METABOLISM OF CLENBUTEROL IN RATS

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ABSTRACT:
The metabolic fate of [14C]clenbuterol was studied in male and female Wistar rats. After a single oral dose of 200 μg/kg [14C]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 [14C]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 14C-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. [14C]CL [4-amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylaminomethyl]benzyl alcohol; NO2-CL, 4-nitroso-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylaminomethyl]benzyl alcohol; SCL, clenbuterol 4-amino sulfonic acid; ADHA, 4-amino-3,5-dichlorobenzoic acid; ADMA, 4-amino-3,5-dichloropurinic acid; ADOA, 2-(4-amino-3,5-dichlorophenyl)-2-oxoacetic acid; ESI, electrospray ionization; FAB, fast atom bombardment; CID, collision-induced dissociation.

Other Chemicals. All solvents (of analytical grade) and formic acid were obtained from Protabo (Paris, France). Chemicals were purchased from the
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-h light/dark cycle. Mean rat weights were 472 ± 21 g (males) and 299 ± 24 g (females). After a 1-week acclimatization period, each animal was force-fed a single oral dose of [14C]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 immediately used.

**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 [14C]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 [14C]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/Radiomatic Labo) before radio-HPLC analysis. The radioactivity in each sample was determined. Approximately 0.5 g of each sample was lyophilized and then homogenized in a Dangoumeau ball-grinder (Pro-labo). 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.** Lungs and kidneys 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 C18 column (250 × 4.6 mm, 5 μm; SFCC, Eragny, France), coupled to an Hypersil BDS C18 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 B, respectively). 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 C18 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, 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 37°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.

**Metabolic 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
M13, using chromatographic separation and C8 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 a prewashed, reverse-phase, Select B C8 cartridge (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 C8 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 C8 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 C8 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 N2, and reconstituted in 10 mM ammonium acetate buffer, pH 3.2/acetoniil (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 C8 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 Analystica 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 (CD3OD and D2O), 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 14C 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 14C 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 14CO2 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. MS 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
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)

<table>
<thead>
<tr>
<th>Metabolitea</th>
<th>Relative Amounts of CL and CL Metabolites in Day 1 Urine</th>
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<tbody>
<tr>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>% of detected radioactivity</td>
<td></td>
</tr>
<tr>
<td>M1 (UM)</td>
<td>10.0 ± 0.9</td>
</tr>
<tr>
<td>M2 (UM)</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>M3 (SCL)</td>
<td>7.0 ± 1.5</td>
</tr>
<tr>
<td>M4 (ADOA)</td>
<td>ND</td>
</tr>
<tr>
<td>M5 (N-OH-CL)</td>
<td>27.4 ± 1.0</td>
</tr>
<tr>
<td>M6 (UM)</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>M7 (M9 glucuronide)</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>M8 (ADMA)</td>
<td>15.9 ± 2.0</td>
</tr>
<tr>
<td>M9 (OH-CL)</td>
<td>ND</td>
</tr>
<tr>
<td>M10 (UM)</td>
<td>ND</td>
</tr>
<tr>
<td>M11 (ADHA)</td>
<td>15.8 ± 0.8</td>
</tr>
<tr>
<td>CL</td>
<td>16.2 ± 0.2</td>
</tr>
<tr>
<td>M12 (NO-CL)</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>M13 (NO2-CL)</td>
<td>1.7 ± 0.9</td>
</tr>
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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 did it 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. 1. Urinary and fecal cumulative radioactivity excretion in male (a) and female (b) rats after a single oral dose of 200 μg/kg [14C]CL.](image)

![Fig. 2. Typical radiochromatographic analysis of urine from a male rat, after an oral dose of 200 μg/kg CL.](image)
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, the major modification of the fecal metabolic profile. For instance, in the rat dosed at 40 mg/kg, M9 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. 5A. 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 14C 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 ± 7.0% of the radioactivity was found to be extractable in males and 32.8 ± 12.2% in females.

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. 5B). Some liver samples were spiked with known amounts of 3H-labeled N-OH-CL before solvent extraction. In these experiments, nearly 100% of the 3H-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. 5C 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 14C detected in extracts of samples obtained from rats sacrificed 12 and 24 hr, respectively (fig. 5D), 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- (NO₂-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. 6A). 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 SO₃, 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 H₂O from the [M+H]⁺ ion of M3 could occur, even if this process was much less favorable than the SO₃ loss (fig. 6A). 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.
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$_9$H$_8$O$_3$N$_2$Cl$_2$ molecular species (fig. 6B). 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$_{43}$−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. 6C). 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$_{43}$−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 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).

![Graph A](image1.png)

**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 14C/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).
Discussion

In rats given a single oral dose of 14C-labeled CL, elimination of radioactivity was relatively rapid and occurred mainly in the urine. In male rats, urinary 14C 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, 14C 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 14CO2 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 14C 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 NO2-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 3H-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 3H-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...
Conjugation to glucuronic acid (M7)

\[
\begin{align*}
\text{H}_2\text{N}-\text{CH}_{2}-\text{NH}-\text{C}_6\text{H}_{13} & \rightarrow \text{H}_2\text{N}-\text{CH}_{2}-\text{NH}-\text{C}_6\text{H}_{13} \text{O} \rightarrow \text{H}_2\text{O} \rightarrow \text{H}_2\text{O} \rightarrow \\
\text{OH-CL (M9)} & \rightarrow \text{OH-CL (M9)} \rightarrow \text{OH-CL (M9)} \rightarrow \\
\text{SCL (M3)} & \rightarrow \text{SCL (M3)} \rightarrow \text{SCL (M3)} \rightarrow \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{N}-\text{CH}_{2}-\text{NH}-\text{C}_6\text{H}_{13} & \rightarrow \text{H}_2\text{N}-\text{CH}_{2}-\text{NH}-\text{C}_6\text{H}_{13} \text{O} \rightarrow \text{H}_2\text{O} \rightarrow \text{H}_2\text{O} \rightarrow \\
\text{ADMA (M9)} & \rightarrow \text{ADMA (M9)} \rightarrow \text{ADMA (M9)} \rightarrow \\
\text{ADOA (M4)} & \rightarrow \text{ADOA (M4)} \rightarrow \text{ADOA (M4)} \rightarrow \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{N}-\text{CH}_{2}-\text{NH}-\text{C}_6\text{H}_{13} & \rightarrow \text{H}_2\text{N}-\text{CH}_{2}-\text{NH}-\text{C}_6\text{H}_{13} \text{O} \rightarrow \text{H}_2\text{O} \rightarrow \text{H}_2\text{O} \rightarrow \\
\text{ABDA} & \rightarrow \text{ABDA} \rightarrow \text{ABDA} \rightarrow \\
\text{ADHA (M11)} & \rightarrow \text{ADHA (M11)} \rightarrow \text{ADHA (M11)} \rightarrow \\
\end{align*}
\]

\[
\begin{align*}
\text{N-OH-CL (M5)} & \rightarrow \text{N-OH-CL (M5)} \rightarrow \text{N-OH-CL (M5)} \\
\end{align*}
\]

\[
\begin{align*}
\text{NO}_2-\text{CL (M13)} & \rightarrow \text{NO}_2-\text{CL (M13)} \rightarrow \text{NO}_2-\text{CL (M13)} \\
\end{align*}
\]

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

was demonstrated only indirectly, i.e. after the identification of ADMA. 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 Manpering, 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. Sulfocojugation 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 (Schmidt 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 NO2-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 (Brés 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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**References**


