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Journal of Chromatography B, 684 (1996) 133–145

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Advances in sample preparation, electrophoretic separation and detection methods for rat cytochrome P450 enzymes

Eugène H.J.M. Jansen*, Catharina A. Laan, Petra de Fluiter

Laboratory for Health Effects Research, National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA, Bilthoven, Netherlands

Abstract

A limited overview is given of the separation and detection of specific cytochrome P450 enzymes of the rat. Separation methods include group-specific chromatographic separation and electrophoretic separation in and elution from polyacrylamide gels. Detection methods that are considered include enzymatic analysis with and without chromatographic step using liquid chromatography and immunochemical methods following separation of the cytochrome P450 enzymes by polyacrylamide gel electrophoresis (Western blotting). The advantages and limitations of the various methods have been compared and discussed.

Keywords: Reviews; Enzymes; Cytochromes

Contents

*Corresponding

0378-4347/96/$15.00
PII S0378-4347(96)00145-4

List of abbreviations

BP	Benzo[*a*]pyrene
EROD	Ethoxyresorufine O-deethylase
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
LAH	Lauric acid hydroxylase
PA	Polyacrylamide
PAH	Polycyclic aromatic hydrocarbons
PAGE	Polyacrylamide gel electrophoresis
PNP	*p*-Nitrophenol
POD	Phenacetine O-deethylase
PROD	Pentoxyresorufine depentylase
SDS	Sodium dodecyl sulphate

1. Introduction

The group of cytochrome P450 isoenzymes constitute an important group of enzymes in the metabolism of both endogenic and exogenic low molecular mass compounds. At present a large number of different families and members within those families have been detected and characterized by their genomic structure [1]. Their main role is the detoxification of compounds by oxidation or dealkylation, resulting in the introduction of attachment sites for phase II enzymes such as glucuronidase, sulfatase or glutathion transferase. Individual cytochrome P450 enzymes are involved in endogenous functions such as hydroxylation of steroids, vitamin D, bile salts, fatty acids and prostanoids. The xenobiotic action includes oxidation and detoxification of pharmaceutical drugs and environmental pollutants. In some cases, however, oxidation can result in the occurrence of metabolites with increased toxic properties. Therefore, the composition of the isoenzyme pattern of cytochrome P450 enzymes in microsomes is an important parameter in biochemical toxicology. For recent reviews on the action and toxicological implications: see references [2–6].[1]

The cytochrome P450 enzymes have been classified into families and subfamilies on the basis of the similarity of their amino acid sequences [1]. The most important families in the rat are 1A, 2A, 2B, 2C, 2E, 3A and 4A. Each family has more members which are indicated by a subnumber e.g., 1A1 and 1A2 [7]. The various members of the cytochrome P450 families can be present constitutively and/or can be induced upon exposure to environmental or food contaminants. Although almost all tissues in the body possess cytochrome P450 enzymes, the liver is the main target organ of study with the highest concentrations of cytochrome P450s.

The goal of this paper is to review the analytical separation and detection techniques of the individual rat cytochrome P450 enzymes. These methods are used frequently in different areas of toxicology, pharmacology and other environmental and biomedical sciences.

This paper is based on a search in the literature until April 1995 and elucidated with examples from own research.

2. Separation methods

2.1. Sample preparations. Fast group-specific purification and detection

Although the cytochrome P450 system is present in almost all tissues of the body, the liver is the main target organ of study because of the large concentrations present in this organ. Since cytochrome P450 enzymes are membrane bound in the endoplasmic reticulum they can be isolated in a microsomal fraction by ultracentrifugation of liver homogenates. This purification step is usually sufficient enough for further analytical characterization studies, such as enzymatic analysis and Western blotting. Other identification procedures such as SDS-PAGE require an additional purification before analysis. This can be simply performed using a kind of non-specific affinity column containing aminohexyl or aminooctyl groups immobilized on Sepharose or agarose, respectively [8]. Jansen and Reinerink [9] used this purification step on a small scale and developed a simple and fast purification procedure for rat liver microsomes. Direct on-column solubilization of a small volume microsomal fraction was performed followed by elution of the cytochrome P450 fraction by an increased detergent concentration. The result was a substantial purifica-

[1] See also the review of Roos in this volume. [J. Chromatogr. B, 684 (1996) 107–131].

tion of the total cytochrome P450 enzyme fraction. This fraction can be analyzed just by SDS-PAGE, which will be discussed in a following section chapter. Jansen et al. [10] reported also a fast detection method for aspecific detection of cytochrome P450 enzymes using chemiluminescence. The chemiluminescence detection makes use of the presence of a haem group in the cytochrome P450 protein moiety. This haem group acts as a catalyst for the luminol derived chemiluminescence upon oxidation with peroxides. Although the specificity for particular cytochrome P450 enzymes was not established, the result was a fast and simple detection method for these enzymes. An example of such an application in column chromatography is shown in Fig. 1.

The cytochrome P450 isoenzymes can be further separated on a DEAE–Biogel A column. The ultraviolet pattern shows about seven possibly different protein peaks (Fig. 2). The corresponding chemiluminescence pattern shows only four different peaks, probably indicating the cytochrome P450 isoenzymes. Because the selectivity of the chemiluminescent determination for cytochrome P450 is not completely established at present, this conclusion may be somewhat preliminary.

An important advantage of this new method of chemiluminescence detection is its enhanced sensitivity (see Fig. 3). The limit of detection is about 0.01 μl of microsomes, which gives a signal ten times the blank value. As a result, the proposed method is about 1000 times more sensitive than conventional methods, such as spectrophotometric assays of cytochrome P450 which require usually 50 μl.

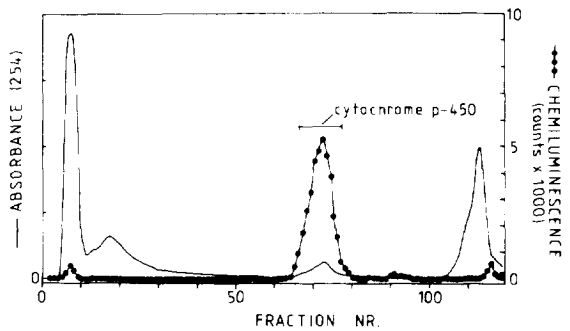

Fig. 1. Chromatographic group-specific purification of rat liver cytochrome P450 on aminooctyl agarose. Detection was performed on-line with UV (254 nm) and off-line with chemiluminescence.

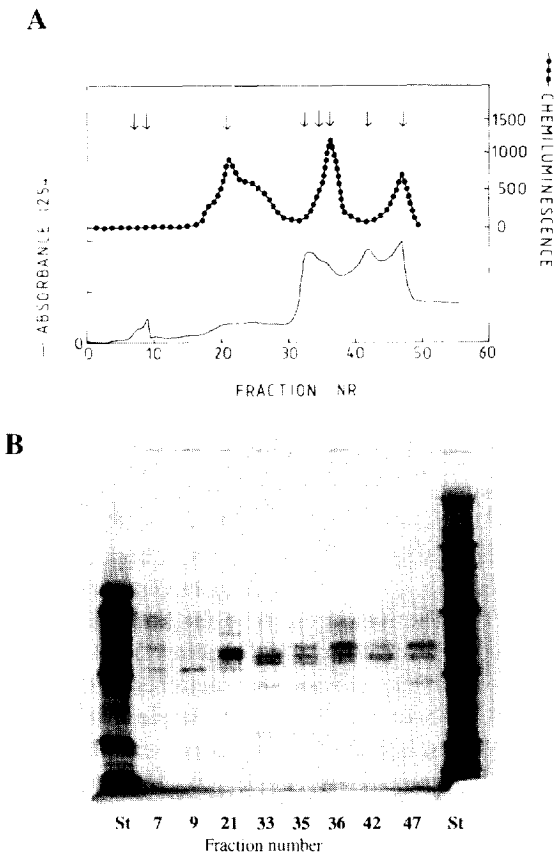

Fig. 2. (A) Elution pattern of the active fraction from the aminooctyl agarose column on a DEAE–Biogel A column (10 cm×1 cm I.D.). The cytochrome P450 isoenzymes were eluted using a gradient of sodium chloride solution from 0 to 0.3 mol l^{-1} in 60 ml. Detection was performed on-line with UV (254 nm) and off-line with chemiluminescence. (B) SDS-PAGE pattern of various fractions from the DEAE column. The fraction numbers correspond with the arrows in Fig. A.

The specificity of the chemiluminescence signal for cytochrome P450 was established by several experiments. Other haem-containing compounds or proteins were tested in the assay system. All compounds tested, such as haemin, microperoxidase (a haem containing decapeptide) and haemoglobin showed kinetics different from the various induced cytochrome P450 fractions, as is shown in Fig. 4.

Also the speed of detection was increased substan-

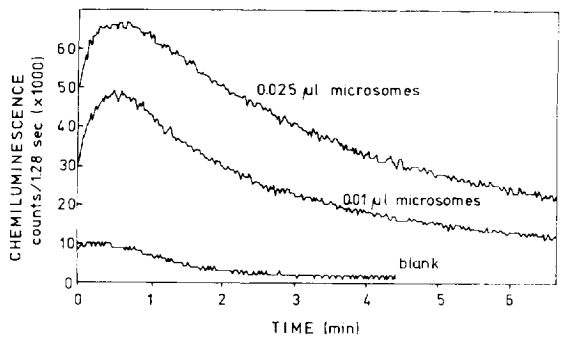

Fig. 3. Kinetics and sensitivity of the chemiluminescence detection of the haem-catalyzed luminol oxidation by cytochrome P450.

tially compared with currently used methods such as the enzymatic degradation of fluorogenic substrates in a reconstituted system or the spectrophotometric determination at 450 nm after treatment with carbon monoxide. The measuring time for both methods is about 10 min per sample and requires 50–100 µl of microsomes. The present chemiluminescent method takes only about 1 to 2 min per sample if the whole kinetic signal is recorded, or 2 s per sample if only one measurement is made at the maximum chemiluminescent signal, between 1 and 2 min after initiation of the oxidation reaction. In a single-tube luminometer, the sample throughput is about 5 s per sample in a large series. With a microtitre plate reader with chemiluminescence detection, 96 samples can be measured in 1 min. If only qualitative measurements are sufficient, simple and fast detection on a photoplate is possible [11], which do not require advanced and expensive instrumentation.

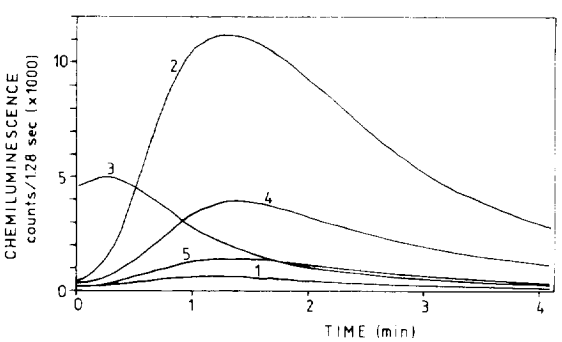

Fig. 4. Kinetics of the chemiluminescence reaction of cytochrome P450s in livermicrosomes from rats induced with: oil (control, curve 1), dexamethasone (2), 3-methylcholanthrene (3), phenobarbital (4) and isoniazid (5).

2.2. Electrophoresis

2.2.1. SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) is one of the most used separation techniques for the analysis of complex protein mixtures in biochemical research. The separation is based on differences in the electrophoretic mobility of proteins caused by differences in size and consequently on differences in molecular mass. The influence of charge differences of the proteins which may disturb the relationship between molecular weight and mobility is eliminated by the addition of negatively charged detergents molecules which give the proteins a uniform charge. The percentage of acrylamide (%T) and the extent of crosslinking of the gel (%C) are the factors which determine the differences in relative mobility of proteins of different molecular mass. The most applications of this technique are performed with acrylamide gels with a single percentage of acrylamide (varying from 5 to 25% depending on the molecular mass range) or with gradient gels containing a linear gradient of acrylamide (e.g., from 5 to 25%). In Fig. 5, SDS-PAGE patterns are shown of liver microsomes of rats treated with different inducers. The cytochrome P450 protein bands are located between 45 and $60 \cdot 10^3$ kDa.

2.2.2. Inversed gradient electrophoresis

Jansen et al. [12] reported a new approach to increase the optical resolution of the separation of proteins in SDS-polyacrylamide gels. The principle of the inversed gradient electrophoresis has been depicted schematically in Fig. 6.

The method is simply based on a decrease in the percentage of acrylamide with increasing migration distance. The gel is composed of a 5% concentration gel (2 cm length) which contains the sample wells and a separation gel of 8, 10, 12 or 15% acrylamide. In this part of the gel the actual separation between the proteins takes place. Then the separated proteins enter again a low percentage acrylamide gel (5%). In this last gel, which we call the running gel, the proteins are not supposed to be retarded in principle.

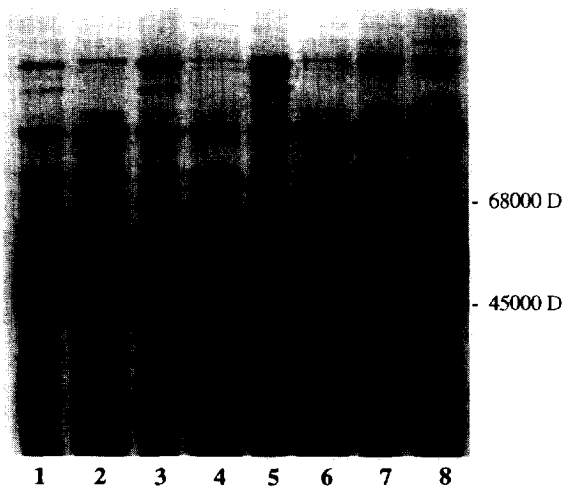

Fig. 5. SDS–PAGE with Coomassie Blue staining of liver microsomes of rats treated with different inducers: (1) phenobarbital, (2) dexamethasone, (3) ethanol, (4) isosafrole, (5) isoniazid, (6) β-naphthoflavone, (7) β-naphthoflavone, (8) oil. All rats except No. 6 were male rats. The protein bands of cytochrome P450 enzymes are located between 45 and 68 kDa.

They just run through this gel with the same high speed. The increased distance between the protein peaks in the running gel is caused by the fact that the proteins reach the boundary between the two gels at different times. As soon as they reach the running gel with a low percentage of acrylamide the speed of migration is increased. The final result is that a relatively small distance between two proteins in the classical SDS-PA separating gel can be increased up until a factor 12. An example of the inversed SDS-PAGE is shown in Fig. 7.

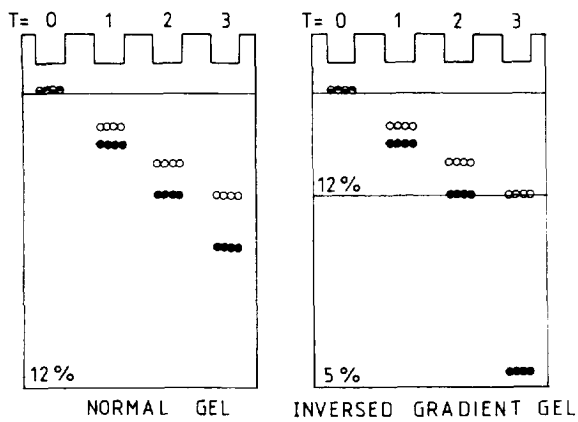

Fig. 6. Schematic representation of the separation between two proteins in a normal SDS-PA gel (left) and an inversed gradient gel (right). The migration of two proteins is shown at four different time intervals.

2.2.3. Elution from gels

The antibodies which are required for immunochemical detection on membranes (Western blotting) can be obtained commercially from several companies or by purification and immunization procedures by the investigators. Since the availability of commercial antibodies and also Western blot kits is still increasing, the need for self-development of antibodies can only be justified by financial reasons. The sources of rat cytochrome P450 antibodies will be considered in the section on Western blotting.

Here, only a brief procedure will be described for the isolation of proteins from electrophoresis gels prior to immunization. Besides the classical purification procedures using a combination of precipitation and low pressure column chromatography with various materials, a fast and simple method is the elution from gels. If the protein bands are separated on a

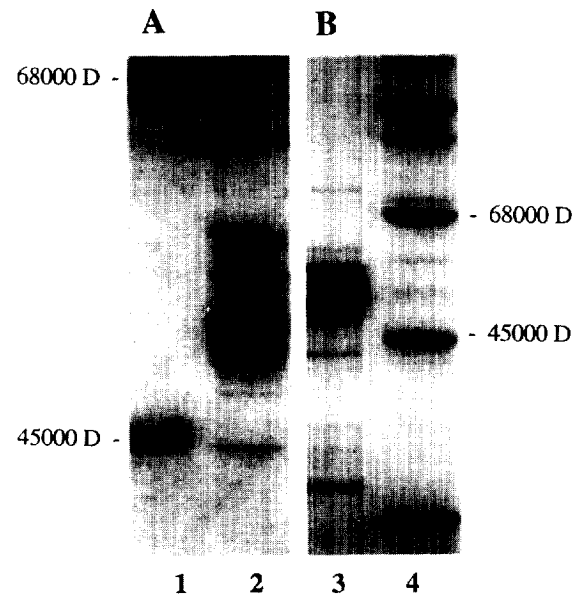

Fig. 7. Comparison of an inversed (from 10 to 5%) (A) and a normal (10%) SDS-PA gel (B) containing a standard mixture (lane 1 and 4) and liver microsomes of a rat treated with β-naphthoflavone (lane 2 and 3). The molecular mass markers have been indicated.

high-resolution gel such as a SDS-PA gel, the elution of such as single band protein may be considered. We have developed a simple procedure which is performed as follows. After a group specific purification on a aminooctyl agarose column (as described in a preceding chapter), the cytochrome P450 fraction was applied onto a inversed gradient PA gel (from 10 to 5% acrylamide). The SDS-PA gel was stained with Phast blue R-350 (for 30 min) for the localization of the protein bands (as shown in Fig. 8A) and destained in a destaining solution (water–methanol–acetic acid, 6:3:1) for 90 min. Then the protein bands were cut out of the gel and frozen at −20°C. The frozen piece of gel was forced through a syringe into a Tris–SDS buffer (10 times the volume of the gel). After incubation for 20 h at 4°C, the buffer was separated from the gel by centrifugation and concentrated in a Centricon centrifugation tube with repeated addition of a saline solution. This resulting solution can be used directly for immunization.

In Fig. 8B the separated cytochrome P450 protein bands have been re-electrophorated on a SDS-PA gel to show the efficiency of the preparative elution procedure. After immunization in rabbits, polyclonal antibodies have been prepared against several bands. In Fig. 9 the result is shown in which these antibodies are used in a Western blot procedure. In this way specific antibodies have been prepared against rat cytochrome P450 1A1, 1A2 and 2B1.

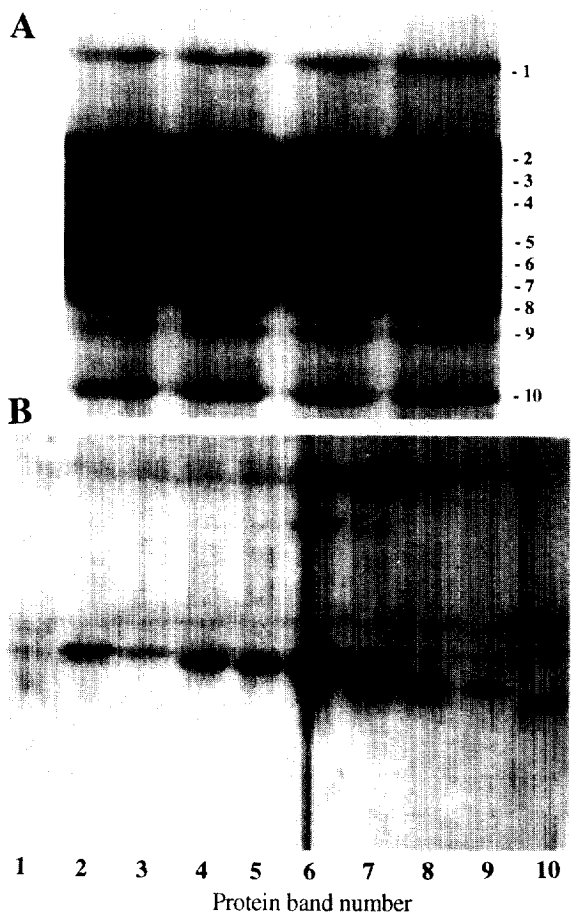

Fig. 8. (A) Part of a inversed gradient SDS–PA gel of the cytochrome P450 pattern (from rat liver microsomes after induction by β-naphthoflavone) after purification on aminoocyl agarose. The protein bands that have been cut out in slices and eluted from the gel have been numbered from 1 to 10. (B) Re-electrophoresis of the 10 fractions eluted from the gel in Fig. A.

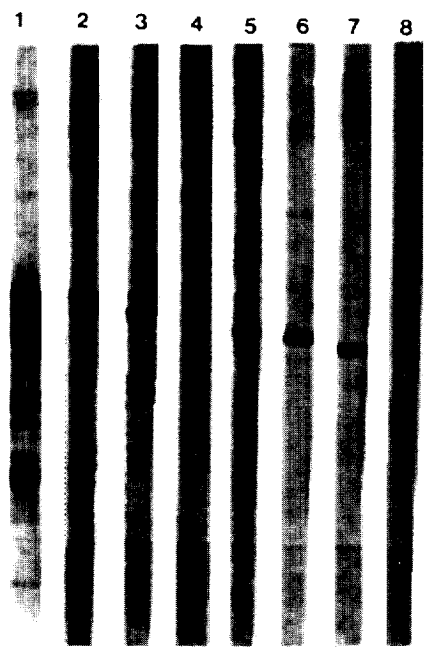

Fig. 9. Western blots of rat liver microsomes after induction by β-naphthoflavone using different antibodies. In lane 1 an antibody was used against the total microsomal fraction of Fig. 8A. In lane 2 to 8 antisera were used raised against the eluted protein bands no. 2, 4, 5, 6, 7, 8 and 10, respectively, from Fig. 8B. Colorimetric staining with diaminobenzidin was performed after incubation with a peroxidase labelled second antibody (sheep anti-rabbit IgG).

3. Detection methods

3.1. Enzymatic activity

3.1.1. Colorimetric and fluorescence methods

The enzymatic activity can be detected in a simple way if the substrate is specific for one of the reactions of the cytochrome P450 isozymes. At least it is required that the substrate can only be transformed into one single product which can be specifically detected by spectrophotometry or fluorescence. The spectrophotometric or fluorescence characteristics of the product must be different from those of the substrate for a specific and sensitive determination. These kind of assays can be performed in semi- or fully-automated systems, such as a spectrophotometer or a (centrifugal) autoanalyzer. The main advantage is the speed of measurements of a large number of samples. The sensitivity depends both on the detection technique, where fluorescence detection is in general more sensitive than spectrophotometrical detection and the spectral characteristics of the product compared to other components in the reaction mixture.

Well-know examples of this kind of assays are the deethylation of ethoxyresorufin (EROD) [13] or ethoxycoumarin [14] and the depentylation of pentoxyresorufin (PROD) [15]. A spectrophotometrical examples is the hydroxylation of *p*-nitrophenol (PNP). Since no chromatographic step is involved in this kind of assay, no extensive review is given here.

3.1.2. Product detection by HPLC

Besides enzyme assays with spectrophotometric or fluorescence detection of products formed by specific cytochrome P450s, a number of applications have been reported using HPLC as chromatographic separation and detection technique of the products formed. With this technique several enzymatic reactions can be monitored if a non-specific substrate is, such as testosterone [16,17], androstenedione [18], cholesterol [19], warfarin [20], benzo[*a*]pyrene [21], caffeine [22], fatty acids [23], aminopyrine [24], etc. In a review by Jansen and de Fluiter [25] in 1992, product analysis of cytochrome P450 reactions by HPLC have been summarized. In this chapter an extension of the literature from 1991 until April 1995 will be given, elucidated with some examples.

Since 1991, additional applications of HPLC assays for the determination of cytochrome P450 enzymes have been reported for N-nitroso-*n*-amylamine [26], dantrolene [27], coumarin [28], S-mephenytoin [29,30], warfarin and acenocoumarol [31], bunitrolol and debrisoquine [32], verlukast [33], ethylmorphine and codeine [34], *p*-nitrophenol [35,36], ochratoxin A [37], 2-ethynylnaphthalene [38], dextromethorphan [39], Trp-P-2 [40], clonazepam [41], ethanol [42], imipramine [43], arachidonic acid and epoxyeicosatrienoic acids [44,45],

Another approach was followed by Jansen et al. [46]. The authors intend to use urinary biomarkers of polycyclic aromatic hydrocarbons (PAH) for risk assessment purposes. They propose to use metabolites of naphthalene as biomarkers for inhalatory exposure and metabolites of pyrene as biomarkers for oral exposure [47]. In order to find correlations between metabolites of these two marker PAHs and the carcinogenic benzo[*a*]pyrene (BP) rat liver microsomes were incubated with the parent compounds. In addition cytochrome P450 enzymes were determined by Western blots, by enzymatic assays using fluorometric detection (EROD, PROD, PNP) or by HPLC analysis (phenacetin O-deethylase, EROD). Naphthalene, pyrene and BP were incubated with 20 different rat liver microsomal preparations. The metabolites have been analyzed by reversed phase HPLC. In Fig. 10 a HPLC chromatogram is shown of an incubation mixture of liver microsomes of rats (treated with 2,3,7,8-tetrachlorodibenzo dioxin) with BP several metabolites can be observed, such as mono-, di- and tetrahydroxy-BPs and BP-diones. After incubation with naphthalene, 1-naphthol was observed and a metabolite identified as naphthalene-1,2-dihydrodiol. Incubation with pyrene gave 1-pyrenol and two pyrene-diones. After correlation analysis important conclusions were drawn on the comparative metabolism of these different PAH compounds.

Also epoxide hydrolase, an important enzyme in the detoxification of the carcinogenic benzo[*a*]pyrene epoxides, was assayed with a new HPLC method using *cis*-stilbene oxide as substrate. A HPLC chromatogram is shown in Fig. 11.

To detect the activity or presence of cytochrome P450 1A1 and 1A2 isoenzymes, the EROD and

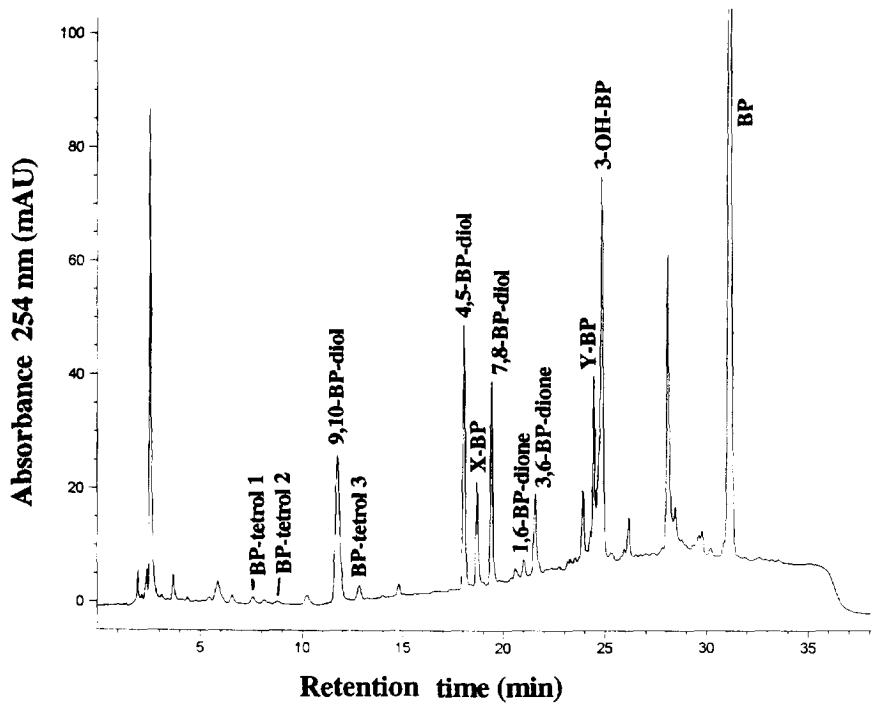

Fig. 10. HPLC chromatogram of the incubation mixtures rat liver microsomes (after treatment with dioxin) with BP. The peaks of substrate and metabolites have been indicated.

phenacetin O-deethylase (POD) assays, respectively, are well established procedures. With HPLC product analysis both assays can be performed by analysis of both decrease of substrate or increase of specific products formed. An example is shown in Fig. 12

3.2. Western blotting

Detection of the protein moiety of the cytochrome P450 enzymes can be done by difference spectrometry after treatment with carbon monoxide. A more specific determination can be done by using specific antibodies against the different isozymes. Since the use of immunoassays is still not very common, more applications have been reported using Western blots [48]. This highly-specific detection technique is a combination of a high-resolution electrophoretic separation of the protein moieties of cytochrome P450 combined with a specific detection by immunochemical techniques. After separation of the proteins by SDS-PAGE, the protein pattern is transferred onto a membrane which is incubated with a specific antibody against a particular cytochrome P450 isozyme. After a second incubation with an enzyme-conjugate coupled to a second antibody or via a biotin–streptavidin system, the proteins which binds to the antibody can be visualized by enzymatic activity of the enzyme bound to the conjugate. The most frequently used enzyme is horseradish peroxidase which can be detected using a colorimetric substrate, such as tetramethylbenzidin, or with chemiluminescence using a luminol- or acridinium-based chemiluminescence. Other examples of enzymes used as label are alkaline phosphatase which has an increasing interest because of the development of new sensitive substrates and xanthine oxidase. Both enzymes can be detected very sensitively with chemiluminescence.

Two parameters that are important in Western blotting are the specificity of the antisera and the sensitivity. The specificity of antisera depends on both the isolation procedure of the cytochrome P450 isozymes and on the way of immunization. The isolation procedure can be performed by a sequence

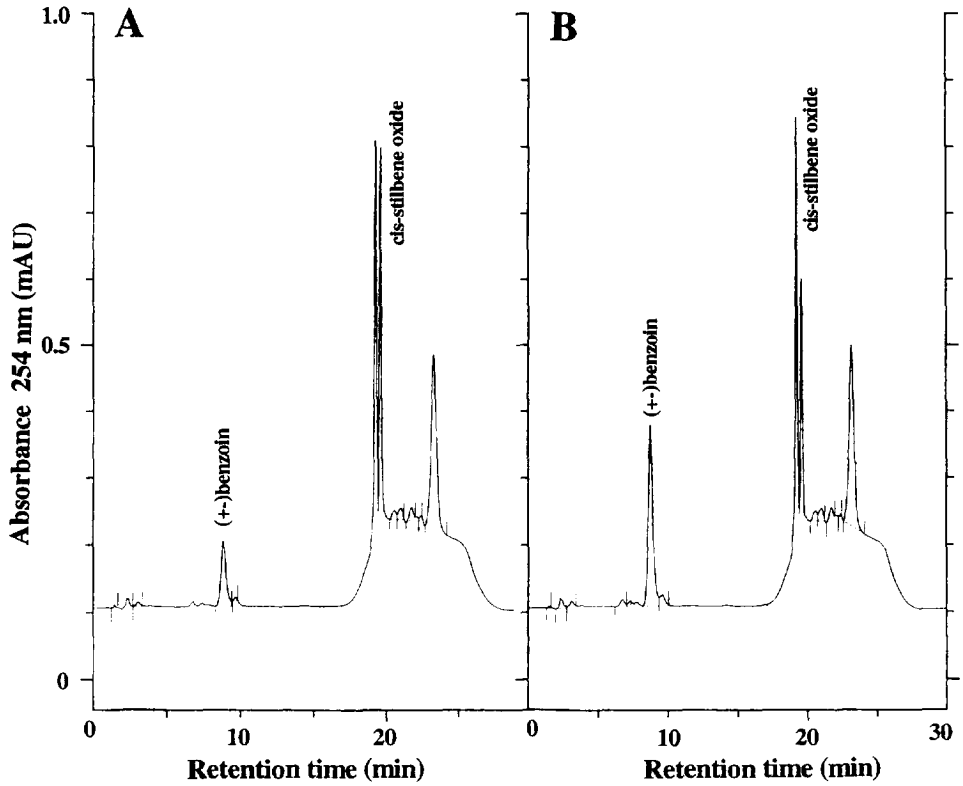

Fig. 11. HPLC chromatograms of the incubation mixtures of liver microsomes of a control rat (A) and a rat treated with phenobarbital (B) with *cis*-stilbene oxide as substrate to detect the epoxide hydrolase activity. The peaks of substrate and metabolite (+ −) benzoin have been indicated.

of conventional purification steps, such as precipitation and column chromatography. On many cases it is difficult to achieve a complete separation of pure isozymes. A combination with the production of monoclonal antibodies can lead to almost 100% specific antibodies. Recently, a number of commercial companies have released antisera or Western blot kits for specific cytochrome P450 isozymes. Amersham Life Science (U.K) has a number of Western blot kits with highly specific antibodies and horseradish peroxidase mediated chemiluminescence detection using the biotine–streptavidin system. Gentest Corporation (USA) has antibodies against cytochrome P450 isozymes for use in Western blot analysis without a detection system. Oxford (USA) has Western blot kits with chemiluminescence detection using a second-antibody labelled alkaline phosphatase conjugate. An example of detection of cytochrome P450 proteins by Western blotting is shown in Fig. 13.

The sensitivity of the various blotting systems depends on the combination of enzyme and substrate used. Although the spectrophotometric detection using horseradish peroxidase and tetramethylbenzidine is rather sensitive, cheap and simple to use, the chemiluminescent systems show an increase sensitivity. In 1991, Jansen et al. [49] compared different systems and concluded that horseradish peroxidase (HRP) with the enzyme enhanced chemiluminescence system was most sensitive. Since than the various systems have been improved, especially the chemiluminescence system using alkaline phosphatase with new substrates which are more sensitive and show faster kinetics. Under development are also new acridinium-like chemiluminescent substrates for HRP which can be used with normal pH which is

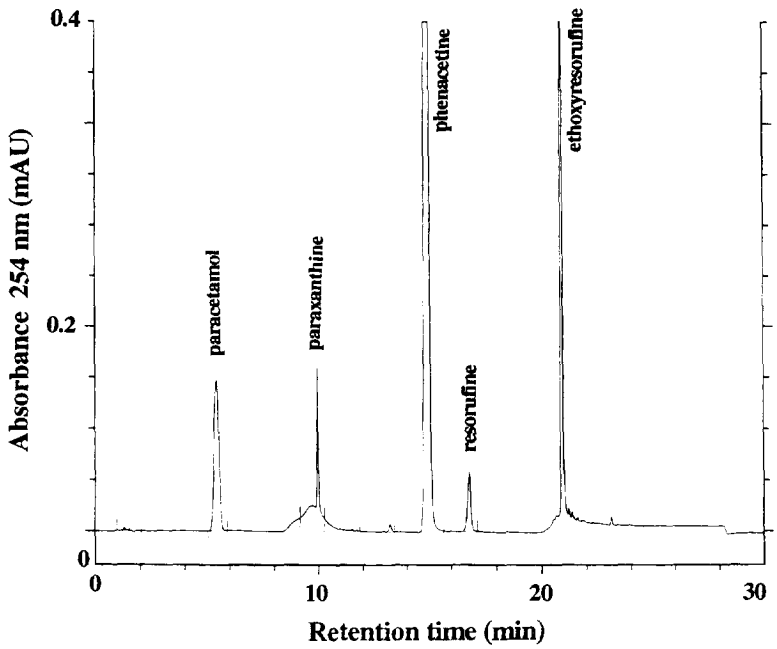

Fig. 12. HPLC chromatograms of the incubation mixtures of liver microsomes of a rat (treated with dioxin) with phenacetine and ethoxyresorufine as substrates to detect the activity of cytochrome P-450 1A1 and 1A2. The peaks of substrates and metabolites formed have been indicated. Paraxanthine was used as internal standard.

very favorable for the optimum activity of the enzyme.

3.3. Correlation of enzyme assays with Western blotting

Since enzymatic activities can be measured more easy than the presence of proteins by Western blotting, it would be helpful if the specificity of enzyme assays is high enough to replace the laborious blotting experiments.

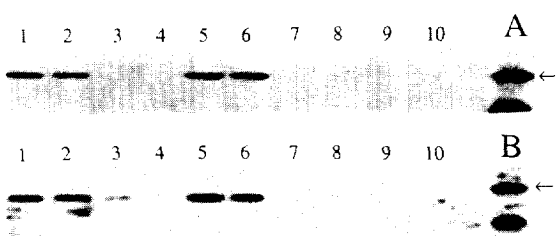

Fig. 13. Western blots of cytochrome P-450 1A1 (A) and 1A2 (B) in liver microsomes of rats treated with different inducers: 1,2, dioxin; 3,4, isoniazid; 5,6, PCB-169; 7,8, PCB-153; 9,10, phenobarbital. The odd numbers are from female rats, the even from male rats. A chemiluminescence detection kit is used with highly specific antibodies against both proteins with the biotine–avidin system. The arrow indicates the position of the MW-standard catalase ($58.1 \cdot 10^3$ kDa).

The correlation of the enzymatic activity of cytochrome P450 enzymes with the occurrence of the protein is in general good for a number of assays. Examples are the EROD activity for cytochrome P450 1A1, the POD activity for cytochrome P450 1A2, the PROD activity for cytochrome P450 2B1 and the PNP activity for cytochrome P450 2E1. In an experiment with 20 differently induced rat liver microsomes, however, some of these correlations are not as good as expected. Especially the correlation between PROD activity and cytochrome P450 2B1 (correlation coefficient 0.691) and between POD activity and cytochrome P450 1A2 (correlation coefficient 0.748) is not very good because of the lack of POD activity in the microsomes of rats treated with isosafrole which may be attributed to an inhibitory effect. Other correlations were as expected, e.g., between EROD activity and cytochrome P450 1A1 (correlation coefficient 0.984), between PNP activity and cytochrome P450 2E1

Table 1
Summary of the advantages and limitations of the various methods to detect cytochrome P-450 isozymes

	Enzymatic analysis	Enzymatic analysis with HPL	Electrophoresis with general staining	Electrophoresis with immunochem. staining
Number of enzymes	One	More possible	A few	One
Specificity	Rather good	Good	Low	Excellent
Sensitivity	Good	Good	Good	Excellent
Quantitation	Excellent	Good	Moderate	Moderate
Dynamic range	High	High	Good	Limited
Number of samples per day	≥100	40	20	10
Time of analysis for 20 samples	1 h	24 h	8 h	24 h
Cost per sample	Low	Rather low	Low	High

(correlation coefficient 0.924). A detailed analysis of this study will be published elsewhere. In another report [50] peroxisomal proliferation was studied by the enzymatic activity of lauric acid hydroxylase and the presence of cytochrome P450 4A1 on blots. Both parameters showed a good correlation with a correlation coefficient of 0.96.

4. Discussion

In this paper a number of methods of analysis of cytochrome P450 enzymes have been considered. They include: (1) detection based on the enzymatic properties of the enzymes both without, and (2) with a chromatographic separation step using HPLC, (3) detection based on the protein moiety of the enzymes by electrophoresis followed by a general staining technique or, (4) by a more specific immunochemical staining (Western blots).

The method of choice for a certain problem depends on the particular problem and the information which is required. This information can include: (1) the number of cytochrome P450 isozymes that must be analyzed, (2) the specificity of the analysis, (3) the speed or the time required for analysis, (4) the number of samples that must be analyzed and (5) the required sensitivity, and (6) the costs of analysis.

In general, enzymatic assays without a chromatographic step is recommended to perform because of the ease of operation, especially if autoanalyzers are used. Some of these reactions are very specific, such as EROD and PNP and PROD to a lower extend.

Enzymatic assays with an additional HPLC analysis of the reaction mixture are more specific with respect to the formation of product(s). In several cases more products can be observed and also the decrease of substrate concentration can be monitored. This kind of analysis is more laborious, however. In addition, although more reactions can be followed by the occurrence of several products, the specificity of these reactions is not always established and the assignment to one specific cytochrome P450 enzyme is often questionable. Useful examples are POD, lauric acid hydroxylase (LAH) and some testosterone hydroxylases.

The Western blot analysis can be a very specific method for the identification and quantitation of specific cytochrome P450 enzymes. The specificity depends on the quality and properties of the antibody used. Besides this high specificity, an additional advantage can be that also rather "old" samples which have probably lost their enzymatic activity, still can be analyzed

with good accuracy by Western blotting. This procedure, however, is much more laborious and expensive than the two other methods mentioned before. Also the precise quantitation and dynamic range is limited, especially when chemiluminescence detection is used.

The advantages and limitations of these methods have been summarized in Table 1.

References

[1] D.W. Nebert, et al., DNA Cell Biol., 10 (1991) 1.
[2] F.P. Guengerich, in A.W. Hayes (Editor), Principles and Methods in Toxicology, Raven Press, New York, 2nd ed., 1989.
[3] M. Barry and J. Feely, Pharmac. Ther., 48 (1990) 71.
[4] T. Porter and M.J. Coon, J. Biol. Chem., 266 (1991) 13469.
[5] C. Ioannides and D.V. Parke, Drug Metab. Rev., 25 (1993) 485.
[6] F.P. Guengerich, Am. Sci., 81 (1993) 440.
[7] W. Tassaneeyakul, D.J. Birkitt, M.E. Veronese, M.E. McManus, R.H. Tukey, L.C. Quarttrochi, H.V. Gelboin and J.O. Miners, J. Pharmacol. Exp. Ther., 265 (1993) 401.
[8] K. Nagata, P. Buppodom, T. Matsunaga, M. Ishimatsu, H. Yamato, S. Yoshihara and H. Yoshimura, J. Biochem., 97 (1985) 1755.
[9] E.H.J.M. Jansen and E.J.M. Reinerink, Anal. Chim. Acta, 255 (1991) 363.
[10] E.H.J.M. Jansen, E.J.M. Reinerink and R.H. van den Berg, Anal. Chim. Acta, 227 (1989) 49.
[11] E.H.J.M. Jansen, R.H. van den Berg, R. Both-Miedema, C. Enkelaar-Willemsen and G. Zomer, Anal. Chim. Acta, 205 (1988) 175.
[12] E.H.J.M. Jansen, C.A. Laan and E.J.M. Reinerink, in B.J. Radola (Editor), Electrophoresis Forum '89, Techn. Univ. Munich, 1989, p. 293.
[13] R.A. Lubet, R.W. Nims, R.T. Mayer, J.W. Cameron and L.M. Schlechtman, Mutat. Res., 142 (1985) 127.
[14] D. Müller-Enoch, P. Churchill, S. Fleischer and F.P. Guengerich, J. Biol. Chem., 259 (1984) 8174.
[15] M.D. Burke and R.T. Mayer, Chem. Biol. Interact., 45 (1983) 243.
[16] E.J.M. Reinerink, L. Doorn, E.H.J.M. Jansen and A.A.J. van Iersel, J. Chromatogr., 553 (1991) 233.
[17] M.P. Arlotto, J.M. Trant and R.W.Estabrook, Meth. Enzymol., 206 (1991) 454.
[18] A.W. Wood, D.E. Ryan, P.E. Thomas and W. Levin, J. Biol. Chem., 258 (1983) 8839.
[19] G.L. Schatzman, M.E. Laughlin and T.R. Blohm, Anal. Biochem., 175 (1988) 219.
[20] L.S. Kaminsky, M.J. Fasco and F.P. Guengerich, Meth. Enzymol., 74C (1981) 262.
[21] J.M. Haake-McMillan and S.H. Safe, J. Biochem. Toxicol., 5 (1990) 203
[22] U. Fuhr, T. Wolff, S. Harder, P. Schymanski and A.H. Staib, Drug Metab. Dispos., 18 (1990) 1005.
[23] E.H.J.M. Jansen and P. de Fluiter, J. Liq. Chromatogr., 15 (1992) 2247.
[24] S. Imaoka, K. Inoue and Y. Funae, Arch. Biochem. Biophys., 265 (1988) 159.
[25] E.H.J.M. Jansen and P. de Fluiter, J. Chromatogr., 580 (1992) 325.
[26] Mirvish, C. Ji, Q. Huang, S. Wang, S.S. Park and H.V. Gelboin, IARC Scientific Publications, 105 (1991) 392.
[27] Z. Jayyosi, J. Villoutreix, J.M. Ziegler, A.M. Batt, F. de Maack, G. Siest and P.E. Thomas, Drug Metab. Dispos., 21 (1993) 939.
[28] D.E. Goeger and K.E. Anderson, Biochem. Pharmacol., 43 (1992) 363.
[29] K. Chiba, K. Manabe, K. Kobayashi, Y. Takayama, M. Tani and T. Ishizaki, Eur. J. Clin. Pharmacol., 44 (1993) 559.
[30] J. Brockmöller, K.L. Rost, D. Gross, A. Schenkel and I. Roots, Pharmacogenetics, 5 (1995) 80.
[31] J.J.R. Hermans and H.H.W. Thijssen, Br. J. Pharmacol., 110 (1993) 482.
[32] T. Suzuki, S. Narimatsu, S. Fujita, Y. Masubuchi, S. Umeda, S. Imaoka and Y. Funae, Drug Metab. Dispos., 20 (1992) 367.
[33] D.A. Nicoll-Griffith, N. Chauret, J.A. Yergey, L.A. Trimble, L. Favreau, R. Zamboni, S.J. Grossman, J. Drey and E. Herold, Drug Metab. Dispos., 21 (1993) 861.
[34] B.Q. Xu, T.A. Aasmundstad, A. Bjørneboe, A.S. Christophersen and J. Mørland, Biochem. Pharmacol., 49 (1995) 453.
[35] R.J. Duescher and A.A. Elfarra, Anal. Biochem., 212 (1993) 311.
[36] W. Tassaneeyakul, M.E. Veronese, D.J. Birkett and J.O. Miners, J. Chromatogr., 616 (1993) 73.
[37] T. Osters, Z. Jayyosi, E.E. Creppy, J.S. el Amri and A.-M. Batt, Toxicol. Lett., 57 (1991) 203.
[38] E.S. Roberts, N.E. Hopkins, W.L. Alworth and P.F. Hollenberg, Chem. Res. Toxicol., 6 (1993) 470.
[39] J.C. Gorski, D.R. Jones, S.A. Wrighton and S.D. Hall, Biochem. Pharmacol., 48 (1994) 173.
[40] S. Minamoto and K. Kanazawa, Anal. Biochem., 225 (1995) 143.
[41] E.J. Seree, P.J. Pisano, M. Placidi, R. Rahmani and Y.A. Barra, Fundam. Clin. Pharmacol., 7 (1993) 69.
[42] S. Kunitoh, T. Tanaka, S. Imaoka, Y. Funae and Y. Monna, Alcohol Alcoholism, 28 (1993) 63.
[43] Y.Masubuchi, S. Fujita, M. Chiba, N. Kagimoto, S. Umeda and T. Suzuki, Biochem. Pharmacol., 41 (1991) 861.
[44] J.H. Capdevila, E. Dishman, A. Karara and J.R. Falck, Methods Enzymol., 206 (1991) 441.
[45] R.T. Okita, J.E. Clark, R.R. Okita and B.S.S. Masters, Meth. Enzymol. 206 (1991) 432.

[46] E.H.J.M. Jansen, E. Schenk, G. den Engelsman and G. van de Werken, Polycycl. Arom. Comp., in press (1996).
[47] E.H.J.M. Jansen, E. Schenk, G. den Engelsman and G. van de Werken, Clin. Chem., 41 (1995) 1905.
[48] K.J. Pluzek and J. Ramlan, in O.J. Bjerrum and N.H.H. Heegaard (Editors), Handbook of Immunoblotting of proteins, CRC Press, Boca Raton, FL, Vol. 1., 1988, p. 177.
[49] E.H.J.M. Jansen, C.A. Laan, R.H. van den Berg and E.J.M. Reinerink, Anal. Chim. Acta, 255 (1991) 359.
[50] E.H.J.M. Jansen, C.A. Laan and P. de Fluiter, Anal. Chim. Acta, 303 (1995) 99.