Identification of sympathomimetic alkylamine agents in urine by liquid chromatography-mass spectrometry and comparison of derivatization methods for confirmation analyses by gas chromatography-mass spectrometry

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Abstract:

Alkylamines agents attracted attention of the sport community due to the number of the adverse analytical findings reported by doping control laboratories in sport competition and for the medical community because the physiological effects which designated as sympathomimetic. The alkylamine’s series are characterized by an amine group (primary or secondary) linked to a short and ramified chain of carbons and has been applied for vasoconstrictor, bhroncholidator or analeptic activity. Nowadays, the WADA prohibited list quotes directly heptaminol, isometheptene, tuaminoheptane and methylhexaneamine. Nevertheless, other alkylamines could be used unduly by athletes in sports. Therefore, a procedure to screen 11 alkylamines is proposed by liquid chromatography-mass spectrometry (LC-MS/MS). Additionally, the comparison of derivatization methods for confirmation analyses by gas chromatography-mass spectrometry (GC-MS) was performed.

Keywords: Stimulants, Alkylamines, Doping, WADA, Derivatization, MSTFA, MBTFA, MTBSTFA, Mosher Reagent, MTPA-Cl, Mass Spectrometry, Liquid Chromatography, Gas Chromatography

1. Introduction

Stimulants are one of the most important classes of substances in the history of medicine, with several clinical applications. On the other hand, stimulants have been the root of great concern for public and sanitary authorities, considering the huge potential for misuse and side effects already documented [1, 2]. As a consequence, great efforts are demanded by the national authorities to restrain their consumption. In sports, stimulants were prohibited by the International Olympic Committee (IOC) in the late 1960’s. This prohibition is maintained by the World Anti-Doping Agency (WADA) until today [3].

The alkylamine stimulants are characterized by an amine group (primary or secondary) linked to a short and ramified chain of carbons. The WADA prohibited list quotes directly heptaminol, isometheptene, tuaminoheptane and methylhexanamine [3]. Isometheptene is an analgesic and potentiator of analgesic effects, when administered in combination with other drugs such as caffeine and dipyrone. Isometheptene is a problem in doping control, particularly in Brazil due to its presence in over the counter medicines. Our laboratory reported 20 positive results in the last five years. Recently methylhexaneamine as well, has received a lot of attention from the media due to recent cases in doping control (31 positives just in 2009) [4]. Moreover, in 2010 laboratories world-wide reported 123 cases to WADA (ref to WADA statistics: http://www.wada-ama.org/en/Anti-Doping-Community/Anti-Doping-Laboratories/Laboratory-Statistics/).

According to the WADA list, other substances with similar chemical structures or similar biological effects are also prohibited (Fig.1). Indeed, other alkylamines were extensively studied in the beginning of the XX century and could be designated as sympathomimetic drugs (Fig. 1) [5]. Among then, hexan-1-amine, hexan-2-amine, 5-methylhexan-2-amine, heptan-1-amine, octan-2-amine and octan-1-amine already had their pharmacological activity evaluated decades ago, but no pharmaceutical preparations are available in the market (ref?). From the best of our knowledge, no pharmacokinetic data is available for these drugs. The potential abuse of these alkylamines as doping agents rely in a similar context observed for the designer steroids [6].

From the analytical point of view, the detectability of the stimulants is well known [7, 8]. The usage pattern contributes to that, since the concentrations expected are normally high, reaching several ng/g or µg/g, depending on the biological matrix. However, as commented by Mueller *et al*. [9] and highlighted by Perrenoud *et al*. [10], from the logistic point of view, the detection of the stimulants would need special attention due to the high diversity of molecular structures. Hence, the implementation in routine of a screening for all stimulants needs the combination of several analytical approaches. A combination of LC-MS/MS and GC-MS seems more suitable, especially considering the strict rules regarding the confirmation of the screening results [11]. Considering the low boiling point observed for the alkylamines, the sample preparation should be led with caution aiming to prevent losses during evaporation.

The dilute-and-shoot (DS) approach is commonly applied to drug analyses in different biological matrices [12], skipping any pre-concentration steps. On the other hand, to lead a GC analysis accordingly, the evaporation steps become necessary aiming at the elimination of water before derivatization. The DS procedure for LC-MS/MS analyses is a fast and practical identification method. However, the mass spectra obtained by LC-MS/MS have fragments of low *m/z* due to the low molecular weight of the aliphatic amines and it is often not satisfactory for unequivocal identification, since the single ion with low *m/z* is common for molecules with low molecular mass. Hence the LC-MS/MS could be applied as screening analyses for alkylamines detection and for their characterization and confirmation GC-MS analyses after derivatization reaction should be used.

 Nevertheless, the GC has been the traditional choice for screening analyses of stimulants. However, considering specifically the requirements of WADA to report an adverse result, a supplementary approach could be necessary, aiming to assure the correct identification of such aliphatic stimulants. Moreover, sympathomimetic amines in general are separated by gas chromatography using non-polar capillary columms such as HP-5® [13, 14] and derivatization techniques are performed to increase the analytical response and the confidence of the identification by increment of the diagnostic ions.

This paper comprises an overview for the detection of the sympathomimetic alkylamines in urine, including four coumponds prohibited by WADA and seven other alkylamines presented for the first time, focusing on human-doping control. First we proposed the LC-MS/MS analyses for screening analyses. Second, we discuss different derivatization approaches aiming to the confirmation by GC-MS.

2. Experimental

2.1. Quality assurance

All analytical and managerial procedures were conducted under ISO/IEC 17025 standard environment, accredited by the Brazilian National Metrological Institute (BNMI) [15], jointly with the WADA International Standard for Laboratories [16].

2.2. Chemicals

The internal standards (IS) propylhexedrine and bromophenetylamine, and the alkylamines heptaminol, methylhexaneamine, tuaminoheptane, hexan-1-amine, hexan-2-amine, 5-methylhexan-2-amine, N-methylhexan-1-amine, heptan-1-amine, octan-1-amine, octan-2-amine and the enzyme β-glucuronidase *Helix pomatia* (116300 units/mL) were from Sigma (Steinheim, Germany). Isometeptene was a kind gift from the Barcelona Doping Control Laboratory. Ammonium formate, potassium hydroxide, anhydrous sodium acetate and formaldehyde solution (37%) were from Merck KGaA (Darmstadt, Germany). Acetonitrile, formic acid 96%, hexane, methanol, tert-butylmethylether (MTBE), glacial acetic acid, isopropyl alcohol, chloroform and ammonium hydroxide and trifluoroacetic acid (TFA) were from Tedia (Fairfield, OH, USA). N-methyl-bis-(trifluoroacetamide) (MBTFA) (99.7%) and N-methyl-N-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (99.1%) from Chemische Fabrik Karl Bucher Gmbh (Waldburg, Germany). R–α-Methoxy-α-trifluoromethylphenylacetic chloride (Mosher reagent) was from Fluka analytical (Buchs, Switzerland). Ultrahigh purified water was obtained from a Milli-Q water dispensing system (Molshein, France). The extraction column BondElut® Certify was purchased from Varian Inc. (Palo Alto, CA, USA).

2.3. Instrumentation

2.3.1. Liquid chromatography conditions

 A Sunfire C8 column 50 mm x 2.1 mm, 3.5 µm (Waters, Zellik, Belgium) was used for chromatographic separation. The column was maintained at a temperature of 20 °C. The mobile phase consisted of water (A) and MeOH (B), both containing 1 mM NH4Ac/0.001% HAc. Gradient elution at a flow rate of 0.3 mL/min was a follows: 100% A for 2 min, linear to 0% A in 4.5 min, holding 100% B for 1 min followed by an increase to the initial concentration of 100% A in 0.1 min. Equilibration time was 2 min, resulting in a total run time of 9.6 min. Samples were cooled at 10 °C in the autosampler.

2.3.2. Triple quadrupole (QqQ)

The LC system consisted of a Surveyor MS-pump and Surveyor autosampler with a 50 µL sample loop (all from Thermo Separation Products, Thermo, San Jose, CA, USA). The LC effluent was pumped to a Triple Quadrupole (QqQ) Quantum Discovery mass spectrometer (Thermo) equipped with an electrospray (ESI) MAX-source. Tuning of the tube lens voltage (TLV) was constant (100 V) for all compounds. The ESI voltage was set to 3500 V. The capillary temperature was 350 °C. The sheath gas flow rate was set at 100 arbitrary units. The auxiliary gas flow was 60 arbitrary units. The mass spectrometer was operated in the SRM mode and the isolation width was 0.7 full width half max. The scan width was maintained at 0.01 Da. For each compound collision energies (CE) were optimized. The collision gas pressure was 200 Pa for all compounds.

The MRM transitions using Q1 and Q3 masses as well as collision energy values were determined for individual compounds. All the compounds were checked in positive and negative modes, prevailing the [M+H]+ form. The optimized tandem mass spectrometry conditions are summarized in Table 1.

2.3.3. Orbitrap

A Q-Exactive (Thermo Fisher Scientific, San Jose, CA, USA) operated in positive–negative polarity switching mode. Parameters were set at 350 °C for ion transfer capillary temperature and (3500 eV) or (−3500 KV) for ESI needle spray voltage in positive or negative ion modes, respectively. Nitrogen sheath gas and auxiliary gas were maintained at 100 and 60 arbitrary units, respectively. MS data were acquired using a calibration in the scan range of *m/z* 100–1250 and processed using Xcalibur software version 2.1. The orbitrap was calibrated every day with MSCAL5-1EA and MSCAL6-1EA for positive and negative ion modes, respectively, and the acceptable range for the deviation mass accuracy was ± 2.5 ppm.

2.3.4. Gas Chromatography-mass spectrometry conditions

The analyses were performed using a Hewlett Packard (HP) (Palo Alto, CA, USA) gas chromatograph (GC) model 6890N equipped with a 7673B HP auto sampler coupled with a quadrupole mass spectrometer (MS), Agilent (MS 5973 Network). Carrier gas was helium (4.5) with initial flow rate of 0.9 mL/min, in constant pressure of 19.00 psi. HP-5MS® capillary column (5% methylsiloxane, 15 m, 0.20 mm I.D., film thickness 0.33 μm) from J&W Scientific, Agilent Technologies Inc. Injector temperature was 250 °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 6mm length×1mm 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 60 °C (held 1min) 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 1min), and to 300 °C at 40 °C/min (held for 3min). The mass spectrometer was operated in the electron impact ionization modes (EI): ion source temperature 250 °C; interface temperature, 280 °C; quadrupole temperature, 180 °C; accelerating voltage, 100 eV higher than the standard tune, in full scan mode within a mass range of *m/z* 50–600.

2.4. Sample preparation

2.4.1. Sample preparation for LC-MS/MS analysis

For LC-MS/MS analysis 0.1 mL of urine was diluted in 900 μL of mobile phase A with XXX ng/mL of bromophenethylamine (IS) at a final concentration of 100 ng/mL. After mixing, the samples were centrifuged at 10,000 x g for 5 minutes. Fifty microliters were injected in the LC-system.

2.4.2. Sample preparation for GC-MS analyses

The solid phase extraction procedure was conducted in 4 mL of urine sample spiked with 2 μg of propylhexedrine (I.S.) and 2 μg of each alkylamines. One milliliter of acetate buffer 1.1 M pH 5.2 (anhydrous sodium acetate/glacial acetic acid) and 50 μL of β-glucuronidase *Helix pomatia* enzyme were added to each sample and incubated for 2 hours at 55 ºC. After cooling, the pH was adjusted to 9 with potassium hydroxide. The samples were centrifuged and added onto a solid phase extraction column, containing 500 mg of bond Elute Certify® phase, previously washed with 2 mL of methanol and 2 mL of Milli-Q water. After application of the samples, the columns were washed with 2 mL of Milli-Q water, 1 mL of acetate buffer pH 4 (potassium hydroxide/glacial acetic acid) and 2 mL of methanol. The columns were dried under vacuum and the samples were eluted with 2 mL of a 80:20 (v/v) mixture of chloroform/isopropanol containing 2% of NH4OH. The eluates were derivatizated.

To obtain the mass spectrum and retention time, from 2 μg of each alkylamines, separately, were performed five different derivatization reactions. In all tubes 4 μg of propylhexedrine was added as internal standard (IS).

2.4.3. Sample preparation for the derivatization procedure

The silyl derivatives were obtained by two reactions: trimethylsilyl derivatives (TMS) obtained with 100 µL of MSTFA at 60 °C for 10min and tert-butyldimethylsilyl (TBDMS) derivatives obtained with 100 µL of MTBDMS at 60 °C for 10min.

Two other derivatization methods were employed using acylation strategies. For trifluoroacetamide/timethylsilyl derivatives (TFA/TMS), 20 µL of MBTFA was 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 was added and dried under nitrogen at 40ºC. 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 all methods the final volume was 100 µL.

The metoxy-α-trifluoromethylphenylacetyl (MTPA) derivatives were formed following a procedure adapted from Shin & Donike [17]. Briefly, 2 mL of urine were alkalinized adding 120 µL of KOH (5M) and vortexed for 10 s. 5 mL of hexane and 60 µL of a 2% (v/v) mixture of R–α-Methoxy-α-trifluoromethylphenylacetic chloride:hexane were added. The sample was vortexed for 10 s, shaken for 20 min and then centrifuged at 4000 rpm for 10 min. The organic phase was transferred to a second tube and dried under N2 flow (under 5 psi) at room temperature. The residue was recovered with 100 µL of ethyl acetate and vortexed for 10 s.

2.4.4. Ion suppression evaluation

The ion supression effect was evaluated by comparison between the average of the areas for each alkylamine spiked in ten different urines and the area of each alkylamine spiked in water at the concentration of 0.5 µg/mL.

The relative standard deviation R.S.D. (%) of the ion suppressions was also calculated in order to evaluate the reproducibility of this suppression.

2.5. Assay validation

2.5.1. Validation of the LC-MS/MS assay

Validation assay for the LC-MS/MS approach was based in ten aliquots of a quality control (QC) spiked with 0.5 μg/mL of each alkylamine in ten different urines. The bromophenethylamine was used as internal standard at a concentration of 100 ng/mL.

The precision assay consisted in the analysis of this ten QC samples in repeatability conditions. The precision was calculated for each compound, as %RSD. Since it was a direct injection approach, the extraction efficiency was not evaluated. For the evaluation of matrix interference, negative urines (n=10) were analyzed to check the presence of endogenous interfering peaks at the expected retention times for the analytes and the I.S.

The limit of detection (L.O.D.) was determined from ten different urines spiked with 1.0, 0.5, 0.25, 0,125 and 0.0625 μg/mL of each compound. The IS quantity (1 μg/mL of bromophenethylamine) was the same in all QC samples. The criterion established was the lowest concentration that could be detected with signal-to-noise > 3.

2.5.2. Validation of the GC-MS assay

 The validation assay for the GC-MS approach was employed only in derivatization reactions that were considered as adequate for each analyte. Ten aliquots of a QC were spiked with analytes and I.S. for final concentrations of 0.5 μg/mL and 1 μg/mL, respectively.

 The precision assay, matrix interference and L.O.D. were the same procedures described for the validation assay of the LC-MS/MS approach. Another ten tubes were spiked with the same concentration of analytes and I.S., without addition of matrix (blank urine), and only including the derivatization step. These tubes were considered as 100% of extraction efficiency. The peak areas on the chromatogram were compared to those of the QC samples extracts (the same QC samples used in the repeatability test).

3. Results and discussion

3.1. Liquid Chromatography analyses

3.1.1. Dilute-and-shoot

The dilute-and-shoot (DS) approach is commonly applied to drug analyses in different biological matrices [12, 18, 19]. For doping control monitoring, the DS procedure has already been applied in equine and human urine for qualitative or quantitative analyses [19, 20, 21]. However, DS procedures can lead to ion suppression in the ESI source and column failure because of the high amounts of protein in the sample. As DS does not apply a preconcetration step nor removal of matrix interferents, ion suppression experiments and the mass spectrometric behavior are the most relevant evaluations for this procedure. On the other hand, due to the high volatility of the alkylamines, the absence of the preconcentration step decreases the possibility of losses of these compounds. Since, the main step for preconcentration is the evaporation of the organic solvent.

3.1.2. Suppression of the ionization

 The ion suppression was evaluated for the most intense transitions of each alkylamine on the QqQ and the [M+H]+ on the ORBITRAP. No differences between the mass spectrometry analyzers were observed, since the molecule ionization occurs in the electrospray source. The most suppressed analyte was heptaminol. The average of the area for this compound showed 68.7% of intensity compared with the area in water. One specific urine showed only 48.1% of intensity in relation to the area in water. Heptaminol is the most polar alkylamine and probably its elution occurs with a larger amount of other polar interferences present in urine.

 Only R.S.D. higher than 10% were observed for the alkylamines with high ion suppression effects (Table 2). This result shows that the variation of repeatability of the DS procedure is substantially affected by the suppression of the ionization of the compounds. The results for analytical precision were within the acceptance limit of R.S.D. < 15% (Table 2).

3.1.3. Qualitative analysis

Usually, the screening analysis by LC is applied to detect different compounds in a single chromatographic run. However, due to the numerous analytes monitored, a time-consuming chromatography gradient is usually required. The LC gradient and the chromatographic column C8 were used to avoid the fast elution of the compounds. Therefore, the co-elution of the alkyalmines with polar matrix components was prevented. Nevertheless, in this procedure it is common the co-elution among the alkylamines. It was not possible to separate all analytes by tR, but all compounds were detected in a 9.6 min run with two transitions each in QqQ and the [M+H]+ in the ORBITRAP analyses. In both mass spectrometry analyzers all alkylamines were detected at the minimum required performance level (MRPL), 500 ng/mL imposed by WADA [22] (figure 2), but only by ORBITRAP analyses all alkylamines were detected at 62.5 ng/mL (Table 2). In particular for ORBITRAP analyses the noise levels were close to the electronic noise, proving the high specificity, which explains the lower LOD for this mass spectrometry analyzer.

The retention times obtained after these analyses are presented in table 2. The elution between isometheptene and heptan-1-amine showed low chromatographic resolution. These compounds can be resolved by mass spectrometry using their specific transitions by QqQ or by differences between [M+H]+ in the ORBITRAP, since for protonated isometheptene the exact mass is 142.159575 and for protonated heptan-1-amine it is 116.143925, a mass difference greater than 0.005 Da is sufficient to allow the discrimination among similar structures. In the QqQ analyses, the main transition is obtained after protonation at the nitrogen followed by C-N cleavage. For both compounds, the deaminated ion is formed after the loss of the nitrogen atom. This pathway produces the ions *m/z* 111 and *m/z* 99 for isometheptene and heptan-1-amine, respectively. However, these ions have low stability and a sequential product ion fragmentation occurs. This second cleavage forms more stable products and generates the *m/z* 69 for isometheptene and *m/z* 57 for heptan-1-amine, with high intensity for both compounds. Therefore, the co-elution between these compounds is not a limitation for their characterization. The co-elution between methylhexaneamine and 5-methylhexan-2-amine cannot be resolved by mass spectrometry, since the molecules do not have differences in molecular mass or in the ions obtained by QqQ.

The mass spectra obtained for methylhexaneamine, tuaminoheptane, 5-methylhexan-2-amine and heptan-1-amine have similar fragmentation pathways resulting in the use of the same ion transitions to monirot their presence (Table 1). This shows the low specificity and highlights a possible limitation for correct identification. Otherwise, the ratios between the ion intensities, at the same collision energies (CE) for these compounds, are different in function of the size of the hydrocarbon chain. At CE of 25 eV the ratio between the *m/z* 41/57 is small for alkylamines with seven-carbon atoms chain without methyl groups. The intensity of the *m/z* 41 is two times greater than the *m/z* 57 in the tuaminoheptane and heptan-1-amine. The displacement of the positive charge in the presence of the methyl substituent in methylhexaneamine and 2-amino-methylhexane increases the intensity of *m/z* 57, because it does not favor a further fragmentation to *m/z* 41 (Figure 3). Therefore, the ratio between *m/z* 41/57 can be used as indicative of which alkylamine will be evaluated by the confirmatory assay.

The ratios between the ion intensities, by QqQ, for hexan-1-amine and hexan-2-amine, alkylamines with a six-carbon atoms chain, do not show significant variations. However, the separation of hexan-1-amine and hexan-2-amine showed baseline chromatographic resolution. Hence, in this case, the tR is the most significant parameter for characterization. Moreover, for these alkylamines, as well as for N-methylhexan-1-amine, the mass spectra obtained with CE of 15 eV showed the characteristic *m/z* 85 ion. This is the deaminated ion formed after the loss of the nitrogen atom, and followed by the formation of the stable ring with six carbons. Similarly, octan-1-amine and octan-2-amine showed one characteristic fragment at *m/z* 71 obtained by formation of a ring with five carbons.

With exception of heptaminol, the only hydroxylated molecule, which showed a mass fragment of *m/z* 168 in high intensity, corresponding to the loss of water, all alkylamines showed a similar fragmentation pathway after the loss of the nitrogen atom. Hence, a low *m/z* is characteristic for these molecules with small molecular mass and different hydrocarbon chains. Nevertheless, co-elutions or interfering ions at low *m/z* are not relevant for screening purposes, since the confirmatory procedure could be applied for unequivocal conclusion. On the other hand, the use of the high mass accuracy for the [M+H]+ of all alkylamines in the ORBITRAP approach allowed higher sensitivity and specificity and increased the confidence for identification of these compounds. Even so, the evaluation of specificity for ten different urine sample was satisfactory for QqQ and ORBITRAP analyses, since no interfering substances at the appropriate retention times were found.

3.2. Derivatization Reactions

 An ancillary tool for identification would be the orthogonal analysis of the derivatives of the amines by GC-MS. In this case, instead of an universal derivatization (suited for screening analysis), it would be better to have specific derivatives for each analyte.

3.2.1 The TMS Derivatives

MSTFA is the most volatile of the silyl reagents [13] and it is the most common derivatization reagent used in GC-MS analyses for doping control and clinical analyses [23, 24, 25, 26]. The reaction with MSTFA leads to a molecular transformation by substitution of the hydrogen in the –OH group and the hydrogen of the amine group of the alkylamine by trimethylsilyl (TMS) group through a nucleophilic attack of these electronegative heteroatoms upon the silicon atom of the silylating reagent [13]. Each TMS derivative added in a molecule, increases 72 Da to its molecular mass. All alkylamines were derivatized with MSTFA and the main fragmentation, observed, is a β-homolytic clevage adjacent to the N atom. For the alkylamines with amino groups in the position 2 of the hydrocarbon chain: heptaminol, tuaminoheptane, methylhexaneamine, hexan-2-amine, 2-amino-5methylhexane, N-methylhexan-1-amine and octan-2-amine, the fragmentation pathway generates the ion *m/z* 116 as most abundant and always the respective methyl loss [M+• - 15] with intensity lower than 20%. Nevertheless, for heptaminol, methylhexaneamine, N-methylhexan-1-amine and octan-2-amine the mass spectra showed low intensity signals at other *m/z-values* (Table 3). Moreover, except for heptaminol, the N-TMS derivatives have a short time of elution and the alkylamines co-elute with a lot of interfering substances present in the urine matrix. Hence, in practice, the mass spectrum of each compound shows unknown and unrelated *m/z*-values due to the co-eluting interferences.

For the alkylamines with amino groups in the position 1 of the hydrocarbon chain, i.e. hexan-1-amine, heptan-1-amine and octan-1-amine, the ion at *m/z* 174 is the most intense. As with the other molecules, the ion [M+• - 15] is present. Three other fragments of *m/z* 86, 100 and 130 are observed for these molecules, which increase the level of confidence for identification of these compounds (Table 4). The difference between the values ​​of the most intense *m/z* between primary and secondary amines occurs due to the presence of a single TMS group for the former and two TMS groups for the later. The alkylamines with the amino groups in position 2 suffer a steric hindrance from the methyl group, which hinders the formation of a product with two TMS. Two TMS groups decrease the alkylamines volatilities and increase the retention time of the derivatives (Table 4). Less volatile molecules interact more with the stationary phase, which allows for a better separation from matrix interferences. Analysis of a blank urine sample spiked with other doping sport agents showed that they do not interfere in the identification of the hexan-1-amine, heptan-1-amine and octan-1-amine. Therefore, the derivatization with MSTFA is suitable for the confirmation of hexan-1-amine, heptan-1-amine and octan-1-amine.

For isometheptene, due to the secondary amine, the most intense ion is *m/z* 130. However, the steric hindrance of the two methyl groups did not allow complete derivatization of the molecule, and the detection of this molecule by this derivatization is not optimal (see section 3.2.3.).

3.2.2. The TBDMS derivatives

MTBSTFA is a reagent very similar to the MSTFA. The structural difference is the replacement of a methyl group by a *tert*-butyl group. The reactivity of the functional group toward TBDMS derivatives follows the order: alcoholic hydroxyl (primary > secondary > tertiary), phenolic hydroxyl, carboxyl, amine and amide. With the exception of isometheptene, all alkylamines were derivatized with this reagent.

The reaction with MTBSTFA is not suitable for sterically hindered sites [27] due to the larger TBDMS which hinders the attack of the electronegative heteroatom to the silyl group [13]. Thus, no TBDMS derivatized alkylamine presented two TBDMS groups on the same nitrogen atom and the OH group from heptaminol was not derivatized.

The hydrogen in the amine groups is replaced by a tert-butyldimethylsilyl group (TBDMS) adding 114 Da in molecular mass for each hydrogen replaced. Due to the stability of the tertiary carbocation, the loss of this *tert*-butyl group is common in mass spectrometric analysis, giving rise to the ion [M+• - 57] [13, 28] and it is the most intense fragment for tuaminoheptane, hexan-1-amine, hexan-2-amine, 5-methylhexan-2-amine, N-methylhexan-1-amine, heptan-1-amine, octan-2-amine and octan-1-amine. The β-homolytic cleavage adjacent to the N atom, is also present in these molecules (Table 5). Nevertheless, for hexan-1-amine, heptan-1-amine, and octan-1-amine, the fragment resulting from the β-homolytic cleavage adjacent to the N atom has an intensity lower than 5% (Table 5)*.*

For hexan-2-amine only the *m/z* 158 is observed, since due to the same *m/z* is obtained after β-homolytic clevage adjacent to the N atom and the loss of the *tert*-butyl group. Therefore, this derivatization could be applied for confirmation of all compounds listed in the first part of the table 5. Analysis of a blank urine spiked with other doping sport agents showed that these do not interfere in the identification of the tuaminoheptane, 5-methylhexan-2-amine, N-methylhexane-1-amine, octan-2-amine, heptaminol and methylhexaneamine.

The mass spectrum obtained for the heptaminol N-TBDMS derivative and for methylhexaneamine N-TBDMS presents other relevant fragments (Fig 4), including the favored loss of the *tert*-butyl group and the β-homolytic cleavage adjacent to the N atom. The use of MTBSTFA gives higher detection specificity for heptaminol and methylhexaneamine and is therefore recommended for their confirmation.

3.2.3. Double derivatization with sylyl and acyl derivatives

Acylation is another usual derivatization method for GC–MS [13, 29]. The reaction of alkylamines with MBTFA consists in the introduction of a trifluoroacetamide group substituting the hydrogen atom in the amine group of the molecule. However, the injection of MBTFA reagent directly in the GC system has adverse effects on the GC column because it decreases their performance and life-time [13]. So, to minimize this adverse effect, the MBTFA is used in small amount, in combination with another derivatizing reagent in large amount. The concomitant use of MBTFA and MSTFA has been described for hydroxyamines [30, 31, 32] and the concomitant use of MBTFA and MTBSTFA was recently described by Sardela *et al.* as a confirmatory procedure for ephedrines [33].

The combined reaction of MSTFA or MTBSTFA, with MBTFA, for the derivatization of alkylamines, leads initially to the substitution of one hydrogen from the nitrogen by one TFA. After that, under the usual experimental conditions, the steric hindrance does not allow the replacement of the other hydrogen in the amino group by a TMS or TBDMS. Therefore, the mass spectra obtained for all alkylamines are the same for the derivatization with MSTFA/MBTFA or for MTBSTFA/MBTFA, except for heptaminol. Because of the tertiary hydroxy group, heptaminol is transformed into the O-TMS derivative when MSTFA is employed and the O-TBDMS derivative is not formed with MTBSTFA. The mass spectra obtained for both derivatization techniques is not recommended in confirmatory procedures for heptaminol (see section 3.2.2.).

The β-homolytic cleavage adjacent to the N atom showed the *m/z* 140 in higher intensity for the alkylamines with amino groups in the position 2 of the hydrocarbon chain and the mass spectra showed low abundance for the characteristic ions. Thus this derivatization is also not recommended for confirmation of these compounds. The loss of the trifluoroacetamide group is most common for the alkylamines with amine in the position 1. The mass spectra obtained showed more than three diagnostic ions. However, the N-TFA derivatives also have the retention time lower than 2 minutes and due to the dipole moment present in the alkylamines molecules N-TFA derivatives, there are strong interactions with active sites present in the capillary wall, which result in broad and asymmetric peaks, generating low sensitivity and poor resolution.

For confirmation of isometheptene the reaction with MBTFA is the most recommended. The N-TFA derivative obtained showed the mass spectra with five fragments for identification, being the *m/z* 95, 110 and 118 the more relevant (Fig 5).

3.2.4. Metoxy-α-trifluoromethylphenylacetyl derivatives

 R–α-Methoxy-α-trifluoromethylphenylacetic chloride (MTPA-Cl) is one of the most popular derivatization reagents for recognition of the absolute stereochemistry of secondary alcohols and amines [34, 35]. For doping control and analytical toxicology in general, the reagent has been applied mainly in the enantiomeric characterization of methamphetamine [36]. This fact is explained by the difference in pharmacological action related to each enantiomer. Another important application already described is in the investigation of the metabolism of MDMA [37]. Although many drugs have different pharmacological activities for each enantiomers [38], the difference in pharmacological activity between the enatiomers of alkylamines has not been evaluated, and the presence of one any of the enantiomers of these molecules could be regarded as an adverse analytical result.

Due to the size of this derivatizing group, the increase in mass of molecules of alkylamines is relevant in the mass spectra as well as in the retention time, increasing the level of confidence in the identification of these molecules. Moreover, the MTPA-Cl derivatization procedure leads to the formation of N-MTPA derivatives simultaneously with the extraction procedure. The MTPA-Cl reagent in hexane is added in the basified urine. The mixture is stirred for 20 minutes in an orbital shaker. During this time, the molecules in urine migrate to hexane and meet the Mosher reagent. The MTPA-Cl derivatization procedure leads to the formation of N-MTPA derivatives simultaneously with the extraction procedure. The derivatization occurs and the polarity of the molecule is decreased even further keeping them in the organic layer. As only primary and secondary amines are derivatized, the procedure have high selectivity.

The reaction of the alkylamines with MTPA-Cl leads to the substitution of one hydrogen from the amino group by the MTPA derivative and the hydrogen from the OH group from heptaminol is not replaced by MTPA. The electron impact ionization of N-MTPA alkylamine derivatives generates a great number of different fragments for identification, the selectivity being higher and no interference being observed. The peak shape is symmetrical and the chromatographic efficiency is higher. For all alkylamines N-MTPA derivatives there are at least three specific *m/z* of high intensity (Table 6). This derivatization technique showed higher confidence for confirmation of the presence of all compounds in urine.

Differently from the LC-MS/MS, the co-elution between methylhexaneamine and 5-methylhexan-2-amine does not occur, inclusive for the enantiomers from these molecules. However, as with LC-MS/MS, the mass spectra obtained for methylhexaneamine, tuaminoheptane and 5-methylhexan-2-amine are similar. The presence of nitrogen leads to one important type of primary ionization and two mechanisms of fragmentation. The first fragmentation pathway is the homolytic clevage in Cα-Cβ adjacent to the N atom leading to the formation of *m/z* 260 observed in low abundance for all compounds. The second pathway is the heterolytic α-clevage leading to the formation of *m/z* 99 and *m/z* 57. For methylhexaneamine and 2-amino-methylhexane, these fragments are more intense than *m/z* 189, on the contrary, for tuaminoheptane the m/z 189 is higher. This difference could be explained because the methylhexaneamine and 5-methylhexan-2-amine have a branched saturated hydrocarbon skeleton and tuaminoheptane has a linear structure (figure 6).

Only hexan-1-amine, heptan-1-amine and octan-1-amine showed the *m/z* 190 in their mass spectra. This fragment is common and exclusive for the alkylamines N-MTPA derivatives with a nitrogen atom in position one (Fig 7). The alkylamines with six-carbon atoms chain: hexan-1-amine, hexan-2-amine and N-methylhexan-1-amine showed the mass spectra with more structural information among all alkylamines N-MTPA derivatives. Even though this compounds have the smaller length of carbon chain among the alkylamines, the values of the *m/z* obtained are higher and only this compounds shows the *m/z* 85 (Fig 8). For the most lengthy alkylamines, octan-1-amine and octan-2-amine, the mass spectra showed higher *m/z* because of the size of the hydrocarbon chain.

The comparison, of heptaminol N-MTPA, with the other derivatives described in this work, showed a different fragmentation pathway when the N-MTPA derivative is formed. This compound has one tertiary alcohol in the hydrocarbon skeleton. The presence of *m/z* 69 in large intensity suggests the ionization of the oxygen of this alcohol and two bonds cleavage could lead to a positive radical in the hydrocarbon fragment (Fig 9). The second most intense fragment is the *m/z* 154. It is formed after an homolytic β-fragmentation clevage, adjacent to the O atom of acetamide group, following a neutral loss of H2O from the tertiary alcohol. The N-MTPA derivative also provides an increase in mass spectrum information for isometheptene, the fragmentation pathway being derived from the reagent group and the *m/z* 69 is also present in large intensity.

For all alkylamines the molecular ions are present in low abundance and for all compounds, the *m/z* 77, *m/z* 105, *m/z* 119, *m/z* 189 and *m/z* 202 are common, since they are characteristic from the N-MTPA moiety.

 The chromatographic separation with MTPA-Cl allows the enantiomers separation for each compound. The identification of the enantiomers of alkylamines may be used in clinical studies for determination of the enantiomeric disposition in humans. The enantiomers of the molecules are separated with resolution superior to 1.5. For methylhexaneamine four peaks were observed due to the two chiral centers in the molecule. For heptaminol, isometheptene, tuaminoheptane, hexan-2-amine, 5-methylhexan-2-amine and octan-2-amine, 2 peaks were observed due to one chiral center and for hexan-1-amine, N-methylhexan-1-amine, heptan-1-amine and octan-1-amine only one peak was observed because of the absence of a chiral center (figure 10).

5. Conclusion

In this paper, alternative strategies for detection of alkylamines in screening analyses by LC-MS/MS are described. For confirmation analyses the chemical conversion of the alkylamines in to an appropriate derivative increases the compound`s vapor pressure, makes their spectra more easily interpretable, fulfill the WADA identification criteria and allow for the unequivocal characterization of alkylamines in urine.

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