Dubey S, Kaur T, Ahi S, Beotra A, Jain S

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 [1]. 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 [(2)]. 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. [(3-4)]

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

GC-MS Analysis	LC-MS/MS Analysis
Urine sample (5ml) pH (14) with 0.5 ml 5N KOH ISTD (Diphenylamine & 10-N-methyl-phenothiazine) Tertiary Butyl Methyl Ether (TBME) (2 ml) Sodium Sulphate (Na_2SO_4) (3 gms) Shake & centrifuge TBME extract Concentrate up to 100 µl Carbon di sulphide 100 µl 15 min at ambient temp.	Urine sample (2/4ml) pH (7) with 1 ml 0.2M Phosphate buffer 50µl β-glucuronidase (*E.Coli.*) Incubate for 1 hours Δ 60°c pH (9-10) with 1 ml 7% K_2CO_3 Add TBME (5 ml); Shake & centrifuge TBME extract Aqueous layer pH (4-5) using 6N HCl 150 µl Ethylacetate (4 ml); Shake & centrifuge Organic extract Combined extract Dried under N_2 stream Reconstitute in mobile phase (50:50 v/v)
GCMS (Agilent 5973 N) Column HP5MS, Fused Silica 0.2mm I.D.X 15.7m Length Column Film thickness 0.33µm Oven Initial 60^0C - Hold 1 min - Rate 20^0C/Min - Next 250^0C - Hold 0.0 min - Rate 40^0C/min - Final 300^0C - Hold 3 min Injector Temperature 250 ^{0}C Detector Temperature 300 ^{0}C Split Ratio 11:1 Carrier Flow 100 kpa He at 60 ^{0}C 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 550^0C

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

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References:

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4. M. Thevis, G. Sigmund, H. Geyer, W. Schänzer, Stimulants and Doping in Sports, *Endocrinol Metab Clin N Am 39, 2010, p 89-105*

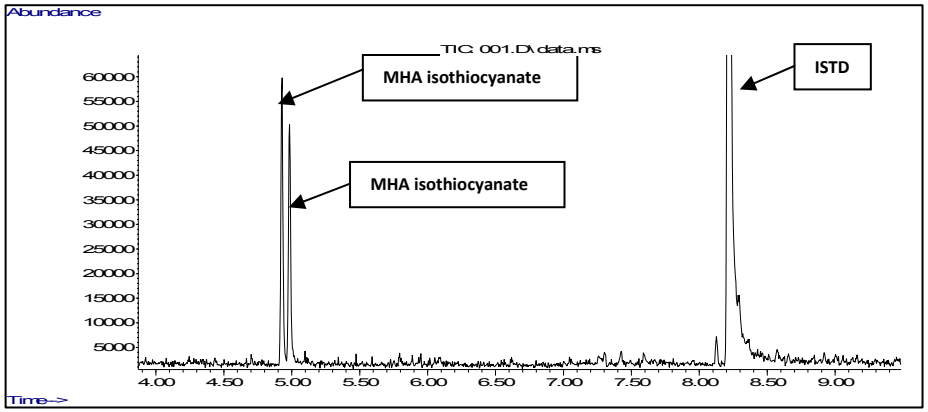

Figure 1: Total Ion chromatogram showing diastereoisomers of 4-methyl-2-isothiocyanate in spiked urine at 500 ng/ml

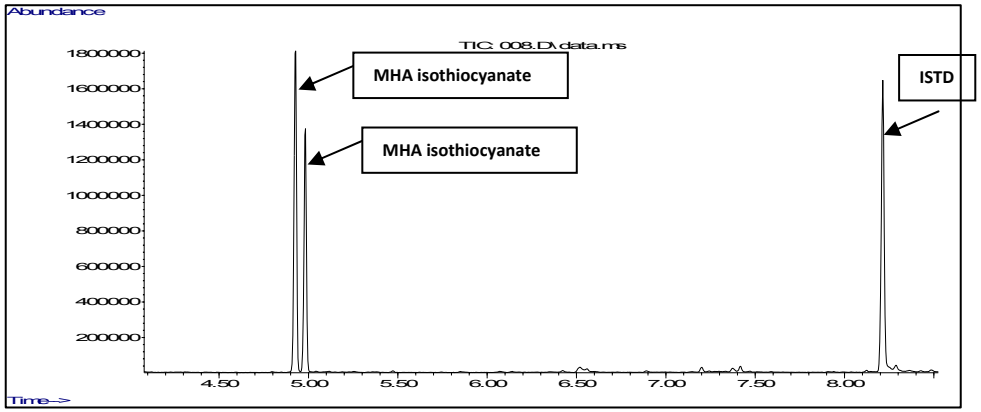

Figure 2: Total Ion chromatogram showing diastereoisomers of 4-methyl-2-hexaneamine hexanamine isothiocyanate in positive urine sample

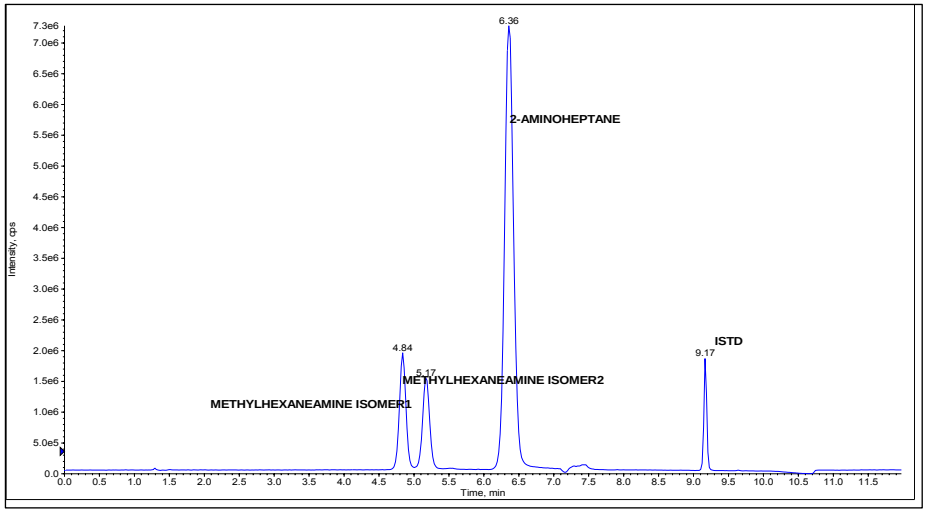

Figure 3: Total Ion chromatogram showing separation of isomers of 4-methyl-2-hexaneamine and 2-aminoheptane in spiked urine at 100ng/ml

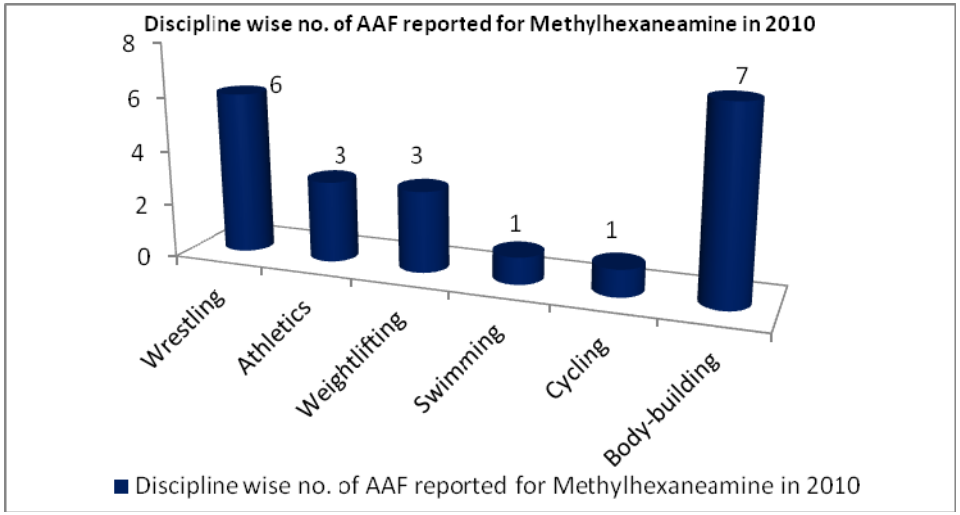

Figure 4: Number of AAF reported in 2010 for methylhexaneamine at NDTL India