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DETERMINATION OF METABOLITES OF CYTOCHROME P-450 MODEL SYSTEMS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatographic techniques were developed for the simultaneous detection of metabolites in a cytochrome P-450 model system composed of NADH, haemoglobin and methylene blue. Monohydroxylated metabolites were determined following aniline, acetanilide and phenol hydroxylations. 4-Aminoantipyrine, 7-hydroxycoumarin and *p*-nitrophenol were determined after dealkylation of 4-N,N-dimethylaminoantipyrine, 7-ethoxycoumarin and *p*-nitroanisole. These substrates are commonly used for measuring cytochrome P-450 activities. Treatment of the samples was minimal, consisting of a simple deproteinization, and did not involve any organic extraction. Separations were carried out on reversed-phase columns and the products were detected by UV adsorption. Separations were completed in less than 15 min and the detection limits were between 0.5 and 4 μM.

INTRODUCTION

Hydroxylation and dealkylation reactions are commonly tested on microsomal cytochrome P-450. Most of the methods traditionally used for the calculation of activities are based on the determination of one major product of the reaction or of one side-product. These methods suffer from two kinds of drawbacks: as they are often spectrophotometric, there may possibly be interference from other compounds in complex mixtures, and with titration of only one major product, other products are assumed to be minor. This might not be a general case and study of the products of reaction could help in differentiating the different forms of cytochrome P-450 by their regioselectivities.

In an attempt to study reactions catalysed by cytochrome P-450 model systems, an approach using high-performance liquid chromatography (HPLC)

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was devised. This allowed both a qualitative and a quantitative method of separation of the reaction products. Both gas and liquid chromatography fitted this requirement, but gas chromatography, although generally more sensitive, was rejected because of the necessary derivatization of aqueous samples. In contrast, HPLC needed minimal preparation of aqueous samples and gave reproducible results within a reasonable time with good sensitivity.

Three hydroxylation reactions on aromatic rings (aniline, phenol and acetanilide) were studied by HPLC. Three oxidative dealkylations were also examined by HPLC: N-demethylation of 4-N,N-dimethylaminoantipyrine, O-de-ethylation of 7-ethoxycoumarin and O-demethylation of *p*-nitroanisole. These chromatographic techniques were applied to the study of activities and regioselectivities of model systems of cytochrome P-450 [1] (an example of which will be given).

The cytochrome P-450 system of liver microsomes is composed of NADPH (electron donor), NADPH cytochrome P-450 reductase (electron carrier) and cytochrome P-450. The model system given here as an example of application is NADH—methylene blue and haemoglobin. The aim was to compare hydroxylations and dealkylations by haemoglobin and cytochrome P-450 and to examine the use of methylene blue as an electron carrier.

EXPERIMENTAL

Materials

Chemicals were obtained from Sigma and Aldrich. Bovine haemoglobin was prepared from freshly drawn blood as described by Heidelberg and Lansteiner [2], with minor modifications. Solutions were concentrated with an Amicon Hollow Fibers system and stored in liquid nitrogen.

Conditions

Experiments were conducted in the dark at 37 °C in 0.1 *M* phosphate buffer (pH 7.4). The model system was composed of 1 m*M* NADH, 10 μM methylene blue, 0.1 *M* haemoglobin and substrate. The concentrations of the different substrates were aniline 30 m*M*, phenol 20 m*M*, acetanilide 10 m*M*, 4-N,N-dimethylaminoantipyrine 20 m*M*, 7-ethoxycoumarin 0.5 m*M* and *p*-nitroanisole 5 m*M*. 7-Ethoxycoumarin and *p*-nitroanisole were previously dissolved in 5% ethanol and 9% polyethylene glycol 400, respectively. Reactions were started by addition of concentrated haemoglobin. Haemoglobin concentrations were measured according to Drabkin [3].

Preparation of samples for HPLC and determination of the products

Samples of 0.5 ml were deproteinized by addition of 40 μl of trichloroacetic acid (TCA) (30%, w/v) or 0.6 ml of methanol (at 4 °C). This was necessary in order to avoid irreversible adsorption of the proteins on the phase of the HPLC column.

Precipitation by TCA to form insoluble salts in an acidic medium was found to be the most efficient method, but oxidative damage of some products occurred. As TCA is rapidly eluted from the column, there is no interference with peaks of products. The use of perchloric acid instead of TCA with subse-

quent precipitation of perchlorate by potassium carbonate did not give any UV absorption but oxidized all the products considerably. Formation of insoluble salts in a neutral medium with heavy metals was inefficient. Dehydration with solvents miscible with water was a mild precipitation method that was used when the use of TCA was prohibited. Methanol was found to be the best precipitating agent after acetone (which absorbs considerably at 280 nm).

Samples were then centrifuged for 5 min at 800*g* and 10—25 µl of the supernatant injected into a Waters Assoc. 6000A HPLC system equipped with an octadecylsilane column. Two types of reversed-phase C_{18} columns were used at 25°C: a Rad-Pak C_{18}, 10-µm (Waters Assoc.) and a Chromatem RP-18, 5-µm (Touzart et Matignon). UV detection was performed at 254, 280 or 340 nm with an M440 detector (Waters Assoc.).

Product identity was confirmed by checking the co-elution of an internal standard. Calibration graphs were established with standards under the same experimental conditions.

RESULTS AND DISCUSSION

Determination of metabolites of aniline hydroxylation

The classical method [4] permits only the determination of 4-aminophenol, which gives indophenol by reaction with phenol and is detected by spectro-

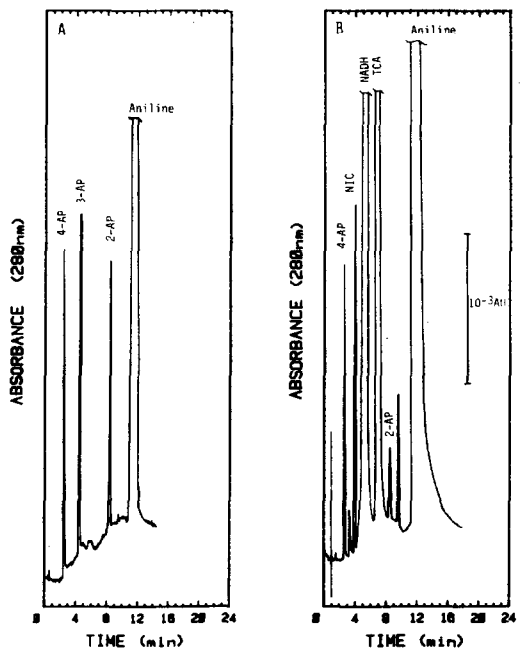

Fig. 1. (A) Chromatogram of standards of aniline and 2-, 3- and 4-aminophenols (2-AP, 3-AP and 4-AP). Deproteinization was accomplished with TCA: 15 µl of the supernatant were injected onto a Chromatem column. Mobile phase 0.2 *M* Tris—acetate buffer (pH 8) plus 0.75% (v/v) triethylamine; flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 1 h. TCA = trichloroacetic acid; NIC = nicotinamide. During the reaction the NADH is partially denatured to nicotinamide. Conditions as stated in the text.

photometry. This very sensitive reaction does not allow the detection of 2- and 3-aminophenols, which were followed by our method during aniline hydroxylation.

A separation of the three isomers on a Dupont Zipax SCX reversed-phase column with 0.1 *M* phosphoric acid (pH 2.9) as mobile phase and a flow-rate of 0.8 ml/min was described by Sakurai and Ogawa [5]. At this pH, substantial tailing of the aminophenols was observed. When the pH was increased to 7.5—8, 4- and 3-aminophenols no longer tailed but 2-aminophenol still did. This might be due to an interaction between the amino group of the aminophenol and the ungrafted silanol remnants in the column, as addition of 0.75% of triethylamine efficiently decreased the tailing. It should be noted that the pH of the mobile phase has to be checked after the addition of this very basic compound.

Fig. 1A shows the chromatogram of aniline plus 2-, 3- and 4-aminophenol standards on a Chromatem column. Fig. 1B shows the chromatogram of an incubation medium after 1 h: 4- and 2-aminophenols are produced. 3-Aminophenol is not detected (detection limits as in Table I).

TABLE I

LINEAR REGRESSION ANALYSIS FOR METABOLITES IN THE HPLC METHODS

x = molarity of the solution injected (μM), y = optical density recorded (O.D. $\times 10^3$).

Compound	Range		Slope	y-Intercept	Correlation coefficient
	μM*	pmol			
4-Aminophenol	2.0—50	30—750	0.052	0.052	0.999
3-Aminophenol	1.0—50	15—750	0.079	0.038	0.999
2-Aminophenol	3.0—50	45—750	0.074	−0.006	0.999
Hydroquinone	1.0—50	12—565	0.080	0.003	0.999
Resorcinol	2.5—50	29—565	0.035	0.027	0.999
Catechol	3.0—50	34—565	0.030	0.006	0.999
4-Acetaminophenol	0.5—25	5—250	0.252	0.035	0.997
3-Acetaminophenol	1.0—25	10—250	0.123	0.052	0.999
2-Acetaminophenol	4.0—25	40—250	0.043	0.039	0.999
4-Aminoantipyrine	1.2—3.5	6—161	0.088	0.016	0.999
7-Hydroxycoumarin	1.0—50	5—230	0.134	−0.037	0.997
p-Nitrophenol	1.0—50	5—230	0.101	−0.026	0.999

*The lower end of the range is the detection limit: optical density = 10^{-4}, equivalent to a signal-to-noise ratio of 2:1. Statistical parameters based on the responses of five standards.

Determination of metabolites of phenol hydroxylation

The possible products examined were the 2-, 3- and 4-monohydroxyphenols, i.e., catechol, resorcinol and hydroquinone. Separation with gradient programming [6] was considered to be too complicated.

Precipitation of the protein was effected with methanol and not TCA, as hydroquinone and catechol are especially sensitive to oxidation in the presence of TCA.

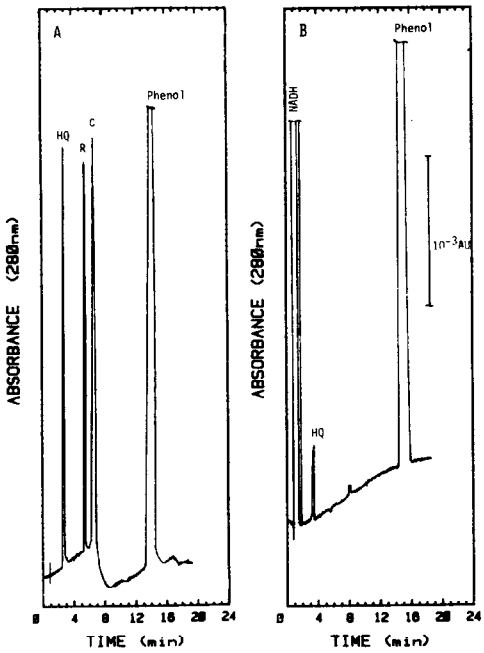

Fig. 2. (A) Chromatogram of standards of phenol, catechol (C), resorcinol (R) and hydroquinone (HQ). Deproteinization was accomplished with methanol: 25 μl of the supernatant were injected onto a Chromatem column. Mobile phase, tetrahydrofuran—0.1 *M* ammonium acetate (pH 5) (2:98, v/v); flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 20 min. Conditions as stated in the text.

Catechol was no longer detectable when the pH of the mobile phase was higher than 5, either for stability reasons or owing to intramolecular bonding between the adjacent hydroxy groups, which increased its affinity for the column. A 0.1 *M* ammonium acetate—acetic acid buffer (pH 5) was therefore chosen.

Fig. 2A shows the separation of standards of monohydroxyphenols and phenol on a Chromatem column. Fig. 2B shows the chromatogram of an incubation medium after 20 min: hydroquinone was found but not catechol or resorcinol (see Table I for detection limits).

Determination of metabolites of acetanilide hydroxylation

Acetanilide and 2-, 3- and 4-acetaminophenols, which are not sensitive to TCA, were separated on a Chromatem column at basic pH. The chromatograms in Fig. 3A and B show separations of standards and metabolites (4-, 3- and 2-acetaminophenols), respectively, in an incubation medium after 31 min (see Table I for detection limits)

Determination of metabolites of 4-N,N-dimethylaminoantipyrine N-demethylation

4-N,N-Dimethylaminoantipyrine is oxidized by cytochrome P-450 into formol and 4-aminoantipyrine. This reaction is usually followed by titration of formol with Nash reagent and detection at 412 nm according to procedures derived from the method of Nash [7].

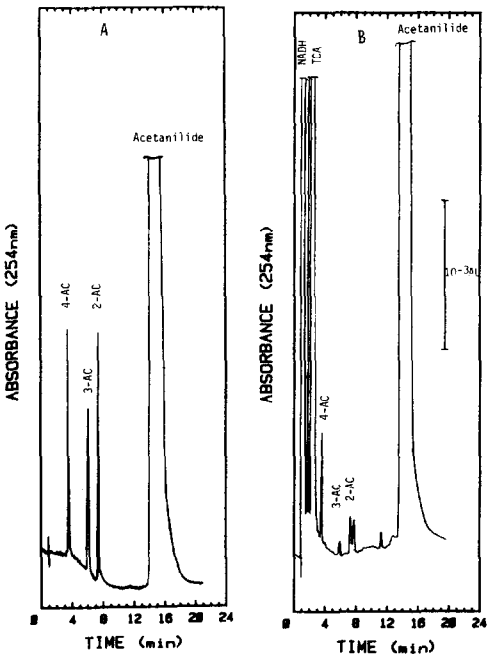

Fig. 3. (A) Chromatogram of standards of acetanilide and 2-, 3- and 4-acetaminophenols (2-AC, 3-AC and 4-AC). Deproteinization was accomplished with TCA: 10 μl of the supernatant were injected onto a Chromatem column. See Table I for detection levels. (B) Chromatogram of an incubation medium after 31 min. Conditions as stated in the text.

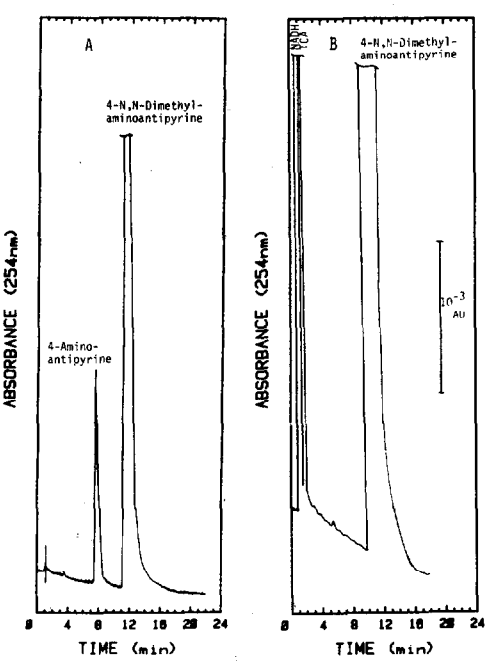

Fig. 4. (A) Chromatogram of standards of 4-N, N-dimethylaminoantipyrine and 4-aminoantipyrine. Deproteinization was accomplished with methanol: 10 μl of the supernatant were injected onto a Rad-Pak column. Mobile phase, methanol—0.1 *M* Tris—acetate buffer (pH 8) (35:65, v/v); flow-rate, 3 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 1 h 25 min. 4-Aminoantipyrine would have been easily detected if it had appeared. Conditions as stated in the text.

Interference was found with derivatives of haemoglobin at this wavelength, so determination of 4-aminoantipyrine by HPLC was preferred. The separation was effected at pH 8 on a Rad-Pak column. Separations of standards and of compounds in an incubation medium (after 1 h 25 min) are shown in Fig. 4A and B, respectively. The expected product, 4-aminoantipyrine, was not found in this model system at our detection limit (1.2 μM or 6 pmol) (Table I). By comparison, activities of different forms of cytochrome P-450 vary from 0.8 to 2.2 μM/min, which is at the level of our detection limit in 1 min [8, 9].

Determination of metabolites of 7-ethoxycoumarin O-de-ethylation

7-Ethoxycourmarin is transformed by cytochrome P-450 into 7-hydroxycoumarin and acetaldehyde. 7-Hydroxycoumarin is commonly determined by spectrofluorimetry (λ_{exc} = 366 nm, λ_{em} = 454 nm) [10]. It was very easily separated from 7-ethoxycoumarin by HPLC at an acidic pH on a Rad-Pak column and detected at 340 nm. A fluorescence detector would decrease the detection limit, which was 1 μM under these conditions. A typical separation of standards is given in Fig. 5A and the chromatogram of the reaction medium after 2 h 30 min in Fig. 5B. 7-Hydroxycoumarin, which was not detected, would have given an easily detected peak (Table I). With different forms of cytochrome P-450 the concentrations of 7-hydroxycoumarin range from 0.2 to 1.3 μM/min [7, 8], which means that in the worst case detection will occur after 5 min.

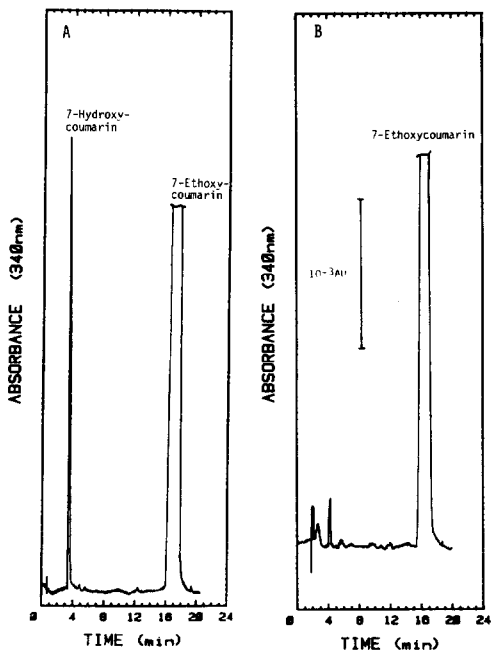

Fig. 5. (A) Chromatogram of standards of 7-ethoxycoumarin and 7-hydroxycoumarin. Deproteinization was accomplished with methanol: 10 μl of the supernatant were injected onto a Rad-Pak column. Mobile phase, methanol—0.1% acetic acid (50:50, v/v); flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 2 h 35 min. 7-Hydroxycoumarin was not found at a detectable level, which would have been very easy. Conditions as stated in the text.

Determination of metabolites of p-nitroanisole O-demethylation

p-Nitroanisole is converted by cytochrome P-450 into *p*-nitrophenol and formol. If *p*-nitrophenol is evaluated by altering the pH to 7.5 to give the yellow phenate form [11], colorimetric interference occurs in our model system. Standards were therefore separated on a Rad-Pak column at acidic pH to give mostly the phenol form and a single peak instead of unresolved peaks (phenol and phenate) (Fig. 6A). Again, the haemoglobin-containing system does not show any detectable *p*-nitrophenol-forming activity (Fig. 6B). *p*-Nitrophenol at concentrations from 0.2 to 0.6 μM/min is obtained from several forms of cytochrome P-450 [7, 8] and it would then be detected in at least 5 min (Table I).

These methods were developed to check the comparison between a cytochrome P-450 model system and a cytochrome P-450 system. Several workers [12—14] have tried to replace cytochrome P-450 with another haemoprotein such as haemoglobin, but until now experiments have been only conducted with aniline as substrate.

Our aim was to determine whether there was similar behaviour of an NADH—methylene blue—haemoglobin system and of a liver microsomal system towards typical substrates of cytochrome P-450, by comparing substrate specificities and reaction regioselectivities [1]. It was found that hydroxylation reactions but not dealkylation reactions occurred in this haemoglobin system

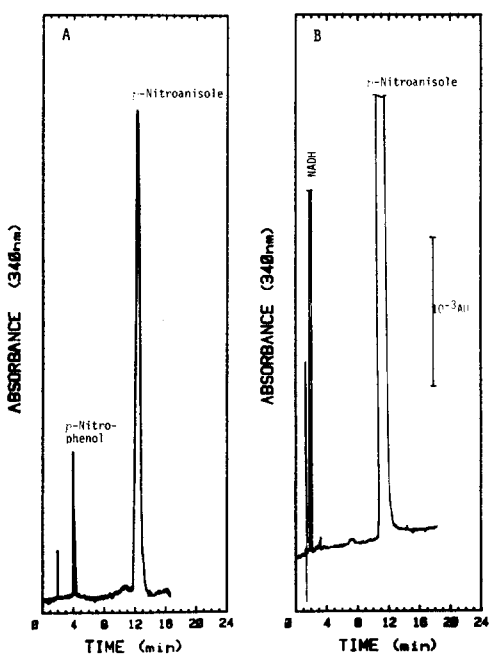

Fig. 6. (A) Chromatogram of standards of *p*-nitrophenol and *p*-nitroanisole. Deproteinization was accomplished with methanol: 10 µl of the supernatant were injected onto a Rad-Pak column. Mobile phase, methanol—0.1% acetic acid (45:55, v/v); flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 2 h 30 min. No detectable activity was found (the peak of *p*-nitrophenol would have been well resolved). Conditions as stated in the text.

TABLE II

REGIOSELECTIVITES IN HYDROXYLATIONS OF ANILINE, ACETANILIDE AND PHENOL

Percentages of isomers formed from aniline, acetanilide and phenol in a model system (30 m*M* aniline, 10 m*M* acetanilide or 20 m*M* phenol, 0.1 m*M* methylene blue, 1 m*M* NADH and 0.1 *M* oxyhaemoglobin) and in a liver microsomal system [15, 16].

Compound	Metabolite	Model system	Cytochrome P-450 system
Aniline	4-Aminophenol	80	85
	3-Aminophenol	< 4	0
	2-Aminophenol	16	15
Acetanilide	4-Acetaminophenol	29	94
	3-Acetaminophenol	11	1
	2-Acetaminophenol	60	5
Phenol	Hydroquinone	100	100
	Resorcinol	< 10	0
	Catechol	< 12	0

(within our detection limits). Phenol was hydroxylated in the *para* positions. Aniline was hydroxylated in the *para* and *ortho* positions with a regioselectivity similar to that known for cytochrome P-450 (Table II). There was substrate specificity towards aniline and phenol. Although acetanilide was hydroxylated by our model system in the *ortho*, *meta* and *para* positions similarly to cytochrome P-450, the regioselectivities were totally different (Table II). The use of these methods allowed us to show that the haemoglobin—cytochrome P-450 comparison was valid only in the particular case with aniline as substrate when hydroxylations were concerned, and was not valid for these dealkylations.

CONCLUSION

Analysis by HPLC prevented interferences from other compounds and allowed the determination of the regioselectivity of a haemoglobin system. These methods might be adjusted for cytochrome P-450 and help to differentiate between different forms according to the different regioselectivities obtained towards the same substrate. They could be of great help in the comparison of model and microsomal systems when studying metabolites of drugs.

With HPLC, pre-treatment of the sample is minimal, no loss of product occurs, in contrast to extraction for gas chromatographic analysis, and there is no need to calculate an extraction yield for each compound. If other products are to be detected, gradients of mobile phase are possible, as was well demonstrated for benzo[*a*]pyrene metabolites [17].

The pH of the sample is very important with regard to the retention of polar compounds on the columns. Detection limits ranged from 1 to 4 μM or from 3 to 40 pmol, depending on the metabolites to be analysed and on the method used (Table II). They may be improved by use of another detection system such as fluorimetry or by decreasing the amount of precipitating agents. With phenol, derivatization with 4-aminoantipyrine could increase the sensitivity ten-fold but after a 40-min reaction [18]. The reproducibility was within 5% and all analyses were completed within 15 min.

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