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Determination of clenbuterol in urine as its cyclic boronate derivative by gas chromatography–mass spectrometry

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ABSTRACT

A rapid and reliable gas chromatographic–mass spectrometric method for the determination of clenbuterol in urine is described. Penbutolol was used as internal standard. Four derivatization procedures have been tested, of which 1-butaneboronic acid gave the best results. The method includes extraction of the alkalinized urine (3 ml) with *tert.*-butyl methyl ether–*n*-butanol (9:1), derivatization with 1-butaneboronic acid (15 min at room temperature), and analysis in the selected-ion monitoring mode of the derivatives of clenbuterol at *m/z* 243, 327 and 342 and of penbutolol at *m/z* 342 and 357. The detection limit is 0.5 ng/ml and the recovery better than 90%.

INTRODUCTION

Clenbuterol, 4-amino-3,5-dichloro-α[(*tert.*-butylamino)methyl]benzyl alcohol hydrochloride, is a β2-adrenoceptor agonist illegally used as a repartitioning agent in meat production and as a doping agent, particularly in horse races. Pharmacological, pharmacokinetic and toxicological properties of this drug have been reviewed [1].

Owing to the high potency of clenbuterol, the concentrations expected in biological fluids are very low. After therapeutic doses of 20–40 μg to humans, the plasma levels are below 0.15 ng/ml; in urine the peak levels are in the range 10–20 ng/ml, and fall to 1–2 ng/ml after 48 h [2,3].

Several methods have been described for the detection of clenbuterol and other β-agonists in biological fluids. Enzyme immunoassay has been used in only a few cases [4,5]; more frequent are the determinations by thin-layer chromatography [5–7], high-performance liquid chromatography [5,6,8–12] and gas chromatography (GC) [2,3,5,13]. Among the chromatographic methods those using mass spectrometric (MS) detection in the selected-ion monitoring (SIM) mode should be preferred because of the high selectivity [2,3,5,7,12,13].

GC analysis requires derivatization. This is always performed by silylation of the clenbuterol hydroxylic group. The electron-impact (EI) mass spectrum of the trimethylsilyl (TMS) derivative, however, shows low abundances of the most

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specific ions (*m/z* 262, 333 and 348), thus ammonia [2,3] or methane [13] chemical ionization is required to achieve suitable detection limits.

This paper describes a simple and rapid GC–MS method, also useful as a screening procedure, based on the formation of a cyclic boronate with 1-butaneboronic acid (Fig. 1). This derivatization technique has already been used successfully for compounds structurally related to clenbuterol, such as β-blocking agents [14,15].

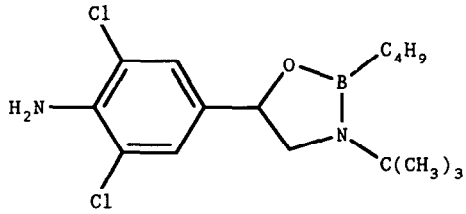

Fig. 1. Structure of the BBA derivative of clenbuterol.

EXPERIMENTAL

Chemicals and reagents

Clenbuterol · HCl, penbutolol · H_2SO_4, and phenylboronic acid (PBA) were kindly supplied by Biomedica Foscama (Rome, Italy), Hoechst Italia (Milan, Italy) and Bracco (Milan, Italy), respectively. 1-Butaneboronic acid (BBA) was purchased from Aldrich, bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Supelco, and trifluoroacetic anhydride (TFAA) from Fluka, all three located in Milan, Italy.

All solvents were of analytical grade: ethyl acetate, methanol and diethyl ether (Fluka), *n*-butanol (Carlo Erba, Milan, Italy), and *tert.*-butyl methyl ether (TBME) (Merck, Milan, Italy).

Separate stock solutions of clenbuterol and penbutolol (1 mg/ml, in methanol) were prepared and stored in the dark at −20°C.

The PBA and BBA solutions were prepared by dissolving 10 mg of the boronic acid in 10 ml of ethyl acetate, dried over anhydrous sodium sulphate (Carlo Erba) and stored in the dark at −20°C for not longer than a week. Some molecular sieves (3 Å, beads *ca.* 2 mm; Merck) were added to the derivatizing solutions as water scavengers.

The derivatization tests were carried out on clenbuterol and penbutolol as free bases extracted from their salts with diethyl ether.

Analytical instrumentation

All the analyses were performed using a Hewlett-Packard (Milan, Italy) 5890A gas chromatograph equipped with a Hewlett-Packard Ultra 2 (5% phenyl, meth-

yl silicone) fused-silica capillary column (12 m × 0.2 mm I.D., 0.33 μm film thickness), coupled with a Hewlett-Packard 5970 mass-selective detector. The injections were made in splitless mode (2.5 min delay before opening the splitter) using helium as carrier gas (0.8 ml/min). The operating temperatures were: injector, 300°C; column, maintained for 2.5 min at 100°C and programmed at 20°C/min to 260°C, then at 5°C/min to 290°C and maintained for 10 min; transfer line, 280°C.

The EI mass spectra of the TMS, PBA and BBA derivatives of clenbuterol were obtained at 70 eV in the mass range 35–450 a.m.u. with a scan-rate of *ca.* 2 scans/s. Analysis of urine extracts was performed in the SIM mode by monitoring specific ions of the BBA derivatives: *m/z* 243, 327, 342 for clenbuterol and *m/z* 342 and 357 for the internal standard. However, for a better characterization of clenbuterol, the chlorine isotopic peak at *m/z* 329, and even that at *m/z* 245, can be measured. All the abundance ratios have to be within 10%.

Extraction procedure

To 3 ml of urine in a 15-ml glass tube, 9 μl of methanol containing 1 ng/μl internal standard were added. After acidification with 0.5 ml of 0.5 *M* sulphuric acid, the sample was washed with 4 ml of TBME on a vortex mixer (1 min), then centrifuged (1000 *g*, 5 min). The organic layer was discarded, and the aqueous phase alkalinized with 0.2 ml of 5 *M* sodium hydroxide and then extracted on vortex mixer (2.5 min) with 5 ml of TBME–*n*-butanol (9:1). After centrifugation (1000 *g*, 5 min), the upper layer was separated and gently evaporated to dryness under a nitrogen stream.

Derivatization procedures

BBA derivatization. The residue from the urine extract was dissolved in 30 μl of the BBA solution. After derivatization (15 min at room temperature), a 2-μl aliquot was injected in the gas chromatograph. The stability of the BBA derivative was checked over a period of 48 h. A methanol solution (50 μl) of clenbuterol and the internal standard, 1 ng/μl each, was evaporated to dryness and, after addition of the BBA solution (50 μl), analysed at 0, 0.25, 0.75, 1.5, 30 and 48 h.

PBA derivatization. The derivatization with PBA was carried out on a methanol solution of clenbuterol (50 ng/μl) as for the BBA derivative.

TMS derivatization. Flash silylation was realized by injection into the gas chromatograph of 1 μl of a methanol solution of clenbuterol (50 ng/μl) together with 1 μl of BSTFA.

TFA derivatization. A 50-μl volume of the clenbuterol methanol solution (50 ng/μl) was evaporated to dryness in a glass micro reaction vial (Supelco). After addition of 50 μl of TFAA, the vial was heated at 70°C for 1 h, then the TFAA was evaporated. The residue was dissolved in 50 μl of ethyl acetate, and 1 μl was injected in the gas chromatograph.

Calibration curve

The calibration curve was prepared by adding known amounts of clenbuterol, equivalent to 1, 2, 5, 10 and 50 ng/ml, to blank human urine. Three determinations were performed for each point. Peak-height ratios (at *m/z* 327 for clenbuterol and *m/z* 342 for the internal standard) were plotted against clenbuterol concentrations.

RESULTS AND DISCUSSION

The use of the mass spectrometer in the SIM mode gives a considerable gain in GC–MS sensitivity and selectivity, particularly when the most abundant and heavier ions in the mass spectrum are selected.

Various derivatization procedures were tested. Fig. 2 shows the EI mass spectra of TMS (A), BBA (B) and PBA (C) derivatives of clenbuterol. Though the TMS derivative exhibits a very good peak shape, the most abundant ion in the EI mass spectrum (*m/z* 86) is not specific, and the heavier ion fragments (*m/z* 262, 333 and 348) have too low abundances. The TFA derivatization procedure was rejected because of the effect of TFAA on a wide range of functional groups and the presence of three possible derivatization sites in the clenbuterol molecule, which led to at least three different derivatization products. The derivatization

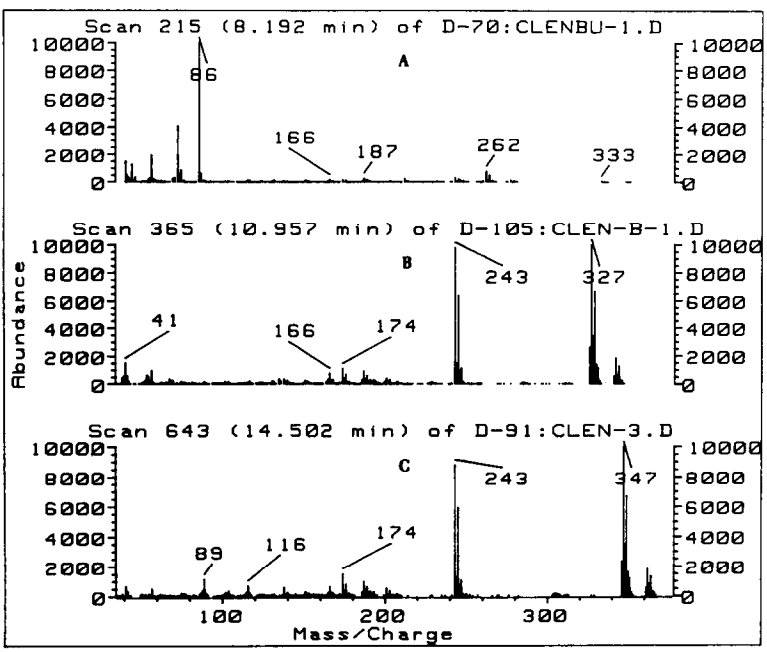

Fig. 2. EI mass spectra of the TMS (A), BBA (B) and PBA (C) derivatives of clenbuterol.

with boronic acids has shown the best results. The BBA and the PBA derivatives of clenbuterol show the most abundant ions in the high-mass range (at *m/z* 243, 327, 342 and at *m/z* 243, 347, 362, respectively). Owing to the better chromatographic properties (peak shape and retention time) of the BBA derivative, this derivatization procedure was finally chosen. Therefore a comparison between the various derivatization procedures as regards yield of the derivatization reactions, time stability, reproducibility was not considered relevant.

The BBA derivative of clenbuterol is stable for at least 48 h. In fact, constant peak heights from 0.25 to 48 h and a peak-height ratio (clenbuterol *versus* internal standard) of 1.19 at 0 h and of 0.94 ± 0.07 from 0.25 to 48 h were measured. Constant peak-height ratios were also observed when urine extracts were injected 24 and 48 h after the addition of the BBA solution.

Fig. 3 shows the ion chromatograms obtained for different ions from a human urine sample spiked with 3 ng/ml clenbuterol. Fig. 4 gives the GC–MS responses for (A) a urine sample collected over a period of 8 h after oral administration of 20 μg of clenbuterol to an healthy volunteer, (B) a blank urine sample spiked with 3 ng/ml clenbuterol, and (C) a blank urine sample.

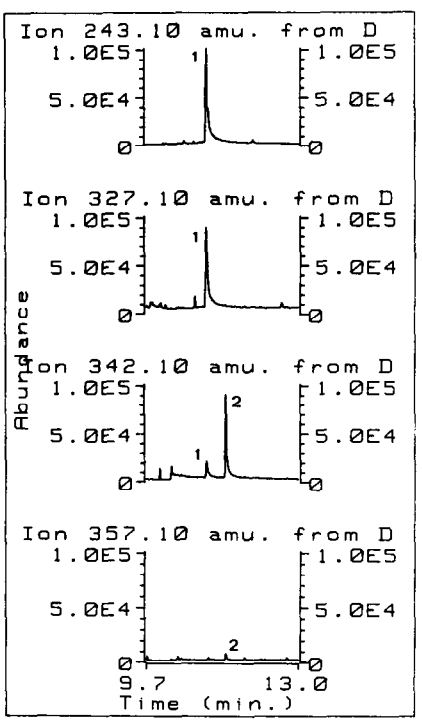

Fig. 3. Ion chromatograms obtained from a human urine sample spiked with 3 ng/ml clenbuterol. Peaks: 1 = clenbuterol; 2 = internal standard (3 ng/ml).

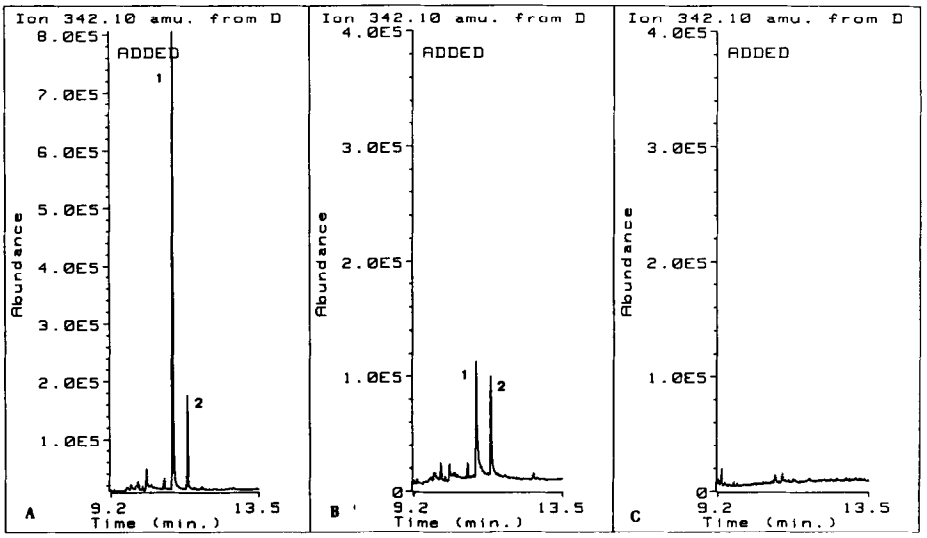

Fig. 4. Reconstructed ion chromatograms (*m/z* 327 and 342 added) obtained from (A) a human urine sample containing 12.9 ng/ml clenbuterol, (B) a human urine sample spiked with 3 ng/ml clenbuterol and (C) a human blank urine. Peaks: 1 = clenbuterol; 2 = internal standard (3 ng/ml).

Table I shows the precision and accuracy data obtained for clenbuterol determinations. The equation of the curve, determined by least-squares regression analysis, was $y = 0.2990x - 0.0142$ (correlation coefficient = 0.9987).

The detection limit in urine is 0.5 ng/ml when 3 ml of biological sample are used. The recovery, assessed by comparing the chromatographic peak height of clenbuterol standard solution with that of an extracted urine spiked with the same amount of clenbuterol, was constantly higher than 90%.

In conclusion, the described GC–MS procedure for the quantitative determination of clenbuterol in urine shows appreciable accuracy and precision, good

TABLE I

ACCURACY AND PRECISION OF THE METHOD

Three determinations for each point.

Amount added (ng/ml)	Amount found (mean ± S.D.)(ng/ml)	Coefficient of variation (%)
1	1.06 ± 0.01	0.9
2	2.14 ± 0.15	7.0
5	4.89 ± 0.15	3.1
10	9.88 ± 0.47	4.8
50	50.03 ± 1.69	3.4

sensitivity and remarkable selectivity. The extraction and derivatization procedures are simple and rapid, and the GC–MS equipment used is available in the majority of the laboratories for drug and doping control. Therefore the method seems applicable to the screening of urine samples for the control of the illegal use of the drug.

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