control (500 ng/mL) - Prepared in blood or urine fresh as needed, 100 µL of the QC Intermediate Standard to 3 mL blank blood or urine.

Sample Preparation

Prepare a calibration curve using the working standard and drug free blood or urine as follows:

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Volume of Standard (µL) and Volume of Blood/Urine (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>10 µL std. and 3 mL blood/urine</td>
</tr>
<tr>
<td>100</td>
<td>20 µL std. and 3 mL blood/urine</td>
</tr>
<tr>
<td>250</td>
<td>50 µL std. and 3 mL blood/urine</td>
</tr>
<tr>
<td>500</td>
<td>100 µL std. and 3 mL blood/urine</td>
</tr>
<tr>
<td>1,000</td>
<td>200 µL std. and 3 mL blood/urine</td>
</tr>
</tbody>
</table>

Pipet 3 mL of samples, negative, and positive controls into labeled 16 × 100 mm culture tubes. Add 50 µL of working internal standard and 2 mL of pH 9.8 Carbonate Buffer, add two drops of NH₄OH to each tube and vortex gently to mix. Add 7.0 mL of n-butyl chloride to each tube, cap, and rotate all tubes for at least 10 minutes. Centrifuge all tubes at 3,000 RPM for 10 minutes. Transfer organic (top) layer to a clean, labeled 16 × 100 culture tube. Add two drops of 0.1% methanolic HCl and evaporate to dryness at 37 °C with nitrogen. Reconstitute dried extracts with 200 µL of ethyl acetate and transfer to autosampler vials with inserts, cap and transfer to the GC/MS/MS for analysis.
GC/MS Ion Trap Analysis

Column: DB-5MS or equivalent 25 m × 200 mm, 0.33 μm
Injection volume: 0.5 μL
Injection mode: Splitless
Inlet temperature: 250 °C
Carrier gas: Helium
Column flow: 1.3 mL/min
Oven program: 70 °C; 1 minute hold
25 °C/min to 310 °C; 4.4 minute hold

Quadrupole Ion Trap MS Conditions

Tune: Auto-tune
Acquisition: EI-MS/MS 50–200 da
Solvent delay: 7.0 minutes
MS temperatures: Trap 210 °C, Manifold 50 °C, Transfer line 310 °C

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rt(min)</th>
<th>Precursor</th>
<th>Quant ion</th>
<th>Qualifiers</th>
<th>Excit volt</th>
<th>Filament</th>
<th>Multiplier</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP</td>
<td>8.1</td>
<td>126</td>
<td>84</td>
<td>124/97</td>
<td>0.5 V</td>
<td>50 μA</td>
<td>+50 V</td>
<td>3,000</td>
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<tr>
<td>Provalerone</td>
<td>8.6</td>
<td>126</td>
<td>84</td>
<td>124/97</td>
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<td>50 μA</td>
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</tr>
<tr>
<td>MDPV</td>
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<td>84</td>
<td>124/97</td>
<td>0.5 V</td>
<td>50 μA</td>
<td>+50 V</td>
<td>3,000</td>
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<tr>
<td>Ropivacaine IS</td>
<td>9.95</td>
<td>126</td>
<td>84</td>
<td>98/56</td>
<td>0.5 V</td>
<td>50 μA</td>
<td>+50 V</td>
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<tr>
<td>Naphyrone</td>
<td>10.38</td>
<td>126</td>
<td>84</td>
<td>124/97</td>
<td>0.5 V</td>
<td>50 μA</td>
<td>+50 V</td>
<td>3,000</td>
</tr>
</tbody>
</table>

Results and Discussion

The following criteria are used to determine the presence and amount of the pyrovalerone analog:

- The chromatography is acceptable (peak resolution, peak symmetry, absence of carryover). The selected ions for quantitation and qualification are present. The retention times of the presumed pyrovalerone analog from the test specimen is within ± 2% of the retention times for the latest calibration.

- The area of the analog and the internal standard quantitative ions are used for quantitative analysis. Quantitation is accomplished by comparison of the relative response of unknowns and controls against a calibration curve produced from the relative responses for each calibrator concentration. The positive controls must be within their target ranges and the pyrovalerone analogs must be absent in the negative control.

- Appropriate m/z ions must be observed. The test specimens and positive controls must exhibit m/z ions resulting from the ionization of 126 m/z at the respective retention times for pyrovalerone, MDPV, PVP, naphyrone, and ropivacaine IS.

- The two ion ratio method is used for identification. For Pyrovalerone, PVP, MDPV, and Naphyrone the abundance for the m/z ion peaks 124 and 97 are divided by the ion abundance of base ion peak 84. Calculated ion ratios are within 20% of the target values determined from the calibration.

<table>
<thead>
<tr>
<th>Linearity</th>
<th>50–1,000 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of detection (LOD)</td>
<td>20 ng/mL</td>
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<tr>
<td>Limit of quantitation (LOQ)</td>
<td>50 ng/mL</td>
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<tr>
<td>Interferences</td>
<td>None were noted</td>
</tr>
</tbody>
</table>
PVP Calibration

\[ R^2 = 0.99827250 \]

Method Limits

Batch Results

Note tags for outliers and below calibration.
Pyrovalerone Calibration

$R^2 = 0.99126376$

500 ng/mL Standard

Batch Results

Note tags for outliers and below calibration.
**MDPV Calibration**

![MDPV Calibration Graph](image)

**500 ng/mL Standard**

![500 ng/mL Standard Graph](image)

**Batch Results**

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Name</th>
<th>Date</th>
<th>Time</th>
<th>MDPV Measured</th>
<th>MDPV Results</th>
<th>Qualifier</th>
<th>Response</th>
<th>Error</th>
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</thead>
<tbody>
<tr>
<td>CAL1</td>
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<td>1/3/2012 3:12 PM</td>
<td>50.000</td>
<td>9.663</td>
<td>126.0</td>
<td>84.0</td>
<td>126.0</td>
<td>124.1</td>
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<td>82.0</td>
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<td>90.0</td>
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<td>3/5/2012 3:15 PM</td>
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<td>3.600</td>
<td>98.0</td>
<td>96.0</td>
<td>98.0</td>
<td>96.0</td>
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<td>HLSH</td>
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<td>92.0</td>
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<td>124.1</td>
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<td>208 BLOOD</td>
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<td>94.0</td>
<td>92.0</td>
<td>94.0</td>
<td>92.0</td>
</tr>
</tbody>
</table>

Note tags for outliers and below calibration.
Naphyrone Calibration

$R^2 = 0.95897912$

500 ng/mL Standard

Batch Results

Note tags for outliers and below calibration.
Conclusions

This application note presents a sensitive, selective, and robust method to determine pyrovalerone analogs in biological samples using ropivacaine as an internal standard. For the analysis of pyrovalerone analogs, the benefits of GC Quadrupole Ion Trap MS/MS cannot be underestimated. In terms of reducing sample matrix interference, improving signal-to-noise, and coupling its high selectivity and sensitivity, the GC Quadrupole Ion Trap MS/MS provides a more confidence driven solution for the analysis of pyrovalerone analogs. GC Quadrupole Ion Trap MS/MS analysis has the potential to reduce false positive and negatives as well as providing an additional degree of confidence in the results obtained. Using the optimized method listed above, a fast, targeted GC/MS/MS method can be used to solve the current pyrovalerone Analog analysis problem facing forensic laboratories. Positive controls were used in conjunction with negative controls to assure accurate quantification and rule out false negatives in the unknown biological samples. Low ng/mL detection limits were observed for pyrovalerone analogs in various sample matrices.

References

5. Saint Louis University Forensic Toxicology-Standard Operation Procedures: Blood Drug Screen by GCNPD.

Acknowledgement

Saint Louis University Forensic Toxicology Laboratory for providing the data used in this study.

For More Information

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