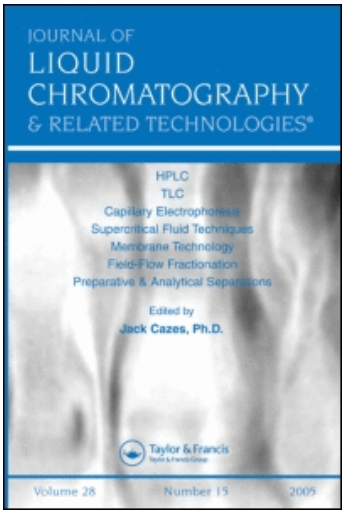

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FRACTIONATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MICROSOMAL CYTOCHROME P-450 INDUCED BY HEXACHLOROBIPHENYL ISOMERS

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ABBREVIATIONS.

High performance liquid chromatography (HPLC), phenobarbitone (PB), β-naphthoflavone (BNF), hexachlorobiphenyl (HCB), hydrophile-lipophile balance (HLB).

ABSTRACT

High performance liquid chromatography has been employed to fractionate rat liver microsomes under nondenaturing conditions. Selective detection at 405 nm allowed resolution of microsomal heme proteins into three peaks (A, B, and C). Cytochromes in the peaks retain their native property of binding CO after HPLC. Peak-A, first eluting, contains P-450 and is rich in cytochrome P-420. Peak-B is largely hemoglobin and peak-C is a major cytochrome P-450. The ratio of peak-C to A is increased by treatment of rats with phenobarbitone, β-naphthoflavone, 2,3,5,2',3',5'-hexachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl as compared to controls. The highest increment in the ratio is observed on feeding 3,4,5,3',4',5'-hexachlorobiphenyl. NADPH cytochrome c reductase elutes earlier than peak-C but cytochrome b_5 is not separated from the major cytochrome P-450 peak. The separations obtained are highly reproducible and considerably faster than conventional gel permeation chromatography. The data presented here are very promising in establishing the role of HPLC in the studies of insoluble proteins and enzymes in general and cytochrome P-450 in particular.

INTRODUCTION

The application of high performance liquid chromatography (HPLC) to the purification of polypeptides and proteins has been recently reviewed (1). The separation mechanism involves partition between a silica gel bonded phase and an aqueous eluant. The highly hydrophobic bonded phase requires in many cases the use of organic solvents in order to displace the polypeptides from the column, a clear limitation of this procedure. A recently introduced bonded phase operates on the principle of molecular exclusion. These columns have been used for the estimation of molecular weights of proteins under denaturing conditions (2). A variation of this latter type involves the use of an ion exchange phase which was used to further separate a homogenous preparation of lipoxygenase (3). Most of the studies on protein isolation and fractionation by HPLC have been confined to soluble proteins only. In the present study, we have made an attempt to fractionate by HPLC an insoluble hydrophobic protein which is localized in the endoplasmic reticulum. Since the mixed function oxidase system plays an important role in the metabolism of endogenous substrates (4), xenobiotics (5) and cytochrome P-450 being the terminal oxidase of microsomal electron transport chain (6), it was relevant to explore the applications of HPLC to the analyses of rat liver cytochrome P-450 in control and hexachlorobiphenyl (HCB) isomers treated rats.

MATERIALS AND METHODS

Charles River CD strain male rats were used in the present study. The treatment of animals, determination of dose, purity of

HCB isomers, preparation of microsomes and assay of enzyme has been previously described (7) except that β-naphthoflavone was used instead of 3-methylcholanthrene. Protein standards and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO 63178.

Solubilization of microsomes. The microsomes were solubilized at room temperature in 100 mM potassium phosphate (pH 7.4), 0.2% Lubrol WX, 0.5% sodium cholate, 0.1 mM ethylene diamine tetraacetic acid, 0.1 mM dithiothreitol and 20% glycerol (buffer A) for a period of one hour. The sample size was so adjusted that each time 100 µg protein in 0.05 ml was applied to the column (Fig. 1).

High Performance Liquid Chromatography (HPLC). Buffer and solubilized microsomes were filtered (0.22 µm, Millipore) prior to use. The equipment used consisted of a model 6000A pump, U6K injector, model 440 dual-channel UV absorbance detector, and a model 730 data module (all from Waters Associates, Inc., Milford, MA 01757). Peak absorbance was simultaneously monitored at 405 nm and 280 nm; peak areas were obtained by integration with the 730 data module. The columns used were a Spherogel TSK-3000SW (0.75 x 60 cm, Altex) and, I-125 and I-250 (0.78 x 30 cm each, Waters Associates). The column was equilibrated for one hour and eluted with buffer A isocratically. The flow rate was 0.5 or 1 ml per min as indicated in the figure legends.

RESULTS AND DISCUSSION

Rat liver microsomes are generally solubilized by using a nonionic detergent such as Emulgen-911 in combination with an ionic detergent, sodium cholate. Emulgen-911 is a polyoxyethylene nonylphenol and its strong absorbance at 280 nm makes the monitoring

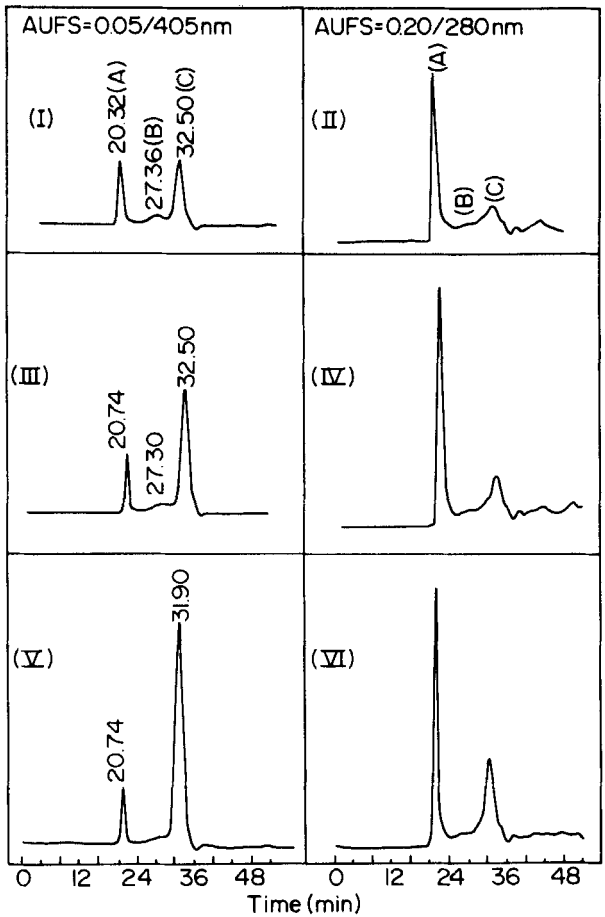

Figure 1. HPLC of solubilized rat liver microsomes from control (I and II), 2,3,5,2',3',5'-HCB (III and IV), and 3,4,5,3',4',5'-HCB (V and VI) treated rats on a Spherogel TSK 3000SW column. The flow rate was 0.5 ml/min and chart speed 0.25 cm/min. Chromatogram I, III and V depict absorbance at 405 nm and II, IV and VI at 280 nm of the respective samples.

of the effluent for protein content impossible. Lubrol WX, a polyoxyethylene alcohol does not have this limitation and its use to solubilize rat liver microsomes has been documented (8). For the HPLC study we chose a combination of sodium cholate and Lubrol WX

which enabled us to monitor the effluent for heme (405 nm) and protein (280 nm) content. Substituting Emulgen-911 for Lubrol WX did not change the separation pattern, as determined by absorbance at 405 nm, a result anticipated from their similar HLB values (9).

As shown in Fig. 1, the rat liver microsomes from control and treated animals were fractionated into three peaks (A, B, and C) when monitored at 405 nm. The peaks from HPLC effluent were collected and their CO binding difference spectra recorded. Peak-B showed a characteristic absorbance at 420 nm when saturated with CO, thus establishing its identity as hemoglobin. Peak-A and C did not show any absorbance when bubbled with CO, however, they showed characteristic absorbance at 420 nm and 450 nm when first reduced with dithionite and bubbled with CO. These studies demonstrate that the cytochrome P-450s were able to retain their native property of binding CO after HPLC analysis. This is noteworthy because cytochrome P-450 is a labile protein in which the heme is not covalently bound, and it is easily converted to its denatured form cytochrome P-420 by factors such as temperature, detergents, etc. Peak-C contained most of cytochrome P-450 and no cytochrome P-420. Peak-A contained cytochrome P-450 and was rich in cytochrome P-420. Since peak-A eluted at the void volume, the solubilized microsomes were centrifuged at 167,000 g for 35 minutes. The supernatant was rechromatographed and no appreciable change in the areas or ratios of peak-C to A were observed, showing thereby that peak-A is not undissolved cytochrome.

To study the pattern of induction by various inducers of cytochrome P-450, microsomes from treated animals were solubilized in

TABLE 1. Effect of Phenobarbitone, β-Naphthoflavone, 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB on Areas of Peaks-A and C and Their Ratios as Obtained by HPLC Analysis. The conditions are described in Fig. 1. Each value is a mean of three separate determinations.

Treatment	Area[a] Peak-A	Peak-C	Ratio Peak C/A
Control	17.12 (1.5)[b]	27.06 (1.67)	1.58
PB	16.04 (0.25)	59.33 (2.09)	3.70
BNF	17.94 (1.10)	49.97 (2.44)	2.79
2,3,5,2',3',5'-HCB	11.52 (0.23)	55.19 (1.42)	4.79
3,4,5,3',4',5'-HCB	10.43 (2.90)	100.49 (11.50)	9.63

[a]Absorbance at 405 nm.

[b]Standard Deviation in ( ).

buffer A for one hour at room temperature and analyzed by HPLC. Each sample was analyzed three times and mean values of the areas obtained and ratios of peak-C to A are presented in Table 1. The ratio of peak-C to A was 1.58 in control microsomes and increased when rats were treated with various inducers. 3,4,5,3',4',5'-HCB caused the highest increase in the ratio of peak-C to A. 3,4,5,3',4',5'-HCB also increased the area of peak-C 3.7 times as compared to peak-C of control microsomes. Similarly, other inducers such as PB, BNF and 2,3,5,2',3',5'-HCB increased the area of peak-C by a relative factor of 2.19, 1.85 and 2.0 respectively. This trend parallels the one determined by estimating the cytochrome P-450 content by CO-difference spectra of reduced microsomes obtained from control and treated rats (7). Consequently an increase in the area of peak-C by an inducer relative to control may be considered a qualitative index of its potency as an inducer of cytochrome P-450.

TABLE 2. Effect of 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB on Specific Activities of Microsomes and Peak-C Obtained After HPLC of Microsomes. Each value is mean of three separate determinations (7). The conditions are described in Fig. 1.

Treatment	Specific Activity nmol Cyt. P-450/mg protein		Recovery of Cyt. P-450 in Peak-C(%)
	Microsomes	Peak-C	
Control	1.02	1.34	78
2,3,5,2',3',5'-HCB	2.34	3.24	88
3,4,5,3',4',5'-HCB	4.31	4.87	99

The capacity of the column is not limited to microgram quantities of protein. Fifty times more protein than used in the initial experiment could be fractionated without any appreciable loss of resolution. Another important feature of the present technique is that peak-C when monitored at 280 nm also showed an increase when microsomes obtained from rat livers treated with 2,3,5,2',3',5'-HCB (Fig. 1, IV) and 3,4,5,3',4',5'-HCB (Fig. 1, VI) were fractionated by HPLC, showing thereby that protein content of peak-C was increased along with heme content leading to the conclusion that inducers increased the de novo synthesis of the associated proteins. These latter experiments were done in triplicate and the specific activities of microsomes and isolated peak-C are presented in Table 2. The specific activities of peak-C showed a modest increase as compared to microsomes indicating the removal of some proteins from the major cytochrome peak. This was further confirmed when sodium dodecyl sulfate polyacrylamide gel electro-

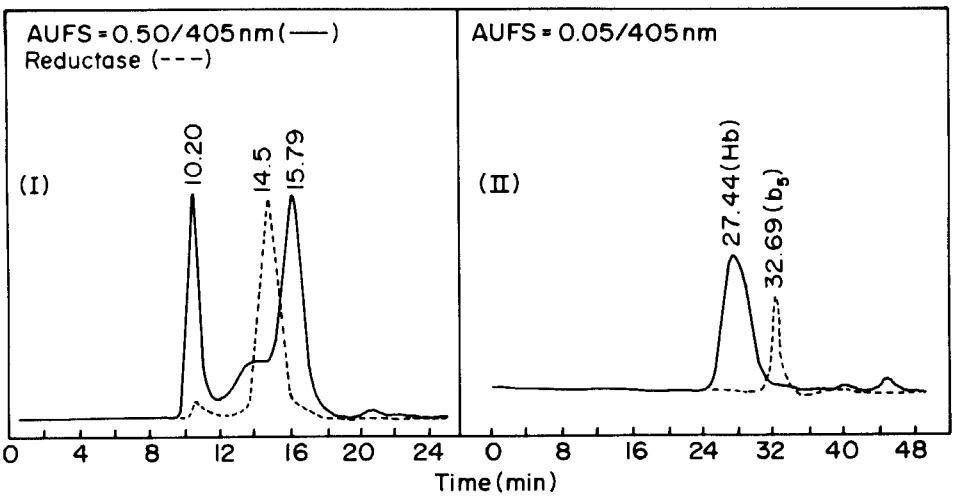

Figure 2. HPLC of solubilized rat liver microsomes on Spherogel TSK 3000SW column. Chart speed was 0.5 cm/min and flow rate 1.0 ml/min. 0.25 ml Fractions were collected and NADPH cytochrome c reductase was assayed in each fraction. Hemoglobin and cytochrome b_5 were chromatographed separately but are shown here in the same chromatogram (II).

phoresis was performed on liver microsomes and peak-C of control, 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB treated rats. Polypeptides corresponding to subunit weights of 80,000, 90,000 and 100,000 were absent in peak-C as compared to their respective microsomes. Since the volumes of the peaks were small, the detergents were not removed prior to protein estimations, thus affecting the specific activities reported. As documented in Table 2, the recoveries of cytochromes in peak-C are excellent.

To study the elution pattern of NADPH cytochrome c reductase, 2.5 mg of microsomal protein was applied to the column. 0.25 ml fractions were collected and enzyme assays were performed in each

fraction. NADPH cytochrome c reductase eluted at 14.5 minutes at 1 ml/min (Fig. 2, I). These results corroborate well with previous observations (10) that NADPH cytochrome c reductase elutes earlier than cytochrome P-450 when solubilized rat liver microsomes are chromatographed on Sephadex G-200. The column used in the present study operates mainly on the principle of molecular exclusion. The entire operation can be performed in minutes as compared to many hours taken on Sephadex. The binding of substrates to cytochrome P-450 monitored by HPLC is an attractive possibility (11).

Hemoglobin and cytochrome b_5 were obtained from rat liver microsomes by chromatography on DEAE-cellulose (10). Hemoglobin had a retention time of 27.5 minutes at 0.5 ml/min, similar to peak-B (Fig. 2, II). Cytochrome b_5 had a retention time of 32.7 minutes (Fig. 2, II) and did not separate from peak-C. Since the content of cytochrome b_5 is not altered on treatment of rats with the inducers studies (unpublished results), the induction demonstrated by the increase in the area of peak-C is real.

When microsomes obtained from untreated rat testis were solubilized and chromatographed by the procedure described for rat liver, the content of peak-A (Fig. 3) was higher as compared to peak-C. Peak-A contained mostly cytochrome P-420 when analyzed spectrophotometrically and peak-C contained cytochrome P-450 and no cytochrome P-420. This experiment illustrates the contrasting stabilities of cytochrome P-450 from different sources. Rat liver microsomes may be prepared and chromatographed as described here, but under similar conditions the cytochrome P-450 in rat testis is rapidly denatured to cytochrome P-420.

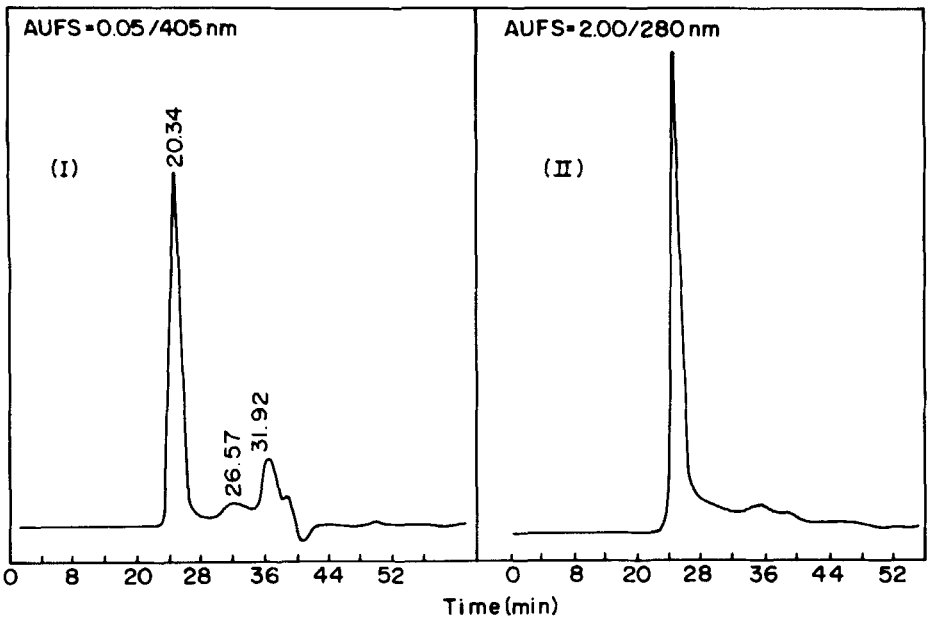

Figure 3. HPLC of solubilized rat testis microsomes on Spherogel TSK 3000SW column. Flow rate was 0.5 ml/min and chart speed 0.25 cm/min. Chromatogram I shows absorbance at 405 nm and chromatogram II at 280 nm.

These studies with rat liver and testis microsomes indicate a very promising role of HPLC in studies of induction, fractionation and denaturation of cytochrome P-450 and extends the application range of HPLC to insoluble proteins. A unique application of this approach is in the study of systems in which cytochrome P-450 content is the limiting factor. The spectral properties of recovered cytochrome P-450, i.e. CO-binding spectrum, are indicative of the structural integrity of the protein (12,13). Reconstitution of the system and demonstration of substrate metabolism are outside the scope of the present manuscript (12,13). This

technique has been successfully employed in our laboratory to monitor the conformational changes in cytochrome P-450 during its conversion to cytochrome P-420 and other applications such as detergent removal are under study.

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