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Rapid determination of clenbuterol residues in urine by high-performance liquid chromatography with on-line automated sample processing using immunoaffinity chromatography

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ABSTRACT

A liquid chromatographic column-switching system for automated sample pretreatment and determination of clenbuterol in calf urine, using an immunoaffinity precolumn with Sepharose-immobilized polyclonal antibodies against clenbuterol, is described. A second precolumn packed with C_{18}-bonded silica was used for the reconcentration of desorbed clenbuterol prior to the analytical separation. Urine, after 2-fold dilution with buffer (pH 7.4), was loaded directly onto the immuno precolumn, where clenbuterol was trapped by the immobilized antibodies. This immuno precolumn has been used for more than 200 runs with standard solutions and samples. Bound analyte was desorbed with 0.01 *M* acetic acid and transferred, via the second precolumn, to the analytical column. The total runtime per sample was 35 min. Using a sample load of 27 ml of dilute urine and UV detection at 244 nm, the detection limit was 0.5 ng/ml. The mean recovery of clenbuterol added to a blank urine sample at the 5 ng/ml level was 82 ± 2% (n = 5) as determined with standard solutions loaded onto the same system. Urine samples from treated animals were analysed and the clenbuterol concentrations were comparable to those obtained by high-performance liquid chromatography using solid-phase extraction for sample clean-up.

INTRODUCTION

Clenbuterol, one of the β_2-agonistic drugs used for the treatment of obstructions of the bronchial tubes [1], has been shown to increase the carcass weight of calves and to improve the carcass quality [1,2]. In The Netherlands, the therapeutic use of clenbuterol has been forbidden for calves older than 14 weeks since November 6th 1988 [3]. For control purposes, mostly urine samples are taken.

In urine samples from male veal calves (aged 12 and 16 weeks) treated orally with the recommended therapeutic dose of clenbuterol · HCl (0.8 μg per kg body weight) twice a day over a period of 2 weeks, the highest concentration of clenbuterol found was 75 ng/ml. Eight days after the final application, the concentrations were lower than 0.5 ng/ml [4]. From this excretion study, a half-live of about 1.5 days was calculated. Therefore, to detect the misuse of clenbuterol over a longer period, highly sensitive methods are necessary.

In our institute, a method based on high-performance liquid chromatography (HPLC) in combination with both ultraviolet (UV) and electrochemical detection has been used, with a simple two-step sample pretreatment involving an Extrelut-3 column

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and a solid-phase extraction column [4]. Clenbuterol in urine can be determined above the 0.5 ng/ml level, which is better than or at least comparable to other reported HPLC methods [5–11]. Positive results obtained with the HPLC method are confirmed by gas chromatography in combination with mass spectrometry (GC–MS) in both the electron impact and chemical ionization modes [12]. In addition to these techniques, other methods have been described, involving high-performance thin-layer chromatography [10,13,14] or GC with electron-capture detection [15,16]. These procedures are not suitable for automation or lack the required sensitivity.

Yamamoto and Iwata [17] described a sensitive enzyme immunoassay (EIA) for the determination of clenbuterol in human plasma with a detection limit of 0.02 ng/ml. However, in principle, immunoassays are prone to cross-reactions, which may lead to less accurate results.

The specificity of an immunoassay can be improved by combination with HPLC for sample clean-up. Such a combination has been applied, for instance, to the determination of the steroid hormone nortestosterone in urine [18]. An alternative to such a combination is HPLC with on-line immunoaffinity chromatography, in which immobilized antibodies are used for the selective preconcentration of the analyte(s) prior to the analytical separation. This technique was successfully applied to the determination of small amounts of nortestosterone in biological samples of calves [19,20].

This paper demonstrates the potential of the above technique for the determination of clenbuterol in urine, without the need for any off-line sample pretreatment. To characterize the applied antibodies with respect to cross-reactivities, a microtitre plate enzyme immunoassay was developed and the results obtained with this procedure are also presented.

EXPERIMENTAL

Apparatus

The set-up of the HPLC system used is shown schematically in Fig. 1. The HPLC

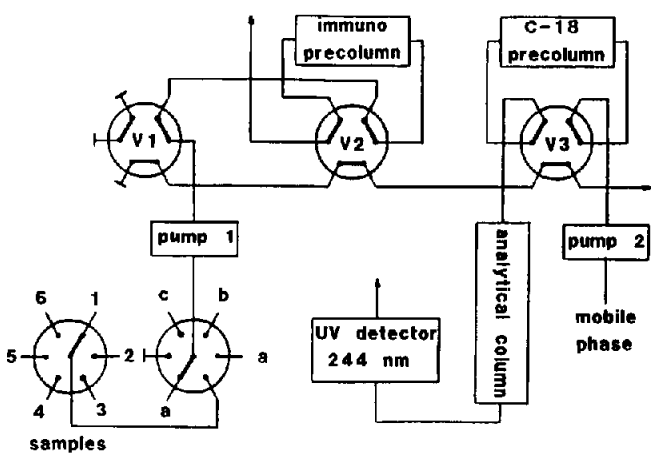

Fig. 1. Set-up of the automated HPLC system for the determination of clenbuterol in urine samples. Valves V1–V3 are all shown in position A (compare Table I).

system consisted of a Merck–Hitachi (Darmstadt, F.R.G.) Model 655-A-11 pump for the analytical column, a Kratos (Ramsey, NJ, U.S.A.) Model 400 pump for sample handling, two Kratos Model Must valve-switching units (containing two solvent-selection valves and three high-pressure six-port rotary valves), a Kratos Spectroflow 450 solvent programmer, a Merck–Hitachi L-4200 UV–VIS detector set at 244 nm and a Merck -Hitachi Model D2000 integrator. The analytical separation was achieved on a Merck 125 mm × 4 mm I.D. LiChrospher 60 RP-select B (5 μm) column with a Merck 4 mm × 4 mm I.D. LiChrospher 60 RP-select B (5 μm) guard column. The C_{18} precolumn was a 10 mm × 2 mm I.D. reversed-phase preconcentration column from Chrompack (Middelburg, The Netherlands). A 10 mm × 10 mm I.D. stainless-steel column equipped with 5-μm stainless-steel screens and PTFE rings at the column inlet and outlet, obtained from the Free University of Amsterdam, was filled with immunosorbent according to the procedure described previously [19]. The preparation of the immunosorbent is described below.

Chemicals

Tresyl-activated Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). HPLC-grade acetonitrile, HPLC-grade methanol, general-reagent grade glacial acetic acid, analytical-reagent grade sodium dodecyl sulphate, horseradish peroxidase and *Helix pomatia* digestive juice (containing a minimum of 40 U/ml β-glucuronidase and 20 U/ml arylsulphatase) were obtained from Merck (Darmstadt, F.R.G.). Sheep anti-rabbit IgG (whole molecule), *o*-phenylenediamine, Tween-20, bovine serum albumin (BSA) and fenoterol hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Clenbuterol hydrochloride was a gift from Boehringer (Ingelheim, F.R.G.) and salbutamol sulphate and terbutaline sulphate were obtained from Bufa-Chemie (Castricum, The Netherlands). Carbuterol hydrochloride was a gift from Warner-Lambert (Bornem, Belgium) and cimaterol was a gift from D.G. Mann Testing Labs. (Mississauga, Ontario, Canada). Flat-bottomed microtitre ELISA plates (96-well) were purchased from Greiner (Nurtingen, F.R.G.). Stock solutions of the β-agonistic drugs were prepared in methanol. Water was clarified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All eluents were filtered through 0.45-μm Millipore filters.

Antibodies

Clenbuterol was conjugated to BSA after diazotization, as described by Yamamoto and Iwata [17]. The conjugate (molar ratio 7.3) was used to elicit antibodies in a New Zealand White rabbit. Antibody titres were determined with an EIA procedure using BSA–clenbuterol as antigen bound to the solid phase. The IgG fraction of the collected antiserum was purified by ammonium sulphate precipitation [21] and stored at −20°C until used.

Preparation of the immunosorbent

Anti-clenbuterol IgG was bound to tresyl-activated Sepharose as recommended by the manufacturer, using 10 mg IgG/ml gel. Until use, the immunosorbent was stored at 4°C in 0.1 *M* sodium phosphate buffer (pH 7.2) containing 0.02% sodium azide as preservative.

Enzyme immunoassays

For cross-reactivity determinations, a competitive EIA was developed, applying clenbuterol–horseradish peroxidase (HRP) as the label. The enzyme conjugate (8.6 mol clenbuterol/mol HRP) was prepared as described above for BSA–clenbuterol. Microtitre plates were coated overnight with 100-μl aliquots of sheep anti-rabbit IgG [10 μg/ml in 50 m*M* sodium carbonate (pH 9.6)] at 4°C. Plates were washed four times with phosphate-buffered saline (PBS)–Tween [5.4 m*M* sodium phosphate–1.3 m*M* potassium phosphate–150 m*M* sodium chloride (pH 7.4)–0.05% Tween-20) with a Wellwash Model 4 microplate washer (Denley Instruments, Billingshurst, U.K.). Aliquots of 50 μl of serially diluted β-agonists were added to the wells, followed by 25 μl of appropriately diluted clenbuterol HRP and finally 25 μl of antiserum (all in PBS–Tween). The plates were incubated for 2 h at 37°C. After washing (as described above), the bound peroxidase was assessed with 100 μl of a freshly prepared solution of 2.2 m*M* *o*-phenylenediamine 0.012% hydrogen peroxide in 100 m*M* citrate–200 m*M* potassium phosphate (pH 5.0). After incubation in the dark for 30 min at 20°C, the reaction was stopped by addition of 50 μl of 12.5% sulphuric acid. The product of the peroxidase reaction was determined at 490 nm with an Argus 400 microplate reader (Canberra Packard, Downers Grove, IL, U.S.A.). The limit of detection of the assay was 100 pg of clenbuterol per well (2 ng/ml). Cross-reactivities were determined at 50% displacement.

Sample materials

Blank samples of calf urine were obtained from the Institute for Livestock Feeding and Nutrition Research (IVVO, Lelystad, The Netherlands). Clenbuterol-containing urine samples were obtained from an animal experiment (at IVVO in March–April 1989), in which five calves (12–16 weeks old) were treated orally with the recommended therapeutic dose of clenbuterol · HCl (0.8 μg/kg body weight) twice a day over a period of 2 weeks. During this treatment and up to 3 weeks after, urine was collected daily in two periods, *i.e.*, from 17.00 p.m. to 6.30 a.m. and from 6.30 a.m. to 17.00 p.m. Two clenbuterol-positive samples were obtained from the Dutch General Inspection Service (Kerkrade, The Netherlands).

Sample preparation

Determination of free clenbuterol. Urine samples were filtered (Type 595 1/2 filter-paper, Schleicher & Schüll, Dassel, F.R.G.) and to 25 ml of filtered urine 25 ml of PBS–buffer (pH 7.4) were added and, if necessary, the pH was adjusted by adding a few drops of 1 *M* hydrochloric acid. Of this mixture, 27 ml were loaded onto the immuno precolumn.

Determination of total clenbuterol. To 10 ml of filtered urine a few drops of 4 *M* acetic acid were added to adjust the pH to 4.8 $\pm$ 0.2. Next, 50 μl of *Helix pomatia* juice were added, the mixture was incubated at 37°C for 2 h and the volume was adjusted to 50 ml by adding PBS buffer (pH 7.4). Of this mixture, 27 ml were loaded onto the immuno precolumn.

HPLC procedure

The analytical procedure is summarized in Table I. The first step involves the preconditioning of the immuno precolumn with water, then the sample is introduced

TABLE I

SCHEDULE OF THE AUTOMATED ANALYSIS

For each valve, position A corresponds to the position shown in Fig. 1. For further explanation, see text.

Step	Event	Valve position		
		Valve 1	Valve 2	Valve 3
1	Flushing capillaries with water (7 ml)	B	A	A
2	Flushing immuno precolumn with water (20 ml)	A	A	A
3	Flushing capillaries with sample (7 ml)	B	A	A
4	Flushing immuno precolumn with sample (27 ml)	A	A	A
5	Flushing capillaries with water (7 ml)	B	A	A
6	Flushing immuno precolumn with water (10 ml)	A	A	A
7	Flushing C_{18} precolumn with water (10 ml)	B	A	B
8	Flushing capillaries with 0.01 *M* acetic acid (7 ml)	B	B	B
9	Flushing immuno precolumn and C_{18} precolumn in series with 0.01 *M* acetic acid (7 ml)	A	B	B
10	Desorbing C_{18} precolumn by on-line switching with the analytical column	A	B	A
11	Flushing capillaries with methanol–water (70:30) (7 ml)	B	A	A
12	Flushing immuno precolumn with methanol–water (70:30) (20 ml)	A	A	A

by pump I via the two solvent selection valves. Next, the immuno precolumn is flushed with water to displace the remaining sample. In the following step, the C_{18} precolumn is switched off-line with respect to the analytical column and preconditioned with water. Subsequently, the immuno precolumn, now containing the trapped analyte, and the C_{18} precolumn are switched in series and the transfer of the analytes is accomplished in the back-flush mode with 0.01 *M* acetic acid (pH 3.5). Subsequently, the actual separation is started by switching the C_{18} precolumn on-line with the analytical column. Simultaneously, the immuno precolumn is reconditioned by flushing with methanol–water (70:30). On the analytical column, clenbuterol is separated by using a mobile phase consisting of a mixture of acetonitrile and an ion-pair buffer (45:55) at a flow-rate of 1 ml/min. The ion-pair buffer contained 25 mmol of sodium dodecyl sulphate and 20 mmol of acetic acid and was adjusted to pH 3.5 with sodium hydroxide (1 *M*). After a total run time of 35 min, the next analysis can be started while the separation in the analytical column is still running. The system is automated for six samples by using a second solvent selection valve as sample selector.

RESULTS AND DISCUSSION

Antibodies

Antibodies were raised against a clenbuterol–BSA conjugate. The specificity of the isolated IgG fraction was determined in a competitive EIA, employing clenbuterol–HRP. Cross-reactions with related β-agonists (Fig. 2) were expressed at a 50% displacement level (Table II, Fig. 3). The observed cross-reactions of the antibodies may be useful in immunoaffinity chromatographic applications, allowing the entrapment of several β-agonists. Despite the relatively high specificity of the

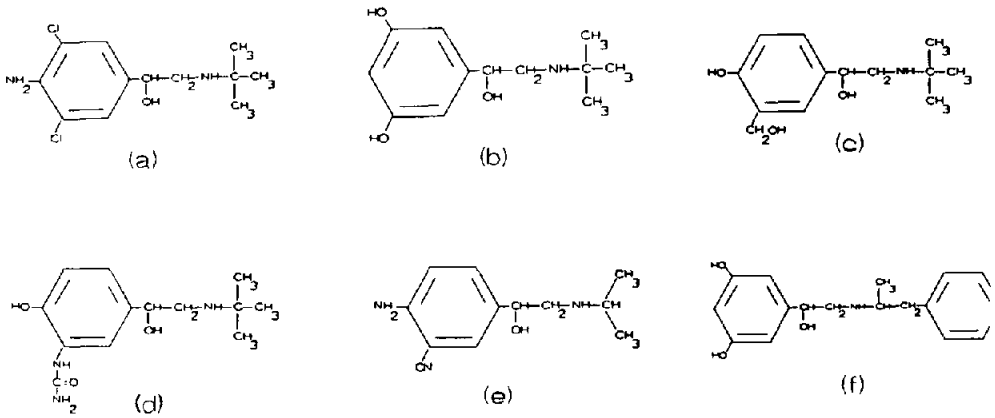

Fig. 2. Structures of (a) clenbuterol, (b) terbutaline, (c) salbutamol, (d) carbuterol, (e) cimaterol and (f) fenoterol.

antibodies for clenbuterol, these data may not be directly extrapolated towards an immunoaffinity chromatographic application. When using anti-clenbuterol IgG for the selective entrapment of analytes from a sample, there is no competition with the peroxidase conjugate, as with the EIA.

HPLC and on-line affinity chromatography

In previous experiments, several HPLC columns and mobile phases were investigated for their applicability to the separation of clenbuterol [4]. The combination of a LiChrosorb 60 RP-select B column (specially designed for basic compounds) and a mixture of acetonitrile and sodium dodecyl sulphate (SDS)–acetate buffer as the mobile phase offered the best results with respect to reproducible retention times of the analyte. The limit of detection for clenbuterol with this system, using UV detection at 244 nm, was 1 ng. The procedure also allowed the separation of clenbuterol from other β-agonistic drugs (Table II).

The column-switching system described here is based on earlier studies of the determination of 19-nortestosterone, in which selective sample pretreatment with an immunoaffinity precolumn on-line with HPLC was developed [19,20]. Agarose-based

TABLE II

RELATIVE RETENTIONS OF SOME β-AGONISTIC DRUGS, COMPARED WITH CLENBUTEROL, ON THE HPLC SYSTEM TOGETHER WITH THEIR CROSS-REACTIVITIES TOWARDS THE ANTIBODIES AGAINST CLENBUTEROL IN AN ENZYME IMMUNOASSAY

β-Agonist	Relative retention	Cross-reactivity (%)
Clenbuterol	1.000	100
Cimaterol	0.407	3.3
Fenoterol	0.386	< 0.01
Terbutaline	0.342	2.8
Carbuterol	0.314	7.9
Salbutamol	0.309	3.3

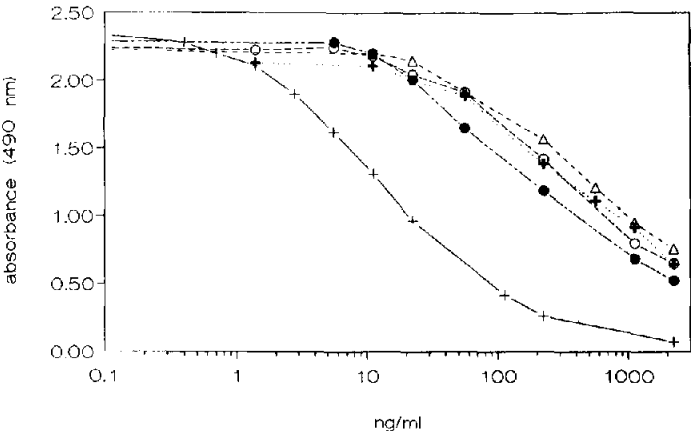

Fig. 3. Cross-reactivities of some β-agonistic drugs in the enzyme immunoassay. +, Clenbuterol; ●, carbuterol; ○, cimaterol; +, salbutamol; △, terbutaline.

precolumns were used because of their proven capability, *i.e.*, they can be loaded, flushed and desorbed with aqueous solutions with or without organic modifiers. The only disadvantage is their compressibility, which requires the application of a dual precolumn system in which the agarose-based precolumn is switched in series with a second precolumn during the desorption step. In this study we used tresyl- instead of cyanogen bromide-activated Sepharose, because of the more stable linkage between the support and the ligand [22]. When using 10 mg IgG/ml Sepharose (at pH 7.8 ± 0.2), the coupling efficiency was 80 ± 5% (n = 5). Columns containing 0.7 ml of Sepharose anti-clenbuterol were applied in the experiments described below.

Desorption of analytes bound to the immobilized antibodies may be achieved by either non-specific elution or by competitive elution with an excess of a related compound. The competitor should preferably have a longer retention time on the analytical column, to allow separation from the analyte.

The cross-reactions of the antibodies against clenbuterol with a number of β-agonistic drugs are all below 10% (Table II), necessitating relatively large amounts for competitive desorption of clenbuterol. Further, their retention times are all shorter than that of clenbuterol (Table II). Hence, a non-specific desorption step is preferred here. In this instance, the requirement for reconcentration of desorbed analyte on a C_{18}-bonded silica precolumn limits the possibilities for non-specific elution. Although clenbuterol is a basic compound that is protonated at pH < 9.5, it can be successfully retained on an RP-18 precolumn from an aqueous solution at pH 3.5. Using this precolumn within the column-switching system, in every run it is switched on-line with the analytical column during the analytical separation using a mixture of acetonitrile and SDS–acetate buffer (pH 3.5) as the mobile phase. SDS is used as an ion-pair reagent to increase the retention time of the polar clenbuterol on the apolar stationary phase. Although the RP-18 precolumn is flushed with water (see step 7 in Table I) prior to the reconcentration of the desorbed analyte, a sufficient amount of SDS apparently remains on the column. Loading up to 10 ml of solutions of clenbuterol in 20 mM citric acid (pH 3.5) or in 0.01 M acetic acid (pH 3.5) onto this precolumn resulted in recoveries higher than 80% compared with small volumes of

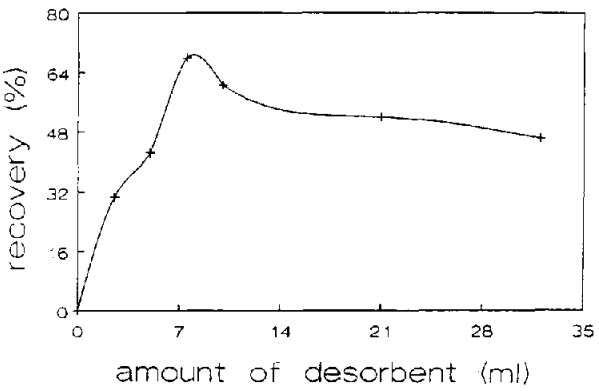

Fig. 4. Recovery of clenbuterol (%) as a function of the amount of desorbent (0.01 *M* acetic acid) in ml.

standard solutions loaded directly onto the analytical column. This positive effect of SDS was confirmed by the low recoveries (10–20%) obtained after loading standard solutions of clenbuterol in 0.01 *M* acetic acid onto a comparable solid-phase extraction column which was never used with this ion-pair reagent. Using dilute acid solutions (pH 3.5) as desorbent, both the immobilized antibodies of the immuno precolumn and the bound clenbuterol will be positively charged, which should facilitate the desorption.

The amount of 7 ml of 0.01 *M* acetic acid needed for the optimum desorption of clenbuterol bound to the immuno precolumn and for the reconcentration onto the RP-18 precolumn was determined by loading 27 ml of a solution of clenbuterol in water (5 ng/ml) onto the system and using different amounts of desorbent (Fig. 4). Under these conditions, loading 27 ml of standard solutions of clenbuterol in water of concentration 1, 2 and 3 ng/ml onto the immuno precolumn resulted in a final recovery of 69 ± 8% (n = 4), compared with small injections directly onto the analytical column. However, the concentrations of clenbuterol in urine will be calculated by a comparison with standard solutions of clenbuterol loaded onto the same system, which should compensate for these losses. After the desorption of the immuno precolumn with 0.01 *M* acetic acid, this column was flushed with methanol–water (70:30) as an extra washing step, to exclude cross-contamination between samples. Executing the procedure described in Table I, cross-contamination between a standard with a concentration of 10 ng/ml and a blank sample was <2%.

For quantitative determinations the capacity of the immuno precolumn must be known. To determine this capacity, first 27 ml of standard solutions of clenbuterol in water with increasing concentrations were loaded onto the system. However, surprisingly high concentrations of clenbuterol (up to 2 μg/ml) still resulted in an increasing peak area. Calculated from the amount of antibodies immobilized to the Sepharose, such an amount of clenbuterol could not be bound specifically, not even when all antigen binding sites would have remained available after coupling of the antibodies. Aspecific binding of clenbuterol to the Sepharose was confirmed by the binding of clenbuterol to an immuno precolumn packed with Sepharose-immobilized polyclonal antibodies against the anabolic hormone 17β,19-nortestosterone. The

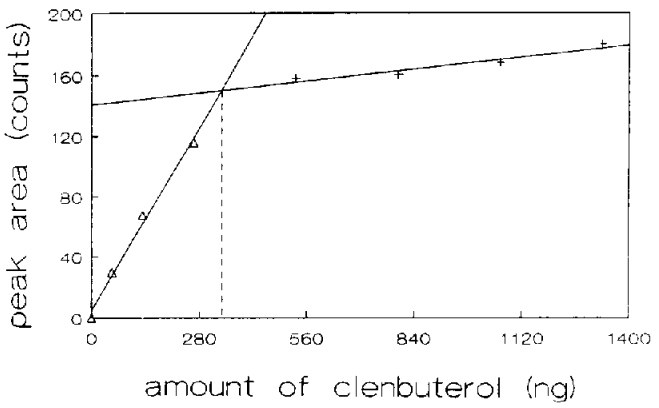

Fig. 5. Peak area as a function of the amount of clenbuterol (ng in 27 ml of standard solution) loaded onto the HPLC system. The total clenbuterol capacity of the immuno precolumn (334 ng) was determined as the intersection of the two straight lines.

aspecific binding to the Sepharose diminishes the degree of selective preconcentration. Hence the latter immuno precolumn was used to explore conditions under which the aspecific binding could be eliminated. Small amounts of organic solutions such as acetonitrile or methanol (up to 10%) added to the sample did not abolish aspecific binding. Dissolving clenbuterol in PBS–buffer was sufficient to eliminate this effect, without affecting the specific binding of clenbuterol to the Sepharose-immobilized antibodies against clenbuterol.

Increasing concentrations of clenbuterol in PBS were loaded onto the system and the peak areas of clenbuterol were measured and plotted against the absolute amount of clenbuterol offered to the system (Fig. 5). The maximum capacity of the immuno precolumn (334 ng) was determined as the intersection of the two straight lines. Comparable experiments with other β-agonistic drugs using the system described here resulted in only very low recoveries of these drugs. As found during experiments using the immunoaffinity column off-line, a number of β-agonistic drugs were bound by this column and could be eluted with 0.01 *M* acetic acid. However, reconcentration of the drugs from this desorbent onto the RP-18 precolumn led to the low recoveries. In off-line experiments, good results were obtained by adjusting the pH of the affinity column eluate to 7.4 prior to reconcentration onto an RP-18 solid-phase extraction column. In our laboratory, this combination was used for the clean-up of urine, feed and tissue samples prior to GC–MS [23]. Hence changes of the system described in this paper have to be made in order to allow the determination of these other β-agonistic drugs.

Urine samples

Urine samples from a blank calf, with and without addition of clenbuterol (5 ng/ml), and urine samples obtained from a calf treated with clenbuterol · HCl (Ventipulmin) were analysed. The only pretreatment was dilution of the sample with an equal volume of PBS–buffer (pH 7.4) and, if necessary, adjustment of the pH to 7.4 $\pm$ 0.2. In total, 27 ml of the diluted sample were loaded onto the immuno-column.

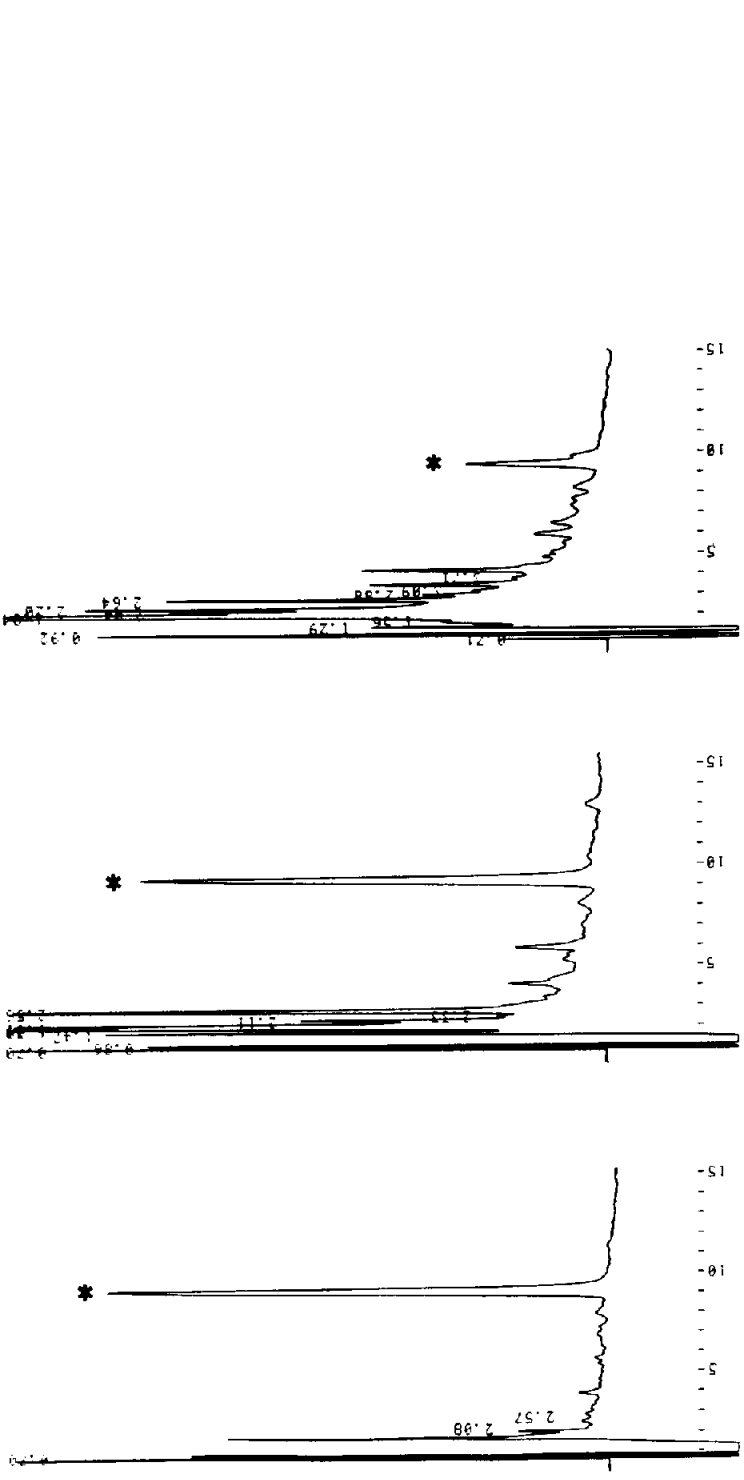

Fig. 6. Chromatograms of (A) a standard solution of clenbuterol (5 ng/ml), (B) a clenbuterol-containing urine sample (26 ng/ml; sample code 14) diluted with PBS (1:4, v/v) and (C) a clenbuterol-containing urine sample (3 ng/ml; sample code 7) diluted with PBS (1:1, v/v). Of the standard solution and dilute urine samples, 27 ml were loaded onto the HPLC system (0.002 a.u.f.s.). The peaks marked with asterisks are clenbuterol. For further conditions, see the text.

As shown in Fig. 6, clenbuterol could be detected clearly and a detection limit of 0.5 ng/ml in urine was achieved. The detection limit may easily be lowered by loading more sample. In addition to clenbuterol, other compounds were detected, most of which eluted between 0 and 5 min. These compounds could be bound non-specifically to the stationary phase backbone, the coupling group or even to the large non-selective surface of the antibodies, or specifically to the immobilized antibodies (unknown cross-reacting compounds). Compared with a standard solution of clenbuterol, (5 ng/ml), the mean recovery of clenbuterol added to the diluted (1:1 with PBS) blank urine sample at the same level was 82% ($n = 5$, S.D. 2%). This could be an effect of the presence of compounds in the urine sample which influence the antigen–antibody interaction. Therefore, for quantitative analyses, a comparison with a calibration graph in urine is preferred.

Clenbuterol was found in urine samples from a calf treated with clenbuterol · HCl (Ventipulmin) at levels between 1 and 5 ng/ml and in two urine samples obtained from the Dutch General Inspection Service. The same urine samples were analysed with an HPLC method using solid-phase extraction [4], and the results are given in Table III. The same urine samples had been analysed 5 months earlier by the latter method and the mean concentration of clenbuterol found at that time was 2.4 times higher, suggesting that the concentration of clenbuterol in urine samples decreases during storage even at temperatures below −20°C. In spite of deviations at low levels of clenbuterol, both methods are suitable for the determination of clenbuterol in urine. However, the advantage of the method presented in this paper is that "off-line" sample preparation is minimized to a dilution only.

The presence of an hydroxyl group in clenbuterol theoretically gives the possibility of conjugation with glucuronic acid or sulphuric acid. Analysis of urine

TABLE III

COMPARISON OF THE CONCENTRATIONS OF CLENBUTEROL FOUND WITH TWO HPLC METHODS USING A DIFFERENT SAMPLE CLEAN-UP

Sample code	Concentration of clenbuterol (ng/ml)	
	Immunoaffinity chromatography on-line with HPLC	HPLC with solid-phase extraction
1	2.2	1.1
2	2.3	1.3
3	2.4	1.2
4	5.9	7.2
5	2.9	1.5
6	2.3	3.8
7	2.7	2.6
8	1.7	1.1
9	1.5	2.0
10	1.6	2.5
11	2.0	2.8
12	1.1	1.9
13	5.0	8.1
14	26.8	27.0

samples from treated calves, with and without enzymatic hydrolysis using *Helix pomatia* juice, showed that hydrolysis did not result in higher concentrations of free clenbuterol.

In fact, the concentrations of clenbuterol were lower after hydrolysis, probably because of degradation of clenbuterol during the 2-h incubation at 37°C.

During the experiments described in this paper, the immuno precolumn has been used for more than 200 runs with standard solutions and samples with no marked decrease in the peak area with the most frequently used highest standard solution of 3 ng/ml.

CONCLUSION

The selectivity of the immuno precolumn, *i.e.*, a column packed with immobilized antibodies against clenbuterol, results in a high clean-up efficiency, allowing the determination of clenbuterol in urine samples at the sub-μg/l level. Such a selective preconcentration in combination with HPLC gives a high probability of excluding false-positive results. By using the immuno precolumn in an automated HPLC system, the required off-line sample pretreatment is limited to a 2-fold dilution with PBS, which leads to a better reproducibility.

The antibodies showed cross-reactions with some other β-agonistic drugs, as determined with an EIA. In experiments using the immunoaffinity column off-line with GC MS, this resulted in methods for the simultaneous determination of clenbuterol, salbutamol, cimaterol and terbutaline in urine and tissue samples [23]. With the HPLC procedure described here, however, the recoveries of these other β-agonists were low ($< 10\%$). Future research will be focused on the determination of these β-agonistic drugs using the immuno precolumn on-line with a modified HPLC procedure.

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