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Analytical fractionation of microsomal cytochrome P-450 isoenzymes from rat liver by high-performance ion-exchange chromatography

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ABSTRACT

Ion-exchange Fast Protein Liquid Chromatography (FPLC) on Mono Q and Mono S was optimized for the analytical separation of microsomal cytochrome P-450 species from rat liver. The effects of detergent, pH, gradient profile and column load on resolution are demonstrated. Successive application of anion- and cation-exchange chromatography leads to eleven separated P-450 fractions. The altered microsomal P450 pattern after treatment of rats with various inducers is reflected by distinct elution profiles. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and enzymatic analysis imply that several FPLC fractions contain more than one P-450 species. Preliminary results are presented showing the suitability of immobilized metal affinity chromatography (IMAC) for general P-450 fractionation and thus for the further resolution of Mono Q and Mono S fractions. Scale-up for preparative P-450 fractionation is easily done by adapting the optimized analytical FPLC procedures to Q- and S-Sepharose Fast Flow.

INTRODUCTION

The cytochrome P-450 enzyme family includes a large number of different protein species [1] which share as a common feature the ability to form a $P\text{-}450_{reduced}$–CO complex with a specific absorption at about 450 nm [2]. The number of known P-450 species in rat liver microsomes is more than 20 and is still increasing, as illustrated by the recent isolation of two new forms [3]. Because of structural and functional similarities of the P-450 isozymes, purification is often difficult and results in low yields. For example, up to six chromatographic steps are needed to obtain purified P-450IIE1 (= P-450j) [4].

The apparent molecular weight [sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)] of the P-450 species is in the range 46 000–57 000 dalton with a clustering at about 51 kilodalton. Therefore, SDS-PAGE is often insufficient as a criterion for P-450 homogeneity and has only a limited value for P-450 identification. In addition to similar molecular weights, P-450 species can exhibit similar or overlapping spectroscopic [5], immunochemical [6,7] and enzymatic properties [8,9]. These circumstances make it difficult to establish a simple method for analysing the pattern of a complex P-450 mixture such as rat liver microsomes. Because individual

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P-450s cannot be identified by a single criterion, any analytical procedure ends up being a combination of different methods.

High-resolution chromatographic techniques such as high-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) have not often been used for P-450 purification. Their analytical application is restricted to only a few studies [10,11] and FPLC has been chosen for this purpose only by Sakaki *et al.* [12] to demonstrate the distinctness of purified P-450 species and by Kastner and Schulz [13] to differentiate inductor effects on the P-450 pattern of liver microsomes of marmoset monkeys.

Because the P-450 pattern in a given species or individual varies with age [14], sex [15], physiological status [16] and/or the action of drugs and xenobiotics [17], a simple analytical system would be useful in follow-up studies of developmental or pathological events or in diagnostics to evaluate xenobiotica exposure. In this paper we present results concerning primarily the first step in the analytical procedure, *viz.*, the chromatographic resolution of a complex P-450 mixture by FPLC.

EXPERIMENTAL

Animals and animal treatment

Sprague–Dawley rats (200–250 g) were purchased from Lippische Versuchstierzucht (Extertal, F.R.G.). They were induced by phenobarbital or β-naphthoflavone as described by Guengerich and Martin [18]. Induction by hexachlorobenzene (HCB) was done by three intraperitoneal injections (20 mg of HCB per 0.5 ml of corn oil) on three subsequent days. Liver dissection followed on the next day.

Preparation and solubilization of liver microsomes

Rat liver microsomes were prepared by the method of Guengerich [19] with additional 0.4 m*M* phenylmethylsulphonyl fluoride (PMSF) in all the solutions used. For microsome solubilization the suspension was adjusted to 2 mg/ml of protein with 100 m*M* Tris–HCl (pH 7.7), 20% glycerol, 1 m*M* EDTA, 1 m*M* dithioerythritol (DTE), 0.4 m*M* PMSF and 0.8% Lubrol PX and stirred for 30 min at 4°C. Insoluble material was removed by centrifugation at 105 000 *g* for 1 h at 4°C. The clear supernatant was passed through a 0.2-μm filter (Minisart NML, SM 16534, Sartorius) prior to FPLC fractionation. To test the solubilization efficiency of detergents we used the same procedure and buffer system including the detergents as specified in Table I. After centrifugation the supernatant was analysed for protein and P-450 content (see below).

Chromatography

Analytical and preparative separations were performed with an automatic FPLC system from Pharmacia (Uppsala, Sweden) equipped with two pumps, a gradient controller, various valves and a fraction collector. Protein and haemoprotein were detected by continuous simultaneous monitoring at 280 and 417 nm. Data processing and storage were performed with a personal computer (Atari PC3) equipped with a PC Integration Pack (Softron, Gräfeling, F.R.G.). For analytical separations 1-ml columns (HR 5/5) of the strong ion exchangers Mono Q (anion) and Mono S (cation) were used. The composition of the equilibration and elution buffers

was as follows: Mono Q-A, 20 m*M* Tris–HCl (pH 7.7), 20% glycerol, 1 m*M* EDTA, 1 m*M* DTE, 0.2 m*M* PMSF, 0.2% Lubrol PX; Mono Q-B, same as Mono Q-A + 1 *M* NaCl; Mono S-A, same as Mono Q-A but with 20 m*M* 3-(N-morpholino)propanesulphonic acid (pH 7.0) instead of Tris–HCl; Mono S-B, same as Mono S-A + 1 *M* NaCl.

Preparative fractionations were carried out in FPLC columns (HR 16/10 and HR 10/10; Pharmacia) filled with 20 ml of Q- or 7.5 ml of S-Sepharose Fast Flow, respectively, using the same buffer systems as for Mono Q or Mono S separations.

Immobilized metal affinity chromatography (IMAC) was performed in 1-ml FPLC columns (HR 5/5) filled with Chelating Sepharose Fast Flow. Charging the gel with metal ions was done according to the manufacturer's user's manual [20]. Buffers for elution by pH gradient were IMAC-A1 for equilibration, consisting of 50 m*M* sodium phosphate (pH 7.0), 500 m*M* NaCl, 20% glycerol and 0.2% Lubrol PX, and IMAC-B1 for elution, with the same composition as IMAC-A1 but adjusted to pH 3.8. Buffers for elution with a competitive ligand were IMAC-A2 for equilibration, consisting of 50 m*M* sodium phosphate (pH 7.2), 500 m*M* NaCl, 20% glycerol and 0.2% Lubrol PX, and IMAC-B2 for elution with the same composition as IMAC-A2 but with an additional 2 *M* NH_4Cl.

Column treatment. The monobead ion exchangers were washed and re-equilibrated immediately after each run by subsequent application of 5 ml of buffer A, 10 ml of buffer B and 5 ml of buffer A (buffers as specified above). Because of increased back-pressure the columns were extensively washed after about 20 runs by the following procedure: (1) alternate application of 0.5 ml of 1 *M* NaOH and 2 ml of water in the reverse flow direction until the back-pressure remained constant; (2) washing with 5 ml of 0.5% trifluoroacetic acid in acetonitrile and subsequently 5 ml of water; and (3) re-equilibration as described above. The Chelating Sepharose was washed with 5 ml of 100 m*M* EDTA (pH 7.2) and 5 ml of water after each run and equilibrated with Zn^{2+} by passing 2 ml of 200 m*M* $ZnSO_4$ solution through the column.

Analytical methods

Column fractions were analysed by SDS-PAGE (8% acrylamide) on slab gels according to Laemmli [21]. Protein bands were rendered visible by silver staining [22]. The P-450 and protein content of the fractions were determined by the methods of Omura and Sato [2] and Lowry *et al.* [23].

Testosterone metabolizing activity

For partial removal of detergent, FPLC fractions were incubated with Biobeads (Bio-Rad Labs., Munich, F.R.G.) (20 mg of beads per mg of protein) for 1 h at 4°C. To evaluate the testosterone-metabolizing activity the sample was supplemented with 10 μg/ml of dilauroylphosphatidylcholine, 125 μM of testosterone and 50 μM of cumene hydroperoxide and incubated for 10 min at 37°C [24]. The steroids were extracted with diethyl ether and the organic phase was evaporated under nitrogen. The residue was dissolved in 0.1 ml of ethanol and analysed by reversed-phase HPLC (Supelcosil RP C_{18}, 5 μm, 150 × 4.6 mm I.D.) with a linear gradient from methanol–acetonitrile–water (43:1.1:55.9) to methanol–acetonitrile–water (75:1.9:23.1). Detection was at 254 nm.

Chemicals and gels

Lubrol PX, benzalkonium chloride, dodecylammoniopropanesulphonate, hexadecyltriethylammonium bromide and other analytical-reagent grade chemicals were purchased from Sigma (Deisenhofen, F.R.G.), except for 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) (Calbiochem, Frankfurt, F.R.G.), Brij 35, sodium cholate, sodium phenobarbital (Serva, Heidelberg, F.R.G.), hexachlorobenzene (Fluka, Neu-Ulm, F.R.G.), cumene hydroperoxide (Aldrich, Steinheim, F.R.G.) and HPLC standards of the hydroxylated testosterones 6β-, 7α- and 16β-hydroxytestosterone (Steraloids, Wilton, NH, U.S.A.).

Gel materials (Mono Q, Mono S, Q-Sepharose Fast Flow, S-Sepharose Fast Flow and Chelating Sepharose Fast Flow) were purchased from Pharmacia.

RESULTS AND DISCUSSION

Selection of suitable detergent

The first step in designing the analytical procedure is screening for a suitable detergent (for membrane solubilization and chromatography), which has to fulfil the following criteria: (1) high efficiency to solubilize microsomal P-450, (2) compatibility with the chromatographic procedure and (3) negligible effects on the enzymatic activity of P-450. Of the different types of detergents, *i.e.*, cationic, anionic, zwitterionic and non-ionic, only members of the last three categories are suitable for P-450 studies because cationic detergents lead to a complete conversion of P-450 to P-420 or irreversible damage of P-450 even at low concentrations ($\leqslant$0.02%). Therefore, we excluded the following detergents from further investigations: benzalkonium chloride, dodecylammoniopropanesulphonate and hexadecyltriethylammonium bromide.

As shown in Table I, acceptable P-450 solubilization is obtained with sodium

TABLE I

EFFICIENCY OF MICROSOME SOLUBILIZATION BY DETERGENTS

Microsomes were solubilized in detergent-containing buffer as described under Experimental. After centrifugation (100 000 *g*), the protein and P-450 content of the supernatant were determined. The figures give the percentage of recovered material.

Detergent	Solubilization (%)	
	Protein	P-450
0.5% Cholate	62	97
0.3% Cholate	72	71
0.1% Cholate	51	35
0.8% Lubrol	69	96
0.4% Lubrol	59	93
0.2% Lubrol	47	79
0.5% CHAPS	43	83
0.3% CHAPS	20	63
0.1% CHAPS	21	40
0.8% Brij 35	74	88

cholate, CHAPS, Lubrol PX and Brij 35. Sodium cholate is widely used in P-450 studies but it is not suitable for analytical separations on the strong anion exchanger Mono Q. Its firm binding to the gel material causes unpredictable shifting of retention times and thus leads to irreproducible chromatographic patterns. This observation is in accordance with the results of Kastner and Schulz [13].

The effect of the three remaining detergents on the chromatographic resolution of P-450 species was further examined using a standard gradient (Fig. 1). Three reasons led us to use Lubrol PX for further investigations: (1) the resolution is superior to that in the presence of CHAPS (Fig. 1); (2) monitoring of protein elution at 280 nm is possible; and (3) enzymatic activities are maintained, as shown by Guengerich and Martin [18] and our own results (see below).

In contrast to our results, Kastner and Schulz [13] found that the chromatographic resolution is higher in the presence of CHAPS than of Lubrol PX. Further, we observed complete binding of P-450 to Mono Q in the presence of CHAPS, whereas the pass-through fraction of Kastner and Schulz [13] contained about 25% of applied P-450. An explanation may be species-specific differences in the chromatographic properties of P-450s from rats and marmoset monkeys.

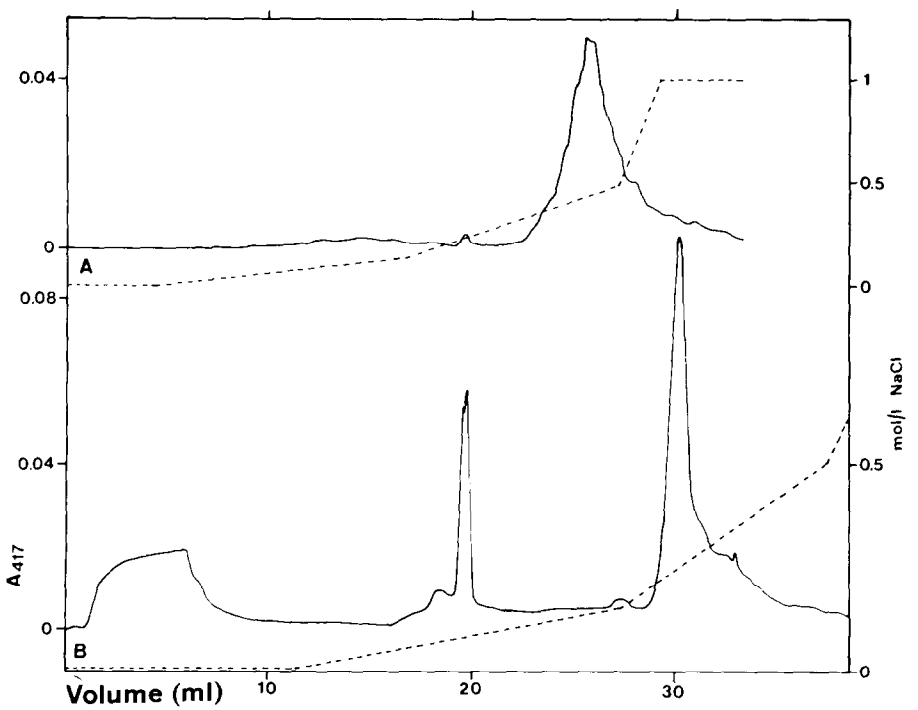

Fig. 1. Separation of P-450 species from detergent-solubilized liver microsomes of phenobarbital-treated rats by anion-exchange FPLC (Mono Q HR 5/5). (A) Sample, 3.5 nmol of P-450 (1.9 mg of protein) in 0.15% CHAPS, 20 m*M* Tris–HCl (pH 7.7), 20% glycerol, 1 m*M* EDTA, 1 m*M* DTE, 0.4 m*M* PMSF. (B) Sample, 4.6 nmol of P-450 (1.7 mg of protein) in the buffer as in (A) but with 0.2% Lubrol PX instead of CHAPS. The equilibration buffer is identical with the corresponding sample buffer. The elution buffers contain 1 *M* NaCl in addition. Flow-rate, 1 ml/min. Solid line, absorbance at 417 nm; broken line, NaCl gradient.

Optimization of the chromatographic procedure

Analytical separations of complex P-450 mixtures by ion-exchange chromatography have been attempted to only a limited extent [10–12]. Preliminary results of our own work were published recently [25]. Prerequisites for a convenient method are reproducibility and high resolution in combination with short separation times. In addition, the sensitivity of detection should be high enough to allow the analysis of small amounts of applied samples.

Anion-exchange chromatography (Mono Q)

Figs. 1B and 2A show that the A_{417} elution profile does not reflect the complexity of the underlying P-450 pattern. Similar gradient systems were also used by

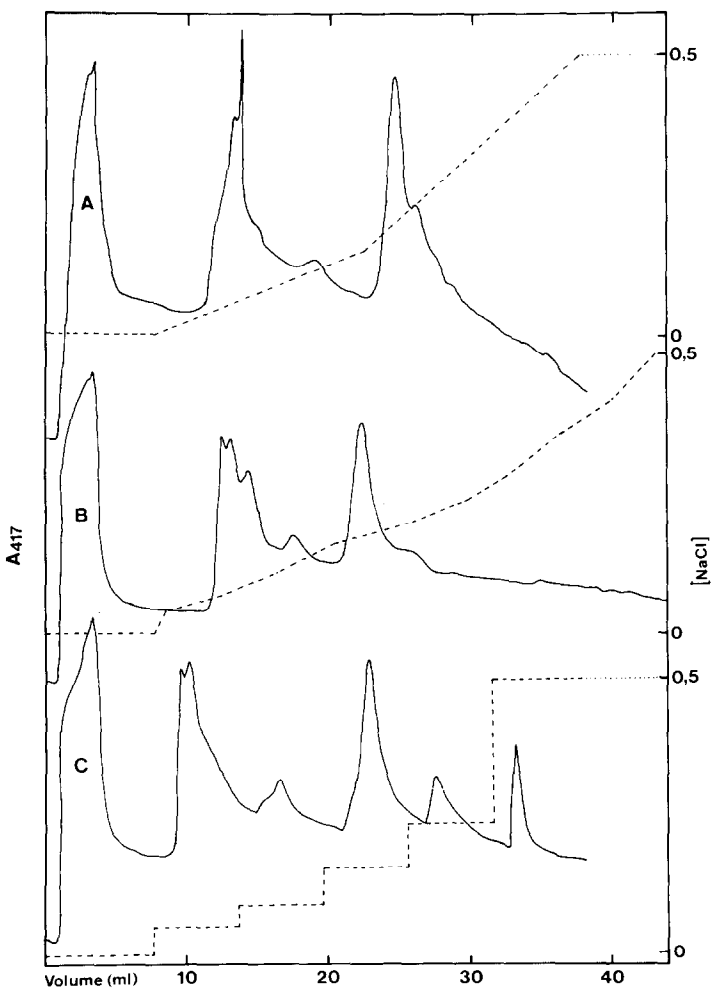

Fig. 2. Effect of the gradient form on the resolution of P-450 species by anion-exchange FPLC on Mono Q HR 5/5. Sample, 1.25 nmol P-450 in Lubrol-containing buffer as specified in Fig. 1A. Rats were treated with phenobarbital and β-naphthoflavone. Flow-rate, 1 ml/min. Solid line, absorbance at 417 nm; broken line, NaCl gradient.

Kastner and Schulz [13] for preparative P-450 separation and by Sakaki *et al.* [12] for the separation of purified P-450 species. The resolution of complex P-450 mixtures, however, requires more complex gradients.

Optimization of resolution is achieved by varying the gradient form as shown in Fig. 2. A pronounced improvement of resolution is obtained by switching from continuous to stepwise gradients. Connecting the discrete concentration steps by linear gradients maintains the analytical character of the elution mode and leads to a further increase in resolution (Fig. 3). Using the optimized method we obtained eight separated P-450 fractions including the material passing through the Mono Q column. P-420 (only sometimes present in our preparations) almost exclusively appears in the Mono Q pass-through fraction. This is in accordance with the observations of Kastner and Schulz [13].

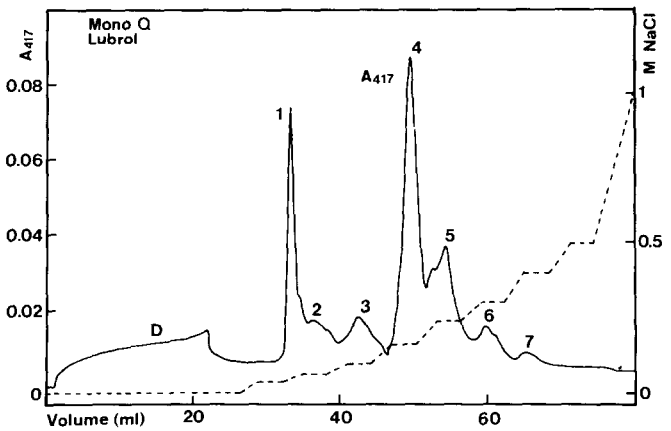

Fig. 3. Chromatographic resolution of P-450 species by optimized gradient elution on Mono Q. Column, Mono Q HR 5/5. Sample, Lubrol-solubilized liver microsomes of rats pretreated with Phenobarbital. P-450 content, 3.8 nmol. Buffers as described under Experimental. D = Unbound material in the pass-through fraction.

The chromatographic resolution especially of peaks 4 and 5 is influenced by the amount of P-450 applied (Fig. 4). Acceptable separation of both peaks is obtained with 1 nmol of P-450, an amount which allows the quantitative spectroscopic determination of P-450 and qualitative analysis by SDS-PAGE of individual Mono Q fractions. Increasing the amount of applied P-450 finally leads to fusion of peaks 4 and 5 (Fig. 4C). Therefore, we routinely use 1–2 nmol of P-450 for analytical separations.

Resolution and reproducibility of chromatographic patterns is maintained even after 20 or more successive runs with simple intermediate reequilibration steps (see Experimental). Retention volumes of individual P-450 peaks are highly reproducible with an averaged variation of 0.6% (n = 8) based on the total gradient volume [25]. As an example, retention times of the Mono Q peaks 4 and 5 obtained with liver microsomes of rats pretreated with various inducers are listed in Table II.

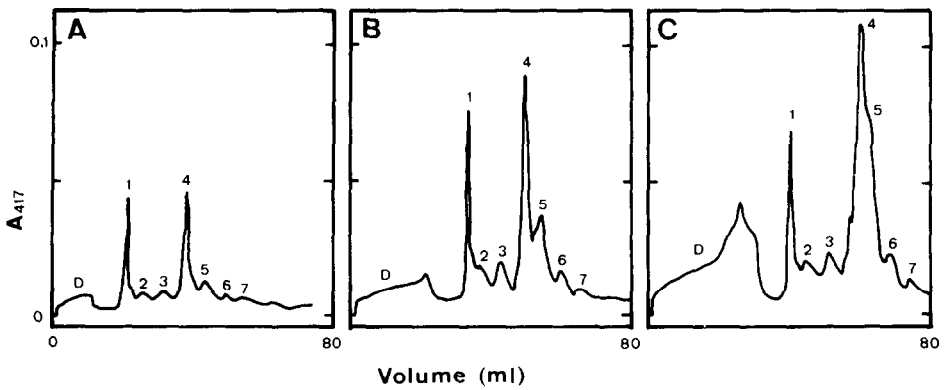

Fig. 4. Influence of the amount of applied P-450 on the elution profile. Column, Mono Q HR 5/5. Sample, Lubrol-solubilized microsomes of phenobarbital-treated rats. (A) 1 nmol of P-450; (B) 3.8 nmol of P-450; (C) 6.5 nmol of P-450. Gradient profile as in Fig. 3. Buffers as specified under Experimental.

Cation-exchange chromatography (Mono S)

The relative amount of P-450 in the Mono Q pass-through fraction depends on the type of induction, *i.e.*, the microsomal P-450 pattern (Table III) and is in the range of 5 to 35% of applied P-450. This fraction can be further separated on the FPLC cation exchanger Mono S. Using MOPS buffer pH 7.0 as described in Experimental the Mono Q pass-through fraction is completely bound to Mono S. This is in agreement with the results of Imaoka *et al.* [26] who used combined anion and cation exchange HPLC for preparative P-450 separation. Chromatographic P-450 resolution of Mono S is more sensitive to pH compared to Mono Q with an optimum at pH 7.0 (not shown). Improvement of resolution is achieved by using a stepwise instead of a linear NaCl gradient (Fig. 5).

Mono S as the first chromatographic step for P-450 separation of Lubrol-solubilized liver microsomes leads to a pass-through fraction containing about 70% of

TABLE II

FRACTIONATION OF MICROSOMAL P-450 (1 nmol) FROM RATS TREATED WITH VARIOUS INDUCERS: COMPARISON OF THE MONO Q PEAKS 4 AND 5

Lubrol-solubilized microsomes (1 nmol P-450) were fractionated on Mono Q HR 5/5 using the optimized chromatographic procedure (Fig. 3). Elution profiles (retention times, peak areas) were analysed by the PC Integration Pack (Softron). P-450 content was determined spectroscopically.

Inducer[a]	Retention time (min)		Area (mV ml)		P-450 (pmol)		Ratio 4:5	
	Peak 4	Peak 5	Peak 4	Peak 5	Peak 4	Peak 5	Area	P-450
PB	47.04	53.54	78.19	25.67	90	80	3.03	1.13
β-NF	47.47	53.92	88.31	40.50	80	250	2.18	0.32
HCB	47.68	53.77	88.29	53.44	74	190	1.65	0.39
m-un	47.48	53.60	72.17	36.95	99	180	1.95	0.63

[a] See Table III.

TABLE III

COMPARATIVE P-450 DETERMINATION IN MONO Q FRACTIONS OBTAINED WITH LUBROL-SOLUBILIZED MICROSOMES OF RATS TREATED WITH VARIOUS INDUCERS

Solubilized microsomes were fractionated on Mono Q HR 5/5 using the optimized chromatographic procedure (see Fig. 3). Peak fractions were pooled and their P-450 contents determined spectroscopically. The figures give the percentage of applied P-450.

Inducer[a]	P-450 (nmol)	P-450 in Mono Q fractions (%)							
		D[b]	1+2	3	4	5	6	7	Total
PB	1.05	24.8	12.4	8.6	8.6	7.6	–[c]	–[c]	63.8
β-NF	0.98	23.5	8.2	10.0	8.2	25.5	3.1	3.1	81.6
HCB	0.96	7.8	10.2	5.1	7.7	19.8	–[c]	–[c]	50.6
INH	0.96	33.6	10.3	6.8	6.8	20.6	1.6	–[c]	80.2
m-un	0.96	15.6	5.1	10.3	10.3	18.8	3.1	–[c]	63.5

[a] PB = Phenobarbital; β-NF = β-naphthoflavone; HCB = hexachlorobenzene; INH = isonicotinic acid hydrazide; m-un = untreated male rats.

[b] D = Pass-through fraction.

[c] P-450 content too low for spectroscopic determination.

applied P-450. Similarly, Kastner and Schulz [13] obtained ⩾80% of unbound P-450 by fractionation of liver microsomes of phenobarbital-induced marmoset monkeys. This amount of unbound P-450 corresponds to the amount bound by Mono Q, provided that the pH of the sample is adjusted to 7.7. Thus, successive combination of Mono Q and Mono S provides a powerful tool for P-450 separation, resulting in eleven P-450 fractions.

Unfortunately, weak FPLC ion exchangers (such as DEAE or CM) based on monobeads are not yet available. Therefore, we had to restrict our studies to the strong ion exchangers Mono Q and Mono S. For protein elution we used NaCl throughout our studies. Kastner and Schulz [13] found that lithium perchlorate gives a better resolution than sodium chloride but results in unstable P-450 fractions.

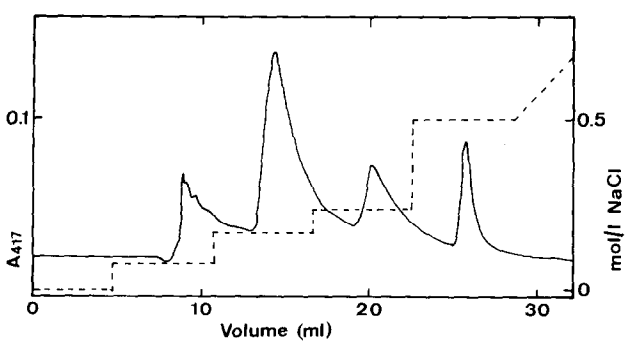

Fig. 5. Subsequent separation of Mono Q pass-through fraction on Mono S HR 5/5. Sample, 0.83 nmol of P-450 of a Mono Q pass-through fraction obtained with liver microsomes of phenobarbital-treated rats. Buffers as indicated under Experimental. Solid line, absorbance at 417 nm; broken line, NaCl gradient. Note that there is no 417-nm absorbing material in the pass-through fraction.

Analysis of FPLC fractions and induction effects

The use of Lubrol PX instead of Emulgen or Triton allows protein elution to be monitored at 280 nm. By simultaneous and continuous detection at 417 nm, haemoprotein-containing fractions are easily recognized. Peak fractions are combined and further analysed by spectroscopy, SDS-PAGE and determination of testosterone metabolizing activity.

Routinely, the P-450 content of individual Mono Q fractions is determined spectroscopically via the $P\text{-}450_{reduced}$–CO complex [2]. Typical results are shown in Table III with the corresponding chromatograms in Fig. 6. Variation of the chromatographic patterns is dependent on the type of P-450 inducer. Three points should be stressed: (1) the amount of unbound P-450 in the pass-through fraction varies with different inducers; (2) chromatograms normalized on a protein basis differ in absolute peak heights, especially for peaks 1, 4 and 5; and (3) the area ratio of peaks 4 and 5 is a good indicator of the induction type (Table II). Detailed data on the latter point will be published in a forthcoming paper [27]. In addition to cytochrome P-450, microsomes contain another haemoprotein, cytochrome *b*5, which elutes in peak 4 at about 160 m*M* NaCl, causing the discrepancy between the peak area at 417 nm and the spectroscopically determined P-450 content relative to other fractions (Table II).

The chromatographic separation of proteins by Mono Q is demonstrated by SDS-PAGE in the P-450 relevant molecular weight range of 46 000–58 000 dalton (Fig. 7). Obviously, some fractions contain more than one P-450 species. Their

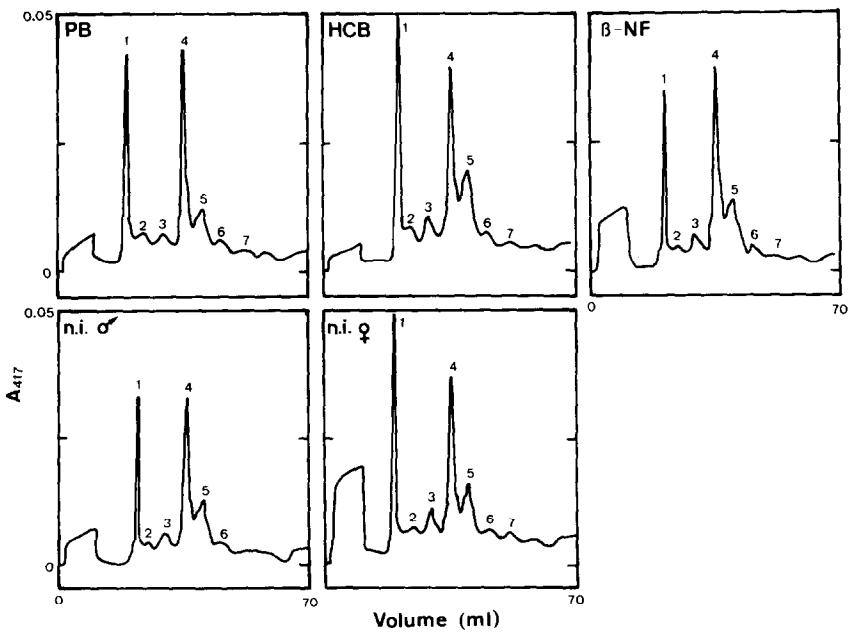

Fig. 6. Analytical fractionation of microsomal P-450 from male rats pretreated with various inducers or untreated rats. Column, Mono Q HR 5/5. Samples, Lubrol-solubilized liver microsomes containing 1 nmol of P-450 each. Gradient profile as in Fig. 3. PB = Phenobarbital; HCB = hexachlorobenzene; β-NF = β-naphthoflavone; n.i. = not induced.

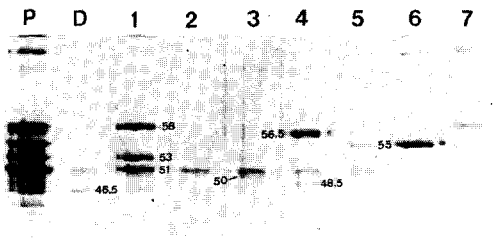

Fig. 7. SDS-PAGE of Mono Q fractions. Samples, fractionated liver microsomes of phenobarbital-treated rats (1 nmol of P-450). Numbers correspond to peaks as in Fig. 3. P = Unfractioned sample. The P-450 relevant molecular weight range between 46 and 58 kilodalton is magnified. Apparent molecular weights are assigned to individual protein bands.

resolution requires a supplementary fractionation step based on a different chromatographic principle (see below). On the other hand, several fractions exhibit similar enzymatic activities and SDS-PAGE patterns (Table IV, Fig. 7). This may be due to the existence of closely related isozyme or allozyme forms. Relevant results by other workers support this possibility. A P-450 preparation homogeneous by SDS-PAGE could be resolved into three fractions by anion-exchange HPLC [28]. Sakai *et al.* [29] detected three immuno-identical P-450 species in rat liver microsomes after phenobarbital induction. These forms probably coincide with multiple P-450b species described by Vlasuk *et al.* [30] and Oertle *et al.* [31].

Data from testosterone metabolism point in the same direction because several fractions catalyse the formation of identical products, especially 6β- and 7α-hydroxytestosterone. This is in partial agreement with data published by Funae and Imaoka [32], who found 6β-hydroxylase activity in five fractions obtained by anion-exchange

TABLE IV

TESTOSTERONE METABOLITES PRODUCED BY MONO Q FRACTIONS OBTAINED WITH LUBROL-SOLUBILIZED MICROSOMES OF RATS TREATED WITH PHENOBARBITAL

Enzymatic activity of Mono Q fractions was determined as described under Experimental. Fraction numbers correspond to those in Fig. 5. Metabolites: testosterone derivatives hydroxylated at the indicated positions.

Fraction	Testosterone metabolite									
	2β-	6α-	6β-	7α-	11β-	15β-	16α-	16β-	18	A[a]
Sample	−	+	+	+	+	+	+	+	+	+
D[b]	−	−	+	+	−	−	−	−	−	−
1	+	−	+	+	−	−	−	−	−	+
2	+	−	−	+	+	−	+	+	−	+
3	+	−	+	+	−	−	−	−	−	+
4	+	+	+	+	−	+	−	−	−	+
5	−	−	+	+	−	−	−	−	−	−
6	−	−	+	+	−	−	−	−	−	−
7	−	−	+	+	−	−	−	−	−	−

[a] A = Androstenedione.
[b] D = Pass-through fraction.

HPLC. Formation of 7α-hydroxytestosterone, however, was restricted to one fraction only. Our finding is especially interesting because 7α-hydroxylation was ascribed to P-450IIA1 (P-450a) only [33]. A recently described new P-450 species (P-450m) [3], however, catalyses the same reaction. The results point to isozymic or allozymic heterogeneity of these P-450 species. Detailed data on testosterone metabolism by individual FPLC fractions will be published in a forthcoming paper [34].

Immobilized metal affinity chromatography (IMAC)

For further resolution of Mono Q fractions containing more than one P-450 species, we chose immobilized metal affinity chromatography. Here we present some preliminary results to demonstrate the applicability of this method to P-450 fractionation.

The chelating gel (Chelating Sepharose Fast Flow) can be charged with different transition metal ions [20]. Of the set tested by us (Ni^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}), only Zn^{2+} proved to be useful for our purposes. The recovery of spectroscopically intact P-450 eluted from the column was very low or zero using any of the other cations. In addition to the nature of the immobilized metal ion, the kind of eluting

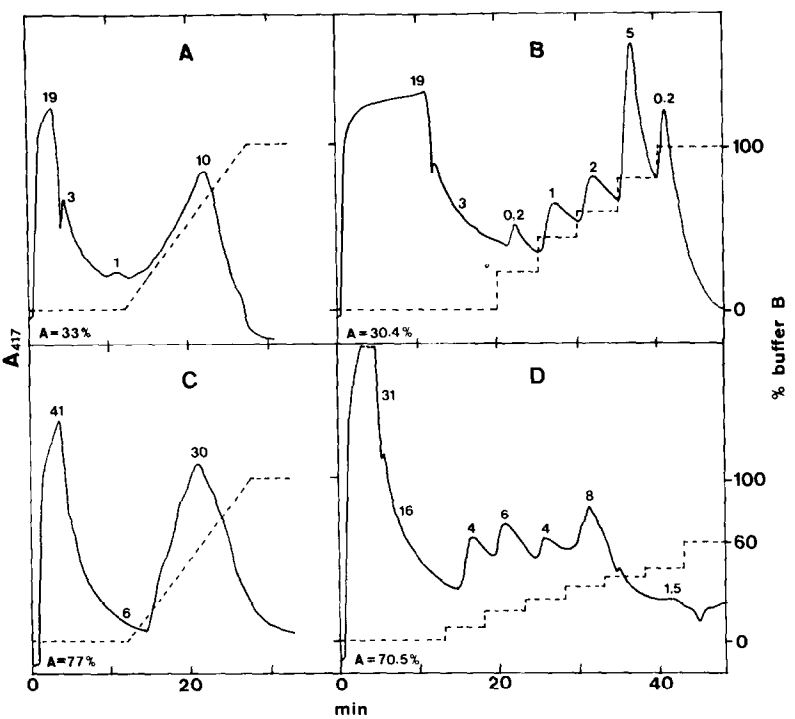

Fig. 8. Fractionation of P-450 species by immobilized metal affinity chromatography (IMAC). Column and gel material, FPLC HR 5/5 filled with 1 ml of Chelating Sepharose Fast Flow. Immobilized cation, Zn^{2+}. Sample, liver microsomes of phenobarbital-treated rats prefractionated on 8-aminooctyl-Sepharose. (A) 0.77 nmol of P-450, linear pH gradient; (B) 3.5 nmol of P-450, stepwise pH gradient; (C) 0.96 nmol of P-450, linear NH_4Cl gradient; (D) 3.1 nmol of P-450, stepwise NH_4Cl gradient. Buffers as specified under Experimental. Gradient composition is given by the broken line as a percentage of buffer B in the eluting buffer. Solid line, absorbance at 417 nm. Numbers, percentage of applied P-450.

agent has a strong effect on recoveries. If elution is performed with decreasing pH (7.0 to 3.8) the P-450 recovery is about 30% compared with 70–100% with an ammonium chloride gradient (0–2 mol/l) (Fig. 8). In analogy with ion-exchange chromatography, improvement of resolution is attained by stepwise gradient elution (Fig. 8B and D).

Scale-up

The results of the optimized analytical ion-exchange procedures suggest their application for preparative purposes. Considering the minute amounts of many P-450

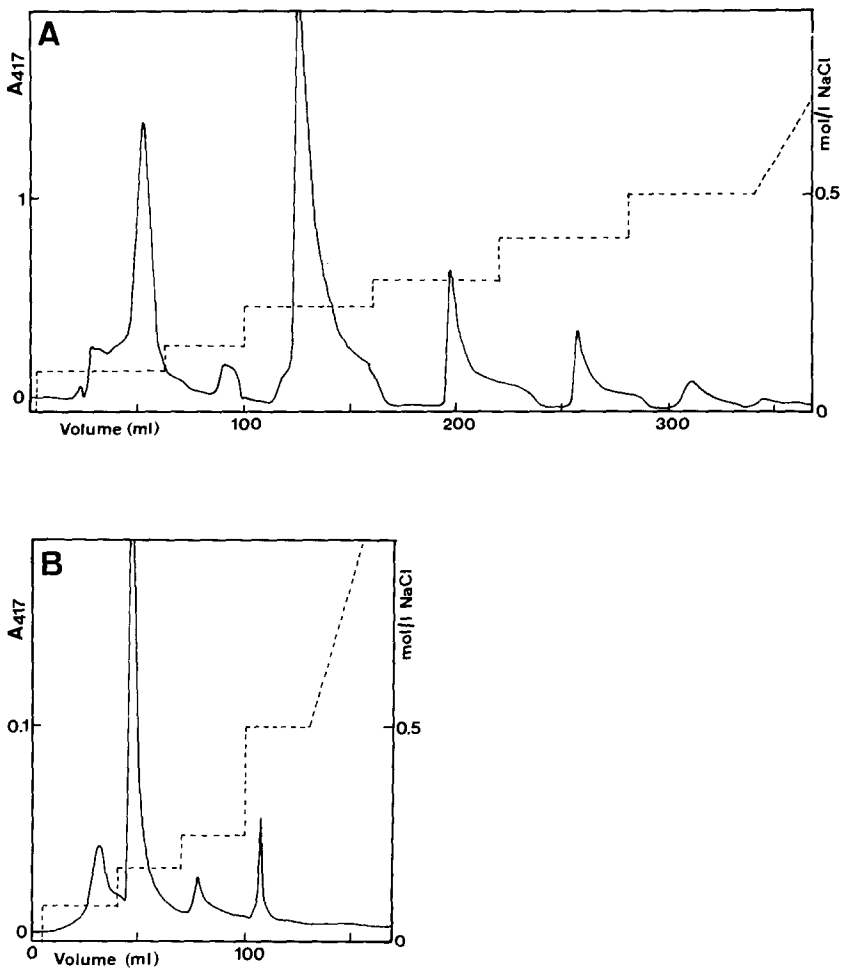

Fig. 9. Preparative fractionation of microsomal P-450 species of phenobarbital-treated rats on Q-Sepharose Fast Flow and S-Sepharose Fast Flow. (A) Column and gel material, FPLC HR 16/10 filled with 20 ml of Q-Sepharose Fast Flow. Sample, 190 nmol of P-450. Buffers as specified under Experimental for Mono Q. Flow-rate, 1 ml/min. Pass-through fraction not shown. (B) Column and gel material, FPLC HR 10/10 filled with 7.5 ml of S-Sepharose Fast Flow. Sample, pass-through fraction of the Q-Sepharose run shown in (A). Buffers as specified under Experimental for Mono S. Flow-rate, 1 ml/min. Solid lines, absorbance at 417 nm; broken lines, NaCl gradient.

species in crude microsomal extracts, columns with high protein binding capacity are required for the initial fractionation steps. The optimized chromatographic procedures for Mono Q and Mono S can be easily adapted to the equivalent Sepharose Fast Flow gels using conventional chromatography columns still taking advantage of the automatic FPLC system. As shown in Fig. 9, gradient steps slightly modified compared with those of the analytical runs can be applied to Q- and S-Sepharose, leading to a comparable resolution. Differences in the elution patterns of Mono Q and Q-Sepharose may be due to the physically and chemically distinct gel matrices, different column geometries and different gradient steps.

CONCLUSION

Fractionation of microsomal proteins by ion-exchange FPLC results in a high resolution of P-450 isoenzymes. This method can be used for both preparative and analytical fractionations. The purpose of these studies is the evaluation of xenobiotically caused effects on the organism by the analysis of microsomal P-450 patterns. We believe that high-resolution FPLC in combination with other more specific analytical methods currently being established will be an appropriate tool for this purpose.

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