COMPARATIVE METABOLISM OF CLENBUTEROL BY RAT AND BOVINE LIVER MICROSONES AND SLICES

DANIEL ZALKO, ELISABETH PERDU-DURAND, LAURENT DEBRAUWER, MARIE-PIERRE BEC-FERTE, AND JACQUES TULLIEZ

Laboratoire des Xénotbiotiques, INRA

(Received May 30, 1997; accepted September 23, 1997)

ABSTRACT:
The metabolism of clenbuterol by liver microsomal fractions and precision-cut liver slices was studied in rats and cattle using a 14C-labeled molecule and radio-HPLC quantitation of the resulting metabolites. 4-N-Oxidation of clenbuterol was found to be an extensive in vitro metabolic pathway in both species. Clenbuterol hydroxylamine was by far the major metabolite characterized from microsomal and slice incubation media. Analysis of incubation media from liver slices also allowed the quantitation of a few additional metabolites, some of which were shown to be conjugated compounds. 4-Amino-3,5-dichlorobenzoic acid, was detected when clenbuterol was incubated with bovine or rat liver slices. Structural characterization of the major metabolites was performed using electrospray ionization-mass spectrometry, either coupled to liquid chromatography or with direct infusion of collected samples. In addition to these compounds, only quantitatively minor metabolites were detected in bovine (but not rat) microsomal incubation media. Analysis of incubation media from liver slices also allowed the quantitation of a few additional metabolites, some of which were shown to be conjugated compounds.
closely related to their structures, particularly to their oxidative status. Therefore, the exact structures of such metabolites need to be established.

The aim of the present study was to compare, qualitatively and quantitatively, CL metabolism in vitro in rats and in the target species, i.e. cattle. The cytochrome P450-dependent oxidation pathway was investigated using liver microsomes. In a second step, CL was incubated with precision-cut liver slices, because it is now well established that this in vitro system constitutes an excellent model for comparative metabolism and toxicity studies (Parrish et al., 1995). Special care was given to the analytical procedure, to avoid chemical oxidation/reduction reactions.

Materials and Methods

**Chemicals.** CL, CL hydrochloride was obtained from Sigma (Saint Quentin Fallavier, France). [benzyl-3 H][CL] and [14C][CL] (labeled at the benzyl carbon) were purchased from Rotem Industries (Beer-Sheva, Israel) and Isotopochim (Ganagobie-Peyruis, France), respectively. The labeled molecules had specific activities of 474 GBq/mmol ([3 H]CL) and 197 MBq/mmol ([14C]CL). Their respective radioiurieties were >97%.

Nevertheless, both were purified using HPLC (see below) with a fraction collector and then RP Select B C8 cartridges (Polylabo, Strasbourg, France), as described elsewhere (Zalko et al., 1997), to reach 99.4% ([3 H]CL) or 99.6% ([14C]CL) purity. The CL structure was confirmed by ESI-MS. CL metabolites used as standards (N-OH-CL, NO-CL, NO2-CL, sulfamate, 4-amino-3,5-dichlorohippuric acid, and the analogue of CL hydroxylated at its tert-butyli moiety) were purified from rat urine as described elsewhere (Zalko et al., 1996, 1997). ADBA was purchased from Pfaltz & Bauer Inc. (Waterbury, CT).

**Other Chemicals.** 7-[3-14C]EC was purchased from Amersham (Buckinghamshire, UK). Formic acid and all solvents (of analytical grade) were obtained from Prolabo (Paris, France). Acetic acid was purchased from Merck (Darmstadt, Germany). 7-EC, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, MgCl2, phosphate buffer, and ascorbic acid were obtained from Sigma.

**Animals.** Six male Wistar rats, weighing about 250 g, were purchased from Ifla Credo (L’arbresle, France). Rats were fed a standard diet (UAR 210; Usine d’Aliments Rationalisés Villemoisson-sur-Orge, France) and had free access to water during a 7-day acclimatization period. They were killed by cervical dislocation, followed by immediate exsanguination. Three untreated male Charolais calves (age, 6 months) were obtained from a local breeder. Calves were fed hay ad libitum, supplemented with an adapted commercial cattle concentrate, and had free access to water. After a 1-week acclimatization period, calves were killed by captive bolt and exsanguination.

**Preparation of Liver Slices.** Three rat livers were perfused with 40 ml of ice-cold (4°C) oxygenated Krebs-Henseleit buffer to remove blood. Liver cores were obtained using a stainless steel tube and a drill press. Cores were immediately placed in ice-cold buffer. A Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL) was used to produce precision-cut slices of 8-mm diameter and 0.2-mm thickness (Krumdieck et al., 1980). An equilibration period of 45 min allowed for the sloughing of damaged liver slices were performed with 25 μM labeled 7-EC for 1, 2, or 4 h. Biotransformation of 7-EC to 7-OHCl, 7-OHCl sulfate, and 7-OHCl glucuronide was studied by radio-HPLC analysis of filtered incubation media. Structures of the conjugated metabolites of 7-EC were determined after enzymatic hydrolysis (Stenisma et al., 1994). Additionally, the integrity of cells in slices was determined by measuring LDH leakage in an aliquot of the culture medium, using a LDH detection kit (procedure 228-UV; Sigma).

**Preparation of Liver Microsomes.** Three rat livers were removed immediately after slaughtering and perfused with 0.9% saline. Liver microsomes were prepared as described elsewhere (Perdu-Durand and Tulliez, 1985) and stored at ~80°C in phosphate buffer (0.1 M, pH 7.4) containing 20% glycerol. The protein concentration was determined by the method of Lowry et al. (1951). Bovine liver samples were obtained from the three Charolais calves described above, and liver microsomes were prepared as described for rats.

**Metabolism of CL in Liver Slices.** Slices were incubated in 12-well, plastic, tissue culture plates (Dortegon, 1993) using Dulbecco’s modified Eagle’s medium. The incubation volume was 1 ml. For both rat and bovine liver slices, assays were performed for each animal with 12.5, 25, 50, or 100 μM labeled CL (10,000 Bq/incubation) for 1, 2, or 4 h. Plates were maintained at ~37°C in a rotary shaker, set at 90 rpm. At the end of each incubation period, media were removed and ascorbic acid (1 mM) was added. Two 50-μl samples were taken for radioactivity determination, before immediate storage at ~80°C until analysis. Slices were gently washed twice with water to allow the measurement of residual radioactivity.

Control incubations were carried out with CL under the same conditions without slices. In addition, for each animal, the 12.5 μM incubations were performed in duplicate, to study concurrently the qualitative distribution of the metabolites remaining inside the slices.

**Metabolism of CL with Liver Microsomes.** Incubations were performed in vials containing 2 mg of microsomal protein, 1 mM NADP, 10 mM glucose-6-phosphate, 5 mM MgCl2, 2 units of glucose-6-phosphate dehydrogenase, and labeled CL, in a final volume of 1 ml of phosphate buffer (0.1 M, pH 7.4). For both species, assays were performed with 12.5, 25, 50, and 100 μM CL, including 10,000 Bq of [14C]CL. For each CL concentration, vials containing microsomal incubation were quickly concentrated under a nitrogen stream and then stored at 80°C in phosphate buffer (0.1 M, pH 7.4) containing 20% glycerol. After a 1-h incubation period, media were removed and ascorbic acid (1 mM) was added. Two 50-μl samples were taken for the determination of radioactivity in the supernatants, which were then stored at ~80°C until analysis.

Microsomal protein samples were individually washed twice with water and then checked for residual radioactivity by combustion. Control incubations were carried out under the same conditions but without a NADPH-generating system and were subjected to the same analytical steps.

**Sample Preparation and Analysis.** All samples were individually thawed immediately before analysis. Aliquots (150 μl) of the supernatants from each microsomal incubation were quickly concentrated under a nitrogen stream and then filtered on a 0.5-g, 0.45-μm, Ultrafree MC filtration unit (Polylabo) before radiochromatographic analysis. Aliquots (100 μl) of each liver slice incubation medium were filtered and analyzed by following the same procedure. All samples were analyzed consecutively within each incubation series (i.e. each animal). To confirm the reproducibility of the chromatograms and the stability of metabolites generated in vitro, several samples were injected twice, with the second analysis taking place at the end of the series. No significant qualitative or quantitative differences were found.

Slices from 12.5 μM CL incubations performed in duplicate were washed with water and homogenized in 0.5 ml of ammonium acetate buffer (10 mM, pH 3.2), using a pellet pestle. Each extract was centrifuged at 10,000 rpm and 4°C for 10 min. The supernatant was filtered and then analyzed by HPLC. The pellet was extracted twice more, with acetonitrile/methanol/ammonium acetate buffer (100 mM, pH 6.8) (60:30:10, v/v/v) and acetonitrile/methanol/sodium hydroxide (1 mM) (60:30:10, v/v/v) successively. Radioactivity was determined by liquid scintillation counting (supernatants) or after combustion (residual pellets).

**Analytical Procedures.** CL Metabolism Studies. Liver microsomal fractions and slice incubation media 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, for radioactivity detection, to a Packard Flo-one ADBA detector (Packard Instruments Co., Meriden, CT) (scintillation cocktail, Flow-scint II; Packard Instruments Co., Downers Grove, IL). For CL purification and ADBA characterization, the Philips 4100 apparatus was connected to an absorbance detector set at 254 nm (P) and to a Gilson model 202 fraction collector (Gilson France, Villiers-Le-
Bel, France). The HPLC system consisted of an Ultrabase C₁₈ column (250 × 4.6 mm, 5 μm; SFCC, Ergany, France) coupled to a Hypersil BDS C₁₈ guard precolumn (18 × 4.6 mm, 5 μm; Shandon/LSI, Cergy Pontoise, France). The mobile phases consisted of ammonium acetate buffer (10 mM) adjusted to pH 3.2 and acetonitrile, at 95:5 (v/v) in solvent B, respectively. The flow rate was 1 ml/min at 35°C. A two-step gradient was used, as follows: 0–5 min, 100% A; 5–25 min, linear gradient from 100% A to 95:5 (v/v) A/B; 25–30 min, linear gradient from 5% B to 40% B; 30–35 min, linear gradient leading to 100% B; 35–45 min, 60:40 (v/v) A/B; 45–47 min, linear gradient leading to 100% B; 47–54 min, 100% B.

The radioactivity in the incubation medium was determined by direct counting in a Packard scintillation counter (model Tricarb 2200CA; scintillation cocktail, Packard Ultima Gold). Metabolite quantitation was carried out by measuring radioactivity in the individual peaks separated by HPLC, using the Packard Flo-one/β A500 detector. Residual radioactivity in microsomal pellets and liver slices was determined by complete combustion in a Packard 306 oxidizer and counting of 14CO₂ in the scintillation counter (scintillation cocktail, Packard Permafluor E+/Packard Carbo-sorb, 2:1, v/v).

Incubation of Slices with 7-EC. Incubation media were studied by radio-HPLC, using the same apparatus as for CL and a C₁₈ Spherisorb ODS2 (250 × 4.6 mm, 5 μm) column (Interchim, Montluçon, France) coupled to a Hypersil BDS C₁₈ guard precolumn. Mobile phases consisted of ammonium acetate buffer (50 mM, pH 5.0) and acetonitrile, 85:15 (v/v) in solvent A and 20:80 (v/v) in solvent B. The flow rate was 1 ml/min. A three-step gradient was used, as follows: 0–4 min, 100% A; 4–10 min, linear gradient from 100% A to 95:5 (v/v) A/B; 10–30 min, 95:5 (v/v) A/B; 30–35 min, linear gradient from 5% B to 40% B; 35–45 min, linear gradient from 50:50 (v/v) A/B to 45:55 (v/v) A/B; 45–47 min, linear gradient leading to 100% B; 47–54 min, 100% B.

Metabolite Identification. Metabolite M₁. The structure of the major metabolite generated by CL incubation with liver microsomal fractions and liver slices was confirmed using HPLC coupled to ESI-MS. Before analysis, samples were filtered on 0.45-μm Ultrafree MC (Polylabo) and 0.22-μm Millex-GS (Millipore, France) filtration units, successively. LC/ESI-MS analyses were performed in the positive-ion mode on a Finnigan LCQ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA). Data were acquired in the selected-ion monitoring mode. In-source collision conditions were used to induce some fragmentation, as described elsewhere (Debrauwer et al., 1997). The MS analysis of M₁ was performed on a Finnigan LCQ quadrupole ion-trap mass spectrometer. ESI was used in the negative-ion mode. The MS as well as MS-MS experiments were used for the characterization of M₁ by comparison with the authentic standard ADBA.

Statistical Analysis. Analysis of variance was performed on the residual radioactivity levels found in rat and bovine microsomal protein pellets, using the general linear-models procedure of the SAS.
Results

Metabolism of CL by Liver Microsomal Fractions and Structural Identification. Radiochromatographic Profiles and M1 Characterization. The metabolism of CL by bovine and rat liver microsomal fractions was studied with four concentrations of CL (12.5, 25, 50, and 100 μM) after 60 min of incubation. In rats, radiochromatographic analysis of the supernatants (fig. 2a) showed only one metabolite (M1), whatever the concentration of the drug. This metabolite was demonstrated to coelute with N-OH-CL, which had been previously purified from rat urine and characterized (Zalko et al., 1997).

The chromatograms obtained from the LC/ESI-MS analysis of a rat microsomal incubation with 100 μM CL are presented in fig. 3. Positive signals were obtained for the four ions monitored for N-OH-CL and CL (table 1), at the same retention times as for the standard compounds (i.e. 16.4 and 21.6 min, respectively). Moreover, the relative signal intensities obtained for each mass fragmentogram were in agreement with those observed in the ESI-MS spectra of both standard compounds in the same conditions. This allowed the unambiguous identification of the unique CL metabolite produced in incubations of CL with rat liver microsomal fractions as N-OH-CL. The CL N-hydroxylase activity was best described by a Michaelis-Menten curve, showing saturation of the enzymatic system (fig. 4).

When CL was incubated with bovine liver microsomal fractions, several metabolites were detected in the supernatants (fig. 2b) but the overall metabolism rate was lower than in rat microsomal incubations. For instance, in 25 μM CL incubations, the metabolism rates for CL were 139.7 ± 19.0 and 46.6 ± 11.8 pmol/min/mg of microsomal protein for rats and cattle, respectively. As observed for rat microsomes, the major metabolite (M1) produced by bovine liver microsomes showed the same chromatographic characteristics as N-OH-CL, but another quantitatively important compound, of lesser polarity than CL, was also detected (M4).

M4 Identification. M4 was first hypothesized to be the 4-nitroso analogue of CL, based on chromatographic characteristics similar to those of the rat urinary metabolite NO-CL. Thus, the products of incubations of 100 μM CL with bovine microsomes were analyzed by LC/ESI-MS, as described for rat incubations except that four additional ions were monitored, to examine the occurrence of NO-CL (table 1). The LC/ESI-MS analysis produced the same results as in rats, i.e. it confirmed the presence of N-OH-CL and CL but no NO-CL was detected. On the other hand, when analyzed by HPLC coupled to radioactivity and absorbance detection, the chemical standard ADBA was found to exhibit a retention time identical to that of both the standard NO-CL and M4. ADBA was also demonstrated to coelute with NO-CL by using the same HPLC apparatus under isocratic conditions (65:35, v/v, A/B). The correspondence between M4 and

---

**Fig. 3.** Ion chromatograms obtained from LC/ESI-MS analysis of rat microsomal incubations with CL (100 μM).

(a), Total ion chromatogram; (b), selected-ion monitoring traces. The retention times of N-OH-CL and CL, using this chromatographic system, were 16.4 and 21.6 min, respectively.
Additional evidence resulted from analysis of the media from incubations of CL with bovine liver microsomes, based on the results of radio-HPLC analyses. Thus, the acidic nature of M4 was confirmed. M4 (as well as ADBA in a separate experiment) was fully retained on such cartridges, whereas M1 and CL were not. Moreover, an MS-MS experiment was performed on the m/z 204 ion, giving rise to a diagnostic daughter ion at m/z 160, resulting from the loss of CO₂ from the selected parent ion (fig. 5b). The same results were obtained from the analysis of standard ADBA, thus enabling the unambiguous identification of M4.

The relationship between the formation of the two major metabolites detected in bovine liver microsomal incubation media (N-OH-CL and ADBA) and the initial concentration of CL is shown in fig. 6. In this species, the saturation of N-OH-CL formation was reached at a lower concentration than for ADBA. Three minor metabolites were observed in incubations of CL with bovine liver microsomes. M2 was found to coelute with the hydroxylated metabolite of CL [4-amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylaminomethylbenzyl alcohol] previously isolated from rat urine (Zalko et al., 1996), for which hydroxylation occurred on one of the methyl groups of the tert-butyl moiety of CL. M5 exhibited the same retention time as NO₂-CL, whereas the very minor metabolite M3 was not identified.

TABLE 1

<table>
<thead>
<tr>
<th>Ions</th>
<th>m/z</th>
<th>a</th>
<th>b</th>
<th>a + b</th>
</tr>
</thead>
<tbody>
<tr>
<td>R = NH₂ (CL)</td>
<td>277/279</td>
<td>259</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>R = NH₂OH (N-OH-CL)</td>
<td>293/295</td>
<td>275</td>
<td>219</td>
<td></td>
</tr>
<tr>
<td>R = NO (NO-CL)</td>
<td>291/293</td>
<td>235</td>
<td>217</td>
<td></td>
</tr>
</tbody>
</table>

ADBA in 12.5 μM incubation media was tested using strong anion exchanger cartridges. M4 (as well as ADBA in a separate experiment) was fully retained on such cartridges, whereas M1 and CL were not. Thus, the acidic nature of M4 was confirmed. Additional evidence resulted from analysis of the media from incubations of [³H]CL with bovine liver microsomes. Indeed, no peak at the retention time of M4 could be detected in the corresponding radiochromatograms, thus demonstrating that, when [³H]CL was used, the radioactivity detected for M4 was not associated with NO-CL. Consequently, M4 was hypothesized to be a metabolite in which the hydrogen atom on the benzylic carbon had been lost, very likely after an oxidative cleavage leading to the loss of the side chain of CL.

In a first step, M4 analysis was performed by LC/ESI-MS in the positive ion mode, with an acidic buffered phase. M4 gave no response, nor did ADBA analyzed in the same way. Therefore, M4 was isolated from [¹⁴C]CL incubation media to be analyzed by negative ESI-MS using direct sample infusion. Under these conditions, the [M–H]⁻ ion of M4 was observed at m/z 204, as indicated in fig. 5a.

A Michaelis-Menten plot of N-OH-CL formation with various concentrations of CL (fig. 8) demonstrated saturation kinetics. Some minor metabolites were also occasionally observed but accounted for <2% of the total radioactivity detected in chromatographic profiles. In bovine slice incubations (fig. 2d), in addition to N-OH-CL (M1), only one metabolite could be detected, showing chromatographic behavior similar to that of 4-amino-3,5-dichlorohippuric acid (M8). Moreover, this metabolite was detected only in the incubation media corresponding to the two lowest CL concentrations. It never accounted for more than 1% of the radio-HPLC profile. The N-oxidation rate (table 2) was found to be much lower than in the rat slice incubations, and no NO₂-CL could be detected.

Rat and bovine slice incubation media (100 μM CL) were analyzed by LC/ESI-MS under the same experimental conditions developed for liver microsomal incubation media. In addition to CL, the occurrence of N-OH-CL was detected in the same manner as described above (data not shown).

Radio-HPLC analysis of the media from incubation of 7-EC with liver slices led to the detection of 7-OHC and the corresponding sulfate and glucuronide conjugates (table 3). As observed for CL, the overall metabolism rate of 7-EC was found to be much more important in rat than in bovine liver slices. Whatever the species, <20% of total slice LDH was recovered in the incubation media after 4 hr.

Residual Radioactivity in Slices. Between 4 and 12% of the initial...
radioactivity remained in the slices. The major part of this radioactivity was found to be extractable. Radio-HPLC analysis of extracts produced profiles similar to those obtained from supernatants. Only about 1% of the initial radioactivity remained in the extracted pellets, whatever the species or the incubation time.

**Discussion**

This work clearly demonstrated that CL was mainly metabolized by liver microsomes to the corresponding arylhydroxylamine. When rat liver microsomes were incubated with 12.5–100 μM CL, >50% of the drug underwent hydroxylation to N-OH-CL in 1 hr. In cattle, in vitro assays were performed only with livers obtained from fully ruminating animals, because several drug-metabolizing activities have been demonstrated to be weaker in veal calves than in older animals with functioning rumens (Kawalek and El Said, 1994). In this species, the hydroxylamine was also the major metabolite formed during incubation of CL with liver microsomes, but to a lesser extent than in rats, and the existence of a second important metabolic route was evident after the identification of ADBA.

LC/ESI-MS was used in the selected-ion monitoring mode for the confirmation of N-OH-CL and CL structures in microsomes as well as slice incubation media. Indeed, ESI-MS is an efficient tool for the structural characterization of β-agonists (Debrauwer and Bories, 1992), and the behavior of CL and its N-oxidation products with ESI and in-source collisionally activated dissociation has been extensively described (Zalko et al., 1997; Debrauwer et al., 1997). A positive signal was recorded when the ions corresponding to N-OH-CL and

---

**Fig. 5.** ESI-MS spectrum (a) and ESI-MS-MS product-ion spectrum of [M−H]− at m/z 204 (b) obtained from the negative-ion ESI-MS analysis of M4.

**Fig. 6.** Michaelis-Menten curves for N-OH-CL and ADBA formation in bovine liver microsomal incubations with various concentrations of CL (1 hr, 37°C, 2 mg of microsomal protein).

Data are mean ± SD obtained from three animals.

**Fig. 7.** Amounts of N-OH-CL detected in media of rat liver slice incubations (1, 2, or 4 hr of incubation) with CL.

Data are mean ± SD obtained from three animals.
CL, for all of the incubation media, were monitored by LC/ESI-MS. Conversely, when bovine microsomal incubation media were analyzed and four more ions corresponding to NO-CL were monitored, no signal was obtained for this compound. This, together with the analysis of duplicate incubations performed using [3H]CL, demonstrated that, despite the fact that the chromatographic characteristics of NO-CL were very close to those of ADBA, no nitroso analogue of CL was present in the media of CL incubations with bovine liver microsomes. The structure of ADBA was determined after direct isolation and subsequent negative-ion ESI-MS and ESI-MS-MS experiments achieved under direct sample infusion conditions.

In CL metabolism assays with liver slices, the ability of liver cells to produce metabolites resulting from oxidation as well as from conjugation reactions was confirmed by the use of 7-EC as a probe. This, together with the analysis of duplicate incubations performed using [3H]CL, demonstrated that 7-EC was metabolized in vivo. Moreover, the incubates were individually thawed just before HPLC analysis, and all samples were analyzed within 3 days after the assays. When samples were analyzed again after an additional storage delay, no significant modification of the radiochromatographic profiles was observed. Therefore, the oxidation of CL to NO-CL detected in bovine liver microsomes as well as rat slice incubation media is not related to the chemical oxidation of N-OH-CL during sample processing. In contrast, NO-CL was not detected in any of the incubation media. Duplicate assays carried out with a tritium-labeled molecule demonstrated that NO-CL was not formed when CL was incubated with bovine liver slices. This is consistent with results of previous in vitro experiments in rats (Zalko et al., 1997), which showed that NO-2-CL (but not NO-CL) was generated in small amounts and excreted in urine.

In incubations of CL with bovine microsomes, a metabolite possessing chromatographic characteristics similar to those of NO-CL was isolated and identified as ADBA. This compound was previously shown to be a urinary metabolite of CL in rats and dogs (Tanabe et al., 1984; Schmid et al., 1990). Moreover, other β-agonists of related structure, e.g., mabuterol (Horiba et al., 1984), have been shown to undergo a similar metabolic pathway.

Some minor metabolites were also identified in the present in vitro assays. 4-Amino-3,5-dichloro-α-(2-hydroxy-1,1-dimethyl)ethylaminomethylbenzyl alcohol was found in incubations of the drug with bovine liver microsomes. Two other compounds, i.e., CL sulfamate (in rats only) and 4-amino-3,5-dichlorohippuric acid, were characterized in incubations of CL with liver slices. Interestingly, although 4-aminoo-3,5-dichlorohippuric acid was observed in both species and results from the glycine conjugation of ADBA, the latter was not detected when CL was incubated with rat liver microsomes.

In work with precision-cut liver slices, no evidence was found for the formation of metabolites resulting from N-OH-CL conjugation. Although not detected, such biotransformations would not be ob-

**TABLE 2**

Quantitation of N-OH-CL in media of liver slice incubations with CL (12.5–100 μM)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Amount of N-OH-CL Detected in Supernatants (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5 μM CL</td>
<td>25 μM CL</td>
</tr>
<tr>
<td>1</td>
<td>0.46 ± 0.12</td>
</tr>
<tr>
<td>2</td>
<td>0.41 ± 0.17</td>
</tr>
<tr>
<td>4</td>
<td>0.29 ± 0.22</td>
</tr>
</tbody>
</table>

**TABLE 3**

Rates of 7-EC biotransformation by rat and bovine liver slices (mean of three animals for each species), as measured by radio-HPLC analysis

<table>
<thead>
<tr>
<th>7-OHC Glucuronide</th>
<th>7-OHC Sulfate</th>
<th>7-OHC</th>
<th>7-EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (pmol metabolite/min/mg protein)</td>
<td>7.9 ± 1.8</td>
<td>29.7 ± 4.0</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td>Bovine (pmol metabolite/min/mg protein)</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>5.7 ± 0.9</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>3.6</td>
<td>5.8</td>
<td>17.0</td>
</tr>
</tbody>
</table>
served in work with microsomes. The enzymatic conjugation of arylamine N-oxidized metabolites can be of major importance for their potential toxicity. Indeed, the formation of a nitrenium ion, considered to be an ultimate reactive species of these compounds, can be highly favored by a previous sulfate or acetate conjugation (Ford and Herman, 1991; Sabbioni and Wild, 1992).

Many β₂-agonists used illegally in meat production are arylamines (Saltron et al., 1996; Leyssens et al., 1993). This is also the case for some other drugs that have been used (Orton and Lowery, 1981; Uetrecht, 1984) or are in development for therapeutic use (Barrow et al., 1995) in humans. The present results indicate that in vitro studies could provide a useful tool for the understanding of N-oxide formation and further putative biotransformations. Moreover, given the structures of several β₂-agonists, these studies may allow simultaneous exploration of the two major metabolic pathways previously demonstrated for CL (Zalko et al., 1996; 1997; Tanabe et al., 1984; Schmid et al., 1990), which are possibly the same for closely related structures, i.e. N-oxidation of the primary amine and oxidative cleavage of the side chain of these molecules.

References