Journal of Chromatography A, 1218 (2011) 1266–1272

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Consequences of the formation of 3,4-dimethyl-5-phenyl-1,3-oxazolidine on the analysis of ephedrines in urine by gas chromatography and a new method for confirmation as N-trifluoroacetyl-O-t-butyldimethylsilyl ether derivatives

V.F. Sardela, P.D.O. Sardela, H.M.G. Pereira, F.R. Aquino Neto *

Universidade Federal do Rio de Janeiro, Instituto de Química, Ilha do Fundão, Avenida Athos da Silveira Ramos, 149, LAB DOP-LADETEC, 21941-909 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 10 September 2010
Received in revised form
28 December 2010
Accepted 30 December 2010
Available online 8 January 2011

Keywords:
Pseudoephedrine
Ephedrine
Artifact
Gas chromatography
MTBSTFA
Doping

ABSTRACT

The compound 3,4-dimethyl-5-phenyl-1,3-oxazolidine can appear as an artifact during the gas chromatographic analysis of ephedrines. Its presence is a risk for doping control and forensic analyses. An evaluation about the consequences of its formation showed the possibility of a false positive for ephedrine, a false negative for pseudophedrine and increased uncertainty in the quantitative approach. Misinterpretations can be avoided with the observation of fragments *m*/*z* 56 and 71 in the ephedrine mass spectrum during GC–MS analysis and also by the formation of N-TFA-O-TBDMS derivatives prior to GC analysis. These N-TFA-O-TBDMS derivatives lead to an increase in the number and mass of diagnostic ions, meet the identification criteria, and provide an improvement in chromatographic resolution, allowing the separation of the ephedrines.

1. Introduction

Ephedrines are banned in sports by the World Anti-Doping Agency (WADA), being classified as stimulant doping agents [1]. Some of them are ingredients of common medicines, being abusively used to reduce tiredness and increase alertness [2]. Thus, threshold values of ephedrines presence in athlete's bodies were established in order to allow the use of these medicines for therapeutic uses without leading to an adverse analytical finding in doping control. Due to their differing biological activity, ephedrine, pseudoephedrine, norpseudoephedrine, norephedrine (currently allowed) and methylephedrine have different threshold values (Fig. 1) [1]. In forensic analyses, ephedrines are potentially interesting, mainly in investigations of accidents involving intoxications [3,4]. Analysis of ephedrines has been a classical procedure in doping control since the 1960s, using gas chromatography (GC) as the preferred technique [5,6]. Since these compounds are diastereoisomers, the characterization based on mass spectral interpretation is not conclusive for identification purposes. Thus, chromatographic separation becomes the key identification step. Van Eenoo et al. provided an important contribution to the simultaneous quantification of ephedrines in urine by GC-NPD with a special temperature program for the separation of diasteroisomers [7]. However, when one of the isomers is in high concentration, it can lead to a co-elution of the analytes over at least part of a chromatographic peak. As these substances have different thresholds, it becomes necessary to identify and separate the diastereoisomers before the quantification. An effective methodology was established for the analysis of the ephedrine's enantiomers [8], but the chiral separation for ephedrine quantitative approach, in routine doping analyses, was not recommended because several peaks can be generated by this method, due to different chiral structures of the same molecule, which can then be different from the proportions of controls available on analysis. Recently, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has been suggested as a quantification procedure [9] by direct injection of the sample. The LC–MS/MS procedure suggested is simple and sensitive, but the direct injection of the urine in LC–MS/MS system could generate ion suppression and retention time instability. Even after dilution, the influence of the matrix will be different if an external quantitative control is used because of the influence of the matrix in the sample. Therefore the effects of suppression will be controlled only if deuterated internal standards are used in all quantitative controls and samples, as suggested by Deventer et al.

After a long absence, pseudoephedrine returned to the prohibited list in 2010 with a considerably high threshold (150 μg/mL).

* Corresponding author. Tel.: +55 21 2260 3967; fax: +55 21 2260 3967.
E-mail address: radler@iq.ufrj.br (F.R. Aquino Neto).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.12.120

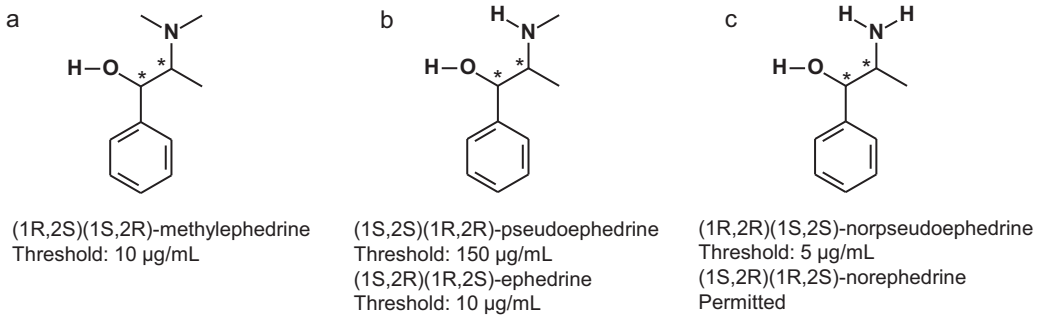

Fig. 1. Chemical structures of methylephedrine (a), pseudoephedrine and ephedrine (b) and norpseudoephedrine and norephedrine (c), with respective threshold values.

Deventer et al. highlighted the increase of consumption of pseudoephedrine in sports during the period its use was permitted (2004–2009) [10]. Indeed, the use of pseudoephedrine is relatively frequent and some cases have been observed in our laboratory in the last five years. In some of these samples it was possible to observe in GC-NPD a minor peak at the ephedrine's expected retention time in a GC-NPD chromatogram. This behavior has also been observed in other anti-doping laboratories (personal communication). In one of these samples analyzed in our lab, the "minor peak" had intensity similar to the expected one for ephedrine when around the WADA's threshold.

The source of this minor peak is controversial and different possibilities have been discussed among the anti-doping specialists. Among them, possible contaminations with ephedrine in the pseudoephedrine tablet, or the possibility of an epimerization reaction, converting pseudoephedrine to ephedrine, have not been completely discarded.

The presence of an unknown peak in sympathomimetic analysis under GC conditions, interfering with the pseudoephedrine identification, was previously described by Lewis et al. [11]. In their experimental conditions for investigating samples from aircraft accidents, the unknown peak co-eluted with pseudoephedrine. After an exhausting characterization process, the interfering peak was identified as a 3,4-dimethyl-5-phenyl-1,3-oxazolidine, a pseudoephedrine–formaldehyde adduct. The formation of this kind of adduct from β-aminoalcohols is well documented when aldehydes are present. Wille and Lambert also observed this adduct, and noted that mass spectral library searches could misidentify it as phenmetrazine [12]. Classified as a stimulant doping agent, phenmetrazine is prohibited by WADA at any concentration detected. The mass spectrum of phenmetrazine is similar to that for 3,4-dimethyl-5-phenyl-1,3-oxazolidine. Therefore, a simple analysis by GC–MS can generate an apparent identification for phenmetrazine due to the formation of the oxazolidine artifact.

The formation of the oxazolidine derivatives as products from condensation between β-aminoalcohols and aldehydes [13,14] has already been observed for pseudoephedrine and ephedrine [15,16], and the differences in stereochemistry of the diastereomers are conserved in the oxazolidines [17,18]. The condensation of aldehydes with β-hydroxyethylamines takes place with primary [19] and secondary amines [20], while tertiary amines are incapable of undergoing such reaction [20]. Therefore, methylephedrine (a tertiary amine), does not form such adducts. The high temperature and aldehydes concentration are an important variables to increase the condensation reaction velocity of pseudoephedrine with aldehydes [21] (Fig. 2). Therefore, GC analyses with high injector temperatures and with extract dissolved in solvents such as methanol, which could be dehydrogenated to formaldehyde [21], increase the oxazolidine formation in the GC injector [22].

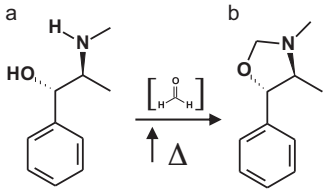

Fig. 2. Pseudoephedrine (a) conversion to 3,4-dimethyl-5-phenyl-1,3-oxazolidine (b).

The stimulants classes derived from epinephrine core structure, including phenmetrazine and ephedrines, have low molecular mass and a mass spectrum, which shows only one ion of low *m*/*z*, for identification (Fig. 3a). Therefore GC–MS analyses adopting derivatization strategies are currently used to increase the mass of the fragments, to add other diagnostic ions for structural characterization, and also to improve the chromatographic peak shapes. However, the strategy of double derivatization, to form N-TFA-O-TMS derivatives, reported by Donike [23] (Fig. 3b) and other derivatives for ephedrines [24,25] (Fig. 3c and d), do not show mass spectra with more than three ions as would be required for current identification criteria [26–28].

The aim of the present work is to characterize the unknown peak, potentially co-eluting with ephedrine, observed in our analyses and other doping control laboratories, and to determine its origin and the variables that may enhance its presence. In addition, the possible impact in the diagnosis of ephedrine's abuse in doping control scope is discussed. Finally, we propose a method based on the O-tert-butyldimethylsilyl-N-trifluoroacetamide, derivative that increases the mass of the fragments and prevents the ephedrine-artifacts, provides an improvement in chromatographic resolution and provides data for unequivocal characterization of ephedrines in human urine analyses.

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) [29], jointly with the WADA International Standard for Laboratories [30].

2.2. Chemicals

The internal standard (IS) diphenylamine (99%), potassium hydroxide and formaldehyde solution (37%) were purchased from Merck KGaA (Darmstadt, Germany), methanol and tert-butylmethylether (TBME) were purchased from Tedia (Fairfield, OH, USA), N-methyl-bis-(trifluoroacetamide) (MBTFA) (99.7%)

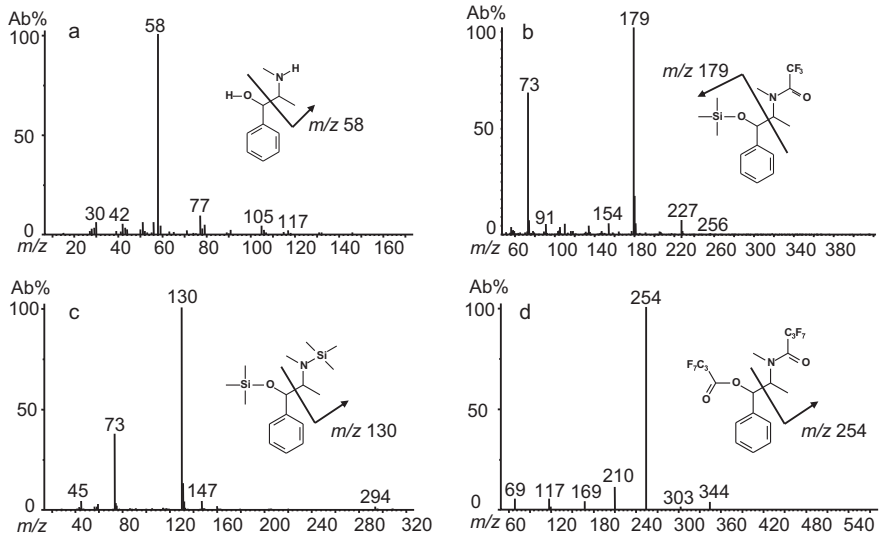

Fig. 3. Comparison between mass spectra (a) non-derivatized ephedrine, (b) N-TFA-O-TMS derivative, (c) N-O-bis-TMS derivative and (d) N-O-bis-(heptafluorobutyryl) derivative, showing their poor diagnostic ions.

and N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (99.1%) from Chemische Fabrik Karl Bucher Gmbh (Waldburg, Germany); the standards ephedrine and pseudoephedrine were purchased from Cerilliant (Austin, TX, USA).

2.3. Sample preparation

For characterization of the unknown peak and identification of its origin, the samples were treated following the method routinely used in our lab for stimulants by GC-NPD [31] adapted for ephedrine's quantification. Briefly, 5 mL of urine was spiked with 20 μL of diphenylamine, used as internal standard at a concentration of 10 μg/mL, followed by the addition of 0.2 mL of KOH (0.5 M), 2 mL of TBME and 1 g of Na_2SO_4. After mixing and centrifugation, the aqueous phase was discarded. For investigation of the causes that increase the presence of the unknown peak, the organic phase was split in two fractions. Fraction (A): 2 μL of organic phase was directly injected in the GC-NPD system. Fraction (B): 1.8 mL of organic phase was split equally in two further fractions: fractions (B1) and (B2). Both these fractions were concentrated under nitrogen flow at room temperature. The dry extract from B1 was re-suspended in 0.1 mL of methanol and the one from B2 was re-suspended in 0.07 mL of methanol + 0.03 mL of formaldehyde. The extracts of fractions B1 and B2 were analyzed by mass spectrometry in both EI and CI ionization modes with variation in GC–MS conditions.

For evaluation of possible impacts on the quantitative analysis of ephedrine, five concentrations (L) of pseudoephedrine were analyzed in triplicate: L1: 15 μg/mL, L2: 30 μg/mL, L3: 60 μg/mL, L4: 90 μg/mL and L5: 180 μg/mL. The total volume of 2.0 mL of solvent was used for all levels (0.2 mL of methanol + 1.8 mL of TBME). Diphenylamine was used as internal standard at a concentration of 10 μg/mL.

In order to increase the number of diagnostic ions in ephedrine analyses, 20 μL of MBTFA were added to fraction (A) after it has been analyzed by GC-NPD, then it was dried under nitrogen at 40 °C. The residue was derivatized by adding 100 μL of MTBSTFA at 60 °C for 10 min, followed by the addition of 20 μL of MBTFA at 60 °C for 10 min, finally the extract was injected in GC–MS.

Seven aliquots of a quality control (QC) were prepared with 2 μg of ephedrine and 2 μg pseudoephedrine in 2 mL of a blank of urine. The final concentration of each compound in urine was 1 μg/mL. Diphenylamine was used as internal standard at a concentration of 10 μg/mL.

The repeatability assay consisted of analysis of the QC samples (1 μg/mL of ephedrine and pseudoephedrine each) including extraction and derivatization step. The repeatability was calculated for each compound, as %RSD. Another seven bottles were spiked (2 μg of ephedrine and pseudoephedrine each) without addition of matrix (blank urine), and only including the derivatization step. These bottles were considered as showing 100% of extraction efficiency. The chromatogram areas were compared to those of the QC samples extracts (the same QC samples used in the repeatability test).

For evaluation of matrix interference, negative urines ($n = 10$) for ephedrine and pseudoephedrine were analyzed to check the presence of interferent peaks at the expected retention times for the analytes and the I.S. For selectivity, one blank of urine was spiked with 0.5 μg/mL of the sympathomimetic amines of similar structure (octopamine, norpseudoephedrine, etilefrine and heptaminol).

Limit of detection (L.O.D.) was determined from a QC sample with 10, 5 and 2.5 ng/mL of ephedrine and pseudoephedrine and the lower limit of quantification (L.L.O.Q.) was determined from a QC sample with 30, 25 and 20 ng/mL. The IS quantity (20 μL of diphenylamine) was the same in all QC samples. The criterion established were: lowest concentration that would be detected with signal-to-noise (s/n) > 3 for L.O.D. and s/n > 10 for L.L.O.Q.

The peak area ratios between the fragment ion m/z 221, for ephedrine and pseudoephedrine, and m/z 167 for I.S. were used for the quantification. The calibration curves were performed at five levels: 5–20 μg/mL for ephedrine and 7.5–30 μg/mL for pseudoephedrine.

For estimation of the stability of the derivatized extract, under the conditions of GC–MS analysis, a QC sample was stored at ambient condition. After ten days the analysis sequence was repeated with the same extract.

2.4. Instrumental analyses

2.4.1. GC 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) and with a

Table 1
Retention time (t_R) and relative retention time (RRT) for ephedrines and its metabolites; diphenylamine as internal standard.

Target compound	t_R (min)	RRT
Norpseudoephedrine (pseudoephedrine metabolite)	11.36	0.50
Norephedrine (ephedrine metabolite)	11.71	0.52
Ephedrine	13.78	0.61
Pseudoephedrine	14.12	0.63
Methylephedrine	16.21	0.72
Diphenylamine (I.S.)	22.52	1.00

nitrogen–phosphorus detector (NPD) (Agilent Technologies Inc., Santa Clara, CA, USA). 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 (100% 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 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 was 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 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).

2.4.2. NPD conditions

Nitrogen–phosphorus detector conditions: detector temperature at 250 °C; hydrogen flow of 2.0 mL/min, compressed air at 60.0 mL/min and make up flow of nitrogen at 30.0 ml/min. Bead voltage of 3400 meV.

2.4.3. MS conditions

The mass spectrometer was operated in dual ionization modes. (i) Chemical ionization (CI): transfer line, 280 °C; ion source temperature 150 °C; interface temperature, 280 °C; quadrupole temperature, 180 °C; methane as ionization gas (7000 mTorr) in the mass spectrometer source, in full scan mode with mass range of *m*/*z* 50–600. (ii) Electron impact ionization (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 with mass range of *m*/*z* 50–600.

3. Results and discussion

3.1. Investigation procedure

The analysis of ephedrines using GC-NPD is based on the determination of the retention time for each compound. This technique is strongly suggested, because the method is selective, since it only detects nitrogen and phosphorus molecules, allowing greater accuracy in quantification. Table 1 shows the expected retention time for each analyte using the chromatographic ramp described. However, the CG-NPD analysis is not considered specific enough to confirm the presence of a substance. So, if a peak presents the same t_R expected for a monitored substance, the sample is dried and the extract is recovered with a small volume of methanol. And then analyzed by GC–MS. The mass spectrum of the peak in question is then compared with the reference mass spectrum (a reference sample collection or a standard analyzed contemporaneously). Frequently, a chromatographic peak with the same t_R of a controlled analyte is generated due to interference. However, it was observed that the same interference with ephedrine's t_R was always present in samples with high concentrations of pseudoephedrine. This co-elution increases the ephedrine's signal. This is critical in the hypothetic situation where ephedrine and pseudoephedrine are present in the same sample, but both in concentrations lower than their respective thresholds. The qualitative criteria for a positive ephedrine result could be fulfilled, since the ephedrine mass spectrum will be present and the apparent ephedrine concentration will be increased by the influence of the interference. Therefore, a false positive for ephedrine could be declared. In quantitative approaches, the presence of an interferent peak may also be responsible for deviations in the linearity of calibration curves and increase of uncertainty values. High uncertainty values are undesirable in quantitative analyses, because they reduce the confidence in measurement and can invalidate a result. Several approaches are described in the literature to evaluate uncertainty in quantitative methods. Recently, WADA provides a technical document to guide the accredited laboratories concerning decision limits and uncertainty evaluation [32]. The inter-laboratory method data approach is described and become an excellent tool to evaluate quantitative analytical methods systematically and in an agile way. The information from a WADA inter-laboratory study for ephedrine analysis were used to evaluate the impact of the adduct in ephedrine's quantification. The uncertainty was estimated using a urine containing ephedrine at 12.6 μg/mL (nominal concentration) using two scenarios: (i) with addition of pseudoephedrine in 100 μg/mL and (ii) without pseudoephedrine. In pseudoephedrine presence, the ephedrine uncertainty was 94.9% due to the interference. Without pseudoephedrine, the same reference urine showed the uncertainty of 8.2%. The total uncertainty was evaluated by inter-laboratory comparison for both reference urines, with $K = 2$ and 95% confidence interval. Quantification by GC-NPD is strongly suggested, because the method is selective, since it only detects nitrogen molecules, which allows greater accuracy in quantification. However, in samples with ephedrine and pseudoephedrine it is suggested the quantification by GC methods with derivatization strategies, which prevents the formation of interferences.

In order to characterize the interferent peak (t_R 13.78 min) observed in the GC-NPD analysis of real samples, a GC–MS analysis was performed on the B1 fraction and the mass spectrum was not from ephedrine (Fig. 4). A CI experiment resulted in the formation of the molecular ions $[M+H]^+$ and $[M+C_2H_5]^+$ with *m*/*z* 178 and 206, respectively, indicating *m*/*z* 177 as the presumable molecular mass. The mass spectrum obtained by GC–MS in EI mode indicated the presence of *m*/*z* 56 and 71 as the highest intensities, unlike the expected ephedrine mass spectrum, where base peak is *m*/*z* 58. Therefore, this mass spectrum matches that described by Lewis et al. for the pseudoephedrine–formaldehyde adduct [11]. Impurities (aldehydes) in the reconstitution solvents, described by Beckett and Moffta [33], increases the possibility of artifact formation in ephedrine analyses. Based in spectral information (EI and CI modes) and molecular mass, it is likely that the artifact present is the 3,4-dimethyl-5-phenyl-1,3-oxazolidine. Slight differences in the chromatographic conditions explain why the artifact mentioned by Lewis co-eluted in the same retention time of pseudoephedrine rather than co-eluting with ephedrine.

Initially, 1.2×10^{-7} mmol of pseudoephedrine was injected using routine GC conditions (injector temperature of 250 °C and TBME as solvent) and the *m*/*z* 58 and 71 were monitored as base peaks for pseudoephedrine and 3,4-dimethyl-5-phenyl-1,3-oxazolidine, respectively. The areas of the *m*/*z* 58 and 71 peaks were 92.9% and 7.1%, respectively (Fig. 5a). When the same amount of pseudoephedrine was analyzed, but with the injector temperature at 300 °C, there was a reduction in the *m*/*z* 58 area to 70.4% and increase in the area for *m*/*z* 71–29.6% (Fig. 5b). Finally, when 1.0×10^{-4} mmol of formaldehyde together with 1.2×10^{-7} mmol of pseudoephedrine were injected at an injector temperature of

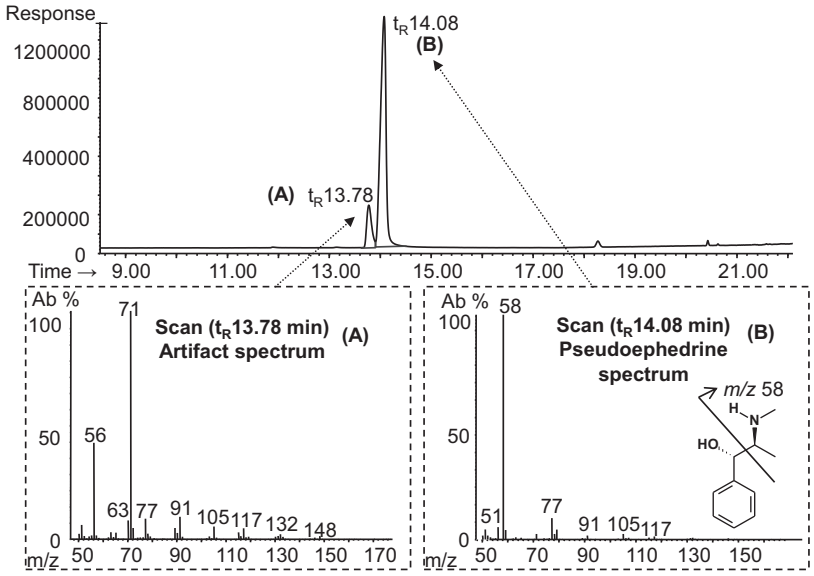

Fig. 4. Mass spectra of the peaks in the same retention time of ephedrine (t_R13.78) and the peak in the retention time of pseudoephedrine (t_R 14.08).

250 °C the *m/z* 58 area reduced to 0.8% and the *m/z* 71 area increased to 99.2% (Fig. 5c). Therefore, the formation of the artifact is favored by increasing the injector temperature and the amount of aldehyde available at the time of injection.

The formaldehyde could be a solvent impurity or originate from other sources such as the matrix itself urine, promoting the conversion of pseudoephedrine into 3,4-dimethyl-5-phenyl-1,3-oxazolidine, which will decrease the real area of pseudoephedrine in the chromatographic peak.

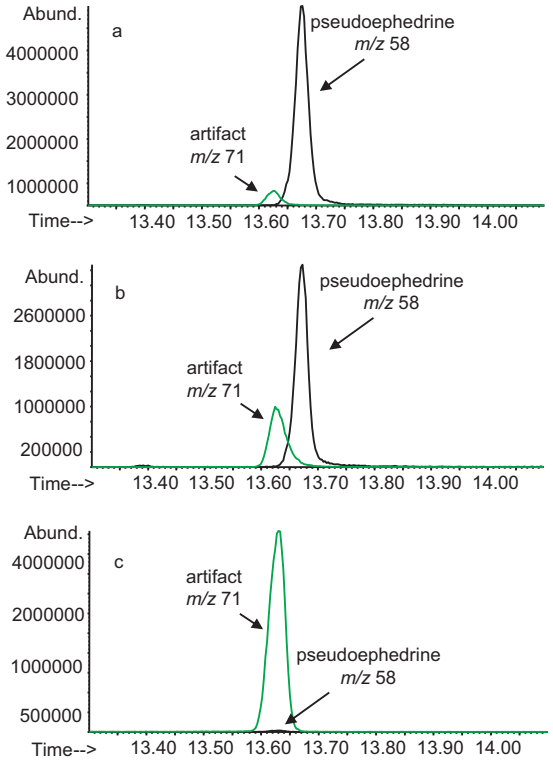

Fig. 5. Extracted ion chromatogram from electron impact full scan GC–qMS for 10 μg/mL of pseudoephedrine. (a) TBME solution with injector temperature at 250 °C, (b) TBME solution with injector temperature at 300 °C and (c) TBME solution containing formaldehyde with injector temperature at 250 °C.

Screening procedures are used for qualitative analysis and quantitative estimation. Typically, more elaborate quantitative methods, and calibration curves are only conducted after concentration estimation in screening. When the artifact is formed, the estimated concentration of pseudoephedrine would be lower than the real value (considering the uncertainty). Therefore, the quantitative confirmation might not be performed. So, in such circumstances, the sample containing pseudoephedrine would be erroneously declared as negative. The formation of the artifact could be a risk for doping control analysis, especially in samples containing pseudoephedrine and ephedrine. The concomitant administration of these substances by athletes might become more common, in an attempt to prevent that both substances reach their respective thresholds, while by synergistic effect they could still enhance performance. GC analysis showed the artifact's formation is not linear with concentration of pseudoephedrine (Fig. 6). The repeatability of the artifact formation was measured with ten injections of an extract containing 60 μg/mL of pseudoephedrine, in the same GC-NPD screening analysis conditions, and the coefficient of variation obtained was 15.12%, showing that the 3,4-dimethyl-5-phenyl-1,3-oxazolidine formation is variable. Therefore, for quantitative analysis by GC-NPD, it is necessary to determine if the artifact is present. To evaluate the formation of

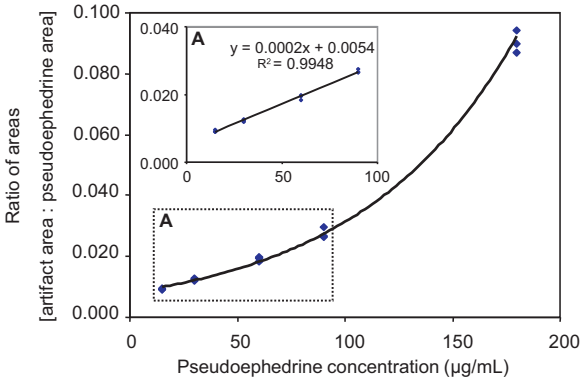

Fig. 6. Artifact formation curve: *X* axis indicates the concentration of pseudoephedrine in μg/mL and *Y* axis the ratio (artifact formation: pseudoephedrine). Insert A shows an expansion of the beginning of the curve.

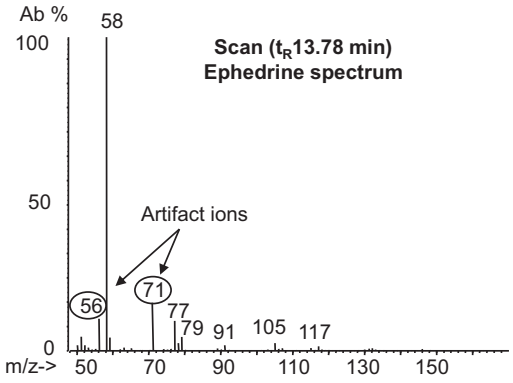

Fig. 7. Ephedrine mass spectrum with the artifact contribution circled.

the pseudoephedrine–formaldehyde adduct, a GC–MS analysis on the same extract analyzed by NPD was performed without any derivatization step. Then, the mass spectrum at the t_R corresponding to ephedrine showed the ions m/z 71 and 56, which indicates the artifact formation (Fig. 7).

Ephedrine analysis by GC–MS, adopting the derivatization strategies previously described in the literature, prevents ephedrine's conversion to the artifact and allows the identification of ephedrines in urine, but do not generate a mass spectrum with three or more diagnostic ions and so do not meet current identification criteria [26–28]. For an identification that meets the WADA's qualitative criteria, a second ionization technique or derivatization is required in these cases.

The compound MTBSTFA is a derivatization reagent with similar reactivity to MSTFA. It forms derivatives, which have chromatographic retention times larger than similar TMS derivatives, which potentially favors the chromatographic resolution of the ephedrine diastereomers. These derivatized products, when formed, usually show the formation of the fragment $[M-57]^+$ due to the loss of the tert-butyl fragment. The formaldehyde adduct formation is prevented when derivatization with MTBSTFA reagent is performed since it inactivates the polar functional groups of pseudoephedrine molecule: –OH and –NH. However, using this reagent alone does not provide an increase in mass spectra information. The difficulty in the interpretation of cases containing such substances (ephedrine, pseudoephedrine, phenmetrazine and other with similar structures) is that their mass spectra show only one ion of low m/z for identification purposes. The use of MBTFA in a two-step derivatization, allows the formation of the trifluoroacetamide group in the secondary amine of ephedrine/pseudoephedrine, which allows a change in the profile of fragmentation, including a β-heterolytic cleavage as discussed by Sardela et al. [34]. Thus, the mass spectra of the N-TFA-O-TBDMS derivatives (Fig. 8a) allow the unambiguous characterization by mass spectrometry, since it increases the mass of the fragments and the number of diagnostic ions. It also provides a gain in chromatographic resolution in samples with high concentrations of the ephedrine diastereoisomers after extraction in urine (Fig. 8b). One blank of urine spiked with sympathomimetic amines showed that they do not interfere in the identification of the ephedrines. The N-TFA-O-TBDMS derivatives of ephedrine and pseudoepehedrine were stable for ten days at room temperature. Derivatives were stable for both compounds (ephedrine and pseudoephedrine) and the extraction efficiencies for ephedrine and pseudoephedrine were of 87.3% and 82.4% respectively, with RSD, for seven aliquots, of 6.6% for ephedrine and 6.7% for pseudoephedrine. For this derivatization, the limit of detection (L.O.D., s/n = 3) was estimated by measuring the signal-to-noise ratio of blank urine spiked with both compounds

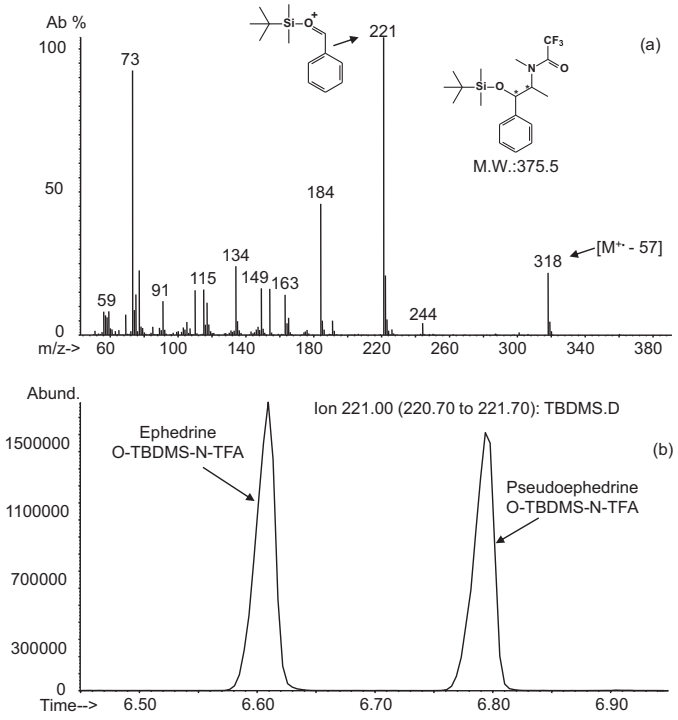

Fig. 8. (a) GC–qMS of ephedrine O-TBDMS-N-TFA derivative, with suggested structures for key ions. (b) Extracted ion chromatogram of m/z 221, from electron impact full scan GC–qMS.

(ephedrine and pseudoephedrine) in 10 ng/mL and for lower limit of quantification (L.L.O.Q., s/n = 10) was 25 ng/mL. The intermediate precisions were 1.4% and 2.1% for ephedrine and pseudoephedrine respectively.

The analytical range was set to 5–20 μg/mL for ephedrine and 7.5–30 μg/mL for pseudoephedrine. For both analytes the linear relationsphips were observed (Fig. 9) with acceptable linearity ($r^2 \geq 0.990$), using a least square fit. One reference sample from WADA External Quality Assessment Scheme (EQAS) in 2008, spiked urine, with nominal concentration of 12.6 μg/mL of ephedrine was analyzed. The quantitative comparison between consensus value of 32 doping control laboratories, for this WADA EQAS reference sample, and the estimated concentration obtained with N-TFA-O-TBDMS derivatives was done. The consensus value was 12.54 μg/mL and RSD of 4.62%. The mean value obtained for triplicate samples with N-TFA-O-TBDMS derivatives was 13.04 μg/mL and RSD 1.49%. The consensus value was used. Robust statistics (median, interquartile mean and Huber's mean) were applied which were not influenced by the presence of "outliers". The z–score gives a bias estimate of the result, z-score ≥3 are unacceptable by WADA. For N-TFA-O-TBDMS derivatives the z-score was 0.96. This z-score value corresponds to excellent results for the concentration determination of ephedrine. The same reference sample, spiked with 100 μg/mL of pseudoephedrine, obtained the z-score of 10.83 when N-TFA-O-TBDMS derivatives was not applied. For pseudoephedrine a reference control was one blank urine spiked with nominal concentration of 60 μg/mL. Three aliquots of this sample were diluted to 15 μg/mL and quantified. The mean value obtained was 14.28 μg/mL and RSD 1.27%.

Ephedrine and pseudoephedrine areas in the blank urine injected after higher concentration level controls in calibration curves represents 0.02% and 0.08%, of these compounds areas in the spiked control, respectively. This carryover is negligible, so does not interfere in the estimation of the concentration or in the linearity of the calibration curve.

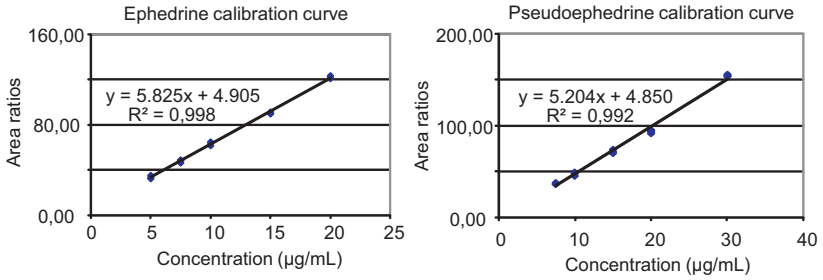

Fig. 9. Quantitative results from the regression analysis for ephedrine and pseudoephedrine O-TBDMS-N-TFA derivatives.

4. Conclusions

A common interferent peak in ephedrines analyses by GC was characterized as an artifact. Its formation occurred in the presence of formaldehyde and was favored by an increase in the injector temperature when TBME was the injection solvent. This artifact was identified as the 3,4-dimethyl-5-phenyl-1,3-oxazolidine by mass spectrometry fragmentation and molecular mass from CI, in agreement with previous literature. The presence of oxazolidine can be determined using GC–MS analysis by observation of the ions *m*/*z* 71 and 56. The combined use of MTBSTFA and MBTFA in two steps for formation of N-TFA-O-TBDMS derivatives of the ephedrines allows unambiguous characterization and quantification by mass spectrometry and hinders the condensation of aldehydes with ephedrine molecules and consequent artifact formation.

Acknowledgments

This work was supported by Fundação Universitária José Bonifácio-FUJB, CNPq, FAPERJ and CBF.

References

[1] The World Anti-Doping Code, The 2010 Prohibited List, WADA, Montreal, 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2010_EN.pdf.
[2] M. Verroken, Balliére's Clin. Endocrinol. Metab. 51 (2000) 1.
[3] D. Samenuk, M.S. Link, M.K. Homoud, R. Contreras, T.C. Theohardes, P.J. Wang, N.A.M. Estes, Mayo Clin. Proc. 77 (2002) 12.
[4] K. Björnstad, P. Hultén, O. Beck, A. Helander, Clin. Toxicol. 47 (2009) 566.
[5] A.H. Beckett, G.T. Tucker, A.C. Moffat, J. Pharm. Pharmacol. 19 (1967) 273.
[6] P.J. van der Merwe, S.E. Hendrikz, J. Chromatogr. B 663 (1995) 160.
[7] P. Van Eenoo, F.T. Delbeke, K. Roels, P. de Backer, J. Chromatogr. B 760 (2001) 255.
[8] S.-M. Wang, R.J. Lewis, D. Canfield, T.-L. Li, C.-Y. Chen, R.H. Liu, J. Chromatogr. B 825 (2005) 88.
[9] K. Deventer, O.J. Pozo, P. Van Eenoo, F.T. Delbeke, J. Chromatogr. B 877 (2009) 369.
[10] K. Deventer, P. Van Eenoo, G. Baele, O.J. Pozo, W. Van Thuyne, F.T. Delbeke, Drug Test. Anal. 1 (2009) 209.
[11] R.J. Lewis, E.F. Huffine, A.K. Chaturvedi, D.V. Canfield, J.J. Mattson, J. Forensic Sci. 45 (2000) 898.
[12] S.M.R. Wille, W.E.E. Lambert, J. Chromatogr. A 1045 (2004) 259.
[13] L. Knorr, H. Mathes: Ber. 34 (1901) 3484.
[14] L. Knorr, P. Rpessler: Ber. 36 (1903) 1278.
[15] E. Schmidt, Arch. Pharm. 89 (1914) 262.
[16] W. Davies, J. Chem. Soc. 213 (1932) 1580.
[17] G. Fodor, K. Koczka, J. Chem. Soc. 155 (1952) 850.
[18] A.F.L. Abdullah, G.M. Miskelly, J. Forensic Sci. 54 (2009) 365.
[19] M. Sennkus, J. Am. Chem. Soc. 67 (1945) 1515.
[20] R.H. Willey, Chem. Rev. 37 (1945) 401.
[21] C. Koppel, K.M. Peixoto-Menezes, J. Tenczer, J. Chromatogr. B 563 (1991) 73.
[22] A.A.F. Lim, G.M. Miskelly, Talanta 81 (2010) 455.
[23] M. Donike, J. Chromatogr. 103 (1975) 91.
[24] A.C. Moffat, E.C. Horning, S.B. Matin, M. Rowland, J. Chromatogr. A 66 (1972) 255.
[25] E.M. Thurman, M.J. Pedersen, R.L. Stout, T. Martin, J. Anal. Toxicol. 16 (1992) 19.
[26] Technical Document TD 2009IDCR, Identification Criteria for Qualitative Assays Incorporating Chromatography and Mass Spectrometry, Version 1.0, WADA, Montreal, 2010, http://www.wada-ama.org/Documents/News_Center/WADA_TD2009IDCR_Identification_Criteria_Qual_Assays_Oct2009.pdf.
[27] Council Directive 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, J. Eur. Commun. L221, (2002) 8–36.
[28] US Department of Health and Human Services, Food and Drug Administration, Guidance for Industry, Mass Spectrometry Confirmation of the Identity of Animal Drug Residues, 2003, Center for Veterinary Medicine, http://www.fda.gov/cvm/guidance/guide118.pdf.
[29] ABNT NBR ISO/IEC 17025:2005, General Requirements for the Competence of Testing and Calibration Laboratories, ABNT, 2005.
[30] Technical Document International Standard for Laboratories, Version 6.0, WADA, Montreal, 2009, http://www.wada-ama.org/Documents/World_Anti-doping_Program/WADP-IS-Laboratories/WADA_Int.Standard_Laboratories_2009_EN.pdf.
[31] H.M.G. Pereira, M.C. Padilha, R.M.A. Bento, T.P. Cunha, N.A.G. Lascas, F.R. Aquino Neto, Trends Anal. Chem. 27 (2008) 648.
[32] Technical Document TD 2010DL, Decision Limits for the Confirmatory Quantification of Threshold Substances, Version 1.0, WADA, Montreal, 2010, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010DLv1.0_Decision%20Limits%20for%20the%20-Confirmatory%20Quantification%20of%20Threshold%20Substances_May%2008%202010_EN.doc.pdf.
[33] A.H. Beckett, A.C. Moffta, J. Pharm. Pharmacol. 20 (1968) 48S.
[34] V.F. Sardela, M.T.R. Motta, M.C. Padilha, H.M.G. Pereira, F.R. Aquino Neto, J. Chromatogr. B 877 (2009) 3003.