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DRUG METABOLISM AND DISPOSITION Vol. 11, No. 3 249-254 **AUTHORS' Version**

SIMPLE AND ION-PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AS AN IMPROVED ANALYTICAL TOOL FOR CHLORAMPHENICOL METABOLIC PROFILING

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Laboratoire de Recherches sur les Additifs Alimentaires I. N. R. A.

ABSTRACT:
Simple and ion-pair reverse phase high performance chromatographic separations combined with selective extraction were developed in order to achieve a qualitative and quantitative analysis of [^{3}H]chloramphenicol (CP) metabolites in rat urine. Complete separation followed by unequivocal identification of ail the metabolites was obtained, especially of the toxicologically significant CP-aryl-amine. Metabolic profiles were established from human and goat urine which confirmed data already available, but was used to identify new acidic metabolites in the latter species. This methodology appears to be a useful research tool in comparative metabolic studies.

Basic data on the fate of CPI in different species were produced by Glazko et al. (1-3) some 25 years ago. A major metabolic route involving glucuroconjugation with enterohepatic circulation, as well as the reduction of the nitro group by the intestinal flora and conjugation of the resulting amines were described in the rat, guinea pig, and dog. Since toxicological problems arise in humans, e.g. the rare but often fatal aplastic anemia, a great deal of work has been devoted to the study of the biochemical mechanisms involved in this toxic process. CP-protein binding observed in rat liver microsomes (4) focused attention on the dichloroacetate moiety of the molecule, and led to the discovery of a new route with CP-alcohol and CP-oxamic acid (fig. 1) as end products. More recently, the role of reduced CP metabolites, i.e., nitroso, hydroxylamine, and amine derivatives, has been emphasized (5, 6). However, the lack of data concerning the occurrence and extent of these complex biotransformations testifies to the fact that the metabolic fate of CP has to be reconsidered in depth in the whole animal. This deficiency must be found out in the analytical problems brought out by extraction, separation, and identification in complex media such as urine, and of numerous metabolites exhibiting extremely different chemical properties. The obtaining of complete metabolic profiles would therefore be of primary interest for pharmacokinetic studies as well as for comparative metabolic investigations. The original methodology developed by Glazko involving colorimetric and microbiological assays, where urinary and fecal metabolites were determined as chemically analogous families, namely aromatic amines, nitro compounds, or CP-conjugates, is now obsolete. Recently, HPLC has been used successfully for the separation of some CP-metabolites during in vitro experiments (7). Based on this powerful separative technique, the aim of this study is to develop an analytical tool suitable for the qualitative and quantitative evaluation of [^{3}H]CP pharmacokinetics in different species. A combination of simple and ion-pair reverse phase HPLC associated with selective extraction is proposed, which give extensive urinary profiles.

Abbreviations used are: CP, chloramphenicol; TBAP, terbutylammonium phosphate; HSA, heptane sulfonic acid; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; EIMS, electron impact MS; CIMS, chemical ionization MS; amu, atomic mass units.
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Materials and Methods

Labeled Molecule: [^{3}H]CP. (1R,2R)-1 p-nitrophenyl-(2,2'-dichlorace-tamido)-1,3-[1-^{3}H]propanediol, 185 mCi/mM, was prepared from CP according to Martin et al. (7), purified by HPLC, and then checked for radiochemical purity and stability.

Reagents. Methanol and ethyl acetate reagents for analysis (Prolabo, Paris) were redistilled before use. TBAP and HSA sodium salt were obtained from Interchim (Montluçon, France). BSTFA came from Pierce, Rockford, IL. Fluorescamine came from Fluka AG, Buchs, Switzerland.

Materials. Materials included the following: a high performance liquid chromatograph (Spectra-Physics 3500) equipped with a column (250 x 4.6 mm) slurry packed with the reverse phase RP-18 (Merck) 5-µm particle size, and a fixed wavelength UY detector operating at 280 nm; a fraction collector (Gilson Microcol TCD 80); a liquid scintillation spectrometer SL 32 Intertechnique and attached Multi 4 computer; and two gas chromatograph-mass spectrometer units: a Hewlett-Packard 5992 for EIMS studies, and a Ribermag R10-10 for CIMS.

Experimental Procedure. 200-g Wistar rats placed in metabolic cages received an im injection of 80 µCi [^{3}H]CP mixed with 20 mg nonradioactive CP in 1 ml propylene glycol. Urine was collected for 24 hr and kept frozen (-20°C) until analysis.

Three goats weighing about 35 kg each and placed in metabolic cages received an im injection of 600 ttCi [^{3}H]CP mixed with 600 mg nonradioactive CP in 5 ml propylene glycol. Urine was collected over a 24-hr period and kept frozen (-20°C) until analysis.

One human volunteer ingested a single dose of 500 mg nonradioactive CP. Urine was collected for 24 hr and kept frozen (-20°C) until analysis.

Urine Analysis. Tritiated water in the urine was determined by com-paring the radioactivity of aliquots measured before and after lyophilization. Urine samples were all lyophilized before analysis. The dry residue was solubilized either in distilled water and adjusted to pH 7.0 using diluted sodium hydroxide solution, or in 0.1 N hydrochloric acid. The final dilution was calculated so that the radioactivity content exceeded 4000 dpm/µl. Extraction of either neutral or acidic urine was carried out by shaking three times in 30 ml ethylacetate; the pooled organic extracts were measured for radioactivity and the residual urine was kept for further analysis. The organic phase was evaporated to dryness under vacuum, and the residue was dissolved in a 1-ml water/methanol (1:1) mixture; 10 µl were chromatographed on the octadecyl reverse phase column using methanol, 0.05 M NaHZP04 (30:70), pH 5.3, as mobile phase at a 0.6 ml/ min flow rate. 150-,u1 fractions were collected in disposable 3-ml scintillation counting tubes using a microcollector. UV absorption (280 nm) was recorded simultaneously. About 100 fractions (15 ml) were necessary for a complete elution of the radioactivity. After addition of 2.5 ml of a fluor mixture (Aqualuma, Kontrou, Velizy, France), each fraction was counted in a scintillation spectrometer. Results were computerized and read out as diagrams constituting urinary metabolic profiles.

The residual urines were neutralized (pH 7.0) using 1 N sodium hydroxide when necessary. Depending on their radioactivity contents, the urine was either diluted by half with methanol prior to direct injection into the chromatograph, or first preconcentrated under vacuum, using absolute ethanol as a water carrier. Ion-pair high performance liquid chromatography was performed on the same octadecyl reverse phase column, using either (i) methanol, 25 mM TBAP in distilled water (30:70), pH 7.5, or (ii) methanol, 0.05 M HSA sodium salt in distilled water (30:70), adjusted to pH 3.3 with H3P04, as mobile phases.

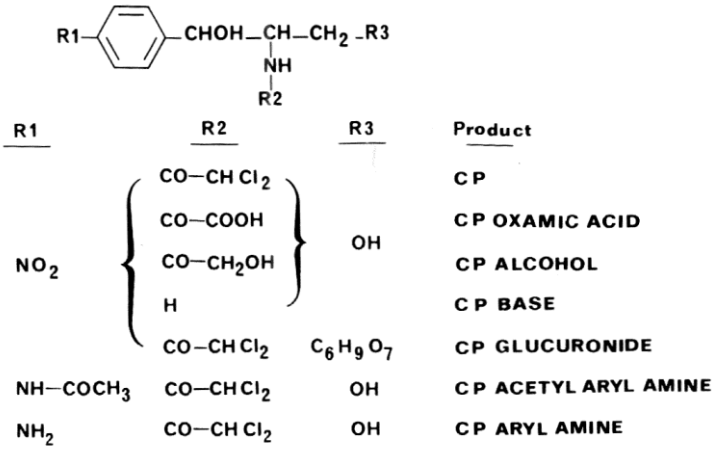

FIG. 1. *Formulas of chloramphenicol metabolites.*

Fraction collection and radioactivity measurements were operated as described for simple reverse phase analysis of the acidic extract. However, due to ion-pairing, retention times were longer, and 150 fractions were collected for a complete recovery of the metabolites.

The quantitation was performed by summation of the radioactivity in the fractions belonging to the same peak. When the separation was incomplete, the quantitation of each peak was obtained by summation of the well separated fractions, and distribution of the common fraction in proportion to peak heights. Recovery rates were evaluated by comparison with the total radioactivity injected into the chromatograph.

Fig.1. Formulas of chloramphenicol metabolites.

Identification of the Metabolites. In order to calibrate the urinary profiles, three labeled standards were used: [^{3}H]CP as previously men-tioned in this section; [^{3}H]CP-glucuronide, prepared from 24-hr rabbit urine following im administration of [^{3}H]CP according to the technique of Kamil et al. (8); [^{3}H]CP -base, resulting from CP-amide bond hydrolysis, and prepared from [^{3}H]CP according to Rebstock et al. (9).

In addition to the HPLC retention times (RT), confirmatory identification was obtained by comparing the EIMS spectra. When CP-glucuronide was concerned, enzymatic hydrolysis using /3-glucuronidase (bacterial origin, Institut Pasteur, Paris) was performed as follows. Ten IU of the enzyme were added to the fraction dissolved in 5 ml phosphate buffer pH 6.5, and then incubated at 37°C for 15 hr. Free CP liberated was analyzed as already described.

Other metabolites were assigned structures based on EIMS and/or CIMS analysis, because synthetic reference compounds were unavailable. Previous isolation and separation were obtained by using TLC on silica

(Silica Gel G, Merck, Darmstadt, Germany) with chloroform/methanol/ water/acetic acid (50:20:2:2) as a developing mixture; the radioactive spots were localized by radio-TLC scanning (Berthold/Friesek, Karlsruhe, Ger-many), the corresponding areas were scraped off, and the metabolites were eluted from the silica for further analysis. Derivatizations of the functional groups were used in order to facilitate GC/MS analysis. Methylation of the carboxylic groups was performed in 1-ml cortical vials in methanol (500 µl) by adding a diazomethane ethereal solution until a persistent coloration was obtained. Silylation of hydroxy groups was realized by using BSTFA directly on the dry residue, and heating 1 hr at 85°C.

Amine groups were detected directly in the HPLC fractions by using fluorescamine. 100 ,ul of the reagent in acetone solution (2 mg/5 ml) and 2 ml acetate buffer, pH 5.0, were added to the 150-l,1 fractions. Fluorescence production was measured at 499 nm under 400 nm excitation. Quantitation was obtained by comparing the emitted light with that of a p-aminohippuric acid fluorescamine derivative prepared in the same conditions, using a calibration curve. Further identification of these metabolites was carried out by derivatization of the amine group with monochloracetylchloride followed by silylation and EIMS final analysis.

[^{3}H]CP -base arylamine was prepared from [^{3}H]CP -base by reduction with zinc and hydrochloric acid. 100 mg [^{3}H]CP -base (106 dpm/mg) were dissolved in 20 ml 3 N HCI; 1 g zinc powder was added, and the mixture was left ai normal temperature for a half-hour. Fractions giving a positive reaction with fluorescamine were submitted to acid hydrolysis at pH 1 and 100°C for 1 hr; the resulting product was compared with the synthetic reference compound using HSA ion-pair reverse phase HPLC.

Results

Analysis of Rat Urine Metabolites. Fig. 2 reports the urinary profiles obtained from 24-hr rat urine using simple reverse phase liquid chromatography of the acidic extract, followed by HSA ion-pair analysis of the residual urine. The identification of the radioactive peaks was performed as follows.

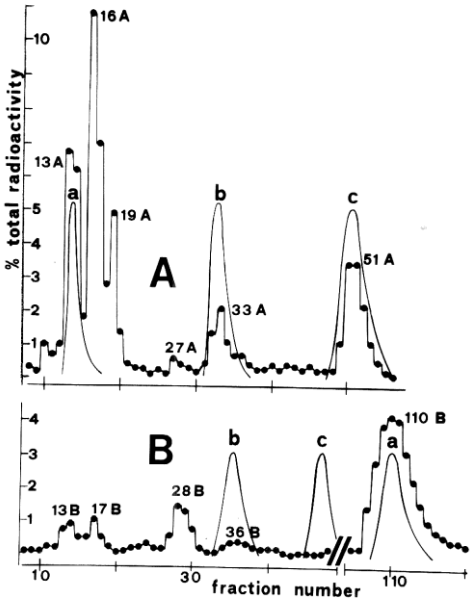

FIG. 2. *Rat urinary metabolite profiles: Radio-HPLC of 0–24-hr urine from rats after a single 20-mg (80 µCi) im dose of [^{3}H]CP.*

A, simple reverse phase HPLC of the acidic extract. *B*, HSA ion-pair HPLC of the residual aqueous phase. Peaks are identified by the number of the most radioactive fraction. Standards: *a*, CP-base; *b*, CP-glucuronide; *c*, CP.

FIG. 2. Rat urinary metabolite profiles: Radio-HPLC of 0-24-hr urine from rats after a single 20-mg (80 µCi) im dose of [^{3}H]CP.

Peak 13A. Radio TLC analysis (fig. 3) indicated a very major radioactive spot at RF 0.20 and a minor one at RF 0.71. Methylation plus trimethylsilylation, then EIMS analysis according to an already published study by the present authors (10), allowed the identification of CP-oxamic acid as being the major spot.

Peak 16A. A single TLC radioactive spot (RF 0.7 I) was obtained. A radio-gas chromatographic analysis of the corresponding trimethylsilylated substance on a 3% OV 17 on Supelcoport (Supelco) column at 280 to 290°C (programmation rate, 2°C/min) exhibited a major peak (I) with a retention time of 4.1 min, and a very minor peak (II) with a retention time of 2 min. CIMS analysis of peak I in the same chromatographic conditions, using isobutane or ammonia as reacting gas, identified the CP-acetylarylamine (fig. 4). Due to the low quantity of metabolite (II), no interpretable mass spectrum was obtained.

Peak 19A. TLC and GC analysis of these fractions were qualitatively identical to those obtained for peak 16A. However the GC peak (II) was by far predominant. EIMS analysis gave a spectrum identical to that of trimethylsilylated CP-alcohol obtained by Martin et al. (7). Confirmation of the CP-alcohol structure was given by using CIMS with ammonia; the spectrum showed the typical [MH]+ molecular ions at m/z = 487 and 489.

Peak 27A. This peak gave a single spot (RF 0.66), but the quantity of either radioactivity or material was too low to accom-plish further structural investigations.

P*eak 33A.* This peak was found identical to CP-glucuronide, either on the basis of RT, RF, or conversion to [^{3}H]CP folîowing P-glucuronidase hydrolysis.

Peak 51A. RT and RF were identical to those of the original [^{3}H]CP. Confirmation was obtained by EIMS analysis following silylation as already described (11).

Peak 13B. The same analysis as for peak 13A indicated CP-oxamic acid.

Peak 17B. An analysis was carried out as for peak 19A. It showed CP-alcohol and only traces of CP-acetylarylamine.

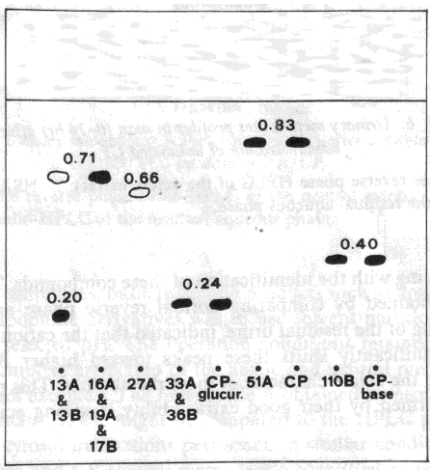

FIG. 3. Radio-TLC analysis of HPLC fractions (Fractions are numbered as in fig. 2.)

FIG. 5. Mass spectrum (EI) of monochloracetylated and trimethylsilylated metabolite from peak 28B.

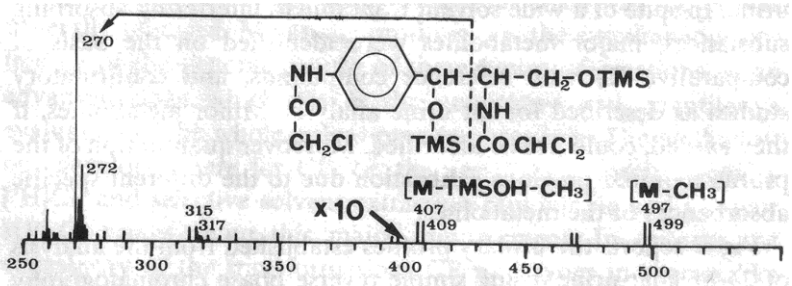

FIG. 5. *Mass spectrum (EI) of monochloracetylated and trimethylsilylated metabolite from peak 28B.*

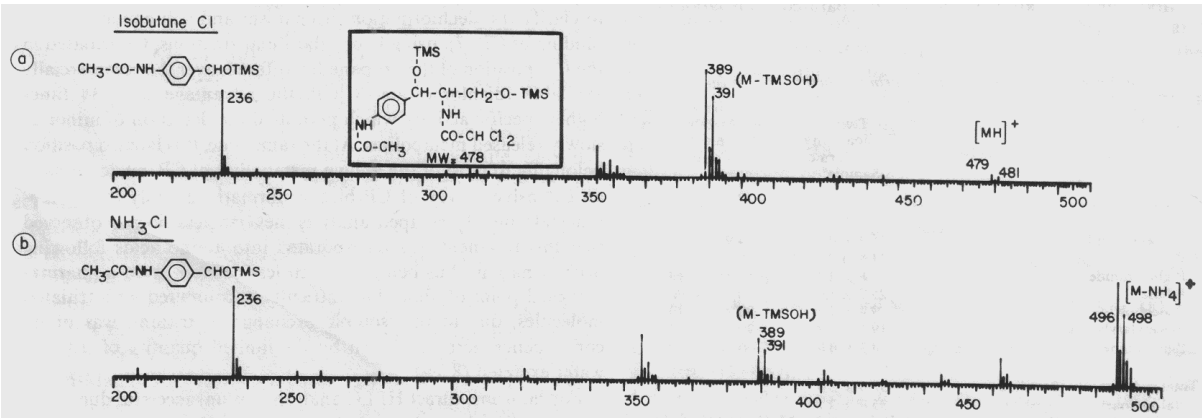

FIG. 4. *Mass spectra of trimethylsilylated (TMS) metabolite I.*

a, isobutane CIMS; *b*, ammonia CIMS.

FIG. 4. Mass spectra of trimethylsilylated (TMS) metabolite I. (a, isobutane CIMS; b, ammonia CIMS.)

Peak 28B. A positive reaction with fluorescamine was obtained from an aliquot, which suggested the presence of amines (12). Note that CP-base treated in the same conditions did not give any fluorescent compound. Monochloracetylation followed by trimethylsilylation, then EIMS analysis, gave a major ionic current peak; the spectrum (fig. 5) showed a base peak at 270 amu, which corresponds to the benzylic moiety normally appearing after CP fragmentation. The difference between this fragment weight and the 225 amu normally obtained with trimethylsilylated CP corresponds exactly to the substitution of the nitro group by the monochloracetylated amine group. A 272 amu and 36.6% base peak abundance fragment confirmed the presence of a chlorine atom. In other respects, the spectrum exhibited weakly abundant fragments of higher mass, mainly [M-15] = 497 and 499 amu and [M-105] = 407 and 409 amu, thus confirming the structure of this major metabolite as CP-arylamine.

Peak 36B. This peak was identified as CP-glucuronide, using the same methodology as for peak 33A.

Peak 110B. RT and RF were identical to those of the [^{3}H]CP -base reference compound. Trimethylsilylation followed by EIMS analysis according to Nakagawa et al. (11) confirmed this identification.

Quantitation of the Profiles. Seventeen per cent of the administered radioactivity was recovered in the 0-24-hr rat urine. Lyophilization of the original urine resulted in the loss of 8.5% radioactivity which corresponds to tritiated water. A quantitative evaluation of the different metabolites identified was established on the basis of the radioactivity contents of the fractions corresponding to the HPLC peaks in both simple reverse phase and ion-pair complementary profiles. The results are shown on table l. The total radioactivity recovered from all the fractions collected along the two runs was slightly higher than that attributed to identified

metabolites. The difference (2.5%) was mainly due to very minor unidentified peaks such as 27A, if it is considered that the radioactivity level reached between the peaks (26.2 ± 2.9 cpm) was similar to the usual background. When calculated from four replicates, the recovery of the radioactivity of the urine samples throughout the two profiles was 95.9 ± 3.1%. These results indicate that the initial partition of the metabolites between the organic phase and the acidified urine was complete for the original CP, CP-base, CP-arylamine, and other fluorescamine reactive products, and CP-acetylarylamine. CP-glucuronide was well separated on both HPLC systems, which means the radioactivity counts were directly additive. CP-oxamic and CP-acetylarylamine, were not completely separated in both profiles; thus, quantitation was carried out as described above. The saine was done for CP-acetylarylamine and CP-alcohol in profile A (fig. 2), but no separation was obtained in profile B and the quantitation was established from a gas chromatographic analysis of the trimethyl-silylated derivatives prepared from the TLC spots; it showed that the interference of CP-acetylarylamine on CP-alcohol determination was only of 1 of 25, which was considered as negligible.

Application to Man and Goat. Fig. 6 reports the urinary profile obtained from 24-hr human urine, using normal reverse phase analysis of the acidic extract plus HSA ion-pairing of the residual urine. In spite of a wide solvent front due to interfering absorbing substances, major metabolites were identified on the basis of comparative RT with reference compounds, and confirmatory studies as described for rat urine analysis. Minor metabolites, if they existed, could not be identified. Moreover quantitation of the profile required previous calibration due to the different specific absorbances of the metabolites.

Fig. 7 reports the urinary profiles established from the analysis of 24-hr goat urine, using simple reverse phase chromatography of the neutral extract, plus TBAP ion-pairing of the residual urine. Apart from CP-metabolites already identified using the procedure described, major unknown peaks were separated. This laboratory is continuing with the identification of these compounds. The first results obtained by comparing normal reverse phase and TBA ion-pairing of the residual urine, indicated that the cationic counter-ion significantly shifts these peaks toward higher RT, thus signifying the acidic character of these metabolites. This property was confirmed by their good extractability in strong acidic medium.

TABLE 1.
Quantitative evaluation of [^{3}H]CP urinary metabolites in the rat

TABLE 1

Quantitative evaluation of [^{3}H]CP urinary metabolites in the rat

	Total Radioactivity in the Urine Sample[a]	Partition Ratio of Each Metabolite between the Two Profiles	
		A	B
	%		
CP-oxamic acid	15.4 ± 0.4	84.9	15.0
CP	11.8 ± 0.6	100	0
CP-glucuronide	7.8 ± 2.4	85.5	14.5
CP-base	25.7 ± 1.0	0	100
CP-alcohol	9.4 ± 2.5	80.9	19.1
CP-acetylarylamine	19.1 ± 0.8	99.2	0.77
CP-arylamine	4.3 ± 0.9	0	100
Total radioactivity identified	93.4 ± 2.6		
Total radioactivity collected	95.9 ± 3.1		

[a] Mean percentage ± SD of four rats (tritiated water was removed by lyophilization prior to analysis and was not taken into account).

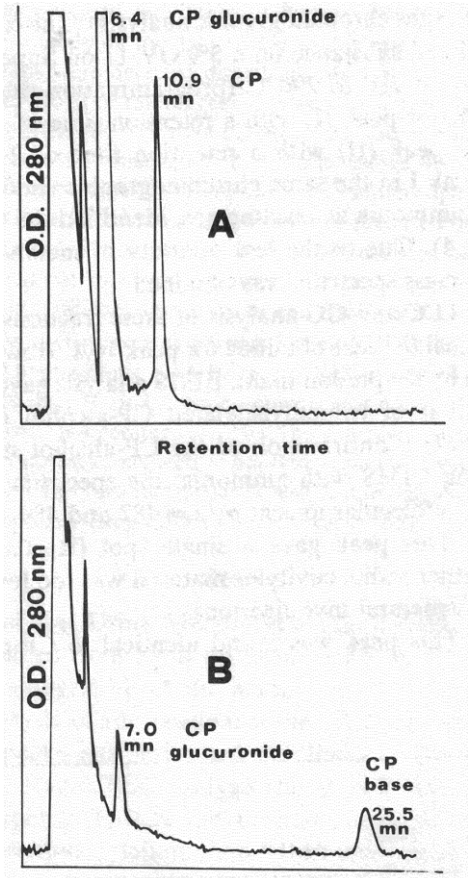

FiG. 6. Urinary metabolites profiles in man (0-24 hr) after po administration of unlabeled CP.
A, simple reverse phase HPLC of the acidic extract; B, HSA ion-pair HPLC of the residual aqueous phase.

FIG. 6. *Urinary metabolites profiles in man (0–24 hr) after po administration of unlabeled CP.*

Discussion

The use of labeled CP for an in vivo study was a first and original approach. [^{14}C]CP and [^{3}H]CP have been used before, but to clarify the dechlorination mechanism and subsequent protein binding of CP (7), using liver subcellular fractions. CP tritiated in the C-1 position of the propanediol offered, over the commercially available [dichloracetate-14C]CP, the advantage of a 34 times higher specific activity which permitted the detection of minor or slowly released metabolites. At the same time, this labeled position belonging to most of the known metabolites of CP, made possible an extensive survey of CP biotransformations. Only the dichloroacetate moiety escaped analysis; nevertheless, it was observed that this fragment was incorporated into amino acids following dechlorination, thus being of much less concern from a pharmacological point of view. The difficulty encountered with tritiated molecules, due to the isotopic exchange of tritium, was of no consequence here, as shown by the limited quantity of tritiated water excreted (8.5%).

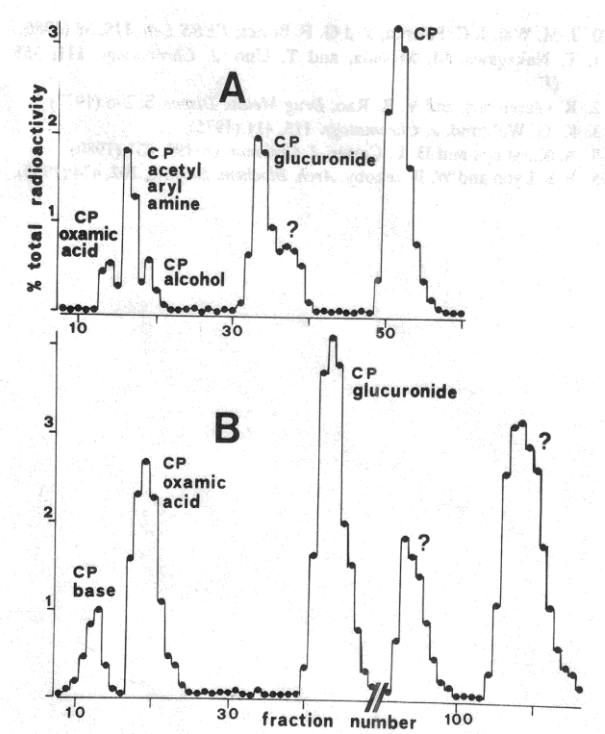

FIG. 7. *Urinary metabolites in the goat (0–24 hr) after a single 600-mg (600 µCi) im dose of [^{3}H]CP.*

A, simple reverse phase radio-HPLC of the neutral extract; *B*, TBAP ion-pair radio-HPLC of the residual aqueous phase.

FIG. 7. Urinary metabolites in the goat (0-24 hr) after a single 600-mg

For rat urine, direct HPLC analysis was unsuccessful due to the too great number of metabolites and insufficient separation. A simple way to discriminate among chemical families as different as acidic conjugates, basic reduction products such as amines, and neutral lipophilic derivatives was to use solvent partition of the urine at selected pH. The operation conditions retained (pH 1) gave a complete extraction of the acidic and neutral metabolites, CP-alcohol excepted. The first profile A obtained by using simple reverse phase HPLC might be compared to the HPLC profile of rat liver cytosol incubations performed in similar conditions (7). CP-oxamic and CP-alcohol were clearly identified in vivo using the same final GC/MS analysis used by these authors. Profile A exhibited two other metabolites: CP-glucuronide whose structure was already definitively established as 3-glucuronide (2), and CP-acetylarylamine. Conjugated amines had already been isolated from rat urine, and identified indirectly by using acid hydrolysis (3). These authors hypothesized that acetylation was the most likely conjugation process that CP-arylamine should undergo from the intestinal flora. However, no further evidence was brought out concerning the exact structure of this major metabolite, until its unequivocal GC/MS confirmation was established by the present authors. Profile B mainly contained the metabolites with basic properties. CP-base was already known as a urinary metabolite in the rat, dog, and man, and its structure was elucidated on the basis of comparative chromatographic behavior and properties with the synthethized reference compound (2). A direct confirmation was given here by using GC/MS analysis. Up to now, the detection of urinary metabolites having an amine function was based on the Bratton-Marshall colorimetric method which is specific for arylamines. However, if CP-arylamine was the most probable metabolite, it has never been clearly identified. Final confirmation was obtained here from the GC/MS analysis.

The recovery and reproducibility of the overall analytical procedure were very satisfactory; this made an exhaustive qualitative and quantitative analysis of the urinary metabolites in the rat possible. In addition, the high specific activity of [^{3}H]CP and sensitivity of the detection of the radioactivity in the HPLC fractions permitted the detection of minor compounds which represented as low as 1 % of total urine. Thus, the distribution of the whole urinary radioactivity among the different metabolites identified has been completely worked out in the rat. This constitutes a major improvement over the previous approach (1) which expressed the results in terms of chloramphenicol equivalents.

For human urine (fig. 6), unlabeled CP followed by UV detection had to be used, involving limited sensitivity and specificity. However, the less complex and perhaps incomplete profile obtained was in good

agreement with the metabolic data already available (1), namely, predominance of CP-glucuronide and the presence of CP-base.

As far as the goat is concerned, the method developed for rat urine profiling failed to resolve a group of metabolites behaving like CP-glucuronide. Cationic ion-pairing was successfully used for the separation of some acidic steroid conjugates, namely glucuronides and sulfates (13, 14). Analogous shifts and finally a similar pattern was obtained here by using TBA-pairing. The hypothesis that one of these metabolites could be CP-sulfate is consistant with the fact that sulfation of CP has been shown to occur in the rat (15). The identification is continuing in the present laboratory; however, this result highlights the interest of this methodology, and the versatility of its applications, in the explo-ration of the metabolic fate of CP in different species.

Detailed knowledge of the metabolic fate of a drug is a prerequisite in the complete understanding of either its mode of action or its adverse effects. Its complete elucidation is a difficult task for the biochemist, due to the long and tedious analytical procedures generally required. Metabolic profiling, i. e. the simultaneous obtaining of the general picture of these biotransformations, is an advanced stage which makes the qualitative and quantitative evaluation of the whole in vivo process accessible. The methodology developed here for CP, i. e. the use of high specific activity [^{3}H]CP and selective solvent extraction plus simple and ion-pair HPLC, brings about this major improvement. In spite of the complexity of the transformations CP undergoes in species like the rat and the goat, a complete separation and quantitation of urinary metabolites was obtained. Structures already established or only hypothesized were discovered, offering new ways of metabolic investigations. However, the isolation of CP-arylamine and arylamine-related derivatives is of particular concern when the attention of most authors is focused on the role of these substances and their intermediary reduction products on CP toxicity. Their origin, fate, and significance in the whole organism, as well as the special ability of certain species or individuals to reduce the CP molecule, may now be investigated.

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