Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (8)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 2000

R.VENTURA, L.DAMASCENO, J.CARDOSO, J.SEGURA:
Confirmation b-Agonists in Urine by GC/MS Using the Diagnostic Evidence of two Consecutive Derivatization Procedures
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (8). Sport und Buch Strauß, Köln, (2000) 183-188

Rosa Ventura [1,2], Lúcia Damasceno [1,3], Jari Cardoso[3], Jordi Segura [1,2]

Confirmation of β-Agonists in Urine by GC/MS Using the Diagnostic Evidence of Two Consecutive Derivatization Procedures

1. Unitat de Farmacologia, Institut Municipal d'Investigació Médica, Barcelona, Spain
2. Universitat Pompeu Fabra, Barcelona, Spain
3. Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil

Introduction

β-agonists (Table 1) are prohibited by the International Olympic Committee [1] due to stimulation on the central nervous system and promotion of certain anabolic effects. Only salbutamol, terbutaline and salmeterol are permitted by inhalation. Due to the low concentrations in urine of some β_2-agonists, identification of these compounds is difficult and requires sensitive and selective extraction and detection methodologies. The most commonly employed procedure is GC/MS [2-4]. The requirements to report the presence of compounds using GC/MS data are based on the comparison of relative retention times and relative abundances of diagnostic ions of the derivatives formed [5]. For some compounds, limited diagnostic information is obtained with the derivative used for screening purposes and availability of additional diagnostic data can be of interest for identification purposes.

This work reports a derivatization method based on the combination of two reagents via the consecutive formation of methylboronate and trimethylsilyl derivatives. Both derivatization methods can be used on the same extract, yielding additional diagnostic data for compound identification.

Experimental

Sample preparation procedure

To 2 ml of urine sample, 10 µL of a methanolic solution of penbutolol (10 µg/mL), used as internal standard, were added. The urines were adjusted to pH 5.2 with acetate buffer, 50 µL of *Helix pomatia* (HP-2) were added, and the samples were incubated at 55°C for 2 h. Bond Elut Certify™ columns were conditioned with methanol (2 mL) and deionized water (2 mL). The hydrolysed samples were adjusted to pH 9.5 with ammonium chloride buffer (100 µL), and were applied to the pre-conditioned columns. The columns were washed with deionized water (2 mL), acetate buffer pH 4 (1 mL) and methanol (2 mL), and eluted with a mixture of chloroform-isopropyl alcohol (80:20, v/v) containing 2% ammonium hydroxide (2 x 2 mL). The extracts were evaporated to dryness and maintained under vacuum for 30 min, before the derivatization procedure was applied.

Derivatization

Two consecutive derivatizations were applied on the same extract:

Derivatization I: Fifty µL of trimethylboroxine solution (2 mg/mL in ethyl acetate) were added to the dried residues, and they were incubated at 60^0C for 30 min. The derivatized extracts (I) were analysed by GC/MS.

Derivatization II: After the first GC/MS analysis, 50 µL of MSTFA were added to the same extract (I), and samples were heated at 60^0C for 20 min and analysed again by GC/MS.

Instrumental Conditions

Analyses were performed using a HP 5890 gas chromatograph equipped with a crosslinked HP methyl/siloxane fused-silica capillary column (17.5m x 0.2mm i.d., 0.11µm), coupled to a HP 5970 MSD. Injections were made in the splitless mode (0.5 min delay) using helium as carrier gas (0.7 $mL.min^{-1}$). Injector and transfer line temperatures were set to 280^0C. Oven temperatures were programmed as follows: initial temperature 100^0C for 2 min, rise at $30^0C.min^{-1}$ to 190^0C, rise at $20^0C.min^{-1}$ to 300^0C maintained for 4 min. Sample injection volume was 1 µL. The analyses were performed in the EI mode (ionization energy = 70eV) using scan acquisition (50-700u), or in SIM acquisition mode, monitoring three characteristic ions for each compound.

Results and Discussion

Derivatization

Formation of cyclic methylboronates (Derivatization I) was effective for bambuterol, clenbuterol, formoterol, salbutamol and salmeterol. For fenoterol and terbutaline, the formation of a cycle in the β-ethanolamine chain is possible; however, the spatial separation of the hydroxyl groups in the *meta* position of the phenyl ring is not adequate for ring formation, and no suitable derivatives were formed.

Addition of MSTFA (Derivatization II) produced silylation of the hydroxy, amino and other groups amenable to derivatization, and no cyclic derivatives were observed in the second GC/MS analysis. The derivatives formed for each compound are listed in Table 2. Mass spectra of these derivatives have been described in the literature [6].

The formation of TMS derivatives after addition of MSTFA to the extract previously subjected to Derivatization I, suggested that cyclic methylboronates did not exist in solution. To study the formation of the methylboronate derivatives, an additional experiment was performed with salbutamol. After Derivatization I, the derivatization reagent (solution of trimethylboroxine in ethyl acetate) was evaporated and the residues were reconstituted with ethyl acetate only. Under these conditions, where trimethylboroxine was not present during the injection process, the formation of salbutamol di-methylboronate was reduced to 1 % .

In summary, the results obtained showed evidence that methylboronate derivatives do not exist in solution and they are mainly formed during the injection process. When MSTFA is added to the reaction mixture, TMS derivatives are formed, blocking all groups amenable to form cyclic derivatives, and only TMS derivatives are observed.

Analysis of urine samples

Limits of detection (LOD) of the two derivatization steps estimated using spiked urines are listed in Table 2. LOD ranged from 0.5 to 5 ng/mL, except for formoterol.

Taking into account the concentrations of the compounds of interest in urine after therapeutic doses [2], only the detection of formoterol and salmeterol could be difficult. However, the detection of these compounds can be improved by increasing the volume of urine or reducing the volume of derivatizating reagents.

Chromatograms of spiked urines showing the disappearance of methylboronates and the appearance of TMS derivatives using the consecutive procedure are shown in Figure 1. The

combination of appearance and disappearance of chromatographic peaks, as well as changes in mass spectra and in retention times of the derivatives, affords additional evidence to confirm the presence of these β-agonists in urine samples.

References

[1] International Olympic Committee. Prohibited classes of substances and prohibited methods. In Olympic Movement Anti-Doping Code, IOC, 1999.

[2] R. Ventura, L. Damasceno, M. Farré, J. Cardoso, J. Segura. Anal. Chim. Acta 2000;418:79-92.

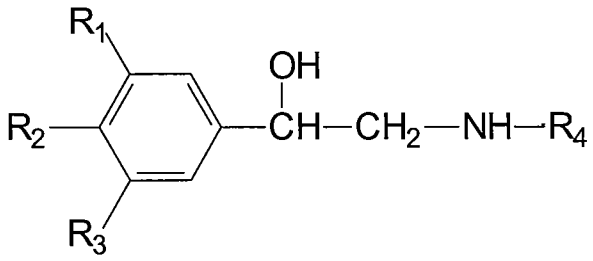
[3] M.-P. Montrade, B. Le Bizec, F. Monteau, B. Siliart, F. Andre. Anal. Chim. Acta 1993; 275: 253.

[4] G. Van Vyncht, S. Preece, P. Gaspar, G. Maghuin-Rogister, E. DePauw. J. Chromatogr. A 1996; 750: 43.

[5] International Olympic Committee. Analytical criteria for reporting low concentrations of anabolic steroids, Internal Communication, IOC, Lausanne, Switzerland, 1998.

[6] L. Damasceno, R. Ventura, J. Ortuño, J. Segura. J. Mass Spectrom. 2000;35:1285-1294.

Table 1. Chemical structures of the compounds studied.



β-agonists	R_1	R_2	R_3	R_4
Salmeterol	$-CH_2OH$	-OH	-H	$-(CH_2)_6-O-(CH_2)_4Ph$
α-Hydroxi Salmeterol	$-CH_2OH$	-OH	-H	$-(CH_2)_6-O-(CH_2)_3CH(OH)Ph$
Fenoterol	-OH	-H	-OH	$-CH(CH_3)-CH_2Ph-OH$
Formoterol	-NH-CHO	-OH	-H	$-CH(CH_3)-CH_2-Ph-OCH_3$
Bambuterol	$-O-CO-N(CH_3)_2$	-H	$-O-CO-N(CH_3)_2$	$-C(CH_3)_3$
Clenbuterol	-Cl	$-NH_2$	-Cl	$-C(CH_3)_3$
Salbutamol	$-CH_2-OH$	-OH	-H	$-C(CH_3)_3$
Terbutaline	-OH	-H	-OH	$-C(CH_3)_3$

Table 2. Relative retention times (RRT) and detection limits (LOD) for different β_2-agonists

Compound	Derivatization I			Derivatization II		
	Derivative	**RRT**	**LOD** (ng/mL)	**Derivative**	RRT	**LOD** (ng/mL)
Bambuterol	MB	1.32	5	O-TMS	1.25	2
Clenbuterol	MB	0.94	0.5	O-TMS	0.89	5
Fenoterol	no suitable derivative			tetrakis-O-TMS	1.34	5
Formoterol	di-MB	1.38	>10	bis-O-TMS	1.45	5
Salbutamol	di-MB	0.89	2	tris-O-TMS	0.94	2
Salmeterol	di-MB	1.63	5	tris-O-TMS	1.70	2
Terbutaline	no suitable derivative			tris-O-TMS	0.89	5
Penbutolol (ISTD)	MB	1.00	-	O-TMS	1.00	-

Figure 1. Chromatograms obtained after analysis of a urine spiked with salbutamol, clenbuterol, bambuterol, salmeterol, salmeterol metabolite, fenoterol and terbutaline, by using Derivatization I (left) followed by Derivatization II (right).