Strategy for identification and confirmation of 4-methyl-2-hexaneamine in human urine in doping control

National Dope Testing Laboratory, Ministry of Youth Affairs and Sports, New Delhi, India.
Email: ndtlindia@nic.in

Introduction

The 4-methyl-2-hexaneamine (MHA) was introduced in WADA prohibited list under the category of non specified stimulants in 2010 which was later shifted under specified stimulants in 2011. It belongs to the class of primary sympathomimetic amines with an active chiral centre. It is an analogue to 2-aminoheptane which has topical decongestant activity. The root extract of the Geranium plant (Pelargonium graveolens) is a constituent of perfumes and edible oils, which is reported to contain 4-methyl-2-hexaneamine.

In National Dope Testing Laboratory (NDTL), India, several doping control urine specimens showed a suspicious peak in the detection time window of 2-aminoheptane in LC-MS/MS analysis but at a different retention time, potentially being 4-methyl-2-hexaneamine. The aim and objective of the present work was to establish an identification and confirmation method for the analysis for 4-methyl-2-hexaneamine in human urine.

Material and Methods

The reference standard of 4-methyl-2-hexaneamine was gifted by the Drug Control Centre, London. The two confirmatory methods were used as detailed in Table 1. Both the methods were validated as per the guidelines of WADA ISL (version 6.0).
<table>
<thead>
<tr>
<th>GC-MS Analysis</th>
<th>LC-MS/MS Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine sample (5ml)</td>
<td>Urine sample (2/4ml)</td>
</tr>
<tr>
<td>pH (14) with 0.5 ml 5N KOH</td>
<td>pH (7) with 1 ml 0.2M Phosphate buffer</td>
</tr>
<tr>
<td>ISTD (Diphenylamine &amp; 10-N-methylphenothiazine)</td>
<td>50µl β-glucuronidase (E.Coli.) Incubate for 1 hours Δ 60°C</td>
</tr>
<tr>
<td>Tertiary Butyl Methyl Ether (TBME) (2 ml)</td>
<td>pH (9-10) with 1 ml 7% K₂CO₃</td>
</tr>
<tr>
<td>Sodium Sulphate (Na₂SO₄) (3 gms)</td>
<td>Add TBME (5 ml); Shake &amp; centrifuge</td>
</tr>
<tr>
<td>Shake &amp; centrifuge</td>
<td>TBME extract</td>
</tr>
<tr>
<td>TBME extract</td>
<td>Aqueous layer</td>
</tr>
<tr>
<td>Concentrate up to 100 µl</td>
<td>pH (4-5) using 6N HCl 150 µl</td>
</tr>
<tr>
<td>Carbon di sulphide 100 µl</td>
<td>Ethylacetate (4 ml); Shake &amp; centrifuge</td>
</tr>
<tr>
<td>15 min at ambient temp.</td>
<td>Organic extract</td>
</tr>
<tr>
<td>Combined extract Dried under N₂ stream</td>
<td>Reconstitute in mobile phase (50:50 v/v)</td>
</tr>
</tbody>
</table>

**Table 1:** Sample preparation steps & instrumental conditions for GC-MS & LC-MS/MS analysis

**GCMS (Agilent 5973 N)**
- Column HP5MS, Fused Silica 0.2mm I.D.X 15.7m Length Column Film thickness 0.33µm
- Oven Initial 60°C - Hold 1 min - Rate 20°C/Min - Next 250°C - Hold 0.0 min - Rate 40°C/min - Final 300°C - Hold 3 min
- Injector Temperature 250 ºC
- Detector Temperature 300 ºC
- Split Ratio 11:1
- Carrier Flow 100 kpa
- He at 60 ºC
- MS Mode Scan Injection Volume 2 µl

**HPLC Agilent 1100 Series**
- Column ZORBAX Eclipse XDB-C-8
- Solvent 1% Formic Acid (A) & Acetonitrile (B)
- Flow 900 µl/minute
- Gradient 0 min-15%B, 6 min-60%B, 9 min-100%B, 10 min-15%B, 12 min-15%B Injection volume 2 µl
- Mass Spectrometer Sciex API 5500, Triple Qudruple QTrap
- Ionization ESI Positive Collision Gas Nitrogen
- Source Temperature 550°C
Results and Discussion

The confirmation methods on GC-MSD and LC-MS/MS showed good separation of two isomers of MHA (Figure 1-2). The LC-MS/MS chromatogram showed distinct separation of 2-aminoheptane peak from the two isomers of MHA (Figure 3). The two confirmation methods were validated and applied to the suspicious samples for the confirmation of MHA at NDTL. A total of 21 adverse analytical findings (AAF) were reported from NDTL, India since August 2010 for various sport disciplines (Figure 4). The percent AAF of stimulants in NDTL raised from 10.5% (2009) to 24.5% (2010) as against international statistics given by WADA which shows worldwide increase of stimulants from 6.4 % (2009) to 10.3% (2010). This may be attributed to sharp increase in MHA cases found in various sports disciplines worldwide. It is revealed from WADA statistics that 21.4% (123 out of 574 AAFs of stimulants) cases were reported for MHA in 2010, whereas, 34% (17 out of 50 AAFs of stimulants) cases were reported by NDTL in 2010.

Conclusion

The combination of two analytical methods was successfully employed for the confirmation purpose of 4-methyl-2-hexaneamine in doping control. Further, work is in progress to study the excretion pattern of 4-methyl-2-hexaneamine following local/oral administration of food supplements containing the drug.

Acknowledgement

The financial support of Ministry of Youth Affairs and Sports, Govt. of India is duly acknowledged. We express our sincere thanks to Prof. David Cowan for gracious gift of reference standard for methylhexaneamine and for the expert opinion on confirmation data.

References:

Figure 1: Total Ion chromatogram showing diastereoisomers of 4-methyl-2-hexanamine isothiocyanate in spiked urine at 500 ng/ml

Figure 2: Total Ion chromatogram showing diastereoisomers of 4-methyl-2-hexanamine isothiocyanate in positive urine sample

Figure 3: Total Ion chromatogram showing separation of isomers of 4-methyl-2-hexanamine and 2-aminoheptane in spiked urine at 100ng/ml

Figure 4: Number of AAF reported in 2010 for methylhexanamine at NDTL India