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Analysis of adrenergic aliphatic amines in urine for doping control

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1. Introduction

Recently, sympathomimetic alkylamines agents attracted attention of the medical and sport community due to the number of the adverse analytical findings reported by doping control laboratories. The alkylamines series are characterized by an amine group (primary or secondary) linked with a short and ramified chain of carbons. Today, the WADA prohibited list quotes directly heptaminol, isometheptene, tuaminoheptane and methylhexaneamine. Often, analyses by GC-MS adopt derivatization strategies that: (i) increase the masses of the fragments, adding other diagnostic ions for structural characterization [1], (ii) improve the chromatographic peak shape and (iii) allows better chromatographic resolution [2]. However, some derivatization techniques, commonly employed for analysis of stimulants, are not suitable for monitoring of alkylamines, because they do not modify sufficiently the original molecular structure or do not change the fragmentation profile. Therefore, the aim of this study was to establish a method for derivatization of alkylamines, classified as stimulants by WADA, for confirmation purposes.

2. Experimental

The alkylamines were extracted from urine samples previously spiked (final concentration of 500 ng/mL) following the method described by Solans *et al*. [3] and adapted in LAB DOP-LADETEC/IQ-UFRJ, for use in stimulants, narcotics and β-blockers screenings [4]. After the extraction procedure three different derivatization reactions were performed.

Two derivatization methods were employed using acylation strategies combined with silyl derivatives. For trifluoroacetamide/timethylsilil derivatives (TFA/TMS), 20 µL of MBTFA were added and dried under nitrogen at 40 ºC. After 1 hour in vacuum the extract reacted with 80 µL of MSTFA at 60 °C for 10 min, followed by 20 µL of MBTFA at 60 ºC

for 10 min. For TFA/TBDMS derivatives similar reaction conditions were applied. Hence, 20 µL of MBTFA were added and dried under nitrogen at 40ºC. After 1 hour in vacuum the extract reacted with 80 µL of MTBSTFA at 60 °C for 10 min, followed by 20 µL of MBTFA at 60 ºC for 10 min. For all methods the final volume was 100 µL. Additionally, a third procedure using R–α-methoxy-α-trifluoromethylphenylacetic chloride (MTPA, Mosher reagent) was performed. Aliquots of 2 mL of urine were spiked with alkylamines in final concentration of 500 ng/mL. Five mL of hexane and 60 µL of 2% Mosher reagent:hexane (v/v) solution were added. After mixing, 120 µL of KOH 5 M were added and the samples were mixed again on a rotary shaker for 20 min. The organic phase was centrifuged and dried under nitrogen at 25 °C, and the extract was dissolved in 100 µL of ethyl acetate. 1 µl was injected into the GC–MS.

The GC-MS system consisted of HP (Palo Alto, CA, USA) GC model 6890N equipped with a 7673B HP auto sampler coupled with a qMS, Agilent (MS 5973 Network) and with a NPD (Agilent Technologies Inc., Santa Clara, CA, USA). Carrier gas was He (4.5) with initial flow rate of 0.9 mL/min, in constant pressure of 19.00 psi. HP-5MS® capillary column (100% methylsiloxane, 15 m, 0.20 mm I.D., film thickness 0.33 µm) from J & W Scientific, Agilent Technologies Inc. Injector temperature was 280 °C. Injection mode: 2 µL split 1/10; septum purge 60 mL/min. A split/splitless in house deactivated glass single liner from HP (cup 6 mm length × 1 mm hole) and an internal volume of 1.1 ml was used. Inside the liner, 0.017 mg of deactivated glass wool were well compacted between 23 and 33 mm measured from its top. The GC temperature programming was set as: initial column oven temperature 100 °C (held 1 min) then programmed to rise to 110 °C at 20 °C/min (held isothermally for 14 min), then to 280 °C at 20 °C/min (held isothermally for 1 min), and to 300 °C at 40 °C/min (held for 3 min).

3. Results and discussion

The concomitant use of MBTFA and MSTFA has been described for hydroxyamines [2,4]. The derivatives formed with reagents MSTFA and MBTFA, except for isometheptene, do not have spectral information for unambiguous identification of the presence of alkylamines in human urine (Table 1). On the other hand, the peak of isometheptene-N-TFA, did not show adequate chromatographic efficiency for this derivative. The secondary amine present in this molecule only reacts with one derivatizing group (TFA). So, the final derivative product remains with considerable basic character. The sympathomimetic amines

usually are separated by GC employing non-polar capillary columms such as HP5® [5]. But in general, the chromatographic resolution for alkylamines is worse than for aliphatic amines with aromatic groups, like amphetamine. In GC-MS analyses, the basic character and the dipole moment present in the alkylamine molecules are responsible for their strong interaction with active sites present in the capillary wall, which results in broad and asymmetric peaks, generating low sensitivity and poor resolution.

Table 1: Diagnostic ions for alkylamines N-TFA, O-TMS in SCAN mode and respective relative intensities. EI ionization.

Target Compound	t_R (min)	Molecular Mass N-TFA, O-TMS	Diagnostic Ions *m/z*(%) EI Intensity order					
Heptaminol	4.27	314.3	131	(100)	298	(16)	186	(9)
Isometheptene	3.83	237.2	95	(100)	168	(85)	110	(78)
Tuaminoheptane	2.59	284.2	212	(100)	254	(9)	268	(7)
Methylhexaneamine	2.59	284.2	214	(100)	186	(11)	254	(9)

The concomitant use of MBTFA and MTBSTFA was recently described by Sardela *et al*. as a confirmatory procedure for ephedrines [2]. The derivative formed allowed to obtain a mass spectrum with higher number of fragments and diagnostic ions and produced a better structural elucidation of the molecules. All the N-TFA-N-TBMDS derivatives obtained with the double derivatization reaction using MTBSTFA and MBTFA showed mass spectra with at least four ions above 20% relative intensity besides the base peak, which meets the WADA criteria (Table 2).

Table 2: Diagnostic ions for alkylamines N-TFA, O-TBDMS in SCAN mode and respective relative intensities. EI ionization.

Target Compound	t_R (min)	Molecular Mass N-TFA, O-TBDMS	Diagnostic Ions [*m/z*](%) EI Intensity order					
Heptaminol	5.00	355.3	69	(100)	158	(49)	202	(25)
Isometheptene	3.83	237.2	95	(100)	168	(85)	110	(78)
Tuaminoheptane	4.00	325.2	268	(100)	170	(46)	120	(45)
Methylhexaneamine	3.99	325.2	57	(100)	268	(51)	254	(28)

Mosher reagent is one of the most popular derivatization reagents for recognition of the absolute stereochemistry of amines [5]. Although, the absolute stereochemistry differences between the enatiomers of alkylamines is not relevant for doping analyses, the derivatizing group of Mosher reagent also leads to an increase in mass and alteration in the fragmentation profile of mass spectra, which results in less spectral interference in the alkylamines´ detection. For heptaminol, tuaminohepthane and methylhexaneamine the

selectivity is higher and no interference was observed. The peak shape is symmetrical and the chromatographic efficiency is higher. The enantiomers of the molecules are separated with resolution superior to 1.5 and the four peaks from methylhexaneamine derivatives were observed due to two chiral centers in the molecules. The retention time for all alkylamines as MTPA-Cl derivatives is greater than for the silyl/acetyl derivatives. The N-MTPA derivative from alkylamines is the most indicated for separation of tuaminoheptane and methylhexaneamine, since this is the only procedure studied capable to chromatographically resolve these compounds. However, the alkylamines are extensively fragmented and the *m/z* are not higher than the ones observed for the other derivatives (Table 3).

Table 3: Diagnostic ions for alkylamines N-MTPA in SCAN mode and respective relative intensities. EI ionization.

Target Compound	t_R (min)	Molecular Mass N-MTPA	Diagnostic Ions [*m/z*](%) EI Intensity order		
Heptaminol	8.30	361.2	69 (100)	154 (95)	346 (6)
Tuaminoheptane	7.03	331.2	57 (100)	170 (46)	99 (45)
Methylhexaneamine	6.74	331.2	57 (100)	99 (60)	170 (48)

4. Conclusion

The N-TFA-N-TBMDS derivatives and Mosher derivatives showed mass spectra which meet the WADA criteria. However, only Mosher derivatives showed higher separation, enabling the separation of the isomers methylhexaneamine and tuaminoheptane with baseline resolution. Therefore, in our opinion, for analyses of heptaminol, tuaminoheptane and methylhexaneamine, the N-MTPA derivative is the best alternative for routine work. For isometheptene analyses the N-TFA derivative can be obtained with employment of MBTFA combined with MSTFA or MTBSTFA reagents.

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