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Determination of clenbuterol in bovine plasma and tissues by gas chromatography–negative-ion chemical ionization mass spectrometry

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ABSTRACT

A highly sensitive and specific assay was developed for the determination of clenbuterol in bovine plasma and tissues. Clenbuterol and the internal standard [$^2H_9$]clenbuterol were measured by gas chromatography–negative-ion chemical ionization mass spectrometry with methane as the reagent gas. Bovine tissues including muscle, liver, heart, kidney, lung, suet, brain, spinal cord and thymus were ground in a buffer of pH 7 and then extracted using ethyl acetate. After two subsequent purification steps, the cleaned-up organic extract was derivatized with pentafluoropropionic anhydride. The mass spectrometer was set to monitor the abundant ions m/z 368 and 377 of the perfluoroacyl derivatives. This assay was performed with 1 ml of plasma or 0.2 g of tissue. The feasibility of this method was demonstrated by the determination of clenbuterol residues as the femtomole level in a variety of tissues.

INTRODUCTION

Many drugs (hormones, antibiotics, anabolic agents) including clenbuterol are widely administered to farm animals for health reasons or to improve meat production. Clenbuterol {4-amino-α-[(*tert.*-butylamino)methyl]-3,5-dichlorobenzyl alcohol hydrochloride} is a broncholytic agent normally used in humans for the treatment of asthma [1]. This potent sympathomimetic drug with selective activity on adrenergic β_2-receptors is also used in veterinary medecine as bronchorelaxant and tocolytic agent. More recently, clenbuterol has been suggested as a bovine growth promotant and is currently, but illegally, used in many countries. Because the active dose is very low and the apparent distribution volume relatively high, the resulting plasma and tissue concentrations of clenbuterol following oral administration of the drug are in the parts per trillion (ppt) range.

The determination of a substance present at the femtomole level in complex biological matrices is a tremendous challenge. In order to investigate correctly the pharmacokinetic behaviour of clenbuterol involving absorption, distribution and elimination of the drug, and to monitor the residue levels in tissues intended for human use, a reliable and robust method is required.

As far as law is concerned, the maximum residue level (MRL) allowed in animal products is zero. Obviously, the MRL is based on the detection limit of the most sensitive analytical method available. Hence the zero level is greatly dependent on the procedure used to quantify, with sufficient specificity and sensitivity, the residue levels of compounds which are most often present in the lower parts per billion (ppb) range in complex matrices.

Most sympathomimetic drugs have been identified or measured in biological fluids by techniques such as high-performance liquid chomatography (HPLC) [2–9], gas chromatography–mass spectrometry (GC–MS) [10–24] or radioimmunoassay (RIA) [25]. High-resolution capillary GC–MS remains the method of choice for the determination of clenbuterol. We have previously reported an isotope dilution method using negative-ion chemical ionization (NICI) mass spectrometry with methane as the reagent gas for the precise and accurate determination of small amounts of clenbuterol following oral administration of the drug to healthy volunteers [26–28]. This assay was performed with 1 ml of plasma or 0.5 ml of urine. The extracts were reacted with pentafluoropropionic anhydride at ambient temperature. The perfluoroacyl derivatives gave rise to characteristic ions of m/z 368 (clenbuterol) and 377 ([$^2H_9$]clenbuterol) which were recorded by selected ion monitoring.

This assay has been successfully adapted to the determination of clenbuterol in bovine plasma and tissues. A study protocol was designed in order to generate a range of levels that illustrates the distribution of clenbuterol in a variety of bovine samples. The feasibility of this highly sensitive and specific assay was demonstrated by preliminary results on the determination of clenbuterol in bovine plasma and tissues from 12 animals receiving oral treatment with clenbuterol.

EXPERIMENTAL

Chemicals

Chemical standards were kindly supplied by Chiesi Farmaceutici Laboratories (Parma, Italy). All solvents and reagents were of analytical-reagent grade from Merck (Darmstadt, F.R.G.) and were used without further purification. Pentafluoropropionic anhydride (PFPA) was obtained from Pierce (Rockford, IL, U.S.A.).

All glassware was cleaned with a mechanical scaling brush, then left overnight in CrO_3–H_2SO_4 and finally rinsed with doubly distilled water. The PTFE caps of the tubes were also carefully cleaned to avoid any subsequent sample contamination.

Calibration graphs

Stock solutions of clenbuterol and [$^2H_9$]clenbuterol (internal standard) were prepared by dissolving each of the pure reference compounds in methanol in order to achieve a primary concentration of 0.1 mg ml^{-1}. Secondary standard solutions, obtained by appropriate dilutions with distilled water, were protected from light by aluminium foil and stored at 4°C until used.

Aliquots of 1 ml of drug-free human plasma were fortified with 50 μl of a 10 ng ml^{-1} [$^2H_9$]clenbuterol solution in water and various amounts of clenbuterol ranging from 5 to 250 pg ml^{-1}. For tissue samples (0.2 g) the added amount of internal standard was the same as for the plasma specimens and a calibration graph was constructed from 10 to 500 pg g^{-1}. Blank samples were prepared in a similar way by spiking 1 ml of control plasma (or 0.2 g of tissue) only with the internal standard solution.

Extraction from plasma samples

Plasma samples (1 ml), spiked as stated above with [$^2H_9$]clenbuterol, were placed in 20-ml screw-capped tubes with 1 ml of 2 *M* sodium hydroxide solution buffered at pH 12 with sodium hydrogencarbonate. After brief mixing, 6 ml of ethyl acetate were added to each tube. The extraction procedure was conducted over a 10-min period using a reciprocating shaker and the tubes were then centrifuged at 1600 *g* for 5 min. The organic layer was transferred into a 10-ml screw-capped tube containing 1 ml of 0.2 *M* sulphuric acid. After extraction and centrifugation as described, the organic solvent was removed under vacuum. The remaining aqueous phase was purified with 4 ml of ethyl acetate–hexane (2:1, v/v). Following a brief extraction and centrifugation, the upper organic layer was discarded. The sulphuric acid phase was made alkaline with 1 ml of 2 *M* sodium hydroxide solution and then extracted with 5 ml of ethyl acetate. The organic solvent was decanted into a 10-ml Quickfit glass tube and evaporated to dryness under nitrogen at 40°C.

Extraction from tissue samples

Tissue samples (0.2 g) were accurately weighed and placed in a 20-ml glas tube with 3 ml of a Sorensen buffer (pH 7) and 500 pg of [$^2H_9$]clenbuterol. Each sample was homogenized for 1 min using a Polytron tissue grinder powered by an overhead stirrer. The tubes were centrifuged for 15 min at 1800 *g* and the supernatant was decanted in another 20-ml screw-capped tube. The aqueous phase was made alkaline with 1 ml of 2 *M* sodium hydroxide solution and then extracted for 10 min with 6 ml of ethyl acetate. The samples were then purified as described above for plasma.

Derivatization procedure

In order to eliminate moisture, the inner sides of the vials were rinsed with 100 μl of acetone, previously dried over anhydrous sodium sulphate. The acetone was evaporated and 40 μl of PFPA were added to the residue in the vials, which were then tightly capped and allowed to stand at room temperature until analysis. A 1-μl aliquot of the derivatization mixture was injected into the gas chromatograph–mass spectrometer.

Precision, accuracy and limit of detection

To assess the precision and accuracy of the method for plasma samples, repeatability assays were carried out at four concentrations (5, 20, 100 and 200 pg ml^{-1}). The spiked plasma samples were analysed on the same day by the same person and for each concentration level the relative standard deviation and mean percentage error were calculated.

The limit of detection (LOD) was defined as the lowest detectable concentration yielding a signal significantly higher than that of blank control specimens [27–30]. First, to calculate the LOD of the method a repeatability assay was performed with ten blank plasma samples. The mean signal ($\bar{Y}_{bl}$) eventually observed at the retention time of clenbuterol and the associated standard deviation (S_{bl}) were used to calculate a theoretical value of the detection limit. Then, a repeatability assay was carried out with ten replicate plasma samples spiked at a concentration corresponding to this theoretical detection limit. The mean signal ($\bar{Y}_{LOD}$) was statistically compared with the mean signal ($\bar{Y}_{bl}$) obtained with the blank samples. After testing the variance homogeneity ($p < 0.001$), a t-test or a Welch test was applied in order to demonstrate that $\bar{Y}_{\mathrm{LOD}}$ was significantly higher than $\bar{Y}_{\mathrm{bl}}$ ($p < 0.05$).

The precision and accuracy of the method for tissue samples were also investigated at two concentrations, 20 and 200 pg g^{-1}, of clenbuterol. The tissue samples were analysed as described above.

GC–MS analysis

The method was developed on an HP 5985 B gas chromatograph–mass spectrometer operated under the SIDS operating system. GC was carried out on a fused-silica capillary column (25 m × 0.35 mm O.D.) wall-coated with OV-1701. The film thickness and I.D. of the column were 0.11 μm and 0.22 mm, respectively.

Samples were injected with a falling needle injector with helium as the carrier gas (inlet pressure 14 p.s.i.). The injection port was maintained at 290°C. The oven temperature was raised from 180 to 230°C at 8°C min^{-1} and from 230 to 290°C at 20°C min^{-1}.

One end of the capillary column was connected to the glass solid injector 1 mm from the bottom of the moving needle. The other end was introduced directly near the ion source of the mass spectrometer via a 1/16-in. stainless-steel transfer line, the temperature of which was held at 280°C.

The GC–MS system was operated in the NICI mode with an electron energy of 100 eV, an emission current of 300 μA and an ion source temperature of 180°C. Prior to the analysis, the instrument was tuned in the NICI mode using the fragments of *m/z* 414, 595 and 633 from the perfluorotributylamine calibrant gas. The reagent gas methane was admitted via a gas-flow controller to an indicated ion source pressure of about 0.8 Torr.

The NICI mass spectra of clenbuterol and [$^2H_9$]clenbuterol perfluoroacyl derivatives were recorded during a GC run by scanning the quadrupole mass filter repetitively every 1.1 s from *m/z* 250 to 450. Determination of clenbuterol was performed by focusing the instrument in the single-ion monitoring mode in order to measure the fragments of *m/z* 368 (clenbuterol) and 377 (internal standard). The dwell time was 100 ms for each mass range.

Quality controls

Throughout the study quality controls were supplied blind to the analyst by spiking blank plasma and tissue samples with known amounts of clenbuterol. The experimenter in charge of the assay ignored the theoretical concentration of these samples, prepared independently by a technician who did not participate in the study. The standard solutions used to spike the control specimens were different from those used to obtain the calibration graphs. The quality control samples were stored at −20°C until analysis. Two samples were assayed each day together with the real life samples collected during the pharmacokinetic study. After calculating the results, the absence of any systematic bias in the assay of clenbuterol was controlled. For that reason, a regression analysis was carried out by plotting the found *versus* the theoretical values. The slope and intercept were then statistically compared with the theoretical values of 1 and 0, respectively, using a Student's t-test.

Drug administration

Twelve Holstein calves received an oral treatment with clenbuterol and during the administration period plasma and urine samples were collected for pharmacokinetic analysis. The calves were subsequently slaughtered and different tissues were collected. All plasma and tissue samples were stored frozen (−20°C) until analysis. Twelve other calves that did not receive clenbuterol were used as control animals.

RESULTS AND DISCUSSION

Sample pretreatment and analysis

The samples must be carefully purified to remove endogenous substances that could interfere during the selected ion monitoring determination of clenbuterol. The multiple-step extraction process leads to a "clean" residue devoid of most impurities. Owing to the use of a glass solid injector and despite this long sample pretreatment, the laboratory throughput can be up to 40 samples per day. The moving needle injection technique, which is very protective for the capillary column, allows an initial oven temperature that is much higher than that required for other sample inlets such as splitless or on-column injectors.

Under the analytical conditions adopted, the retention times of clenbuterol and [$^2H_9$]clenbuterol were 5.22 and 5.16 min, respectively. The two chromatographic peaks followed a Gaussian distribution and each derivative was totally eluted from the column in less than 4 s. The peak width at half-height was *ca.* 1.3 s. A dwell time of 0.1 s per mass unit led to a minimum of 18 measurements for each analyte.

Derivatization procedure

The determination of underivatized clenbuterol is unsuitable owing to the presence of hydroxy and amino groups, which are mainly responsible for tailing peaks in GC. We have already proposed to derivatize clenbuterol and the internal standard with hexamethyldisilazane (HMDS) [31,32]. This silylating reagent gave a low chemical background with highly volatile by-products. The extracts were heated with 30 μl of HMDS for 1 h at 80°C. A faster derivatization step yielded low sensitivity and poor reproducibility, with the formation of small amounts of reaction products. Moreover, the O-trimethylsilyl ether derivative of clenbuterol was prone to hydrolysis: glassware and solvents were carefully protected from moisture in order to hinder this interfering reaction process.

We have now developed another derivatization procedure that offers a more favourable starting point for a highly sensitive and specific assay of clenbuterol. Under the mild conditions applied, clenbuterol and [$^2H_9$]clenbuterol reacted completely with PFPA. A subsequent study of this derivatization process has shown that, at ambient temperature, the hydroxy and amino groups of the side-chain were acylated with PFPA to give the dipentafluoropropionyl derivative. Because of the immediate loss of one PFP substituent in the reaction medium followed by internal dehydration, the final and highly stable reaction product was the monosubstituted derivative.

When the reaction temperature was raised, the 4-amino function also reacted with PFPA, and another chromatographic peak corresponding to the disubstituted derivative was observed. From a quantitative point of view, this reaction at higher temperature was not reproducible and the relative proportions between the two products differed day by day.

Mass spectra

The NICI mass spectra of clenbuterol and [$^2H_9$]clenbuterol mono-PFP obtained with methane as the reagent gas exhibit characteristic ions in the high-mass region, as shown in Fig. 1.

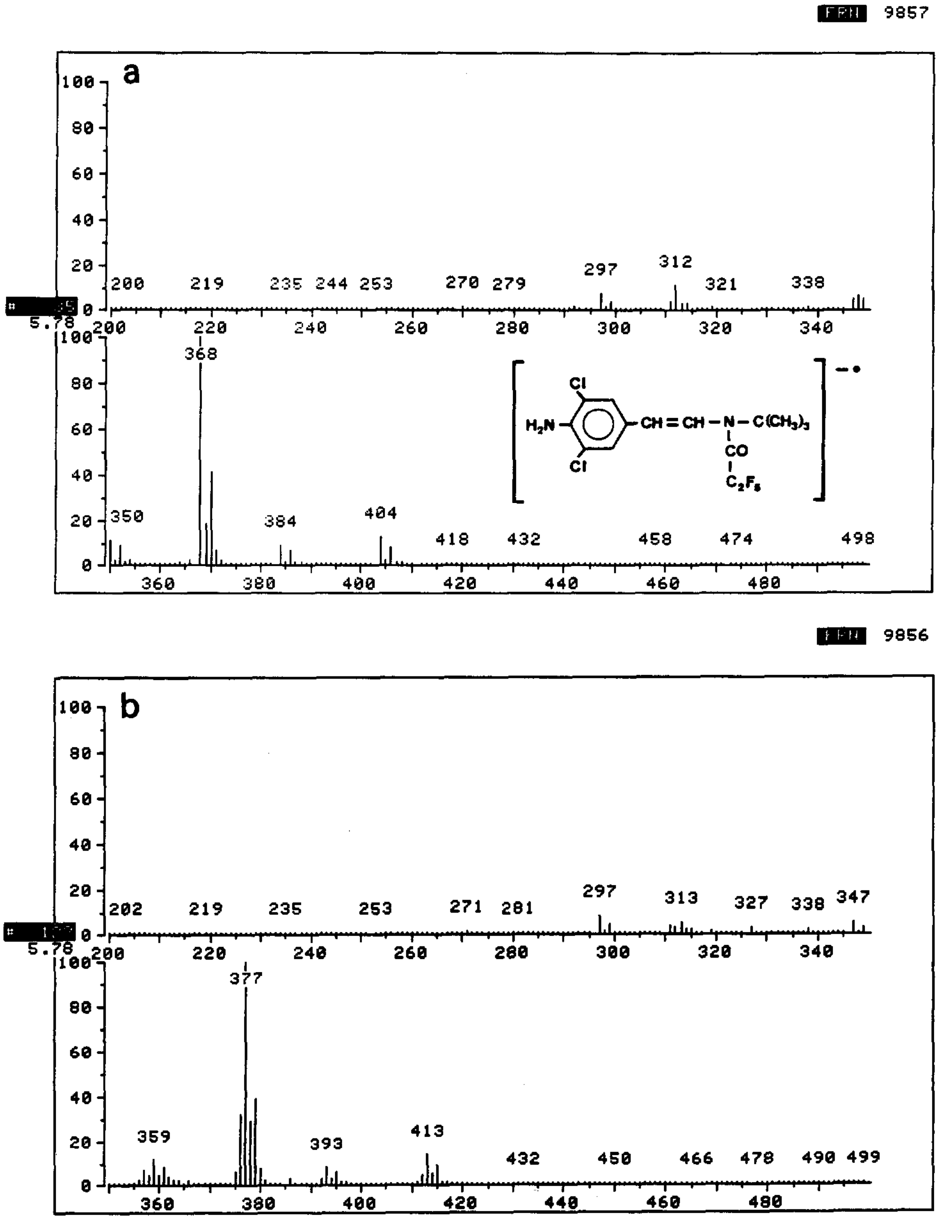

Fig. 1. Methane NICI mass spectra of the mono-PFP acyl derivatives of (a) pure clenbuterol and (b) [$^2H_9$]clenbuterol.

The molecular ions of m/z 404 and 413 are present with a relative intensity of about 15%. The two spectra are similar except for a mass shift of 9 u for most of the ions. The loss of one 36 u fragment ($[M-HCl]^-$) from the molecular ions could proceed from internal cyclization and gave rise to the base peaks observed at m/z 368 and 377. These two ions successively undergo the elimination of *tert.*-butyl and amino groups, leading to the formation of the ions of m/z 312, 313 and finally 297.

Calibration graphs

The seven-point plasma calibration graphs obtained each day of the assay by plotting the peak-area ratios of m/z 368 and 377 *versus* known plasma concentrations of clenbuterol were straight lines ($r = 0.9998$) over the concentration range 5–250 pg ml^{-1}. The slopes of the plasma calibration curves were reproducible from day to day, with a relative standard deviation of less than 4%. The least-squares regression analysis passed near the origin, with a mean intercept value close to zero.

The corresponding tissue calibration graphs were linear over the concentration range 10–500 pg g^{-1} for each tissue tested.

Precision and accuracy

The yield of the extraction was *ca.* 80% and all sources of variability were considerably reduced owing to the use of a stable isotopically labelled analogue as the internal standard. The relative standard deviations were less than 4% and the mean percentage error ranged from -1.9 to $+0.2$% for clenbuterol plasma concentrations between 20 and 200 pg ml^{-1} (Table I).

The precision and accuracy of the method were also tested with bovine tissue samples at two different concentrations. The relative standard deviations were 9.0 and 7.3%. The mean percentage error ranged from -5.5% to $+6.4$% for tissue spiked with 20 and 200 pg of clenbuterol (Table II).

Limit of detection

A signal-to-noise ratio $\geqslant 2.5$ is commonly used as a criterion for a significant response. Unfortunately, even if the low electronic noise is relatively constant day by day, the same does not apply to the chemical background, which is sometimes very different from one determination to another. This is principally due to the sample itself, the solvents, the glassware and/or the chromatographic system. In order to take

TABLE I

PRECISION AND ACCURACY OF THE CLENBUTEROL ASSAY PROCEDURE FOR PLASMA SAMPLES

Theoretical concentration (pg ml^{-1})	n	Mean observed concentration (pg ml^{-1})	S.D.	R.S.D. (%)	Error (%)
200	10	196.27	5.89	3.0	−1.9
100	5	99.30	3.41	3.4	−0.7
20	5	20.03	0.74	3.7	+0.2
5	10	4.78	0.61	12.8	−4.3

TABLE II

PRECISION AND ACCURACY OF THE CLENBUTEROL ASSAY PROCEDURE FOR TISSUE SAMPLES

Theoretical concentration (pg g^{-1})	*n*	Mean observed concentration (pg g^{-1})	S.D.	R.S.D. (%)	Error (%)
200	10	212.7	15.6	7.3	+6.4
20	10	18.9	1.7	9.0	−5.5

into account all of these soures of variation, we have developed a statistical determination of the detection limit based on the results obtained after GC–MS analysis of ten different blank plasma extracts. The mean signal ± S.D. (1.3482 ± 0.1114) calculated from the repeatability assay performed at 5 pg ml^{-1} was significantly different ($p<0.001$) from that observed with the ten blank specimens (0.4320 ± 0.0602). Owing to the relative standard deviation (12.8%) and mean percentage error (−4.3%), a 5 pg ml^{-1} concentration level was validated as the detection limit of the method for plasma samples.

For tissue samples the limit of detection was 10 pg g^{-1}. A representative selected ion chromatogram obtained after GC–MS analysis of a blank tissue specimen (kidney) is shown in Fig. 2. As examplified by this chromatogram, no interference from endogenous compounds was observed in the single-ion monitoring of the fragment of *m*/*z* 368.

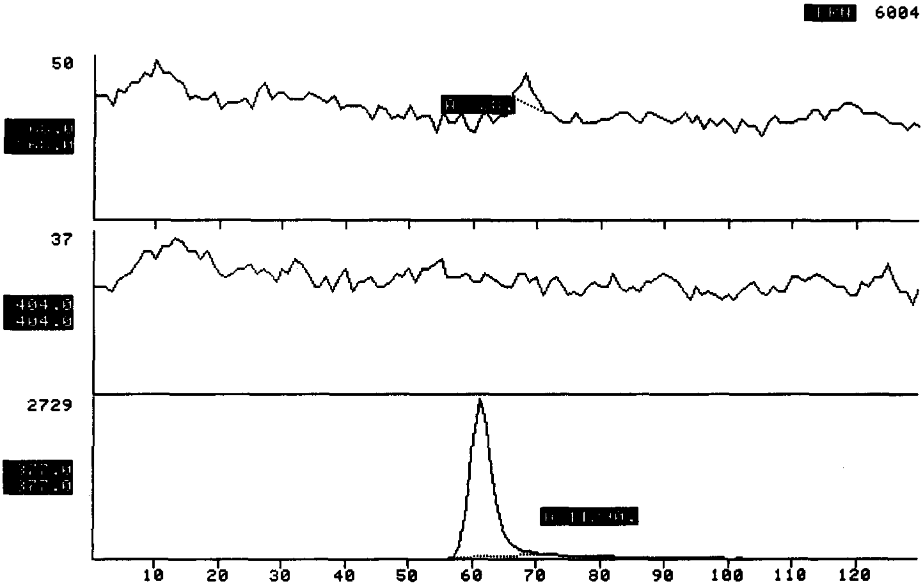

Fig. 2. Representative mass chromatogram obtained from a control kidney specimen (0.2 g) spiked with 500 pg of [$^{2}H_{9}$]clenbuterol.

Results for control tissue samples (back muscle and spinal cord), spiked with 10 and 250 pg of clenbuterol, respectively, are presented in Fig. 3.

Quality controls

Quality control analysis ($n=36$), showed that the found concentrations were

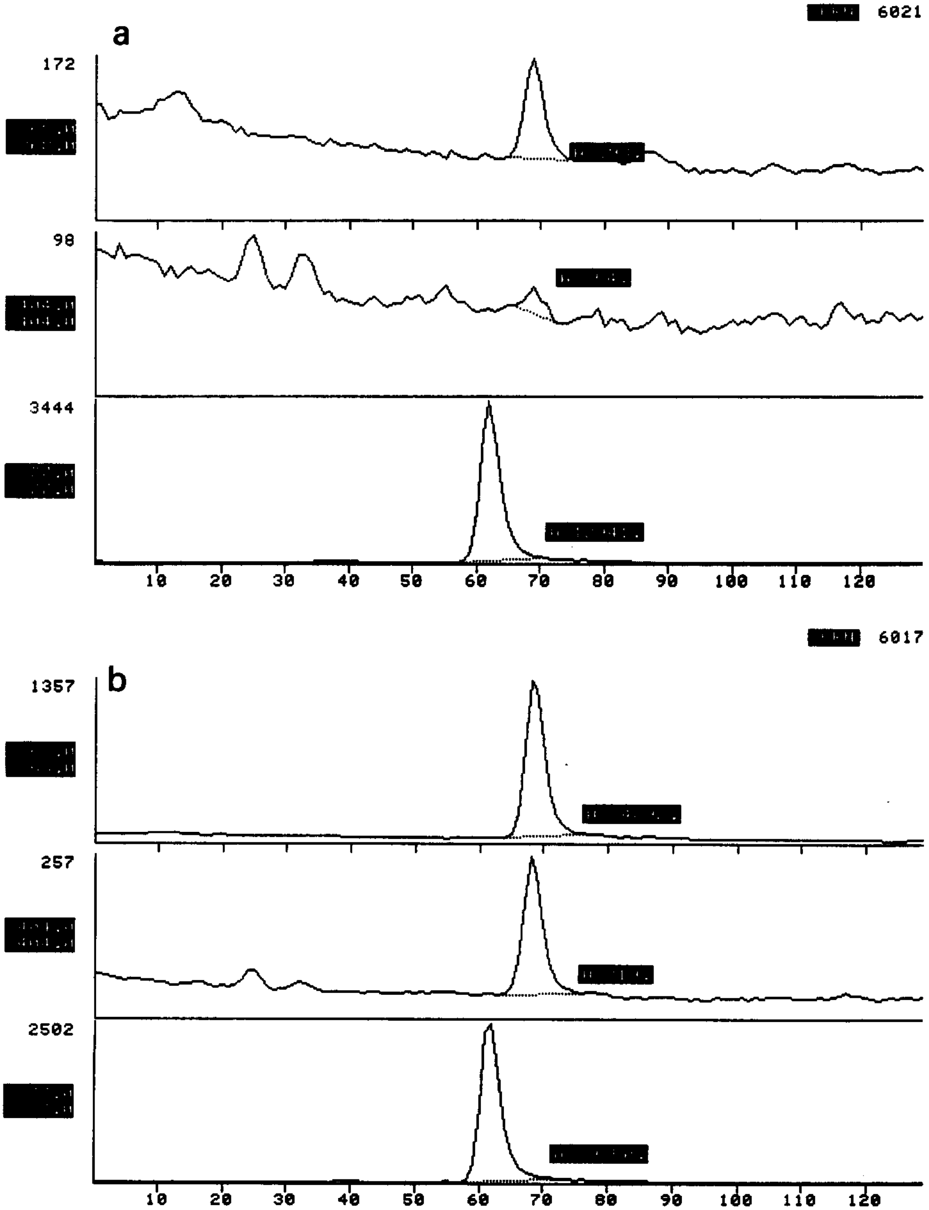

Fig. 3. Selected ion monitoring traces obtained from (a) a back muscle fortified with 10 pg of clenbuterol and (b) a spinal cord specimen spiked with 250 pg of clenbuterol and 500 pg of [$^2H_9$]clenbuterol.

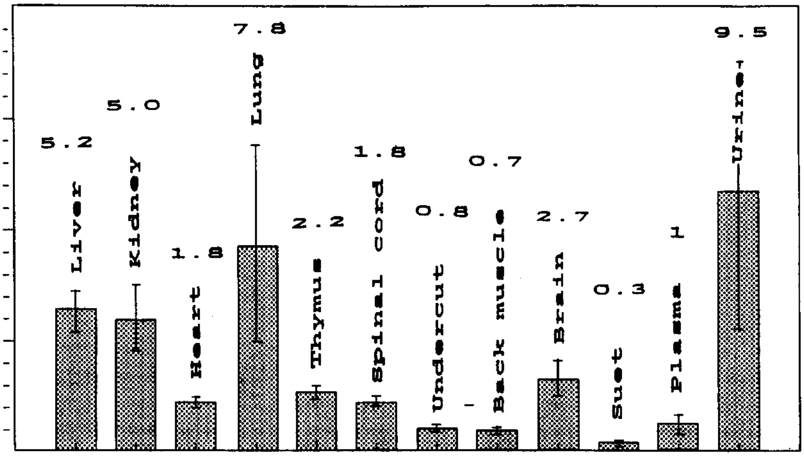

Fig. 4. Distribution of clenbuterol in different target tissues measured after oral administration of the drug.

correlated with the theoretical values ($r=0.999$). A Student's t-test was carried out in order to demonstrate that the slope and intercept of the regression analysis were not significantly different from the theoretical values of 1 and 0, respectively, at a probability level of 95% for $n-2$ degrees of freedom.

The values of $t_{\text{slope}} = 1.312$ and $t_{\text{intercept}} = -0.289$, were lower than the limit (1.960) of the t-table. Consequently, this assay was applied to the quantitative measurement of clenbuterol without any constant of proportional systematic error. The mean difference between the theoretical and actual values was 5.2% for clenbuterol plasma concentrations ranging from 0 to 250 pg ml^{-1}.

Distribution of clenbuterol in target tissues

The levels observed in different tissues are summarized in Fig. 4. The factor 1 was arbitrarily assigned to plasma and the other tissues were calculated on this basis.

Clenbuterol is a β_2-agonist drug initially used for the treatment of asthma in human, which is why the lungs normally contained the highest levels of clenbuterol. High concentrations of clenbuterol were also measured in liver, kidney and thymus, where the affinity of the drug for the β_1-adrenergic receptors of the heart was of minor importance. The muscles and the suet contained the lowest levels of clenbuterol. Clenbuterol is a lipophilic compound crossing the blood–brain barrier; relatively high levels of this drug were observed in the brain and spinal cord.

These results were in good agreement with those obtained previously when we compared the distribution of clenbuterol and salbutamol after intravenous administrations of the two drugs to six dogs [31,32].

CONCLUSION

The correct pharmacokinetic behaviour and the distribution of clenbuterol in target tissues can only be achieved if accurate and precise analytical data are obtained.

The method used to determine clenbuterol in biological matrices should be carefully optimized, so that the reliability of the analytical procedure depends greatly on the criteria used to validate the assay. The isotope dilution mass spectrometric assay described here afforded a sensitive and specific technique for measuring clenbuterol at the femtomole level in plasma and tissue. The monopentafluoropropionyl derivative of clenbuterol gave an intense signal and when the fragment of m/z 368 was monitored under NICI conditions the detection limit of this assay was 5 pg ml^{-1}. The relative standard deviations and mean percentage errors calculated during the different repeatability assays demonstrated that, even around this limit of detection, the precision and accuracy of the method were suitable for routine analysis for clenbuterol.

This method was successfully applied to the analysis of bovine tissues including muscle, liver, heart, kidney, lung, suet, brain, spinal cord and thymus after the administration of an oral dose of clenbuterol to twelve calves. This assay has been routinely applied for 4 years in many pharmacokinetic and bioavailability studies following oral or intravenous administration of clenbuterol to humans or animals.

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