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

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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).

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doi:10.1016/j.chroma.2010.12.120

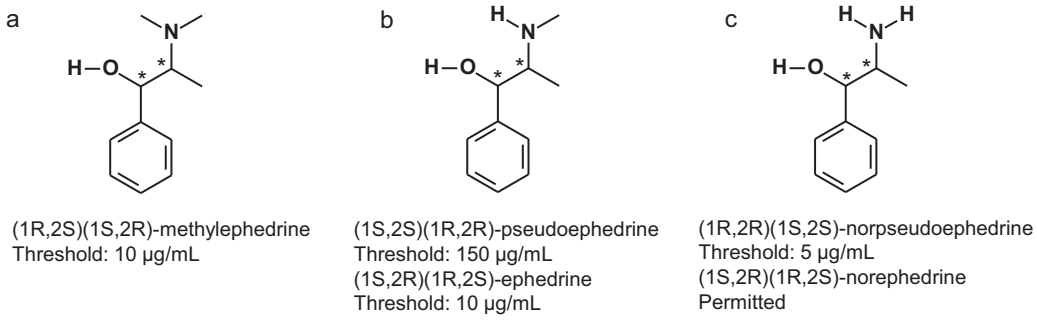

Fig. 1. Chemical structures of methylephedrine (a), pseudoephedrine and ephedrine (b) and norpseudoephedrine and norephedrine (c), with respective threshold values.

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].

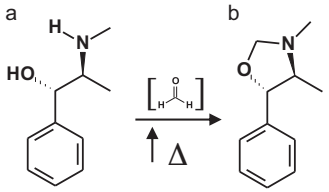

Fig. 2. Pseudoephedrine (a) conversion to 3,4-dimethyl-5-phenyl-1,3-oxazolidine (b).

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%)