Journal of Chromatography B, 877 (2009) 3767–3770

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Detection in urine of 4-methyl-2-hexaneamine, a doping agent

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ARTICLE INFO

Article history:
Received 11 May 2009
Accepted 12 September 2009
Available online 17 September 2009

Keywords:
Doping
Stimulant
Mass spectrometry
Liquid chromatography
Gas chromatography

ABSTRACT

Stimulants are banned in-competition for all categories of sports by the World Anti-Doping Agency. A simple liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay employing electrospray ionisation in positive mode was developed in that work for the quantification in urine specimens of 4-methyl-2-hexaneamine, a primary amine exhibiting sympathomimetic properties. Following a simple pretreatment procedure, the analyte was separated using a gradient mobile phase on reverse phase C8 column. Selected reaction monitoring m/z 116.2 → 57.3 was specific for detection of 4-methyl-2-hexaneamine and the assay exhibited a linear dynamic range of 50–700 ng/mL. The validated method has been successfully applied to analyze the target compound in food supplements as well as in urine specimens. The administered drug (40 mg) was detected at the level of 350 ng/mL in the urine up to 4 days.

1. Introduction

Stimulants have found a widespread use in sport, in particular to improve the performance on the day of competition [1]. It is expected that administration of these drugs will result in an increase of alertness, competitiveness and aggression. Stimulants include 2-heptylamine (tuaminoheptane) on the list of substance prohibited in-competition for all categories of sports by the World Anti-Doping Agency (WADA) [2]. In the pharmaceutical point of view, this compound is a primary amine used in its sulfate form for the relief of nasal congestion [3]. Except for strychnine, the minimum required performance limit (MRPL) for the detection of stimulants at which laboratories accredited by WADA must operate is established at 500 ng/mL [4]. Recently, Thevis et al. developed an assay for the determination of tuaminoheptane in doping control urine samples [5]. Following liquid–liquid extraction, gas chromatography–mass spectrometry (GC–MS) equipped with an additional nitrogen-phosphorus detector (NPD) was applied with and without derivatization for the quantification of tuaminoheptane in the concentration range from 100 to 4000 ng/mL.

The list of stimulants reported as prohibited substances by WADA is not exhaustive since it is also mandatory for anti-doping laboratories to determine other substances displaying a similar chemical structure or similar biological effects [2]. In that respect, 4-methyl-2-hexaneamine, an analog of tuaminoheptane, has received a particular attention in the media [6]. Indeed, this compound is contained in some dietary supplements and consequently, its ingestion may potentially result in an adverse analytical finding depending on the metabolism of the drug and its detection window in urine. As a result of this concern, a LC–MS/MS assay for the quantification of 4-methyl-2-hexaneamine in human urine specimens was validated in terms of specificity, precision, linearity to investigate the inter-individual variability in excretion kinetic of the drug.

2. Experimental

2.1. Chemicals and reagents

All solvents and reagent were of analytical grade purity. Tert-butyl-methyl-ether (TBME), potassium hydroxyde, sodium chloride and potassium carbonate anhydrous were obtained from Acros Organics (Geel, Belgium). Ethyl acetate was purchased from Panreac (Barcelona, Spain). Acetonitrile was supplied by Biosolve (Valkerswaard, The Netherlands). Cyclohexanone, 2-heptylamine (tuaminoheptane) and sodium sulfate anhydrous were obtained from Fluka (Buchs, Switzerland). Reference compound 4-methyl-2-hexaneamine was purchased from Sigma–Aldrich (Sigma–Aldrich Library of Rare Chemicals, Milwaukee, USA). Sodium hydrogen carbonate was supplied by Merck (Darmstadt, Germany). Ultrapure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a Quantum™ EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA).

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doi:10.1016/j.jchromb.2009.09.013

2.2. Urine samples

A urine specimen was found to contain 4-methyl-2-hexaneamine during a routine doping procedure carried out in the doping control laboratory of Lausanne (Switzerland). The A-sample and B-sample were stored at −20 °C until confirmation and counter-analysis, respectively.

Specificity tests were conducted on 1200 different urine samples from elite athletes (in- or out-competition testing). These specimens were stored without additives at −20 °C until analysis.

Two Caucasian healthy male volunteers were self-administered a food supplement (Geranamine from Predator Nutrition, UK) containing about 40 mg of 4-methyl-2-hexaneamine as indicated by the manufacturer. The study was in accordance with the Helsinki declaration of 1975 and all of the subjects gave their written informed consent. Baseline urine samples were obtained before initial administration, and subsequent spot urine samples were collected over a period of 140 h after the capsule administration. Each urine sample was divided into 20-mL flasks and stored without additives at −20 °C until analysis.

2.3. LC–MS/MS analysis

Urine samples were thawed at 37 °C and centrifuged at 1350 × *g* for 5 min before sample treatment. Internal standard tuaminoheptane (1 μg) was added to 2 mL of urine. The sample was saturated with solid potassium carbonate and further extracted at basic pH with 3 mL ethyl acetate by shaking for 10 min. After centrifugation at 1350 × *g* for 5 min, the organic layer was collected. Extraction of the aqueous layer was repeated twice with 3 mL of ethyl acetate. Then, the organic fractions were pooled and one drop of EtOH with 1% (v/v) HCl was added before evaporation. The residue was dissolved in 125 μL of ammonium formiate buffer 10 mM pH 4/acetonitrile (90/10). Finally, the solution was transferred into a vial prior to LC–MS/MS analysis.

A portion (20.7 mg) of the dietary supplement contained in a cap (200 mg) was transferred into a 250-mL volumetric flask. Internal standard tuaminoheptane (5 μg) was added together with a volume of about 100 mL methanol. After sonication for 10 min, the flask was filled up with methanol. A volume of this solution (20 μL) was mixed with 180 μL of ammonium formiate buffer 10 mM pH 4 into a vial prior to LC–MS/MS analysis.

The LC–MS/MS system consisted of a Rheos 2200 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and a HTS Pal autosampler (CTC analytics AG, Zwingen, Switzerland) coupled to a triple stage quadrupole mass spectrometer TSQ Quantum Discovery Max (ThermoFinnigan, San Jose, CA, USA), equipped with an atmospheric pressure ionisation interface Ion MAX™ operated in ESI mode. The separation was performed on a Sunfire™ C8 column (100 mm × 2.1 mm, 3.5 μm) from Waters (Milford, MA, USA). A low dispersion in-line filter (0.2 μm frits) from Agilent (Palo Alto, CA, USA) was installed between the injector and the column. For the analysis of 4-methyl-2-hexaneamine, elution solvents were ammonium formiate buffer 10 mM pH 4 in water (A) and acetonitrile (B). The gradient percentage of organic solvent (B) started with 10% for 3 min, changed linearly to 40% during a period of 2 min, then linearly to 80% during a period of 1 min followed by an isocratic elution for 0.5 min. After returning to initial conditions, the system was equilibrated for 1 min. The flow rate was set to 350 μL/min, and the injected volume was 10 μL. The column temperature and the autosampler tray were set at 25 °C and 12 °C, respectively. The mass spectrometer was operated in positive ionisation mode, collision offset voltage was set at 18 V for both 4-methyl-2-hexanamine and tuaminoheptane (IS). The first (Q1) and third (Q3) quadrupoles were set at 0.7 amu mass resolution. Scan time and scan width were 0.15 s and 1.0 amu, respectively, and each chromatographic peak was the result of at least 10 scans. The ionisation conditions were the following: the capillary temperature 320 °C and ESI spray voltage 4 kV. The sheath and auxiliary gas (nitrogen) flow rate was 40 and 30 (arbitrary units), respectively. The tube lens voltages were set to 100 V and the Q2 collision gas (argon) pressure was 0.2 Pa.

2.4. GC–MS analysis

Urine samples were thawed at 37 °C and centrifuged at 1350 × *g* for 5 min before sample treatment. Internal standard tuaminoheptane (1 μg) and 0.2 mL KOH 5 M were added to 2 mL of urine. Approximately 200 mg of solid sodium chloride were dissolved, and the sample was further extracted with 0.8 mL TBME by shaking for 10 min. After centrifugation at 1350 × *g* for 5 min, the organic layer was collected. Finally, 4-methyl-2-hexanamine and tuaminoheptane were derivatized with 20 μL cyclohexanone for 20 min at 25 °C. Prior to GC–MS analysis, the phase was dried on a minimum amount of anhydrous sodium sulfate and the solution transferred into a vial.

Samples were analyzed in full-scan mode for identification purpose only. The gas chromatograph was a Hewlett–Packard 6890 (HP\Analytical division, Waldbronn, Germany) coupled to a HP 5973 mass selective detector (MSD). Chromatography was achieved on a ZB-5 capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) from Phenomenex (Torrance, CA, USA). The initial oven temperature set at 40 °C for 1 min and thereafter increased 25 °C/min to 310 °C, and finally held for 1.5 min. Samples (1 μL) were injected in the splitless mode. The injector temperature was set at 200 °C. Helium was used as carrier gas at a constant flow of 2 mL/min. The quadrupole and ion source temperatures were set at 150 °C and 230 °C, respectively. The MS instrument was operated in the electron ionisation mode at 70 eV. The scan range was *m*/*z* 40–300 with a scan rate of 2.78 scans/s.

3. Results and discussion

3.1. Chromatography and mass spectrometry

At the occasion of a routine LC–MS/MS analysis of a urine sample collected in-competition, two unresolved peaks were observed in the detection window of tuaminoheptane corresponding to a single reaction monitoring (*m*/*z* 116.2 → 57.3). However, none of the peaks did match the retention time (RT) of tuaminoheptane, though the MS/MS spectra were identical. Actually, two peaks were found to elute roughly 1 min earlier than tuaminoheptane using the applied HPLC conditions (Fig. 1). Furthermore, all of these signals exhibited identical MS/MS spectra in the positive mode, thereby indicating that there were likely isomers of similar structure. Based on a brief search in the literature [6], we suspected the primary amine 4-methyl-2-hexaneamine, an analog of tuaminoheptane contained in a dietary supplement, to be the unknown compound. Remarkably, an authentic standard of 4-methyl-2-hexaneamine exhibited by LC–MS/MS two unresolved peaks with identical retention times as those obtained for the unknown compound in the urine specimen. Alltogether, these results demonstrated that the compound with two stereogenic centres consisted actually of a mixture of diastereoisomers in both the standard preparation and the biological sample. Structural information provided by ^{1}H, ^{13}C NMR experiments measured at a 300 MHz ^{1}H Larmor frequency further confirmed the presence of the diastereoisomers. The excellent match of the experimental ^{13}C data with literature data [7] confirms the assignment (see Table 1). The proton spectra of the two diastereoisomers nearly perfectly overlap. The expected two nearly degenerated triplets of quartet of H-C^{α} between 3.05 and 2.9 ppm were observed. Furthermore, the terminal methyls (C^{ε}) coming

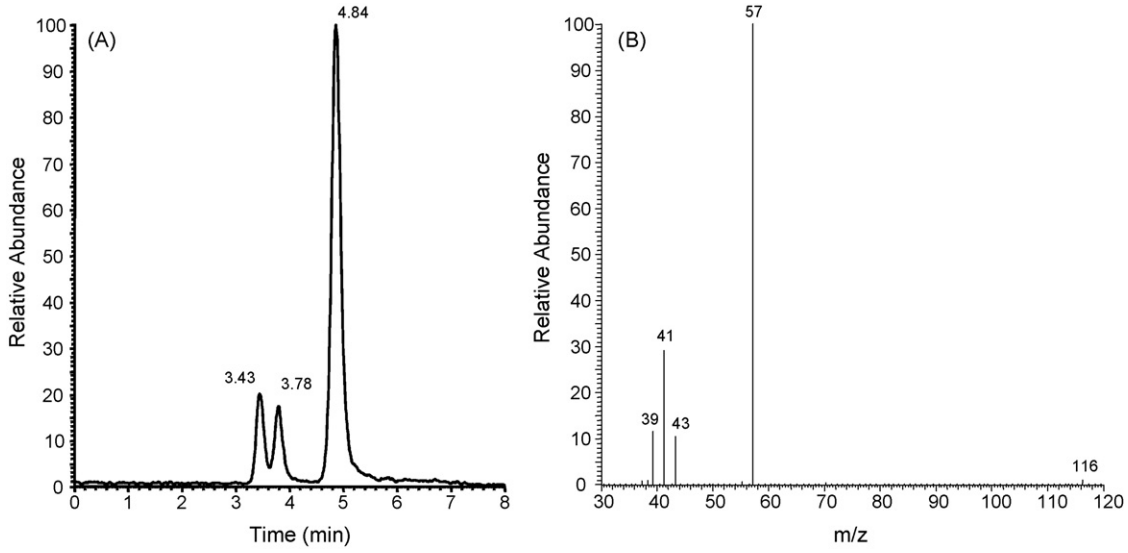

Fig. 1. The primary amine 4-methyl-2-hexaneamine was extracted from a urine specimen at a concentration of 300 ng/mL and injected into the LC–MS/MS system. (A) The LC–MS/MS chromatogram in ESI(+) of tuaminoheptane (R_T = 4.8 min) and 4-methyl-2-hexaneamine (R_T = 3.4 and 3.8 min, peak dedoubling). In these LC conditions, the peak resolution of the diastereoisomer of 4-methyl-2-hexaneamine was of about 0.8. In the MS/MS spectrum of 4-methyl-2-hexaneamine (B), the release of ammonia (−17 Da) followed by fragmentation along the hydrocarbon chain of the pseudomolecular ions $[M+H]^+$ at *m*/*z* 116.2 yield the prominent ion at *m*/*z* 57.3.

out as triplets at 1.035 ppm and the doublets and triplets of the $[CH_3{}^{\beta'}]$ and $[CH_3{}^{\delta'}]$ methyls overlaping between 0.9 and 0.82 ppm also confirmed the structure. The CH_2–CH–CH_2 fragments between 1.45 and 1.07 ppm has the right number of protons but the NMR signal resulted as broad multiplets with second-order effects making it impossible to verify this part of the coupling networks. No further investigations were conducted in that study for the identification of possible metabolites of the different stereoisomers of 4-methyl-2-hexaneamine.

The detection of the target compound was also performed by means of GC–MS, in particular for confirming adverse analytical findings. However, these highly volatile amines are known to elute rapidly after the solvent when standard GC conditions are applied [5]. The formation of a Schiff base between tuaminoheptane and cyclohexanone was found to significantly prolong the GC retention time of the resulting compound [8]. Chemical modification of 4-methyl-2-hexaneamine improved also considerably the chromatography, even though the diastereoisomer forms were not completely resolved (data not shown). The EI mass spectra of both peaks were identical, characterized in particular by the presence of the $[M]^+$ ion and a most prominent fragment ion at *m*/*z* 124 resulting from the homolytic cleavage of the C–C bond next to the nitrogen (α-cleavage) (Fig. 2). As already described in a previous report, molecular ions of the Schiff base derivatives of tuaminoheptane will react in the source by the loss of alkyl radicals [5,9]. The fragmentation of the Schiff base derivatives of 4-methyl-2-hexaneamine and tuaminoheptane yielded identical ions, hence the information provided by mass spectrometry could not be used alone for the detection of both primary amines.

Table 1
^{13}C NMR of the two diastereoisomers in $CDCl_3$.

	Multiplicity	Chemical shifts (ppm)		
		Diastereoisomer 1	Diastereoisomer 2	Reference[a] [7]
α	d (CH)	44.25	44.51	44.57
β	t (CH_2)	47.34	47.55	48.02
β′	q (CH_3)	24.78	23.94	25.14
γ	d (CH)	31.33	31.53	31.67
δ	t (CH_2)	30.02	29.44	30.06
δ′	q (CH_3)	18.93	19.43	19.43
ε	q (CH_3)	11.26	11.16	11.34

[a] In C_6D_6 using TMS as reference.

3.2. Assay validation

Validation parameters for the quantification of 4-methyl-2-hexaneamine in urine specimens by means of LC–MS/MS were assessed after considering the optimal conditions for extraction, chromatography and mass spectrometry. Calibration curves were built from the ratio of the integration of the two unresolved peaks area of 4-methyl-2-hexaneamine versus the peak area of tuaminoheptane.

Specificity tests did not show any interfering peaks at the retention times of the target analyte and internal standard. On each of the 6 validation days, the calibration and the QC sample at 300 ng/mL were prepared in duplicate. The calibration covering the range of

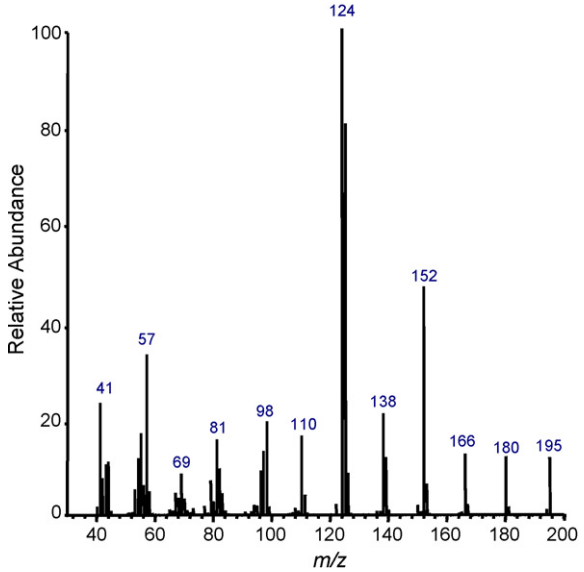

Fig. 2. EI mass spectrum of the Schiff base of cyclohexanone and 4-methyl-2-hexaneamine (mol wt = 195 Da).

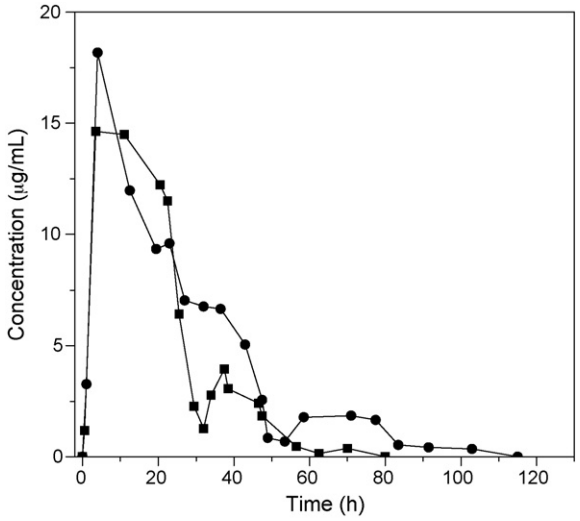

Fig. 3. Urinary concentrations of 4-methyl-2-hexaneamine after a single administration at $t = 0$ min of a food supplement containing 4-methyl-2-hexaneamine 40 mg in two healthy volunteers.

50–700 ng/mL (50, 100, 200, 400 and 700 ng/mL) was found to be linear with correlation coefficient higher than 0.9951. Alternatively, the quantification of the analyte in the μg/mL range would have required a quadratic regression. The interday precision and accuracy were of 5.8% and 3.5%, respectively. The matrix effect was not found to be significant for the target compounds in the QC sample, as the signals observed were of 102% (SD = 3%, $n = 6$) and 106% (SD = 3%, $n = 6$) for 4-methyl-2-hexaneamine and I.S., respectively, in comparison with the neat solution. Finally, using the extraction presented herein, the recovery of 4-methyl-2-hexaneamine in the QC sample was determined at 78% (SD = 6%, $n = 6$) and the I.S. at 82% (SD = 9%, $n = 6$).

Quantification of 4-methyl-2-hexaneamine contained in 5 different caps yielded a mean amount very close to that indicated by the manufacturer ($m = 38.3$ mg, SD = 1.4 mg). Based on these data, the recovery of 4-methyl-2-hexaneamine extracted from the food supplement is of 96%.

3.3. Application to real samples

Excretion samples and the food supplement were analyzed for quantification purpose using a 5-point calibration curve ranging from 50 to 700 ng/mL. For 4-methyl-2-hexaneamine levels out of the linear range, the urine specimen was diluted with an appropriate volume of water and subsequently re-extracted. All concentration values of the parent compounds in urine specimens were corrected for a target specific gravity value of 1.020.

Both peaks corresponding to the diastereoisomeric forms of the analyte displayed similar area in the food supplement as well as in all the urine specimens analyzed in this study. Intake of 40 mg 4-methyl-2-hexaneamine was found to be excreted unchanged up to 105 h post-administration in one subject, whereas for the other volunteer the parent compound was not detected anymore after 80 h. The resulting concentrations of the compound excreted in the urine are shown in Fig. 3. The maximum concentration was reached after 4 h with level up to 18 μg/mL.

These preliminary results show that the detection window of the parent compound is relatively long. However, the presence of 4-methyl-2-hexaneamine in urine should be interpreted with care as there is no urinary threshold established to declare a doping offence with a stimulant, except in the case of ephedrines. A recent study dealing with the detection of a doping with stimulants concluded that a discrimination between recent administrations of a small dose and the end-stage excretion following intake an higher dose may not be achieved based on low concentration of the metabolite [10]. In agreement with this conclusion, the time of administration of 4-methyl-2-hexaneamine could hardly be deduced from a positive sample containing levels of 4-methyl-2-hexaneamine slightly higher than 500 ng/mL. Indeed, for one subjects who self-administered 40 mg of the stimulant, the parent compound was detected over 50 h at levels ranging from 500 to 2000 ng/mL. In contrast, our finding tend to show that a urinary concentration of about 15,000 ng/mL, as determined in a doping sample, would likely indicate a recent administration of the substance prior to urine collection, provided that the quantity ingested was not well above 40 mg.

4. Conclusion

The class of stimulants has a long history of new or analogue compounds being abused (Bromantan, Carphedon, Mesocarb, etc.). Although an extensive screening of all stimulants analogues seems unpractical [11], the combination of the various analytical techniques available nowadays appears to be an interesting and relevant challenge for anti-doping laboratories. We described in this work a LC–MS/MS assay to detect the presence and quantify an analogue of tuaminoheptane in urine specimens together with a GC–MS procedure employed for confirmation purpose. The current study presents the first published data on the excretion of 4-methyl-2-hexaneamine following administration of a food supplement. However, additional studies should be conducted to estimate with accuracy the time elapsed between drug use and sampling. Based on these preliminary results, this parameter seems difficult to estimate. In turn, the analysis of the substance in blood could predict the time of exposure with more confidence, as already demonstrated in forensic toxicology for the interpretation of cannabinoid plasma levels [12].

Acknowledgments

This project has been carried out with the support from the Centre Hospitalier Universitaire Vaudois and University of Lausanne. The authors will thank Sabine Dominé for her precious technical assistance and Dr. Elia Grata for scientific support. Dr. Yves Allenbach and Dr. Damien Jeannerat (Department of Organic Chemistry, University of Geneva, Geneva, Switzerland) are gratefully acknowledged for NMR measurements and data interpretation of 4-methyl-2-hexaneamine.

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