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Metabolism of isometheptene in human urine and analysis by gas chromatography–mass spectrometry in doping control

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Abstract

A study of the metabolism of isometheptene, an antispasmodic drug, in man and comparison with heptaminol metabolism, is presented in this paper. Isometheptene and two metabolites were detected in human urine after oral administration of a tablet containing isometheptene mucate. The urine level of the parent drug, which is excreted during the first 24 h, was determined using gas chromatography–mass spectrometry, after alkaline extraction with organic solvent. A minor metabolite of isometheptene was converted to heptaminol in vitro under the acidic hydrolysis conditions used for the screening procedure of stimulants and narcotics in doping control analysis.
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Keywords: Doping control; Isometheptene; Gas chromatography–mass spectrometry; Heptaminol

1. Introduction

Isometheptene, 6-methylamino-2-methylheptene (Fig. 1a), is an antispasmodic drug belonging to the aliphatic amine series of adrenergic agents. It was introduced into clinical practice for the treatment of spastic conditions of biliary and urinary tracts. It is an indirect-acting agent given for vasoconstrictor effect in the treatment of migraine administered in combination with dichloralphenazone and acetaminophen, or with caffeine and dipyrone. It is also used in the management of smooth muscle spasm [1]. It has been shown that i.v. isometheptene elicits: (i) increases in heart rate via an indirect (tyramine-like action) mechanism; and (ii) increases in blood pressure via a predominantly indirect (tyramine-like action), as well as a minor direct (α_1-adrenoceptor), sympathomimetic mechanism [2]. Isometheptene is considered as a prohibited substance for doping control, under the S6 section (stimulants) of the List of Prohibited Substances [3]. In the countries where isometheptene is marketed for migraine, it is indicated as a prohibited substance on the websites of the National Antidoping Organizations (for example, www.uksport.gov.uk/did).

A literature search revealed that the metabolic fate of isometheptene in human has not been reported. Taylor and Couts have studied the metabolism of isometheptene in rat urine and found that it is metabolized through a trans-allylic-hydroxylation [4]. Furthermore, isometheptene has a very close structure to heptaminol, a well-known sympathomimetic substance considered as stimulant and as such is prohibited in doping control under the S6 section of the List of Prohibited Substances. Heptaminol, 6-amino-2-methyl-heptan-2-ol (Fig. 1b), has been used for many years as a cardiotonic agent with a positive inotropic action and as a corrector of the hypotensive effect of neuroleptics [5]. Heptaminol has been analyzed in human urine by gas chromatography (GC) [6], in human plasma by thin layer chromatography and in situ fluorimetry [7], in human plasma and urine by liquid chromatography [8,9], in rat plasma and urine using radiochemical procedures [10] and in horse urine using GC [11]. Heptaminol is well screened in doping control analysis both with GC and gas chromatography–mass spectrometry (GC–MS) [12]. All the investigations in human urine have concluded that

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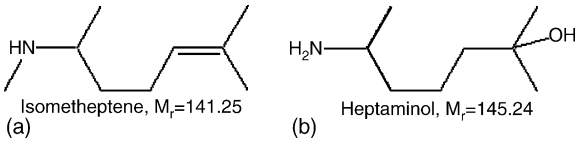

Fig. 1. Structure of (a) isometheptene and (b) heptaminol.

it is well absorbed and excreted rapidly, mainly unchanged in urine about 90% of the dose.

This article describes the study on the metabolism of isometheptene in human urine, the determination of isometheptene and its metabolites by GC and GC–MS and the relation between isometheptene metabolites and heptaminol detection in doping control analysis.

2. Experimental

2.1. Chemicals

The formulation Neosaldina tablets from Abbott Laboratories Colombia, was kindly provided by the Colombian Olympic Committee. Each Neosaldina tablet contains 30 mg of isometheptene mucate, 30 mg caffeine and 300 mg dipyrone. Heptaminol hydrochloride was purchased from Sigma–Aldrich, Steinheim, Germany. Standard methanolic solutions were prepared from the above materials. Codeine and diphenylamine (internal standards) were purchased from Sigma–Aldrich. All reagents and organic solvents used in the extraction procedure were of analytical grade. Derivatization reagents: TFAA (trifluoroacetic acid anhydride) was purchased from Sigma–Aldrich, Steinheim, Germany, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), *N*-methyl-bis(trifluoroacetamide (MBTFA) were purchased from Chem Fabrik Karl Brucher, Waldstetten, Germany. β-Glucuronidase from Helix pomatia (H. pomatia), 134,300 units/mL, pyridine and acetic anhydride were purchased from Sigma–Aldrich. Cysteine and toluene-4-sulfonic acid (TsOH) were purchased from Fluka.

2.2. Instrumentation

An Agilent 6890 GC (Agilent Technologies, Palo Alto, CA, USA) coupled with a nitrogen–phosphorus detector (NPD) with a cross-linked methylsiloxane capillary column (HP ULTRA 1, 12 m length × 0.200 mm internal diameter (i.d) × 0.33 μm film thickness) was used. Nitrogen was used as carrier gas at a flow rate of 1.1 mL min^{-1}. Two microliters of sample were injected in the splitless mode. Temperatures of the injector and the detector were set at 250 and 310 °C, respectively. The hydrogen and the air flows of the detector were 3.8 and 60.0 mL min^{-1}, respectively. Initial oven temperature was 90 °C, ramped to 40 °C min^{-1} to 240 °C, 30 °C min^{-1} to 310 °C and held for 2.00 min.

An Agilent 6890 GC (Agilent Technologies, Palo Alto, CA, USA) coupled with a 5973 quadrupole mass spectrometric detector (MSD) with a cross-linked methylsiloxane capillary column (HP ULTRA 2, 12 m × 0.200 mm (i.d) × 0.33 μm (film thickness)) was used. Helium was used as carrier gas at a flow rate of 1.1 mL min^{-1}. One microliter of sample was injected in the split mode (15:1). Temperatures of the injector and the transfer line were set at 250 and 300 °C, respectively. Initial oven temperature was 100 °C, then ramped at 20 °C min^{-1} to 290 °C, held for 5.0 min. MS parameters: ionization energy 70 eV, full scan mode (mass range 40–400 amu).

2.3. Drug administration

A Neosaldina tablet (30 mg of isometheptene mucate) was administered to a healthy volunteer and the urine samples were collected for 5 days. After each collection, the urine samples were placed in a freezer (−20 °C) and kept until the end of the excretion study. Furthermore, to compare isometheptene and heptaminol metabolism, an heptaminol excretion study was performed using an ethanolic solution of 30 mg heptaminol administered orally to a healthy volunteer. Urine samples were collected for 2 days.

2.4. Sample preparation

Firstly, the samples from the two excretion studies were analyzed according to the standard analytical procedures of the Athens Doping Control Laboratory: Procedure I (non conjugated volatile stimulants and narcotics) and Procedure II (free and conjugated non volatile stimulants and narcotics). For the Procedure I, 2.5 mL of urine were extracted with 1 mL of *t*-butylmethylether (or diethylether), at pH approximately 14 using KOH 5M solution, in the presence of 2 g of anhydrous sodium sulphate, after shaking for 20 min. The samples were centrifuged at 2000 × *g* for 10 min. Two microliters were subjected to GC-NPD analysis. For the GC–MS analysis, the organic phase was evaporated at room temperature and the sample was reconstituted with 100 μL ethyl acetate. Diphenylamine was used as internal standard. For the Procedure II, 2.5 mL of urine were hydrolyzed either with acidic or enzymatic hydrolysis. The acidic hydrolysis was performed with 250 μL HCl 6 M, at 100 °C for 30 min. Cysteine, 50 mg, was added before the incubation as antioxidant. For the enzymatic hydrolysis, pH was adjusted to 5.2 with acetate buffer 1.0 M, and 50 μL of β-glucuronidase from H. Pomatia were added. Hydrolysis was performed overnight at 37 °C. Codeine was used as internal standard. Anhydrous sodium sulphate (2 g) was added to the hydrolyzed urine and the pH was adjusted to 9–10 with the addition of a 10:1 mixture of solid sodium hydrogen carbonate–disodium carbonate. The solution was shaken for 20 min and extracted with 5 mL of diethylether: isopropanol (5:1). In the case of acidic hydrolysis, 250 μL of NaOH 6 M were added prior to the carbonate buffer. The samples were centrifuged at 2000 × *g* for 10 min. The organic phase was evaporated to dryness under a stream of nitrogen at room temperature. The samples were derivatised by adding 100 μL of MSTFA, heating at 80 °C for 10 min and 30 μL of MBTFA, heating at 80 °C for 5 min. The GC–MS analysis was performed with 1 μL of injection.

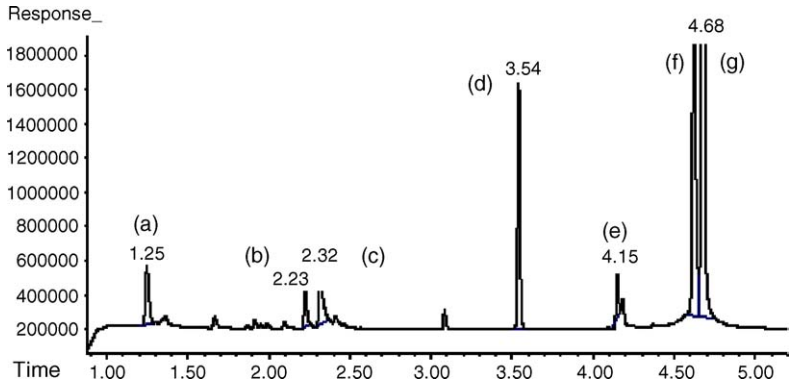

Fig. 2. GC-NPD chromatogram from Neosaldina excretion urine (5 h post administration), according to Procedure I (see Section 2.4). (a) Isometheptene, (b) possible metabolite of dipyrone, (c) major metabolite of isometheptene, (d) diphenylamine (istd), (e) caffeine, (f) ampyrone and (g) dipyrone.

Acidic extraction was also performed to hydrolyzed urine with ethyl acetate at pH 4.0 using HCl 1.0 M, for detecting possible acidic metabolites.

The trifluoroacetyl derivatisation was performed with 50 μL of ethylacetate and 50 μL TFAA, at 60 °C for 1 h, evaporation to dryness, and reconstitution with 100 μL ethyl acetate.

Dehydration of heptaminol was performed with TsOH in toluene using microwaves of 900 W for 40 min.

3. Results and discussion

The chromatograms from the excretion study of isometheptene free fraction (Procedure I) revealed that isometheptene parent compound is excreted during the first 24 h (Fig. 2). Furthermore, the chromatograms showed the presence of one major compound with a longer retention time than isometheptene, fully excreted at 35 h. The mass spectra of the underivatised and the

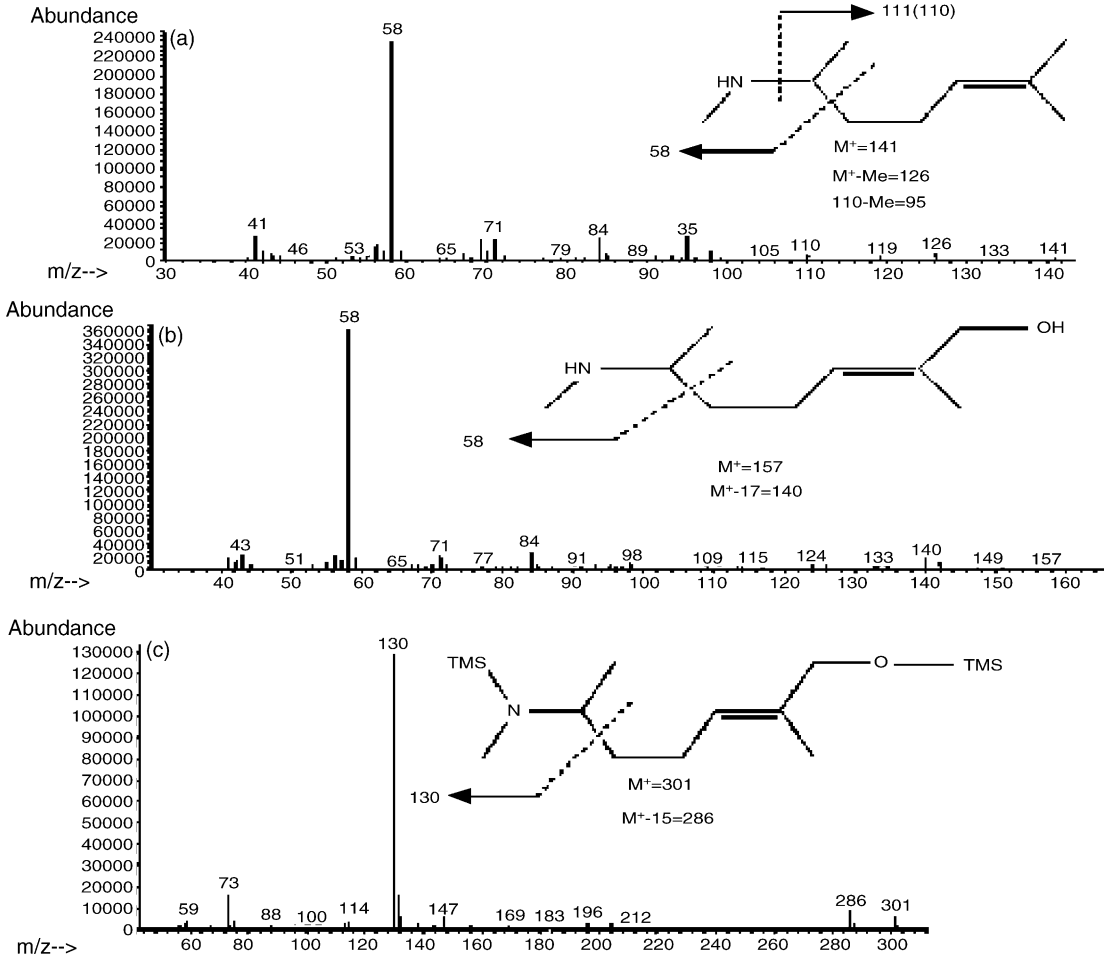

Fig. 3. Full scan mass spectra and fragmentation patterns of (a) isometheptene, (b) major metabolite of isometheptene and (c) diTMS derivative of major metabolite of isometheptene.

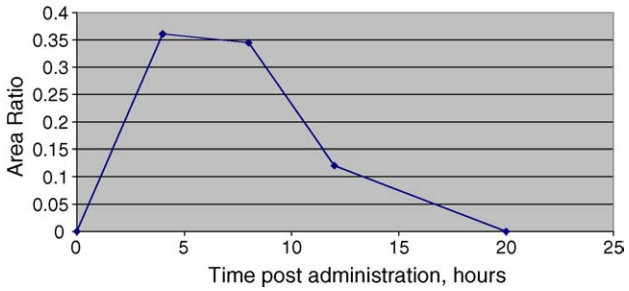

Fig. 4. Excretion profile of isometheptene following oral administration of a tablet of Neosaldina, containing 30 mg of isometheptene mucate.

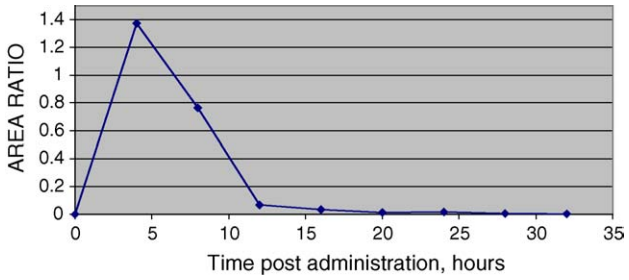

Fig. 5. Excretion profile of the major metabolite of isometheptene (2-methyl-6-methylamino-2-hepten-1-ol) following oral administration of a tablet of Neosaldina, containing 30 mg of isometheptene mucate.

di-TMS derivative of this metabolite are presented in Fig. 3, together with the spectrum of underivatised isometheptene. Both are consistent with the previously reported metabolite of isometheptene in rat urine (2-methyl-6-methylamino-2-hepten-1-ol) [4]. The trans and the cis isomers of the metabolite were detected when silylated (the one in 10-fold abundance relative to the other), but we have not confirmed which isomer is the one that is preferred; they present exactly the same spectrum. Cross experiments with and without hydrolysis of urine showed that isometheptene and its major metabolite are excreted unconjugated in urine. Excretion curves for isometheptene and its major metabolite are presented in Figs. 4 and 5, respectively. The presence of isometheptene in urine was semi quantified using a sample of isometheptene 2.0 μg/mL prepared by spiking blank urine with isometheptene solution. Relative areas in excretion urine samples were compared to those of the sample of isometheptene. In addition, four calibration curves of isometheptene, using spiked urine samples, proved to be linear through the apparent range 0.1–2.0 μg/mL, with a mean correlation coefficient of 0.9991 ($N = 4$).

The chromatograms from the excretion study of isometheptene from Procedure II showed that when acidic hydrolysis is performed, isometheptene undergoes a full hydration of the double bond following Markovnikov's rule (Fig. 6). The parent compound disappears and only the hydrated product is present. The full scan mass spectrum of the –OTMS–NTFA derivative of the hydrated product illuminates the proposed structure (Fig. 7). The major metabolite of isometheptene is not affected by the acidic hydrolysis conditions. It seems that the hydroxy group, which is already attached to the adjacent carbon atom of the double bond, subtracts electronic density, and the addition of the water molecule is no more favorable.

The screening Procedure II (acidic hydrolysis) from the excretion urine of isometheptene gave also a small signal for heptaminol. Heptaminol was not detected in the excretion urine of isometheptene when enzymatic hydrolysis or no hydrolysis was used. Hydration of *N*-desmethyl-isometheptene, a possible metabolite of isometheptene was suspected. To elucidate this hypothesis, the retention time and the mass spectrum of the dehydrated product recovered after the treatment of heptaminol with TsOH in toluene were compared with those of a minor product of isometheptene excretion (5 h post administration, 25 mL of urine) and found identical. The spectra of the underivatised substance and the TFA derivative are presented in Fig. 8. *N*-Desmethyl-isometheptene is hydrated under the acidic

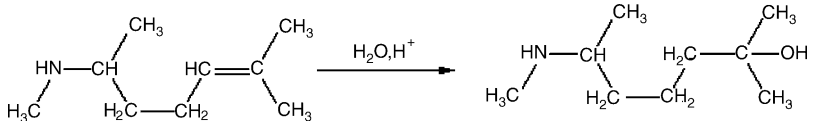

Fig. 6. Isometheptene hydration under acidic hydrolysis conditions.

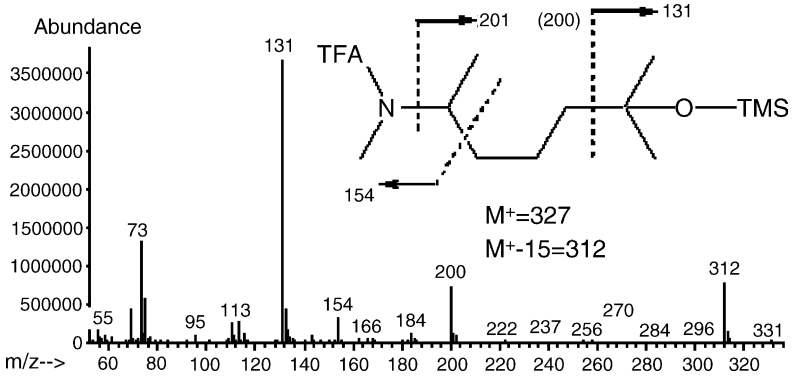

Fig. 7. Full scan mass spectrum and fragmentation pattern of the –OTMS–NTFA derivative of the hydrated product of isometheptene.

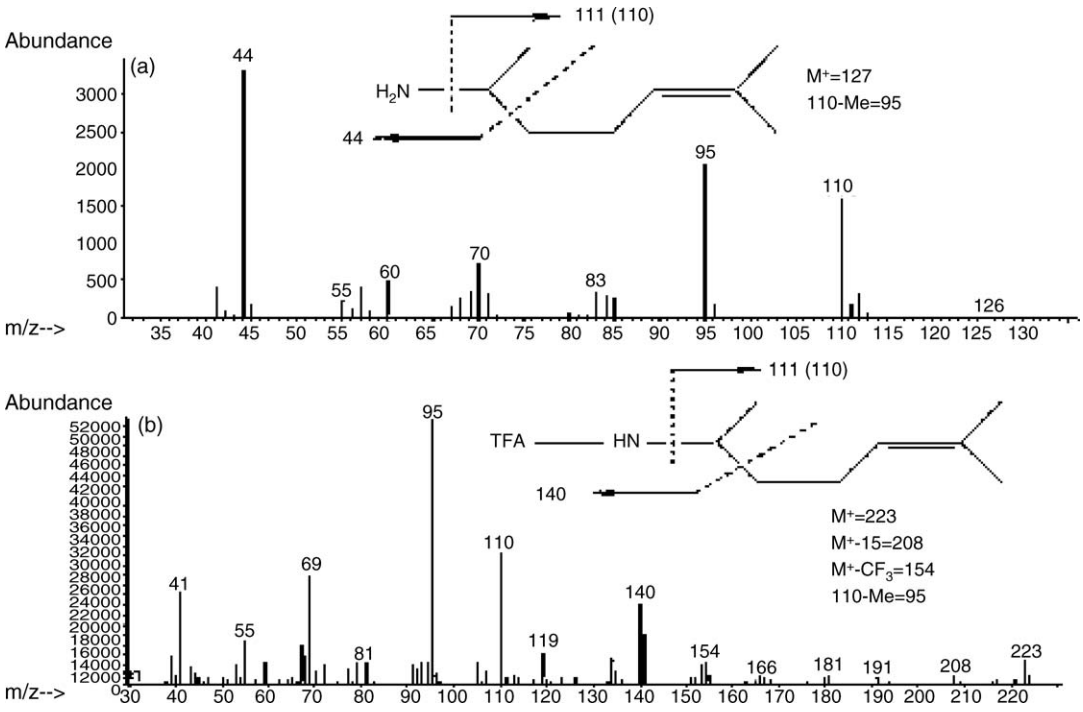

Fig. 8. Full scan mass spectra and fragmentation patterns of (a) *N*-desmethyl-isometheptene and (b) TFA derivative of *N*-desmethyl-isometheptene.

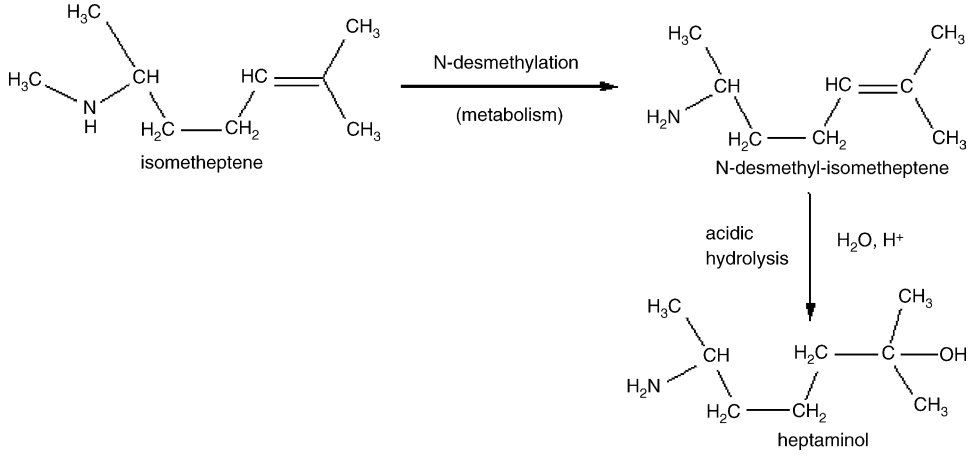

Fig. 9. Proposed mechanism of heptaminol production from isometheptene excretion urine after acidic hydrolysis.

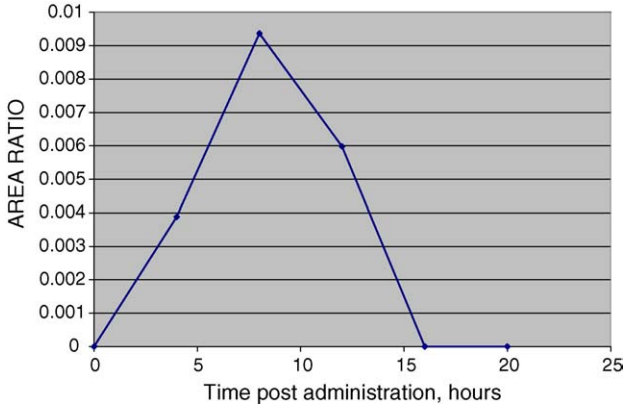

Fig. 10. Excretion profile of *N*-desmethyl-isometheptene following oral administration of a tablet of Neosaldina containing 30 mg of isometheptene mucate.

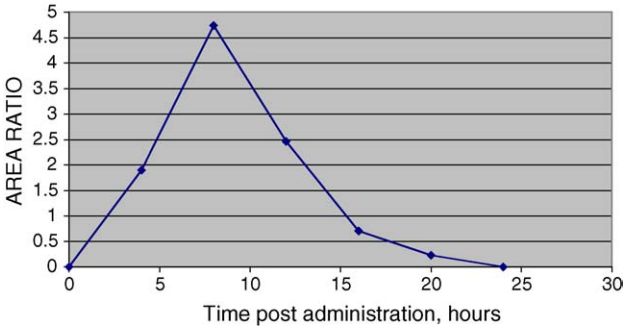

Fig. 11. Excretion profile of heptaminol following oral administration of an ethanolic solution containing 30 mg of heptaminol.

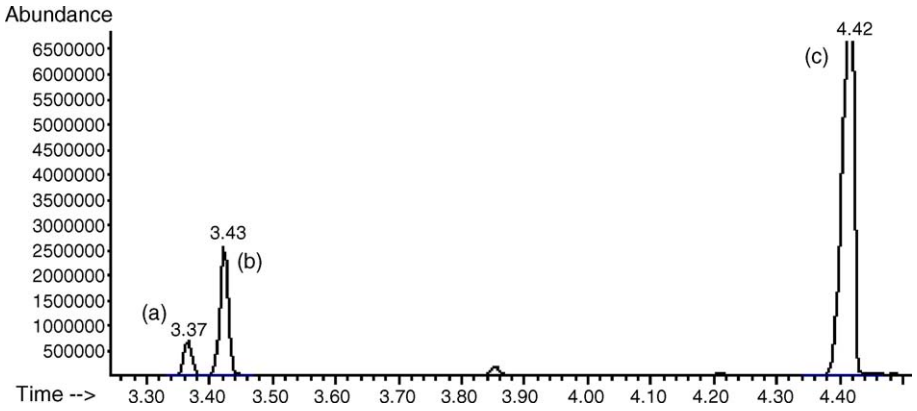

Fig. 12. GC–MS chromatogram from TFA derivatisation of heptaminol: (a) 6-methylhept-6-en-2-amine-NTFA (N-desmethyl-isometheptene-TFA isomer); (b) 1,5-dimethyl-hex-4-enylamine-NTFA (N-desmethyl-isometheptene-TFA); (c) heptaminol-diTFA.

hydrolysis conditions of Procedure II (Fig. 9), and hence heptaminol is not a metabolite of isometheptene but an artifact. This minor metabolite is excreted free in urine during the first 16 h post administration (Fig. 10), and cannot be detected using the normal screening Procedures I and II, because of its volatility and its low quantity.

On the other hand, the acidic extraction, also carried out in urine, did not give a signal of any possible acidic metabolite. In order to make sure that the detected metabolites are not urinary oxidation artifacts, a control experiment was performed, where isometheptene was added to human urine in a quantity of 4.0 μg/mL. The urine solution was left at room temperature for 5 days and then extracted according to Procedure I. Isometheptene was recovered unchanged, while none of the metabolites was detected.

The metabolic study of heptaminol showed that the substance is excreted during the first 24 h (Fig. 11), mainly as unchanged drug. The presence of heptaminol in urine was quantified using Procedure I. A standard sample of heptaminol 1.0 μg/mL was prepared by spiking blank urine with heptaminol standard solution. Relative areas in excretion urine samples were compared to those of the standard sample of heptaminol. In addition, four calibration curves of heptaminol, using spiked urine samples, proved to be linear through the range 0.1–5.0 μg/mL, with a mean correlation coefficient of 0.9987 ($N = 4$). A typical equation for the calibration curve, y (relative area) = $f(x$, concentration μg/mL) is $y = 0.01743(\pm 0.00030)x + 0.00028(\pm 0.00075)$, $N = 5 \times 2$ (five scalar points × two injections) with mean analytical error of 4.6%. It was assessed that heptaminol is excreted in a conjugated form at a less than 10% ratio relative to the unchanged drug. These results are in agreement with the literature [8,9]. Neither isometheptene, nor any of its metabolites was detected in the excretion urine of heptaminol. However, it is worth to mention that trifluoroacetyl derivatisation of heptaminol, aside from the diTFA derivative, yields two more peaks attributed to the dehydrated products of heptaminol, following Saytzeff's rule. One of these dehydrated products is *N*-desmethyl-isometheptene (Fig. 12).

In conclusion, the marker of an isometheptene administration in doping analysis is the hydroxy-metabolite, which is excreted unconjugated and is detectable for 35 h after the administration. In the screening Procedure II for stimulants and narcotics, this metabolite is detected as the diTMS-derivative. Under acidic hydrolysis conditions the desmethylated metabolite of isometheptene is hydrated to heptaminol.

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