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High-performance liquid chromatographic determination of clenbuterol and cimaterol using post-column derivatization

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ABSTRACT

A general high-performance liquid chromatographic method for the simultaneous and rapid determination of cimaterol and clenbuterol is described. Solid samples, such as animal tissues, faeces and feedingstuffs, are extracted with dilute acid saturated with ethyl acetate. The resulting extracts or liquid samples, such as urine, plasma, blood and bile, are purified via Chem Elut columns. Separation is achieved by ion-pair chromatography on a Nova-Pak C_{18} column, and highly specific detection is obtained with an adapted version of the post-column derivatization described previously for the determination of clenbuterol in urine and animal tissues. Detection limits for liquids and solids are 0.1 ng/ml and 0.2 ng/g, respectively. The results are in complete agreement with analyses by high-performance thin-layer chromatography and gas chromatography–mass spectrometry, applied for confirmation after the same sample pretreatment. With this simple method, complete analysis of a liquid sample needs about 30 min and, even without an automatic sampler, 40 samples can be completely analysed in one day. This method has been used on a routine scale for nearly two years.

INTRODUCTION

In addition to the well known therapeutic use of clenbuterol for relaxation of bronchial and uterine smooth muscles, this β-adrenergic agonist also causes a significant repartition of feed energy into more carcass lean tissue. Experiments have shown that addition of β-agonists to the diet of cattle, sheep, pigs and poultry leads to a reduced fat content in favour of a higher percentage of muscle and to a positively affected feed conversion [1]. With ruminants and poultry an improved growth rate is also usually observed. For this reasons some β-agonists are used on a large scale as growth promoters. The possible adverse effects on the health of consumers of meat originating from treated animals has led in most countries to a total ban of clenbuterol, cimaterol and other β-agonists for fattening purposes.

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Owing to the very low residue levels of *ca.* 1 μg/kg, monitoring of possible misuse demands sensitive methods. Several methods for clenbuterol based on gas chromatography–mass spectrometry (GC–MS) [2–5] are capable of detection and identification at such low levels. However, they are not suitable for fast and cheap control. Most methods for the detection of clenbuterol in urine and plasma are based on high-performance liquid chromatography (HPLC). Especially electrochemical detection has been applied [6–9]. The detection limits are *ca.* 1 ng/ml. Methods with ultraviolet absorbance detection [10,11] are less sensitive and have been used for pharmaceutical formulations [11]. In comparison with HPLC, thin-layer chromatographic (TLC) methods [12] are less suitable for automation. Henion *et al.* [13] described a TLC–MS–MS method, and with high-performance thin layer chromatography (HPTLC) with detection using modified Ehrlich's reagent [14,15] low detection limits can be reached.

Although cimaterol has been mentioned in some papers as an illegally used compound for growth-promoting purposes, no method for its determination has been published.

This paper describes a routine method for the simultaneous determination of clenbuterol and cimaterol in a wide variety of samples. A rapid purification is performed by using a Chem Elut column under alkaline conditions and subsequent extraction of the β-agonists from the organic eluate with a very small volume of dilute acid. In this way concentration by evaporation can be avoided. Both steps will be discussed in detail. Optimization of the extraction of solid samples has also been performed. The presence of an aromatic amino group in both β-agonists allowed a very specific transformation into diazo dyes in a Bratton–Marshall reaction [16]. This was achieved by post-column derivatization after ion-pair reversed-phase HPLC.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile, analytical-reagent grade, toluene, dichloromethane, ethyl acetate, ammonium amidosulphonate, N-(1-naphthyl)ethylenediamine dihydrochloride, 37% hydrochloric acid and 32% sodium hydroxide solution and sodium *n*-dodecyl sulphate for biochemistry were obtained from Merck (Darmstadt, Germany). Glacial acetic acid (99–100%), 28% ammonia solution and sodium nitrite were supplied by UCB (Leuven, Belgium), Chem Elut CE 1020 columns by Analytichem International (Harbor City, CA, U.S.A.) and modified Ehrlich's TLC reagent by Alltech (Deerfield, IL, U.S.A.). Demineralized water prepared with an Elgastat UHQ system (Elga, High Wycombe, U.K.) was used.

Standards

Clenbuterol · HCL [2-*tert.*-butylamino-1-(4-amino-3,5-dichlorophenyl)ethanol hydrochloride] and cimaterol [2-isopropylamino-1-(4-amino-3-cyanophenyl)-

ethanol] were gifts from Boehringer (Ingelheim, Germany) and the State Institute for Quality Control of Agricultural Products (RIKILT, Wageningen, The Netherlands), respectively. 2-Cyclohexylamino-1-(4-amino-3,5-dichlorophenyl)ethanol synthesized at the Laboratory of Organic Chemistry of the Faculty of Agricultural Sciences (R.U.G., Ghent, Belgium), was used as the internal standard.

Standard solutions

Each standard was dissolved in 0.001 *M* hydrochloric acid at concentrations of 0.3 and 3 μg/ml. These solutions were stored in the dark at 4°C.

Mobile phase

The eluent consisted of a mixture of an ion-pair buffer and acetonitrile (53:47, v/v). The buffer contained 0.025 mol/l sodium *n*-dodecyl sulphate and 0.02 mol/l glacial acetic acid and was adjusted to pH 3.5 with 1 mol/l sodium hydroxide. The solution was filtered through a 0.2-μm membrane filter (Schleicher & Schüll, Dassel, Germany) with the aid of a solvent clarification kit (Schleicher & Schüll).

Reagents for post-column derivatization

The three reagents for the post-column reaction were prepared freshly on day of use. Reagent A consisted of 0.3 g of sodium nitrite, 125 ml of distilled water and 25 ml of nitric acid 65% (Suprapur, Merck). Reagent B was made by dissolving 2.25 g of ammonium amidosulphonate in 150 ml of distilled water. Reagent C (coupling reagent) contained 0.15 g of N-(1-naphthyl)ethylenediamine dihydrochloride in 150 ml of distilled water.

Apparatus

The HPLC system consisted of a Merck–Hitachi Model L-6200 solvent-delivery system, a Rheodyne Model 7125 syringe-loading injection valve with a 100-μl sample loop, a Merck-Hitachi 655 A-13 reaction pump for the post-column derivatization and a Waters 484 tunable absorbance detector (Millipore–Waters, Milford, MA, U.S.A.). Chromatograms were recorded on a Waters 745 data module. The analytical column was a Nova-Pak C_{18} (4 μm) column (15 cm x 4.6 mm I.D.) supplied by Millipore–Waters.

Sample application for HPTLC analysis was performed using a Linomat IV (Camag, Muttenz, Switzerland), and plates were developed in Camag twin trough chambers for 10 x 10 cm plates.

The GC–MS system (Hewlett-Packard, Rockville, MD, U.S.A.) consisted of a Model 5890 gas chromatograph, a Model 5970 mass-selective detector and a Model 7673-A autoinjector. A HP Ultra 1 column (25 m x 0.2 mm I.D.) (Hewlett-Packard) with a film thickness of 0.11 μm was used with helium as the carrier gas at a flow-rate of 1.0 ml/min. The oven temperature was maintained at 80°C for 2 min and programmed at 20°C/min to 160°C, then at 2°C/min to 190°C and finally at 30°C/min to 300°C and maintained at 300°C for 10 min. The injector and transfer-line temperatures were set at 290 and 280°C, respectively.

Extraction procedure

In addition to the tissue preparation by enzymatic digestion as described previously [14], a facile extraction under acid conditions was worked out. In a centrifuge tube, 5 g of minced animal tissue, 100 μl of a standard solution containing 30 ng of internal standard and 10 ml of 0.5 *M* hydrochloric acid, saturated with ethyl acetate, were homogenized for 3 min by means of an Ultra-Turrax T 25 (Janke und Kunkel, Staufen, Germany). After centrifugation for 20 min at 4000 *g*, the supernatant was transferred into a 50-ml beaker. To the centrifugate another portion of 10 ml of acid solution was added, homogenized for 1 min and centrifuged as above. Both supernatants were combined.

For the analysis of faeces and feedingstuffs the same acid extraction conditions were used; after addition of 30 ng of internal standard, 5 g of faeces or feedingstuffs were magnetically stirred for 15 min in a 100-ml centrifuge tube with 20 and 50 ml 0.5 *M* hydrochloric acid, respectively, saturated with ethyl acetate.

The tubes were centrifuged as described previously. For the faeces the supernatant was decanted into a 50-ml beaker, whereas for the feedingstuffs 20 ml of the supernatant, corresponding to 2 g of feedingstuffs, were accurately transferred into a 50-ml beaker.

Purification

The supernatant, obtained after extraction of the animal tissue, faeces or feedingstuffs, was treated with 32% sodium hydroxide solution in order to obtain a pH of 12. In the analysis of urine, bile or plasma, a sample of 16 ml was transferred into a beaker, internal standard was added, the pH was adjusted to 12. For blood analysis a sample of 10 ml was diluted with 8 ml of water followed by alkalinization. For further purification the same procedure was followed for all types of samples. The basic solution obtained was transferred onto the Chem Elut CE 1020 column and allowed to stand for *ca.* 10 min. With extracts of feedingstuffs it was sometimes necessary to apply a slight pressure to the column in order to distribute the sample over the packing material.

The sample beaker was rinsed with 20 ml of a mixture of toluene–dichloromethane (3:1, v/v) and the solution was applied to the column. The eluate was collected into a screw-topped 50-ml conical-bottomed centrifuge tube. After an interval of *ca.* 5 min the column was eluted with two further portions of 20 ml of the toluene–dichloromethane mixture.

A volume of 160 μl of 0.1 *M* hydrochloric acid was added to the tube, which was then shaken vigorously by hand for 1 min and placed in an ultrasonic bath for 5 min prior to centrifugation. A complete separation of both layers was achieved in 10 min at 3000 *g*.

High-performance liquid chromatography

Before and after each series of samples the HPLC system was calibrated by means of two standard solutions containing 30 and 300 ng per 100 μl of cimate-

rol, clenbuterol hydrochloride and internal standard. From the 0.1 *M* hydrochloric acid phase a portion of 100 μl was injected into the system. Chromatography was done using a flow-rate of 1.3 ml/min at ambient temperature on the Nova-Pak C_{18} column. The three reagents for the post-column derivatization were subsequently added to the eluate by the reaction pump at a flow-rate of 0.2 ml/min. The length of the reaction coil, consisting of PTFE tubing (0.33 mm I.D.), between each addition was 1, 3 and 1 m, respectively. After diazotization with reagent A, the excess of nitrite was eliminated with reagent B. Finally, the diazo dyes were obtained with reagent C. The absorption maxima for the reaction products with cimaterol and clenbuterol were 537 and 493 nm, respectively.

High-performance thin-layer chromatography

Two chromatographic runs were performed in opposite directions on one silica gel 60 TLC plate of 10 x 10 cm (Merck, Art. No. 5633). From the aqueous extracts a portion of 125 μl was made alkaline by addition of 25 μl of 1 *M* sodium hydroxide solution and extracted with 150 μl of dichloromethane. After centrifugation for 1 min at 4000 *g*, aliquots of 60 μl were spotted on a starting line 1 cm from one edge. On the opposite side of the plate, again 1 cm from the edge, the spotting was repeated. The standards (5 ng) were spotted in different lanes also on both starting lines.

Development was carried out in non-saturated tanks. The plate was eluted in one direction as far as the middle of the plate with ethyl acetate–methanol–propionic acid (8:1:1, v/v) (solvent system I). After drying, the opposite half of the plate was eluted with ethyl acetate–methanol–ammonia (8.5:1:0.5, v/v) (solvent system II).

The dried plate was sprayed with modified Ehrlich's reagent and heated at 80°C for 5 min. Clenbuterol and cimaterol appeared as red spots with R_F values for 0.40 and 0.16, respectively, in solvent system I and 0.51 and 0.38, respectively, in solvent system II.

Gas chromatography–mass spectrometry

After using the same extraction procedure as described for the TLC analysis, an aliquot of 120 μl of the dichloromethane phase was evaporated to dryness at 40°C under a stream of nitrogen. The analytes were converted into their trimethylsilyl derivatives and analysed according to the RIKILT Standard Operating Procedure A 546 (17).

The derivatization procedure consisted in adding 100 μl of a freshly prepared solution of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and ethyl acetate (1:1, v/v) to the dried extract. The mixture was thoroughly mixed and heated at 70°C for 30 min. After cooling, the mixture was evaporated to dryness at 35°C under a gentle stream of nitrogen. The residue was dissolved in 25 μl of ethyl acetate containing 1% BSTFA. A 3-μl aliquot was injected for GC–MS analysis.

For screening purposes, the mass spectrometer was operated in the selected ion

monitoring (SIM) mode. The following selected ions were monitored, with a dwell time of 100 ms, within three discrete ion groups. (1) clenbuterol-mono-TMS, *m/z* 86, 243, 262, 264, 266, 277; (2) cimaterol-mono-TMS, *m/z* 72, 143, 203, 219; (3) cimaterol-di-TMS, *m/z* 72, 158, 219, 291.

RESULTS AND DISCUSSION

Extraction of solid samples

The extraction of solid samples was originally performed using 0.5 *M* hydrochloric acid. In order to decrease adsorption, better solvation of the apolar part of the analytes was attempted by addition of an organic solvent. From a variety of solvents ethyl acetate was chosen, as it was compatible with the subsequent step in which this solution was extracted with toluene–dichloromethane. With other solvents more miscible with water, *e.g.*, methanol, some of the basic aqueous solution was co-eluted with the toluene–dichloromethane. The addition of ethyl acetate in an experiment with ten different feedingstuffs led to an increase in the overall recovery of clenbuterol from an average of 73% to 84%.

The saturated solution has to be prepared freshly on the day of use because of the instability of ethyl acetate in the presence of acid.

Purification

Because of the higher polarity of cimaterol in comparison with clenbuterol, some adaptations from the originally described purification procedure [14] were necessary. In order to achieve better elution of the compounds of interest from the Chem Elut column, two modifications were examined. First, in order to achieve more complete deprotonation, more basic conditions (pH $>$ 10) were investigated. In addition, more polar eluents than the hexane used were tried. As a higher polarity might have an adverse influence on the subsequent extraction with the small amount of dilute acid, a large gradation in the polarity of the solvents was examined. These needed to be immiscible with and lighter than water. Toluene and mixtures of toluene and dichloromethane seemed to be well suited for this purpose.

During the preliminary work, problems were encountered owing to breakthrough of the Chem Elut columns. Especially at higher pH even a non-observed minor breakthrough resulted in zero recoveries. Indeed, the 160 μl of 0.01 *M* hydrochloric acid, used in the subsequent extraction, became alkaline, so that extraction into the aqueous phase was impossible. Although the Chem Elut Type 1020 columns or Extrelut 20 columns (Merck) are described as suitable for the retention of 20 ml of aqueous solutions, a reduction of the volume to 16 ml for urine, bile and plasma were necessary in order to prevent the problem of breakthrough.

Also in a few instances with samples containing basic compounds the diluted acid became alkaline. Therefore, a solution of 0.1 *M* hydrochloric acid was pre-

ferred instead of the 0.01 *M* described previously [14]. This concentration still had no influence on the chromatographic step. Prior to injection into the HPLC system the aqueous solution was always monitored with litmus paper and, if necessary, acid was added and the organic solvent re-extracted.

For the optimization of both pH and polarity of the eluent, a complete factorial experiment on urine was set up. The pH was examined at two levels, 10 and 12, and the following eluents were compared: hexane, toluene, toluene–dichloromethane (9:1, v/v) and toluene–dichloromethane (3:1, v/v). For urine the elution of the Chem Elut columns proceeded very rapidly. As this phenomenon might influence the recovery, a decrease in the elution velocity, by the application of a tap at the end of the column, was examined. The higher pH, the use of solvents with higher polarity and the application of a tap were taken as the three factors in the complete factorial experiment. This resulted in a total of sixteen combinations as shown in Table I. All combinations were performed twice. The above-described procedure was used with the following modifications: for combinations 1–4 and 9–12 the pH of the urine was adjusted to 10 with 28% ammonia solution; in combination 9–16 after the appearance of the first drops of eluent the tap was closed for 15 min and subsequently adjusted to an elution velocity of one drop per 2 s; the elution solvent in each trial was that given in Table I.

TABLE I

RECOVERIES OF CLENBUTEROL AND CIMATEROL IN URINE WITH DIFFERENT COMBINATIONS OF THE FACTORS TAP, pH AND ELUENT

Combination	Factors			Recovery (%)			
	Tap (A)	pH (B)	Eluent (C)[a]	Clenbuterol		Cimaterol	
1	Without	10	Hexane	0	0	0	0
2		10	Toluene	81.5	74.9	13.7	12.7
3		10	T–D (9:1, v/v)	95.5	82.4	19.7	16.9
4		10	T–D (3:1, v/v)	97.8	96.4	25.7	25.4
5		12	Hexane	4.9	3.5	0	0
6		12	Toluene	95.7	93.2	46.5	46.4
7		12	T–D (9:1, v/v)	91.6	92.8	59.2	59.3
8		12	T–D (3:1, v/v)	96.6	96.5	85.9	86.6
9	With	10	Hexane	0	0	0	0
10		10	Toluene	92.5	90.6	16.3	16.7
11		10	T–D (9:1, v/v)	86.2	89.0	21.5	23.1
12		10	T–D (3:1, v/v)	92.5	86.8	46.9	44.1
13		12	Hexane	13.3	14.1	0	0
14		12	Toluene	92.6	89.3	42.1	41.5
15		12	T–D (9:1, v/v)	94.1	94.7	76.9	76.1
16		12	T–D (3:1, v/v)	92.1	92.6	90.3	91.0

[a] T–D = toluene–dichloromethane.

The urine used in the experiment originated from an untreated heifer and was spiked with cimaterol and clenbuterol at a level of 100 ppb. The blank urine was previously analysed according to combination 8 and showed no interferences at the retention times of either β-agonist. The recoveries of the 32 experiments are given in Table I.

The results were subjected to an analysis of variance in order to examine the effects of and the interactions between the three factors. The analyses of variance for cimaterol and clenbuterol are given in Tables II and III, respectively, after elimination of the results obtained in the combinations with hexane as the eluent. These were in many instances equal to zero, leading to heterogeneity of the variances. The mean square error which was used as the denominator in the *F*-test was composed of the sum of squares for the three-factor interactions and of the sum of squares for the error. In Table IV the results for all combinations are compiled. The application of a tap gave a significant improvement for cimaterol of 5.5% (7.4% on deleting the recoveries with hexane), and a non-significant change in the recovery for clenbuterol of 1.1% (−0.2% on deleting the results with hexane).

A higher pH gave a significant improvement for cimaterol and clenbuterol of 32.4 and 4.7%, respectively (43.3 and 5.7%, without the results for hexane). Finally, the recoveries for cimaterol using hexane, toluene, toluene–dichloromethane (9:1, v/v) and toluene–dichloromethane (3:1, v/v) as successive eluents were 0, 29.5, 44.1 and 62.0%, respectively. They are each significantly different from themselves at the $P = 0.01$ level (Duncan test). Concerning clenbuterol, toluene–dichloromethane (3:1, v/v), giving an 93.9% recovery, is significantly better than toluene and hexane, giving 88.8 and 4.5%, respectively, although not significantly different from toluene–dichloromethane (9:1, v/v) giving 90.8%. The

TABLE II

ANALYSIS OF VARIANCE FOR CIMATEROL (AFTER ELIMINATION OF THE COMBINATIONS 1, 5, 9 AND 13)

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Tap (A)	1	325.5	325.5	19.5	<0.005
pH (B)	1	11 228.5	11 228.5	673.5	<0.005
Eluent (C)	2	4242.6	2121.3	127.2	<0.005
AB	1	17.2	17.2	1.0	>0.25
AC	2	196.8	98.4	5.9	<0.025
BC	2	614.8	307.4	18.4	<0.005
ABC	2	222.9	111.5	6.7	<0.01
Error	12	10.5	0.9		
ABC + error	14	233.4	16.7		

TABLE III

ANALYSIS OF VARIANCE FOR CLENBUTEROL (AFTER ELIMINATION OF THE COMBINATIONS 1, 5, 9 AND 13)

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Tap (A)	1	0.2	0.2	0.01	>0.25
pH (B)	1	129.7	129.7	6.55	<0.025
Eluent (C)	2	106.3	53.2	2.68	>0.10
AB	1	16.7	16.7	0.84	>0.25
AC	2	117.2	58.6	2.96	>0.05
BC	2	45.9	22.9	1.16	>0.25
ABC	2	136.4	68.2	3.44	>0.05
Error	12	140.9	11.7		
ABC + error	14	277.3	19.8		

last value is significantly ($P = 0.01$ level) better than that for hexane, but not toluene. Both differ significantly from themselves. Therefore, using the non-polar hexane, the strongly polar cimaterol remains completely and the less polar clenbuterol partly adsorbed in the unwetted region at the bottom of the column.

Tables II and III show that for cimaterol no interaction occurred between the

TABLE IV

MEAN RECOVERIES OF CIMATEROL AND CLENBUTEROL IN THE THREE-FACTOR EXPERIMENT PER FACTOR

Factor	Recovery (%)			
	Cimaterol	Cimaterol (minus combinations 1, 5, 9 and 13)	Clenbuterol	Clenbuterol minus combinations 1, 5, 9 and 13)
Tap				
Without	31.1	41.5	69.0	91.2
With	36.7	48.9	70.0	91.1
pH				
10	17.7	23.6	66.6	88.8
12	50.1	66.8	72.4	93.5
Eluent[a]				
Hexane	0		4.5	
Toluene	29.5	29.5	88.8	88.8
T–D (9:1, v/v)	44.1	44.1	90.8	90.8
T–D (3:1, v/v)	62.0	62.0	93.9	93.9

[a] T–D = toluene–dichloromethane.

use of a tap and the change in pH, so that the effect of pH is not influenced by the use of a tap and *vice versa*. On the other hand, interaction occurred between the use of a tap and the variation in the eluent and between the pH and the eluent. The effect of more polar solvents was more pronounced with a lower elution speed, and still larger effects on the recovery, using more polar eluents, were observed at a higher pH. With clenbuterol no interactions between factors were observed.

For routine analyses optimum recoveries were obtained using toluene–dichloromethane (3:1, v/v) as the eluent at pH 12. As the use of a tap turned out to be of minor importance, it was omitted in further procedures.

HPLC analysis

The Nova-Pak (Waters) and RP-select B columns (Merck) in our hands were superior to all other reversed-phase columns tried for the separation of the basic analytes. In order to retard the β-agonists in comparison with matrix compounds ion-pair conditions were used.

The post-column reaction described previously [14] made use of nitrous acid prepared with sodium nitrite and hydrochloric acid. Owing to the highly corrosive effect of chloride ions on stainless steel, other acids were tried. The best alternative of orthophosphoric, sulphuric and nitric acid was found to be nitric acid.

With the same molarity even a slightly better response was obtained than with hydrochloric acid. The response was almost linearly correlated with the acid concentration. The maximum absorbance was dependent on the acidity of the final eluent–reagents mixture and values of 493 and 537 nm were determined for the reaction products with clenbuterol and cimaterol, respectively. Figs. 1 and 2 show typical chromatograms for the analysis of urine and faeces, originating from a bull treated with clenbuterol.

The linearity of the response was excellent: correlation coefficients of >0.99999 for injected amounts of 1–1000 ng of the analytes were observed. The relative standard deviation (R.S.D.) on eleven repeated injections of 100 ng was 1.4%. The detection limit for clenbuterol as determined by the method of Mücke [18] was as low as 0.32 ng. With a volume injected of 100 μl, from a volume of 10 ml for urine, bile and plasma, this resulted in a detection limit of *ca.* 0.05 μg/l. Recoveries for these samples were in the range 94–99%. Analysis of ten separate extractions on different days of 10 μg/l clenbuterol-spiked urine samples gave values of 9.68 $\pm$ 0.20 μg/l (mean $\pm$ S.D.; $n = 10$; R.S.D. = 2.1%). For liver, kidney and edible animal tissues the detection limit for clenbuterol was 0.2 μg/kg with recoveries between 60 and 70%. Five-fold analyses on different days of a faeces sample spiked with clenbuterol at a level of 10 μg/kg gave values of 6.65 $\pm$ 0.27 μg/kg (R.S.D. = 4.1%).

Both TLC and GC–MS were used for confirmation of the results obtained with the HPLC procedure. No case of false-positive results with the HPLC proce-

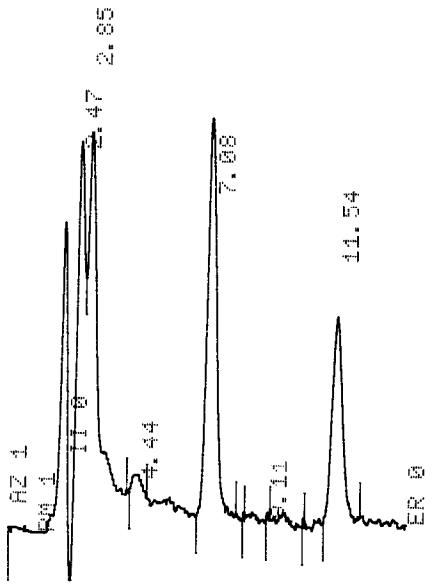

Fig. 1. Chromatogram of a urine sample from a bull treated with clenbuterol, containing 1.3 ng/ml β-agonist. For chromatographic and post-column derivatization conditions, see Experimental. Detection at 494 nm, 0.004 a.u.f.s. Retention times of clenbuterol and internal standard are 7.08 and 11.54 min, respectively.

dure have been encountered so far. The results obtained from the same samples of urine and faeces from the treated bull are shown in Figs. 3 and 4.

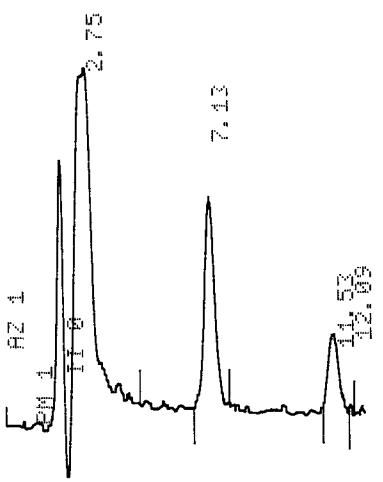

Fig. 2. Chromatogram of a sample of faeces from the same animal as in Fig. 1, containing 2.6 ng/g clenbuterol. Chromatographic conditions as in Fig. 1. Retention times of clenbuterol and internal standard are 7.13 and 11.53 min, respectively.

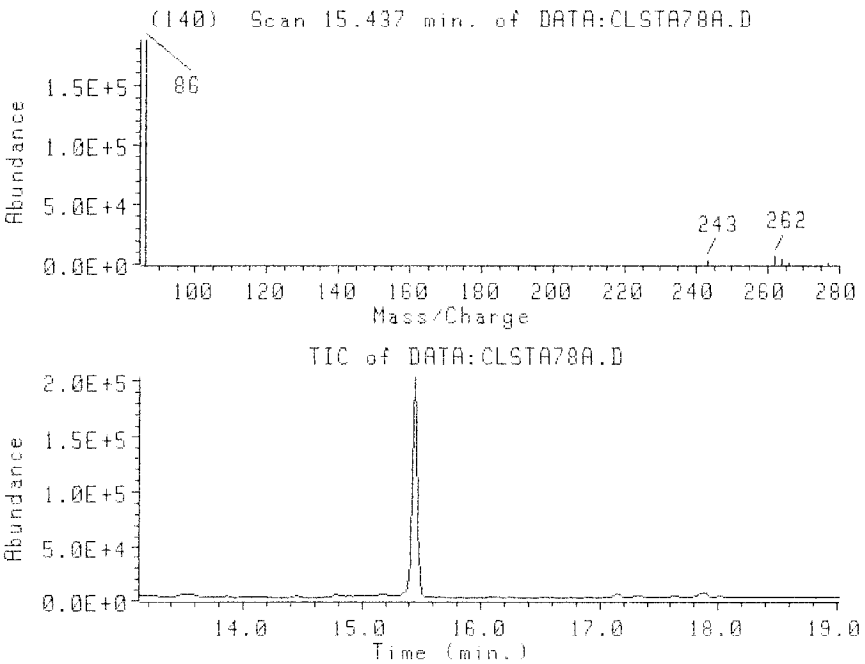

Fig. 3. Response and mass spectrum, utilizing selected ion monitoring, of the urine sample in Fig. 1. The retention time of clenbuterol is 15.42 min.

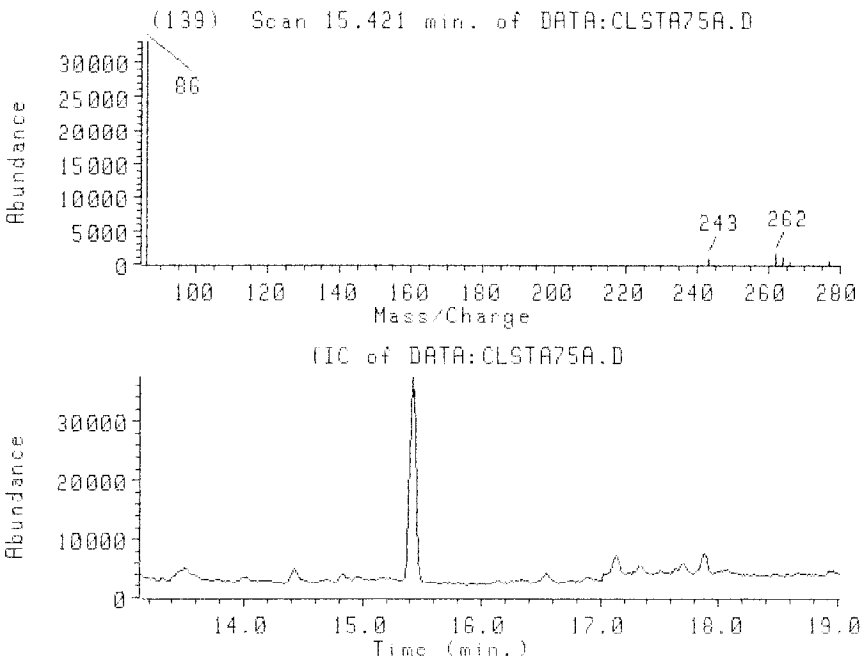

Fig. 4. Response and mass spectrum, utilizing selected ion monitoring, of the sample of faeces in Fig. 2. The retention time of clenbuterol is 15.44 min.

CONCLUSIONS

The proposed HPLC method is well suited for the rapid and simultaneous determination of clenbuterol and cimaterol. The method is simple, cheap and reliable. A very high selectivity is achieved by the post-column reaction, allowing the determination of the β-agonists in almost any kind of sample. The sensitivity obtained is sufficient for residue determination, even in meat. The selectivity is much better than that achieved with a sensitive electrochemical detector. The extraction and purification procedure is very suitable for TLC and GC–MS analysis. Both methods are appropriate for confirmation purposes.

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