Determination of Clenbuterol in Bovine Tissues and Urine by Enzyme Immunoassay

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We have developed an enzyme immunoassay (EIA) for clenbuterol using an antiserum raised in rabbits by immunization against a clenbuterol diazo derivative coupled to human serum albumin. Horseradish peroxidase coupled to the clenbuterol diazo derivative was selected as a tracer. The dose of clenbuterol that caused 50% binding inhibition was 18 pg per well, and the limits of detection of the EIA were 0.15 and 0.3 ppb in urine and liver, respectively (when the variability of blank values in samples from untreated animals was taken into account). This assay was used to test urine and tissue samples collected from veal calves and cows that had received in their feed a number of concentrations of clenbuterol over a range of time periods.

INTRODUCTION

β-Adrenergic agonist drugs have been used for a long time in human medicine for the treatment of asthma. Clenbuterol [4-amino-α-[(*tert*-butylamino)methyl]-3,5-dichlorobenzyl alcohol hydrochloride] is a β-adrenergic drug approved for use in veterinary medicine for the treatment of respiratory conditions (Ventipulmin, Boehringer Ingelheim Vetmedica GmbH) and for obstetric purposes (Planipart, Boehringer Ingelheim Vetmedica). In recent years, it has been established that a number of β-agonists, including clenbuterol, may have repartitioning effects in meat-producing animals. These effects on the lipid metabolism have been described when the β-agonists are administered at dosages in excess of 5–10-fold the recommended therapeutic dose (>1 μg/kg of body weight per day) (Hanrahan et al., 1986). The flow of nutrients is apparently shifted from adipose tissue toward muscle tissue. The result is an improved lean meat deposition and higher production efficiency (Hanrahan, 1987). An advantage of these molecules is that they are orally active and may be mixed with feed. The use of β-agonists as feed additives is by definition not permitted in the European Community, due to the fact that no compound from this class has at this time been listed in any of the Annexes to Directive 70/524/EEC. Nevertheless, there have been several reported incidences of its use by unprincipled veal calf or adult cattle producers in a number of European countries. The enforcement agencies in the various countries have pursued the illegal producers to protect the interests both of the farming community at large and of the consumer. The substance most often mentioned in connection with the illegal use of β-agonists as feed additives is clenbuterol. Accordingly, we have developed an enzyme immunoassay to detect this substance and to measure its concentration in urine, blood, feces, and edible tissues (muscle, liver, kidney, fat). The assay has been validated in urine and liver. This immunochemical analytical method is more simple, more rapid, and cheaper than the physicochemical methods described until now.

MATERIALS AND METHODS

Reagents and Equipment. Clenbuterol and cimaterol were generously provided by Boehringer Ingelheim Vetmedica. Terbutaline, salbutamol, isoproterenol, pirbuterol, adrenaline, noradrenaline, fenoterol, DL-3,4-dihydroxymandelic acid, and DL-4-hydroxy-3-methoxymandelic acid were obtained from Sigma (St. Louis, MO). Human serum albumin (HSA) was from Serva (Heidelberg, Germany), horseradish peroxidase (HRP) from Boehringer (Mannheim, Germany), and *o*-phenylenediamine from Sigma. Tween-20 and thimerosal were from Merck (Darmstadt, Germany). Freund's complete adjuvant was from Difco (Brussels, Belgium). All other chemicals were of analytical grade or better and were used as obtained. Deionized water was purified on a Milli Q system (Waters). Buffers were prepared as follows:

A. Carbonate/Bicarbonate Buffer Solution (Coating Buffer). 50 mM pH 9.6 Na_2CO_3 (1.59 g), $NaHCO_3$ (2.93 g), and distilled water (1 L).

B. Phosphate-Buffered Saline. Bovine serum albumin solution (0.01 M PBS/BSA, pH 7.4) (EIA buffer), NaCl (8.6 g), $Na_2HPO_4 \cdot 2H_2O$ (1.6 g), $NaH_2PO_4 \cdot H_2O$ (0.14 g), thimerosal (0.1 g), BSA (1 g), and distilled water (1 L).

C. Washing Solution. NaCl (8.76 g), Tween 20 (0.5 mL), distilled water (1 L).

D. Citrate/Phosphate Buffer (Substrate Buffer, pH 5.0). Citric acid (9.32 g), $Na_2HPO_4 \cdot 2H_2O$ (18.34 g), thimerosal (0.1 g), and distilled water (1 L).

E. Substrate Solution. *o*-Phenylenediamine (60 mg), perhydrol (10 μL), and substrate buffer (15 mL).

F. Stopping Solution. 6 M H_2SO_4.

All stock and standard solutions were stored at 4 °C. ELISA plates were obtained from Nunc (Roskilde, Denmark).

Absorbance of plate wells was measured with a Multiscan MCC/340 microplate reader from Flow Laboratories.

Immunogen Preparation. Clenbuterol was diazotized and coupled to human serum albumin (HSA) (to prepare the immunogen) or to horseradish peroxidase (preparation of the enzymatic tracer) following a procedure adapted from the method described by Yamamoto and Iwata (1982).

A. Preparation of the Diazo Derivative. Clenbuterol hydrochloride (3 mg) was dissolved in 400 μL of distilled water, and the pH was adjusted to 1.5 by addition of 100 μL of 1 N HCl. A $NaNO_2$ (3 mg) solution in distilled water (200 μL) was added dropwise to this mixture under stirring in the dark at 4 °C.

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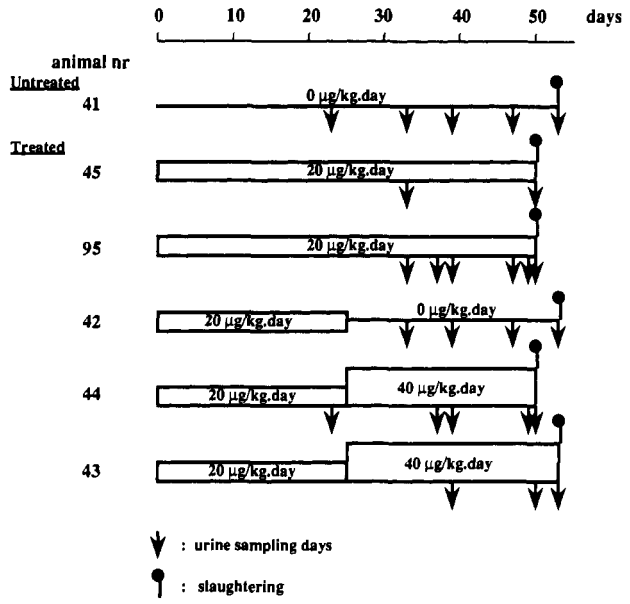

Figure 1. Treatment protocol applied to the five veal calves of experiment 1 and time schedule of collection of samples.

Table I. Percentage of Cross Reaction of the Antiserum Antidiazotized Clenbuterol Coupled to Human Serum Albumin

substance	% cross reaction[a]	substance	% cross reaction[a]
clenbuterol	100	adrenaline	<0.01
terbutaline	5.6	noradrenaline	<0.01
salbutamol	2.7	fenoterol	<0.01
cimaterol	1.4	DL-3,4-dihydroxy-mandelic acid	<0.01
isoproterenol	0.04	DL-4-hydroxy-3-methoxymandelic acid	<0.01
pirbuterol	0.02		

[a] % cross reaction = (pmol of clenbuterol that leads to 50% binding inhibition/pmol of substance that leads to 50% binding inhibition) × 100.

The formation of diazotized clenbuterol was detected by the appearance of a deep yellow color after reaction with *N,N*-dimethylaniline.

The formation of the diazo derivative was also monitored by thin-layer chromatography (TLC) on silica gel 60 Alu Rol sheets (Merck) after migration in the solvent mixture of ethyl acetate/methanol/25% ammonium hydroxide (85:10:5 v/v). After treatment of the sheet by spraying a solution of 2,7-dichlorofluoresceine (0.1% in ethanol) (Merck), it appeared that the clenbuterol R_f in this system was 0.39, while its diazo derivative did not migrate. The reaction was continued until the disappearance of the clenbuterol spot. The reaction was stopped by addition of an ammonium sulfamate (7 mg) solution in water (140 µL).

B. Immunogen Preparation. The solution of the diazo derivative was added by 50-µL portions to 1 mL of 0.1 M phosphate buffer, pH 7.5, containing human serum albumin (65 mg). During this operation, the pH was kept at a value of 7.5 by addition of 1 N NaOH. The reaction mixture was left for 18 h at 4 °C. (The solution was dialyzed against 3 L of 0.01 M phosphate buffer solution containing 0.15 M NaCl for 3 days with six changes of buffer.) The dialyzed solution of immunogen was frozen at −20 °C until use.

C. Antibody Preparation. The immunogen was used to prepare an antiserum. It was raised in rabbit subcutaneously injected with an emulsion of the immunogen preparation in Freund's complete adjuvant. This antiserum is now commercially available (Laboratoire d'Hormonologie Animale, Centre d'Economie Rurale, Marloie, Belgium).

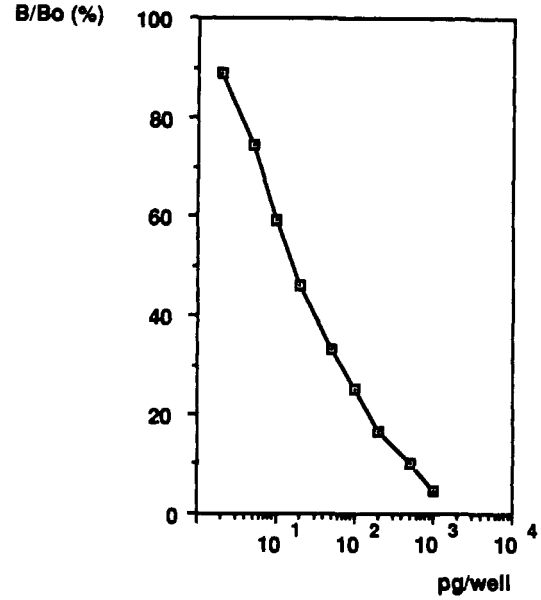

Figure 2. Calibration curve of the enzyme immunoassay of clenbuterol.

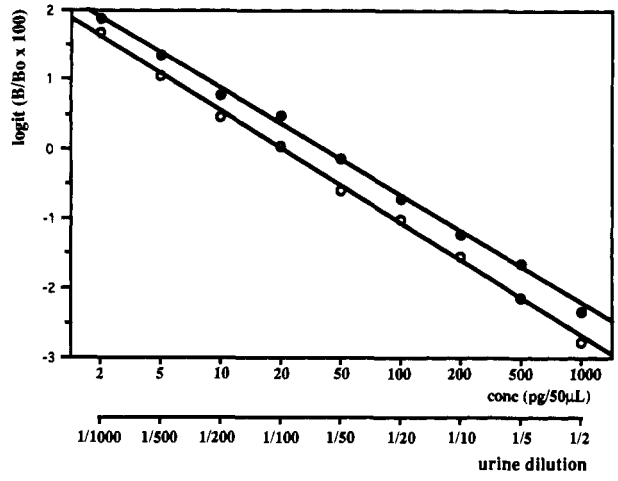

Figure 3. Calibration curve established using standard solutions of clenbuterol in the assay buffer (open circles) and binding inhibition curve obtained with serial dilution of a urine sample collected from a treated animal [veal calf 44 treated for 25 days with 20 µg kg^{-1} day^{-1} and veal calf 14 treated with 40 µg kg^{-1} day^{-1} (see Table IV)] with a blank urine sample from untreated animals (solid circles).

Enzyme Conjugate Preparation. The diazo derivative of clenbuterol was prepared as described above. The whole solution (740 µL) was added dropwise, at 4 °C, to a horseradish peroxidase solution (8 mg of HRP) in 0.01 M phosphate buffer, pH 7.5 (500 µL). The pH was continuously adjusted to 7.5 with 0.1 N NaOH. After standing for 2 h at 4 °C, the preparation was dialyzed against 0.01 M phosphate buffer, and unreacted material was separated from the conjugate by chromatography on Sephadex G-25.

Plate-Coating Procedure. The crude antiserum was aliquoted and stored at −20 °C after dilution in 0.1 M PBS/glycerol mixture (serum/PBS/glycerol 1:9:10) to give a final dilution of 1:20.

A working solution of the antiserum was prepared by dilution of 7 µL of the above stock solution (1:20) in 7 mL of coating buffer A.

Table II. Accuracy and Reproducibility of the Assay in Urine Samples

added, μg/L	Accuracy[a] measured, μg/L	recovery, %
1.0	0.9 ± 0.2	86
2.0	2.3 ± 0.3	115
5.0	5.2 ± 0.3	104
	Reproducibility in Samples Containing Known Concentrations of Clenbuterol coefficient of variation, %	
concn of clenbuterol, μg/L	intraassay (n = 8)	interassay (n = 8)
2.0	6.5	8.7
5.0	7.1	11.4

[a] Clenbuterol concentrations and recoveries were determined by enzyme immunoassay after addition of known amounts of clenbuterol to a pool of urine samples from untreated veal calves. Values are means of eight determinations (blank values subtracted) with standard deviations.

Table III. Accuracy and Reproducibility of the Assay in Liver Samples

added, μg/kg	Accuracy[a] measured, μg/kg	recovery, %
1.0	1.1 ± 0.2	113
2.0	1.9 ± 0.2	95
5.0	5.2 ± 0.4	105
	Reproducibility coefficient of variation, %	
concn of clenbuterol, μg/kg	intraassay (n = 8)	interassay (n = 8)
2.0	8.9	10.4
5.0	7.7	11.1

[a] Clenbuterol concentrations and recoveries were determined by enzyme immunoassay after addition of known amounts of clenbuterol to a pool of liver samples from untreated veal calves. Measured values are expressed as means of six determinations (blank values subtracted) with standard deviations.

This antibody solution (100 μL) was added to the inner 60 wells of the polystyrene microtiter plate (the values for well to well variation are greatly reduced by omitting results from the perimeter wells).

The plate was covered with a plastic film (flow plate sealer) and stored at 4 °C for 16 h or at 37 °C for 2 h.

The plate was washed four times with washing solution C to remove unbound antibodies.

Pretreatment of the Samples. *Urine.* Five hundred microliters was mixed with 1 N NaOH (50 μL), and *tert*-butyl methyl ether (1 mL) was added. This mixture was vortexed for 30 s and centrifuged at 2750*g* for 15 min at 4 °C. An aliquot (200 μL) of the organic phase was evaporated to dryness under nitrogen. The dry residue was dissolved in PBS/BSA buffer B (500 μL), and 50 μL of the solution (corresponding to 10 μL of the original urine sample) was used in the assay.

Liver. A portion of liver was homogenized in 3 volumes of distilled water using an Ultraturrax homogenizer at maximum speed (20 s). An aliquot (1 g) of homogenate was mixed with 1 N HCl. This suspension was vortexed for 30 s and centrifuged at 30000*g* for 30 min at 4 °C. The supernatant was collected in a tube containing 75 μL of 1 N NaOH. Clenbuterol residues were extracted as described for urine samples. The aliquot of the extract used in the assay corresponded to 5 mg of liver.

Enzyme Immunoassay. Fifty microliters of standard solutions of clenbuterol (range 0.04–10 μg/L), urine, plasma, or tissue extracts from controls (untreated) or

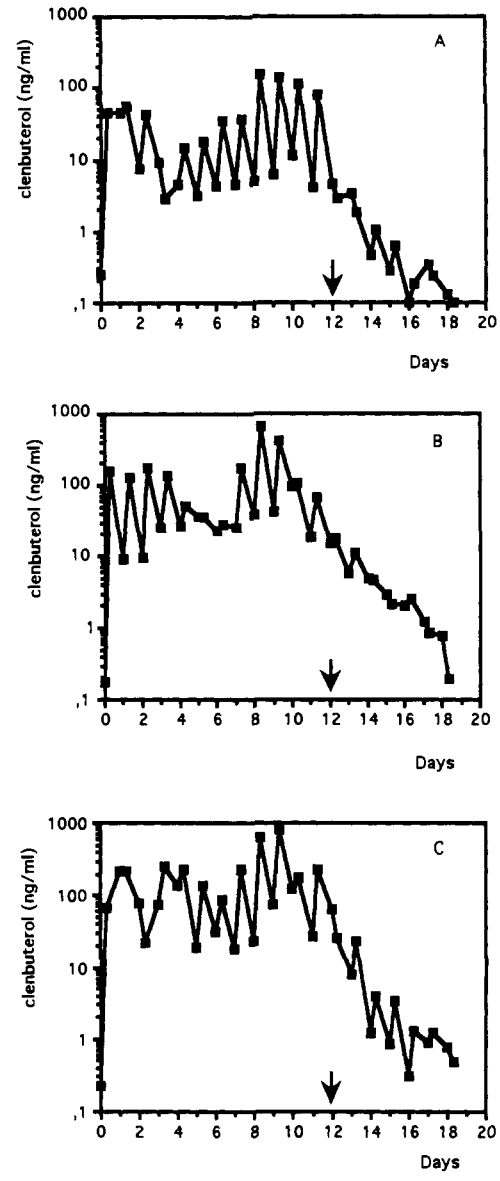

Figure 4. Elimination of clenbuterol (measured by enzyme immunoassay) in three cows orally treated from day 0 to day 12 with 2 (A), 5 (B), and 10 (C) μg/kg of body weight per day. The arrow indicates the end of the treatment (experiment 2).

treated animals were dispensed, in duplicate, into individual wells of the microtitration plate already coated with the specific antibody. Then, 100 μL of the clenbuterol–enzyme conjugate solution (8000-fold dilution in PBS/BSA buffer B) was added. The plate was covered with plate sealer and, after shaking, incubated overnight at 4 °C. The wells were washed four times as described above in the coating procedure. Buffered enzyme substrate solution E (150 μL) was then added to each well, and the plate was incubated at room temperature (18–20 °C) in the dark. After 30 min, the reaction was stopped by adding 50 μL of 6 M sulfuric acid to each well, in the same order and at the same rate as the substrate solution was added to keep the reaction time constant for all of the samples. The plate was gently shaken before absorbances were measured at 492 nm with the microtiter plate reader.

Calculation. Results were calculated according to the method of Rodbard (Rodbard and Frazier, 1973) by interpolation from a calibration curve where the bound enzyme activity, expressed as the logit of the ratio (in percent) between absorbance increase per 30 min, at each concentration of clenbuterol (B) divided by the bound

Table IV. Levels of Clenbuterol Determined in Urine Samples Collected from Clenbuterol-Treated and Untreated Veal Calves (See Figure 1 for Treatment Schedule)

	treatment with clenbuterol, days, at			
animal	20 μg kg^{-1} day^{-1}	40 μg kg^{-1} day^{-1}	0 μg kg^{-1} day^{-1}	urinary concn of clenbuterol, μg/L
untreated				
41			53	<0.2
treated				
45	33			25
	50			72
95	33			15
	37			13
	39			21
	47			14
	49			12
	50			7.5
44	23			6
	25	12		>100
	25	14		>100
	25	24		75
	25	25		42
42	25		8	75
	25		14	<0.2
	25		22	<0.2
	25		28	<0.2
43	25	14		76
	25	25		>100
	25	28		84

activity in the absence of unlabeled clenbuterol (B_0) was plotted vs log of clenbuterol concentration. The Rodbard procedure of calculation was adapted to enzyme immunoassay using EXCEL (Microsoft) on a Macintosh computer.

Animal Treatments. *Experiment 1.* Five veal calves (about 40 kg) (animals 45, 95, 44, 42, 43) were orally treated over a period of 25 days with a dosage of clenbuterol corresponding to 20 μg/kg of body weight per day. The drug was dissolved in their morning ration of reconstituted milk. Veal calf 41 received no clenbuterol. After this first period of 25 days, the treatment protocol was as described in Figure 1. After injection of a diuretic drug (Dimazon Hoechst 0.5 mL, 25 mg, Munich, Germany), urine samples were collected at regular intervals during the period of the experiment (see Figure 1). At slaughtering, the following samples were taken: muscles (biceps femoris, longissimus dorsi, psoas), liver, kidney, spleen, brain, adrenal gland.

Experiment 2. Three cows (about 500 kg) received per os three different doses of clenbuterol: 2, 5, and 10 μg/kg of body weight per day for 12 days. The clenbuterol was added to the morning ration. Urine samples were collected by vesical catheterizing at 8 a.m. and 4 p.m. during the treatment period and for 8 days after the treatment was stopped.

Experiment 3. As in the first experiment, three veal calves (about 40 kg) were orally treated but with a dosage of clenbuterol corresponding to 2 μg/kg of body weight per day that was closer to the dosage suspected to be used in illegal treatments of cattle for meat production. One animal was treated for 11 days and slaughtered 4 days after treatment was stopped. The second and third animals were treated for 15 days and slaughtered 3 and 7 days, respectively, after treatment was stopped. Liver, bile, kidney, spleen, and different types of muscle samples (biceps femoris, psoas, and longissimus dorsi) were collected at slaughtering.

RESULTS

This competitive enzyme immunoassay is similar to that previously described (Degand et al., 1989) for the analysis of artificial anabolics in urine.

The specificity of the antiserum was established by EIA and is shown in Table I.

The calibration curve of the EIA for clenbuterol is presented in Figure 2. The day-to-day variability of the standard curve was determined from 10 calibration curves. The mean slope ± standard deviation and coefficient of variation were −(0.67 ± 0.02) (3%). The midpoint of the curve (ED50) was 18.3 ± 1.4 (7.8%) (n = 10).

When used to analyze urine samples, the limit of detection of this assay was estimated to be 0.15 μg/L (ppb) (mean determined concentration of 20 blank urine samples collected from untreated veal calves + 3 times standard deviation), and the limit of determination (mean of 20 blank urine samples + 6 times standard deviation) was 0.19 μg/L (Commission Decision 89/610/EEC). To show that the response of the assay was linear with endogenous clenbuterol concentration, we compared (Figure 3) a calibration curve determined using standard solutions of clenbuterol in the assay buffer and a binding inhibition curve obtained with serial dilution of a urine sample collected from a treated animal (veal calf 44 treated for 25 days with 20 μg kg^{-1} day^{-1} and for 14 days with 40 μg kg^{-1} day^{-1}; see Table IV) with a blank urine sample (from untreated animals). The difference in the slopes (−0.69 in buffer vs −0.67 for urine samples) of these two curves was found to be compatible with the day-to-day variability of the standard curve.

In liver tissue, the limit of detection and limit of determination were estimated to be 0.3 and 0.45 ppb, respectively.

Evaluations of the accuracy and reproducibility of the assay in urine and liver samples are given in Tables II and III, respectively.

Results of the analysis of urine samples from veal calves in experiment 1 (see Figure 1 for treatment schedule) are given in Table IV. Clenbuterol levels in urine from treated animals differed largely even when the animal received the same treatment: for example, animals 45 and 95 received 20 μg kg^{-1} day^{-1} for 50 days and the clenbuterol concentration was found to be 10 times higher in urine of veal calf 45 than in urine of veal calf 95.

Large differences were also observed with time in the same animal: for example, animal 44 gave a urine sample on day 49 that contained a clenbuterol concentration (75 μg/L) almost twice that found on day 50 (42 μg/L). Eight days after treatment was stopped (20 μg kg^{-1} day^{-1} for 25 days), urine of veal calf 42 had a concentration of 75 μg/L, whereas 6 days later, this level had dropped below 0.2 μg/L.

A quantitative relationship did not appear between clenbuterol concentration in urine and the level of the administered dose: for example, urine from animal 45 showed a concentration of 72 μg/L, whereas animal 44 that received 40 μg kg^{-1} day^{-1} in place of 20 μg kg^{-1} day^{-1} during the second 25-day period presented a urinary level in clenbuterol of 42 μg/L.

These results must be interpreted with two points in mind: (1) the number of animals was limited and did not allow a statistical analysis of the results; (2) the minimal dose (20 μg kg^{-1} day^{-1}) administered in this experiment was high compared to the doses suspected to be used in illegal treatment of cattle for meat production (probably about 1–2 μg kg^{-1} day^{-1}).

Furthermore, it must be noted that urine samples were not whole urine collected during 24 h but were taken following the injection of a diuretic drug. The aim of this experiment was not the determination of an elimination profile of clenbuterol in urine, but it was to provide samples

Table V. Levels of Clenbuterol Determined in Tissues Collected from Veal Calves at Slaughtering (Experiment 1)

animal	treatment with clenbuterol, days, at 20 μg kg^{-1} day^{-1}	40 μg kg^{-1} day^{-1}	0 μg kg^{-1} day^{-1}	clenbuterol concn, μg/kg liver	psoas major	longissimus dorsi	biceps femoris	kidney	spleen	brain
untreated										
41			53	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
treated										
42	25		28	1.4	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
43	25	28		60	14	11	21	39	44	9
44	25	25		30	9	13	15	21	26	11
45	50			24	3	7	13	24	31	7
95	50			28	6	11	8	29	37	4

Table VI. Levels of Clenbuterol Determined in Tissues Collected from Veal Calves at Slaughtering (Experiment 3)

animal	treatment with clenbuterol	slaughtering	clenbuterol concn, μg/kg liver	bile	kidney	spleen	longissimus dorsi	biceps femoris
373	15 days, 2 μg kg^{-1} day^{-1}	3 days after treatment was stopped	0.8	<0.3	<0.3	<0.3	<0.3	<0.3
374	11 days, 2 μg kg^{-1} day^{-1}	4 days after treatment was stopped	all values <0.3					
376	15 days, 2 μg kg^{-1} day^{-1}	7 days after treatment was stopped	all values <0.3					

from clenbuterol-treated animals for the validation of our immunoassay and to estimate its applicability in control.

At the three dosages used in experiment 2 for the treatment of cows, the appearance of clenbuterol in urine (collected by vesical catheterizing) was rapid after its administration in feed (Figure 4). The urinary elimination showed large differences between concentrations measured in morning and afternoon samples. This was probably due to the fact that clenbuterol was mixed with the morning meal. This reflects the rapidity of clenbuterol excretion. During the treatment period, values ranged from 2 to 800 μg/L.

Table V shows the clenbuterol levels determined in tissues from veal calves treated or not treated as described in Figure 1. The clenbuterol concentrations in tissues, with the exception of liver, of animal 42 were too low to identify it as a treated animal 28 days after treatment was stopped. On the other hand, the veal calves treated with clenbuterol during the whole period (animals 43, 44, 45, 95) were easily identified owing to the presence of detectable clenbuterol in the various tissues examined, higher concentrations being observed in liver, kidney, and spleen.

These results contrast with that presented in Table VI. In this experiment (experiment 3), veal calves had been treated with a much lower dose of clenbuterol (2 μg kg^{-1} day^{-1}) that is probably closer to the dose suspected to be used in illegal treatment for meat production. In this case, the concentration of clenbuterol in tissues was lower than the detection limit of our assay when the withdrawal period was equal to or longer than 4 days. If the daily administration of 2 μg/kg of live weight of clenbuterol is stopped 3 days before slaughtering, levels of clenbuterol residues are close to the limit of detection of our immunoassay in liver, whereas they were undetectable in bile, kidney, spleen, and muscles.

DISCUSSION

The quantitative assay of β-agonists in biosamples collected in human patients for pharmacokinetic studies or in animals treated for therapeutic or zootechnical purposes is not easy due to the very low concentrations of these drugs in blood, urine, or tissue samples of treated human or animal patients. Two types of analytical methods have been applied to this problem: physicochemical methods (spectrometry, chromatography, GC/MS) and immunoassays.

Kopitar and Zimmer described in 1976 a radioimmunoassay of clenbuterol using a tritium labeled tracer to study the pharmacokinetics of the drug after administration to dogs and rats.

The first enzyme immunoassay of clenbuterol was described by Yamamoto and Iwata (1982). It was used to determine clenbuterol plasma levels in human patients treated by oral route with this drug. It was a highly sensitive double-antibody and heterologous immunoassay based on a competition for binding to a clenbuterol-specific antibody between a diazotized clenbuterol analogue (NA 1141) labeled with β-galactosidase and unlabeled standard or sample clenbuterol. The antibody-bound enzyme hapten was separated from free hapten by anti-rabbit IgG immobilized to a polystyrene ball. The enzymatic activity was determined by fluorometry. This estimation of the activity of the enzyme bound to the solid phase allowed the detection of 0.5 pg of clenbuterol per tube. Compared to the Yamamoto system, our homologous single antibody was easier to develop as the same diazo derivative of clenbuterol was used for coupling to human serum albumin to produce the immunogen and to horseradish peroxidase to prepare the tracer. Our simple system had a sensitivity that was found to be suitable to detect clenbuterol use in animals by analyzing urine or liver samples. The limit of detection of our assay is well below the maximum residue level proposed by the EEC (1 μg/kg in liver) (personal communication) for the control of meat-producing animals.

The enzyme immunoassay presented here is more sensitive than a radioimmunoassay recently described by Delahaut et al. (1991), the amount of clenbuterol inhibiting 50% of the tracer binding to antibodies being 18 pg/well and 101 pg/tube in our enzyme immunoassay and in the radioimmunoassay, respectively, although the same anti-clenbuterol antiserum was used in both assays. Meyer and Rinke (1990) also reported an enzyme immunoassay based on a competition for binding to an antibody raised against clenbuterol–diazo-BSA between salbutamol–carboxymethyl ether–horseradish peroxidase and several structurally related β-agonists: clenbuterol, salbutamol, or cimaterol. These authors applied this nonspecific assay as a screening method to identify animals illegally treated for meat production, the confirmation of positive results being done by a combination of HPLC and EIA.

Cross reactivity of antibodies raised against β-agonist conjugates to serum albumin was also independently

exploited by two groups of Dutch authors (Haasnoot et al., 1990; van Ginkel et al., 1989): they determined β-agonist drugs in urine, tissues, and feed samples with immunoaffinity chromatography on columns filled with polyclonal anti-clenbuterol antibodies immobilized on an activated solid matrix. This "multiple immunoaffinity chromatography" was used in combination with GC/MS or HPLC. Schilt et al. (1990) briefly described in their paper an enzyme immunoassay of clenbuterol with a limit of detection of 2 ng/mL in urine; this corresponds to a value 10 times higher than that reached in our assay (about 0.2 ng/mL).

In conclusion, the enzyme immunoassay of clenbuterol described here may be used in the routine analysis of clenbuterol in urine and animal tissues. Physicochemical methods are needed to confirm samples that were found to be positive by EIA. Among the most recently published methods, we can mention GC/MS (Fürst et al., 1989; Förster et al., 1988; Schmitz et al., 1989; Girault et al., 1990; Schilt et al., 1990; van Ginkel et al., 1989), LC/MS (Blanchflower and Kennedy, 1989), GC/MS–MS (Leyssens et al., 1991), HPLC (Degroodt et al., 1989; Courtheyn et al., 1991; Ali Qureshi and Ericksson, 1988), and GC-ECD (Brunn, 1988). The limits of detection reported for these methods ranged from 5 pg/mL to 5 ng/mL, the most sensitive physicochemical method being that described by Girault et al. (1990).

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