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High-performance liquid chromatography technique for resolving multiple forms of hepatic membrane-bound cytochrome *P*-450

(high-performance liquid chromatography of rat hepatic microsomes/solubilized membrane protein/selective induction of cytochrome *P*-450)

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ABSTRACT Solubilized hepatic microsomal cytochrome *P*-450 from rats was resolved into 12 different heme-containing components by ion-exchange high-performance liquid chromatography. Separation was accomplished by the utilization of Anpac ion-exchange resin. Phenobarbital (PB) and 3-methylcholanthrene (3-MC) treatment of rats resulted in selective increases or decreases of specific components observed in the elution profile of solubilized microsomes prepared from untreated rats. Treatment with polychlorinated biphenyls increased peaks that were selectively increased by PB or 3-MC treatment. Proteins collected from the major peaks induced by PB or 3-MC treatment were found to retain spectral characteristics of cytochrome *P*-450. The recovered cytochrome b_5 and NADPH–cytochrome *c* reductase retained spectral properties and enzymic activity, respectively. Purified *P*-450_b obtained from PB-treated rats was found to cochromatograph with one of the peaks selectively induced by PB treatment. These results suggested that it is possible to separate membrane-bound proteins without dramatically altering their physical properties.

During the past 20 years the cytochrome *P*-450 liver enzyme system was found to be responsible for the oxidation of a large number of lipid-soluble compounds via side chain and aromatic oxidation, deamination, *N*-, *O*-, and *S*-dealkylation, and *N*- and *S*-oxidation. Important biological reactions include biotransformation of steroids, the activation and deactivation of drugs, and involvement in xenobiotic toxicity, carcinogenesis, and teratogenesis.

Membrane-bound cytochrome *P*-450 is composed of an undetermined number of monooxygenases with molecular weights between 43,000 and 60,000 (1). The basic methods utilized to evaluate the number of isoenzymes of cytochrome *P*-450 are kinetic and enzyme substrate specificity (2, 3), chromatography (4–6), sodium dodecyl sulfate/polyacrylamide gel electrophoresis (7), antibody recognition (8), amino acid composition and sequence determination (9), and peptide mapping (10). Selective induction of presumably different enzymes suggests that cytochrome *P*-450 can be divided into two broad categories on this basis (11). One group of inducing substances, typified by phenobarbital (PB), increases the amount of cytochrome *P*-450. The second group of inducing substances includes a number of polycyclic aromatic hydrocarbons, which induce a modified cytochrome *P*-448 or P_1-450 with spectral and biochemical properties different from those of the cytochrome *P*-450 induced by PB. More recent studies utilizing both induction and a combination of techniques described above confirm the existence of more than two isoenzymes of cytochrome *P*-450 (12).

Ion-exchange chromatography is the most common technique utilized in the isolation and characterization of isoenzymes of cytochrome *P*-450. In this procedure it is possible to isolate sufficient quantities of homogeneous enzymes for evaluation of physical and biochemical properties. Unfortunately, the poor resolving properties and physical limitations of this method hampered the development of separation procedures capable of rapidly resolving small samples. In the last 5 years, advances in high-pressure liquid chromatography (HPLC) have made possible the rapid resolution of complex mixtures of soluble proteins and peptides. A number of papers recently reported the use of 5- to 10-μm particle reverse-phase (13), permeation (14, 15), and ion-exchange (16) supports in the separation of soluble proteins and peptides. The separation of solubilized proteins by column chromatography often requires high concentrations of glycerol, detergents, and salts incorporated into the elution buffer to maintain the solubility of the proteins and effect their elution from the column. The use of such solutions for HPLC was assumed to result in excessively high pressures or interfere with the monitoring of eluting proteins by UV absorbance. Therefore, the utilization of the HPLC technique to resolve membrane-bound proteins was ignored. To examine the feasibility of this technique to resolve membrane-bound proteins, we selected hepatic microsomal cytochrome *P*-450 as a model protein, because it is known to consist of multiple isoenzymes and its elution profile can be monitored by UV absorbance at 417 nm. In this paper we describe a HPLC technique for obtaining a 25-min chromatographic profile of multiple forms of cytochrome *P*-450 from less than 1 mg of solubilized hepatic microsomal *P*-450.

METHODS

Washed liver microsomes were prepared from untreated male Sprague–Dawley rats (180–200 g); rats had been treated with PB, 40 mg/kg in saline for 4 days; 3-methylcholanthrene (3-MC), single 40 mg/kg dose in corn oil; or polychlorinated biphenyls (PCB) (Aroclor 1254), 80 mg/kg in corn oil for 3 days. Microsomal pellets were layered with 20% (vol/vol) glycerol and frozen at −20°C. The pellets, frozen for less than 7 days, were solubilized in a Tris/acetate buffer containing 10 mM sodium phosphate, 0.5% sodium cholate, 0.1 mM EDTA, 20% (vol/vol) glycerol, and 0.2% Emulgen 911 adjusted to pH 7.4 as described by Warner *et al.* (17). Each milliliter of sample was adjusted to contain 10 mg of microsomal protein. One hundred-microliter samples were subjected to chromatography. All samples were free of hemoglobin.

Two Altex model 100 high-pressure liquid chromatographs equipped with a model 400 solvent programmer and a Hatachi

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Abbreviations: PB, phenobarbital; 3-MC, 3-methylcholanthrene; PCB, polychlorinated biphenyls; HPLC, high-performance liquid chromatography.

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100-20 spectrophotometer fitted with an 8-μl flow cell were used in this study. The separation was performed on a 250 × 4.6 mm stainless steel column packed with 10 μm Anpac anion-exchange resin (Anspec, Warrenville, IL), and proteins were monitored by absorbance at 417 nm. The ion-exchange support prepared for this study was subject to large variability among batches. Only those supports showing good resolving properties for solubilized cytochromes P-450 and b_5 were used in this study. Mobile phase A was a 20 mM Tris/acetate buffer containing 0.2% Emulgen 911 and 20% (vol/vol) glycerol adjusted to pH 7.2 (buffer A). The chromatogram was developed with a 30-min linear gradient of 0.8 M sodium acetate incorporated into buffer A (buffer B) at a flow rate of 1.6 ml/min. Initial and final column pressures were 1750 and 2400 pounds/inch^2 (1 pound/inch^2 = 690 pascals), respectively. Except for the preparation of washed microsomes, which was performed at 4°C, the remainder of the experiment was performed at room temperature. One-minute fractions were collected and monitored for cytochrome b_5 (18), NADPH–cytochrome c reductase (12), and cytochrome P-450 (18). Purified hepatic cytochrome P-450$_b$ (15.5 nmol/mg of protein) was prepared from PB-treated rats (12) and was generously supplied by Wayne Levin and Dene Ryan (Hoffmann–La Roche).

RESULTS

Fig. 1 presents the HPLC elution profile of solubilized rat liver microsomes prepared from control and PB- and 3-MC-treated rats monitored at 417 nm. Solubilized microsomes from control rats were resolved into 12 heme-containing peaks and shoulders. PB treatment resulted in a large increase in peaks 1a and 4b, an increase in peak 5, and a decline in peak 2. There were no changes in peaks 1b, 1c, 3, 3a, 4a, 6, and 7. Treatment with 3-MC resulted in a dramatic increase in peaks 5 and 6. There were no marked changes in peaks 1a, 1b, 1c, 3a, 3b, 4, and 7, but there was a decline of peak 2.

Fig. 2*A* presents an HPLC elution profile obtained from a mixture of cytochrome P-450s prepared by combining solubilized microsomes from two groups of animals treated separately with PB or 3-MC. This profile resembled a composite of Fig. 1*B* and *C*. Fig. 2*B* illustrates an HPLC profile obtained from a group of rats treated with PCB. This profile showed increases in peaks 4b, 6, and possibly 5 with a concomitant loss of peaks in 1a, 1b, and 1c. An additional component was observed between peaks 2 and 3.

Fig. 2*C* shows the elution profile of NADPH–cytochrome c reductase activity. Both NADPH–cytochrome c reductase and cytochrome b_5 eluted at peak 8 at a concentration of 0.8 M acetate. No cytochrome P-450 contamination was observed in these fractions. Furthermore, no cytochrome b_5 or cytochrome c reductase contamination was detected in the region of cytochrome P-450 elution. The HPLC elution patterns of cytochrome b_5 and cytochrome c reductase were similar to the pattern obtained by Warner *et al.* (17), using ion-exchange chromatography.

Fig. 3 shows the reduced CO binding difference spectra of samples collected from peaks 4b and 6 of the chromatography of solubilized microsomes prepared from PB- and 3-MC-treated rats, respectively. As expected, peak 4 displayed a maximum at 450 nm and peak 6 displayed a maximum at 447 nm. The reduced difference spectra of cytochrome b_5 derived from peak 8 of the chromatography of the samples described above also are shown in Fig. 3. Cytochrome b_5 obtained from PB- and 3-MC-treated rats have identical reduced spectra.

Purified P-450$_b$ obtained from PB-treated rats was evaluated by the HPLC method. The purity of this preparation was examined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and found to exist as a single component representing 95% of the protein applied to the gel (12). HPLC of 66 pmol (4.3 μg of protein) of cytochrome P-450$_b$ resulted in the elution of a single peak slightly before peak 4b obtained from

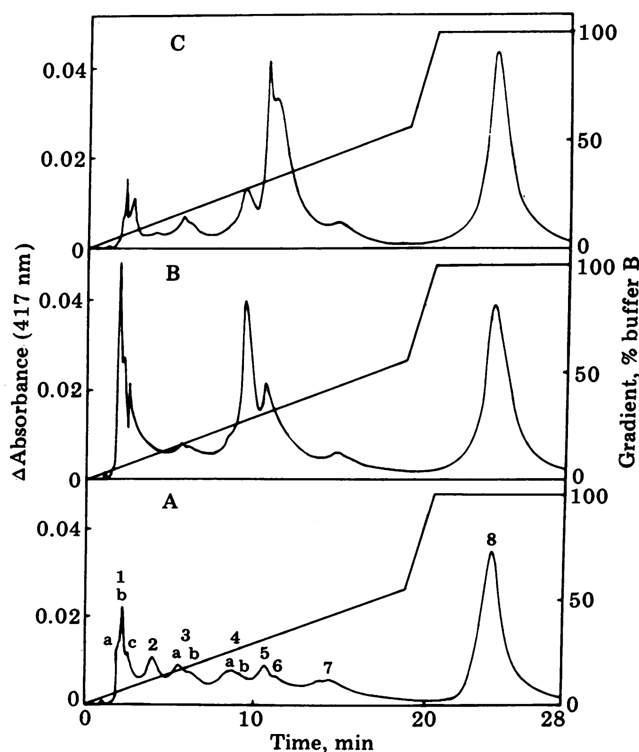

FIG. 1. Anion-exchange HPLC of solubilized cytochrome P-450. Hepatic microsomes from untreated (*A*), PB-treated (*B*), and 3-MC-treated (*C*) rats were used.

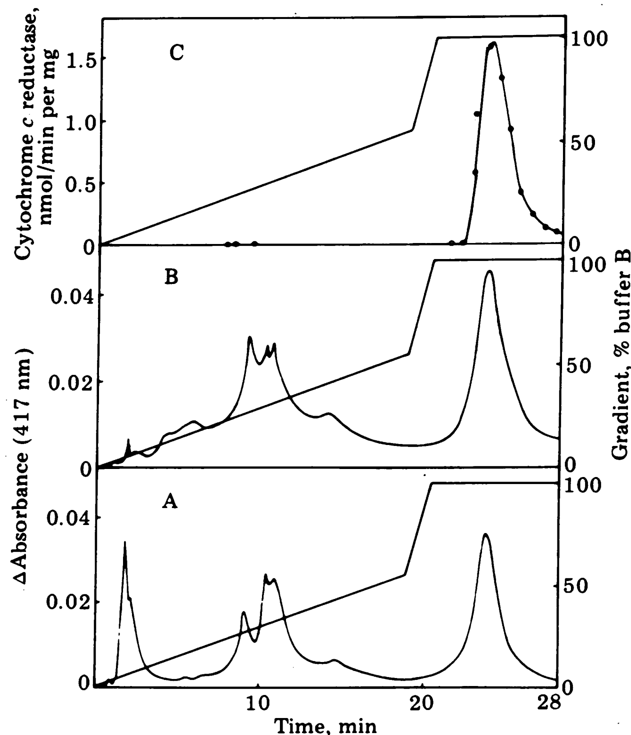

FIG. 2. Elution profiles of cytochrome P-450 and NADPH–cytochrome c reductase. Microsomes were prepared by combining solubilized microsomes obtained from PB- and 3-MC-treated rats (*A*) and microsomes prepared from PCB-treated rats (*B*). (*C*) NADPH–cytochrome c reductase was monitored in samples eluted from the column.

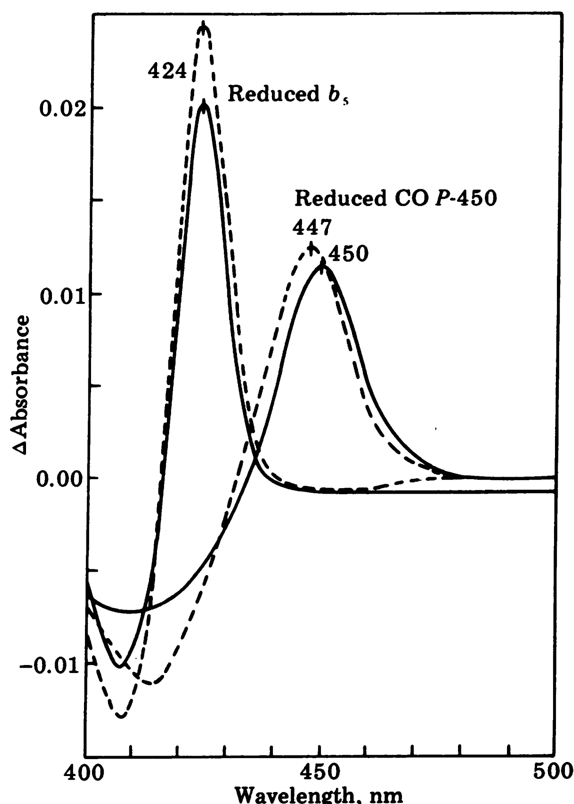

FIG. 3. Difference spectra of samples collected from the major fractions contributing to peaks 4b and peaks 6 and 8 of the HPLC obtained from solubilized microsomes prepared from PB- and 3-MC-treated rats, respectively.

solubilized microsomes prepared from PB-treated rats. When a mixture of purified P-450$_b$ and solubilized microsomes prepared from PB-treated rats was subjected to HPLC, the purified P-450$_b$ enhanced peak 4b.

DISCUSSION

The chromatograms prepared by this HPLC technique demonstrated greater resolution than those obtained by other chromatographic techniques used for the purification of cytochrome P-450. The evidence presented in this paper indicated that the multiple peaks observed in the HPLC profile were different forms of cytochrome P-450 and not artifacts induced by the procedure. First, pretreatment with xenobiotics known to induce different forms of cytochrome P-450 resulted in increases in different peaks in the chromatographic profile. Second, mixtures of microsomes prepared from rats pretreated with two of the xenobiotics, PB and 3-MC, showed composite chromatographic patterns with characteristics of each inducing agent. Third, pretreatment with a third xenobiotic, PCB, reported to possess PB- and 3-MC-inducing properties (19, 20), resulted in chromatographic profiles similar to the profile obtained with a mixture of PB and 3-MC microsomes. Fourth, purified P-450$_b$, prepared from PB-treated rats, cochromatographed with one of the major peaks increased by PB pretreatment. The shorter retention time for purified P-450$_b$ when chromatographed alone may have been due to the lack of lipids in this preparation.

The HPLC method did not seem to affect the physical and chemical properties of the solubilized proteins. Cytochrome P-420 and b_5 eluting from the column retained spectral properties, and no sign of cytochrome P-420 (inactive P-450) was detected. In addition, recovered NADPH–cytochrome c reductase retained enzymic activity.

The advantages of the HPLC technique compared with sodium dodecyl sulfate/polyacrylamide gel electrophoresis are that the heme proteins were intact and proteins could be recovered intact. When compared with column chromatography (12, 17, 21, 22), the method displayed better resolution of cytochrome P-450 isoenzymes and was more rapid (30–40 min per sample), and small samples (less than 0.5 mg) could be used for analysis. Because of the rapidity of this technique, optimal chromatographic conditions were more easily obtained.

Additional studies will be required to directly compare the results of P-450 separation by the HPLC technique with other separation procedures. Little similarity was observed when comparing the HPLC chromatogram with DEAE-cellulose chromatograms. However, both the HPLC technique and the DEAE separation technique demonstrated selective induction of certain peaks by PB and 3-MC, suggesting that similar proteins were being observed. Further refinement of our technique may permit separation of larger quantities of proteins so that precise characterization can be accomplished by electrophoresis and enzyme kinetic analysis.

Finally, this paper demonstrates a high-resolution HPLC technique capable of separating closely related membrane-bound proteins. Accordingly, this technique may be of value for resolving other membrane-bound proteins of pharmacological or physiological importance.

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