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Analysis of sibutramine metabolites as N-trifluoroacetamide and O-trimethylsilyl derivatives by gas chromatography–mass spectrometry in urine

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ABSTRACT

A method for identifying the metabolites of sibutramine 1-(4(chlorophenyl)-N,N-dimethyl-α-(2-methylpropyl))cyclobutanemethanamine) in urine, utilizing a double derivatization strategy, with N-methyl-N-(trimethylsilyl)-trifluoroacetamide and N-methyl-bis-(trifluoroacetamide), in gas chromatography/mass spectrometry is proposed. This methodology results in mass spectra with at least three fragments in abundance superior to 20%, attending the World Anti-Doping Agency identification criteria for qualitative assays. The characterization of the derivatives was obtained through two ionization modes: Chemical Ionization and Electron Impact ionization, both in full scan mode. Sibutramine was administered to 5 (five) volunteers and the excretion profile followed for 92 h. Routine analytical, hydroxy-cyclobutane-bis-nor-sibutramine which becomes the more abundant metabolite in the first 10 h and hydroxy-isopropyl-bis-nor-sibutramine which becomes the most abundant after 40 h, were proposed for doping monitoring.

1. Introduction

Sibutramine (SIB, 1-(4(chlorophenyl)-N,N-dimethyl-α-(2-methylpropyl))cyclobutanemethanamine) was originally developed as an antidepressant drug [1]. SIB and its main metabolites are serotonin and noradrenalin re-uptake inhibitors, usually involved in the regulation of food intake in humans [2]. However, because of the potential abuse in sports, SIB was banned in 2006 by the World Anti-Doping Agency (WADA), having been classified as a stimulant doping agent (class S6) [3].

Brazil holds the first position in the world ranking of consumption of stimulants, mainly used as anorexic agents, and the sale of this kind of medicine, without prescription, is usual [4]. Obviously, in the sport scenario this social problem is also observed. Recently in 2007, thirteen Adverse Analytical Findings (AAF) for appetite suppressant drugs were reported by the WADA accredited Brazilian doping control laboratory. Eleven of those were due to detection of SIB metabolites.

Thevis et al. made the first contribution for the detection of SIB abuse for doping purposes, after the synthesis and detection of the N-desmethyl (nor-SIB) and N-bisdesmethyl (bis-nor-SIB) metabolites by LC-MS-MS [5].

One year later, Strano-Rossi et al. [6] reported the characterization of SIB urinary metabolites (Fig. 1). In addition to the de-methyl metabolites presented previously, other four metabolites were reported: hydroxy-isopropyl-nor-sibutramine (OH-nor-SIB1), hydroxy-isopropyl-bis-nor-sibutramine (OH-bis-nor-SIB1), hydroxy-cyclobutane-nor-sibutramine (OH-nor-SIB2) and hydroxy-cyclobutane-bis-nor-sibutramine (OH-bis-nor-SIB2). All of them were described as glucuronide conjugates. The authors used the classical trimethylsilylation strategy [7,8] to study and characterize those metabolites by gas chromatography–mass spectrometry (GC-MS). Due to the use of trimethylsilyl derivatives, this contribution created the basis for the SIB metabolites detection in the screening of steroid anabolics by GC-MS, in spite of the differences in biological activity and structure.

In 2003, WADA published a technical document where the criteria of qualitative identification of doping agents were described [9]. According to this document, at least three diagnostic ions, with more than 5% relative intensity, should be present in the spectrum; otherwise a second derivative would have to be prepared.

Typically, the GC-MS detection of stimulants is usually performed in routine through the one pot double derivatization strategy, with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and N-methyl-bis-(trifluoroacetamide) (MBTFA), proposed by Donike [10].

After inspection of the results of O-TMS derivatives from SIB metabolites, it may be concluded that many of these derivatives revealed mass spectra with only one ion showing reasonable

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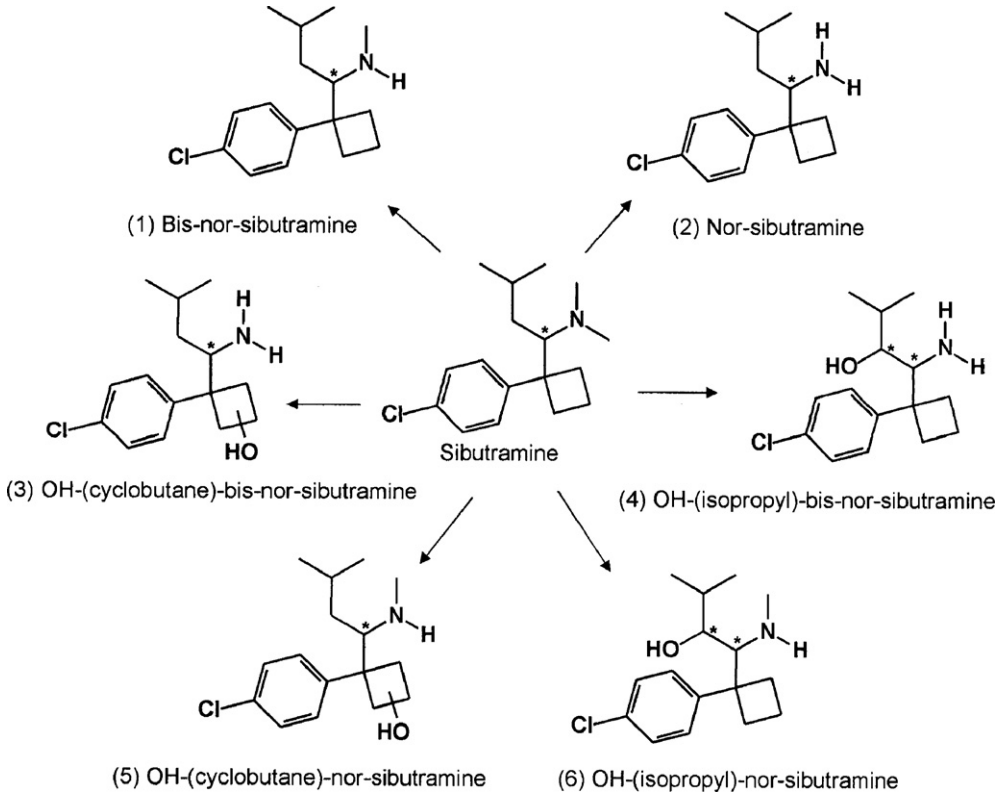

Fig. 1. The structure of sibutramine and its main metabolites. An asterisk indicates the location of a chiral center.

intensity (Table 1). Hence, they would not completely fulfill the identification criteria stipulated by WADA. Therefore, following WADA rules, in a case of a presumptive analytical finding of SIB metabolites, an orthogonal method of confirmation becomes necessary.

The aims of this work were: (i) to evaluate the formation and to characterize the urinary SIB metabolites previously described, using N-TFA-O-TMS as alternative derivatives. (ii) To compare the mass spectra profiles of N-TFA-O-TMS and O-TMS, following the qualitative WADA criteria. (iii) To evaluate, through analysis of real samples, the urinary excretion profiles of SIB metabolites, looking for the best confirmation target compound.

2. Experimental

All analytical and managerial procedures were accredited for the ISO/IEC 17025 standard, by the Brazilian National Metrological Institute (BNMI) [11], jointly with the WADA International Standard for Laboratories [12].

2.1. Reagents and standards

The internal standard (I.S.) codeine (99%) was purchased from Radian International LLC (Austin, TX, USA); methyl alcohol, glacial acetic acid, isopropyl alcohol, chloroform and ammonium hydroxide were purchased from Tedia (Fairfield, OH, USA), N-methyl-bis-(trifluoroacetamide) (MBTFA) (99.7%) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) (98.1%) from Chemische Fabrik Karl Bucher–Gmbh (Waldstetten, Germany). Anhydrous sodium acetate and potassium hydroxide were obtained from Merck KGaA (Darmstadt, Germany) and the enzyme β-glucuronidase *Helix pomatia* (116,300 units/mL), from Sigma (Steinheim, Germany). The extraction column BondElut® Certify was purchased from Varian Inc. (Palo Alto, CA, USA). Sibutramine chloridrate, purchased from Abbot GmbH and Co. KG (Ludwigshafen/Reno, Germany).

2.2. Sample preparation

Urine samples were collected after administration of a single dose of 15 mg of monohydrated sibutramine chloridrate, to three healthy males and two healthy females. The excretion study was carried out according to International and Brazilian regulations and approved by the University's Ethics Committee (protocol 168/02). A total of 37 aliquots per volunteer were collected, performing approximately 90 h of excretion study. The samples were analyzed by the method described by Solans et al. [13] adapted in LAB DOPLADETEC/IQ-UFRJ, for use in stimulants, narcotics and β-blockers

Table 1
Diagnostic ions for sibutramine metabolites N-TMS-O-TMS (6) derivatives by GC-qMS detection and their respective relative intensities.

Target compound	Diagnostic ions [m/z] (%) EI							
Bis-nor-SIB 2TMS	158	(100%)	102	(5%)	115	(2%)	128	(1%)
Nor-SIB TMS	172	(100%)	137	(2%)	102	(1%)	129	(1%)
OH-(cyclo)-bis-nor-SIB 2TMS	158	(100%)	102	(2%)	129	(1%)	115	(1%)
OH-(cyclo)-nor-SIB TMS	172	(100%)	129	(3%)	114	(3%)	410	(1%)
OH-(isopropyl)-bis-nor-SIB 2TMS	246	(100%)	156	(48%)	340	(4%)	266	(1%)
OH-(isopropyl)-nor-SIB TMS	247	(100%)	170	(25%)	114	(4%)	129	(1%)

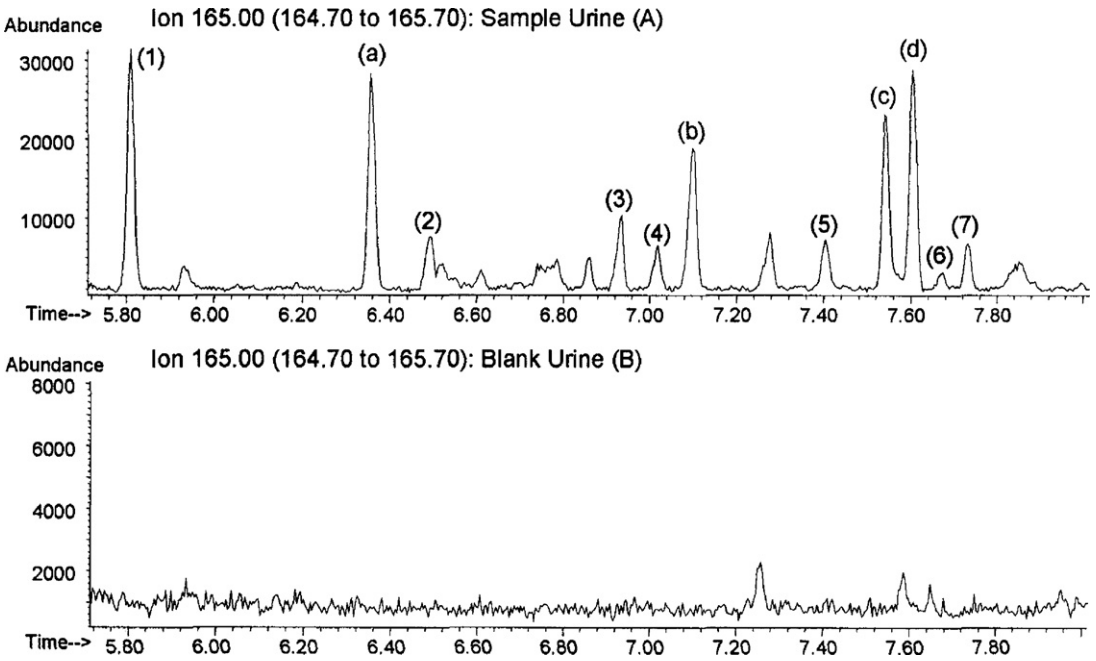

Fig. 2. Ion chromatogram of *m/z* 165, from electron impact full scan GC-qMS. (A) Sample urine, 2 h after administration of 15 mg of chloridrate of sibutramine; (B) blank urine of the same volunteer. Peaks of sibutramine metabolites: (1) bis-nor-SIB, (2) nor-SIB, (3) OH-bis-nor-SIB2, (4) OH-bis-nor-SIB1, (5) OH-nor-SIB2, (6) OH-nor-SIB1, (7) isomeres of OH-nor-SIB1. Peaks (a), (b), (c) and (d): possible SIB metabolites not yet described.

screenings [14]. Following the addition of 83.6 μmol of codeine to 5 mL of each urine sample, 1 mL 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 h at 55 °C. After cooling, the pH was adjusted to 9 with potassium hydroxide. The samples were centrifuged and poured 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 NH_4OH. Then, 20 μL of MBTFA was added to the eluate and the solution dried under nitrogen at 40 °C. The derivatization was performed initially with 100 μL of MSTFA at 60 °C for 5 min, followed by 20 μL of MBTFA at 60 °C for 10 min.

2.3. GC-MS analysis

Analyzes were performed using an Agilent 6890N gas chromatograph coupled with a 5973Network® mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with an 7683B series autosampler (Agilent Tecnhnologies Inc.), using a capillary column HP-5MS® (15 m × 0.2 mm ID, 0.33 μm film thickness; J&W Scientific, Agilent Technologies Inc.) operated in the splitless mode. Injector and detector temperatures were maintained at 280 °C. Helium (4.5) was used as carrier gas, with initial flow of 0.9 mL/min, at constant pressure of 1.53 BAR. The oven temperature was initially set at 100 °C, maintained for 1 min, then raised to 290 °C at a rate of 20 °C/min and finally maintained at 290 °C for 5 min.

The mass spectrometer was operated in dual ionization modes: (i) Chemical Ionization (CI) mode in full scan, using methane as ionization gas, and (ii) Electron Impact ionization (EI) in full scan. The acquisition time was 15.5 min. A volume of 2 μL of each sample was injected with an autosampler.

2.4. Assay validation

The experimental assay is restricted due to lack of standards of the metabolites of sibutramine in the study. Six metabolites were validated with special emphasis on the aspects of repeatability and specificity. The experimental design used for the validation experiments was based on WADA's Technical Document International Standard for Laboratories [12] and the one proposed by the BNMI [11].

2.4.1. Preparation of QC sample

For preparation of QC sample, 5 mL of a blank of urine was spiked with 53.7 μmol of sibutramine standard and 83.6 μmol of codeine. The QC sample was analyzed together with excretion study aliquots.

2.4.2. Specificity

All negative cases concerning sibutramine, in the year of 2007, were analyzed to control that no peaks interfere with the detection of analytes or the I.S. Blank samples spiked with sympathomimetic amines (ephedrine, norpseudoephedrine, phentermine and methoxyphenamine) were evaluated in order to recognize any possible interference.

2.4.3. Analytical repeatability and reproducibility

QC samples generated with the selection of a sample from the excretion study had all metabolites of sibutramine. The sample was distributed into seven aliquots of 5 mL. Analytical repeatability was calculated for each metabolite, as R.S.D.%. Reproducibility (within-lab) was calculated by one-way ANOVA using data as the grouping variable.

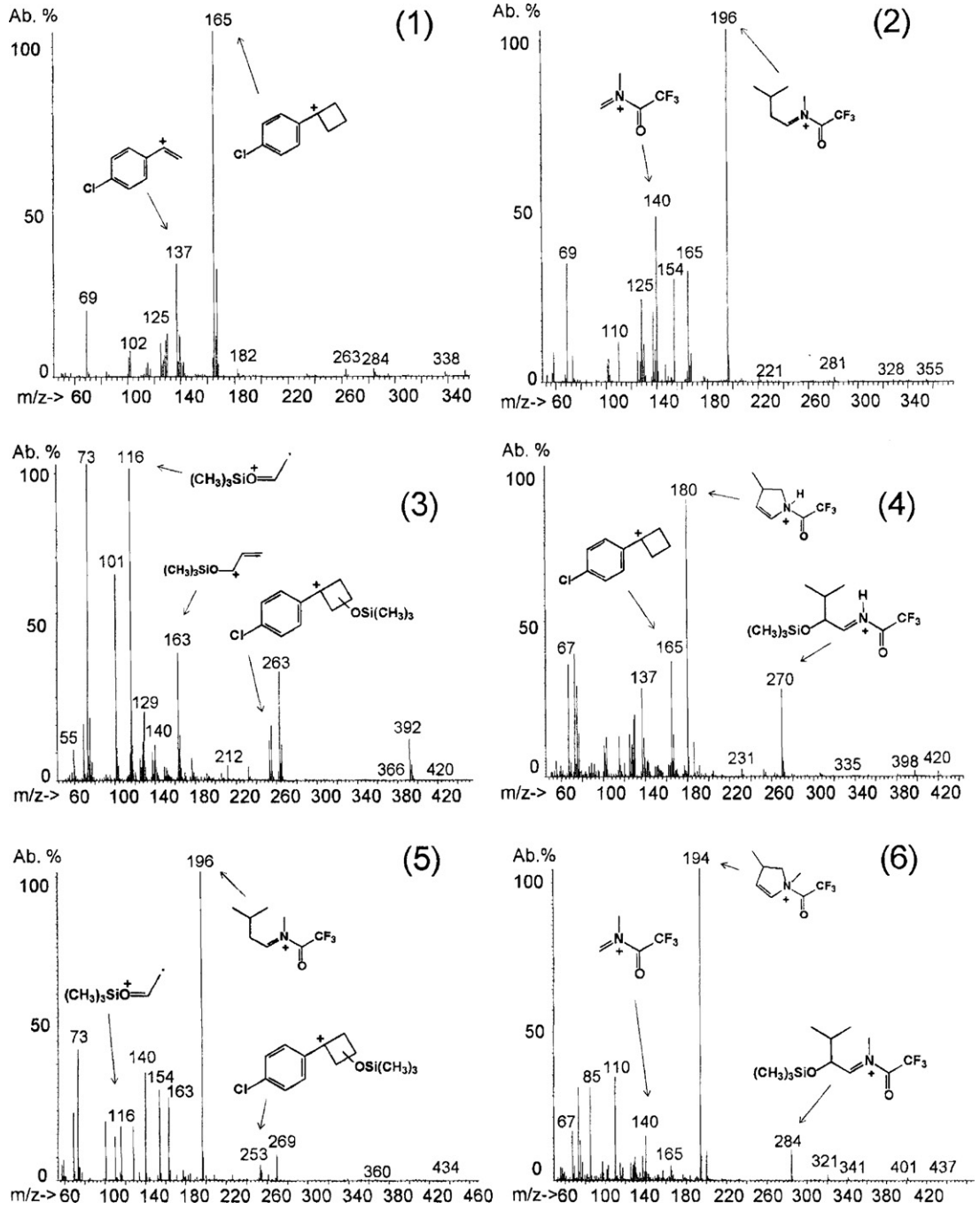

Fig. 3. GC-qMS mass spectra of sibutramine metabolites N-TFA-O-TMS derivatives, with suggested structure for key ions. (1) Bis-nor-SIB, (2) nor-SIB, (3) OH-bis-nor-SIB2, (4) OH-bis-nor-SIB1, (5) OH-nor-SIB2 and (6) OH-nor-SIB1.

2.4.4. Verification of limit of detection (L.O.D.)

Samples of the excretion study with lower concentration for each metabolite were analyzed to determine the L.O.D., the criteria established were: lowest concentration that would be detected with signal-to-noise >3.

2.4.5. Stability

For estimation of the stability of the solution to be injected, under the conditions of GC-MS analysis, all QC samples were extracted, derivatized, and transferred to autosampler vials. Injection followed conditions of a regular run. The QC samples were stored at ambient conditions (25 °C) and after five days (120 h), the analysis sequence was repeated.

3. Results and discussion

3.1. Identification strategy

Due to the lack of commercially available standards, it was necessary to characterize the metabolites by means of urine from excretion studies. Following the extraction and derivatization of the samples, the residues were analyzed by EI-mass spectrometry.

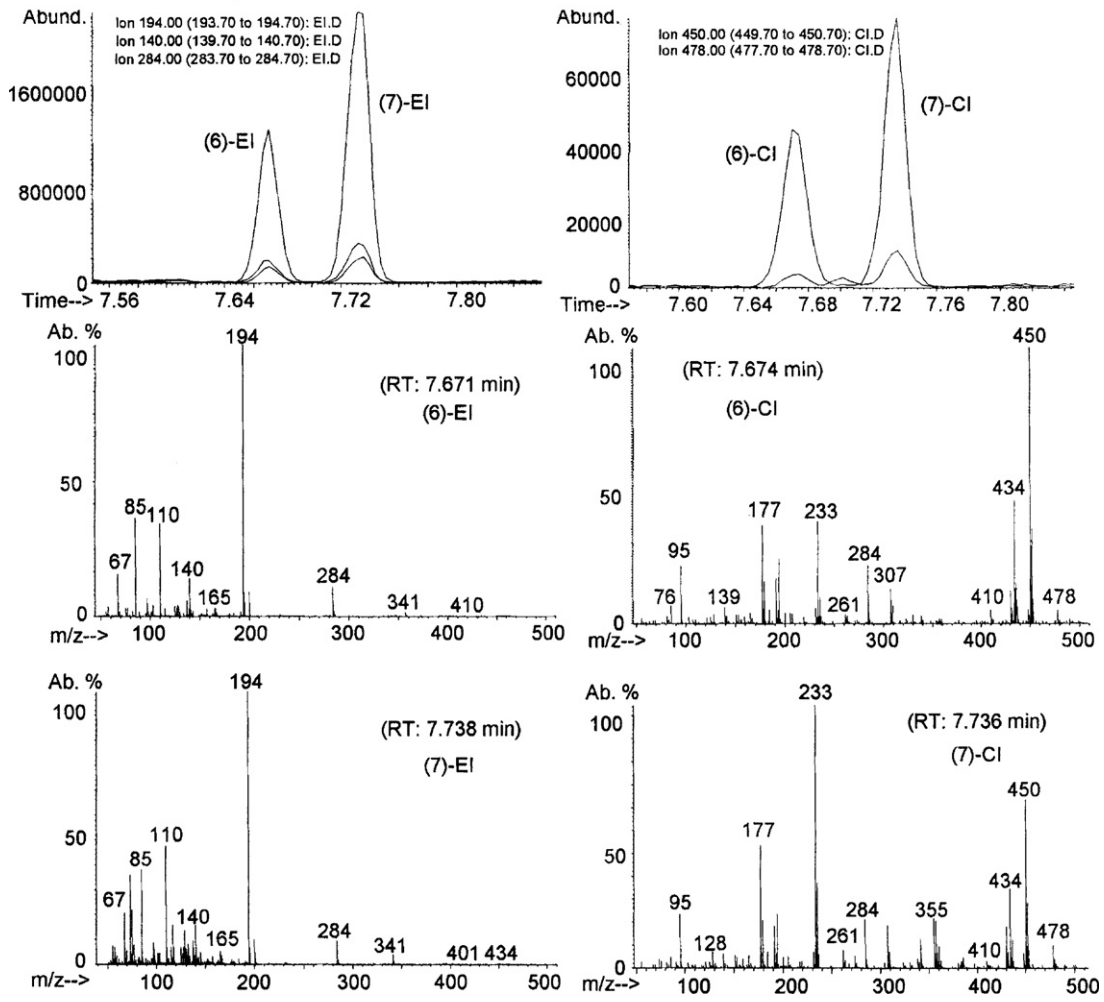

Fig. 4. Probable isomers for OH-isopropyl-nor-sibutramine-N-TFA-O-TMS derivative. (6) and (7) peaks and spectra obtained in Electron Impact ionization (EI) and in Chemical Ionization mode (CI).

In the ion chromatogram of the fragment *m*/*z* 165, obtained with EI mode in full scan, the alteration in the background of the urinary profile, collected 2 h after administration of the sibutramine chloridrate, was remarkable (Fig. 2). The *m*/*z* 165 is a characteristic fragment, resulting from a charge-induced α-bond cleavage to the nitrogen atom, observed in all metabolites except for the ones hydroxylated at the cyclobutane ring (Fig. 3).

A priori, Donike's double derivatization strategy allows the identification of all six metabolites described by Strano-Rossi et al., as N-TFA-O-TMS derivatives. However, due to the stability of the fragments formed after EI experiments, the N-TFA-O-TMS derivatives disfavor the observation of the molecular ion for all metabolites.

On the other hand, in the CI experiments, the pseudo-molecular ions $[M+H]^+$ and $[M+C_2H_5]^+$ were observed for all metabolites previously described. Matching data from EI and CI experiments was performed by calculation of the relative retention times of metabolites.

Using this strategy, more than ten chromatographic peaks, including the six metabolites previously described, were highlighted. Peak 6 and 7, in Fig. 2, represents two isomers for OH in the isopropyl group. These can also be observed in the chromatogram obtained by Strano-Rossi et al. [6], but no comment was made about their nature. Peak 7 has EI and CI mode spectra identical to OH-nor-SIB1 (Fig. 4). Other peaks observed had similar spectrum for SIB metabolites in EI mode, but only with EI and CI spectra it was not possible to determine the structure.

The fragmentation behavior of N-TFA-O-TMS derivatives from SIB metabolites is evidently influenced by the coupling of the trifluoracetamide group with the nitrogen of SIB metabolites. For the N-TMS-O-TMS derivatives, cleavage adjacent to the N atom is the more favored pathway, as demonstrated by Strano-Rossi et al. [6]. On the other hand, all N-TFA/N-TFA-O-TMS derivatives formed from SIB metabolites show different pathways of fragmentation, including a heterolytic β-cleavage.

The heterolytic cleavage is initiated by the electron ionization, being favored by the considerable stability of the tertiary carbon cation, formed on the cyclobutane ring present in the sibutramine (Fig. 5 (part 1)). Migration of the two electrons to the asymmetric carbon of the molecule is justified by the inductive effect, caused by electrophilic nature of the trifluoromethyl group attached to the nitrogen atom (Fig. 5 (part 2)). This induces the formation of a *m*/*z* 165 ion (Fig. 5 (part 3)), common to the four metabolites without OH in the cyclobutane ring. With the formation of a stable cation (Fig. 5 (part 4)), a simultaneous or consecutive break of the carbon bond in the cyclobutane occurs, also favored by the elimination of a stable neutral fragment (Fig. 5 (part 5)) and resonance stabilization of the cation.

Fragments that follow the formation of this ion, such as *m*/*z* 137 and *m*/*z* 125, are responsible for the rise of other peaks in the mass spectrum of these compounds. A fragmentation pathway also occurs in metabolites with OH in cyclobutane. However, in cases which O-TMS derivatives, in cyclobutane ring, are formed, the *m*/*z*

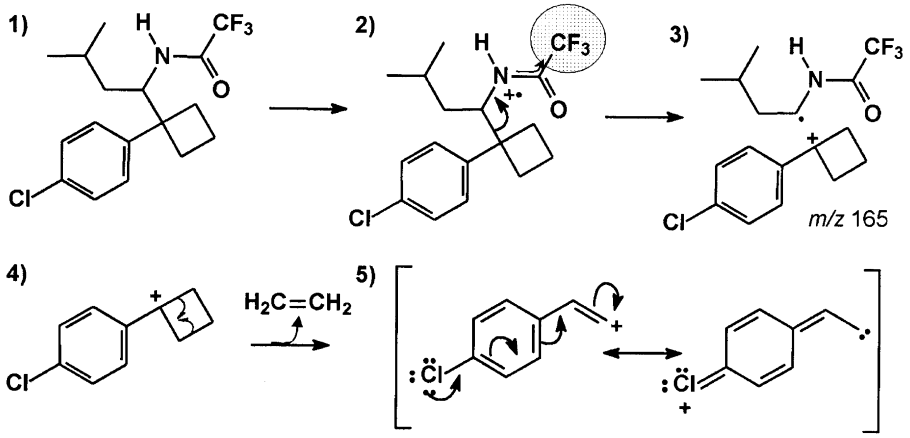

Fig. 5. Proposal for formation of ion *m*/*z* 165, through the heterolytic cleavage of the C–C bond beta to the nitrogen. (1) Bis-nor-sibutramine N-TFA derivative, (2) influence of the electrophilic field formed by the trifluoromethyl group (hatched area), arrows indicate the migration of electrons, (3) formation of fragment *m*/*z* 165, (4) proposal of the formation of the fragment *m*/*z* 137 through the homolytic fragmentation of cyclobutane and (5) release of a neutral fragment.

253 is the ion present. The subsequent formation of *m*/*z* 163 and *m*/*z* 116 in higher abundance is observed. This explain the reason for the change of profile of fragmentation when the changed N-TMS derivative is replaced by N-TFA (Fig. 6).

As a consequence of the formation of the single fragment in N-TMS-O-TMS derivatives, the resultant fragments show great intensity, by not allowing the formation of other fragments with high abundance. However, the formation of a single abundant ion, does not allow the unambiguous characterization of the compound.

Therefore, as the mass spectra for N-TFA/N-TFA-O-TMS derivatives present at least three fragments in abundances higher than 20%, as can be observed in Table 2, the WADA criteria for identification is easily fulfilled.

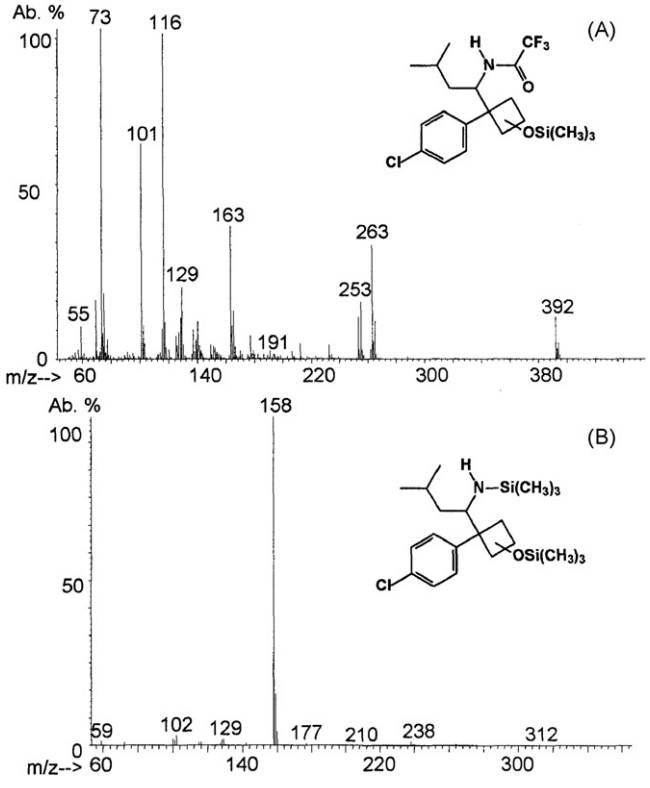

Fig. 6. Comparison between mass spectra (A) N-TFA-O-TMS derivative and (B) N-TMS-O-TMS derivative for OH-bis-nor-SIB2.

3.2. Validation results

3.2.1. Specificity and matrix interference

The specificity and matrix interference are factors that, according to the International Standard for Laboratories (WADA, 2008), must be investigated in the validation of confirmation methods to non-threshold substances. Specificity is defined as the ability of the assay to detect only the substance of interest and can be understood as the ability of the method to discriminate between the analyte of interest and others with structural similarities.

On the other hand, matrix interference is defined as possible interference of an endogenous compound, in the detection of the prohibited substance.

3.2.1.1. For N-TFA-O-TMS derivatives. No matrix interference was verified in the detection of any N-TFA-O-TMS SIB metabolite. Considering all compounds described in the List of Prohibited Substances (WADA, 2008) classified as stimulants, it is possible to affirm that none of those are able to interfere. This affirmation can be achieved in a variety of doping agents currently used by athletes, since no interferences in N-TFA-O-TMS derivatives had been observed in 5547 samples analyzed in our lab, in 2007, utilizing the described strategy.

Using the urine collected before the administration of the drug as a basis for comparison and the same 5547 samples analyzed in 2007 with N-TFA-O-TMS methodology, we did not observe any endogenous interference at the same retention time of the analytes.

3.2.1.2. For N-TMS-O-TMS derivatives. The N-TMS-O-TMS derivatives of SIB metabolites were included in our routine for anabolic steroid screening [7,8]. In our lab, an interference peak was frequently observed, close to the retention time of N-TMS-O-TMS hydroxy-cyclobutane-bis-nor-sibutramine (Fig. 7). When present in high concentration, it becomes hard to have an accurate diagnosis related to the SIB abuse. This metabolite is the most abundant in the first 10 h (see Section 3.3). Due to WADA criteria, where the variation in retention time shall be 1% or ±0.2 min (whichever is smaller) [9], the interference near the retention time of the main SIB metabolite, would require additional evaluation with an alternative method to avoid false positive results, thus increasing the delay in results delivery, as well as the workload. As an alternative to circumvent this problem, it is always possible to include the characteristic ions of other metabolites in the acquisition method. However, it is necessary to consider the impact of this inclusion in the method sensitivity, since the steroid screening works in Selection Ion Mon-

Table 2
Diagnostic ions for sibutramine N-TFA-O-TMS metabolites in SCAN mode, with CI and EI techniques.

Target compound	t_R (min)	Diagnostic ions [m/z] CI		Diagnostic ions [m/z](%) EI					
		$[M+H]^+$	$M+[H]^+ + C_2H_4$	Intensity order					
Bis-nor-SIB	5.803	348	376	165	(100%)	137	(36%)	263	(3%)
Nor-SIB	6.488	362	390	196	(100%)	140	(55%)	165	(39%)
OH-bis-nor-SIB2	6.940	436	464	116	(100%)	163	(48%)	263	(42%)
OH-nor-SIB2	7.391	450	478	196	(100%)	140	(37%)	154	(30%)
OH-bis-nor-SIB1	7.104	436	464	180	(100%)	165	(44%)	137	(34%)
OH-nor-SIB1	7.740	450	478	194	(100%)	110	(37%)	140	(20%)

itoring (SIM) acquisition mode. The inclusion of more ions would reduce acquisition times, resulting in low sensitivity. Indeed, from the eleven AAFs obtained with the N-TFA-O-TMS methodology in 2007, only nine were detected when the N-TMS-O-TMS derivatives were used. Therefore, for two AAFs with SIB metabolite in low concentration, only N-TFA-O-TMS avoided false negative reporting.

3.2.2. Analytical repeatability and L.O.D.

The results for analytical repeatability were within the acceptance limit of R.S.D. < 20% (Table 3). The highest R.S.D. was 18.2% for OH-nor-SIB1. All metabolites showed L.O.D. < 1 ng/mL, and this concentration was estimated for the peak-area ratio (sibutramine versus codeine and corresponding metabolite versus codeine). Considering the response factor for all metabolites in the total ion chromatogram as similar, it was possible to estimate the concentration for all metabolites. This allowed to infer the limit of detection for this methodology as 0.3 ng/mL, for hydroxy-cyclobutane-bis-nor-sibutramine.

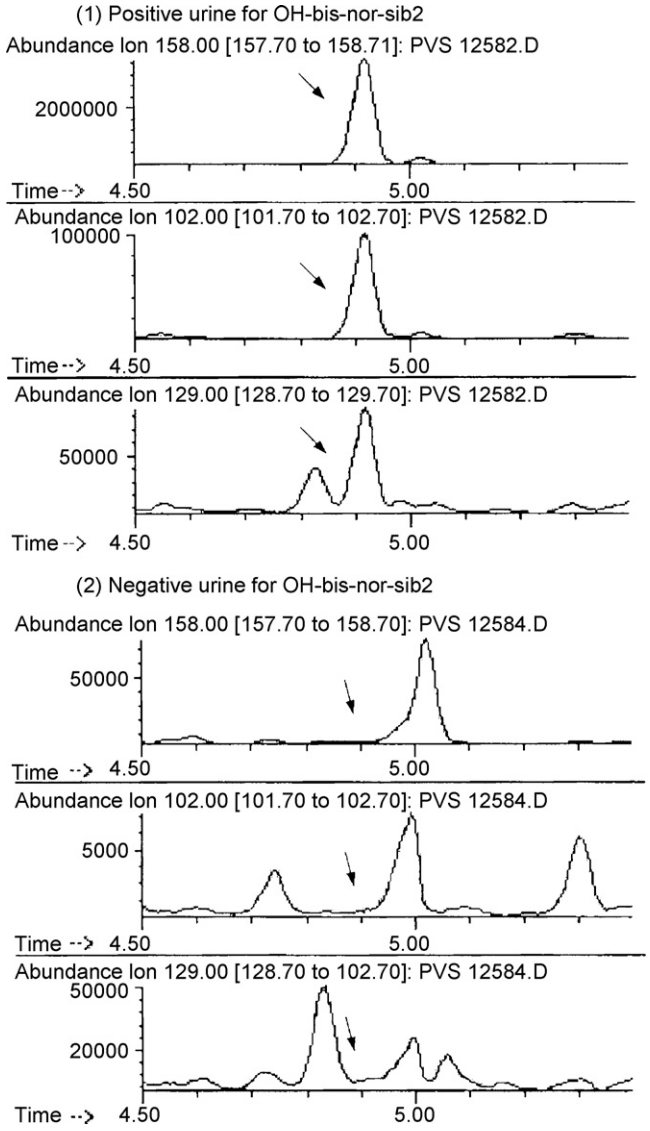

Fig. 7. Comparison between positive urine, after 10 h of sibutramine administration (1) and negative urine, before sibutramine administration (2), for the N-TMS-O-TMS derivative.

Table 3
Repeatability (R.S.D.%) and limit of detection (L.O.D.) results for SIB metabolites.

Sibutramine metabolites	L.O.D. (ng/mL)	Repeatability R.S.D.%
Bis-nor-SIB	0.57	11.7
Nor-SIB	0.24	10.5
OH-bis-nor-SIB2	0.29	9.8
OH-nor-SIB2	0.76	5.7
OH-bis-nor-SIB1	0.56	9.6
OH-nor-SIB1	0.41	18.2

3.2.3. Stability

The stability of SIB metabolites was performed with the confrontation of mass spectra and ratio of areas [SIB metabolites × ISTD] of extracts injected in different days, up to the fifth day after sample preparation. The derivatized SIB metabolites stored at ambient conditions were stable for at least 120 h at room temperature (25 °C).

3.3. Analytical target for routine screening

Since the parent drug SIB becomes extensively biotransformed, it could not be detected in the urine samples. The concentration of each SIB metabolite was estimated by using a blank of urine spiked with SIB in known concentration. The ratio of areas with the internal standard (codeine) was calculated, the value found was compared with the ratio of areas of each metabolite with the same internal standard present in each aliquot of the excretion study. The choice

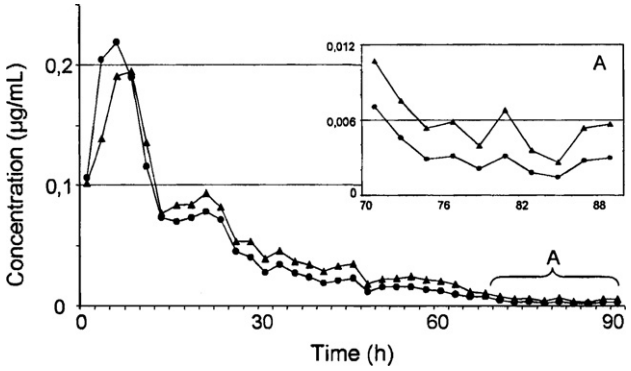

Fig. 8. Excretion study for main sibutramine metabolites, average of concentration was determined with $n = 5$ volunteers. *Y* axis indicates the concentration in µg/mL and *X* axis the time in hours. ●: OH(cyclobutane)-bis-nor-SIB; ▲: OH (isopropyl)-bis-nor-SIB. Insert A shows an expansion of the excretion study for the last 20 h.

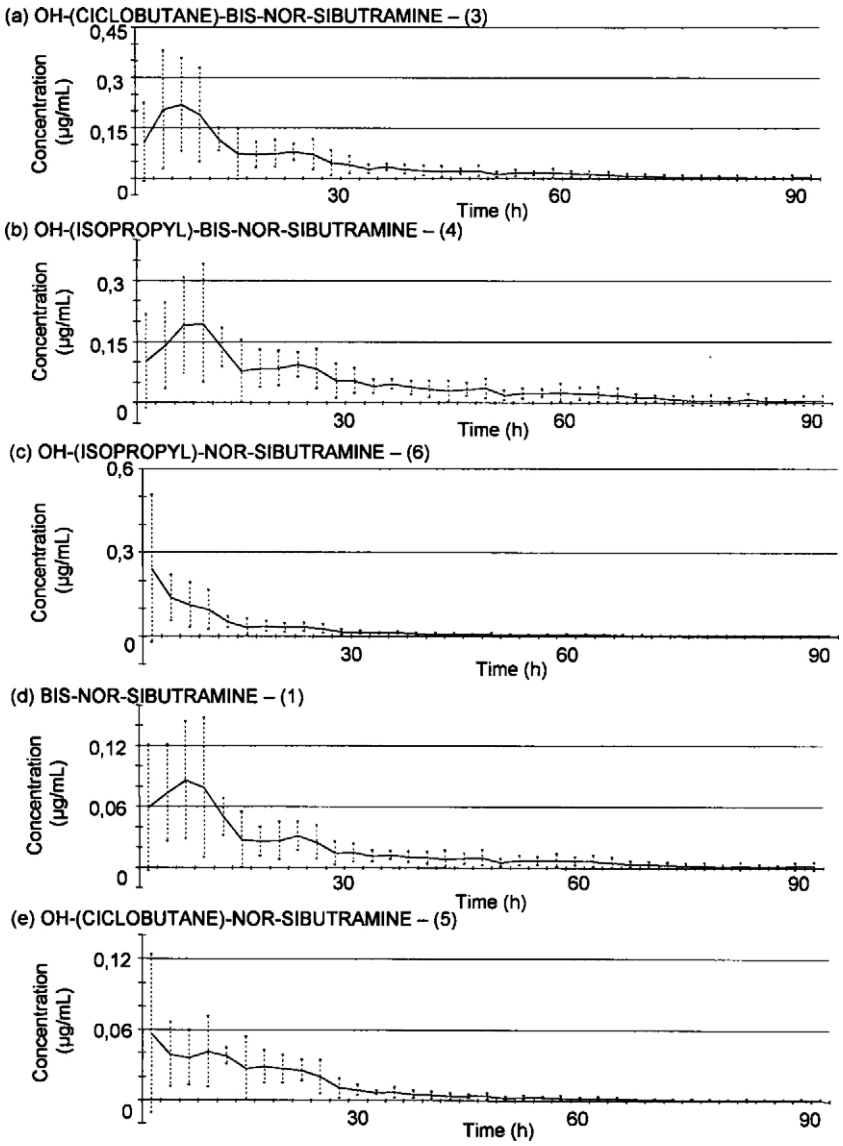

Fig. 9. Excretion in urine for five main SIB metabolites. The *Y* axis indicates the concentration in μg/mL and *X* axis the time in hours. Dashed line represents the standard deviation for the average, with $n = 5$ volunteers.

of the analytical target for SIB screening was done through the plot of concentration × time, for each metabolite detected. Hydroxy-cyclobutane-bis-nor-sibutramine was more abundant in the first 10 h after administration, with maximum average values of concentration estimated as 0.2 μg/mL.

However, after 40 h, hydroxy-isopropyl-bis-nor-sibutramine becomes the most abundant metabolite for all volunteers, since its concentration is at least two times higher than the second most abundant metabolite (hydroxy-cyclobutane-bis-nor-sibutramine). Both could be detected up to 90 h after administration (Fig. 8).

The results indicate that the analytical target for the detection of the recent abuse of SIB could be hydroxy-cyclobutane-bis-nor-sibutramine. In the long term, the results suggest the monitoring of hydroxy-isopropyl-bis-nor-sibutramine, since this metabolite shows the larger detection window. The lack of commercially available standards for SIB metabolites hinders the complete evaluation of the sensitivity of the method.

3.4. Disposition in the body

Rapidly absorbed after oral administration and distributed to the tissues, sibutramine is almost completely metabolized by microsomal CYP3A4, the principal isoenzyme of cytochrome P450 found in the liver [15]. Considering only the fraction of drug excreted in urine during the first 90 h, about 30% of the dose is excreted as hydroxy-cyclobutane-bis-nor-sibutramine (Fig. 9(a)) and other 30% as hydroxy-isopropyl-bis-nor-sibutramine (Fig. 9(b), as glucuronide conjugates, 20% as hydroxy-isopropyl-nor-sibutramine (Fig. 9(c)), 15% as bis-nor-sibutramine (Fig. 9d) and 5% as hydroxy-cyclobutane-nor-sibutramine (Fig. 9(e)). Nor-sibutramine is excreted in amounts smaller than 0.1%, being observed only in the first 6 h of excretion.

4. Conclusion

In this paper, we describe an alternative strategy for the detection of sibutramine metabolites, which fulfills the WADA identification criteria. The N-TFA-O-TMS derivatives formed are interesting for routine screening, decreasing the number of re-analyzes due to the presence of interference in the N-TMS-O-TMS derivatives. Alternatively, since they originate mass spectra with more than three diagnostic ions, it can also be used as an orthogonal confirmatory procedure. A second surge of the

main metabolites was observed 40 h after their major excretion.

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