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METABOLISM OF CLENBUTEROL IN RATS

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ABSTRACT:

The metabolic fate of [^{14}C]clenbuterol was studied in male and female Wistar rats. After a single oral dose of 200 μg/kg [^{14}C]clenbuterol, in an 8-day study period, approximately 60% of the radioactivity was eliminated in urine; 20 and 30% of the radioactivity was excreted in feces by male and female rats, respectively. HPLC coupled to on-line radioactivity detection allowed the separation and quantitation of clenbuterol metabolites, some of which were found to be poorly stable in urine. Most of the urinary and fecal metabolites of clenbuterol were isolated and identified using various MS techniques. Analytical methods were also developed to establish the metabolic profiles in feces and tissues, up to 72 hr after clenbuterol administration. Clenbuterol was mainly metabolized by *N*-dealkylation (secondary amine), as well as *N*-oxidation and sulfate conjugation (primary amine). Gender-related differences in the rates of clenbuterol *N*-dealkylation were observed. 4-*N*-Hydroxylamine was the major metabolite detected in urine, whereas more than one half of the radioactivity in feces was associated with clenbuterol sulfamate.

The β-agonist CL,[1] which was developed more than 20 years ago, is registered in many countries as a veterinary bronchospasmolytic and tocolytic drug. In several animal species, it was demonstrated that CL could also act as a "repartitioning agent," able to decrease fat deposition and enhance protein accretion, when administered orally at high doses (Williams, 1987; Guggenbuhl, 1996). In the past decade, the illegal use of this compound in cattle led to a number of reports of human intoxication (Martinez-Navarro, 1990; Pulce *et al.*, 1991). Indeed, residual levels of CL in bovine tissues (primarily liver) can be high enough to produce pharmacological effects in human consumers. However, CL elimination is considered to be quite rapid, and such incidents are thought to occur only after premature slaughtering of the treated animals or accidental distribution of high CL doses.

Many European and North American laboratories have developed various efficient methods to detect CL in urine and tissue samples. In contrast, only a few studies have been carried out to elucidate metabolic pathways for CL in animals. Some of the urinary and fecal metabolites of CL have been quantified in rats, dogs, and rabbits (Kopitar, 1976; Kopitar and Zimmer, 1976). In rats and dogs, Tanabe *et al.* (1984a,b) characterized the structures of some of the urinary metabolites of [^{14}C]CL, based on a TLC comparison of CL metabolites with a limited number of chemical standards. Tanabe *et al.* (1984a,b) identified several metabolites originating from the oxidative cleavage of the side chain of CL, as well as one hydroxylated metabolite of CL. In 1990, a nearly complete metabolite pattern for CL in dog urine was reported by Schmid *et al.* (1990). Those authors stated that the biotransformations of CL observed in dogs were qualitatively rather similar to those in rabbits and rats, as well as in humans. Unfortunately, because of the lack of information on the administered doses and the analytical conditions used, those statements could not be adequately assessed.

We recently demonstrated that a previously unknown, important metabolic pathway of CL, *N*-oxidation, occurred both *in vivo* (Zalko *et al.*, 1996, 1997) and *in vitro* (Zalko *et al.*, 1998) in rats and cattle. The metabolism of CL was further investigated in rats using a ^{14}C-labeled molecule and radio-HPLC detection, with the aim of establishing the complete metabolic pathways of this β-agonist in a laboratory animal model before studying CL biotransformations in cattle. Our previous studies indicated that the characterization of several CL metabolites was complicated by their weak stability in biological matrices. Therefore, additional analytical methods were developed to separate, quantify, and isolate rat CL metabolites from urine, feces, and tissues and to determine their chemical structures.

Materials and Methods

Chemicals. *CL and Labeled Molecules.* [^{14}C]CL [4-amino-3,5-dichloro-α-[(*tert*-butylaminomethyl)]benzyl alcohol, labeled on the benzylic carbon] was purchased from Isotopchim (Ganagobie-Peyruis, France) and had a specific activity of 1997 MBq/mmol. Based on HPLC and TLC analyses, its radiopurity was verified to be >97%. CL hydrochloride was purchased from Sigma (Saint Quentin Fallavier, France). The CL structure was confirmed by ESI/MS. ^{3}H-labeled *N*-OH-CL was isolated and purified from rat urine as described elsewhere (Zalko *et al.*, 1997).

Other Chemicals. All solvents (of analytical grade) and formic acid were obtained from Prolabo (Paris, France). Chemicals were purchased from the

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[1] Abbreviations used are: 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; SCL, clenbuterol 4-aminosulfonic acid; ADBA, 4-amino-3,5-dichlorobenzoic acid; ADHA, 4-amino-3,5-dichlorohippuric acid; ADMA, 4-amino-3,5-dichloromandelic acid; ADOA, 2-(4-amino-3,5-dichlorophenyl)-2-oxoacetic acid; ESI, electrospray ionization; FAB, fast atom bombardment; CID, collision-induced dissociation.

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following sources: acetic acid, Merck (Darmstadt, Germany); *Helix pomatia* juice (Helicase), IBF (Villeneuve-La-Garenne, France); type VI sulfatase and D-saccharic acid 1,4-lactone, Sigma; ADBA, Pfaltz and Bauer (Waterbury, CT).

Animals. *Metabolic Balance in Rats.* Eight adult Wistar rats (four males and four females) were individually housed in stainless steel metabolism cages. Animals were allowed free access to water and to a standard diet (UAR 210; UAR, Villemoisson-Sur-Orge, France). They were maintained on a 12-hr light/dark cycle. Mean rat weights were 427 ± 21 g (males) and 299 ± 24 g (females). After a 1-week acclimatization period, each animal was force-fed a single oral dose of [^{14}C]CL, adjusted to 200 μg/kg of body weight. Urine and feces were collected daily over an 8-day period. At the end of the study, rats were killed by exsanguination. Cages were individually washed with 500 ml of methanol/water (1:1, v/v), from which 1-ml aliquots were taken for radioactivity determination. Livers were separated from the rest of the carcasses. All samples were stored at −20°C if not used immediately.

Metabolic Profiles in Tissue Extracts. Four male Wistar rats (age, 10 weeks) were housed as described above. After a comparable acclimatization period, animals were force-fed a single oral dose of 200 μg/kg of body weight [^{14}C]CL. Rats were sacrificed by exsanguination 12, 24, 48, or 72 hr after CL administration. Livers, lungs, and kidneys were excised and stored at −20°C until analysis.

Influence of CL Dose. Three male rats (age, 10 weeks) were housed and handled as described above. They were dosed orally with 5, 20, or 40 mg/kg of body weight [^{14}C]CL (diluted with unlabeled CL to specific activities of 650 Bq/ng for the two lower doses and 300 Bq/ng for the highest dose). Urine and feces were collected three times each day for the 96-hr study and were stored at −20°C until use.

Apparatus. Samples were analyzed by HPLC with a Philips 4100 apparatus (Pye Unicam, Cambridge, UK) equipped with a Rheodyne model 7125 injector (Rheodyne, Cotati, CA) and connected to a Radiomatic Flo-one/β A500 radioactivity detector (Radiomatic, La-Queue-Les-Yvelines, France) (scintillation cocktail, Flow-scint II; Packard Instruments Co., Downers Grove, IL), to establish metabolic profiles, or to a UV detector set at 254 nm (PU 4021 multichannel detector; Pye Unicam) and a Gilson model 202 fraction collector (Gilson France, Villiers-Le-Bel, France), to purify metabolites. Radioactivity in urine and all other liquid samples was determined by direct counting in a Packard liquid scintillation counter (Tricarb 2200CA; Packard, Meriden, CT), using Packard Ultima Gold as the scintillation cocktail. Radioactivity in rat carcasses, tissues, fresh or lyophilized feces, and extraction pellets was determined by complete combustion using a Packard 306 oxidizer, followed by $^{14}CO_2$ quantitation in the Packard liquid scintillation counter (scintillation cocktail, Packard Permafluor E+/Packard Carbosorb, 2:1, v/v). Four replicates were analyzed for each sample.

Sample Processing. *Urine.* Urine samples were mixed with methanol (1:2, v/v), stirred, and centrifuged for 10 min at 10,000 rpm (4°C). The pellet was discarded. The supernatant was concentrated under vacuum and was filtered with Millipore Ultrafree-MC filtering units (0.5 g, 0.45 μm; Polylabo, Strasbourg, France) before radio-HPLC analysis.

Feces. Preliminary assays were carried out with feces obtained from the four rats used to establish the metabolic profiles in tissues, with the aim of investigating the possible occurrence of labile or volatile fecal CL metabolites. The radioactivity contained in these samples was determined by combustion and liquid scintillation counting of aliquots obtained before and after lyophilization. The radiochromatographic analyses of fecal extracts obtained from fresh or lyophilized material were also compared.

All feces collected daily during the metabolic balance study were individually lyophilized and then homogenized in a Dangoumeau ball-grinder (Prolabo). The radioactivity in each sample was determined. Approximately 0.5 g of lyophilized material was vortex-mixed for 2 min with methanol/100 mM ammonium acetate, pH 3.2 (1:6:3, w/v/v), and then centrifuged for 10 min at 10,000 rpm (4°C). The pellet was extracted twice more with the same solvent mixture and once with methanol/1 mM ammonium hydroxide (1:6:3, w/v/v). After radioactivity counting, the four supernatants were pooled, delipidated with isooctane, and concentrated under N_2. The resulting extract was filtered with filtering units (0.5 g, 0.45 μm) before radio-HPLC analysis. Radioactivity remaining in the last centrifugation pellet was determined by combustion analysis. For each rat, day 2 and day 3 feces (as well as day 4 feces for female rats) were separately processed and analyzed to establish the fecal metabolic profiles. Feces from rats given higher CL doses were subjected to the same procedure.

Tissues. Liver metabolic profiling was carried out after the extraction of approximately 5 g of tissue. Samples were cut into small pieces, homogenized with 6 ml/g of acetonitrile/methanol/50 mM ammonium acetate buffer, pH 3.2 (6:3:1, v/v/v), using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland), and then centrifuged for 10 min at 10,000 rpm and 4°C. The supernatant was stored immediately at −20°C, and the pellet was subjected to five additional extractions, *i.e.* two with the same solvent mixture, one with acetonitrile/methanol/50 mM sodium bicarbonate buffer, pH 8.35 (6:3:1, v/v/v), and finally two using acetonitrile/methanol/1 mM sodium hydroxide (6:3:1, v/v/v). The residual radioactivity remaining in the last centrifugation pellet for each sample was determined by combustion analysis. The six supernatants were pooled, delipidated using acetonitrile-saturated isooctane, concentrated under vacuum, and filtered, as described above, before HPLC analysis.

N-OH-CL was previously shown to be a poorly stable CL metabolite (Zalko *et al.*, 1997). To determine whether *N*-OH-CL was present in rat livers, the procedures described above were performed in duplicate with rats sacrificed 12 or 48 hr after CL administration, with the aim of shortening the extract concentration step. In this case, only the first extraction supernatant was analyzed by radio-HPLC. Both procedures were also repeated after initial addition of known amounts of radioactive *N*-OH-CL. In these experiments, small liver pieces were spiked with ^{3}H-labeled *N*-OH-CL (500 Bq) before solvent extraction with the Polytron homogenizer, and radio-HPLC analyses were conducted to detect ^{3}H and ^{14}C simultaneously, with adjusted double-labeling detection conditions.

Lung and kidney metabolic profiling was performed by using the same extraction schemes. Kidney samples were washed twice with water before extraction.

Analytical Procedures. *Metabolic Profiling in Urine, Feces, and Tissue Extracts.* HPLC with on-line radioactivity detection was used for metabolic profiling, using chromatographic condition 1. An Ultrabase C_{18} column (250 × 4.6 mm, 5 μm; SFCC, Eragny, France), coupled to an Hypersil BDS C_{18} guard precolumn (18 × 4.6 mm, 5 μm; Shandon/LSI, Cergy Pontoise, France), was used. Mobile phases consisted of 10 mM ammonium acetate buffer, pH 3.2/acetonitrile (95:5, v/v, in A and 30:70, v/v, in B). The flow rate was 1 ml/min at 35°C. In HPLC system 1, a three-step gradient was used, as follows: 0–4 min, 100% A; 4–10 min, linear gradient from 100% A to 95% A/5% B (v/v); 10–30 min, 95% A/5% B (v/v); 30–35 min, linear gradient from 5% B to 40% B; 35–45 min, 60% A/40% B (v/v); 45–47 min, linear gradient to 100% B; 47–54 min, 100% B.

Metabolite Isolation. The Philips 4100 apparatus was connected to a fraction collector. Separations were achieved using both HPLC systems 1 and 2. The latter consisted of the equipment described above, except that the analytical C_{18} column was a Capcell Pak column (250 × 4.6 mm, 5 μm; Interchim, Montluçon, France). Mobile phases consisted of water/acetonitrile/acetic acid (93:5:2 and 28:70:2, v/v/v, in A and B, respectively). The flow rate was 1 ml/min at 35°C. 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.

Enzyme Hydrolysis. Twenty microliters of crude urine (or 2 μg of purified metabolite in 20 μl of water) were mixed with 480 μl of 0.1 M sodium acetate buffer, pH 4.8, and 20 μl of *H. pomatia* juice (*i.e.* 2,000 Fishman units of β-glucuronidase and 20,000 Roy units of sulfatase activity) and incubated for 16 hr at 42°C. Incubations were centrifuged and then filtered with Ultrafree-MC units (0.45 μm) before radiochromatographic analysis. Duplicate assays were performed using the same conditions, except that 10 mM D-saccharic acid 1,4-lactone was added to inhibit the β-glucuronidase activity. More specific incubations were conducted with some of the purified metabolites to test the occurrence of sulfate conjugates. Samples (2 μg) of each metabolite were added to 450 μl of 20 mM Tris buffer, pH 7.1, and 50 μl of *Aerobacter aerogenes* type VI sulfatase, incubated for 16 hr at 37°C, and analyzed similarly.

Metabolite Isolation from Urine. Metabolite isolation was carried out on 0–24- and 24–48-hr urine samples collected from rats dosed with 5–40 mg/kg CL. Two different procedures were developed, in combination with the HPLC systems described above. The isolation of metabolite **M5**, as well as **M12** and

TABLE 1

Metabolic balance (radioactivity recovery) in male (N = 4) and female (N = 4) rats after a single oral dose of 200 μg/kg [^{14}C]CL in an 8-day study

	Radioactivity Recovered					
	Urine	Feces	Liver	Carcass	Cage	Total
	% of dose					
Males	60.0 ± 3.7	22.8 ± 3.1	0.4 ± 0.1	2.0 ± 0.3	2.0 ± 0.7	87.1 ± 2.5
Females	56.6 ± 5.4	30.4 ± 2.3	0.4 ± 0.1	1.3 ± 0.4	1.6 ± 0.7	90.2 ± 4.2

Values are mean ± SD.

M13, using chromatographic separation and C_8 cartridges, has been described and extensively discussed previously (Zalko *et al.*, 1997).

Procedure 1. Aliquots (1 ml) of urine were diluted with methanol (1:2, v/v) and centrifuged for 10 min at 10,000 rpm (4°C). Supernatants were concentrated under vacuum to eliminate methanol and then were filtered with Millipore Ultrafree-MC units (0.45 μm). The resulting extracts (approximately 1 ml) were individually mixed with 9 ml of 50 mM ammonium acetate buffer, pH 6.8, and applied to prewashed, reverse-phase, Select B C_8 cartridges (0.5 g; Merck). Cartridges were eluted successively with 2 ml of water, water/methanol (1:1, v/v), methanol, and finally methanol/formic acid (19:1, v/v) twice.

The methanol eluates were concentrated, taken up in mobile phase, and separated by HPLC using chromatographic system 1, with a fraction collector. The fraction containing CL was concentrated to approximately 10% of its initial volume, taken up in 2 ml of 50 mM ammonium acetate buffer, pH 6.8, and applied to a prewashed, reverse-phase, Select B C_8 cartridge (0.2 g; Merck), which was successively eluted with water (0.5 ml) and methanol (1 ml). The methanol eluate was concentrated and subjected to structural analysis.

Water/methanol eluates were similarly processed and separated using chromatographic system 1. The fraction corresponding to metabolite **M11** was prepared for MS studies using a 0.2-g, reverse-phase, Select B C_8 cartridge, as described for CL, whereas fractions containing **M7**, **M9**, or **M10** were subjected to an additional purification step. These metabolites were separated using chromatographic system 2, with a fraction collector, before final concentration with 0.2-g, reverse-phase, Select B C_8 cartridges, as described for CL.

Procedure 2. Day 1 and day 2 urine samples from a rat dosed with 20 mg/kg CL were acidified to pH 2 with HCl. The mixture was added to ethyl acetate (1:3, v/v) and stirred for 5 min. The aqueous phase was recovered using a MN 616 WA separation-phase paper filter (Macherey-Nagel, Hoerdt, France) and was similarly extracted a second time. Aliquots of both ethyl acetate phases were used for radioactivity determination. The two extracts were pooled, dried under N_2, and reconstituted in 10 mM ammonium acetate buffer, pH 3.2/acetonitrile (95:5, v/v). Metabolites were separated using chromatographic systems 1 and 2. **M3** and **M8** were obtained as described above for **M10**. **M4** was subjected to an additional purification step on C_8 cartridges.

Metabolite Isolation from Feces. Metabolite isolation from feces was carried out with samples from a rat dosed with 40 mg/kg CL. Lyophilized day 2 and day 3 feces samples were pooled and extracted as described above in *Sample Processing*. Only the first two acidic extracts were used. After delipidation, concentration (to eliminate methanol), and filtration, an aliquot was taken for radio-HPLC analysis, and the rest of the sample was divided in two equal parts. The first portion was processed using procedure 1 described for urine, allowing the isolation of **M9**, **M10**, and CL. The second portion was subjected to procedure 2, allowing **M3** isolation.

Metabolite Identification. MS analyses of the various metabolites were performed using FAB ionization or ESI techniques. FAB/MS analyses were performed with a Nermag R-10-10-H single-quadrupole mass spectrometer (Delsi-Nermag Instruments, Argenteuil, France) fitted with an M-Scan (Ascott, UK) FAB gun. Xenon gas was used for bombardment at an accelerating voltage of 8 kV, with 1–2 mA as the discharge current. Typically, 0.5–1 μg of sample was deposited on the FAB target, using magic bullet as the matrix. Instrument control and data collection were achieved using an HP Chem Station data system interfaced to the Nermag mass spectrometer (Quad Services, Poissy, France).

ESI/MS analyses were performed either with the aforementioned instrument fitted with an Analytica of Branford (Branford, CT) ESI source or with a Finnigan LCQ (Thermo Quest, Les Ulis, France) quadrupole ion-trap mass spectrometer. In both cases, solution samples (typically 10 ng/μl in methanol/water, 1:1, v/v) were directly infused into the ESI source at a flow rate of 1–3 μl/min, with a syringe pump. Additional structural information was obtained using either in-source CID (with the Nermag single-quadrupole instrument) (Debrauwer and Bories, 1992; Debrauwer *et al.*, 1997) or MS/MS experiments (with the LCQ ion-trap spectrometer). To determine some structural features, H/D exchange experiments were also performed using deuterated solvents (CD_3OD and D_2O), as described elsewhere (Debrauwer *et al.*, 1997).

Statistics. Comparisons between percentages were performed using the Student *t* test, after data transformation into square roots to correct for unequal variances.

Results

Metabolic Balance in Rats. After a single oral dose of 200 μg/kg CL and an 8-day study period, 87.1 ± 2.5% of the ^{14}C dose was recovered in males and 90.2 ± 4.2% was recovered in females. Radioactivity was excreted mainly in urine and to a lesser extent in feces (table 1), for which higher values were found in females ($p < 0.05$). In female rats, excretion of radioactivity in urine was slower, compared with that in male rats, and ^{14}C excretion in feces remained relatively important until day 5 (fig. 1).

At the end of the study, residual radioactivity levels found in carcasses corresponded to 3.62 ± 0.36 ng of CL equivalents/g in males but only 2.20 ± 0.62 ng of CL equivalents/g in females ($p < 0.05$). Expired $^{14}CO_2$ was quantified in two additional rats and accounted for <1% of the administered dose.

Radio-HPLC Profiling and Quantitation of CL Urinary Metabolites. *Profile of Urinary Metabolites.* Fourteen different radiochemical peaks were detected in rat urine. Some of the peaks were never detected (or were detected in only trace amounts) in freshly collected urine. A urinary radiochromatographic profile from a rat given a single oral dose of 200 μg/kg CL is presented in fig. 2, to demonstrate the qualitative distribution and complete pattern of CL metabolites. This analysis was performed with urine that had been stored for 24 hr at 4°C. Therefore, compounds **M9** and **M12** appear in the chromatogram; they would have not been detected if the same sample had been analyzed immediately after collection.

Radiochromatographic analysis of urine was performed with samples collected during the metabolic balance studies. Table 2 presents the results obtained for each group of animals in the 0–24-hr collection period after CL administration. **M5** and **M11**, and to a lesser extent **M1** and **M3**, were the main metabolites detected, with **M5** being the major metabolite in both genders. A fifth compound (**M8**) accounted for >15% of the radioactivity in male rat urine, whereas only background signal was recorded for female urine at the corresponding elution time (approximately 26 min). Additional gender-related differences were observed. Metabolite **M2** was never detected in female rat urine. In this group, the proportion of unchanged CL was considerably greater than that in males (37 and 16%, respectively). In contrast, analysis of urine collected in the later days of the study demonstrated that **M4**, **M7**, and **M10** were formed by both genders. In addition, this analysis showed that the contributions of CL and **M5** to

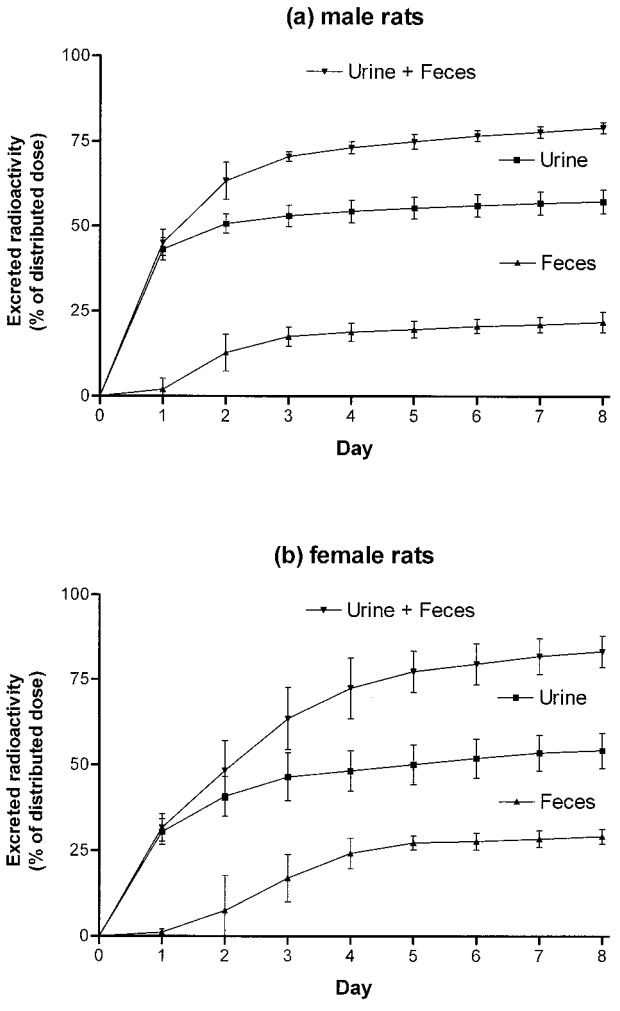

FIG. 1. *Urinary and fecal cumulative radioactivity excretion in male (*a*) and female (*b*) rats after a single oral dose of 200 μg/kg [^{14}C]CL.*

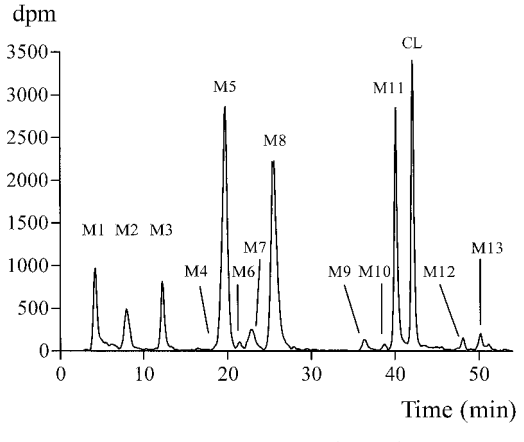

FIG. 2. *Typical radiochromatographic analysis of urine from a male rat, after an oral dose of 200 μg/kg CL.*

Urine was collected for 12 hr after CL administration and was stored for 24 hr at 4°C before analysis.

the urinary radioactivity quickly decreased, whereas all other metabolites were better represented in the later samples. In these later samples, the minor urinary metabolites **M4** and **M10** were present in greater amounts than in day 1 urine.

TABLE 2

Metabolic balance in rats; radio-HPLC quantitation of CL metabolites in male and female rat urine collected 0–24 hr after an oral dose of 200 μg/kg CL (N = 4 in both groups)

Metabolite[a]	Relative Amounts of CL and CL Metabolites in Day 1 Urine	
	Males	Females
	% of detected radioactivity	
M1 (UM)	10.0 ± 0.9	12.8 ± 0.7
M2 (UM)	2.6 ± 0.1	ND[b]
M3 (SCL)	7.0 ± 1.5	12.2 ± 2.9
M4 (ADOA)	ND	ND
M5 (*N*-OH-CL)	27.4 ± 1.0	23.2 ± 8.1
M6 (UM)	0.9 ± 0.2	T[c]
M7 (**M9** glucuronide)	1.0 ± 0.6	ND
M8 (ADMA)	15.9 ± 2.0	0.7 ± 0.5
M9 (OH-CL)	ND	ND
M10 (UM)	ND	T
M11 (ADHA)	15.8 ± 0.8	12.3 ± 2.2
CL	16.2 ± 0.2	36.9 ± 6.4
M12 (NO-CL)	1.4 ± 0.7	T
M13 (NO_2-CL)	1.7 ± 0.9	1.5 ± 1.3

Values are mean ± SD.
[a] UM, unidentified metabolite.
[b] ND, not detected.
[c] T, trace (<0.5% of the total radioactivity detected).

Influence of CL Dose on Urinary Metabolite Pattern. For three rats given high single oral doses of CL, urine samples were collected during the first 96 hr after drug administration. Before metabolite isolation, these samples were analyzed by radio-HPLC. The respective contributions of the parent compound and its major metabolites to the overall radioactivity in urine were calculated and are presented in fig. 3. In addition to unchanged CL, **M5** was by far the main metabolite detected in the urine of rats receiving 5–40 mg/kg CL. Moreover, the proportion of **M5** in urine was greater with higher doses

Enzyme Hydrolysis. When male rat urine was incubated for 16 hr with *H. pomatia* juice, almost all of the radioactivity initially detected as **M5** shifted to the retention time of CL. In addition, **M7** was no longer detected, and a corresponding amount of radioactivity was associated with **M9**. **M5** was also found to be transformed (partially or totally) into CL when incubated with sulfatase or with *H. pomatia* juice and D-saccharic acid 1,4-lactone, but similar results were obtained in control incubations carried out under the same conditions with no enzyme.

Radio-HPLC Profiling and Quantitation of CL Fecal Metabolites. Preliminary experiments demonstrated that lyophilization did not cause the loss of radioactivity from feces, nor it did modify the results of radio-HPLC analyses. Therefore, all feces collected during metabolic studies were lyophilized before solvent extraction, which was carried out separately on samples containing the highest radioactivity levels, *i.e.* days 2 and 3 for males and days 2–4 for females. In male rat feces, 85% of the radioactivity was found to be extractable at day 2 or 3 of the study. Results were similar in females (86% at day 3) and did not vary significantly through days 2–4. The first two acidic extracts always accounted for >95% of the total radioactivity recovered.

M3 was the major metabolite characterized in male and female rat fecal extracts. In male rats, **M3** and CL represented 67.6 ± 12.4 and 28.3 ± 13.2%, respectively, of the radioactivity detected by radio-HPLC analyses (day 3). Very small amounts of one (or more) polar metabolite(s) were also observed, with a retention time of 4–5 min. Radiochromatographic profiles obtained from females rat feces were quite similar (fig. 4). At day 3, **M3** and CL accounted for 78.1 ± 3.5 and 15.8 ± 4.0%, respectively, of the radioactivity detected in female rat feces. In this group, a minor metabolite was also present and was

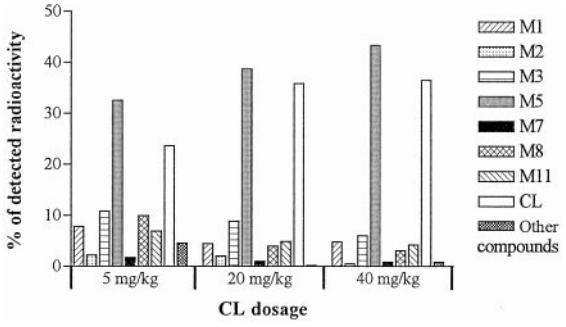

FIG. 3. *Quantitation of the major urinary metabolites of CL in male rats orally dosed with 5, 20, or 40 mg/kg CL.*

Results were calculated on the basis of radio-HPLC analyses of samples collected from day 1 to day 4 after CL administration and are expressed as percentages of the overall urinary radioactivity.

shown to coelute with **M10**, which had been previously isolated from urine. Limited amounts of **M9**, **M11**, and polar metabolites were observed in some of the extracts. Day 2 and day 4 feces analyses produced very similar results. A high CL dose did not produce any major modification of the fecal metabolic profile. For instance, in the rat dosed at 40 mg/kg, **M3** alone still represented nearly 60% of the radioactivity in the extracts of pooled day 1–4 feces, whereas unchanged CL accounted for >30%. In addition, small amounts of **M9** and approximately 4% **M10** were detected.

Residual Radioactivity in Tissues. *Quantitation and Extraction*. Results obtained from four male rats sacrificed 12, 24, 48, or 72 hr after an oral dose of 200 μg/kg CL are presented in fig. 5*A*. Liver was the target organ, but significant residue levels were also measured in the lungs and kidneys. Radioactivity quickly decreased in all tissues over 48 hr but was still detectable in liver and lungs on day 3. To precisely determine the amounts of bound residues in tissues, six successive extractions were performed at different pH values. For all samples, >90% of extractable radioactivity was recovered in the first two acidic extracts. Bound radioactivity in liver accounted for 32% (12 and 24 hr), 39% (48 hr), and 64% (72 hr) of CL residues. On day 8, results from rats in the metabolic balance study indicated that residual ^{14}C levels in liver corresponded to 14.2 and 12.8 ng of CL equivalents/g in males and females, respectively ($p > 0.05$). At that time, only 24.2 $\pm$ 7.0% of the radioactivity was found to be extractable in males and 32.8 $\pm$ 13.2% in females.

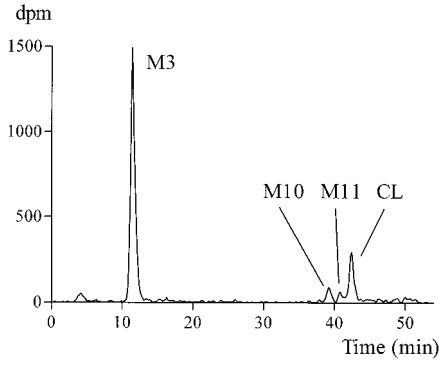

FIG. 4. *Fecal metabolic profiling in female rats after a single oral dose of 200 μg/kg CL.*

An example of radiochromatographic analysis after extraction of lyophilized feces collected on day 2 of the study is shown (metabolite **M11** was not systematically detected when other extracts were analyzed).

Metabolic Profiles in Tissues. All radiochromatograms obtained after liver extract analyses were qualitatively similar. In addition to CL (which always represented more than one half of the detected radioactivity), two metabolites, possessing the same retention times as **M10** and **M13**, were observed. Forty-eight hours after CL administration, the metabolites accounted for 32 and 17%, respectively (fig. 5*B*). Some liver samples were spiked with known amounts of ^{3}H-labeled *N*-OH-CL before solvent extraction. In these experiments, nearly 100% of the ^{3}H-labeled *N*-OH-CL was reduced to CL when the entire extraction procedure was performed, but only approximately 80% was reduced when a quick preparation method was used.

Analyses of kidney and lung extracts were carried out only for the first two animals. The corresponding radiochromatograms are presented in fig. 5, *C* and *D*. Most of the radioactivity in kidneys was associated with unchanged CL (approximately 70%) and one other compound, which coeluted with **M11**. In lung extracts, only one metabolite was observed together with CL. The elution time of this metabolite was the same as that of **M13**. The metabolite accounted for 32 and 24% of the ^{14}C detected in extracts of samples obtained from rats sacrificed 12 and 24 hr, respectively (fig. 5*D*), after CL administration.

Metabolite Isolation and Identification. *CL*. CL was isolated from both rat urine and feces. Its structure was confirmed by ESI/MS analysis, on the basis of its quasimolecular $[M+H]^+$ ion (*m/z* 277) and some diagnostic fragments (*m/z* 259, 203, and 168), which have been extensively described elsewhere (Debrauwer and Bories, 1993; Doerge *et al.*, 1993; Debrauwer *et al.*, 1997).

Metabolites **M5**, **M12**, *and* **M13**. The isolation procedure and the identification of these compounds were reported previously (Zalko *et al.*, 1997; Debrauwer *et al.*, 1997). Metabolites **M5**, **M12**, and **M13** were identified as the 4-*N*-hydroxylamine (*N*-OH-CL), 4-nitroso (NO-CL), and 4-nitro- (NO_2-CL) analogues of CL, respectively. The chemical instability of these compounds, which was extensively discussed previously (Zalko *et al.*, 1997), explains misleading results such as those observed after incubation of rat urine with *H. pomatia* juice, *i.e.* reduction of *N*-OH-CL to CL.

Metabolite **M3**. **M3** was isolated from rat urine as well as from fecal extracts. Using negative-ion FAB/MS, the $[M-H]^-$ ion of **M3** was observed at *m/z* 355; it exhibited the characteristic isotopic pattern of a species with two chlorine atoms. No fragmentation was observed in this case (data not shown). When analyzed by means of positive-ion ESI/MS, **M3** exhibited $[M+H]^+$ (*m/z* 357), $[M+Na]^+$ (*m/z* 379), and $[M+K]^+$ (*m/z* 395) quasimolecular ions, thus allowing the unambiguous confirmation of its molecular mass (fig. 6*A*). Some fragmentation was induced by means of CID occurring in the capillary-skimmer region of the ESI source (Debrauwer and Bories, 1992; Debrauwer *et al.*, 1997). The main decomposition process observed was loss of SO_3, leading to CL and subsequent CID products described elsewhere (Debrauwer and Bories, 1993; Doerge *et al.*, 1993; Debrauwer *et al.*, 1997). Nevertheless, a low-intensity fragment was observed at *m/z* 339, indicating that loss of H_2O from the $[M+H]^+$ ion of **M3** could occur, even if this process was much less favorable than the SO_3 loss (fig. 6*A*). The occurrence of the *m/z* 339 fragment ion indicated that the benzylic alcohol function of CL remained free in **M3**, thus allowing identification of this metabolite as SCL. SCL was found to be stable in rat urine stored below −20°C. It was not hydrolyzed by *H. pomatia* juice, in incubations with crude urine, nor was it hydrolyzed by *H. pomatia* juice, or *A. aeruginosa* sulfatase after purification from urine or feces. Interestingly, when isolated and stored in methanol at −20°C, SCL was slowly deconjugated to CL. Under these conditions, the estimated half-life of SCL was approximately 4 months.

Delay after clenbuterol administration (hours)	ng/g of clenbuterol equivalent		
	Liver	Lung	Kidney
12	158	58	45
24	107	53	34
48	41	6	14
72	31	5	ND

A

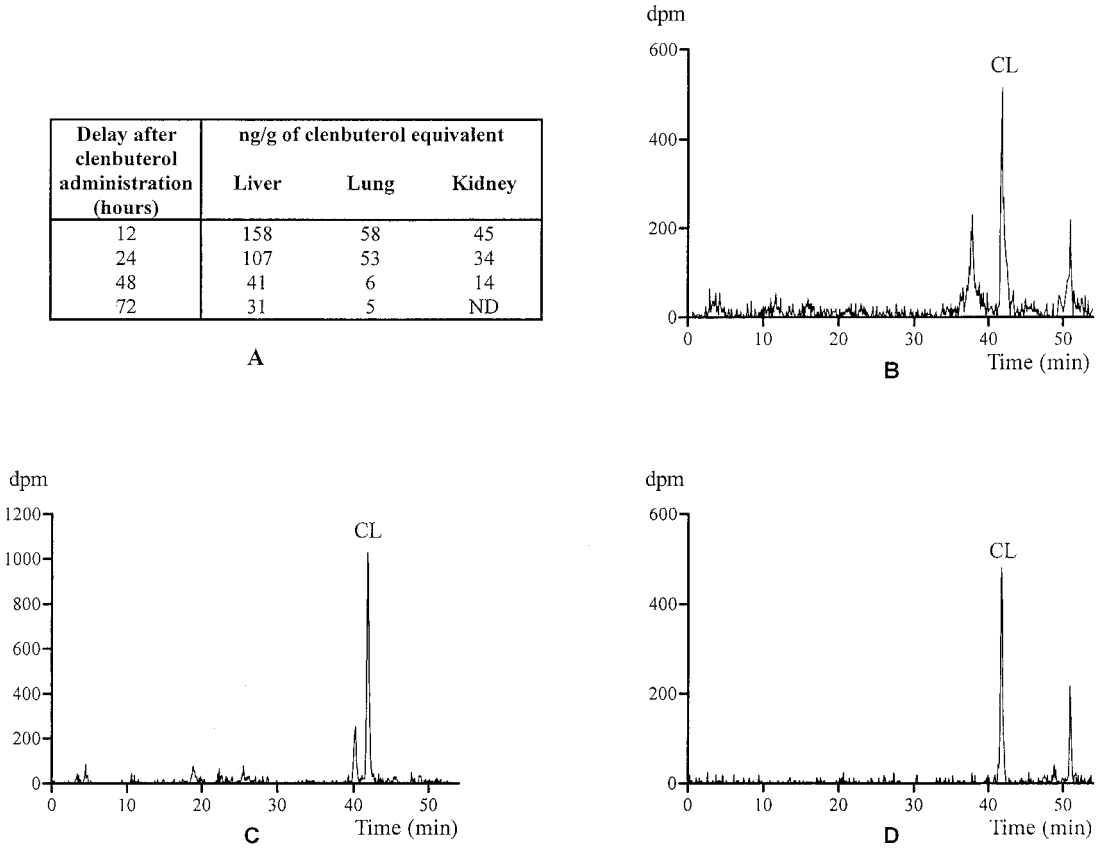

FIG. 5. A, *radioactivity levels measured in liver, lung, and kidney of rats sacrificed 12–72 hr after a single oral dose of 200 μg/kg [^{14}C]CL (results are expressed in nanograms of CL equivalents per gram of tissue);* B–D, *typical radiochromatographic profiles obtained after solvent extraction for liver (48 hr) (*B*), kidney (24 hr) (*C*), and lung (24 hr) (*D*).*

Metabolite **M11**. The MS analysis of **M11** was performed by positive-ion ESI/MS. Under these conditions, the $[M+H]^+$ ion of **M11** was observed at *m/z* 263, which was consistent with a $C_9H_8O_3N_2Cl_2$ molecular species (fig. 6*B*). Using negative-ion FAB/MS with thioglycerol as the matrix, a weak signal was also observed at *m/z* 261, thus confirming the molecular mass of **M11** (data not shown). As for **M3**, ESI/MS in-source CID was used to obtain more structural information. The main decompositions observed consisted of neutral CO_2 and glycine losses, giving rise to *m/z* 219 and 188 fragment ions, respectively. On these bases, **M11** was characterized as ADHA. The unconjugated analogue of **M11**, namely ADBA, was not detected in rat urine analyzed soon after collection, regardless of the rat gender or the CL dose (the retention time of standard ADBA in chromatographic system 1 is approximately 48 min).

Metabolites **M4** *and* **M8**. At pH 2, both compounds were almost completely extracted from urine with ethyl acetate. Because of their acidic natures, both metabolites gave no response when analyzed by FAB/MS or ESI/MS in the positive-ion mode. When **M4** was analyzed by negative-ion ESI/MS, an $[M-H]^-$ ion was observed at *m/z* 232, with an $[M_{d3}-D]^-$ ion at *m/z* 234 being generated under deuterium-labeling conditions. This was in agreement with a molecule of molecular mass 233 bearing three active hydrogen atoms. The MS/MS spectrum of the *m/z* 232 quasimolecular ion exhibited fragment ions at *m/z* 204 and 160, corresponding to the consecutive losses of CO and CO_2, respectively. From these observations, **M4** was identified as ADOA.

Using negative-ion ESI/MS, **M8** yielded a positive signal at *m/z* 234 ($[M-H]^-$ species). When subjected to in-source CID processes as well as MS/MS experiments in the ion-trap analyzer, the $[M-H]^-$ ion decomposed mainly into *m/z* 190 and 154 fragment ions, corresponding to consecutive losses of CO_2 and HCl, respectively (fig. 6*C*). This allowed tentative identification of **M8** as ADMA. Additional H/D exchange experiments resulted in the shifting of the *m/z* 234 ion to *m/z* 237, corresponding to an $[M_{d4}-D]^-$ species (data not shown). This was consistent with a molecule with four mobile hydrogen atoms, thus eliminating the option of the isobaric 4-nitro-3,5-dichlorobenzoic acid molecule in the identification of **M8**.

Metabolites **M7** *and* **M9**. Very small amounts of **M7** were isolated from rat urine, because the major portion of **M7** was found to be transformed into **M9** during the various isolation steps, regardless of the separation method used. **M9** was characterized by positive-ion ESI/MS, using in-source CID. As observed for metabolite **M5**, **M9** exhibited an $[M+H]^+$ ion at *m/z* 293, corresponding to an hydroxylated form of CL. In H/D exchange experiments, the quasimolecular ion was shifted from *m/z* 293 to *m/z* 299, indicating that **M9** contained one more exchangeable hydrogen than did CL (data not shown). From the $[M+H]^+$ ion (*m/z* 293), the main informative fragment ions obtained by in-source collisional activation consisted of *m/z* 275 and 203 ions (fig. 6*D*). According to previously established fragmentation pathways for CL (Debrauwer and Bories, 1992, 1993; Debrauwer *et al.*, 1997), the occurrence of the *m/z* 275 to *m/z* 203 transition indicated that the hydroxylation site was located on the *tert*-butyl moiety of the CL molecule, and **M9** was identified as OH-CL. In urine, **M7** was deconjugated to **M9** by *H. pomatia* extract (with or without D-saccharic acid 1,4-lactone), but not by type VI sulfatase, and it was consequently hypothesized to be a glucuronide conjugate of **M9**.

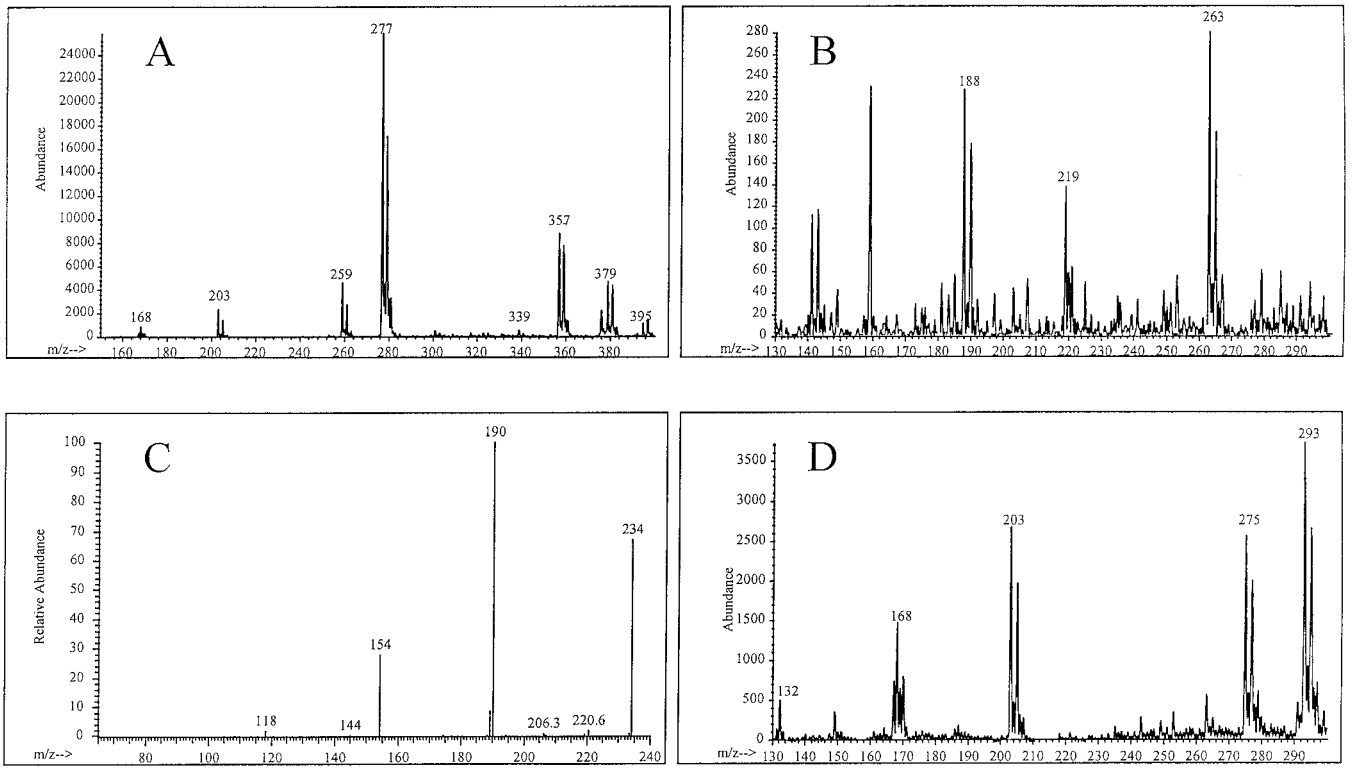

FIG. 6. *ESI mass spectra of CL metabolites, recorded for metabolite* **M3** *under positive-ionization conditions without in-source CID (*A*), for metabolite* **M11** *under positive-ionization conditions with in-source CID (*B*), for metabolite* **M8** *under negative-ionization conditions using MS/MS of the* m/z *234 precursor ion (*C*), and for metabolite* **M9** *under the same conditions as for* **M11** *(*D*).*

Discussion

In rats given a single oral dose of ^{14}C-labeled CL, elimination of radioactivity was relatively rapid and occurred mainly in the urine. In male rats, urinary ^{14}C excretion in the first 48 hr (*i.e.* nearly 90% of the radioactivity recovered in urine in the whole study) accounted for more than one half of the distributed dose. These results were quite similar to those described in two previous reports (Kopitar and Zimmer, 1976; Tanabe *et al.*, 1984b). In the same experimental group, 18% of the administered radioactivity was eliminated in feces in the first 72 hr (23% by the end of the study). Again, these results were in agreement with those of Tanabe *et al.* (1984b), who previously demonstrated that fecal excretion of CL was relatively important in rats. No other work was available for comparison.

Metabolic studies in female rats revealed gender-related differences in CL pharmacokinetics. Although levels were quantitatively similar by the end of the study, the excretion of radioactivity in urine by female rats was much slower, compared with that by male rats, and remained relatively important until day 5. Moreover, ^{14}C elimination in feces lasted longer for females and consequently was significantly more important than for males at the end of the experimental period (>30% of the initial radioactive dose). In male and female rats, 87 and 90%, respectively, of the initial radioactive dose was recovered by day 8 of the study. This rather incomplete metabolic balance was not related to significant $^{14}CO_2$ expiration, because that route accounted for only an additional 1%, nor it was related to residual radioactivity remaining in the metabolism cages. Therefore, the loss of ^{14}C may be associated with the occurrence of volatile urinary metabolites, which would have not been characterized in the present study.

Using specific HPLC conditions, 13 different metabolites were detected in male rat urine (11 in female rat urine). The structure of the main metabolites of CL was elucidated. A metabolic scheme of CL biotransformations in this species is presented in fig. 7 and should complete the results previously published by Schmid *et al.* (1990). The major pathway of CL metabolism, as discussed extensively elsewhere (Zalko *et al.*, 1997), was found to be *N*-oxidation of the parent drug to the corresponding hydroxylamine and NO_2-CL, with the latter being formed in very small amounts. NO-CL, resulting from the chemical autoxidation of *N*-OH-CL (Zalko *et al.*, 1997), could also appear in urine samples after storage. The present study confirmed the importance of *N*-oxidation reactions in CL metabolism, although these biotransformations were not observed previously (Schmid *et al.*, 1990). This major metabolic pathway, for which no gender-related difference was observed, was clearly favored when high oral doses of CL were used in rats. Nevertheless, although *N*-OH-CL was recently demonstrated to be a major metabolite of CL produced when the drug was incubated with rat liver microsomes (Zalko *et al.*, 1998), it was not detected in liver extracts of rats dosed with 200 μg/kg CL. Using ^{3}H-labeled *N*-OH-CL, it was possible to show that this compound would be reduced to CL during the liver extraction procedure. However, when sample preparation for HPLC analysis was shortened, some ^{3}H-labeled *N*-OH-CL was recovered unchanged, thus demonstrating that *N*-OH-CL was very likely not present in liver extracts.

The second important pathway of CL metabolism involved oxidative cleavage of the side chain of the molecule, with the formation of ADMA and further biotransformations leading to ADOA and ADBA (fig. 7). The occurrence of these metabolites, as well as that of ADHA (resulting from the conjugation of ADBA with glycine), was previously reported (Tanabe *et al.*, 1984a; Schmid *et al.*, 1990). In the present work, very small amounts of ADOA were detected in rat urine. Moreover, no radioactive peak corresponding to ADBA (on the basis of the retention time of the commercial standard) was observed in freshly collected urine samples. Consequently, ADBA formation

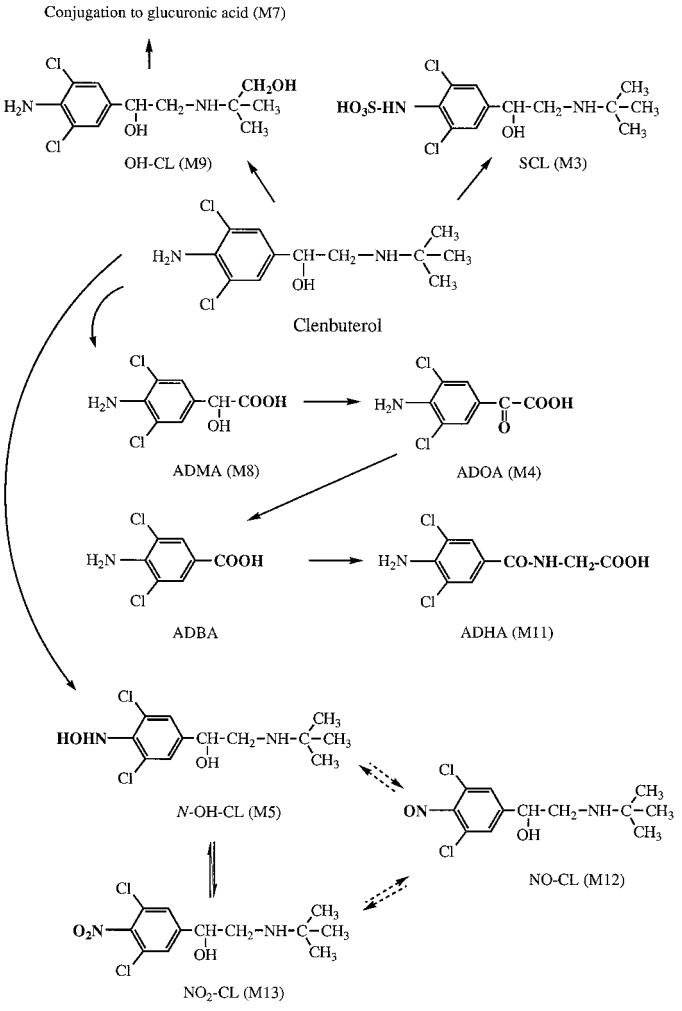

FIG. 7. *Proposed metabolic pathways for CL in rats.*

was demonstrated only indirectly, *i.e.* after the identification of ADHA. Only little (if any) ADMA was detected in female rat urine, whereas this metabolite accounted for 16% of the urinary radioactivity in males treated with 200 μg/kg CL. Nevertheless, because the formation of ADHA (which implies that of ADBA and ADOA) is thought to depend on the initial *N*-dealkylation of CL to ADMA and because ADHA is a major urinary metabolite of CL in both male and female rat urine, it is concluded that ADMA production is very likely not male-specific. A possible explanation for the absence of ADMA in female rat urine is suggested by previous experiments, in which the rates of some *N*-dealkylation reactions were shown to be much lower in female rats than in mature male rats, *e.g.* in the case of ethylmorphine (Nerland and Mannering, 1978).

Oxidation of the *tert*-butyl group of CL was observed. The resulting metabolite, namely OH-CL, was characterized from rat urine but was not detected in samples analyzed soon after collection. Indeed, only an OH-CL conjugate (**M7**) was present in fresh urine. **M7** was spontaneously labile and was tentatively identified as a glucuronic acid conjugate of OH-CL, based on enzymatic hydrolysis tests. Sulfoconjugation of CL on the primary amine was found to be another important biotransformation reaction for the drug. SCL was characterized from rat urine, thus confirming previous findings (Schmid *et al.*, 1990). The development of a procedure for extraction from lyophilized feces allowed the identification of SCL as the major compound present in these samples, regardless of the rat gender or the CL dose administered. SCL was not deconjugated when it was incubated with *H. pomatia* juice or a specific sulfatase, a finding that is probably related to the sulfation site.

Extraction methods were also developed to study the metabolic profiles of CL in liver, lung, and kidney. These three tissues were previously demonstrated to be the sites in which detectable CL levels lasted longest in Wistar rats after a 200 μg/kg dose of the drug (Tanabe *et al.*, 1984b). The amounts of CL equivalents found in these organs were in full accordance with the results of the more specific study of Tanabe *et al.* (1984b), except that the values we obtained for the lung were slightly higher. Radio-HPLC studies of lung extracts indicated the presence of only one metabolite. This compound, which was also observed in all liver extracts, exhibited chromatographic characteristics similar to those of NO_2-CL but could not be isolated in sufficient amounts to allow MS studies and direct structural confirmation. Another metabolite, of lower polarity than CL, was detected in liver extracts, and limited amounts of ADHA were found in kidney.

This work has clearly confirmed that the metabolism of CL is very different from that of β-agonists sharing the catechol structure, for which conjugation reactions were demonstrated to be the most important detoxication pathways (Brès *et al.*, 1985; Morgan, 1990). In the case of CL, as well as mabuterol (a β-agonist of closely related structure) (Horiba *et al.*, 1984), oxidative cleavage of the side chain of the molecule results in the production of several compounds, most of which possess an acidic function. On the other hand, important biotransformations (*N*-oxidation and sulfate conjugation) occur on the primary amine group of CL. The quantitation of *N*-oxidized compounds was difficult, because of their chemical instability. Consequently, low-pH extraction procedures and HPLC were useful for the study of CL metabolism. The methods developed for this study should be suitable for the investigation of CL metabolism in the target species, *i.e.* cattle, and may be adapted for the study of the metabolism of CL analogues (Leyssens *et al.*, 1993; Saltron *et al.*, 1996).

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