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Journal of Chromatography B, 693 (1997) 468–478

JOURNAL OF
CHROMATOGRAPHY B

Short communication

Application of a sequential analytical procedure for the detection of the β-agonist brombuterol in bovine urine samples

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Received 7 October 1996; revised 18 December 1996; accepted 30 December 1996

Abstract

The multistep analytical procedure routinely applied in our laboratory for the detection of the aryl amine β-agonists clenbuterol, mabuterol and mapenterol in bovine matrices has been extended to the analysis in urine samples of brombuterol, a new clenbuterol-like compound. In the screening steps, the urine samples were first enzyme-linked immunosorbent assay (ELISA)-tested, then the positive samples were analyzed by thin-layer chromatography (TLC) and the β-agonists detected with the Bratton-Marshall color reaction. The TLC spots corresponding to the suspected compounds were scraped off the plates, collected and extracted separately with methanol. The β-agonists in the extracts were detected by HPLC–Vis (limits of detection: 0.5 ng/g). In the confirmatory step the presence and the concentration of the compounds of interest in the samples were established by GC–MS with two different ionization techniques, EI and CI (limits of detection: 1.0 ng/g). The use of this procedure has made possible the detection of brombuterol in officially sampled bovine urine.

Keywords: Brombuterol

1. Introduction

Despite the EEC ban on β-agonists as growth promoters in meat production, these compounds are still widely used all over Europe. Clenbuterol is certainly the best known representative of the class of aryl amine β-agonists [1], and most of the analytical methods is centered on its detection in animal matrices. In order to elude the official controls, in recent years a number of new compounds has been developed from clenbuterol. One of these molecules, which has received the trivial name of brombuterol (4-amino-3,5-dibromo-α[(*tert.*-butylamino)methyl]benzyl alcohol) (Fig. 1), maintains the typical aryl amine structure, but differs from clenbuterol in the substitution of the benzene ring with two bromine atoms instead of two chlorine [2]. Presently, very few studies on brombuterol are available in the scientific literature. In general, they report brombuterol determination by GC–MS in illegal pharmaceutical preparations for animal use [2] or in blank urine samples spiked with the standard compound [3,4]. No paper has been published so far on the detection of brombuterol in urines collected in an official sampling plan. The protocol for the detection of brombuterol presented here employs a sequential multistep combination of four different analytical techniques, namely enzyme-linked im-

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PII S0378-4347(97)00056-X

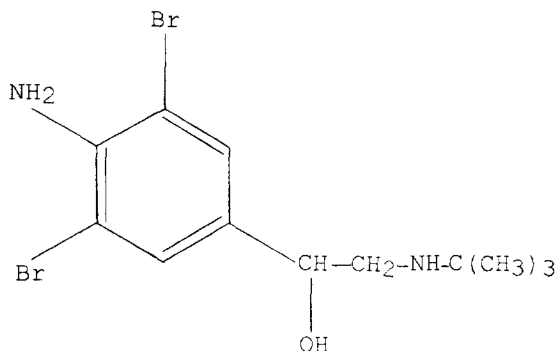

Fig. 1. Structure of brombuterol.

munosorbent assay (ELISA), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in the screening steps, and gas chromatography–mass spectrometry (GC–MS) (and GC–MS–MS) in the confirmatory step.

2. Experimental

2.1. Chemicals

Sodium hydroxide, *n*-hexane, methylene chloride, ethyl acetate, methanol, 85% *o*-phosphoric acid, 37% hydrochloric acid and acetic acid were purchased from Carlo Erba (Milan, Italy). Triethylamine (TEA), sodium nitrite, *N*-(1-naphtyl)-ethylendiamine dihydrochloride, HPLC-grade acetonitrile, Extrelut columns and refills, silica gel 60 TLC plates (10×20 cm) were purchased from Bracco Merck (Milan, Italy). "Generic Bronchodilators" and "Clenbuterol" ELISA kits (manufactured by Neogen, Lexington, KY, USA) were purchased from Dieschem (Milan, Italy). SPE Bakerbond cyano columns, each with 500 mg of stationary phase and a 3-ml volume, were purchased from J.T. Baker-Schilling (Milan, Italy). The columns were connected to a Supelco SPE vacuum manifold block, from Supelchem (Milan, Italy). Water for HPLC analysis was prepared with a Barnstead Nanopure Ultrapure water system from International PBI (Milan, Italy). *N,O*-Bis (trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Alltech (Alltech Italia, Milan, Italy).

2.2. Standards, standard solutions and spiked samples

Clenbuterol, terbutaline and metoprolol (used as internal standard in the GC–MS analyses) were purchased from Aldrich (Milan, Italy). Brombuterol, mapenterol and mabuterol were kindly provided by the Istituto Superiore di Sanità (Rome, Italy). The stock standard solutions of each β-agonist were prepared in methanol at the concentration of 1.0 mg/ml (stability 6 months at −20°C). The working standard solutions were prepared diluting the stock standard solutions on the day of use to the concentration of 10 μg/ml in methanol. Blank control urine (stability 1 month at −20°C) was prepared by filtering and pooling the urine samples tested negative at the routine ELISA tests for β-agonists and was used to prepare two sets of standard solutions ("standard curves") – one of clenbuterol and the other of terbutaline at concentrations increasing from 0.312 ng/ml to 10.0 ng/ml.

For the recovery studies, 2 ng/ml and 4 ng/ml clenbuterol and brombuterol spiked urine samples were prepared on the day of use by fortifying aliquots of blank control urine with the working standard solutions and were processed as described in Section 2.4. The recovery percentages for the screening steps were calculated by comparing the clenbuterol and mabuterol HPLC peak heights of the extracts from both the samples and the standard compounds spotted directly on the TLC plates. The recovery percentages for the confirmatory step were calculated by comparing the GC–MS area peak data of both the internal standard metoprolol and clenbuterol and brombuterol in the spiked samples.

2.3. Instrumentation

ELISA absorbances were read at 450 nm on a Multiskan MCC microplate reader of Titertek (Dasit, Milan, Italy).

The HPLC system consisted of a Series 4 pump equipped with a Rheodyne loop injector, an LC-95 UV–Vis detector and a Model 561 chart recorder (all supplied by Perkin-Elmer Italia, Milan, Italy). The HPLC separation was performed at room temperature on a 10 μm LiChrospher 100 RP-8 (250×4 mm

I.D.) column from Merck. The mobile phase A was a 0.1 *M* *o*-phosphoric acid solution buffered to pH 3.0 with TEA; the mobile phase B was pure acetonitrile. The gradient program was set as follows: 80:20 (v/v) A:B for 10 min, then 70:30 (v/v) A:B for 15 min. The flow-rate was 1.2 ml/min and the recorder chart speed was 5 mm/min. The Vis detector wavelength was 440 nm, with 0.01 AUFS. The injection volume was 20 μl.

The GC–MS and GC–MS–MS analyses were performed on a Model 3400 gas chromatograph (Varian Italia, Milan, Italy) equipped with a DB5 fused-silica column (30 m×0.32 μm I.D., 0.25 μm film thickness) from J&W Scientific (Fisons, Milan, Italy) and coupled to a Model TSQ 700 mass spectrometer (Finnigan Italia, Milan, Italy) in the EI and CI mode with methane N5 as reagent gas. Data acquisitions were performed in the multiple-ion detection mode. The GC temperature settings were as follows: injector at 250°C, transfer line at 290°C. The GC oven program was: 2 min at 50°C, from 50°C to 230°C at 15°C/min, from 230°C to 290°C at 8.0°C/min and 10 min at 280°C. The injection volume in the splitless mode was 2 μl. The GC–MS–MS experiments were carried out with a shortened GC oven program in the product ion mode using argon as collision gas at a cell pressure of 0.20 Pa and with a collision energy of −90 eV.

2.4. Sample analysis

2.4.1. Screening steps

The urine standard curves and samples were kept at −20°C until the day of analysis. After thawing, aliquots of 20 μl were tested simultaneously with the ELISA kits "Clenbuterol" and "Generic Bronchodilators" following the producer's specifications. The ELISA-positive urine samples were evaluated by comparing their absorbance values to those of the blank urine standard solutions. To the samples suspected of the presence of phenolic compounds the HPLC confirmation method reported by Fedrizzi and Riberzani [5], based on the work of Ong et al. [6], was applied. To the samples suspected of the presence of aryl amine compounds the continuation of this protocol, based on the works of Degroodt et al. [7] and Brambilla et al. [8], was applied. Aliquots of the urine (20 ml), ELISA-tested positive for aryl amine compounds, were adjusted to pH 12 with 40% aqueous sodium hydroxyde, passed through the Extrelut columns and extracted with 20 ml and 2×35 ml 50:50 (v/v) methylene chloride–*n*-hexane. Each extract was dried, redissolved in 3 ml 20% aqueous methanol, and applied to a SPE cyano column, preactivated with 3 ml methanol and 3 ml water. Each column was washed with 2 ml 50% aqueous methanol and the β-agonists were eluted with 2 ml methanol containing 1% TEA. The eluates were dried under nitrogen, redissolved in 60 ml methanol, and spotted on the TLC plates, which were developed with ethyl acetate–methanol–acetic acid (80:10:10, v/v/v) as mobile phase. The presence of β-agonists in the urine extracts was detected by visual comparison of the sample spots with the standard spots obtained by spraying the plates with the following sequence of reagents: 5 *M* hydrochloric acid–0.4% aqueous sodium nitrite–0.4% aqueous *N*-(1-naphthyl)ethylendiamine dihydrochloride (Bratton-Marshall spray reaction). The colors and the R_F values of clenbuterol, mabuterol and mapenterol spots were respectively purple red at 0.41, pale orange at 0.48 and dark orange at 0.56. Finally, the color spot zones on the TLC plates were grasped off with a sharp blade, collected separately and extracted with methanol. After centrifugation, the extracts of each sample were combined, evaporated under nitrogen, redissolved with 100 μl methanol, and injected onto the HPLC system. The identification of the suspected β-agonist was performed by comparison with the standards processed in the same way.

2.4.2. Confirmatory step

HPLC-positive urine samples (20 ml) were purified and extracted on Extrelut and cyano SPE columns as described in Section 2.4.1. The methanolic extracts resulting from the SPE elution were then transferred to 1 ml glass vials. To each vial 80 ng of the internal standard metoprolol was added and, after evaporating the solvent under a gentle stream of nitrogen, 40 μl pure BSTFA was also added. Then the vials were firmly closed, heated at 65°C for 1 h, and cooled to room temperature. Finally, 2 μl of the resulting mixtures were injected onto the GC–MS system.

3. Results and discussion

In our laboratory the urine samples for the detection of β-agonists are routinely processed with a screening-confirmation protocol. In the first screening step separated aliquots of the same samples are simultaneously assayed with two ELISA kits, which, according to the producer and to the results of our own experiments, exhibit good sensitivity (from 0.5 to 2.0 ng/ml depending from the compound), but different cross-reactivity patterns. By combining the two ELISA data sets, we are able to direct the analyses to follow. The urine samples are then purified through an Extrelut partition followed by a cyano-column solid-phase extraction. This passage is fast and effective enough to eliminate from the extracts the interfering substances which might have produced the "false positive" ELISA-samples. In the second screening step, the TLC test, preceded by an additional SPE cyano extraction, is applied to the samples. After the chromatographic run the β-agonists are converted on the plate into color azo dyes with the Bratton-Marshall spray reaction; this TLC method has a limit of detection of 0.5 ng/ml in urine for each of the three aryl amine compounds mentioned and it provides an extra-purification of the sample extracts. In the third screening step, the clean methanolic extracts of the TLC spots are analyzed by HPLC with Vis spectrophotometric detection at 440 nm [9]. The combination between the high absorbance of the color azo dyes and the reduction of the interfering compounds in the extracts results in very plain chromatographic profiles. Under these conditions the limits of detection for each of the three β-agonists are of about 0.5 ng/ml in urine. In the confirmatory step, new aliquots of the samples screened positive for aryl amine compounds are purified as described in Section 2.4.2, and the compounds of interest are derivatised with BSTFA and detected as TMS-products by GC–MS in EI and CI modes; the limits of detection for both the techniques were 1 ng/g for the two β-agonists. The application of two different ionisation techniques to the same derivatives provides a number of ions high enough to comply with the requirements proposed by the European Reference Laboratory for Residues of Veterinary Drugs [10] avoiding sample splitting. The EI can be regarded as a screening technique for the β-agonists because it lacks in specificity. Actually, the EI fragments of the TMS derivatives are generated in the aliphatic moiety of the molecules and tend to occur in the low part of the spectra. Therefore, the EI results need to be combined with the CI data, in which the molecular ions represent the diagnostic peaks. Table 1 lists the m/z values of the brombuterol, clenbuterol and metoprolol ions monitored in the EI and CI modes.

In 1996, in compliance with the Italian national plan of control on anabolic residues in animal matrices, a set of five bovine urine samples was sent to our laboratory for the routine analysis of β-agonists. After the ELISA-positive screening, the TLC results showed the presence of the typical clenbuterol spot, but, as shown in Fig. 2A, the HPLC profiles of the spot extracts presented two different peaks, one of clenbuterol and the other with the same retention time as brombuterol, as demonstrated by co-chromatography (Fig. 2B). Therefore, clenbuterol and brombuterol, which exhibit the same TLC behaviour (purple red spot at R_F 0.41) can be distinguished by HPLC. Fig. 3 shows the HPLC chromatograms of the TLC extracts of the blank urine (A) and of the blank urine spiked with 2 ng/ml of clenbuterol and brombuterol (B). The presence of the "new" β-agonist brombuterol was also confirmed by GC–MS. Fig. 4 shows the full scan EI spectrum of bromobuterol–TMS derivative, which is very similar to the clenbuterol one, with the dominant fragment at m/z 86 and the dibromo cluster centered at m/z 352. Fig. 5 shows the EI mode chromatograms of the blank urine (A) and of one of the urine screened positive (B). Fig. 6 shows the CI chromatograms of the blank urine (A) and of one of the urine screened positive (B). The recovery percentages in the screening steps in urine samples were 65% with S.D. 5% for clenbuterol and 75% with S.D. 4.3% for brombuterol (three replicates for each

Table 1
EI and CI m/z values of brombuterol, clenbuterol and metoprolol ions using TMS derivatives

Compound	EI (m/z)	CI (m/z)
Brombuterol	86.352	437, 439, 441
Clenbuterol	86.262	347, 349, 351
Metoprolol	72.223	340

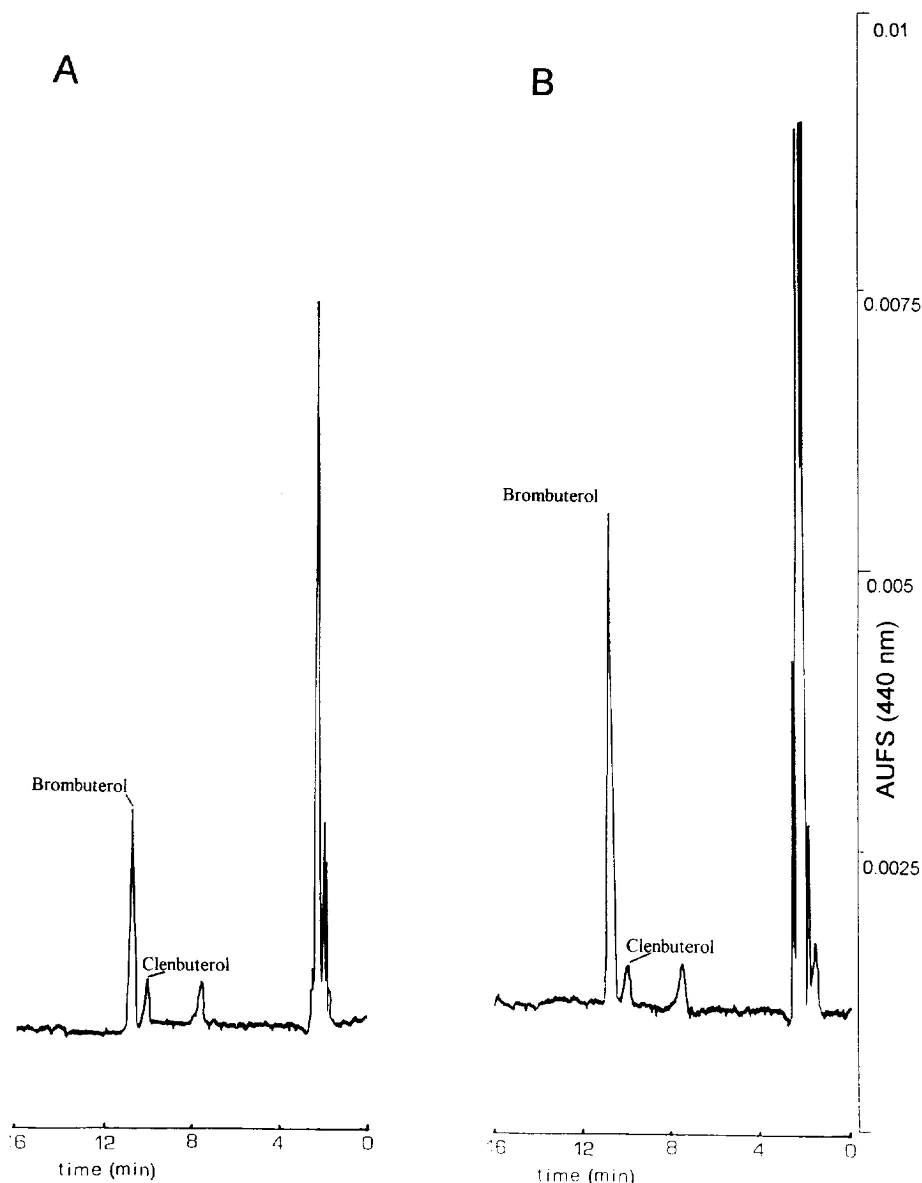

Fig. 2. HPLC chromatograms of the TLC extracts of (A) brombuterol-positive bovine urine at a concentration of 1.8 ng/ml (about 7 ng brombuterol injected) and (B) co-chromatography of the same urine with 10 ng brombuterol injected.

sample). The recovery percentages in the EI confirmatory step were respectively 69% with S.D. 8% and 76% with S.D. 9%. Also, the sample with the highest brombuterol value was analyzed by GC–MS–MS in the product ion mode from the m/z 352 precursor ion to the m/z 86 product ion. With this experiment an extremely clean single peak spectrum could be obtained (Fig. 7).

4. Conclusions

In this paper we report the extension of the analytical procedure used in our laboratory for other aryl amine β-agonists to the detection of brombuterol in bovine urine. Owing to the simple and effective combination of Extrelut partition and cyano column solid-phase extraction, this protocol lends itself to

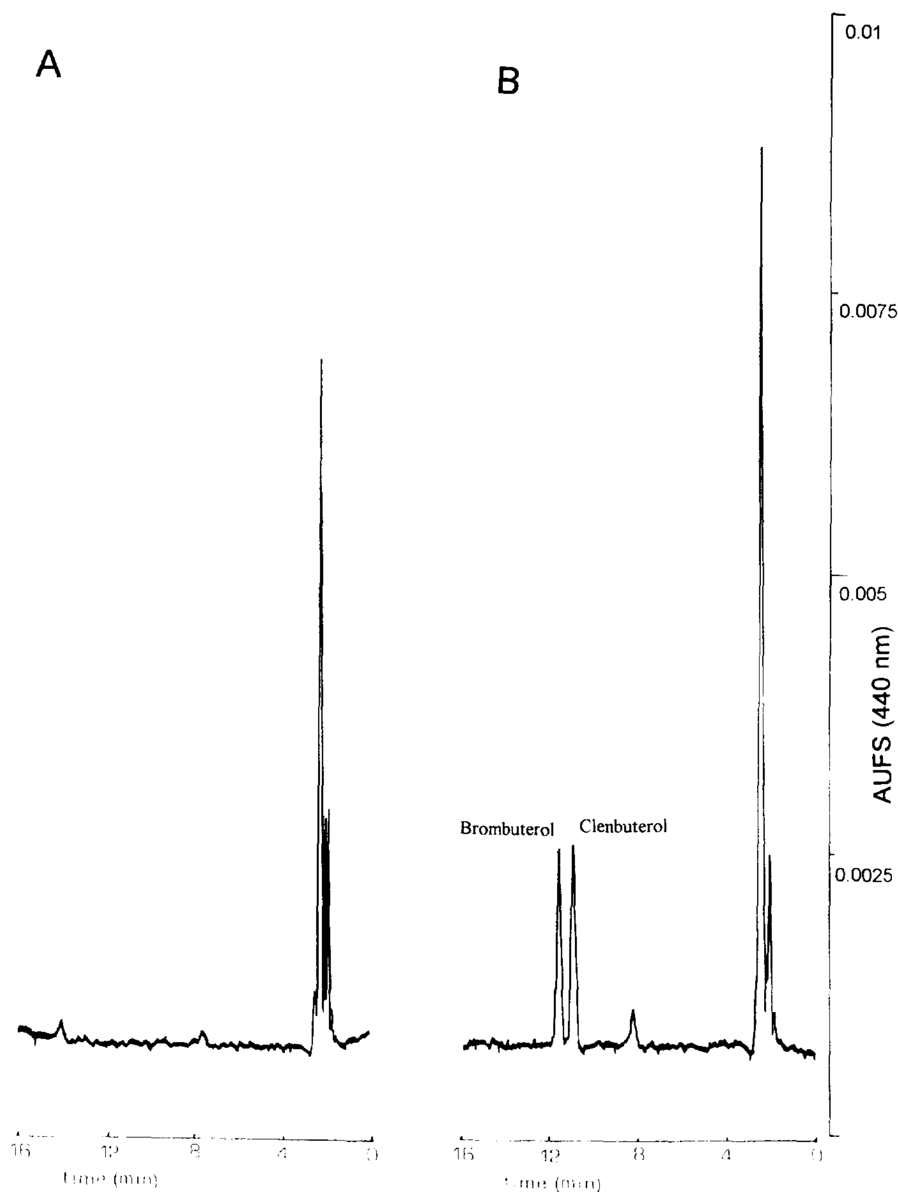

Fig. 3. HPLC chromatograms of the TLC extracts of (A) blank bovine urine and (B) blank bovine urine spiked with 2 ng/ml clenbuterol and 2 ng/ml brombuterol.

time-saving routine analyses. Moreover, to complete a single sample analysis, it requires only one 20-ml urine aliquot for the ELISA, TLC, and HPLC screening steps, and just another 20 ml one for the GC–MS confirmatory step. Also, the application to the same sample of three sequential screening tests – one immunoenzymatic and two chromatographic – followed by the GC–MS confirmation, confers to the whole protocol a very high degree of precision and specificity. In particular, the use of two different

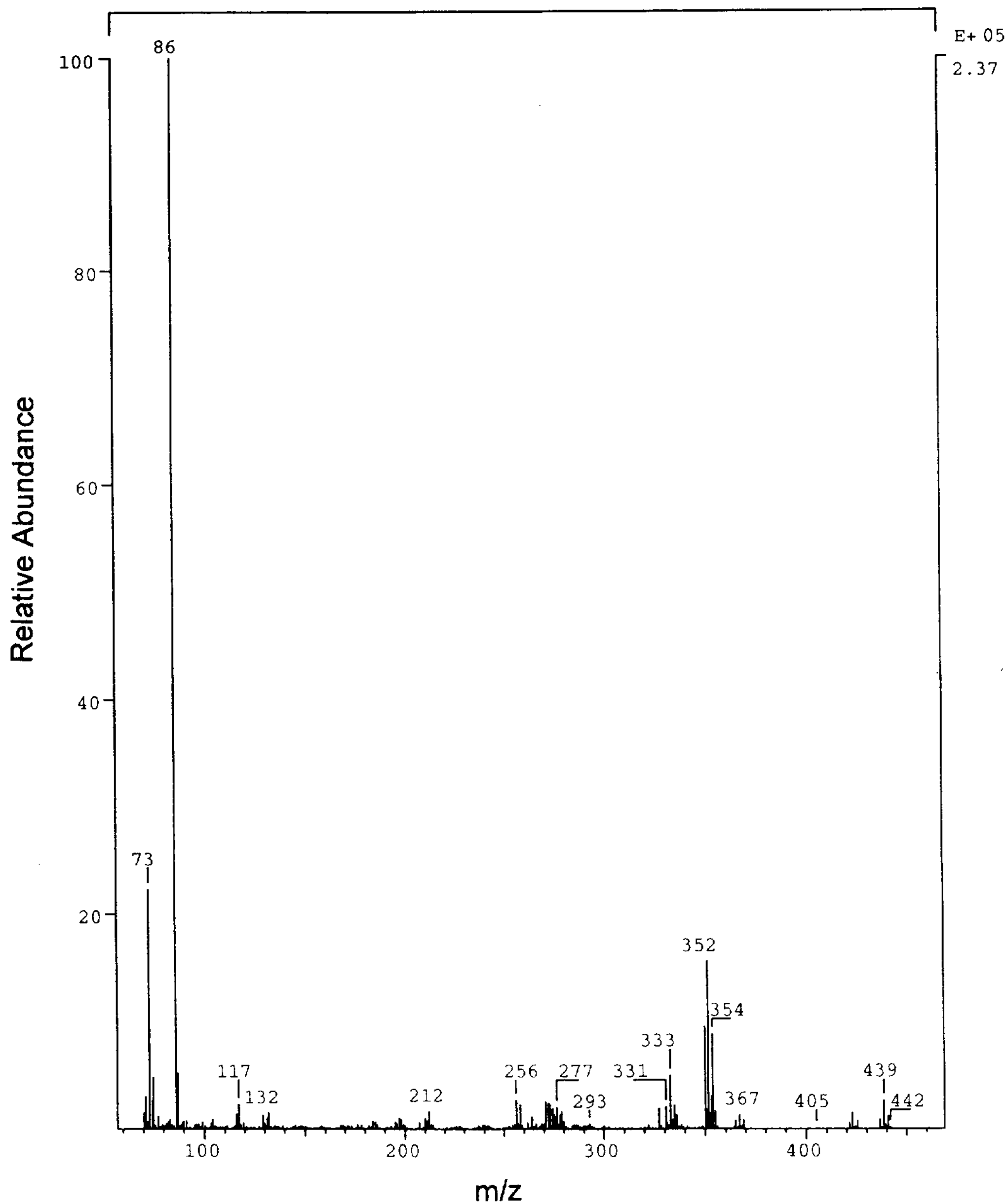

Fig. 4. Full scan EI mass spectrum of 5 ng brombuterol TMS derivative.

GC–MS ionization modes for the total generation of five diagnostic ions, completely fulfils the EEC recommendations and is in full agreement with the most important analytical requirements issued by the EURL (European Reference Laboratory) on the detection of β-agonists in import products [10].

Acknowledgments

The authors thank Dr. Angeletti of Istituto Zooprofilattico delle Venezie, Padua, and Dr. Brambilla of Istituto Superiore di Sanità, Rome, for their precious advice. They also wish to thank Miss M.

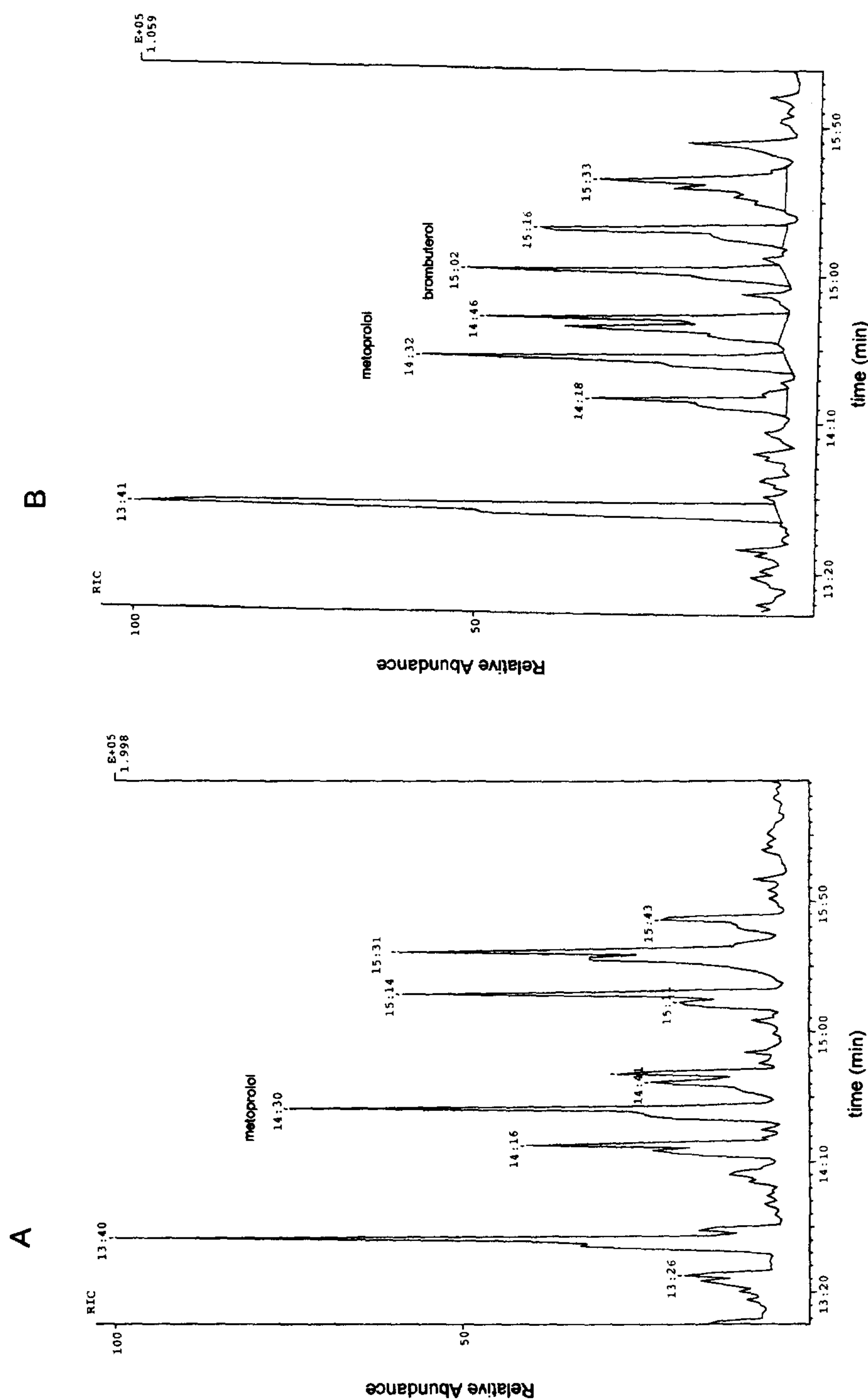

Fig. 5. EI selected ion chromatograms of (A) blank bovine urine and (B) brombuterol-positive bovine urine at a concentration of 1.8 ng/ml (m/z values of the monitored ions: 72, 86, 223, 262 and 352).

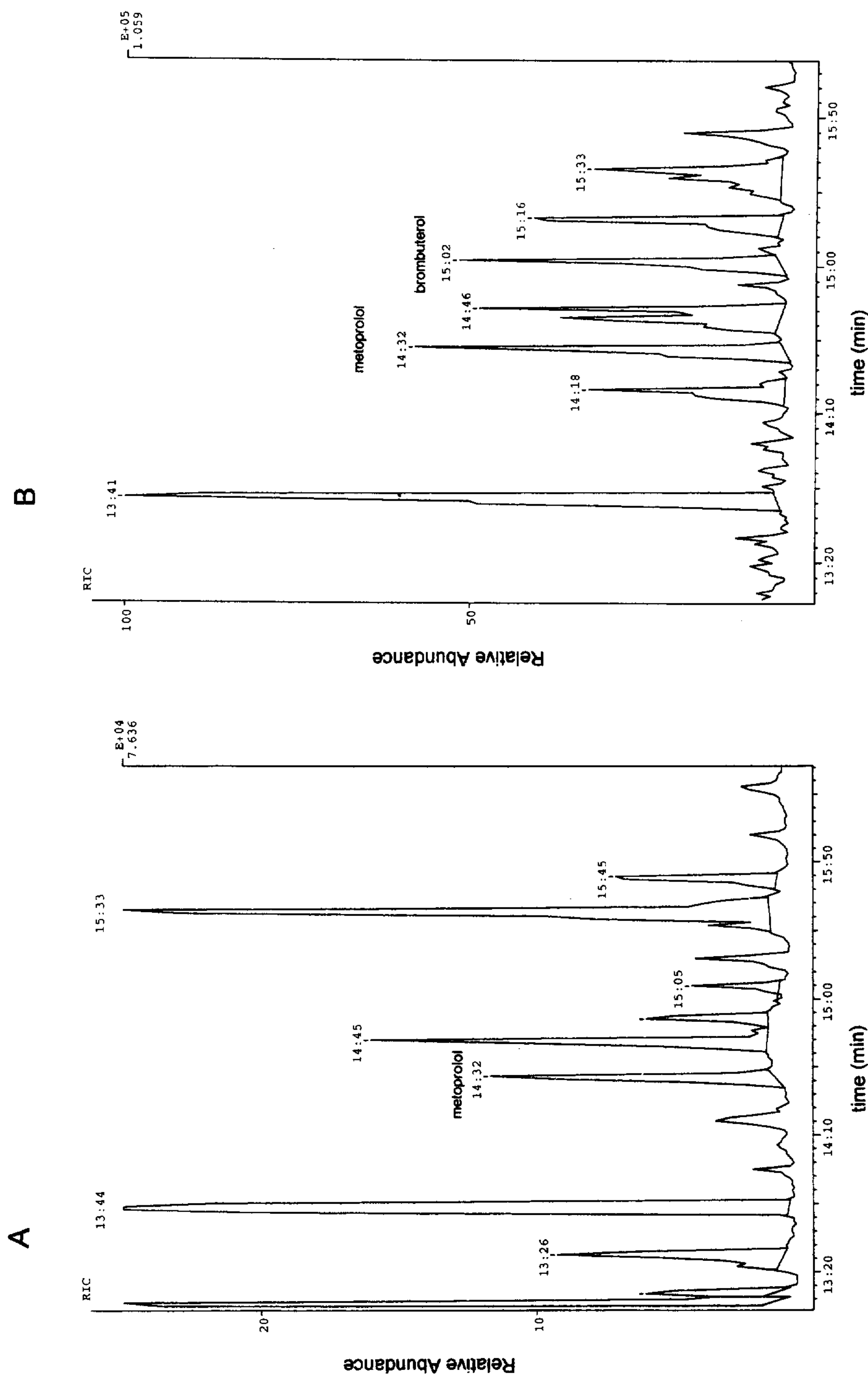

Fig. 6. CI selected ion chromatograms of (A) blank bovine urine and (B) brombuterol-positive bovine urine at a concentration of 1.0 ng/ml (m/z values of the monitored ions: 340, 347, 349, 351, 437, 439 and 441). Clenbuterol is not detectable being below the detection limit.

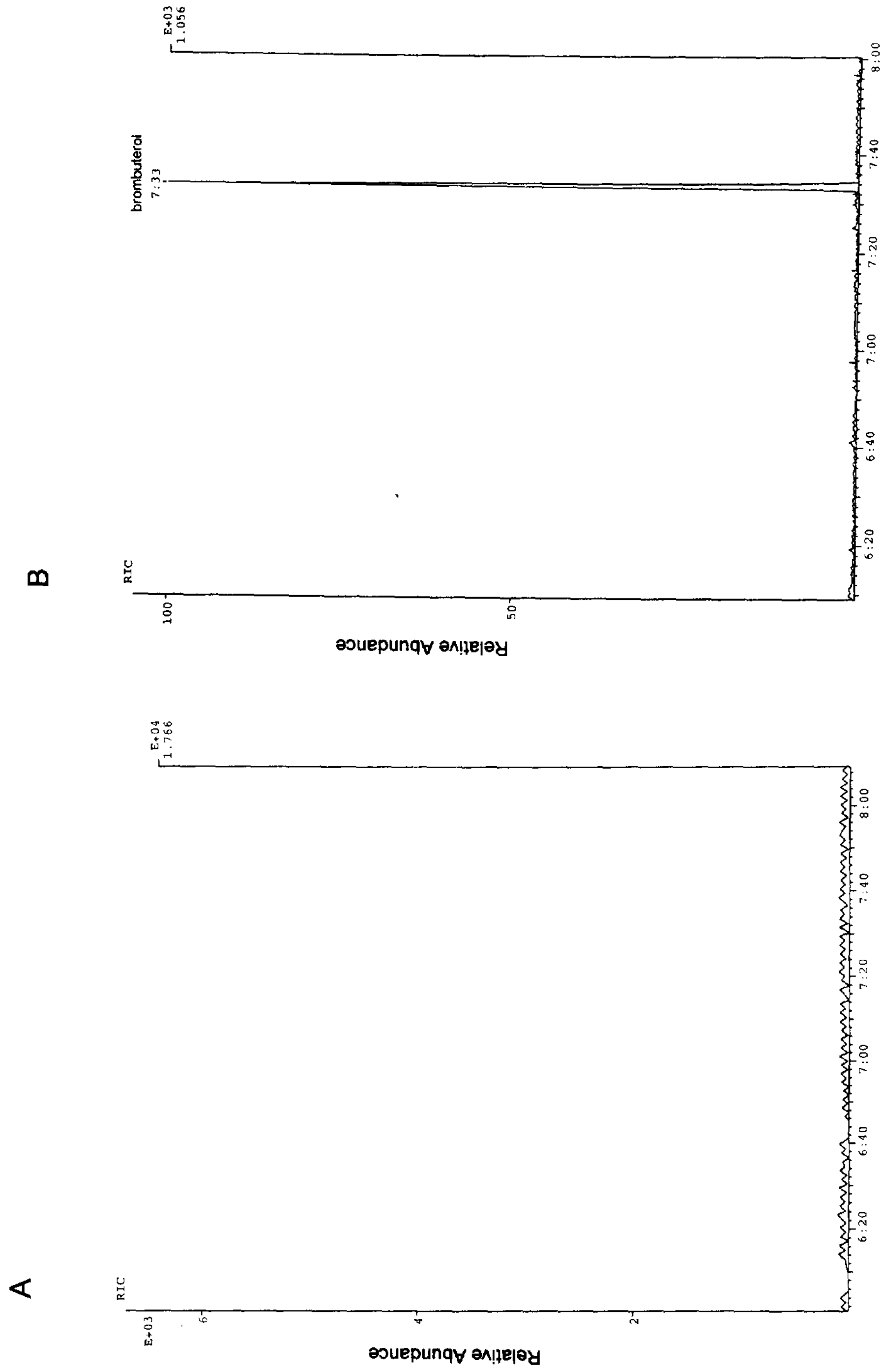

Fig. 7. GC–MS–MS chromatograms of (A) blank bovine urine and (B) bromobuterol-positive urine (m/z 352 precursor ion and m/z 86 product ion). The chromatograms have been obtained with a shorter GC oven program.

Luciani and Mr. M. Mezzana for their skilful technical help.

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