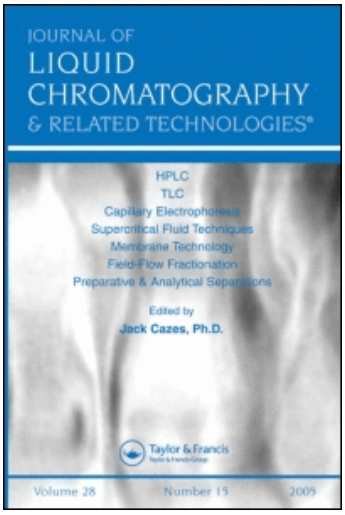

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF CHLORPROPHAM AND ITS METABOLITES IN ISOLATED RAT HEPATOCYTE INCUBATIONS

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ABSTRACT

Free chlorpropham (CIPC) and its metabolites (4-hydroxy CIPC, 3-chloroaniline and 3-chloroacetanilide) were separated by reverse phase high performance liquid chromatography (H.P.L.C.) using an isocratic procedure. The separation was used to quantify the metabolism of CIPC in rat hepatocyte incubations. The free metabolites were diethylether-extracted at pH 7.4. The sulfate and glucuronide conjugates of 4(OH)CIPC were enzymatically split and the aglycone determined by HPLC. Beside the unmetabolized CIPC which accounts for 33 % of the dose, 4(OH)CIPC as the sulfoconjugate and glucuroconjugate represents 35 % and 20 % respectively, while free 4(OH)CIPC accounts for only 3 %. About 10 % of the CIPC is hydrolytically split yielding 3-chloroaniline (2 %) which further transformed into 3-chloroacetanilide (5 %).
In spite of the great sensitivity of the proposed method (0.2 µg/ml incubation medium) no other metabolites were recorded. This extraction and separation procedure could be applicable to future metabolic studies in cellular incubation systems.

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INTRODUCTION

The metabolic fate of CIPC in animals has been the subject of several investigations carried out *in vivo* in the rat (1, 2, 3) using high doses of CIPC and TLC for the determination of metabolites in urine samples. In these studies a number of metabolites were positively identified and others speculatively. Investigations carried out with soybean plants treated with CIPC showed the presence of two arylhydroxylated metabolites and a metabolite resulting from alkylhydroxylation was characterized in soybean plant extracts (4).

This analytical study was based upon HPLC determination in isolated hepatocytes. In the scope of a toxicological study related to the increase of CIPC toxicity, with the conjugation process partially or completely inhibited (5).

EXPERIMENTAL

Reagents :

All chemicals and solvents were analytical grade reagents.

Acetate buffer pH 5 : 5 ml of glacial acetic acid were added to about 90 ml of glass-distilled water and then brought to pH 5 by addition of 1 M NaOH dropwise.

Glucuronidase (20 U/mg) and arylsulfatase (5 U/mg) were obtained from Boehringer (ref. 127680 and 102890 respectively). Chlorpropham was purchased from Sigma and 3-chloroaniline from Merck.

The metabolites which were not commercialy avalaible were synthesized as follows :

. 3-chloro-4-hydroxyaniline was prepared by reduction of 2-chloro-4-nitrophenol with stannous chloride. Tin was precipitated at pH 1.5 by H_2S. Stannous and stannic sulfides were separated by centrifugation. The aqueous phase was brought to pH 7 and the 3-chloro-4-hydroxyaniline was extracted with diethyl ether and recristallized from chloroform (mp = 152° C).

. 3-chloro-4-hydroxyaniline was acetylated with a stoechiometric amount of acetic anhydride to give 3-chloro-4-hydroxy-N-acetylaniline (mp = 145° C).

. 3-chloro-N-acetanilide (mp = 79° C) was prepared by acetylation of 3-chloroaniline at 160° C using an excess of acetic anhydride.

The isopropyl N-(3-chloro-4-hydroxyphenyl) carbamate 4(OH)CIPC was synthesized by refluxing 3-chloro-4-hydroxyaniline (0.02 M) and isopropyl chloroformiate (0.01 M) in a mixture (200 ml) of anhydrous diethylether and dichloromethane (1/1) at room temperature for 12 hours. The extracts were centrifuged at 4000 r.p.m. for five minutes. The ether phases were collected and evaporated to dryness under a N_2 flow. The residues were taken up with 1 ml of methanol and a 10 µl aliquot was injected into the HPLC.

As traces of diethylether remaining in the aqueous phase inhibit the enzymatic process, they need to be eliminated under N_2 flow. As isolation of conjugates is normally hampered by the presence of large amounts of naturally occurring compounds such as proteins the cellular fraction was centrifuged. The supernatant and the aqueous phase from the cell centrifugation were then ajusted to pH 5 with 0.3 M HCl dropwise and 0.5 ml of acetate buffer. Two aliquots of 1 ml were then incubated with β-glucuronidase (200 µl) and arylsulfatase (20 µl) respectively at 37° C for 24 hrs. The incubations were extracted twice with 10 ml of diethylether after ajusting the pH to 7 with 0.1 M NaOH dropwise. Then the previously described procedure was used.

RESULTS AND DISCUSSION

Diethylether was used as extractant since all the compounds investigated were very soluble in it and it is a solvent which is easy to evaporate under N_2 flow. In effect previous experiments carried out in order to study the recovery of 3-chloroaniline had shown that for complete recovery the etheral phase must be evaporated at low temperature. The use of a water bath thermostated at 55° C gives a recovery of only 15 %.

Moreover if chromatograms are recorded at 240 nm no peak occurs when diethylether is used as the extractant.

A recovery study of several metabolites of CIPC was carried out at different pH values since previous in vivo metabolism investigations had shown that CIPC is transformed into compounds with very different fonctional groups arising from OH ring substitution, oxidation of the esteralkylgroup, cleavage of the carbamate linkage.

Supernatant fractions from the control group (without CIPC) were spiked with known amounts (100 µg) of various metabolites which were determined using the described procedure.

Table 1 gives the mean value of recovery over a wide range of pH.

The excess of 3-chloro-4-hydroxyaniline was precipitated under chlorhydrate form and then eliminated by filtration. The organic phase was washed with 0.1 M HCl ; then evaporated to dryness. The product had a melting point of 90° C after recrystallization from chloroform-petroleum spirit. The isomeric isopropyl N-5(chloro-2-hydroxyphenyl) carbamate or 2(OH)CIPC (mp = 122° C) was prepared in the same manner from 2-hydroxy-5-chloroaniline.

For each synthesized compound the purity was verified by the presence of a single peak using HPLC and the corresponding product identified by mass spectrometry (Varian Mat 3311 A).

Instrumentation :

The liquid chromatograph consisted of a Waters model 6000 A solvent delivery system equipped with an Uvikon 722 LC detector set at 240 nm. The sensitivity was fixed at 0.1 AUFS for the analyses. An analytical reverse phase (30 cm x 3.9 mm, 5 µm particle size) C-18 column from Waters Associates was used for all analyses carried out at ambiant temperature. Chart speed 0.5 cm/mn. Integration time 1s.

Two mobile phases were used :

- Eluant I consisted of 90 % methanol and 10 % water with a final pH of 4 by dropwise addition of 0.1 M HCl. It was used at a flow rate of 0.8 ml/min.

- Eluant II consisted of 70 % methanol and 30 % water, with 2 ml of ethanolamine and 0.5 M HCl added dropwise until pH 7.8 Eluant II was used at a flow rate of 0.6 ml/min.

Procedure :

Isolation of hepatocytes from the rat was performed by collagenase perfusion as previously described (6). Incubations were carried out at 37°C for 2 hours using 10 ml of cell suspension (1.5 x 10^7 cells) in Hank's buffer pH 7.4 and adding 1 µM of CIPC (213 µg CIPC in 25 µl DMSO).

Cells were separated from the extracellular medium using the procedure described by Schwenk (7) with a few minor modifications. After separation from the supernatant the cells were resuspended in 5 ml of water and

TABLE 1

Recovery (μg) as a function of extraction pH for 100 μg of each compound added.

pH	3-Chloroaniline	3-Chloro-4-hydroxy-aniline	3-Chloro-acetanilide	CIPC	4(OH)CIPC
1.0	3 ± 2	0	95 ± 5	100± 5	90 ± 5
7.4	85 ± 5	70 ± 10	95 ± 5	100± 5	95 ± 5
13.0	95 ± 5	0	90 ± 5	100± 5	3 ± 2

sonicated for 15 s. The supernatant and hepatocyte suspension were separately extracted twice with a 20 ml volume of diethylether at pH 7.4.

Table 1 shows that-except for amino compounds the recovery at pH 7.4 can be considered as satisfactory since the percentages are of the same order of magnitude as those which are generally obtained using extraction procedures. It can be expected that the slight difference between complete recovery and the values reported in Table 1 are due to the sum of the experimental errors. On the other hand for amine derivatives the recovery is not complete at pH 7.4. Further recovery tests with real samples showed that, especially in acidic medium (pH = 1), about 50 % of the sulfate ester of 4(OH)CIPC is split hydrolytically giving erroneous results when the free 4(OH)CIPC is determined. Therefore a compromise might be found between the introduction of a corrective factor for the quantification of amino compounds and the use of multiple extractions at different pH values giving a more complicated and time-consuming procedure. Thus extraction in neutral medium was adopted for all the analyses.

Although the molar absorption was about two-fold higher at 210 nm than at 240 nm for all compounds investigated, this latter wavelength was chosen as the biological medium is complex and at 210 nm several peaks occur which are also present in chromatograms from control incubations without any CIPC added.

Separation of CIPC and metabolites was achieved by performing optimization of solvent strength and selectivity by testing several solvent mixtures.

Using a 5 µm C-18 column and eluant I pH 7.8 at a flow rate of 0.8 ml/min. gives a good separation of CIPC, 4(OH)CIPC and 3-chloroacetanilide with acceptable retention volumes. But in these conditions 3-chloroaniline is not separated from the 4(OH)CIPC. Fortunately the 3-chloroaniline peak can be displaced by modifying the pH value of the eluant I. Descreasing the pH to 4, close to the pK of 3-chloroaniline (pK = 3.46), leads to partial protonation and delays the peak occurrence. In these latter conditions most of the compounds are correctly separated except the 4(OH)CIPC peak which overlaps that of 3-chloro-4(OH)acetanilide. Moreover at pH 4 the molar absorption of 3-chloro-4(OH)aniline is low. Increasing the water content of the eluant improves separation of the two phenolic compounds also using eluant II at pH 7.8 enhances the peak height of 3-chloro-4(OH)aniline. Table II gives the retention volumes and capacity factor of CIPC and its metabolites using the two proposed mobile phases. For the determination of K' column void volume was determined by the injection of sodium nitrate (8).
Fig. 1 shows typical chromatogram shapes of free metabolites. After enzymatic hydrolysis the chromatogram is given in Fig. 2. Each peak

TABLE 2

Retention Volumes and Capacity Factor.

COMPOUND	Mobile phase I pH 4 flow rate 0.8 ml/min		Mobile phase II pH 7.8 flow rate 0.6 ml/min	
	Retention volume ml	K'	Retention volume ml	K'
3-Chloro-4(OH)aniline	2.80	0.13	2.88	0.14
3-Chloro-4(OH)acetanilide	3.14	0.23	4.08	0.62
4(OH)CIPC	3.36	0.31	4.44	0.76
2(OH)CIPC	4.0	0.56	11.52	3.60
3-Chloroaniline	4.64	0.81	4.68	0.86
3-Chloroacetanilide	3.68	0.44	4.92	0.95
CIPC	4.16	0.63	-	-

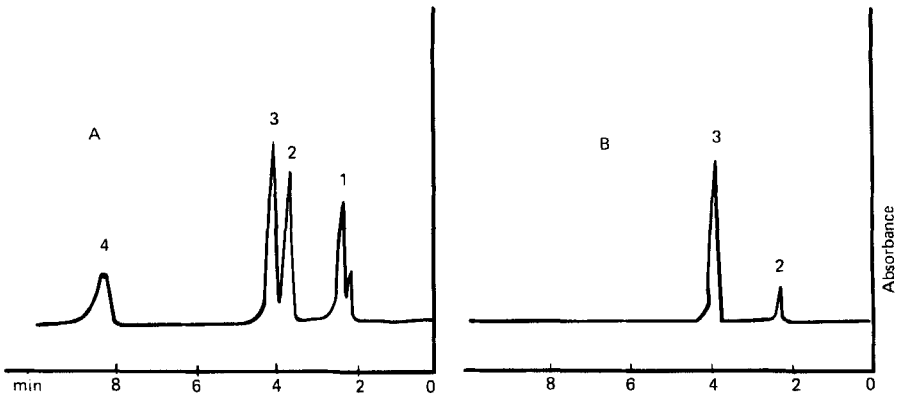

Figure 1 : Free metabolite assay (Mobile phase I 0.8 ml/min.)
Mixture of standards (A)
1 : 4(OH)CIPC, 2 : 3-chloroacetanilide, 3 : 2(OH)CIPC,
4 : CIPC, 5 : 3-chloroaniline.
Extracellular Fraction (B)
1 : 4(OH)CIPC, 2 : 3-chloroacetanilide, 4 : CIPC, 5 : 3-chloroaniline, 6 : Tris.
Intracellular Fraction (C)
1 : 4(OH)CIPC, 2 : 3-chloroacetanilide, 4 : CIPC, 6 : Tris.

TABLE 3

Metabolite Profile of CIPC (1 μM) following Incubation with Isolated Rat Hepatocytes (2 hrs).

COMPOUND	EXTRACELLULAR (μg)	INTRACELLULAR (μg)
CIPC (unmetabolised)	40 ± 10	26 ± 5
4(OH)CIPC (free)	12 ± 2	8 ± 4
3-Chloroacetanilide	10 ± 2	3 ± 1
3-Chloroaniline	2.5 ± 1	0
4(OH)CIPC (sulfate ester)	70 ± 10	0
4(OH)CIPC (glucuronide)	30 ± 10	0
3-Chloro-4(OH)aniline	0	0
3-Chloro-4(OH)acetanilide	0	0
2(OH)CIPC	0	0

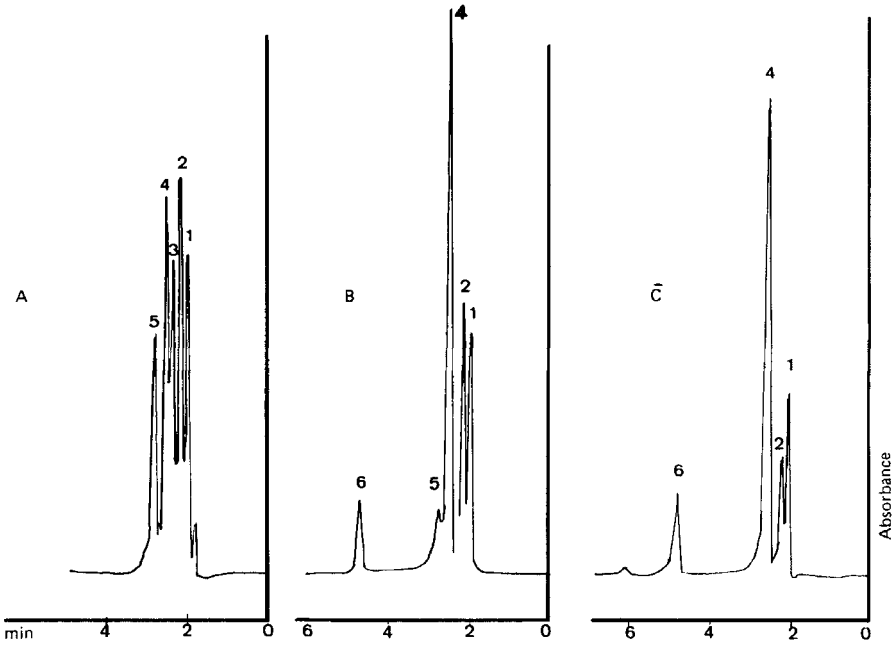

Figure 2 : Conjugate assay (Mobile phase II 0.6 ml/min.)
Mixture of standards (A)
1 : 4(OH)3-chloroaniline, 2 : 4(OH)3-chloroacetanilide,
3 : 4(OH)CIPC, 4 : 2(OH)CIPC.
Conjugate sample (B)
2 : Exogeneous peak, 3: 4(OH)CIPC.

was isolated and the fonctionnal group of the corresponding compound qualitatively characterized : the phenolic fonction using Folin-Ciocalteu (9) reagent and the amino group by the yellow color given after adding dimethylaminobenzaldehyde reagent. Moreover each compound was characterized by Mass Spectrometry. Table 3 gives the mean value of the amounts found for four replicata incubations with 1 μM of CIPC added.

Table 3 shows that the sulfate ester of 4(OH)CIPC was the predominant metabolite accounting for two-thirds of the conjugate 4(OH)CIPC. However the glucuronic acid conjugate was also of importance. Unchanged CIPC accounts for about one third of the dose, this fact which does not confirm the earlier in vivo experiments (1, 2) can be explained by the large difference in the dose tested and especially in experimental duration

(2 hrs instead of 24 or 48 hrs). None of the peaks recorded for free or conjugated metabolites coincided with 2(OH)CIPC. This fact is in agrement with the in vivo results of Bobik (2) for chlorpropham and those of Paulson (10) for propham.

The fact that no unknown peak is recorded and the satisfactory total obtained with characterized compounds rules out the presence of other metabolites at significant levels. Therefore the absence of hydroxy derivatives of the alkyl chain is to be expected in our experimental conditions. Approximately 10 % of the metabolism of chlorpropham involved cleavage of the carbamate side chain giving 3-chloroaniline which is further N-acetylated, but hydroxylation of these two metabolites did not occur in our experiments with CIPC added.

However further investigation carried out in the same experimental conditions with 3-chloroaniline (0.15 μM) showed that microamounts of 3-chloro-4(OH)acetanilide under glucuronide conjugated form and 4-aminophenyl sulfate ester are formed after 2 hrs of incubation. When 4(OH)CIPC (0.5 μM) was added to the incubation medium for 2 hrs, only the conjugation process occurs without any side chain cleavage. This fact proves that the conjugate forms, of 3-chloro-4(OH)aniline and 3-chloro-4(OH)acetanilide found in in vivo studies carried out with CIPC (1) result from hydroxylation of 3-chloroaniline and of 3-chloroacetanilide rather than from the hydrolysis of the side chain of 4(OH)CIPC.

Table 3 shows that the cellular fraction retains the unmetabolized herbicide a great deal longer than the other compounds, this fact can be connected with toxicological properties and a study is being carried out to evaluate the toxicological properties and the fate of the CIPC metabolites using isolated hepatocytes and the proposed HPLC procedure. Based upon recovery of spiked samples and replicata determinations on real incubations, the proposed methodology can be considered as simple reliable, sensitive, and well adapted to in vitro metabolism investigations with isolated cells or cell cultures.

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