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Determination of clenbuterol in urine of calves by high-performance liquid chromatography with in series ultraviolet and electrochemical detection

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Abstract: A method for the determination of clenbuterol in calf urine is described. After a simple two-step sample pretreatment, involving an Extrelut-3 column and a solid-phase extraction column (C2), the separation of clenbuterol from interfering compounds present in urine samples was performed with ion-pair chromatography on a LiChrospher RP-Select-B column with a mixture of acetonitrile and sodium dodecyl sulphate/acetate buffer (pH 3.5) as mobile phase. To obtain a higher specificity, two different physico-chemical detection techniques, i.e. UV-absorption (244 nm) and electrochemical detection (+1250 mV), were applied in series. The lowest limit of determination was 0.5 ng ml^{-1} and the mean recovery of clenbuterol spiked at 10 ng ml^{-1} level was 79.9% (RSD = 6.3%; n = 9). The analysis of one urine sample, including sample preparation, took less than 2 h. Results obtained with this method correlated well with GC–MS analysis. With the described method about 400 urine samples were analysed. In a pilot experiment, in which a calf received orally 4 μg clenbuterol.HCl per kilogram body weight twice a day (five times the therapeutic dose for oral application) for 5 days, the highest concentration of clenbuterol found in urine was 73 ng ml^{-1}. In a second experiment, in which two calves received the therapeutic dose of clenbuterol.HCl twice a day over a period of 2 weeks, the highest concentration of clenbuterol was 75 ng ml^{-1} of urine. Eight days after the final application, concentrations of clenbuterol were lower than 0.5 ng ml^{-1}. From this excretion study for clenbuterol a half-life value of approximately 1.5 days was calculated.

Keywords: *Clenbuterol; ion-pair reversed-phase chromatography; UV-detection; electrochemical detection; urine analysis; excretion study; beta-agonists; GC–MS.*

Introduction

Clenbuterol, a beta-sympathomimetric or beta-agonistic drug, is used for the treatment of obstructions of the bronchial tubes of animals. Clenbuterol decreases bronchial and uterine smooth muscle activity and as a bronchodilator it increases the airway size and decreases airway resistance [1]. Next to the actions on smooth muscle, beta-agonists have other effects, in particular when added to the diet of animals at various levels (0.25–4 ppm) they effect the live-weight gain. Experiments with animals have shown improvements in feed conversion, changes in carcass quality, meat structure and composition. There is a significant change in the fat:meat ratio and the weight-increase of calves. The fat:meat ratio turns out to be favourable in terms of the production of lean meat. The increase in carcass weight of calves, orally treated with clenbuterol as growth promotor, is about 10%, without any change in food intake [2]. Since 6 November 1988 the (therapeutic) use of clenbuterol has been forbidden in The Netherlands for calves older than 14 weeks [3]. Control of possible misuse demands the availability of sensitive methods capable of detecting the expected low concentrations of the drug in biological samples. Furthermore, data about the excretion of clenbuterol after a legal therapeutic application, have to be obtained before any conclusions can be drawn about possible misuse.

Several authors have described methods for the determination of clenbuterol, such as enzyme-linked immunosorbent assay (ELISA) [4], high-performance liquid chromatography (HPLC) [5–9], high-performance thin-layer chromatography (HPTLC) [10], gas chromatography with mass spectrometric detection (GC–MS) [10–12] gas chromatography with electron capture detection (ECD) [13, 14].

The ELISA [4] has a limit of detection of

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about 3.0 ng ml^{-1} in plasma and requires antibodies that are not (yet) commercially available. The HPLC methods which use UV-detection [5, 6], lack the sensitivity required to monitor the low ppb levels (<10 ng ml^{-1}). HPLC with electrochemical detection has already been utilized by Diquet [7], Qureshi [8] and Papillon [9] for the determination of clenbuterol in plasma and urine. They reported limits of detection of clenbuterol of 3.0 ng ml^{-1} and 0.5 ng ml^{-1} in plasma samples and 1.0 ng ml^{-1} in urine samples. HPTLC methods [8] are more tedious and not as suitable for automation as HPLC methods. Methods which use gas chromatography in combination with mass spectrometry (GC–MS) [9, 11, 12] are highly specific but expensive and more suitable for confirmation purposes, whilst gas chromatography in combination with ECD detection [13, 14] lacks the sensitivity required.

Within the author's laboratory, HPLC was chosen as the technique to analyse urine samples for the presence of clenbuterol. However the methods described in the literature have been found unsuitable. In particular, irreproducible retention times for clenbuterol were observed and the recommended sample clean-up procedures were found to result in too many interfering substances that gave signals comparable to the analyte.

This paper presents an assay for clenbuterol in calf urine that employs ion-pair reversed-phase liquid chromatography with in series UV-detection and oxidative electrochemical detection, after a two-step sample pretreatment procedure. The method has been applied to the determination of clenbuterol in the urine of treated calves and in urine from calves destined for export.

Experimental

Chemicals and reagents

Tertiary-butyl methyl ether (HPLC grade) was obtained from FLUKA (Buchs, Switzerland), Acetonitrile (HPLC/Spectro grade) from Alltech (Deerfield, USA). Bondelut C2 cartridges (1.0 cm^3) were supplied by Analytichem International (Harbor City, USA). Clenbuterol.HCl (4-amino-α[(tert-butylamino) methyl]-3,5-dichlorobenzylalcohol-hydrochloride) and Ventipulmin were gifts from Boehringer Ingelheim (FRG). Extrelut-3 and all other chemicals used were of analytical-reagent grade and were obtained from Merck (Darmstadt, FRG). Water was clarified by means of a Milli-Q system (Millipore, Bedford, USA).

Apparatus

The high-performance liquid chromatographic system consisted of a Model L-6200 solvent delivery system, Merck-Hitachi (Darmstadt, FRG), a Model 231/402 autosampler, Gilson (Villiers-le-bel, France) and a L-4200 UV-detector, Merck-Hitachi Model or a Model 1040-M diode array detector with a chem station, Hewlett–Packard (Rockville, USA). Chromatograms were recorded using a model D 2000 dual-channel integrator-plotter, Merck-Hitachi. A Model SpH 99 column thermostat, Spark (Emmen, The Netherlands) was used. The flow rate of the HPLC system was adjusted to 1.0 ml min^{-1} and the injection volume of the autosampler was varied between 10 and 200 μl. All separations were performed at 30°C. The wavelength of the UV-detector was set at 244 nm.

Electrochemical detection

A Model 656/641 electrochemical detector, Metrohm (Herisau, Switzerland), equipped with a glassy carbon working electrode, a glassy carbon auxiliary electrode and a silver–silver chloride electrode [filled with a lithium chloride solution (3 M in water)] as reference electrode was used. The potential was set at + 1250 mV versus the reference electrode. The electrochemical detector was set to 50 nA full scale.

Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile and an ion-pair buffer (45:55, v/v). The ion-pair buffer contained 25 mM sodium dodecyl sulphate and 20 mM acetic acid and was adjusted to pH 3.5 with 1 M sodium hydroxide. The mobile phase was filtered through a type GVWP 0.2 μm membrane filter (Millipore, Bedford, USA) with the aid of a solvent clarification kit (Millipore). Separation was achieved on a LiChrospher 60 RP-select B analytical column (250 mm × 4 mm i.d., 5 μm, Merck) with a LiChrospher 60 RP-select B guard column (4 mm × 4 mm i.d., 5 μm, Merck).

Sample materials

Urine samples were obtained from two

animal experiments and from several slaughterhouses in The Netherlands.

In Experiment 1, urine samples were obtained from a 6-month old male veal calf which had been treated with clenbuterol.HCl (Ventipulmin, Boehringer, Ingelheim, FRG). The animal received a relatively high oral dose (five times the therapeutic dose) of 4 μg clenbuterol.HCl per kilogram body weight, twice a day (8.00 a.m. and 8.00 p.m.), for a period of 5 days. During this treatment, urine was collected in two periods, i.e. from 8.00 a.m. to 8.00 p.m. and from 8.00 p.m. to 8.00 a.m. On the sixth day the animal was slaughtered. This animal experiment was performed at ILOB/TNO (Wageningen, The Netherlands).

In Experiment 2, two male calves (14 and 16 weeks old) were treated orally with the recommended therapeutic dose of clenbuterol.HCl (0.8 μg per kilogram 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 5.00 p.m. to 6.30 a.m. and from 6.30 a.m. to 5.00 p.m. This animal experiment was performed at the Institute for Livestock Feeding and Nutrition Research (IVVO, Lelystad, The Netherlands).

Other urine samples were taken by inspectors of the National Inspection Service for Meat, Livestock and Animal Products at several slaughterhouses in The Netherlands. All urine samples were stored at −20°C prior to analysis.

Sample pretreatment

To a 10 ml test tube, containing 4 ml of the urine sample, a few drops of 10 M sodium hydroxide were added to obtain a pH between 10 and 11. After centrifuging at 200**g** for 5 min, the supernatant was transferred to an Extrelut-3 column. After 5 min, which was sufficient to enable the absorption and distribution of the aqueous sample as a thin film over the hydrophilic packing material, the column was eluted with three 5 ml volumes of tert-butyl methyl ether. The combined organic fractions were evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 500 μl of a 0.01 M borate buffer (pH 9.5) and transferred to a C2 solid-phase extraction column. This column had previously been activated with one column-volume of methanol and subsequently two column-volumes of 0.01 M borate buffer (pH 9.5). Next, the test tube was rinsed with another 500 μl borate buffer (pH 9.5), which was also transferred to the solid-phase extraction column. The column was washed twice with 250 μl methanol–water (35:65, v/v), dried, by suction of air for about 5 min, and subsequently eluted with 2 × 1 ml of a mixture of methanol and 1 M ammonium acetate (99:1, v/v). The eluates were combined and evaporated to dryness at 45°C under a stream of nitrogen. Prior to liquid chromatography the residue was dissolved in 250 μl of the mobile phase.

Results and Discussion

Extrelut-column

Clenbuterol, when deprotonated at a high pH, may be extracted from urine samples with tert-butyl methyl ether (TBME). However, with most urine samples, phase separation was complicated by the formation of an emulsion. The problem, however, was avoided by extraction from urine by means of an Extrelut column. Clenbuterol was eluted from the hydrophobic packing material with TBME and recoveries, measured by applying standard solutions of clenbuterol in a buffer (pH 11), were 96.4 ± 3.5% ($n = 9$). Other advantages of using such columns as a first step in the analysis is that it acts as a filter by which particles present in the urine samples are removed and hydrophilic compounds present in the urine sample remain on the column.

Solid-phase extraction

In the last decade the importance of solid-phase extraction has grown tremendously. In the past, solid-phase extraction columns have been used in the author's laboratory to clean-up samples such as urine, meat, etc. [15]. For the clean-up of clenbuterol, several types of extraction columns in principle can be used. Clenbuterol, a basic compound, can be retained, if deprotonated at a high pH, on reversed-phase materials. Therefore, the residue, obtained after evaporation of the Extralut-column eluate, was dissolved in a borate buffer (pH 9.5) before transferring to a reversed-phase column.

As was shown in previous experiments, using C-2 and C-18 materials, the retention of clenbuterol was independent of the chain-length of the column packing. This might be due to interaction between the amino-group of clenbuterol and the free silanol groups of the solid-phase column. For the clean-up of urine

samples C-2 columns were preferred to C-18 columns, because with the latter more interfering substances also were concentrated.

Chromatography

A number of methods exist in the literature, for the separation of clenbuterol by liquid chromatography [5–9]. In all the author's experience none of the described methods fulfil the requirements of the current analysis. Especially the separation of clenbuterol on a CN-column [5, 6, 9] showed an enormous shift in retention time under the chromatographic conditions prescribed. Experiments with an endcapped C-18 column and an ion-pair salt also resulted in non-reproducibile retention times. The combination of a LiChrospher 60 RP-select-B column, especially designed for basic compounds, and a mobile phase consisting of a mixture of acetonitrile and sodium dodecyl sulphate and acetate buffer resulted in a stable system, in which retention times were reproducible during a sequence of more than 20 samples.

From the UV-spectrum of clenbuterol, with maxima at 244 and 300 nm, the wavelength with the highest absorbance (244 nm) was chosen for the detection of clenbuterol.

To obtain the optimal signal-to-noise ratio in electrochemical detection, the potential was set at +1250 mV. The ratio of electrochemical to UV-absorbance signals can be used to confirm the presence of clenbuterol in the urine sample. However, contamination of the working electrode of an electrochemical detector is a well known problem. In experiments, using an auto-sampler in which more than 20 samples were analysed overnight, the sensitivity of the electrochemical detector was found to decrease rapidly. After electrochemical cleaning of the electrodes, for 5 min at +1750 mV and at −1000 mV for 1 min, the sensitivity increased to its original level. Therefore it would seem that, to use an electrochemical detector for the assay of clenbuterol over prolonged periods (>10 h) is not practicable. In most samples however, due to a good separation, clenbuterol could be analysed with UV-detection only. Figure 1 shows the chromatograms of a standard solution of clenbuterol.

Sample pretreatment with an Extrelut-3 column and a C-2 solid-phase column appeared

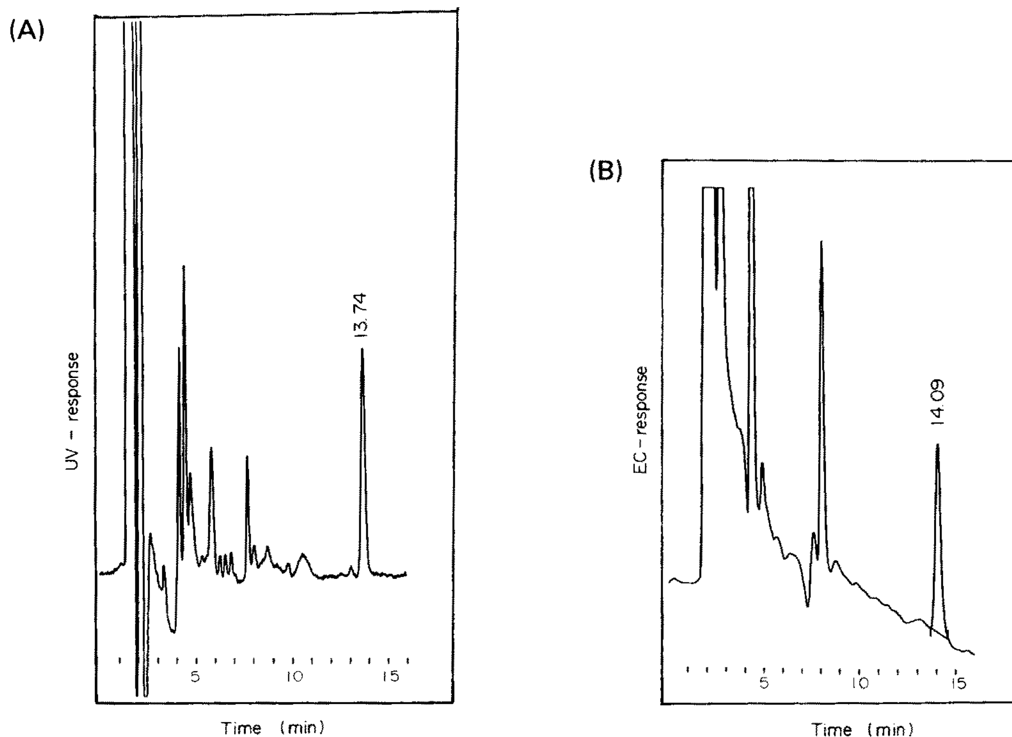

Figure 1
Chromatograms of a standard solution of clenbuterol.HCl (10 ng injected). (A) UV-detection (244 nm), 0.002 AUFS, retention time of clenbuterol 13.74 min. (B) EC-detection (+1250 mV), attenuation 10, 50 nA full scale, retention time of clenbuterol 14.09 min.

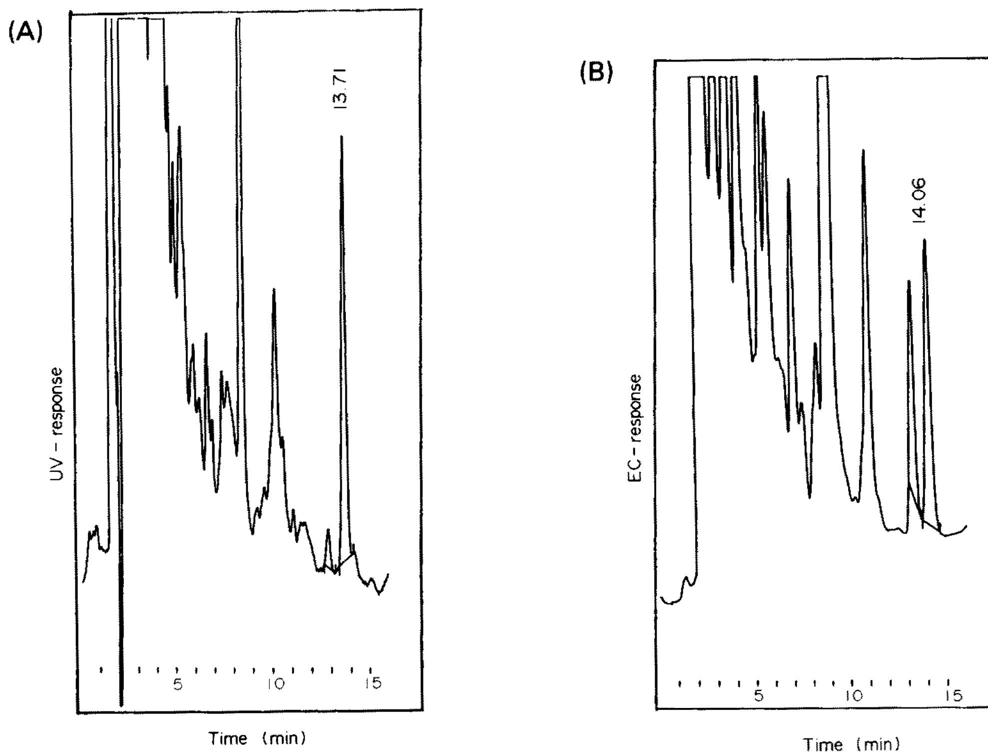

Figure 2
Chromatograms of a urine sample from Experiment 1 (96 h after the first dose) containing 61 ng clenbuterol per millilitre. (A) UV-detection, 0.002 AUFS, retention time of clenbuterol 13.71 min. (B) EC-detection, attenuation 10, 50 nA full scale, retention time 14.06 min.

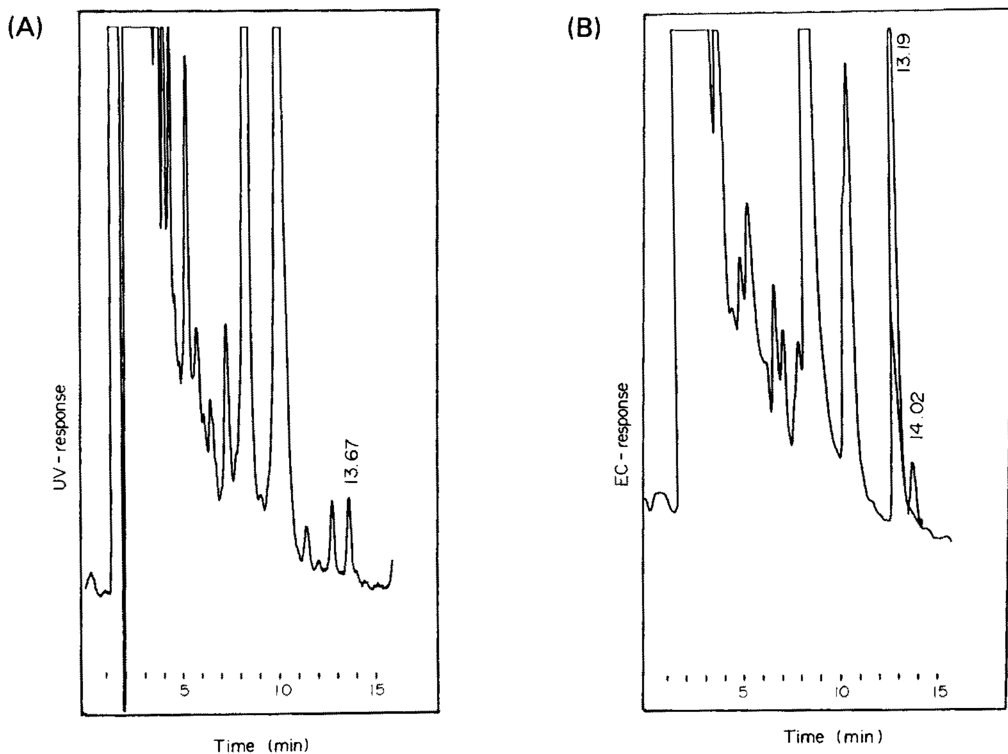

Figure 3
Chromatograms of a urine sample, obtained from a slaughterhouse, containing 2.0 ng clenbuterol per millilitre. (A) UV-detection, 0.002 AUFS, retention time of clenbuterol 13.67 min. (B) EC-detection, attenuation 10, 50 nA full scale, retention time of clenbuterol 14.02 min.

to be sufficient to enable analysis of clenbuterol at the low ppb level in urine of calves. In Fig. 2 the chromatograms of a urine sample from a calf treated with clenbuterol (Experiment 1) are shown. In Table 1, the concentrations of clenbuterol found in the urine samples taken from this calf during the whole treatment are given.

In Fig. 3 the chromatograms of calf urine obtained from a slaughterhouse are presented to demonstrate the purity of a urine extract at the low ppb level. This urine sample contained 2.0 ng clenbuterol per millilitre of urine.

Calibration and linearity

Under the conditions described in the experimental section, the linear range of the method and the recoveries of clenbuterol added to urine were determined. A calibration curve for clenbuterol, in the range of 1.0, 2.5, 5, 10 and 25.0 ng ml^{-1}, was obtained by adding standard solutions of clenbuterol to 4.0 ml of blank calf urine. In this linearity study, the correlation coefficient was 0.991 (n = 12). Recovery experiments were performed by adding 50 ng clenbuterol to urine samples. The within-day relative standard deviation for the determination of clenbuterol in urine was 10.2% (n = 9) with a recovery of 101.6%. The between-day relative standard deviations for the determination of clenbuterol in urine and water were 18.1 (n = 37) and 12.7% (n = 44) respectively with recoveries of 81.2 and 83.8%.

Excretion study

In Fig. 4, the concentrations of clenbuterol found in urine from the two calves treated with the therapeutic dose (Experiment 2), are plotted against the number of days after the first application. The highest concentration of clenbuterol found in this experiment was 75 ng ml^{-1} of urine. Eight days after the final application, concentrations of clenbuterol were lower than 0.5 ng ml^{-1}. From this excretion study, half-life values of 1.6 and 1.0 days, for clenbuterol added at the therapeutic dose to two male veal calves of 12 and 16 weeks old, were calculated. In Experiment 1 a half-life value of 1.6 days was obtained. During treatment and the withdrawal period large deviations in the excretion levels of clenbuterol were found. In both experiments (1 and 2), the concentration of clenbuterol was mostly two to three times higher in the urine collected during the night when compared to that collected during the day. Regarding its chemical structure, clenbuterol can be metabolized by both phase I and phase II reactions. Horiba *et al.* [16] studied the metabolism of the closely related substance mabuterol. They observed oxidation of the tertiary butyl group, oxidative deamination, oxidation of the secondary hydroxyl-group and conjugation with glycine to a hippuric acid metabolite. Also, theoretically, direct conjugation of clenbuterol to a glucuronide or sulphate metabolite is possible. In an experiment, with urine samples from Experiment 2, the possibility of clenbuterol being excreted as the free form or as the glucuronide or sulphate conjugate was investigated. A known amount of urine was hydrolysed using a mixture of β-glucuronidase and

Table 1
Excretion of clenbuterol in urine from a calf treated with clenbuterol for 5 days (see Experiment 1)

Time after first dose (8.00 am) (h)	Clenbuterol concentration (ng ml^{-1})
0	<0.5
12	33.9
24	39.2
36	35.4
48	20.2
60	32.2
72	41.5
84	23.0
96	61.3
108	20.2
120	72.9

In this experiment a 6-month old calf was treated with 4 μg clenbuterol.HCl per kilogram body weight, orally twice a day. Urine samples were collected from 8:00 am to 8:00 pm and 8:00 pm to 8:00 am.

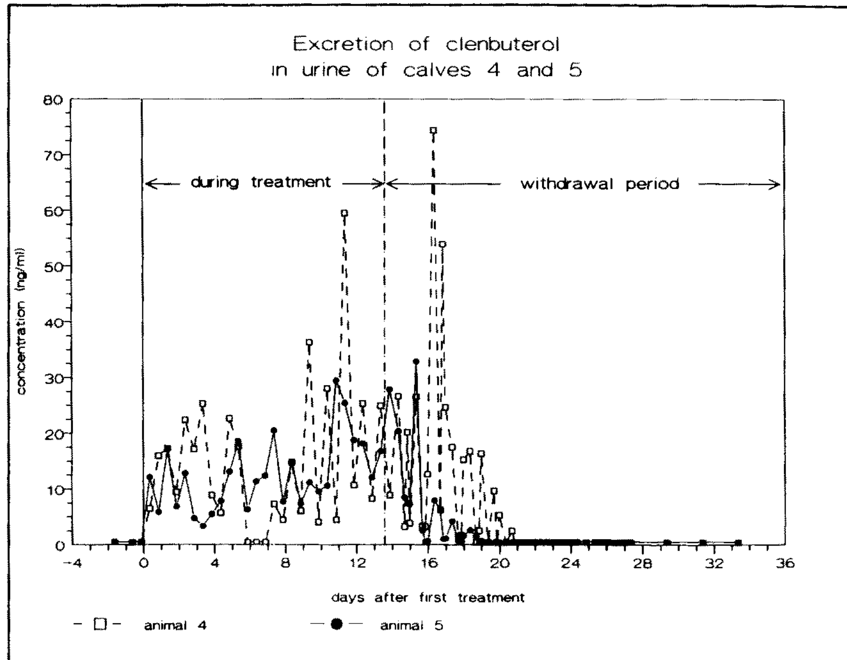

Figure 4
Concentrations of clenbuterol found in the urine samples of two calves treated with clenbuterol (Experiment 2) plotted against the number of days after the first application of clenbuterol.

aryl-sulphatase (*Helix pomatia* juice) under the conditions described previously [17]. The amount of clenbuterol found following hydrolysis was not significantly higher than that found previously.

The method has been applied to determine clenbuterol in more than 400 urine samples from calves destined for export. In three of the urine samples, clenbuterol could be detected (8–13 ng ml^{-1}) and the presence of clenbuterol was confirmed by GC–MS. With some of the urine samples from both animal experiments the presence of clenbuterol was also confirmed by GC–MS. After sample clean-up the samples were converted to their trimethylsilyl derivatives and analysed by GC–MS using both electron-impact and chemical ionization.

A GC–MS method for the determination of clenbuterol in urine, meat, liver, etc. in combination with immunoaffinity chromatography as selective clean-up, will be published elsewhere [18].

Conclusions

The sample pretreatment developed in combination with the reproducible HPLC-separation resulted in a valuable screening method for the determination of clenbuterol in urine samples from calves above a level of 0.5 $\mu g\ l^{-1}$. With this method, using an autosampler, one technician can handle about 30 samples per day. In most samples, clenbuterol can be analysed with UV-detection only. The ratio of electrochemical to UV-absorbance signals was used to increase specificity. However, the electrochemical detector can only be applied for a few consecutive analyses, because of a progressive decrease of the electrochemical signal with time. So, the latter detection technique was used only in case of suspected positive samples.

In general, positive results in regulatory control obtained with a HPLC-method should be confirmed by a more specific detection technique like mass spectrometry in combination with, for instance, gas chromatography. For this purpose, as was found in the authors' laboratories, almost the same sample pretreatment as that described in this paper can be used prior to derivatization and GC–MS analysis.

By analysing urine samples obtained from male calves treated with clenbuterol (therapeutic doses), a half-life value of about 1.5 days was calculated. More animal experiments,

such as, for instance, the treatment of female calves, are necessary to enable firm conclusions about misuse of clenbuterol.

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