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Multiresidue analysis of β_2-agonists in human and calf urine using multimodal solid-phase extraction and high-performance liquid chromatography with electrochemical detection

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Abstract

Various β_2-agonists are used as illegal growth promoters in man and in animals. We developed a multiresidue procedure for the analysis of four β-agonists in human and calf urine. The sample was pre-extracted with an Extrelut column at alkaline pH. The β-agonists were eluted with a mixture of *tert*.-butylmethyl ether and hexane. Then the extract was further cleaned with a mixed mode SPE column, or with a combination of immunoaffinity chromatography (IAC) and the mixed mode SPE column. The IAC column contained antibodies against salbutamol, which were suitable for multiresidue extractions. The extract was then brought onto a mixed mode SPE column at an acidic pH. The column was washed with 70% methanol in water. Thereafter, the β-agonists were eluted with ammoniated ethanol–hexane. The extract was analysed with an HPLC method with electrochemical detection. The β-agonists were separated on a reversed-phase column using a mobile phase buffered at pH 5.5 and containing an ion-pair reagent. Recoveries were higher when the IAC procedure was not performed (90–105% vs. 65–75%), but the extracts were cleaner when the latter step was included. Detection limits in human and calf urine were in the low ng/ml range. The study indicated that β_2-agonists can be analysed in human and calf urine without the selectivity of a mass spectrometer, but that comprehensive clean-up is required to avoid the interference of urine matrix components.

Keywords: β_2-Agonists; Clenbuterol; Brombuterol; Mabuterol; Mapenterol

1. Introduction

β_2-Agonists were originally developed for the treatment of chronic obstructive pulmonary diseases. More recently, they have drawn substantial attraction because of their abuse as a repartitioning agent in the fattening of cattle [1–3]. The misuse of β_2-agonists by athletes has been demonstrated and the use of clenbuterol as well as of other β_2-agonists has been banned by the International Olympic Committee. In addition, the misuse of these substances by adolescents has been suggested [4]. When cattle are treated illegally with a β-agonist, residues may remain in the meat and liver. Residues of clenbuterol, the most widely used β-agonist to date, have already caused a number of food poisonings [5,6]. Therefore, the use of β-agonists in meat-producing animals is now banned [7]. For the control of this ban, samples taken during fattening at the farm or at slaughter houses are analysed for the presence of illegal growth

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promoters. Urine is the sample mostly used for the analysis of the β-agonists, although concentrations are higher in retina, liver and hair [8,9]. Recently, an extensive review covering sample pre-treatment and detection methods for the analysis of the β-agonists in biological samples has appeared [10]. Clenbuterol has been extracted from urine using liquid–liquid extraction [11–14], solid-phase extraction (SPE) [12,15–21] and immunoaffinity chromatography (IAC) [22–24]. For SPE, reversed-phase sorbents [12,15,18–21] and mixed-mode sorbents containing both reversed-phase and cation-exchange functional groups [16,17] have been used. For the detection of the β-agonists, HPLC [11–14,18–22] and GC–MS [15–17,23,24] methods have been reported. The HPLC methods all used columns with reversed-phase sorbents with buffered mobile phases, either with [11–13,19,22] or without [14,18,20,21] an ion-pair reagent. Detection methods included UV absorbance [11,19,20,22], electrochemical detection (ED) [12–14,19,21] and enzyme immunoassay [18]. HPLC–MS methods have been reviewed recently [25]. The HPLC–ED methods were developed for the analysis of only one or two β-agonists in calf urine [19] or in serum or plasma [12–14,21].

Here, the development of a multiresidue method for four β-agonists in human and calf urine is described, which does not require the use of an MS instrument. The sample is successively extracted with an Extrelut column, an IAC column and a mixed-mode SPE column. The procedure was also evaluated without the IAC extraction. The β-agonists were detected with a newly developed reversed-phase HPLC method with electrochemical detection (ECD) in the amperometric mode.

2. Experimental

2.1. Chemicals

The following β-agonists were used: clenbuterol hydrochloride was obtained from Karl Thomae (Biberach and der Riss, Germany). Cimaterol, brombuterol, mabuterol and mapenterol were supplied by RIVM (Community Reference Laboratory/Laboratory for Analytical Residue Research, National Institute for Public Health and the Environment, Bilthoven, Netherlands).

Octanesulphonic acid sodium salt and acetic acid were obtained from Acros (Geel, Belgium). Sodium chloride, disodium edetate (Titriplex® III), potassium hydroxide, potassium dihydrogen phosphate, anhydrous sodium acetate, dibasic sodium phosphate dihydrate and tribasic sodium phosphate dodecahydrate were analytical grade from Merck (Darmstadt, Germany). Thimerosal was bought from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). Acetonitrile was HPLC purity reagent from Fisher Scientific (Loughborough, UK). Methanol, ethanol, *n*-hexane, 25% ammonia and 85% phosphoric acid were at least analytical grade from Merck. *tert.*-Butylmethyl ether was of Suprasolv quality (Merck). Water was demineralised in-house and was purified with a Maxima ultrapure water instrument (Elga, obtained from Salm & Kipp, Breukelen, The Netherlands). Helium, nitrogen and vacuum were available through in-house facilities.

2.2. Prepared solutions

Standard solutions of the β-agonists were prepared with a concentration of 0.2 μg/ml in demineralised water.

For the 1 *M* potassium phosphate buffer (pH 5.5, I), 68 g potassium dihydrogen phosphate was dissolved in 500 ml demineralised water and the pH was adjusted to 5.5 with solid potassium hydroxide. The 1 *M* 1-octanesulphonic acid solution (II) was prepared by dissolving 11.71 g octanesulphonic acid sodium salt in 50 ml demineralised water. For the 1 *M* sodium chloride solution (III), 5.84 g sodium chloride was dissolved in 100 ml demineralised water. The disodium edetate solution (IV) contained 5 g disodium edetate per 100 ml demineralised water. These four solutions were filtered over a 0.20-μm RC 58 membrane filter (Schleicher and Schuell, Dassel, Germany) and were stored in a refrigerator at 4°C. Mobile phase component A was prepared by mixing 25 ml I, 2.5 ml II, 2.5 ml III, 1 ml IV and 970 ml demineralised water. Mobile phase component B was pure acetonitrile. Different mixtures of mobile phase components A and B were used as indicated in Section 2.5. Mobile phases were ultrasonically degassed under vacuum (the ultrasonic

cleaner, model B2210-E-MT, was from Bransonic Ultrasonics, Danbury, CT, USA). During measurements the mobile phase was continuously deaerated with helium.

For the 0.11 *M* acetate buffer pH 4.0, 0.49 g sodium acetate and 0.94 ml acetic acid were dissolved in 200 ml demineralised water. If necessary, the pH was adjusted with some additional acetic acid. The multi-immunoaffinity chromatography (mIAC) buffer was prepared by mixing 10 ml acetate buffer pH 4.0, 30 ml demineralised water and 160 ml ethanol.

The 0.1 *M* potassium phosphate buffer pH 4.0 was prepared by mixing 1.35 ml phosphoric acid with 200 ml demineralised water and adjusting the pH to 4.0 with a 10% potassium hydroxide solution. The 0.5 *M* potassium phosphate buffer pH 4.0 was prepared by mixing 7.75 ml phosphoric acid with 200 ml demineralised water and adjusting the pH to 4.0 with a 10% potassium hydroxide solution. For phosphate buffered saline (PBS), 2.78 g dibasic sodium phosphate, 0.42 g monobasic potassium phosphate, 9 g sodium chloride and 50 mg thimerosal were dissolved in 1 l demineralised water. The pH of this buffer was 7.5. It should be stored in the refrigerator at 4°C. The 0.5 *M* tribasic sodium phosphate solution was prepared by dissolving 0.95 g tribasic sodium phosphate in 5 ml demineralised water.

2.3. Extraction columns

LiChrolut® TSC columns, 3 ml/300 mg, were from Merck. These columns contain a mixed-mode material with both C_8 and cation-exchange functional groups.

Extrelut® columns were filled in-house or were standard 3-ml columns (Merck). They contain kieselguhr material. For the prepared columns a small piece of silanised glass wool (Serva, Heidelberg, Germany) was put at the bottom of a glass column with an I.D. of 14 mm, with on top of the glass wool a paper filter. The column was filled with 4.4 g of Extrelut (Merck), under continuous ticking against the wall. When all the material was in the column, the Extrelut was further compressed with a thick glass rod.

IAC columns contained antibodies raised against salbutamol (CER, Marloie, Belgium), which were coupled to Fractoprep® (Merck) at CER. The 100× 12 mm glass columns contained 1 g gel in PBS buffer and were obtained from RIVM. When the columns were not used, they were filled with PBS buffer and stored in the refrigerator at 4°C. It should be noted that the capacity of the IAC columns used was at least 300 ng per ml gel as determined with radiotracers [26]. No significant carryover was observed.

2.4. Equipment

The HPLC system consisted of a L-6200 gradient pump (Merck) with a AS-2000 autosampler (Merck). The T-6300 column thermostat (Merck) was set at 30°C. The AMOR amperometric detector (Spark Holland, Emmen, The Netherlands) had a glassy carbon working electrode operated at a potential of +0.80 V and an Ag/AgCl in situ reference electrode. The filter was set at 3 s. Results were integrated with a HP 3396A integrator (Hewlett-Packard, Avondale, PA, USA). The HPLC columns were LiChroCART® 125-4 or 250-4 HPLC cartridges containing LiChrospher® 60 RP-Select B material, 125×4 mm I.D. or 250×4 mm I.D. respectively (Merck), protected by a LiChroCART 4-4 guard column with LiChrospher 60 RP-Select B material, 4×4 mm I.D. (Merck).

The other equipment used were a Megafuge 1.0 (Heraeus Sepatech, Osterode, Germany), a vacuum concentrator (Uni Equip, Martiensried, Germany) and a Pierce Reacti-Therm heating module (Pierce, Rockford, IL, USA).

2.5. Methods

2.5.1. Extraction and analysis using a mixed mode SPE column only

To 5 ml human urine 1 ml of 0.5 *M* potassium phosphate buffer pH 4.0 was added and the sample was shaken by hand for a few seconds. The sample was centrifuged at 3000 *g* for 10 min. A TSC-column was pre-treated with 2 ml methanol, followed by 2 ml demineralised water and finally with 1 ml of 0.1 *M* potassium phosphate buffer pH 4.0. Of the sample 4.8 ml (80%) was applied to the TSC-

column in two portions of 2.4 ml at a flow of about 0.5 ml/min. Then the column was dried for 10 s under high vacuum and it was quickly (in about 1 min) washed with 2 ml of 70% methanol in water. The column was dried under high vacuum (>20 in Hg) for 5 min. Then the β-agonists were eluted with two portions of 2 ml ethanol–*n*-hexane–ammonia (70:25:5, v/v/v). The solvent was evaporated at 40°C under a continuous flow of nitrogen. The residue was redissolved in 200 μl 25% acetonitrile in water and 10 μl was injected in the HPLC–ED system.

A 125-mm column with a 76:24 mixture of mobile phase components A and B at a flow-rate of 0.9 ml/min was used for the analysis of the β-agonists.

2.5.2. Final extraction and analysis procedure

2.5.2.1. Extrelut extraction. To 5 ml urine, 0.5 ml of a solution of 0.5 *M* tribasic sodium phosphate, pH 12.7, was added. The solution was gently mixed and carefully poured on top of the Extrelut packing. After the urine had gone fully into the packing, the column was allowed to stand for 15 min. The β-agonists were eluted with 14 ml of *tert*.-butylmethyl ether (TBME)–*n*-hexane (2:8, v/v). The eluate (about 9 ml) was collected in a tube and evaporated to dryness in a vacuum concentrator at 50°C and 1000 *g* (this takes about 20 min). The sample was further treated either by TSC extraction, in which case the residue was redissolved in 2 ml 0.1 *M* potassium phosphate buffer pH 4.0, or by IAC extraction, in which case the residue was redissolved in 2 ml 20% ethanol in water.

2.5.2.2. IAC extraction. In every step the column was prevented from running dry. The column was pre-treated with 10 ml 20% ethanol in water. The Extrelut extract was carefully applied and was run as far as possible through the column. Then the column was washed with 10 ml demineralised water. Thereafter, the column was eluted with 4 ml mIAC buffer. The IAC column was regenerated with 15 ml PBS buffer. The collected eluate was evaporated to approximately 1 ml in about 80 min by using a vacuum concentrator at 40°C and 1000 g. To the remaining fluid 2 ml 0.1 *M* potassium phosphate buffer, pH 4.0, was added and then SPE was performed.

2.5.2.3. TSC extraction. A TSC-column was pre-treated with 2 ml methanol, followed by 2 ml demineralised water and finally with 1 ml 0.1 *M* potassium phosphate buffer, pH 4.0. The complete sample was applied to the column and totally run through it in about 6 min. The drying, washing and elution was then carried out as described in Section 2.5.1. The residue was redissolved in 200 μl 25% acetonitrile in water and 20 μl was injected in the HPLC–ECD system.

2.5.2.4. HPLC–ED analysis. For the analysis of clenbuterol extracted from human urine, a 250-mm HPLC column was used with a mixture of mobile phase components A and B at a ratio of 76:24 at a flow of 0.8 ml/min and for the analysis of clenbuterol, brombuterol, mabuterol and mapenterol from calf urine, a 250-mm HPLC column was used with a mixture of A and B (70:30, v/v) at a the same flow-rate.

2.6. Calculation of results

Recoveries were calculated by comparison of the peak area obtained with a spiked sample, if necessary corrected for peaks in the blank sample, with the average peak area obtained with an external standard containing the analyte of interest at the concentration under study.

3. Results and discussion

3.1. HPLC system

A new HPLC system was developed to separate the β-agonists of interest on a reversed-phase column. The mobile phase contained an ion-pair reagent, octanesulphonic acid, to increase the retention times and to obtain sharp, symmetrical peaks. It was buffered at pH 5.5 to ensure that the β-agonists were only protonated at the aliphatic amine functional group. Sodium chloride and disodium EDTA were necessary for a proper functioning of the amperometric detector. Typical retention times obtained with the final system are given in Table 1. The retention time of the more polar cimaterol was rather short so that matrix components interfered with a reliable

Table 1
Retention times (t_R) of five β-agonists in the final HPLC system[a]

β-Agonist	t_R (min)
Cimaterol	5.5
Clenbuterol	14
Brombuterol	17
Mabuterol	22
Mapenterol	33

[a] Conditions: the β-agonists were separated on a 250-mm long column with LiChrospher RP-Select B material with a mobile phase containing components A and B (70:30, v/v) at a flow-rate of 0.8 ml/min.

quantitation. Therefore, if cimaterol is present, adaptation of the mobile phase to give longer retention times is recommended.

3.2. Extraction of human urine using a mixed mode SPE column only

The procedure developed earlier for the extraction of clenbuterol from bovine urine [27] was adapted to allow extraction of the other β-agonists. The sample volume was halved to decrease interference. The pH was adjusted with a buffer of pH 4.0, because previous experiments showed that this resulted in more reproducible recoveries [27]. The TSC column was now also conditioned with 0.1 *M* phosphate buffer. The percentage of methanol in the wash solvent had to be decreased to 70% to ensure good recoveries. Finally, the percentage of ethanol in the eluting solvent was increased. This resulted in satisfactory recoveries for the four β-agonists tested (Table 2). At the spiking level of 50 ng/ml, these β-agonists could be extracted rather well from human urine; only mabuterol had a slightly lower recovery. This may have been caused by overlapping of an endogenous urine peak. Representative chromatograms are shown in Fig. 1.

Table 2
Recoveries of four β-agonists spiked at a level of 50 ng/ml in human urine ($n=2$)[a]

β-Agonist	Recovery (%)	
Clenbuterol	109	98
Brombuterol	99	90
Mabuterol	79	67
Mapenterol	85	95

[a] Conditions: as described in Section 2.5.

Additional clean up of the samples was deemed necessary to avoid interference from matrix components and to decrease the detection limits (see Fig. 1). IAC columns appeared to be a suitable option. However, it was found to be necessary to use an Extrelut pre-extraction prior to the IAC analysis to achieve quantitative recoveries.

3.3. Extraction using combinations of Extrelut, IAC and mixed mode SPE columns

The IAC column contained salbutamol antibodies, which were shown to have good cross-reactivities with the other β-agonists [26]. The extraction procedure was derived from literature [24,28]. This procedure was changed in that the sample was now applied in 20% ethanol in water, but this did not affect the recoveries. However when the combined

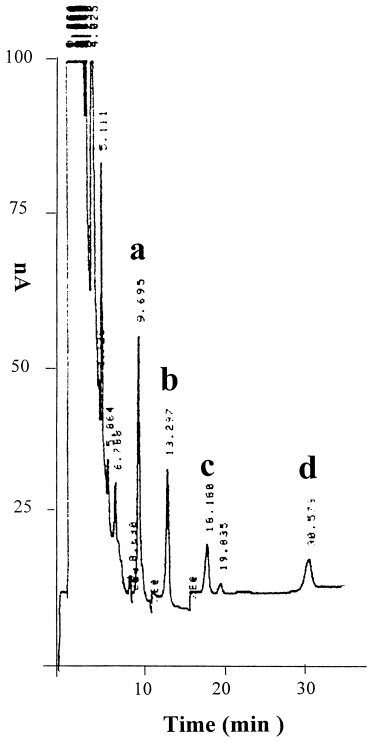

Fig. 1. Chromatogram of a spiked human urine sample after TSC extraction. The sample was spiked at the 50 ng/ml level with clenbuterol (a), brombuterol (b), mabuterol (c) and mapenterol (d). The detector was operated at 100 nA full scale.

extraction IAC–TSC was tested with urine samples, the recoveries were substantially lower. Conceivably, remaining matrix components had a negative effect on the performance of the IAC column. It was found necessary to insert a pre-extraction on an Extrelut column (kieselguhr), prior to the IAC step to ascertain suitable recoveries.

It was very important to densely pack the Extrelut column, because otherwise the recoveries were poor. Several solvents were tested as eluting solvent for the β-agonists. A TBME–hexane (1:1, v/v) mixture gave the best recoveries of clenbuterol, but for the other β-agonists a 2:8 mixture of these solvents resulted in better recoveries and cleaner extracts. Therefore, this solvent mixture was used in the final procedure.

The total extraction procedure was evaluated both with and without the IAC extraction. Human urine samples were spiked with clenbuterol at the 4 ng/ml level. The cleanest extracts were obtained when the Extrelut extraction was followed by IAC and then by a TSC extraction (Fig. 2). The recovery of clenbuterol was only slightly lower than after the combination Extrelut-TSC (65% versus 72%, $n=2$).

Next, the four β-agonists were extracted together from calf urine. The combination Extrelut–TSC provided reasonably clean chromatograms (Fig. 3) and good recoveries of the analytes (Table 3). The larger standard deviations for clenbuterol and brombuterol were conceivably due to background variations in the early part of the chromatogram. When IAC was included, the chromatograms were cleaner (Fig. 3). The cleaner chromatograms now resulted in smaller standard deviations for clenbuterol and brombuterol, but the overall recoveries dropped by about 25% (Table 3). Apparently, calf urine still contained interferences that caused losses in the IAC step.

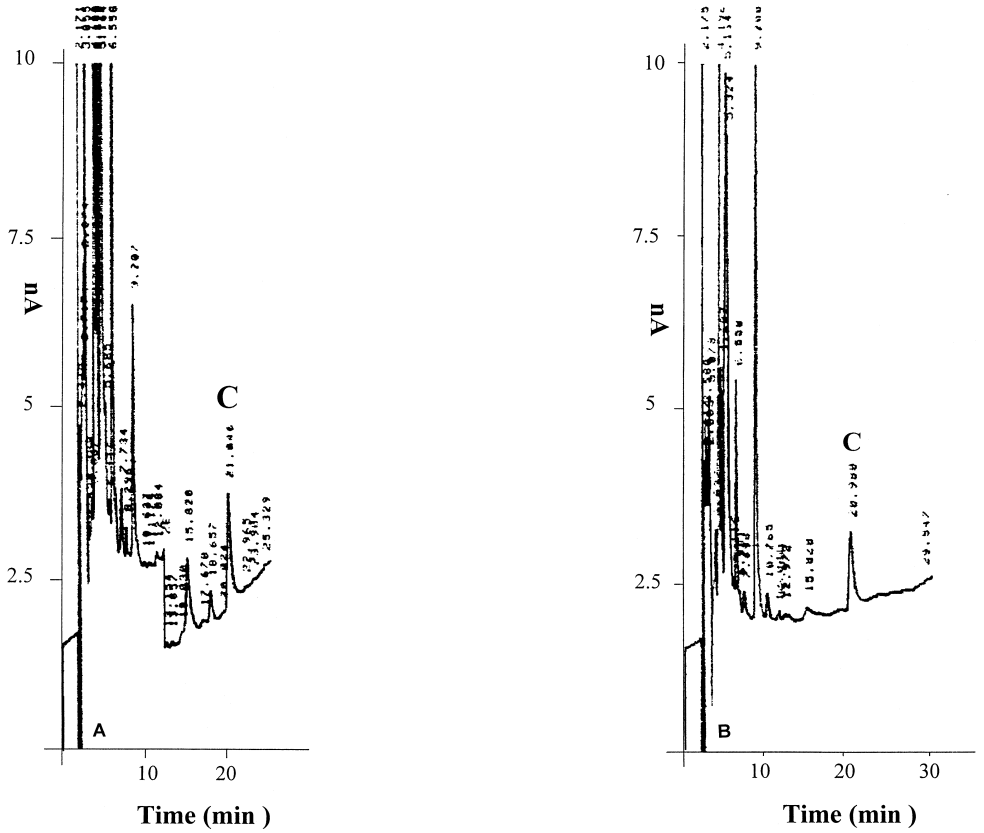

Fig. 2. Chromatograms of human urine spiked at 4 ng/ml level with clenbuterol after extraction with a combination of Extrelut–TSC (A) or Extrelut–IAC–TSC (B). The clenbuterol peak is indicated with a C. The detector was operated at 10 nA full scale.

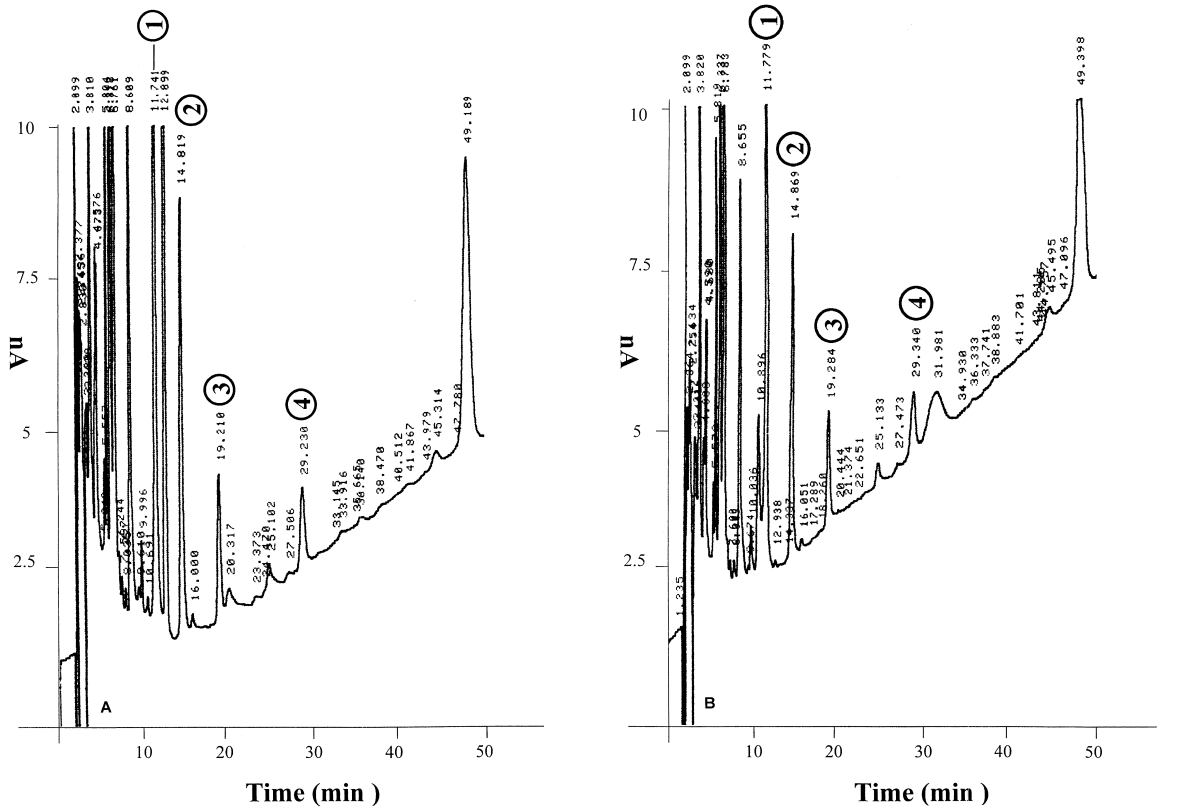

Fig. 3. Chromatograms of spiked calf urine after extraction with a combination of Extrelut–TSC (A) or Extrelut–IAC–TSC (B). The sample was spiked at the 4 ng/ml level with clenbuterol (1), brombuterol (2), mabuterol (3) and mapenterol (4). The detector was operated at 10 nA full scale.

Cimaterol could be extracted from the matrix with an acceptable recovery (90% at an 8 ng/ml spiking level), after the ratio of the *n*-hexane and TBME in the elution step of the Extrelut extraction was brought to 3:2 and the IAC procedure was left out. Including IAC in the method meant a drop of the cimaterol recovery to under 50%.

It should be noted that the polar phenolic β-agonists (salbutamol, terbutaline and fenoterol) could not be retrieved at all. Probably these substances did

Table 3
Day-to-day accuracies and precisions for four β-agonists from calf urine spiked at a concentration of 4 ng/ml with a combination of Extrelut and TSC ($n=4$) or with Extrelut–IAC–TSC ($n=6$)[a]

β-Agonist	Recovery (%)			
	Without IAC		With IAC	
	Average	S.D.	Average	S.D.
Clenbuterol	106	14.6	73	8.7
Brombuterol	101	12.1	68	7.8
Mabuterol	90	3.7	68	9.8
Mapenterol	94	4.8	64	9.7

[a] Conditions: as described in Section 2.5.2.

not elute from the Extrelut columns, because the phenolic hydroxy groups were ionised at the alkaline pH of the sample [10]. However, the phenolic β-agonists are less likely candidates for growth promotion.

In conclusion, a suitable multiresidue extraction procedure was developed for four β-agonists, either by using a combination of Extrelut and TSC or by using the combination Extrelut–IAC–TSC. Clenbuterol, brombuterol, mabuterol and mapenterol could all be retrieved from human and calf urine at a level of 4 ng/ml. Cimaterol could be extracted quantitatively using a slightly adapted procedure. Absolute recoveries were in the order of 90% when using the combination of Extrelut and TSC extraction. The combination of Extrelut, IAC and TSC for the extractions gave somewhat cleaner extracts, but also a significant drop in recoveries of about 25% for the less polar β-agonists and of even 40% for cimaterol. The use of a suitable internal standard, for example a structurally related β-blocker, may help to circumvent this phenomenon for quantitative analyses. On the other hand, it should be noted that the IAC procedure is rather time-consuming. The final extracts were analysed by a new, sensitive HPLC method with electrochemical detection. Again, for the determination of cimaterol the mobile phase composition was slightly different from that for the other four β-agonists. In urine extracts absolute detection limits determined at $S/N>3$ were in the order of 100 pg (cimaterol, clenbuterol), 250 pg (brombuterol) or 500 pg (mabuterol, mapenterol), respectively.

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