Journal of Chromatography, 580 (1992) 325–346
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6358

Review

Detection of the enzymatic activity of cytochrome P-450 enzymes by high-performance liquid chromatography

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(First received January 23rd, 1992; revised manuscript received May 5th, 1992)

ABSTRACT

The reactions catalysed by the various cytochrome P-450 enzymes are reviewed with respect to the analysis of products by high-performance liquid chromatography (HPLC). Especially biotransformation reactions of purified cytochrome P-450 enzymes in a reconstituted system and in microsomes mainly of rat liver origin are considered. Emphasis is put on the specificity of product formation due to the individual isozymes of cytochrome P-450. It is shown that the presence of eight cytochrome P-450 isozymes can be monitored and determined by specific product formation after HPLC analysis, which is an important parameter in toxicological studies.

CONTENTS

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0378-4347/92/$15.00

LIST OF ABBREVIATIONS

DMBA	Dimethylbenz[*a*]anthracene
EROD	Ethoxyresorufin O-deethylase
Glu-P-1	2-Amino-6-methyldipyrido[1,2,-*a*:3′,2′-*d*]imidazole
HPLC	High-performance liquid chromatography
IQ	2-Amino-3-methylimidazo[4,5-*f*]-quinoline
4-MA	17β-N,N-Diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one
3-MC	3-Methylcholanthrene
NADPH	Reduced β-nicotinamide adenine dinucleotide
PB	Phenobarbital
SCC	Side-chain cleavage
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Trp-P-2	3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole

1. INTRODUCTION

1.1. Scope of the review

The main purpose of this review is to summarize and evaluate the high-performance liquid chromatographic (HPLC) studies of product formation that have been performed to determine the enzymatic activity of specific cytochrome P-450 enzymes.

It will be clear that the composition of the cytochrome P-450 enzyme pattern in a given tissue can be an important toxicological parameter. It can provide specific information about the exposure to xenobiotic compounds. The information can be of both qualitative (the kind of compound) and quantitative origin (the concentration of the compounds).

Cytochrome P-450 is involved in the metabolism of both endogenous and exogenous compounds. In this review, we shall summarize the compounds that have been used in studies to establish isozyme specificity of cytochrome P-450 by product analysis by chromatographic methods. Much attention will be paid to cytochrome P-450 isozymes from rat liver microsomes. The *in vivo* detection of metabolites in urine and other body fluids will not be considered.

Because of the broad scope of the subject and in spite of an extensive literature search, a certain selection of references was necessary.

1.2. Properties and function of cytochrome P-450

Cytochrome P-450 is the name for a family of proteins that belong to the class of enzymes

called monooxygenases. Monooxygenases transfer one oxygen atom into the product of the enzymatic reaction. Cytochromes P-450 are haeme proteins with protoporphyrin as cofactor. In contrast to other haeme proteins, the haeme in cytochrome P-450 is not covalently bound to the protein moiety. Its name originates from the maximum in the absorption spectrum of 450 nm in the presence of carbon monoxide. The native absorption spectrum shows a large peak at 420 nm. Cytochrome P-450 is usually found in membranes of the endoplasmic reticulum and mitochondria. The relative molecular mass range of the different enzymes is between $45 \cdot 10^3$ and $53 \cdot 10^3$. This enzyme system is present in all living organisms. The rat acts as a suitable model system for cytochrome P-450. In the rat about 30 different cytochrome P-450 genes have been located. Although the liver contains the highest concentration of cytochrome P-450, isozymes are present in almost all organs and tissues of the body except in striated muscle and erythrocytes.

Cytochrome P-450 is involved in the so-called phase I reactions of the biotransformation process in which the substrate is converted into a more water-soluble compound. This is often done by hydroxylation, epoxidation, dealkylation or oxidation, which makes the substrate suitable for the phase II conjugation reactions. The cytochrome P-450 system has a twofold function in the phase I reactions. On one hand, it is involved in the biosynthesis and metabolism of endogenous compounds, such as steroids, fatty acids, bile acids, prostaglandins and vitamins, and on the other hand it becomes involved in the detoxification of xenobiotic compounds.

1.3. Different cytochrome P-450 isozymes and nomenclature

The cytochrome P-450 biotransformation system consists of a large number of enzymes. Although most investigators used a nomenclature in which the cytochrome P-450 isozymes were indicated with letters, such as *a, b, c, d, h, j, p,* etc., up to 1987 some research groups used their own nomenclature. From 1987, Nebert and co-workers [1–3] presented a unified classification. The cytochrome P-450 isozymes have been classified into families and subfamilies on the basis of the similarity of their amino acid sequences. The most important subfamilies in rats are IA with members IA1 (formerly *c*) and IA2 (formerly *d*); IIA with members IIA1, IIA2 and IIA3 (formerly *a*); IIB with members IIB1 (formerly *b*), IIB2 (formerly *e*) and IIB3; IIC with members IIC6, IIC7 (formerly *f*), IIC11 (formerly *h*), IIC12 (formerly *i*) and IIC13 (formerly *g*); IIE with member IIE1 (formerly *j*); IIIA (formerly *p*) with members IIIA1 and IIIA2; and IVA (formerly P-452) with members IVA1, IVA2 and IVA3.

1.4. Induction of cytochrome P-450 isozymes

The various cytochrome P-450 enzymes can be induced by different xenobiotica. In principle, every compound can induce a specific cytochrome P-450 enzyme pattern. On the one hand a compound can induce only one particular cytochrome P-450 enzyme or can induce a number of different cytochrome P-450 enzymes, each to a different extent. As a result, a more or less specific enzyme pattern will be induced. In microsomes of control rats, mainly the isozymes IA2 and members of the families IIE and IIIA are present.

In general, the inducers can be divided into five classes.

(a) Polycyclic aromatic hydrocarbons with a planar structure, such as 3-methylcholanthrene, β-naphthoflavone and dioxins. These xenobiotic compounds induce members of the family IA of cytochrome P-450 (or cytochrome P-448) and suppress members of the IIIA family.

(b) A number of structurally unrelated compounds, such as phenobarbital and non-planar aromatic hydrocarbons. These compounds give an induction of cytochrome P-450 enzymes of the family IIB (sometimes also indicated as cytochrome P-450 in contrast to cytochrome P-448).

(c) Xenobiotic steroids such as dexamethasone and pregnenolone-16α-carbonitrile. These steroids induce cytochrome P-450 enzymes of family IIIA and IIB.

(d) A group of small molecules, such as ethanol

and isoniazid. Induction of the cytochrome P-450 IIE family takes place by these compounds.

(e) A number of hydrophobic compounds interfering with fat metabolism, such as clofibrate, phthalate esters and chlorophenoxy esters. These compounds induce members of the cytochrome P-450 IVA family (or cytochrome P-452) and to a lesser extent members of the IIB and IIE families.

1.5. Methods of analysis of specific cytochrome P-450 isozymes

To obtain the required toxicological information, the composition of the cytochrome P-450 enzyme pattern must be known. Therefore, the concentration or induction or suppression factors must be known for each individual enzyme. Methods of identification and determination of the individual enzymes are as follows.

(a) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). Although a number of enzymes differ sufficiently in their molecular masses (in the region of relative molecular mass 45 000–53 000) in order to allow their separation and determination (*e.g.*, cytochrome P-450 IA1 and IA2), not all cytochrome P-450 enzymes can be determined by this method.

(b) SDS-PAGE followed by Western blotting. The specificity of the bands in the SDS-PAGE gel can be increased by Western blotting. In this method the cytochrome P-450 proteins are transferred onto thin membranes which can be incubated with specific antibodies against the different proteins. After incubation with a so-called second antibody (*e.g.*, goat anti-rabbit immunoglobulin G) which has been coupled to a detector enzyme, such as horseradish peroxidase, xanthine oxidase or alkaline phosphatase, the presence of the particular cytochrome P-450 enzyme can be detected semi-quantitatively. Detection can be performed by development of colour on the blot or by chemiluminescence detection by exposure to a sensitive instant film of 20 000 ASA or an X-ray film.

(c) Direct enzymatic assays. So far only a few examples of these assays are known. The most commonly used assays are the ethoxyresorufin O-deethylase assay and the corresponding O-depentylase assay. These fluorimetric assays detect the enzymatic activity of cytochrome P-450 IA1 and IIB1, respectively.

(d) Detection of enzymatic activity of cytochrome P-450 by chromatographic analysis of the products formed after incubation with a certain substrate molecule. The chromatographic techniques that have been used for this purpose are predominantly HPLC but also thin-layer chromatography and gas chromatography–mass spectrometry.

Here only *in vitro* determinations will be considered, such as incubations of substrates with liver microsomes of mainly the rat or with purified cytochrome P-450 isozymes in a reconstituted system. Although most of the studies used microsomes or a reconstituted system with an NADPH-generating system, some workers used other oxidation systems, such as sodium periodate, sodium hypochlorite, hydrogen peroxide, cumene hydroperoxide, linoleic acid hydroperoxide and *tert.*-butyl hydroperoxide [4] in combination with partially purified cytochrome P-450 enzymes. Especially with sodium periodate and cumene hydroperoxide higher hydroxylation rates of testosterone were observed, which might be useful in an identification procedure or in the preparation of standard compounds.

2. STRATEGIES TO ASSIGN PRODUCT FORMATION TO SPECIFIC CYTOCHROME P-450 ISOZYMES

The specificity of the product formation or the assignment of a specific cytochrome P-450 isozyme to the formation of a certain product by HPLC can be assessed by a number of methods.

(a) Product formation studies with purified cytochrome P-450 isozymes in a reconstituted system. The pure enzyme will produce one or more products if a particular substrate is added to a system in which the purified enzyme can express its enzymatic activity. For the cytochrome P-450-mediated monooxygenase reaction the following components are required: NADPH or an NADPH-generating system, NADPH–cyto-

chrome P-450 reductase and membranes such as phospholipid particles. To be able to assign the formation of a particular metabolite to the occurrence of a particular cytochrome P-450 isozyme, a number or preferably all known isozymes must be available in pure form.

(b) Cytochrome P-450 induction studies. One of the main features of the cytochrome P-450 biotransformation system is the inducibility of particular cytochrome P-450 isoenzymes by certain inducer molecules. Well known examples are 3-methylcholanthrene (3-MC), β-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, phenobarbital (PB), dexamethasone, pregnenolone-16α-carbonitrile, isosafrole, isoniazid, clofibrate and di(2-ethylhexyl)phthalate. Most of these compounds give rise to a known induction pattern of the cytochrome P-450 isozymes after incubation of microsomes with a certain substrate molecule. These reactions can be performed with microsomes obtained from both *in vivo* and *in vitro* experiments. Combination of this knowledge with specific product formation of a cytochrome P-450-catalysed reaction can supply the required information about the specificity of these reactions.

(c) Incubation studies of microsomes or purified cytochrome P-450 preparations with specific antibodies raised against particular cytochrome P-450 isozymes. If the antibody is directed to a part of the active site or to the vicinity of the active site, the reaction catalysed by that particular cytochrome P-450 isozyme can be inhibited. As a result, the product formation is decreased or inhibited completely.

3. SUBSTRATES FOR CYTOCHROME P-450 ISOZYMES

3.1. Steroids

It is well known that liver microsomes contain a number of cytochrome P-450 isozymes that catalyse the oxidation of numerous xenobiotic compounds. Several of these cytochrome P-450 isozymes also catalyse the oxidation of endogenous substrates, such as steroid hormones. Extensive reviews concerning the role of cytochrome P-450 isozymes in the interactions and biosynthesis of steroids have been published by Waxman [5] and Hall [6], respectively.

Rat liver microsomes can also catalyse the 5α-reduction of steroids. The steroid 5α-reductase activity (Fig. 1) is predominantly present in microsomes of female postpuberal rats. For instance, testosterone is reduced to 5α-dihydrotestosterone ten to twenty times faster by liver microsomes from mature female rats than by microsomes from mature male rats [7]. The 5α-reductase activity can convert the hydroxylated testosterone products into their 5α-analogues. As a result, the hydroxylated metabolites disappear from the HPLC trace because the 5α-metabolites have no absorbance at 240 nm. These compounds, such as 5α-dihydrotestosterone and its hydroxylated metabolites, can be separated on a C_{18} column using water–methanol–acetonitrile as eluent. Detection was effected at 220 nm [7].

Not all hydroxylated testosterone metabolites are good substrates for this 5α-reductase activity. Very good substrates are testosterone itself, 6β-, 14α-, 16α- and 16β-hydroxytestosterone and androstenedione. The metabolites 2β-, 11β-, 18- and 19-hydroxytestosterone are degraded by 5α-reductase to a lesser extent. The metabolites 1α-, 1β-, 2α-, 6α- and 7α-testosterone show almost no sensitivity towards 5α-reductase activity [7]. In addition to products, the substrate testosterone can also be subject to degradation to 5α-dihy-

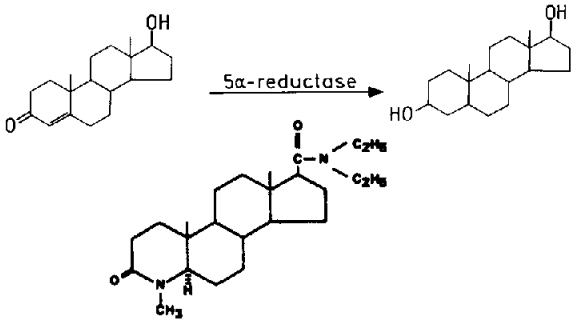

Fig. 1. Enzymatic reaction of testosterone to androstanediol by microsomal 5α-reductase and structure of the 5α-reductase inhibitor 4-MA.

drotestosterone during the incubation period with rat liver microsomes. To overcome these problems, the substrate testosterone can be added in excess in order to occupy all possible sites of action of the 5α-reductase pathway. A second, more elegant way is to block the 5α-reductase activity by the addition of a specific 5α-reductase inhibitor, such as 17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA) [8] (Fig. 1). It was shown [7] that despite its marked effect on the accumulation of hydroxylated testosterone metabolites, 4-MA had no effect on their initial formation by liver microsomes. More details can be found in an extensive study of Sonderfan and Parkinson [7].

A second phenomenon that has to be taken into account is the difference between the assays with (induced) microsomes and with purified cytochrome P-450 isozymes in a reconstituted system. First, the microsomes contain a number of isozymes in different ratios and second, the amount of NADPH cytochrome P-450 reductase in microsomes can be a limiting factor for the total expression of the activity, whereas with reactions in a reconstituted system an excess of reductase is added. An example is the difference in activity of cytochrome P-450 1A1 in purified form and in microsomes. Purified 1A1 catalyses the 6β-hydroxylation of testosterone whereas in microsomes of rats induced with β-naphthoflavone a decrease in 6β-hydroxylase activity is found compared with microsomes of control rats [9]. Most probably a limitation of cytochrome P-450 reductase in microsomes can account for this effect.

3.1.1. Testosterone

Rat liver microsomes can oxidize testosterone to 1β-, 2α-, 2β-, 6α-, 6β-, 7α-, 15β-, 16α-, 16β- and 18-hydroxytestosterone and androstenedione (Fig. 2). Several HPLC studies have been reported in which these metabolites of testosterone were separated and determined [10–25]. The various workers all used a reversed HPLC gradient system with the usual organic modifiers such as methanol, acetonitrile and tetrahydrofuran. One

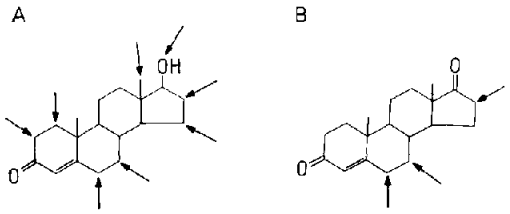

Fig. 2. Structures of (A) 17β-testosterone and (B) androstenedione. The arrows indicate the site of enzymatic attack by cytochrome P-450 enzymes.

of the best separations was reported by Wood *et al.* [21] (see also Fig. 4), who achieved an HPLC separation of seventeen hydroxylated metabolites of testosterone with a methanol–acetonitrile mobile phase. The use of tetrahydrofuran changed only the position of the 6β-hydroxytestosterone peak. This was shown also by Van der Hoeven [14], who used methanol–tetrahydrofuran. Sonderfan *et al.* [18] reported an almost identical separation also using methanol–acetonitrile. Only the order of elution of 6β-hydroxytestosterone and 19-hydroxytestosterone was changed and no separation between 1α- and 1β-hydroxytestosterone was achieved. Arlotto *et al.* [20], Gemzik *et al.* [22], Wortelboer *et al.* [11] and Reinerink *et al.* [12] reported similar separations.

A typical chromatogram of several hydroxylated metabolites of testosterone from our own studies is shown in Fig. 3 [12]. The metabolites were separated on a Chromspher C_{18} column (200 mm × 3.0 mm I.D., particle size 5 μm). The elution conditions were as follows: isocratic elution for 10 min with 11% mobile phase B [methanol–acetonitrile (90:10, v/v)] and 89% mobile phase A [methanol–water (30:70, v/v)], both containing acetic acid (pH 4.5). From 10 to 40 min a linear gradient was applied to 28% B. The flow-rate was 0.8 ml/min at 50°C.

The separation of all hydroxylated testosterone metabolites can be achieved by reversed-phase HPLC using gradient elution with common organic modifiers. Sometimes problems can

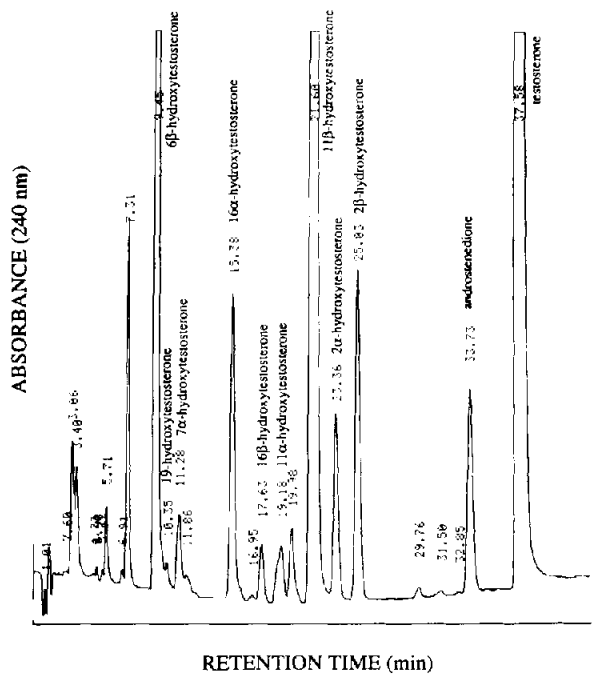

Fig. 3. HPLC separation of the incubation mixture of testosterone with microsomes from a male rat treated with dexamethasone. A reversed-phase HPLC column (C_{18}) was used, eluted with a gradient of methanol–water.

be encountered in separating 6β- and 15α-hydroxytestosterone [22], 1α- and 1β-hydroxytestosterone [18,22] or 7α-, 15α- and 15β-hydroxytestosterone [23] from each other. Therefore, a good combination of C_{18} packing material, mobile phase composition and the shape of the gradient have to be found by trial and error, starting from one of the conditions described in the literature.

The main aim of this review is to survey recent HPLC assays for specific cytochrome P-450 isozymes. This is complicated for testosterone not only by the formation of several hydroxylated metabolites of testosterone by one specific isozyme but mainly by the formation of one metabolite by several cytochrome P-450 isozymes. To assign the formation of hydroxylated testosterone metabolites to specific cytochrome P-450 isozymes, a large number of data from the literature were combined, and the following conclusions were drawn from these data.

Isozyme cytochrome P-450 IIA1 is specifically involved in the formation of 7α-hydroxytestosterone [13,15,16,21,25,26]. Isozyme cytochrome P-450 IIB1 metabolizes testosterone to the metabolites 16α- and 16β-hydroxytestosterone and androstenedione. The formation of 16β-hydroxytestosterone is specific for this isozyme.

Cytochrome P-450 IA1 hydroxylates testosterone at the 2β- and 6β-positions [5,21,27]. Because this isozyme has a very low hydroxylase activity towards testosterone, the contribution to the metabolism will be low. The same is valid for cytochrome P-450 IA2, which has an even lower metabolic activity. Isozyme IIB2 shows the same metabolic pattern as cytochrome P-450 IIB1, but has only 10% of its activity [21].

Cytochrome P-450 IIC13 can produce the metabolites 6β-, 15α- and 16α-hydroxytestosterone, but in small amounts [5]. Cytochrome P-450 IIC11 catalyses specifically the formation of 2α-hydroxytestosterone and also of the less specific metabolite 16α-hydroxytestosterone [5,15,16,26].

Cytochrome P-450 IIIA1 forms four hydroxylated metabolites of testosterone, 2β-, 6β- 15β- and 18-hydroxytestosterone [16,26]. As this isozyme is responsible for more than 85% of the 6β-hydroxylase activity, the occurrence of this metabolite can be ascribed to cytochrome P-450 IIIA1.

3.1.2. Androstenedione

Similarly to testosterone, androstenedione can also be used as a versatile substrate for cytochrome P-450 isozymes (Fig. 2). Product analysis by reversed-phase HPLC has been reported in a number of studies [5,15,17,28–31]. In Fig. 4 chromatograms are shown of available reference metabolites of testosterone and androstenedione [21]. The androstenedione metabolites were separated on a Zorbax C_{18} column (150 mm × 4.6 mm I.D., particle size 5 μm). A concave gradient was used from 15 to 36% tetrahydrofuran in water for 20 min at a flow-rate of 1.5 ml/min. The effluent was monitored at 254 nm. The metabo-

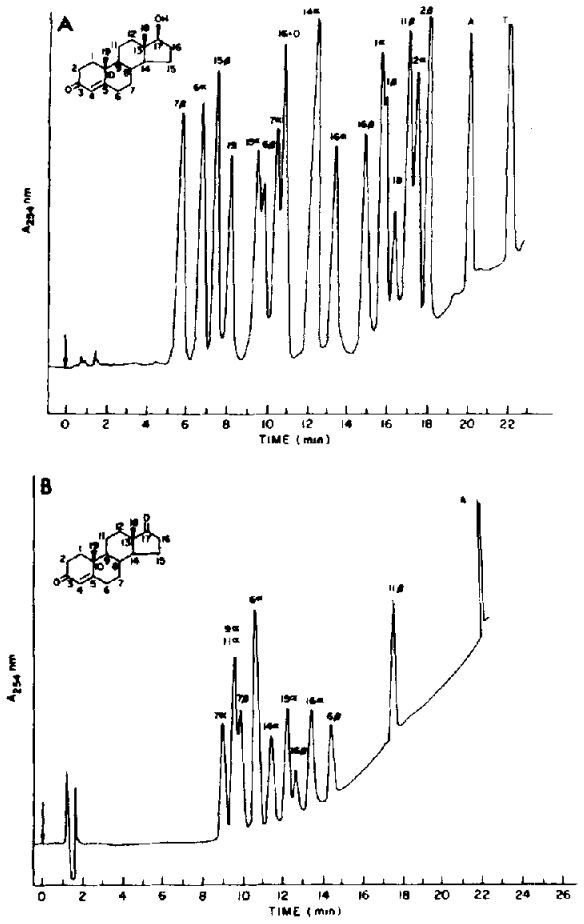

Fig. 4. HPLC separation of available metabolites of (A) testosterone and (B) androstenedione on a reversed-phase C_{18} column using different mobile phases. Reprinted from ref. 21 with permission.

lites of interest are the 6α-, 6β-, 7α-, 16α- and 16β-hydroxy derivatives of androstenedione. The workers who reported retention times all obtained the same order of elution for the hydroxylated metabolites of androstenedione.

From literature data [21,28,32], the following metabolites show more or less specificity for one of the cytochrome P-450 isozymes. Hydroxylation at the 7α-position of androstenedione is catalysed by cytochrome P-450 IIA, 16α-hydroxylation by cytochrome P-450 IIC11 and to a lesser extent by cytochrome P-450 IIB1, 16β-hydroxylation by cytochrome P-450 IIB1 and 6β-hydroxylation by cytochrome P-450 IIIA. These specific reactions make androstenedione a good substrate for the simultaneous determination of the activity of four cytochrome P-450 isozymes.

3.1.3. Cholesterol

Cholesterol can act as a substrate in the determination of two distinct activities of cytochrome P-450 isozymes: the side-chain cleavage (SCC) activity and the 7α-hydroxylase activity. The SCC of cholesterol to pregnenolone is catalysed by cytochrome P-450 XIA1 and is assumed to exist with two different enzyme activities: 17α-hydroxylase and C_{17-20} lyase. It is the initial step in the biosynthesis of various steroid hormones. Schatzman *et al.* [33] used a normal-phase column in combination with gradient elution mixtures of tetrahydrofuran and hexane. Three variants were used for the separation of 17α-hydroxypregnenolone and its metabolites, for the separation of 17α-hydroxyprogesterone and its metabolites and for the separation of pregnenolone and its metabolites. The detection was performed by on-line radioactive monitoring. The cytochrome P-450 SCC activity was reported also by Sugano *et al.* [34]. They described an elegant way to avoid the use of radioactivity. The saturated 3-hydroxysteroids, such as cholesterol and pregnenolone, which are only visible in the UV region at 215 nm with low sensitivity, were converted into their 4-ene-3-one analogues by enzymatic treatment using cholesterol oxidase (Fig. 5). The reaction products can be detected at 240 nm with a ten-fold increased sensitivity. In addition, it was shown that both (22*R*)-hydroxycholesterol and (22*R*)-hydroxycholest-4-ene-3-one were good substrates to monitor the SCC activity of cytochrome P-450 (Fig. 6). The advantage of the last substrate is that it can be monitored directly at 240 nm.

The 7α-hydroxylation of cholesterol is the initial and rate-limiting step of the synthesis of bile acids in the hepatocyte. This cytochrome P-450-dependent activity can be monitored by the HPLC analysis of the conversion from cholesterol to 7α-hydroxycholesterol. A normal-phase HPLC method was reported in which the 3-hydroxycholestane steroids were converted into

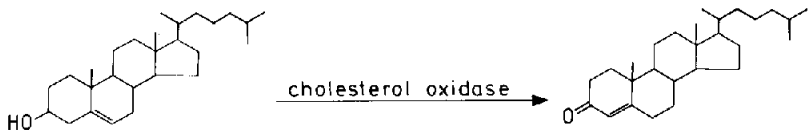

Fig. 5. Enzymatic reaction of cholesterol oxidase in the transformation of cholesterol derivatives to 4-ene-3-one steroids in order to obtain steroids which have a high absorbance at 240 nm.

their 3-one-4-ene analogues [35]. A few modifications were published by Hylemon *et al.* [36], who used an internal standard (7β-hydroxycholesterol) and a reversed-phase HPLC system. The column used was an Altex Ultrasil-ODS (250 mm × 4.6 mm I.D., particle size 10 μm). The eluent was acetonitrile–methanol (70:30, v/v). The metabolites were eluted at an initial flow-rate of 0.8 ml/min for 18 min, which was then increased to 2.0 ml/min. The effluent was monitored at 240 nm. A baseline separation was achieved between the 7α- and 7β-isomers. No assignments were made, however, to one of the family classes of cytochrome P-450.

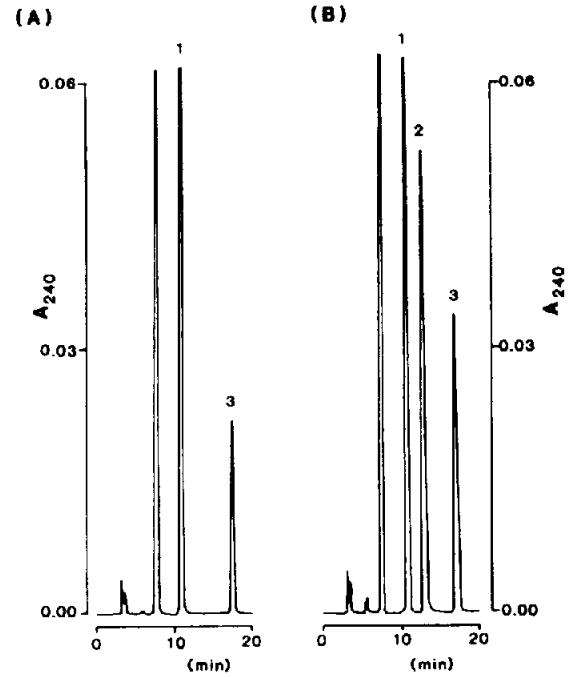

Fig. 6. HPLC separation (normal-phase) of the incubation mixtures of (A) (22*R*)-hydroxycholesterol and (B) (22*R*)-hydroxycholest-4-ene-3-one in a reconstituted cytochrome P-450 SSC system. Reaction mixture A was further incubated with cholesterol oxidase. Peaks: 1 = progesterone; 2 = (20*R*,22*R*)-dihydroxycholest-4-ene-3-one; 3 = deoxycorticosterone acetate (internal standard). Reprinted from ref. 34 with permission.

3.1.4. Other steroids

Waxman [17] reported an isocratic reversed-phase HPLC system for the separation of several hydroxylated derivatives of progesterone. It was shown that progesterone acts as a substrate for cytochrome P-450 in a similar way to testosterone and androstenedione with respect to the 2α-, 6α-, 6β-, 7α- and 16α-hydroxy metabolites. The same conclusion was drawn by Yoshihara *et al.* [24] in a study with highly toxic polychlorinated biphenyl and dibenzofuran. They used radioactive progesterone as substrate for cytochrome P-450 from rat liver microsomes with both radioactive and UV detection after separation by reversed-phase HPLC.

We have studied the metabolism of 17α-methyltestosterone and 19-nortestosterone (unpublished results). In contrast to testosterone, 17α-methyltestosterone showed only one major metabolite, whereas 19-nortestosterone showed a number of metabolites as shown in Fig. 7. To separate the metabolites of both steroids, reversed-phase gradient HPLC systems were used with UV detection at 240 nm. Induction with PB and dexamethasone both showed some qualitative and a number of quantitative changes in the metabolism pattern. This study was used to indicate the possibilities of *in vitro* studies to detect and identify metabolites of the parent compounds *in vivo*, which can be of great help in forensic analytical investigations. The different metabolites have not been identified, however.

3.2. Coumarin derivatives

The structures of several coumarins are shown in Fig. 8.

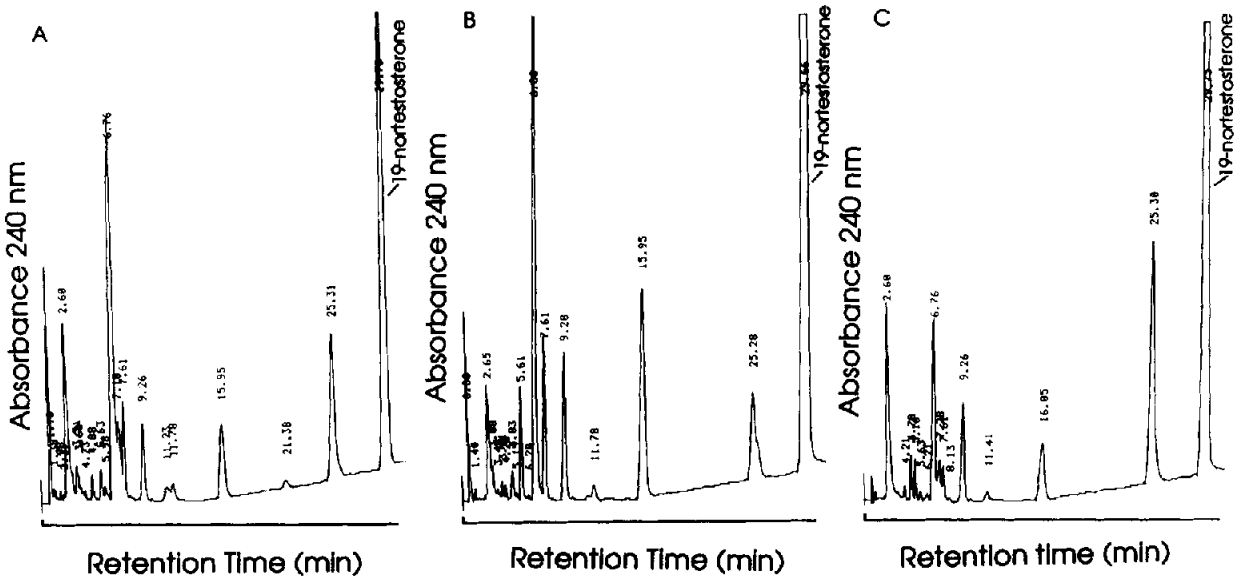

Fig. 7. HPLC separation of incubation mixtures of 19-nortestosterone with liver microsomes of (A) a control rat and rats treated with (B) phenobarbital and (C) dexamethasone. A C_{18} HPLC column was used which was eluted with a gradient of methanol–water.

3.2.1. Warfarin

A reversed-phase method to resolve a number of metabolites of warfarin (3α-phenyl-β-acetylethyl-4-hydroxycoumarin) have been reported by Fasco *et al.* [38], Spink *et al.* [39], Kaminsky *et al.* [40] and Hermans and Thijssen [41]. The following metabolites were analysed: dehydrowarfarin and the 4′-, 6-, 7- and 8-hydroxy derivatives of warfarin (Fig. 9). Hermans and Thijssen [41] compared the metabolism of the enantiomers of warfarin and 4′-nitrowarfarin (acenocoumarol).

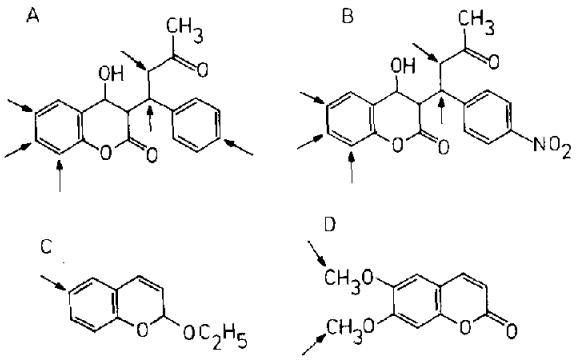

Fig. 8. Structures of coumarins: (A) warfarin, (B) acenocoumarol, (C) ethoxycoumarin and (D) scoparone. The arrows indicate the sites of enzymatic attack by cytochrome P-450 enzymes.

In Fig. 10 two chromatograms are shown of the separation of (*R*)-warfarin and (*S*)-acenocoumarol [41]. An isocratic reversed-phase HPLC system was used to separate all metabolites of both

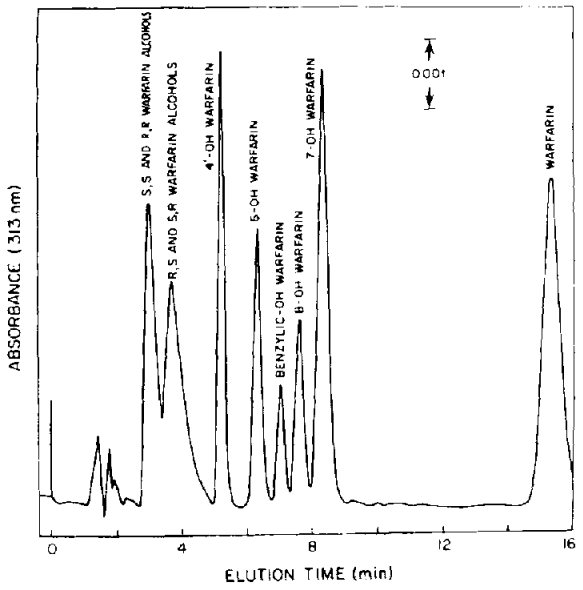

Fig. 9. HPLC separation of warfarin and its synthetic metabolites on a C_{18} column eluted with 1.5% acetic acid (pH 4.7)–acetonitrile (69:31, v/v). Reprinted from ref. 38 with permission.

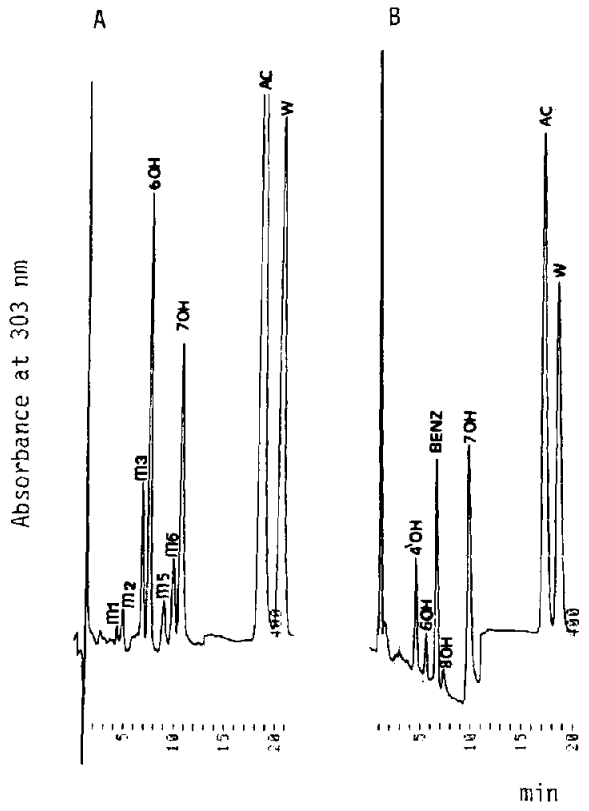

Fig. 10. HPLC separation of extracts of incubations of (A) (*S*)-acenocoumarol and (B) (*R*)-warfarin with liver microsomes of rats treated with phenobarbital. The peaks marked m1–m6 are unknown metabolites; W = warfarin; AC = acenocoumarol. Reprinted from ref. 41 with permission.

compounds. The column used was Chromspher C_{18} (200 mm × 3 mm I.D.) and the eluent was 0.1% aqueous acetic acid–acetonitrile (7:3, v/v) at pH 4.7. UV detection was applied at 303 nm. It was concluded that the metabolism of warfarin and acenocoumarol are similar with respect to the hydroxylations at the 6-, 7- and 8-positions and at C-9 and C-10, except for the 4'-hydroxylation. Differences were found between the *R* and *S* isomers of both coagulants. It was concluded that these differences were caused by the involvement of different cytochrome P-450 enzymes, but, without an assignment to particular isozymes. The specificity for the different cytochrome P-450 isozymes has been reported elsewhere [42]. Although it was stated that cytochrome P-450 IA1 metabolizes (*R*)-warfarin to 6- and 8-hydroxywarfarin [40,42] and the 7-hydroxylase activity to cytochrome P-450 IIC6 [43], it was not indicated whether these enzyme activities originated only from the enzymes mentioned.

3.2.2. Ethoxycoumarin

Rosenberg *et al.* [44] reported the HPLC determination of the O-deethylase activity of ethoxycoumarin (Fig. 8). 7-Hydroxycoumarin can be detected by UV (336 nm) or by fluorescence detection (λ_{ex} 368 nm, λ_{em} 456 nm) using the on-line change in the protonated state of this compound. The HPLC method with fluorescence detection turned out to be 200–400 times more sensitive (less than 0.05 μg of total protein) than the direct fluorescence method and at least 500 times more sensitive than that reported by Jung *et al.* [45] using HPLC with UV detection.

3.2.3. Dimethoxycoumarin

Scoparone (6,7-dimethoxycoumarin) (Fig. 8) is metabolized by several isozymes of cytochrome P-450 into the two monomethoxy derivatives 7-hydroxy-6-methoxycoumarin and 6-hydroxy-7-methoxycoumarin [46]. Exposure to either PB or polycyclic aromatic compounds results in an increase in the O-demethylase activity and in an alteration of the ratio of both products. A gradient reversed-phase HPLC method was published by Legrum *et al.* [47], with long retention times. Van Pelt *et al.* [48] reported an isocratic reversed-phase chromatographic method suitable for the determination of the metabolites in cell cultures of hepatocytes.

A rapid isocratic reversed-phase HPLC system (total analysis time 10 min) was described by Muller-Enoch and Greischel [43]. They used a Hypersil RP-18 column (142 mm × 4.6 mm I.D., particle size 5 μm). The mobile phase was 0.05 *M* ammonium acetate buffer (pH 4.25)–acetonitrile (80:20, v/v). The flow-rate was 1 ml/min and the compounds were detected at 345 nm. It was claimed that this method allows the direct determination and differentiation of cytochrome P-450 activities of 3-MC-like and PB-like induction states by the determination of the ratios between 7-hydroxy-6-methoxycoumarin and 6-hy-

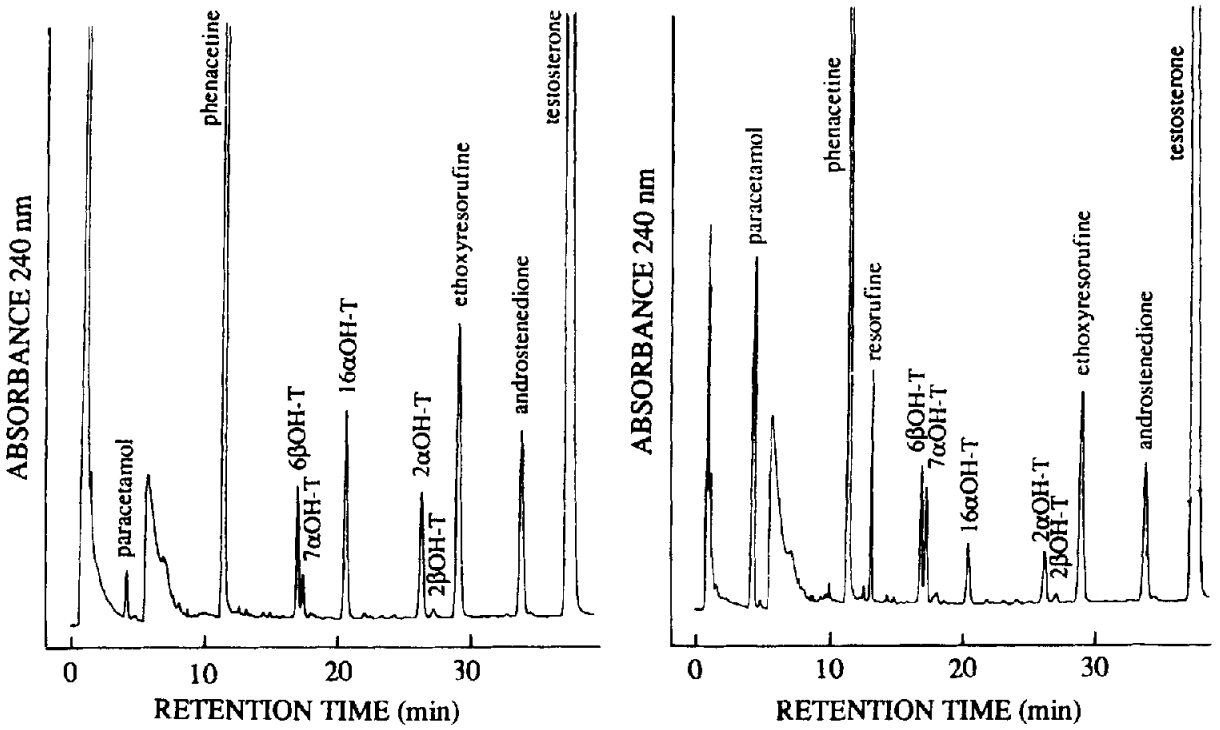

Fig. 11. HPLC separation of the combined incubation mixtures of testosterone, ethoxyresorufin and phenacetin with liver microsomes from (A) a male control rat and (B) a male rat treated with β-naphthoflavone. In this assay the testosterone hydroxylase, the ethoxyresorufin O-deethylase and the phenacetine O-deethylase reactions can be monitored simultaneously.

droxy-7-methoxycoumarin. This ratio ranged from 0.5 for microsomes from PB-treated rats to 3.4 for microsomes from 3-MC-treated rats, whereas microsomes from control rats had a ratio of 2.6.

3.3. Resorufins

One of the well known and most often used homogenous enzymatic assays is the deethylation of ethoxyresurofin to resorufin [37]. The direct fluorimetric determination of the resulting resorufin can be performed in an easy and sensitive way. The ethoxyresorufin O-deethylase (EROD) reaction can also be followed by reversed-phase HPLC with UV or preferably fluorescence detection. Although the HPLC method is much more laborious, the EROD reaction can be incorporated in the HPLC separation of testosterone metabolism studies or other cytochrome P-450-catalysed reactions. An example from our research is shown in Fig. 11.

The dealkylation of other alkoxyresorufins, such as methoxy- and pentoxyresorufins, can also be monitored by this HPLC method. Because these very frequently used reactions yield the same end-product (resorufin), they cannot be monitored simultaneously.

3.4. Polycyclic aromatic hydrocarbons

Yang's group [49] published a number of HPLC separations of cytochrome P-450-induced oxidation products of polycyclic aromatic hydrocarbons, both planar compounds, such as benz[*a*]anthracene, benzo[*a*]pyrene and chrysene, and non-planar compounds, such as benzo[*c*]phenantrene, 12-methylbenz[*a*]anthracene and 7,12-dimethylbenz[*a*]anthracene (Fig. 12). The oxidation products [(*S*, *R*)-epoxides and (*R*,*R*)-dihydrodiols] could be separated by HPLC with a chiral stationary phase. More information can be obtained in an extensive publication [50] about the stereoselectivity and the references cit-

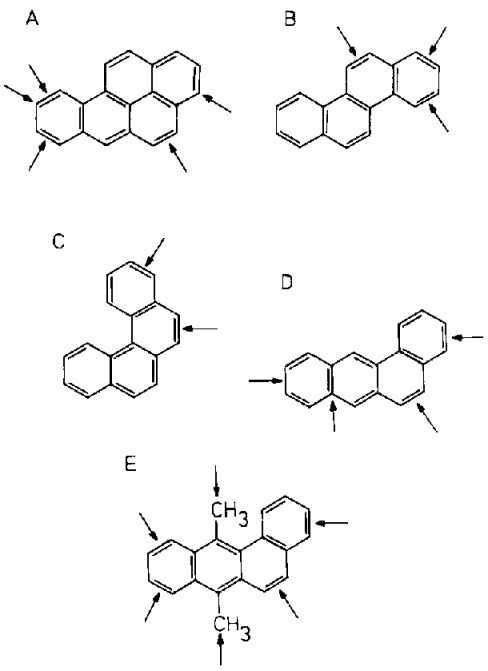

Fig. 12. Structures of polycyclic aromatic hydrocarbons: (A) benzo[*a*]pyrene, (B) chrysene, (C) benzo[*c*]phenanthrene, (D) benz[*a*]anthracene and (E) 7,12-dimethylbenz[*a*]anthracene. The arrows indicate the sites of enzymatic attack by cytochrome P-450 enzymes.

ed therein. Although for some substrates a clear difference was observed between 3-MC and PB or control microsomes, a specific assignment to cytochrome P-450 isozymes was not indicated. Haake-McMillan and Safe [51] used a reversed-phase HPLC system with a linear gradient to separate metabolites of benzo[*a*]pyrene. The following metabolites were separated and determined with fluorescence detection (at an exitation wavelength of 254 nm and an emission range of 430–700 nm): the 3- and 9-hydroxy- and the 4,5-, 7,8- and 9,10-diol derivatives of benzo[*a*]pyrene. It was concluded that both the control and induced activities of benzo[*a*]pyrene hydroxylase were carried out by different cytochrome P-450 isozymes.

O'Dowd *et al.* [52] and Morrison *et al.* [53] reported the metabolism of 7,12-dimethylbenz[*a*]anthracene (DMBA) in rat and human liver. The products 7-hydroxy-MBA, 12-hydroxy-MBA, DMBA-3,4-diol, DMBA-5,6-diol, DMBA-8,9-diol and DMBA-10,11-diol were separated using a normal-phase system but with a long analysis time (80 min). Determination was effected by off-line radioactive counting of the different fractions [53] or by UV detection at 254 nm [52]. Antibody inhibition studies [53] indicated that the proximate carcinogen DMBA-3,4-diol was formed for 90% by members of the family IIC, which also contributed significantly to the formation of DMBA-8,9-diol. The formation of DMBA-5,6-diol can be accounted for entirely by the combined activity of members of the IIC and IIB families.

3.5. Caffeine and metabolites

The increasing interest in caffeine metabolism originates from the versatile potential information about the status of enzyme systems. The determination of metabolites or ratios of metabolites can give information about the induced activity of some cytochrome P-450 isozymes involved in the metabolism of xenobiotics, about the activity of xanthine oxidase and about the acetylator phenotype by the activity of N-acetyltransferase. In addition, a number of drugs interacting with caffeine may have toxicological relevance for the general population [54], although

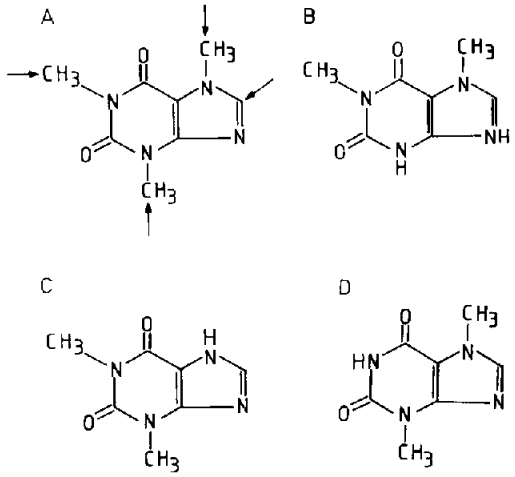

Fig. 13. Structures of metabolites of caffeine: (A) caffeine, (B) paraxanthine, (C) theophylline and (D) theobromine. The arrows indicate the sites of enzymatic attack by cytochrome P-450 enzymes.

the drug may be considered to be harmless and to have a widespread daily consumption. The primary pathways of caffeine metabolism in rat and man are the N-demethylations (Fig. 13), which are catalysed by cytochrome P-450 in the liver. It has been suggested that the cytochrome P-450 IA family, inducible by polycyclic aromatic hydrocarbons, is responsible for the major route of the caffeine metabolism, the N-3-demethylation.

The detection of the metabolites of caffeine by cytochrome P-450 *in vitro* required highly sensitive detection systems because of the low turnover of caffeine. Valero *et al.* [55] and Berthou *et al.* [56] reported an isocratic reversed-phase HPLC method with on-line radiometric detection of the radioactively labelled substrate and products. It was observed in both studies that the N-3-demethylation of caffeine to paraxanthine was linked to the activity of cytochrome P-450 IA2, an enzyme with considerable toxicological concern as it is involved in the activation of a

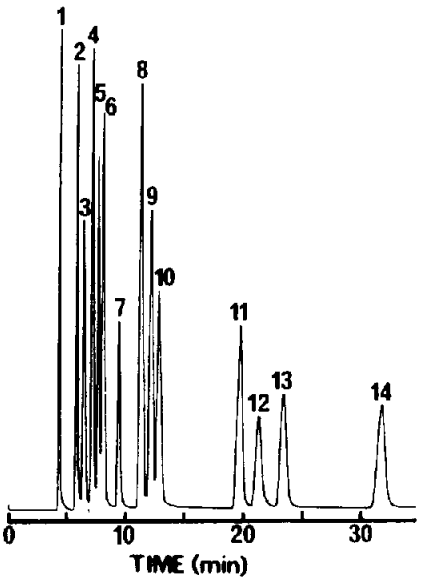

Fig. 14. HPLC separation of thirteen caffeine metabolites plus internal standard (N-acetyl-4-aminophenol). A reversed-phase HPLC column was used with isocratic elution with 0.05% acetic acid–methanol (88:12, v/v), with detection at 280 nm. Peaks: 1 = 3U; 2 = 7U; 3 = 7X; 4 = 1U; 5 = 3X; 6 = 37U; 7 = 1X; 8 = internal standard; 9 = 13U; 10 = 37X; 11 = 17U; 12 = 17X; 13 = 13X; 14 = 137U (where 37U means 3,7-dimethyluric acid and 1X means 1-methylxanthine). Reprinted from ref. 62 with permission.

number of carcinogenic compounds to their genotoxic products [57]. Butler *et al.* [65] also showed a strong correlation between the caffeine 3-demethylation and other activities strongly related to the presence of human cytochrome P-450 IA2.

Fuhr *et al.* [58] studied caffeine metabolism using reversed-phase HPLC with UV detection at 278 nm. The detection limits (three times the noise fluctuation) was between 0.19 and 0.24 μM. Other HPLC separations with baseline separation of the numerous urinary metabolites of caffeine have been reported [59–62]. In all instances reversed-phase HPLC was used. Because of the large differences in the polarities of the metabolites, often two different extraction methods and also different HPLC elution methods have to be used to determine all metabolites. An example of the reversed-phase separation of caffeine metabolites is shown in Fig. 14 [62].

Campbell *et al.* [63] reported isocratic reversed-phase HPLC separations of caffeine, paraxanthine, theobromine and theophylline. For each compound an HPLC assay was developed to ensure adequate separation and determination without interferences. The percentage of methanol (88–95%) in the mobile phase (methanol 0.05% acetic acid), the flow-rate (0.8–1.3 ml/min), the internal standard and the column temperature (32–40°C) were optimized. From correlation analysis human liver microsomal preparations, it was concluded that the ratio of 8-hydroxylase to 7-demethylase activity was strongly correlated with the EROD activity.

Another reversed-phase gradient HPLC system was reported by Shively and Vesell [64]. They studied the biotransformation of theobromine using a radioactive substrate. To verify the retention times of the radioactive peaks, UV detection at 263 nm using standard compounds was applied. A number of metabolites were shown to increase upon induction with both PB and 3-MC.

3.6. Lauric acid and derivatives

The NADPH- and oxygen-dependent ω-oxidation is catalysed by the microsomal cyto-

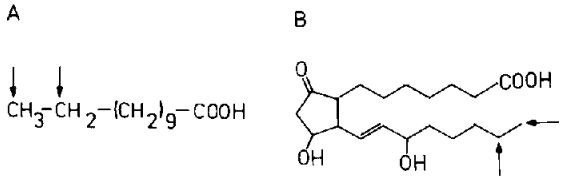

Fig. 15. Structures of (A) lauric acid and (B) prostaglandin E_1. The arrows indicate the sites of enzymatic attack by cytochrome P-450 IVA1.

chrome P-450 IVA1 and leads to the formation of both ω- and (ω − 1)-hydroxylated products (Fig. 15). The ratio is dependent on external parameters such as drug administration (hypolipodaemia, phthalate esters), starvation and diabetes. A number of reversed-phase HPLC methods have been developed for the detection of ω- and (ω − 1)-hydroxylated derivatives of fatty acids as their methyl esters. In most of these studies [66–74] radioactive lauric acid was used as substrate in order to obtain a sensitive detection method for the determination of the hydroxylated products. For confirmation of the retention times of standards a refractive index detector was used [73,74]. In one study dodecylthioacetic acid was used as substrate [75].

Recently a number of reports using HPLC with non-isotopic detection methods, mainly precolumn derivatization of the carboxylic groups have been published. Azerad *et al.* [66] used both radiolabelled lauric acid to yield both hydroxylated products, but also used derivatization of the hydroxylic group with the diastereoisomeric compound methoxytrifluoromethyl phenylacetate. The corresponding racemic esters of methylated (ω − 1)-hydroxylauric acid have been separated with an isocratic normal-phase HPLC method. Dirven *et al.* [76] determined the hydroxylated products of lauric acid [ω- and (ω − 1)-hydroxylauric acid] by precolumn derivatization of the carboxylic groups with the fluorescent re-

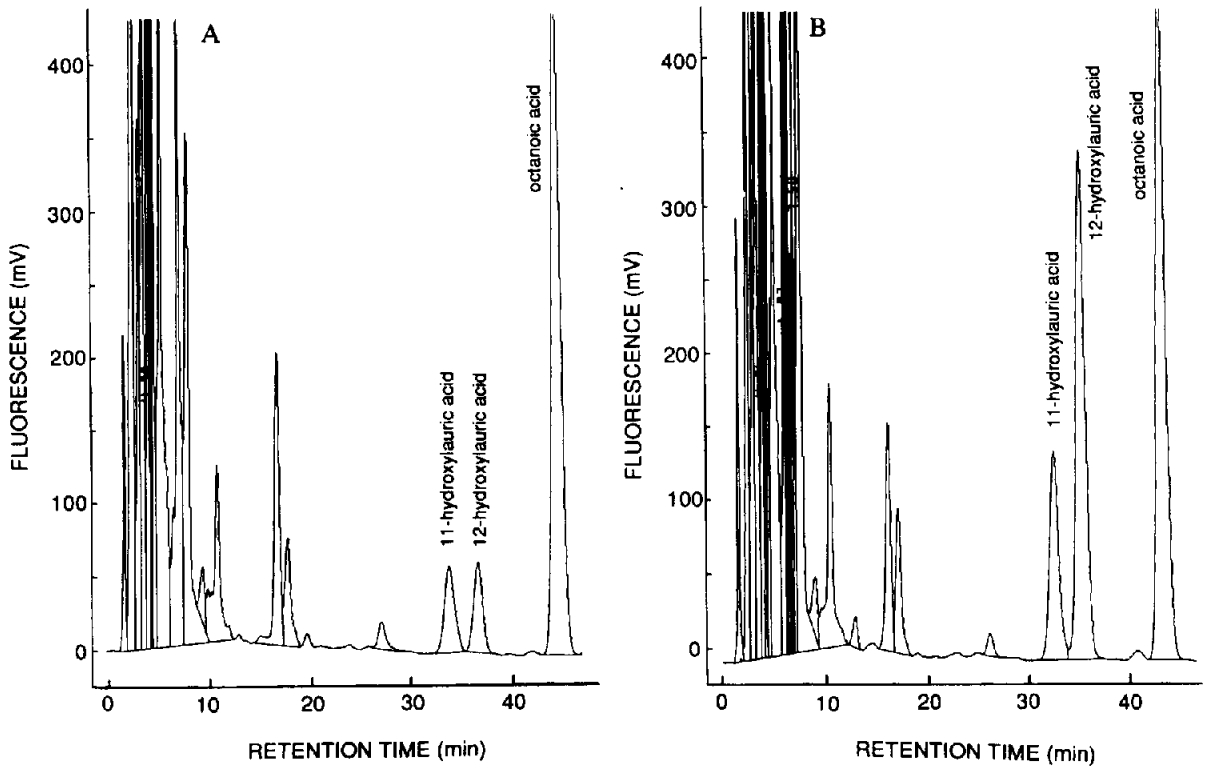

Fig. 16. HPLC separation of the lauric acid 11- and 12-hydroxylase assay by cytochrome P-450 IVA1. (A) Chromatogram of a liver homogenate from a control male rat; (B) chromatogram of a liver homogenate from a male rat which had received 200 mg DEHP per kg diet.

agent 4-bromomethyl-6,7-dimethoxycoumarin. Subsequent HPLC analysis using a C_{18} column with gradient elution (methanol–water) gave a baseline separation of both hydroxylated products. Almost the same procedure but with an internal standard (octanoic acid) was used by Jansen and De Fluiter [77]. They used 4-bromomethyl-7-methoxycoumarin as a pre-column derivatization reagent followed by isocratic reversed-phase HPLC analysis using a Chromspher C_{18} column (250 mm × 4.6 mm I.D., particle size 5 μm). The eluent was methanol–water (67:33, v/v). Fluorescence detection was applied with λ_{ex} = 330 nm and λ_{em} = 396 nm. An example of the resulting chromatograms is shown in Fig. 16. The critical step in the derivatization procedure is the solvent acetonitrile, which has to be dried before use [77]. Dirven *et al.* [76] used acetone, which might be less suitable to achieve a quantitatively complete derivatization of the carboxylic group. Both studies were conducted to determine the activity of cytochrome IVA1 as a result of the treatment with the plasticizer di(2-ethylhexyl) phthalate.

Aoyama and Sato [78] used another derivatization reagent for carboxylic acids, the UV-absorbing compound *p*-bromophenacyl bromide. HPLC separation occurred both with a isocratic normal-phase system (Porasil) with 0.5% isopropanol in hexane as solvent and a gradient reversed-phase system with a mixture of methanol and phosphate buffer. Although the reversed-phase system gave a complete baseline separation, in the normal-phase separation the retention times differed by about 10 min. In this study myristic acid, palmitic acid and stearic acid were also used as substrates in a reconstituted monooxygenase system containing purified cytochrome P-450 enzyme.

Probably the same cytochrome P-450 isozyme is involved in the ω- and (ω − 1)-hydroxylation of prostagladins. Holm and Kupfer [79] described the hydroxylation of prostaglandin E_1 and E_2 on the 19- (ω − 1) and 20- (ω) positions (Fig. 15), which was followed by isocratic reversed-phase HPLC with detection at 280 nm as described previously [80,81].

3.7. Miscellaneous compounds

3.7.1. Riboflavin

The hydroxylation of the 7- and 8-methyl groups of riboflavin (Fig. 17) and riboflavin tetrabutyrate was reported by Ohkawa *et al.* [82]. Both hydroxylated metabolites, the parent compound riboflavin and also the standards flavin mononucleotide and lumiflavin could be separated in a gradient reversed-phase HPLC system using fluorescence detection (λ_{ex} = 440 nm; λ_{em} = 530 nm). Microsomes from PB- and 3-MC-treated rats both increased the formation of the 7α-metabolite about two-fold, which means that probably no specific cytochrome P-450 isozymes can account for this small effect.

3.7.2. Acetylaminofluorene

The metabolism of 2-acetylaminofluorene (Fig. 17) was studied in detail by Åström and DePierre [83], who used eight different forms of purified rat liver cytochrome P-450 enzymes. The

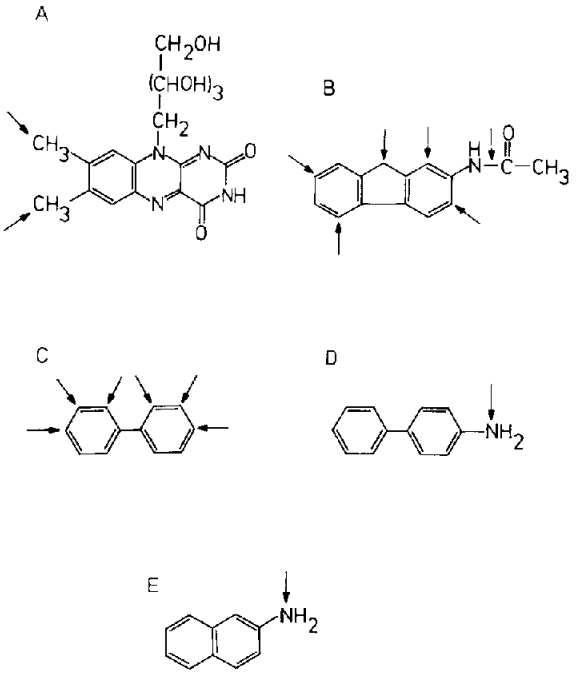

Fig. 17. Structures of (A) riboflavin, (B) 2-acetylfluorene, (C) biphenyl, (D) 4-aminobiphenyl and (E) 2-naphthylamine. The arrows indicate the sites of enzymatic attack by cytochrome P-450 enzymes.

HPLC system used was able to separate the 1-, 3-, 5-, 7-, 9- and N-hydroxy derivatives of 2-acetylaminofluorene as developed by Smith and Thorgeirsson [84]. They used a radioactive substrate for detection. The 3-, 5-, 7- and 9-hydroxy metabolites were formed mainly by cytochrome P-450 IA1, although other isozymes, such as IIA1, IIB1, IIB2, IA2 and IIC11, can also contribute to their formation. The 1-hydroxylase activity originates mainly (90%) from IA1 and only 10% from IIC11. The N-hydroxylase activity was produced by both IA1 and IA2 at almost equal rates. Both the 1- and 3-hydroxylase activity of 2-acetylaminofluorene can be used for monitoring the activity of cytochrome P-450 IA1. Combination with other hydroxylase activities of this compound can also give information about the presence of cytochrome P-450 IA2.

3.7.3. Biphenyls

The HPLC separation of biphenyl (Fig. 17) metabolites was reported by Weaver and Van Lier [85] for unconjugated monohydroxy derivatives, by Powis *et al.* [86] for free and conjugated monohydroxylated metabolites, by Burke and Prough [87] for the parent compound and the major free and conjugated mono- and dihydroxy metabolites. The last method was improved by Mole *et al.* [88] with respect to the extraction procedure, the chromatography (the use of an amino-bonded phase with both isocratic and gradient elution using different mobile phases) and the sensitivity of detection (with fluorescence). Unfortunately, no HPLC traces were shown, but the retention times were indicated with ranges. A simple method of analysis was developed with direct HPLC injection after a single filtration step. For all metabolites (2-, 3- and 4-hydroxybiphenyl and 2,2′- and 4,4′-dihydroxybiphenyl), good recoveries between 97 and 103% were obtained. Assignments to specific cytochrome P-450 isozymes were not mentioned.

The N-oxidation of 4-aminobiphenyl and also 2-naphthylamine (Fig. 17), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 3-amino-1-methyl-5*H*-pyrido[4,3,-*b*]indole (Trp-P-2) was investigated by Butler and co-workers [65,89]. The metabolites were identified by their UV spectra and by comparison of retention times with standards. The HPLC assays [89,90] were performed with on-line radioactive counting to determine the metabolites. It was concluded that these activities were closely related to cytochrome P-450 IA2 but also to other isozymes to a lesser extent [89]. The 2′-hydroxylase activity was reported to be specific for IA2, but the activity was very low [89].

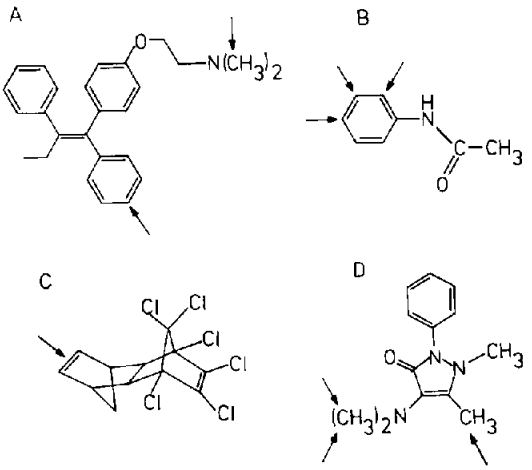

Fig. 18. Structures of (A) tamoxifen, (B) acetanilide, (C) aldrin and (D) aminopyrine. The arrows indicate the sites of enzymatic attack by cytochrome P-450 enzymes.

3.7.4. Tamoxifen

Jacolot *et al.* [91] described the isocratic reversed-phase HPLC separation of tamoxifen and eight metabolites from the 4-hydroxylase and N-demethylase (Fig. 18) activity in rat and human liver microsomes. The nine related compounds could be baseline-separated with a LiChrosorb C_{18} RP-Select B column (250 mm × 4 mm I.D.) with methanol–water–triethylamine (90:10:0.1, v/v/v) as eluent. The flow-rate was 0.8 ml/min and the eluate was monitored at 238 nm. The metabolites were identified on the basis of their chromatographic behaviour and UV spectra. It was shown that the tamoxifen N-demethylase activity was related to the erytromycin N-demethylase and the testosterone 6β-hydroxylase activity. Also from the results of antibody inhibition ex-

periments it was concluded that the N-demethylase activity of tamoxifen originates from a cytochrome P-450 enzyme of the IIA subfamily.

3.7.5. Acetanilide

Guenthner *et al.* [92] and Esclade *et al.* [93] reported the separation of acetanilide (Fig. 18) and its 2-, 3- and 4-hydroxylated metabolites using an isocratic reversed-phase HPLC system with UV detection at 254 nm and off-line radioactive detection [92]. An excellent correlation was found between the 4-hydroxy product detected optically and radiometrically when control microsomes were incubated with the radioactively labelled substrate acetanilide. The 4-hydroxylase assay was used [93] to determine the catalytic activity of a 3-MC-induced cytochrome P-448 enzyme, probably IA2.

3.7.6. Aldrin

Pantaleoni *et al.* [94] followed the epoxidation of aldrin (Fig. 18) to dieldrin by isocratic reversed-phase HPLC. Both components were separated with a retention time difference of 4 min. The limit of detection was 0.1 ng as detected at 215 nm. The epoxidation activity was shown to be increased in liver microsomes of rats treated with PB and Aroclor 1254.

3.7.7. Phenols

A number of HPLC methods were developed by Esclade *et al.* [93] for the simultaneous detection of phenolic products of cytochrome P-450 using a model system containing NADH, haemoglobin and methylene blue. Both monohydroxylated metabolites of phenol, aniline and dealkylation products of 4-N,N-dimethylaminoantipyrine and *p*-nitroanisole were separated on reversed-phase columns. The prepurification of the samples was minimal, consisting of a deproteinization without organic extraction. HPLC separations were completed in less than 15 min. The detection limits were between 0.5 and 4 μM and were not always sufficiently low to observe the formation of products in the model incubation system. It remained unclear if these quantitative analyses can also be performed on microsomal samples with possible matrix disturbances.

3.7.8. Aminopyrine

The generation of four aminopyrine (Fig. 18) metabolites by rat liver microsomes was reported by Imaoka *et al.* [95]. The metabolites 4-methylaminopyrine, 4-aminoantipyrine, 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazoline-5-one and one unidentified metabolite were separated on an isocratic reversed-phase (C_8) column (Fig. 19) with water–methanol–triethylamine (79:20:1, v/v/v) at pH 5.4 as mobile phase. The unknown metabolite apparently contained no primary or secondary amino groups, which was concluded from acetylation experiments. With this procedure, all metabolites with amino groups were acetylated, which was checked by a change in the HPLC retention times. The retention time of the unknown metabolite remained unchanged on acetylation. Therefore, it was concluded that the compound was not a product of the N-demethylation reaction of the tertiary amino group at the 4-position of aminopyrine. Although an extensive study was per-

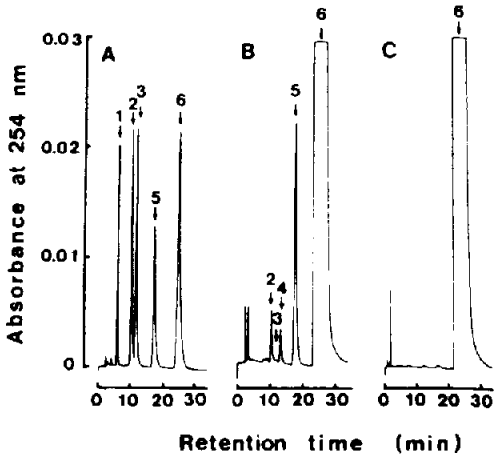

Fig. 19. HPLC separation of aminopyrine and its metabolites with a C_8 reversed-phase column. (A) Chromatogram of standards; (B) chromatogram of aminopyrine metabolites after incubation with microsomes of control male rats; (C) chromatogram of the blank assay without NADPH. Peaks: 1 = 4-formylaminoantipyrine; 2 = 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazoline-5-one; 3 = 4-aminoantipyrine; 4 = an unknown metabolite; 5 = 4-methylaminoantipyrine; 6 = aminopyrine. Reprinted from ref. 95 with permission.

formed with purified cytochrome P-450 isozymes in a reconstituted system, the formation of the metabolites could not be assigned to a particular cytochrome P-450 isozyme.

3.7.9. Bufural, debrisoquine, dextromethorphan

Kronbach *et al.* [96] reported a method to determine the polymorphism of drug metabolism of the debrisoquine/sparteine-type. The *in vitro* metabolism was assayed with bufuralol (1′-hydroxylase activity), debrisoquine (4- and 6-hydroxylase) and dextromethorphan (O-demethylase) as substrates (Fig. 20) using purified cytochrome P-450 isozymes of human origin. The reaction products were analysed by reversed-phase HPLC with fluorescence detection using different excitation and emission wavelengths for each class of compound. Because of the basic characteristics of the compounds due to the presence of ionizable nitrogen atoms, the application of reversed-phase HPLC is limited. Therefore, the authors used a combination of chaotropic effects and ion-pair formation of perchlorate. This approach had

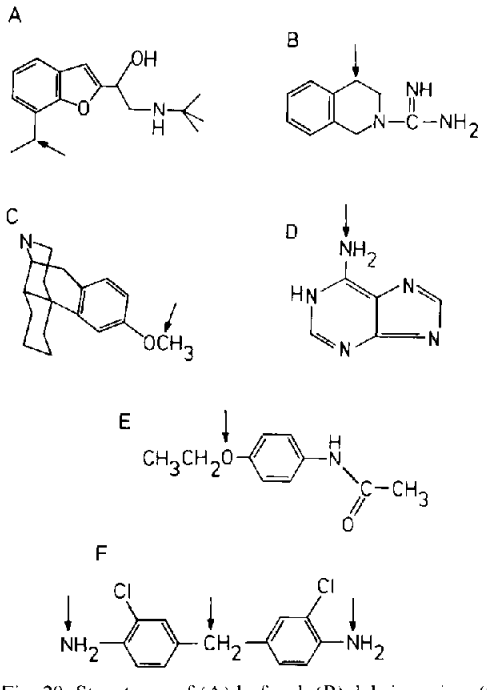

Fig. 20. Structures of (A) bufural, (B) debrisoquine, (C) dextromethorphan, (D) adenine, (E) phenacetin and (F) 4,4′-methylenebis(2-chloroaniline). The arrows indicate the sites of enzymatic attack by cytochrome P-450 enzymes.

the advantage of a non-extractive sample preparation with external standardization and allowed the use of acidic eluents and the investigation of additional metabolites. Although it was suggested that these substrates are metabolized by the same cytochrome P-450 isozyme, it remained unclear to what extent other isozymes contribute to the microsomal metabolism of each substrate.

3.7.10. Adenine

Clement and Kunze [97] showed the microsomal N-hydroxylation of the nucleic base adenine (Fig. 20) to 6-N-hydroxyaminopurine, which is known to be genotoxic and carcinogenic. The metabolite was identified by comparison of the retention time and the UV spectra using diode-array detection. An HPLC sysem was used with a cation-exchange column (200 mm × 4 mm I.D.) containing Nucleosil SA (particle size 5 μm). The mobile phase was a mixture of methanol and ammonium formate buffer (0.1 *M*, pH 3.0) (67:33, v/v) at a flow-rate of 0.8 ml/min. Detection was performed at 270 nm. Although the production of the metabolite was very low (0.01–0.06 nmol/min · mg protein), the difference in retention times between the parent compound and the N-hydroxy metabolite (8.3 and 6.4 min, respectively) was sufficient for determination. The formation of the N-hydroxy metabolite was increased five- to ten-fold with microsomes from a rat treated with isosafrole and 3-MC, respectively. Other inducers, such as PB and ethanol, had a suppressing effect on the hydroxylation activity. Therefore, it was concluded that cytochrome P-450 isozymes from the IA subfamily can account for this activity.

3.7.11. Phenacetin

A well known activity of cytochrome P-450 IA2 is the phenacetin O-deethylase reaction (Fig. 20), yielding paracetamol [65]. This reaction can be followed easily by reversed-phase HPLC with UV detection as shown in Fig. 11, in which this assay is included in the testosterone hydroxylase and the ethoxyresorufin O-deethylase assays (our unpublished work). Butler *et al.* [89] also used

TABLE I

CYTOCHROME P-450 ISOZYME SPECIFIC REACTION MONITORED BY HPLC OF PRODUCTS

CP-450 isozyme	Substrate/reaction	Section
IA1	Ethoxyresorufin O-deethylase	3.3
IA1	2-Acetylaminofluorene 1-hydroxylase	3.7.2
IA1	2-Acetylaminofluorene 3-hydroxylase	3.7.2
IA1/IA2	Adenine N-hydroxylase	3.7.10
IA2	Acetanilide 4-hydroxylase	3.7.5
IA2	Caffeine 3-demethylase	3.5
IA2	Phenacetin O-deethylase	3.7.11
IIA1	Testosterone 7α-hydroxylase	3.1.1
IIA	Androstenedione 7α-hydroxylase	3.1.2
IIB1	Testosterone 16β-hydroxylase	3.1.1
IIB1	Androstenedione 16β-hydroxylase	3.1.2
IIC11	Testosterone 2α-hydroxylase	3.1.1
IIIA1	Testosterone 6β-hydroxylase	3.1.1
IIIA	Androstenedione 6β-hydroxylase	3.1.2
IVA1	Lauric acid 12-hydroxylase	3.6
XIA1	22-Hydroxycholest-4-ene-3-one SCC	3.1.3
XIA1	(22*R*)-Hydroxycholesterol SCC	3.1.3

reversed-phase HPLC to determine the phenacetin O-deethylase activity. The retention times were 5.3 and 13.4 min for paracetamol and phenacetin, respectively. The rate of metabolite formation was determined with a radioactive flow detector.

3.7.12. Methylenechloroaniline

The metabolism of 4,4′-methylenebis(2-chloroaniline) (Fig. 20) to its 6-, N- and C-4-hydroxy derivatives was described by Butler *et al.* [89]. The derivatives were separated on a Waters μBondapak C_{18} column (300 mm × 3.9 mm I.D.). The mobile phase consisted of 20 m*M* diethylamine acetate (pH 6.2) and methanol in a linear gradient mode. All three reactions, however, were catalysed by various cytochrome P-450 isozymes.

4. CONCLUSIONS

The biotransformation system of cytochrome P-450 is very important in both pharmacological and toxicological studies. Especially the composition of the cytochrome P-450 isozyme pattern can give valuable information about the nature and concentration of the exposed compounds to a certain target tissue. In this paper, a large number of reactions of cytochrome P-450 isozymes have been reviewed. After incubation with a suitable substrate, HPLC can supply detailed information about product formation and consequently about the composition of the cytochrome P-450 isozyme pattern. It appears that detection limits using fluorescence and even UV detection are sufficient in most instances to observe and measure metabolite formation at a low level. Special attention has been paid to the specificity of product formation by individual cytochrome P-450 isozymes. It was found that the presence and concentration of eight cytochrome P-450 isozymes can be determined by the specific formation of a product after incubation with a certain substrate. These cytochrome P-450 isozymes are IA1, IA2, IIA1, IIB1, IIC11, IIIA1, IVA1 and XIA1. The corresponding reactions are listed in Table 1.

From the studies reviewed here it is clear that HPLC is an important tool in the analysis of metabolic patterns and consequently in the assessment of cytochrome P-450 isozyme patterns. It is hoped that this review of HPLC applications will provide a useful contribution to this rapidly developing toxicological topic.

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