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Could a pseudoephedrine artifact lead to a misinterpretation? A pitfall in ephedrines analysis

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1. Introduction

Ephedrines are banned in sports by the World Anti-Doping Agency (WADA) with different threshold values. Since these compounds are diastereoisomers, the characterization based on mass spectral interpretation is not conclusive for identification purposes. Thus, chromatographic separation becomes the key in the identification step.

After a long absence, pseudoephedrine returned to the prohibited list in 2010 with a considerably high threshold (150 μg/mL). Indeed, the use of pseudoephedrine at bellow threshold level is relatively frequent.An interfering peak which co-elutes with ephedrine 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 [1].

The ephedrines have low molecular mass and show a mass spectrum with only one ion of low *m/z* for identification. Therefore, derivatization strategies are currently used to increase the mass of the fragments. However, the strategy of double derivatization to form N-TFA-O-TMS derivatives, reported by Donike [2], and other derivatives for ephedrines [3,4] do not show mass spectra with more than three ions as required by the current identification criteria. Recently, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been suggested as a quantification procedure by direct injection of the sample [5]. The LC-MS/MS procedure suggested is simple and sensitive, but the direct injection of the urine could generate ion suppression and retention time instability. Even doing preceding dilution, the influence of the matrix will be different in the quantitative control for the influence of the matrix in the sample, so 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*.

The aim of the present work is evaluate and discuss the possible impact in the diagnosis of ephedrine's abuse in doping control scope. 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

Briefly, 5 mL of urine was spiked with 20 μL of diphenylamine (I.S.) at final concentration of 10 μg/mL, followed by the addition of 0.2 mL of KOH (0.5M), 2 mL of TBME and 1 g of Na_2SO_4. After mixing and centrifugation, 0.2 mL of organic phase was directly injected in the GC-NPD (Fraction A) and another 0.2 mL of the same organic phase was analyzed by mass spectrometry (Fraction B).

For evaluation of possible impacts on the quantitative analysis of ephedrine, a calibration curve for ephedrine was generated using blank urine spiked at 5, 7.5, 10, 15 and 20 μg/mL. One reference sample, spiked urine with nominal concentration of 12.6 μg/mL of ephedrine, was analyzed without and with the addition of 100 μg/mL of pseudoephedrine.

In order to increase the number of diagnostic ions in ephedrine analyses, 20 μL of MBTFA was added to fraction (B), 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.

GC conditions: The analyses were performed using a Hewlett Packard (HP) (Palo Alto, CA, USA) GC model 6890N equipped with a 7673B HP auto sampler coupled with a quadrupole MS, Agilent (5973 Network) and with a NPD (Agilent Technologies Inc., Santa Clara, CA, USA). Carrier gas was He (4.5) 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: 2μL split 1/10; septum purge 60 mL/min. The GC programming: initial column oven temperature 60 °C (1 min) then programmed to rise to 110 °C at 20 °C/min (isothermally for 14 minutes), then to 280 °C at 20 °C/min (isothermally for 1 minute), and to 300 °C at 40 °C/min (3 minutes).

<u>MS conditions:</u> Electron Impact ionization with following operations conditions: 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.

3. Results and discussion

The interference of the 3,4-dimethyl-5-phenyl-1,3-oxazolidine increases the signal of ephedrine. If ephedrine and pseudoephedrine are present in the same sample, but both with 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 increasing of uncertainty values. The uncertainty for quantification of a reference urine with ephedrine in 12.6 µg/mL was evaluated with addition of pseudoephedrine in 100 µg/mL and without pseudoephedrine. In pseudoephedrine presence, the ephedrine uncertainty was of 94.9% due to the oxazolidine interference. Without pseudoephedrine, the same reference urine showed the uncertainty of 8.2%. The total uncertainty was evaluated by interlaboratory comparison for both reference urines, with K=2 and 95% confidence interval.

The formaldehyde could be a solvent impurity or originated from other sources such as the matrix itself, promoting the conversion of pseudoephedrine into oxazolidine artifact, which will decrease the real area of pseudoephedrine in the chromatographic peak.

Screening procedures are used for qualitative analysis and quantitative estimation. Typically, more elaborated 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 that the sample containing pseudoephedrine would be erroneously declared as negative. 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.

The compound MTBSTFA is a derivatization reagent with similar reactivity to MSTFA. The formaldehyde adduct formation is prevented when derivatization with MTBSTFA reagent is performed. However, using this reagent alone does not provide an increase in mass spectra information for identification. The use of MBTFA in a two-step

derivatization allows the formation of the TFA group with the secondary amine of the molecules of ephedrine/pseudoephedrine, which allows a change in the profile of fragmentation, whose mechanism was discussed by Sardela et al. [6]. Thus, the N-TFA-O-TBDMS derivatives allow the unambiguous characterization by mass spectrometry, since it increases the mass of the fragments and the number of diagnostic ions (fig 1a). It also provides a gain in chromatographic resolution in samples with high concentrations of the ephedrine diastereoisomers (fig. 1b).
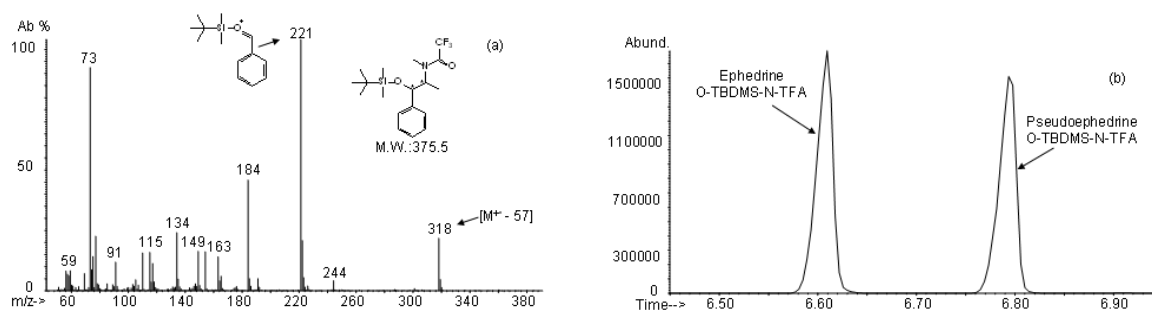

Fig. 1. GC-qMS mass spectra of ephedrine O-TBDMS-N-TFA derivative, with suggested structure (a). Extacted ion chromatogram of m/z 221, from EI full scan GC-qMS (b).

4. Conclusion

The interferent peak in ephedrines analyses by GC is the 3,4-dimethyl-5-phenyl-1,3-oxazolidine and its presence could induce a misinterpretation in ephedrines. The concomitant use of MTBSTFA with MBTFA in two steps for formation of N-TFA-O-TBDMS derivatives in ephedrines, allows unambiguous characterization by mass spectrometry and hinders the condensation of aldehydes with ephedrine molecules and consequent artifact formation.

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