RECENT ADVANCES
IN DOPING ANALYSIS
(9)

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(Editors)

Sport und Buch Strauß, Köln, 2001

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Diuretics Screening and Confirmation Using LC/MS/MS
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in
doping analysis (9). Sport und Buch Strauß, Köln, (2001) 321-326
Diuretics Screening and Confirmation using LC/MS/MS

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INTRODUCTION

Diuretics promote the excretion of water and electrolytes by the kidneys and have been banned in sport since 1988. Diuretics can therefore be used as masking agents for banned substances as a result of dilution effects in urine. They may also be used to allow rapid weight loss simply by passing water and so have been used by athletes to participate in a lower weight class.

Diuretics have been screened using a number of methods such as HPLC and GCMS of methylated derivatives (1-12). Studies in our laboratory have shown that some diuretics such as benzthiazide are difficult to detect by GCMS using traditional methylation methods so this method of screening is limited and also while they may be detected using HPLC their confirmation is difficult. It was thus useful to investigate the use of LCMS as a multiresidue procedure for diuretics. The data for four diuretics which are the most difficult to detect using extractive alkylation are presented here.

The procedure evaluated involved:

- Combining a high recovery extraction method\(^1\) with a relatively new, powerful analytical tool.
- pH controlled liquid-liquid extraction and wash.
- RP HPLC with MS/MS detection using internal standard quantification.
- Confirmation according to IOC criteria.
EXPERIMENTAL

Sample preparation was as follows:

- Urine (2mL) to which methaneside internal standard had been added was buffered at pH 5 with acetate buffer (2M),
- Extract diuretics by shaking for 30mins with ethyl acetate (4mL), centrifuging and removing the organic layer.
- Add 1 ml of phosphate buffer 0.2 M (pH 6) to the organic layer and shake for 15 minutes, centrifuge and remove organic layer.
- Evaporate to dryness under nitrogen
- Reconstitute in 50:50 methanol/water (200uL)
- Analyse by LC/CM/SMS

LC/MS/MS Instrumentation and Conditions

<table>
<thead>
<tr>
<th>HPLC System</th>
<th>Waters 2690 Separations Module – Alliance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass Spectrometer</td>
<td>Micromass Quattro LC triple quadrupole</td>
</tr>
<tr>
<td>Sample inlet mode</td>
<td>Electrospray</td>
</tr>
<tr>
<td>Ionisation mode</td>
<td>Positive</td>
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</tbody>
</table>

HPLC Conditions

<table>
<thead>
<tr>
<th>SCREEN RUN</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC Column</td>
</tr>
<tr>
<td>Guard column</td>
</tr>
<tr>
<td>Mobile Phase</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
</tr>
<tr>
<td>Injection volume</td>
</tr>
<tr>
<td>Column Temperature</td>
</tr>
<tr>
<td>HPLC run time</td>
</tr>
<tr>
<td>LC run program</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
</tbody>
</table>
**Multiple Reaction Monitoring**

| Mass Scanning Mode for screening | Multiple Reaction Monitoring (MRM)  
m/z: 295.82 > 278.89 (Chlorothiazide)  
m/z: 348.95 > 264.03 (Torasemide)  
m/z: 354.90 > 121.69 (Xipamide)  
m/z: 383.13 > 81.00 (Mefruside ISTD)  
m/z: 431.83 > 91.01 (Benzthiazide) |
|---------------------------------|----------------------------------------------------------------------------------|
| Mass Scanning Mode for          | Multiple Reaction Monitoring (MRM)  
m/z: 295.82 > 279, 205, 215, 296 (Chlorothiazide)  
m/z: 348.95 > 349, 264, 125, 168, 183, 219, 230, 290 (Torasemide)  
m/z: 354.90 > 355, 122, 107, 234 (Xipamide)  
m/z: 383.13 > 81.00 (Mefruside ISTD)  
m/z: 431.83 > 91, 432 (Benzthiazide)  
confirmation                      | monitoring includes parent ion                                                    |
| Retention Time                  | Chlorothiazide - 0.94 min  
Torasemide ~ 2.48 min  
Xipamide - 4.45 min  
Mefruside ISTD - 2.46 min  
Benzthiazide - 2.93 min |

**RESULTS**

The mass spectral data for the screening process is shown in the example below (FIG 1) for a 0.1μg/mL spiked urine sample. This is shown at the minimum level at which diuretics detection is monitored in the IOC reaccreditation. It can be seen that the signal to noise at this concentration is excellent and the final detection limits are well below this. The mass spectral fragmentation for these transitions can be monitored by measuring several ions or by scanning the appropriate range.
FIG 1 Example LC/MS/MS Chromatograms (0.1 µg/ml<sup>-1</sup> spike in urine)

Name: CF_000628_25
Text: 100/5

1: mefruside  2: benzbthiazide  3: chlorothiazide

4: torasemide  5: xipamide

The results for detection limits, repeatability and recovery are shown in the Table1 below.

Table 1  Validation Data

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Recovery</th>
<th>Method Detection Limit (µg/ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Instrument Repeatability (%CV, n=20)</th>
<th>Method Repeatability (%CV, n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzthiazide</td>
<td>99</td>
<td>0.005</td>
<td>2.3</td>
<td>6.2</td>
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<td>Chlorothiazide</td>
<td>54</td>
<td>0.020</td>
<td>6.8</td>
<td>9.0</td>
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<tr>
<td>Torasemide</td>
<td>86</td>
<td>0.002</td>
<td>3.5</td>
<td>7.3</td>
</tr>
<tr>
<td>Xipamide</td>
<td>99</td>
<td>0.002</td>
<td>3.4</td>
<td>8.7</td>
</tr>
<tr>
<td>Mefruside (IS)</td>
<td></td>
<td></td>
<td>3.7</td>
<td></td>
</tr>
</tbody>
</table>

Recovery based on 0.1 µg/ml<sup>-1</sup> fortified urines. Method repeatability determined using low level fortified urines (0.010 or 0.020 µg/ml<sup>-1</sup>).
DISCUSSION

The method has been found to have a number of advantages:

- Rapid, low-cost sample preparation and handling
- High sample throughput
- Combines detection and confirmation methods (when required)
- High sensitivity, low method detection levels (sensitivity varies for individual diuretics and each needs to be determined separately).
- ISO G25 accredited
- Analytes are fully quantifiable
- While the data for four diuretics is presented here the method can be extended to include all currently monitored diuretics.

Bibliography


