Metabolic Fate of Clenbuterol in Calves

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The metabolic fate of [^{14}C]clenbuterol was studied in two calves, following single or repeated oral administration of the drug at an anabolic dosage (5 μg/kg). Analytical methods were developed to evidence the nature of clenbuterol's metabolites in excreta and tissues. The major biotransformation pathways of clenbuterol were 4-*N*-oxidation, 4-*N*-sulfation, and oxidative *N*-dealkylation of the parent compound. Unchanged clenbuterol accounted for about 20 and 50% of the radioactivity detected in urine and feces, respectively, and for a very large part of the radioactivity detected in organs (≥90%). Retina extracts contained only clenbuterol. Two different extraction techniques and the use of ^{3}H-labeled clenbuterol hydroxylamine demonstrated that this labile compound was not present in liver extracts. Therefore, the toxicity of clenbuterol residues remaining in tissues of cattle treated with this β-agonist toward the consumer is expected to be solely related to unchanged clenbuterol. The implications of the present results on the monitoring of illegal clenbuterol use in cattle, following detection in matrixes such as urine, liver, or retina are discussed.

Keywords: *Clenbuterol; β-agonist; bovine; metabolism; biotranformation; in vivo*

INTRODUCTION

The β-agonist clenbuterol (CL) has been illegally used in cattle to improve carcass characteristics and productivity rates (Williams, 1987). In several European countries, this rather β_2-selective drug (Main, 1990) is approved in animals as a bronchospasmolytic (horses, calves) and tocolytic (cows) agent. When administered at high dosage (~5−10-fold the 0.8 μg/kg therapeutic dosage) over a few weeks, it was demonstrated to decrease fat deposition and enhance protein accretion (Williams, 1987; Miller et al., 1988). As a result of illegal CL use in cattle, several cases of human intoxication have been reported (Martinez-Navarro, 1990; Pulce et al., 1991). Indeed, a low dosage of CL is sufficient to produce pharmacological effects on the heart (positive inotropic and chronotropic response), the lung (bronchodilation), and the muscular system (muscle tremors) (Wiegand et al., 1991). Corresponding symptoms, together with headaches, were observed in humans that had been intoxicated by consumption of liver from bovine treated with CL (Pulce et al., 1991).

Many laboratories have successfully developed powerful analytical tools to detect CL in various biological matrixes, with the aim to enable a sensitive detection of its illegal use in livestock. Fewer studies have been carried out to determine the amount of residues remaining in tissues of cattle treated with CL (Meyer and Rinke, 1991; Elliott et al., 1993a; Sauer et al., 1995). These studies were conducted using various CL dosages (ranging from 1.6 to 10 μg/kg) and withdrawal times before slaughter. Therefore, these studies have provided valuable data for scientists dealing with CL detection. However, the nature of CL residues and the metabolic fate of CL in cattle were never investigated. Neither the occurrence of metabolites possessing a β-agonist activity nor the possibility that a metabolite could serve as a marker of CL use has been investigated.

We have previously reported CL metabolic studies in rats, using ^{14}C-labeled CL (Zalko et al., 1997, 1998a). Analytical techniques, sufficient to achieve HPLC separation and further characterization of CL metabolites in urine, feces, and tissues, were developed in these studies. Several ^{3}H- and ^{14}C-labeled metabolites were isolated and purified from rat urine (Zalko et al., 1998a). We now report results obtained from calves fed [^{14}C]-CL. This pilot study was designed to examine the metabolic fate of CL in cattle treated orally at a commonly admitted anabolic dosage (Miller et al., 1988; Meyer and Rinke, 1991).

MATERIALS AND METHODS

Chemicals. Clenbuterol hydrochloride was purchased from Sigma Chemical Co. (St. Quentin Fallavier, France). Clenbuterol hydrochloride purity was >95%, and its identity was confirmed by electrospray MS as previously described (Zalko et al., 1997). [^{14}C]CL (Figure 1) [4-amino-3,5-dichloro-α-(*tert*-butylaminomethyl)benzyl alcohol, labeled on the benzylic carbon], was synthesized by Isotopchim (Ganagobie-Peyruis, France) with a specific activity of 1997 MBq/mmol. Its radiopurity, checked by HPLC and TLC analyses, was >97%. [^{14}C]Clenbuterol-labeled metabolites, namely CL 4-aminosulfonic acid (SCL), CL-hydroxylamine (*N*-OH-CL), 4-nitroso-CL (NO-CL), 4-nitro-CL (NO_2-CL), 4-amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylaminomethylbenzyl alcohol (OH-CL), 4-amino-3,5-dichlorohippuric acid (ADHA), 4-amino-3,5-dichloromandelic acid (ADMA), and 2-(4-amino-3,5-dichlorophenyl)-2-oxoacetic acid (ADOA), were isolated from rat urine and directly identified by mass spectrometry as described elsewhere (Zalko et al., 1997, 1998a). Labeled 4-amino-3,5-dichloro benzoic acid ([^{14}C]ADBA) was purified from the supernatants of CL incubations with bovine liver microsomal fractions, as described by Zalko et al. (1998b). [^{3}H-*N*-OH-CL was purified, following methods described elsewhere (Zalko et al., 1997), from the urine of a rat dosed orally with [^{3}H]CL, which was purchased from Rotem Industries (Beer-Sheva, Israel).

Other chemicals were obtained from the following sources: acetic acid, Merck (Darmstadt, Germany); formic acid and analytical grade solvents, Prolabo (Paris, France); *Helix pomatia* juice (Helicase), IBF (Villeneuve-La-Garenne, France).

Animals. Two 6-month-old male Charolais calves weighing 200 kg (calf I) and 190 kg (calf II) were housed in separate

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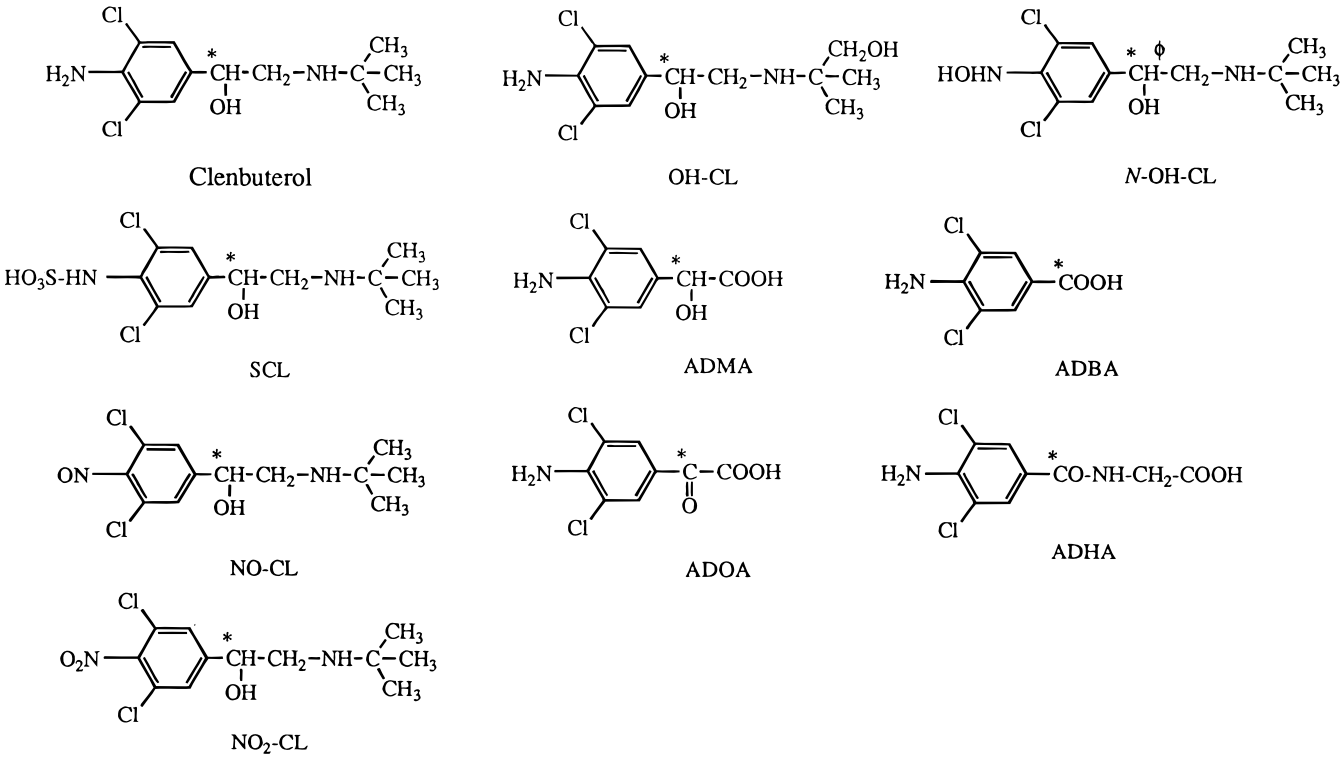

Figure 1. [^{14}C]Clenbuterol and clenbuterol metabolites. Asterisk indicates ^{14}C position; (ϕ) indicates ^{3}H position for [^{3}H]-*N*-OH-CL, used only in specific extraction assays.

stalls, with free access to water. They were allowed ad libitum access to hay and were fed an adapted commercial cattle concentrate (2 kg per day per animal) distributed twice daily in a plastic container. After a 10-day acclimatization period, calf I was fed a single oral dosage of 5 μg/kg of it body weight [^{14}C]CL (7.21 MBq) and was slaughtered 12 h later by stunning with a captive bolt, followed by exsanguination. Calf II was fed [^{14}C]CL (5 μg/kg of body weight, 6.85 MBq) twice a day (8 a.m. and 8 p.m.) for 14 days and was slaughtered 12 h after the last CL administration, as described for calf I. Labeled CL (in ethanol solution) was administered after being mixed with the commercial cattle feed. Labeled CL was completely consumed within a maximum delay of 15 min. Residual radioactivity remaining in containers was quantified after dosing and never exceeded 0.1% of the radioactivity.

Apparatus. Samples were analyzed by HPLC on a Philips 4100 apparatus (Pye Unicam, Cambridge, U.K.) equipped with a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA) and connected for radioactivity detection to a Radiomatic flo-one/β A500 instrument (Packard Instrument Co., Downers Grove, IL) using Flow-scint II (Packard) as the scintillation cocktail to establish metabolic profiles or to a Gilson model 202 fraction collector (Gilson France, Villiers-Le-Bel, France), for metabolite purification. When appropriate, radio-HPLC analyses were conducted with simultaneous detection of ^{3}H and ^{14}C. Radioactivity in liquid samples was determined by direct counting on a Packard Tricarb 2200CA scintillation analyzer (Meriden, CT) using Packard Ultima Gold as the scintillation cocktail (each sample was counted for 10 min). Radioactivity in feces, tissues, and pellets was determined by complete combustion in an oxidizer (Packard 306) followed by quantitation of ^{14}-CO_2 by liquid scintillation analysis (scintillation cocktail: Packard Permafluor E+ and Packard Carbosorb, 2:1 v/v).

Analytical Procedure. Two gradient elution systems, developed for the investigation of CL metabolism in the rat (Zalko et al., 1998a), were applied in this study. Both chromatographic systems used an Ultrabase C_{18} column (250 × 4.6 mm, 5 μm) (SFCC, France) coupled to a Hypersil BDS C_{18} guard precolumn (18 × 4.6 mm, 5 μm) (Shandon/LSI, Cergy Pontoise, France). For chromatographic system 1, mobile phases consisted of ammonium acetate buffer (10 mM, pH 3.2) and acetonitrile, respectively 95:5 v/v in reservoir A and 30:70 v/v in reservoir B; a three-step gradient was used: 0−4 min, 100% A; 4−10 min, linear gradient from 100% A to 95% A; 10−30 min, 95% A; 30−35 min, linear gradient from 95% A to 60% A; 35−45 min, 60% A; 45−47 min, linear gradient leading to 0% A (100% B); 47−54 min, 100% B. For chromatographic system 2, mobile phases were composed of water/acetonitrile/acetic acid (respectively 93:5:2 and 28:70:2 v/v/v, in A and B). A one-step gradient was used as follows: 0−5 min, 100% A; 5−15 min, linear gradient from 100% A to 100% B; 15−25 min, 100% B. In both HPLC systems, flow rate was 1 mL/min at 35 °C. Metabolite profiling was always conducted using chromatographic system 1; system 2 was used for metabolite isolation [see Zalko et al. (1998a) for additional details]. All samples or extracts were filtered on 0.5 g Ultrafree-MC 0.45 μm Millipore filtering units before HPLC analysis.

Metabolism Studies. For calf I, feces and urine were not collected during the 12 h study. At slaughter, urine and bile were immediately collected into a syringe from the bladder and the gall bladder, respectively. Lung, liver, spleen, and kidney samples were quickly frozen in liquid nitrogen and stored at −80 °C. Eyes, muscle (longissimus dorsi), and perirenal fat were stored immediately at −20 °C if not used quickly after slaughter. Blood was collected in heparinized tubes and was immediately centrifuged. An identical procedure was applied when calf II was slaughtered. In this second study, feces and urine excreted over a 24 h period were collected on days 8 and 14 of the experiment. Exhaustive collection of excreted urine was carried out using a ventral bag equipped with a filter and connected to a glass flask and a pump. Urine was received in the glass flask at ambient temperature, from which it was sampled several times a day. Blood samples were collected before the first CL administration, as well as 12 h after CL administration on days 3, 6, 10, and 14 of the study.

Sample Preparation. *Urine.* Urine samples were mixed with 2 volumes of methanol, stirred, and centrifuged for 10 min at 10000*g* (4 °C). Methanol was evaporated under vacuum. The supernatant, containing >95% of the initial radioactivity measured in urine, was filtered with 0.5 g

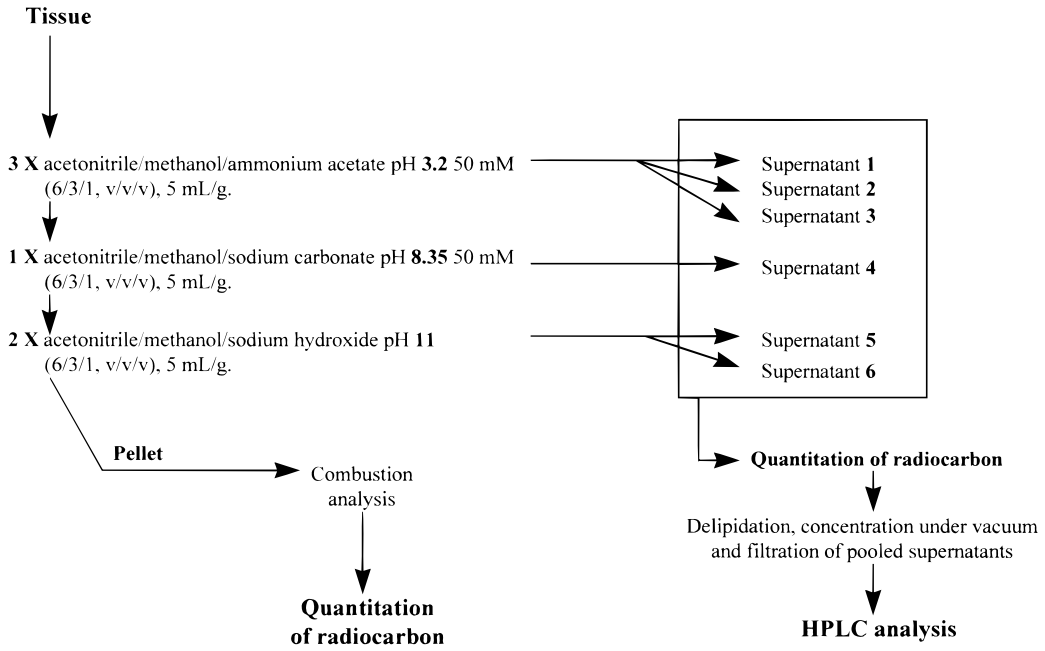

Figure 2. Extraction procedure applied to bovine tissues and preparation of extracts for radiochromatographic analysis.

Ultrafree-MC 0.45 μm Millipore filtering units (Polylabo, Strasbourg, France) and then mixed with 2 volumes of mobile phase A. HPLC (chromatographic system 1) coupled to on-line radiodetection was used for metabolite profiling and quantitation.

Feces. Calf II feces (day 8) were weighed, and then 2 kg of water was added. The mixture (8.73 kg) was homogenized for 30 min using a blender. Four samples were removed for combustion and liquid scintillation analysis, and 1 kg was lyophilized. Four aliquots of the lyophilized feces were taken for radioactivity determination. Metabolite extractions were performed with 1 g samples of lyophilized material. Briefly, samples were homogenized in 9 mL of ammonium acetate buffer (100 mM, pH 3.2) and methanol (1:2 v/v) using a Polytron homogenizer (Kinematica AG, Luzern, Switzerland) and were centrifuged at 10000*g* for 15 min (4 °C). The supernatant was removed, and the pellet was extracted twice more following the same procedure and then once using 9 mL of sodium hydroxide (1 mM) and methanol (1:2 v/v). Radioactivity remaining in the pellet was determined by combustion analysis. The four extraction supernatants were pooled, back extracted with isooctane, and concentrated under vacuum. The concentrated extracts were centrifuged, and the supernatant was filtered before HPLC analysis. The same procedure was followed with day 14 feces (5.85 kg of feces + 2 kg of water).

Bile. Bile samples were diluted with 3 volumes of methanol and were stirred for 5 min using a magnetic stirrer. Samples were centrifuged for 10 min at 2600*g* (room temperature). The pellet was discarded, and the supernatant was concentrated and filtered before chromatographic analysis.

Retina. For each calf, one eye was dissected immediately after slaughter, and the second was processed similarly after 2 weeks of storage at −20 °C (the radiochromatographic analyses of retina extracts were identical in both cases). Following dissection, the retina (including the pigmented epithelium) was obtained. Three aliquots of retina were assayed by combustion analysis. Extractions were conducted with retina pieces weighing ~0.2 g. Extraction samples were added to ~2 mL of ethanol, sonicated for 5 min using a Vibra Cell 72434 ultrasound homogenizer (Bioblock Scientific, Illkirch, France) (25 W and 40% power), and then were centrifuged for 10 min at 10000*g* (4 °C). Radio-HPLC analyses were performed on supernatants after concentration under N_2 and filtration. Duplicate extractions were conducted using the same protocol, except that 1 M NaCl/ethanol (1:1 v/v) was substituted for ethanol.

Plasma. Blood samples were centrifuged at 900*g* for 5 min (4 °C). Plasma samples were mixed with 3 volumes of methanol and centrifuged (2600*g*, 5 min, room temperature). The resulting supernatant was delipidated with isooctane, filtered, and then submitted to HPLC analysis. Radioactivity associated with erythrocytes and with the plasma protein pellet was measured using combustion analysis. Prior to the first centrifugation step, combustion analysis was also performed on whole blood.

Other Tissues. Tissue pieces (typically 4−8 g) were processed using a Polytron homogenizer and were extracted six times under three different pH conditions (Figure 2). Samples were homogenized with acetonitrile/methanol/ammonium acetate buffer, 50 mM pH 3.2 (6:3:1 v/v/v, 5 mL/g), and were centrifuged for 10 min at 10000*g* (4 °C). Radioactivity in the supernatant was measured, and the pellet was used for the next extraction steps. At the end of the extraction procedure, residual radioactivity in the pellet was determined by combustion analysis. For each tissue, extracts containing >2% of the radioactivity were pooled, delipidated (using acetonitrile-saturated isooctane), concentrated, centrifuged, and then filtered before radio-HPLC analysis, following the methods described for feces extracts. Duplicate analyses were performed with liver samples from both animals, after addition of [^{3}H]-*N*-OH-CL (200 Bq/g of tissue) before homogenization, with the aim to test the stability of this metabolite throughout the extraction procedure, by means of a double-labeling method.

Matrix solid-phase dispersion (MSPD) extraction assays were also conducted with liver, lung, kidney, and spleen samples from calf II. In brief, 1 g of tissue was blended and then mixed with 3 g of C_{18} packing material using a pestle pellet. The mixture was transferred into a syringe, between two filter paper disks. The resulting cartridge was washed with isooctane (20 mL) and CH_2Cl_2 (20 mL) and then eluted successively with methanol (10 mL) and methanol/formic acid [19:1 v/v (30 mL)].

Enzymic Test. The enzymic hydrolysis of CL metabolites in bovine urine was tested as follows: 20 μL of *Helix pomatia* juice (corresponding to 2 000 Fishman units of β-glucuronidase and 20 000 Roy units of sulfatase activity) was mixed with 480 μL of 0.1 M sodium acetate buffer adjusted to pH 4.8 and 20 μL of freshly collected urine. The mixture was stirred and incubated at 42 °C for 16 h. Incubations were filtered and analyzed, using the radiochromatographic conditions described

above. Control incubations were carried out under the same conditions but with no enzyme added.

Metabolite Isolation and Characterization. Metabolites were individually purified from bovine urine using methods detailed previously (Zalko et al., 1998a). The characterization of bovine CL metabolites was achieved by comparison of their chromatographic behavior with that of radiolabeled metabolites previously isolated from rat urine (Zalko et al., 1997, 1998a), following cochromatographic analysis and/or retention time comparison using chromatographic systems 1 and 2. ADBA and NO-CL exhibited identical retention times using these chromatographic conditions. Distinction between them was achieved by testing their retention on a strong anion exchanger (Chromabond SB cartridges; Macherey Nagel, Eckbolsheim, France). Cartridges were prewashed successively with methanol (5 mL) and 0.1% sodium carbonate (5 mL). Metabolite samples were concentrated under vacuum, reconstituted in 3 mL of water, and applied to the cartridge after pH verification (and when necessary, adjustment to pH 7). Cartridges were washed with 0.1% sodium carbonate (9 mL), dried under N_2, and eluted with 7 mL of methanol/formic acid (9:1 v/v).

RESULTS

Radioactivity Excretion in Urine and Feces. *Single Administration Study (Calf I).* Twelve hours after [^{14}C]CL administration, urinary radioactivity was associated with two major metabolites (Figure 3A) and unchanged CL (which accounted for 24% of the radiochromatographic profile). On the basis of retention time comparisons with ^{14}C-labeled metabolites previously purified from rat urine, the major metabolites were identified as *N*-OH-CL and ADHA. They accounted for 37 and 19% of the radio-HPLC profile, respectively. SCL and ADMA were also detected. Retention times of metabolites B1, B2, and B4, present in lower amounts in urine, did not correspond to those of known compounds. When [^{3}H]-*N*-OH-CL was added to the samples before analysis, it was found to elute at the same retention time as the urinary [^{14}C]-*N*-OH-CL (data not shown).

Two Week Administration Study (Calf II). Total urinary and fecal outputs were collected on days 8 and 14 of the study. It was established that 46.9 ± 6.0% of the radioactivity distributed daily to calf II was eliminated in urine, while 33.5 ± 1.1% was recovered in feces. The radiochromatographic profile obtained from urine collected directly from the bladder at the end of the study (Figure 3B) showed that unchanged CL was associated to less than 11% of the radioactivity detected by radio-HPLC, and *N*-OH-CL contribution was much lower than in calf I. Conversely, metabolite B2 was detected in higher amounts than in urine obtained after a single CL administration.

Both *N*-OH-CL and B2 were unstable during sample processing. *N*-OH-CL was readily reduced to CL, and B2 transformed very quickly into CL, even when urine was stored at −20 °C. Consequently, in samples taken from the urine collection flask, these two metabolites were detected in low quantities, and greater amounts of radioactivity were associated with unchanged CL. This instability was confirmed in enzymic hydrolysis assays with *H. pomatia* juice. In these experiments, both compounds were transformed into CL, but similar results were obtained when urine was incubated under the same conditions with no enzyme (data not shown). Conversely, B3 was enzymatically deconjugated by *H. pomatia* juice but left unchanged in the control assay.

Fecal radio-HPLC profiles were obtained after extraction of lyophilized feces. Lyophilization did not cause

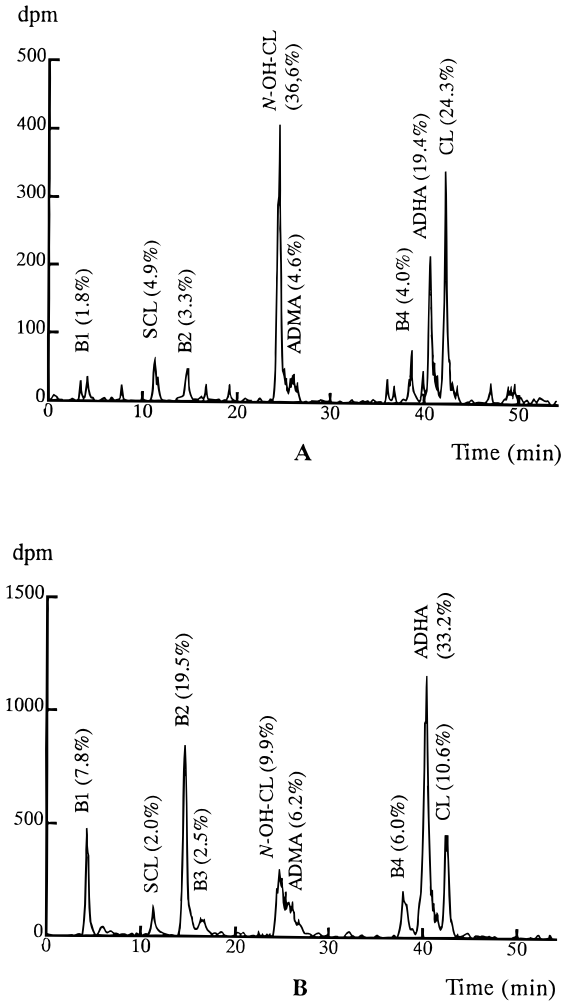

Figure 3. Radio-chromatographic analyses of bovine urine following single (calf I, A) or repeated (calf II, B) oral administration of 5 μg/kg of body weight CL. Both analyses were performed on urinary samples collected directly from the bladder 12 h after (the last) CL administration. Percentages indicate the respective contributions of metabolites to the HPLC profile.

the loss of radioactivity from feces. Most of the radioactivity was extractable (80% at day 8, 76% at day 14). Day 14 fecal radioactivity was mainly associated with CL, CL sulfamate, and metabolite B5 (Figure 4). In addition, limited amounts of polar metabolites were detected. Results were qualitatively similar for day-8 feces. The retention time of metabolite B5 (48 min) corresponded to that of NO-CL and ADBA. Both compounds were previously found to possess very close chromatographic properties despite their structural differences (Zalko et al., 1998b). Nevertheless, B5 in fecal extracts was well retained on strong anion cartridges, while standard NO-CL was not (data not shown). On this basis, it was concluded that B5 possessed an acidic function and thus corresponded to ADBA.

Residual Radioactivity in Tissues. Radioactivity levels measured in tissues after single or repeated CL dosage are given in Table 1. Liver and kidney samples from both animals, as well as lung and spleen samples from calf II, were extracted with various solvent systems, at pH ranging from 3.2 to 11. Acidic extracts (1−3), always contained >95% of the total extractable radioactivity (Table 2). Low levels of bound residues occurred after a single oral dose. Following repeated

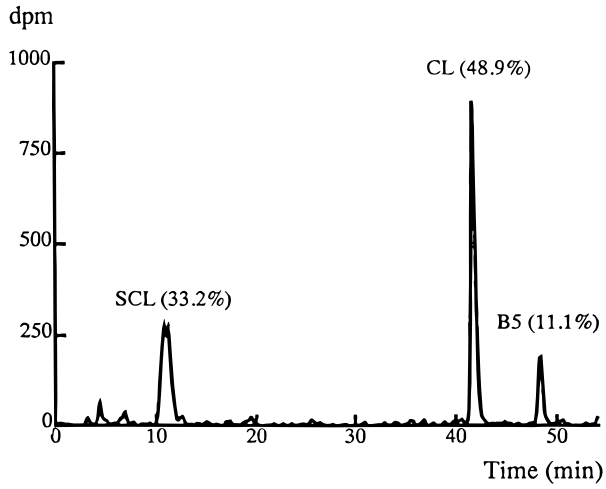

Figure 4. Radio-HPLC profile of fecal extract from a calf dosed twice daily with 5 μg/kg CL for a 2 week period. The radiochromatogram corresponds to an aliquot of feces excreted on day 14 of the study.

Table 1. Total Radioactive Residues (CL Equivalents) in Tissues of Bovine Dosed with 5 μg/kg CL[a]

	ppb of CL equivalent	
tissue	calf I	calf II
liver	39.7	245.7
kidney	24.9	210.1
lung	7.5	111.0
spleen	7.2	88.6
heart	2.2	27.1
muscle (longissimus dorsi)	1.6	11.7
fat (perirenal fat)	0.3	6.7
gland (scapular gland)	5.1	47.5
retina	338.0	3720.0

[a] Calf I was slaughtered 12 h after a single oral CL administration. Calf II was dosed with CL twice a day for 14 days and was slaughtered 12 h after the last administration of the drug. Results are expressed in ng CL equivalents per g of tissue (ppb).

administration of CL, bound residues represented one-sixth (lung and liver) to one-fourth (spleen) of the radioactivity in tissues. A second extraction procedure, MSPD, was applied (calf II tissues only) to shorten sample preparation delay before HPLC analysis. The total amount of MSPD extractable radioactivity from tissues paralleled total recoveries of radioactivity by solvent extraction (Table 2).

For both calves, nearly 100% of the radioactivity within retina samples was recovered after NaCl/ethanol extraction, but recovery was slightly less when ethanol only was used (~80%). Blood levels of radioactivity measured during the 2 week study (calf II) are presented in Figure 5. About 15% of blood ^{14}C was associated with erythrocytes, and 85% was located in the plasma. Fifteen percent of the plasma radiocarbon precipitated with plasma proteins when methanol was added. Total radioactivity extracted from blood for radio-HPLC analysis thus averaged 70%.

Radio-HPLC Profiling in Plasma, Bile, and Tissues. About 85% of the plasma radioactivity was associated with unchanged CL in calf II blood collected 12 h after the last CL dosage (Figure 6A). Clenbuterol sulfamate and ADHA were also detected, together with polar compounds; each accounted for ~5% of the radioactivity. Biliary excretion of ^{14}C was observed mainly as SCL, B4, and unidentified metabolites of low polarity. The radio-HPLC analysis of calf II bile (Figure 6B) was very similar to the radiochromatogram of calf I bile. The metabolite exhibiting a retention time of 48 min co-eluted with both NO-CL and ADBA. The use of a strong anion exchanger failed to provide sufficient resolution for structural characterization of this metabolite.

Radio-HPLC profiles in tissues obtained from the analysis of pooled pH 3.2 solvent extractions (extracts 1−3) are summarized in Table 3. Most of the radioactivity in tissues was associated with unchanged CL. Analysis of residual radioactivity extracted at basic pH (extracts 4−6, data not shown) indicated no qualitative difference when compared to the results obtained following the radio-HPLC analysis of acidic extracts. Radiochromatographic analyses of liver extracts are presented for calf I (Figure 6C) and calf II (Figure 6D), respectively. Unchanged CL, ADOA, B4, and an unidentified metabolite (B6) were detected. In calf II, compounds possessing the same retention times as ADHA and NO_2-CL, as well as a polar peak, were also observed, but in low amounts. In other tissues, CL and trace amounts of polar metabolites were detected; calf I kidney extracts contained ~4% ADHA (see Table 3). Radioactivity in retina was exclusively associated with unchanged CL.

Tissue MSPD extracts were analyzed for calf II. Regardless of the tissue, the resulting radiochromatograms were similar to those obtained after solvent extraction. Additional experiments were conducted with liver samples spiked with ^{3}H-labeled *N*-OH-CL, to determine its stability during extraction. Less than 10% of the [^{3}H]-*N*-OH-CL added to samples was detected by radio-HPLC, after the solvent extraction procedure, as most of it was reduced to [^{3}H]CL. Using MSPD extraction, ^{3}H mainly eluted with CH_2Cl_2 (47%) and methanol (46%). The CH_2Cl_2 eluate contained NO_2-CL (58%) and CL (40%), and the methanol fraction consisted of *N*-OH-CL (74%) and CL (23%).

DISCUSSION

Previous studies have provided valuable data about CL pharmacokinetics and residues in cattle (Meyer and Rinke, 1991; Elliott et al., 1993a; Sauer et al., 1995). Most of these studies were conducted following single or repeated administration(s) of 1.6−20 μg/kg CL, to mimic as much as possible the conditions in which CL is supposed to be illegally used in cattle. The present work was designed to investigate the qualitative aspects of CL metabolism in cattle. A 5 μg/kg oral dosage was chosen. Using ^{14}C-labeled CL, such a dosage could not allow the simultaneous determination of radiochromatographic profiles in tissues and the direct characterization of metabolites detected in bovine biological samples, by mass spectrometric methods. Therefore, structural identifications were achieved on the basis of retention time comparison with metabolites previously purified from the urine of rats fed higher dosages of [^{14}C]-CL and identified by mass spectrometry (Zalko et al., 1998a).

When [^{14}C]CL was administered twice a day over 2 weeks (calf II), about 47 and 33% of the daily ^{14}C dosage were recovered in urine and feces, respectively, corresponding to approximately 300 ppb (urine) and 100 ppb (feces) CL equivalents. In a previous study, Smith and Paulson (1997) found that only ~2% of a 3 mg/kg ^{14}C-labeled CL dose was eliminated in the feces of cattle within 48 h after dosing. We have also reported similar values from a preliminary study on one calf, dosed with

Table 2. Percentage of Extractable Radioactivity in Tissues of Cattle Fed Single (Calf I) or Repeated (Calf II) Oral Dosage(s) of 5 μg/kg CL, after Extraction at pH 3.2 (Extracts 1−3), pH 8.35 (Extract 4), and pH 11 (Extracts 5 and 6)[a]

	solvent extraction					MSPD				
	extracted radioactivity (%)				bound	extracted radioactivity (%)				
animal/tissue	1−3	4	5−6	total	residues (%)	IS	D	M	M/AF	total
calf I										
liver	95.2	1.2	0.4	96.8	3.2					
kidney	96.4	0.8	0.3	97.5	2.5					
calf II										
liver	81.4	0.8	0.6	82.8	17.2	0	3.2	55.1	21.5	79.8
kidney	96.1	0.7	0.4	97.2	2.8	0	4.2	64.2	30.0	98.4
lung	82.7	1.6	1.1	85.4	14.6	0.1	3.3	59.3	15.4	78.1
spleen	74.6	1.0	0.6	76.2	23.8	0.2	2.3	43.6	12.8	58.9

[a] Results are also presented for tissues from calf II after MSPD extraction; MSPD cartridges were eluted successively with isooctane (IS), CH_2Cl_2 (D), methanol (M), and methanol/formic acid (M/AF).

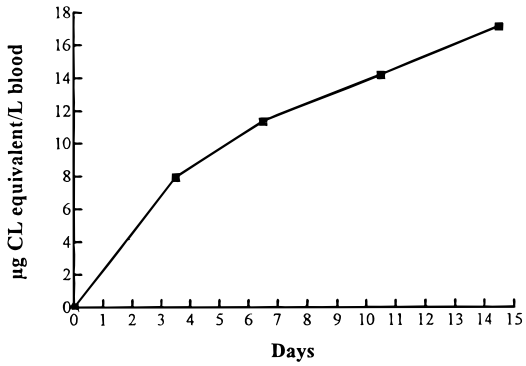

Figure 5. Total radioactive residues (CL equivalents) in calf II blood during the 2 week study. Results were calculated from radioactivity levels measured in blood, on the basis of CL molecular weight.

5 μg/kg ^{3}H-labeled CL (Zalko et al., 1996), for which <5% of the ^{3}H dose was eliminated in feces during the first 48 h. The present results have demonstrated that fecal excretion of CL residues in cattle was more important than previously reported, but occurred after more than 48 h.

Biotransformations of clenbuterol in cattle (Figure 7) were quite similar to those previously well evidenced in the rat; that is, the two major metabolic pathways were *N*-oxidation of the primary amine (*N*-OH-CL) and oxidative cleavage of the side chain of CL (ADMA, ADOA, ADBA) followed by glycine conjugation (ADHA). In addition, sulfate *N*-conjugation produced a minor urinary metabolite (SCL), which was better represented in feces. In cattle, hydroxylation of the *tert*-butyl group of CL was not demonstrated. Conversely, one unidentified CL metabolite (B2) was detected in calf urine samples after repeated administration of the drug. Metabolite B2 was readily degraded to CL in crude urine and during enzymic tests, thus impeding its identification. It was a rather polar metabolite, and as its structure was directly related to that of parent CL, it may be a labile CL conjugate; however, further work is necessary to confirm such a hypothesis.

Radio-HPLC analysis of urine collected at slaughter (calf II) revealed that only 10% of the radioactivity was associated with unchanged CL. Most of the screening procedures developed by laboratories involved in the control of CL misuse in cattle have focused on the identification of the parent compound in urine (Boyd et al., 1996). In calf II urine, however, *N*-OH-CL and B2 were highly labile and decomposed spontaneously into CL in unprotected samples. They respectively accounted for about 10 and 20% of the total urinary residues of CL. Most existing methods for CL detection in bovine urine use sample alkalanization (to pH 9−11) as a first step of the analytical procedure, to enable an efficient extraction of the drug (Delahaut et al., 1991; Hooijerink et al., 1991; Collins and O'Keeffe, 1994). Under such conditions, *N*-OH-CL was demonstrated to reduce to CL (Zalko et al., 1997). Among these analytical methods, only a few include an enzymic deconjugation step (Haasnot et al., 1990). However, the high lability of B2 observed in the present study suggests this metabolite should be completely converted into CL during the extraction procedure, whatever its structure. Thus, the monitoring of CL illegal use, based on the quantitation of the parent compound in bovine urine, very likely results in the detection of more CL than originally present in samples (depending on the extraction and detection methods applied). Similar conclusions may be drawn from the analysis of fecal extracts, if one is attempting to detect CL residues in this matrix, easily available at the farm (Batjoens et al., 1996). Indeed, parent CL only accounted for half of the fecal radiochromatographic profile, while the remaining radioactivity was associated with metabolites SCL and ADOA. Clenbuterol sulfamate remained unchanged when urine was incubated with *H. pomatia* juice, as previously observed (Zalko et al., 1998a).

Though the monitoring of CL illegal use by means of CL characterization in bovine urine may be satisfactory, an additional urinary metabolite, ADHA, may also be used for result confirmation. Indeed, ADHA accounted for about one-third of CL residues in bovine urine following a multidose CL treatment. Consequently, the additional search of ADHA in samples could be used to strengthen screening results, for instance, when detection is achieved by mass spectrometry in the single ion monitoring mode (Blanchflower and Kennedy, 1989; Blanchflower et al., 1993; Solans et al., 1995). Moreover, urinary metabolic profiles have not been established for bovines slaughtered >12 h following repeated CL administrations, even though unchanged CL levels were previously measured after various withdrawal delays (Meyer and Rinke, 1991). Thus, further experiments are required to examine whether ADHA could be a useful urinary marker of CL illegal use in bovine, when CL is detected only in trace amounts.

Consistent with previous results (Elliott et al., 1993b; Dürsch et al., 1995), relatively high concentrations of CL accumulated in bovine retina, even after a single administration of the drug. Accumulation of CL in pigmented tissues (Dürsch et al., 1995) is related to the high binding affinity of melanin toward basic drugs (Howells et al., 1994). Different extraction procedures

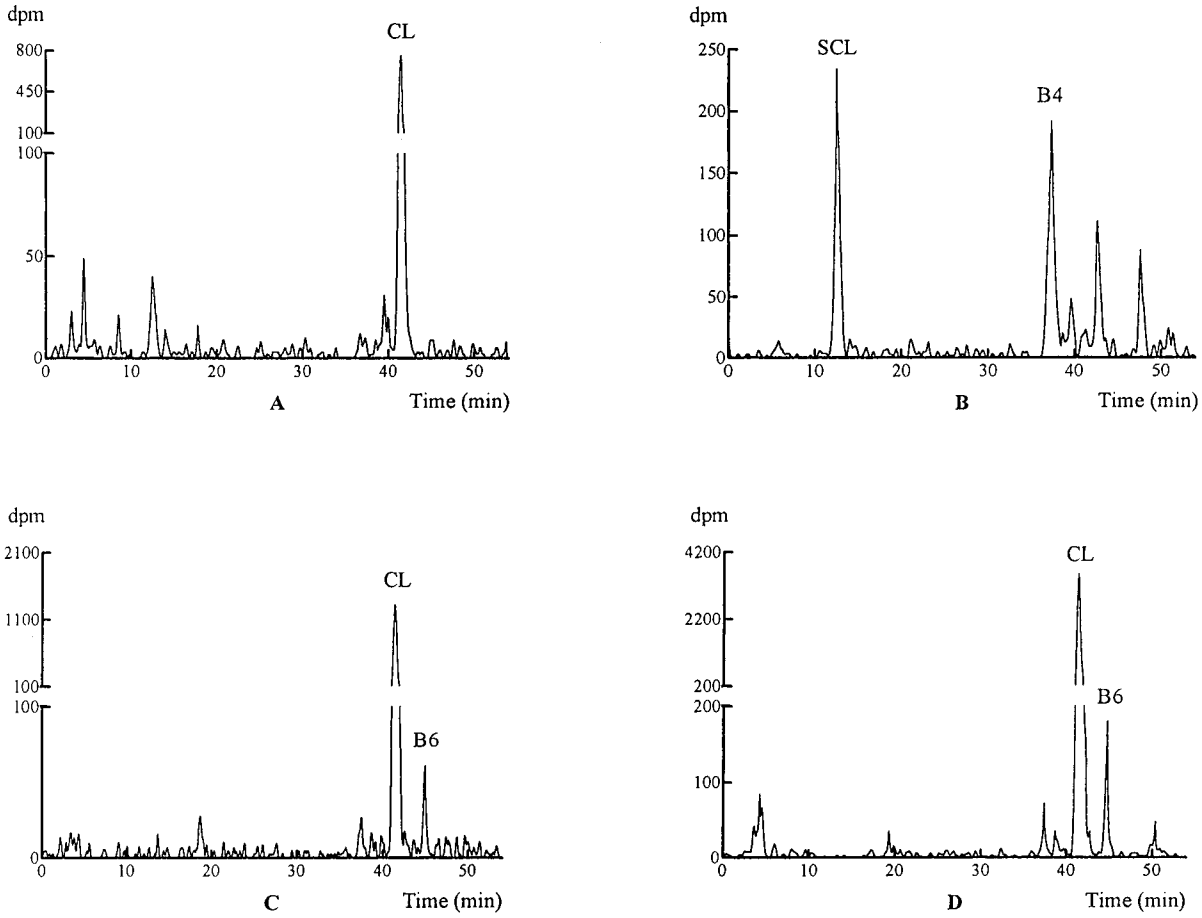

Figure 6. Radio-chromatographic analysis of calf II plasma (A), bile (B), and liver (D) and calf I liver (C) extracts. Liver metabolic profiles correspond to fractions extracted at acidic pH (extracts 1−3). Blood and bile were collected at slaughter.

Table 3. Qualitative Distribution of Radioactivity in Calf I and Calf II Tissue Extracts[a]

		% of radioactivity in tissue extract							
		calf I			calf II				
metabolite	retention time (min)	liver	kidney	retina	liver	kidney	lung	spleen	retina
B1	4		0.7	0.2	3.4	2.2	2.1	1.9	
ADOA	19.5	2.2			0.6				
B4	38	2.6	1.7		1.4				
ADHA	39.5		4.1		0.9	0.9			
CL	42	90.8	92.0	99.8	89.1	96.9	97.9	98.1	100
B6	45	4.4	0.9		3.8				
NO_2-CL	50		0.6		0.8				

[a] Results were obtained by radiochromatographic analysis of acidic extracts 1−3, containing >95% of the tissue extractable radioactivity.

allowed us to determine that only the parent compound was present in retina samples. These results confirmed that retina is a matrix of choice for CL monitoring, though available only at the slaughterhouse. Comparative extraction assays using either ethanol or ethanol/NaCl supported the hypothesis that ionic bonds were involved in the binding of CL to retina (Howells et al., 1994).

Radioactive residue levels measured in blood of calf II were consistent with values published previously (Stoffel and Meyer, 1993; Sauer et al., 1995), and 85% of the extractable plasma radioactivity was associated with unchanged CL. Clenbuterol residues detected in lung and spleen (calf II) and liver and kidney (calf I and calf II) extracts were consistent with previous studies, in which 1.6−10 μg/kg CL were administered to cattle (Elliott et al., 1993a; Stoffel and Meyer, 1993; Sauer et al., 1995). Regardless of the tissue, a large part of the radioactivity was extractable, and most of it was unchanged CL. *N*-OH-CL, detected in calf urine, was previously demonstrated to be a major in vitro CL metabolite produced when the drug was incubated with bovine liver microsomes (Zalko et al., 1998b). Because of the potential toxicity of this structure toward cell proteins (Zalko et al., 1997) and because the pharmacological activity of *N*-OH-CL is unknown, its presence in tissues (especially liver) had to be checked carefully. Though the characterization of *N*-OH-CL in biological samples was difficult due to its instability, the use of MSPD and [^{3}H]-*N*-OH-CL, in addition to solvent extraction, clearly evidenced that this metabolite was not detectable in bovine liver.

Recently, Smith and Paulson (1997) reported results stating that unchanged CL accounted for 40−70% of the extractable residues in liver, kidney, and spleen of calves treated with a single oral dosage of 3 mg/kg CL.

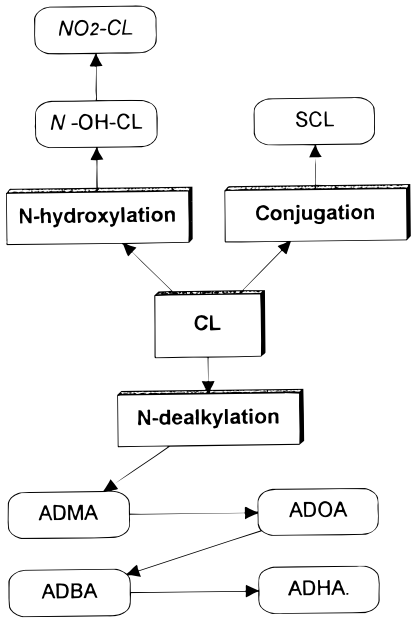

Figure 7. Proposed metabolic pathways of CL metabolism in the bovine.

These results have to be confirmed, but the present data indicate that when using a "classical" anabolic CL dosage, at least 90% of the extractable radioactivity located in bovine tissues is associated with unchanged CL. Therefore, from our results, the toxicity of CL residues toward the consumer is expected to be broadly, if not solely, related to the pharmacological properties of the parent compound. Nevertheless, further biotransformations of the drug may occur in humans.

ABBREVIATIONS USED

CL, clenbuterol; *N*-OH-CL, 4-hydroxyamino-3,5-dichloro-α-(*tert*-butylaminomethyl)benzyl alcohol; NO-CL, 4-nitroso-3,5-dichloro-α-(*tert*-butylaminomethyl)benzyl alcohol; NO_2-CL, 4-nitro-3,5-dichloro-α-(*tert*-butylaminomethyl)benzyl alcohol; OH-CL, 4-amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylaminomethylbenzyl alcohol; ADHA, 4-amino-3,5-dichlorohippuric; ADBA, 4-amino-3,5-dichlorobenzoic acid; ADMA, 4-amino-3,5-dichloromandelic acid; ADOA, 2-(4-amino-3,5-dichlorophenyl)-2-oxoacetic acid; SCL, clenbuterol 4-aminosulfonic acid; MSPD, matrix solid-phase dispersion.

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