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Short communication

β_2-Adrenergic agonist residues: simultaneous methyl- and butylboronic derivatization for confirmatory analysis by gas chromatography–mass spectrometry

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Abstract

A derivatization procedure for confirmatory residue analysis of β_2-agonists is described. Methyl (MBA) and butyl (BBA) boronic acids are simultaneously used for the derivatization of tulobuterol, mabuterol, mapenterol, salbutamol, clenproperol, clenbuterol, clenpenterol and bromobuterol by GC–MS determination. A temperature of 55°C during 60 min was selected as optimal temperature–time condition for simultaneous MBA and BBA β_2-agonists derivatization. It was also observed that stability of boronic derivatives was maintained at −20°C over a period of four days. The proposed methodology was tested in urine and it could be applied for confirmatory residue analysis of clenbuterol-like compounds. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, GC; Tulobuterol; Mabuterol; Mapenterol; Salbutamol; Clenproperol; Clenbuterol; Clenpenterol; Bromobuterol

1. Introduction

Since the last decade, clenbuterol and other β_2-agonists (Fig. 1) have been illegally used as growth promoters in meat-producing animals by increasing protein deposition and decreasing body fat accumulation [1–3].

In order to control this abuse, techniques are required for the detection and identification of these drugs. Gas chromatography–mass spectrometry (GC–MS) was usually the preferred methodology for confirmatory analysis because of its superior specificity [4–7]. However, the most used MS was based on low-resolution techniques and that requires a minimum of four diagnostic ions for confirmatory analysis [8–11]. As a rule, two ionization modes, electronic impact (EI) and chemical ionization (CI), or, alternatively, two different derivatives are necessary to meet the European Union (EU) criteria for β_2-agonist confirmatory residue analysis under the most commonly used derivatization procedure: silylation [12–14].

This paper describes the simultaneous methyl- and butylboronic derivatization of tulobuterol, mabuterol, mapenterol, salbutamol, clenproperol, clenbuterol, clenpenterol and bromobuterol for confirmatory residue urine analysis. Validation data of the proposed

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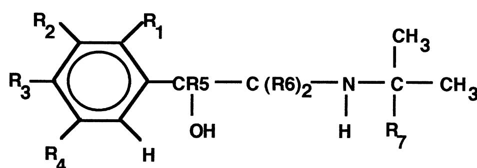

Compound	R1	R2	R3	R4	R5	R6	R7
Tulobuterol	Cl	H	H	H	H	H	CH_3
Mabuterol	H	Cl	NH_2	CF_3	H	H	CH_3
Mapenterol	H	Cl	NH_2	CF_3	H	H	CH_2CH_3
Salbutamol	H	CH_2OH	OH	H	H	H	CH_3
Clenproperol	H	Cl	NH_2	Cl	H	H	H
Clenbuterol	H	Cl	NH_2	Cl	H	H	CH_3
ClenbuterolD3	H	Cl	NH_2	Cl	D	D	CH_3
Clenpenterol	H	Cl	NH_2	Cl	H	H	CH_2CH_3
Bromobuterol	H	Br	NH_2	Br	H	H	CH_3

Fig. 1. Structural formula of β_2-adrenergic agonists.

methodology were presented and their use in urine analysis was also discussed.

2. Experimental

2.1. Reagents and apparatus

The standards of clenpenterol, clenproperol, mabuterol and bromobuterol were a gift of Professor François André (National Veterinary School of Nantes, France). Clenbuterol was acquired from Interchim (Montluçon, France), salbutamol was provided by Sigma (Madrid, Spain), tulobuterol and tri-deuterated (D3) clenbuterol (internal standard) were kindly offered by Doctor Henri Vranckx (UCB Pharma, Braine l'Alleud, Belgium) and by Doctor Jan Rud Andersen (Danish Meat Institute, Roskilde, Denmark), respectively.

Methyl- and butylboronic acid (MBA and BBA) and ethyl acetate over molecular sieve used were bought from Fluka (Buchs, Switzerland), and purified water was obtained through a Millipore Milli-Q Plus system (Bedford, MA, USA). The nitrogen and helium N55 were supplied by Sofager (Coimbra, Portugal). Vials, cups for derivatization and other materials used for automatic injection were from Chromacol (Dias de Sousa, Lisbon, Portugal).

Determination of β_2-agonists by GC–MS was performed on a Hewlett-Packard (HP) apparatus, composed of HP5890 series II gas–liquid chromatograph, HP6890 autosampler, HP5972 MSD detector, HP Vectra VL2 4/50 computer, HP Deskjet 520 printer and a 15 m$\times$0.25 mm I.D., 0.25 μm film thickness HP1 column (Soquímica, Lisbon, Portugal).

Heating aluminium blocks for dryness evaporation under nitrogen and derivatization procedure were purchased from Reagente 5 (Oporto, Portugal).

Urine solid-phase extraction (SPE) was carried out in a vacuum manifold with Clean Screen DAU 1000 mg columns (Worldwide Monitoring), which were supplied by Reagente 5.

All the other necessary reagents were acquired from Merck (J.M. Vaz Pereira, Lisbon, Portugal).

2.2. Derivatization

Fifty μl of the standard methanolic solution in a concentration of 2.5 μg/ml for tulobuterol and salbutamol and of 1.0 μg/ml for the other β_2-agonists and 50 μl of clenbuterol D3 (1.0 μg/ml) were put in a derivatization vial and were evaporated to dryness under a nitrogen stream at 45°C. The residue was taken up in 50 μl of ethyl acetate MBA+BBA solution at a concentration of 5 mg/ml of each one. The derivatization then took place, after vortexing. Times and temperatures of derivatization are discussed in the following sections.

2.3. GC–MS

A 2-μl portion was injected into the GC–MS system in splitless mode (1 min delay), using helium as carrier gas, at a flow-rate of 1 ml/min. Oven temperatures were programmed as follows: 120°C (0.1 min)→15°C/min→245°C (0.0 min)→30°C/min→300°C (4.0 min). The detector and the injector temperatures were set at 280°C and 260°C, respectively. The analyses were performed in the EI mode and the ionization voltage was fixed at 70 eV. Acquisition was made in the selected ion monitoring (SIM) mode. Selected ions, as well as the retention

Table 1
Retention times (t_R) and monitored ions (m/z) of β_2-adrenergic agonist boronic derivatives

Compound	No.[a]	MBA		No.[a]	BBA	
		t_R (min)	Ions (m/z)		t_R (min)	Ions (m/z)
Tulobuterol	1	3.4	**236**; 194	3	5.0	**278**; 194
Mabuterol	2	4.7	319; **277**	7	6.2	361; **277**
Mapenterol	4	5.3	319; **277**	8	6.7	361; **277**
Salbutamol	5	5.5	**272**; 230	15	8.8	356; **272**
Clenproperol	6	5.6	271; **229**	10	7.1	313; **229**
Clenbuterol D3 (I.S.)	7	6.2	288; **246**	12	7.6	330; **246**
Clenbuterol	7	6.2	285; **243**	12	7.6	327; **243**
Clenpenterol	9	6.8	285; **243**	13	8.2	327; **243**
Bromobuterol	11	7.2	375; **333**	14	8.6	417; **333**

[a] See Fig. 2.

times of the β_2-agonist methyl- and butylboronic derivatives, can be seen in Table 1.

2.4. Validation

To validate the proposed methodology, bovine urine blank samples were used as matrix. The extraction procedure was previously described [12]. In brief, 20 ml of spiked samples were passed through an activated Clean Screen DAU column, previously conditioned with methanol, water and phosphate buffer. The columns were washed with acetic acid and methanol. The elution of β_2-agonists was carried out with ethyl acetate–ammonia (97:3). The eluate was evaporated to dryness under a nitrogen stream and the residue was taken up in methanol for a derivatization vial. After vortexing, the methanol was evaporated to dryness and the residue was derivatized by 50 μl of MBA+BBA solution. A 2-μl volume was injected as described above.

Fig. 2 shows a chromatogram of a calibration solution containing both β_2-agonist derivatives at 5.0 ng injected for tulobuterol and salbutamol and at 2.0 ng for the other compounds.

A urine sample of a clenbuterol treated animal is shown in Fig. 3 (m/z 243 was for clenbuterol and m/z 246 was for clenbuterol D3 internal standard).

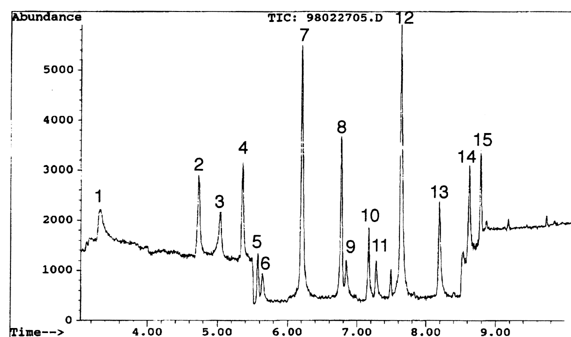

Fig. 2. Chromatogram of standard β_2-adrenergic agonist boronic derivatives. For the identification of the different peaks see Table 1.

3. Results and discussion

Four ions for each one of the boronic derivatives can be determined when MBA or BBA were used alone on β_2-agonist analysis (for instance, m/z 300, 285, 245 and 243 or m/z 342, 327, 245 and 243 for MBA and BBA clenbuterol ions, respectively). However, a better determination limit could be

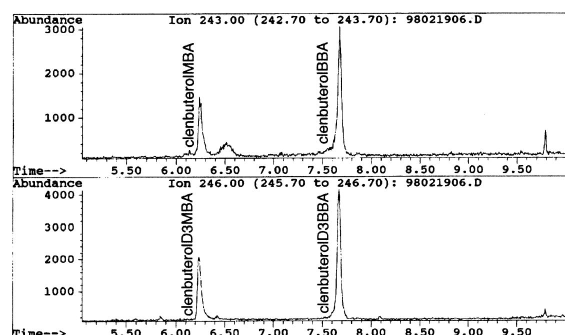

Fig. 3. Chromatogram of a clenbuterol urine sample (2.7 μg/l).

obtained if still less m/z ions were detected by MS. The proposed double simultaneous derivatization was developed in consideration that only two m/z ions were selected for each one β_2-adrenergic agonist derivative, to fulfil EU requirements for confirmatory analysis. A double GC retention time was obtained as an additional advantage with this procedure when compared to single MBA or BBA derivatization.

Five temperatures (room temperature, 35°C, 45°C, 55°C and 65°C) were assayed with a fixed 30 min MBA+BBA derivatization period. Under these circumstances, 45°C, 35°C and room temperature showed similar results for all β_2-agonists studied. A significant improvement on detector response was achieved at 55°C and no better results were obtained when temperature was increased to 65°C.

At a temperature of 55°C, the times of 15, 30, 45, 60 and 75 min for the MBA and BBA derivatization were tested. Only clenbuterol showed different best times for MBA and BBA derivatization, respectively 60 and 45 min. Nevertheless, the little difference of detector response between 45 and 60 min for clenbuterol BBA derivatives was not sufficient to select 45 instead of 60 min for derivatization, because 60 min was the best for all other MBA and BBA β_2-agonist derivatization times.

These considerations were sufficient to suggest that 55°C for 60 min was a good condition for the β_2-agonist MBA+BBA derivatization procedure.

Table 2 shows data, reported for urine samples, on repeatability, within-laboratory reproducibility, recovery (spiked urines at 2 μg/l for clenbuterol, mabuterol and mapenterol, and at 5 μg/l for the other β_2-agonists), and detection limits (3:1, signal-to-noise ratio, referred to base peak). Data of tulobuterol and salbutamol precision and recovery were not recommendable to suggest this procedure for urine analysis. Furthermore, salbutamol precision did not agree with EU recommendations [11,15,16]. However, salbutamol was the only one phenolic β_2-agonist that boronic derivatization could be used. Precision and recovery for the other β_2-agonists studied were according EU requirements [11,15,16]. Detection limit data, excluding tulobuterol and salbutamol, were sufficient to warrant that a program for confirmatory analysis could be performed with this procedure under the most common β_2-agonist determination limit in urine, 1 μg/l.

Stability of the β_2-adrenergic agonist boronic derivatives was also studied. Room temperature and −20°C were assayed. Room temperature was not advised to conserve the referred derivatives. Stability of MBA derivatives begin to decrease after derivatization. Only stability of tulobuterol and mabuterol remained for a maximum period of 8 h. For BBA derivatives, all the β_2-adrenergic agonists essayed maintain their stability over an 8 h period. However, a good stability of β_2-agonist MBA and BBA derivatives was achieved when a congealing temperature (−20°C) was used. In this case, boronic derivatives maintain their detector responses over a period of four days, after GC–MS determination. Only at the fifth day after derivatization did stability begin to decrease.

The possibility of a signal on the ion-trace (m/z= 246) deriving from native bis-chlorinated isotope compound which is indistinguishable from clen-

Table 2
Validation data for MBA and BBA β_2-adrenergic agonist derivatives

Compound	Precision (n=5) R.S.D.				Recovery (%)		Detection limit (μg/l)	
	Repeatability		Reproducibility (%)					
	MBA	BBA	MBA	BBA	MBA	BBA	MBA	BBA
Tulobuterol	15.4	11.2	29.5	18.2	29.7	32.1	3.0	1.2
Mabuterol	7.6	5.2	12.2	9.8	58.6	77.3	0.7	0.3
Mapenterol	10.2	4.0	18.3	13.0	64.1	76.9	0.6	0.3
Salbutamol	13.8	16.6	25.2	36.6	22.8	28.7	2.6	2.6
Clenproperol	8.2	6.6	11.0	10.4	60.9	64.1	2.3	0.9
Clenbuterol	4.4	3.3	8.6	7.8	60.1	78.8	0.7	0.3
Clenpenterol	8.0	5.9	16.1	11.2	54.5	86.8	1.4	0.6
Bromobuterol	5.3	9.4	9.1	15.0	70.7	91.2	1.0	0.5

buterol D3 suggest the use of clenbuterol D6 as internal standard (I.S.). However, taking into account that the purpose of this study is the determination of illegal clenbuterol in urine, the use of clenbuterol D3 as I.S. is also correct.

van Rhijn and co-workers [13,14] have previously described a simultaneous derivatization procedure using two different silylation derivatives, BSTFA [*N*,*O*-bis(trimethylsilyl)trifluoracetamide] and MTBSTFA [*N*-methyl-*N*(*tert*.-butyldimethylsilyl)trifluoracetamide], to confirmatory analysis of β_2-adrenergic agonists in urines. Nevertheless, boronic derivatives show always high abundances of the most specific ions when compared to the corresponding EI silyl derivatives. A faster procedure and a saving of helium were another advantage, due to the use of a 15-m GC column for boronic derivatives, instead of a 30-m column usually used for corresponding silyl compounds.

According previous remarks, the proposed methodology could be used for confirmatory bovine urine residue analysis of clenbuterol-like compounds, except tulobuterol.

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