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Detection of sibutramine metabolites as N-TFA and N-TFA, O-TMS derivatives

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The drug sibutramine (SIB,1-(4-(chlorophenyl)-N,N-dimethyl-α-(2-methylpropyl) cyclobutanemethanamine) was developed to moderate appetite. However because of its potential abuse in sports it was banned in 2006 by WADA, classified as a stimulant. Brazil is the world leader in sales of medicines without prescription. In 2007, thirteen adverse analytical findings for appetite suppressant drugs were reported, where eleven were from SIB.

Thevis *et al*. made the first contribution for the detection of SIB abuse for doping purposes, after the synthesis of the N-desmethyl (nor-sib) and N-bis-desmethyl (bis-nor-sib) metabolites and their detection by LC-MS-MS [1].

One year later, Strano Rossi *et al*. [2] reported the characterization of other four metabolites N,O-bis-TMS derivatives in biological fluids: 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). However many of these derivatives have poor spectra, so that they would hardly fulfill the identification criteria stipulated by WADA [3].

The purpose of the present work was: 1) to evaluate the formation and to characterize the urinary SIB metabolites previously described, using N-TFA, O-TMS as alternative derivatives. 2) to compare the mass spectra profiles of N-TFA, O-TMS and N, O-bis-TMS, following the qualitative WADA criteria. 3) to evaluate, through analysis of real samples, the urinary excretion profiles of SIB metabolites, looking for the best confirmation target compound.

The samples were prepared following our screenings II and IV, and analyzed by gas chromatography coupled to mass spectrometry (Agilent 5973), in electron impact and chemical ionization modes. Urine samples were collected after administration of a single dose

of 15 mg of monohydrated sibutramine hydrochloride, to three healthy males and two healthy females.

A priori, Donike's double derivatization strategy [4] 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.

All derivatives showed mass spectra with three or more ions in abundance superior to twenty percent (Table 1); on the contrary to the N-TMS, O-TMS derivatives, where only one ion is significant, and the others are smaller than five percent in the spectra.

Table 1: 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 Intensity order					
		$[M+H]^+$	$M+[H]^++C_2H_4$						
(1) bis-nor-sib	5.80	**348**	376	165	(100%)	137	(36%)	263	(3%)
(2) nor-sib	6.49	**362**	390	196	(100%)	140	(55%)	165	(39%)
(3) OH-bis-nor-sib2	6.94	**436**	464	116	(100%)	163	(48%)	263	(42%)
(4) OH-nor-sib2	7.39	**450**	478	196	(100%)	140	(37%)	154	(30%)
(5) OH-bis-nor-sib1	7.10	**436**	464	180	(100%)	165	(44%)	137	(34%)
(6) OH-nor-sib1	7.74	**450**	478	194	(100%)	110	(37%)	140	(20%)

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, O-bis-TMS derivatives, cleavage adjacent to the N atom is the more favored pathway, as demonstrated by Strano-Rossi *et al*. [2]. 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 (fig. 1.A and 1.B).

Both screenings permitted the observation of six metabolites in the first five hours. However the SIB metabolites included in screening IV showed an interference peak, frequently observed, close to the retention time of N, O-bis-TMS hydroxy-cyclobutane-bis-nor-sib (fig. 1.C and 1.D) the preferred ion for screening purposes. Indeed, from the eleven adverse analytical findings (AAFs) obtained with the N-TFA, O-TMS methodology in 2007, two were not detected when the N, O-bis-TMS derivatives were used, due to poor mass

spectra. Therefore, for these two AAFs with SIB metabolite in low concentration, only N-TFA, O-TMS avoided false negative reporting.

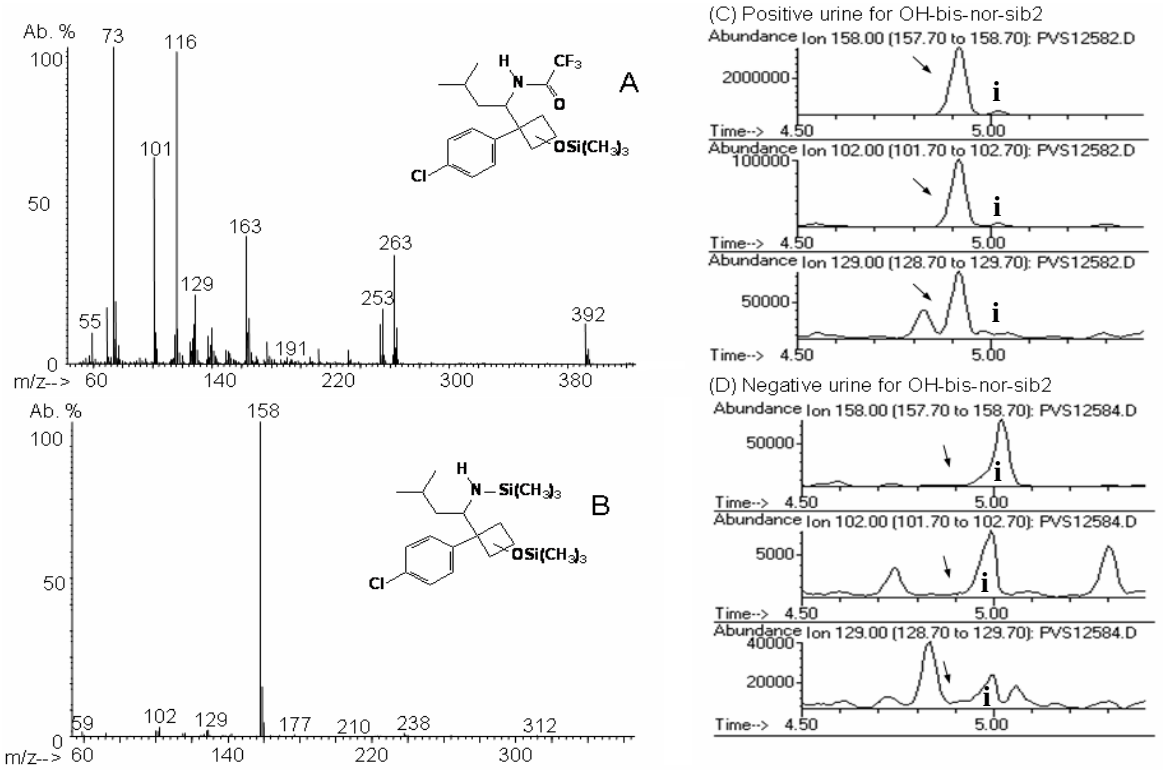

Fig. 1. Comparison between mass spectra (A) N-TFA, O-TMS derivative and (B) N, O-bis-TMS derivative for OH-bis-nor-sib2 (3) and comparison between positive urine, after 10 h of sibutramine administration (C) and negative urine, before sibutramine administration (D), for the N, O-bis-TMS derivative. Arrow shows t_R for OH-bis-nor-sib2 N, O-bis-TMS derivative; "i" shows the interferent.

The heterolytic cleavage is initiated by the electron ionization, being favored by the considerable stability of the benzylic carbocation, formed on the cyclobutane ring present in the sibutramine (fig 2.1). The migration of the two electrons to the asymmetric carbon of the molecule is justified by the inductive effect, caused by the electrophilic nature of the trifluoromethyl group attached to the nitrogen atom (fig 2.2). This induces the formation of a *m/z* 165 ion (fig 2.3), common to the four metabolites without OH in the ciclobutane ring. With the formation of a stable cation (fig. 2.4), a consecutive break of the carbon bond in the ciclobutane occurs, also favored by the elimination of a stable neutral fragment (fig 2.5) 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 presence of other peaks in the mass spectrum of these compounds.

In the screening II, the metabolite hydroxy-cyclobutane-bis-nor-sib was more abundant in the first 10 hours. It was replaced after 40 hours, by the metabolite hydroxy-isopropyl-bis-nor-sib even though both can be seen, at least, up to 90 hours.

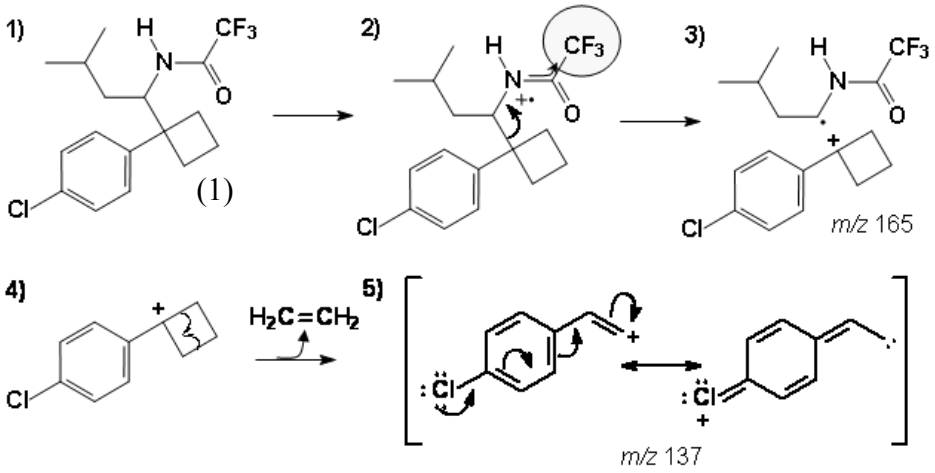

Fig. 2. Proposal for formation of ion *m/z* 165, through the heterolytic cleavage of the C-C bond beta to the nitrogen. 1) bis-nor-sibutramine N-TFA derivative (1), 2) Influence of the electrophilic field formed by the trifluoromethyl group (hatched area), arrows indicate the migration of electrons,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

The full experimental and instrumental conditions and other considerations about sibutramine metabolites analysis as N-trifluoroacety and O-trimethylsilyl derivatives by gas chromatography-mass spectrometry in urine are described elsewhere [5].

Acknowledgements: FUJB, FAPERJ, CNPq, CBF.

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