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Short Communication

A high-performance liquid chromatographic method for the evaluation of aldrin epoxidation by cytochrome P-450 dependent monooxygenase in small liver samples

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Introduction

The majority of xenobiotics that enter body tissues are lipophilic, a property that enables them to penetrate lipid membranes and to be transported by lipoproteins present in body fluids. The metabolism of xenobiotics by a complex of enzymes usually involves two distinct phases. In the first phase, a polar reactive group is introduced into the molecule (this provides increased water solubility) and in the second, the altered compounds combine with an endogenous substrate to produce a water soluble conjugated product that is readily excreted. Microsomal mixed-function oxidation reactions are catalysed by a multienzyme system located in the endoplasmic reticulum of the cell, which may be isolated by homogenization and ultracentrifugation of the postmitochondrial supernatant fraction. This system, which has cytochrome P-450 as the terminal oxidase, has been studied in many tissues and organisms. In fact, it is known that various forms of cytochrome P-450 exist in bacterial, plant and animal organisms.

Cytochrome P-450 participates in the oxidative metabolism of xenobiotic compounds such as drugs, foodstuffs, dyes, pesticides, mutagens and carcinogens. Its rôle is well established in the metabolism of endogenous substances such as steroids [1], in the syntheses of vitamin D [2], bile acids [3] and in the metabolic breakdown of fatty acids including arachidonic acid [4]. Another function of cytochrome P-450 may be to participate in the metabolism of endogenous toxic substances formed in pathological conditions such as uremia [5].

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Cytochrome P-450 exists in multiple forms which differ in spectral characteristics, molecular weight, substrate specificity and sensitivity to inducers and inhibitors. Sensitive assays are required in order to study the properties of these cytochrome forms, and especially when low monooxygenase activities or only small amounts of biological material are available for investigation. A reaction which can be monitored sensitively and selectively is the epoxidation of aldrin which is known to be mediated by monooxygenases. Epoxidation is an extremely important microsomal reaction since not only can stable epoxides be formed, but arene oxides, the epoxides of aromatic rings, are intermediates in aromatic hydroxylations. The epoxidation of aldrin to dieldrin is the best known example of the metabolic formation of a stable epoxide. The results of studies by Gilieen [6] and Van Cantfort [7] suggested that epoxidation is specific for the activity of cytochrome P-450 forms induced by phenobarbital (PB) treatment of animals and that it was independent of cytochrome P-448 species induced by pretreatment with 3-methylcholanthrene (3-MC). Furthermore, it was found that P-450 catalysed aldrin epoxidation by a factor of more than 100-fold more efficiently than P-448. Studies published by Wolff *et al.* [8] on aldrin epoxidation activity, confirmed that aldrin is a highly selective substrate for cytochrome P-450 and a poor substrate for cytochrome P-448.

The present report describes a high-performance liquid chromatographic (HPLC) method for the determination of aldrin epoxidation by cytochrome P-450 in small liver samples.

Experimental

Chemicals

Aldrin and dieldrin of Organic Analytical Standard grade, methanol, acetonitrile and hexane, HPLC grade, were purchased from Riedel-de-Haën (Hoechst, Italy); glucose-6-phosphate, G-6-P dehydrogenase and NADP were obtained from Calbiochem Behring Corp. (Hoechst, Italy).

Animals and pretreatments

Male Sprague-Dawley rats (Charles River, Italy), weighing about 230–270 g, were used, housed in cages with free access to laboratory chow and tap water. The animals were divided into six groups each of eight animals, and each group received one of the following treatments at the doses here indicated:

(i) controls received vehicle only;
(ii) a group was treated by i.p. injection with PB 80 mg kg^{-1} daily for 4 days;
(iii) PB by gavage 12.5 mg kg^{-1};
(iv) Aroclor 1254 by gavage at three doses (10–20–40 mg kg^{-1} in corn oil) daily for 7 days.

Animals were starved overnight before being sacrificed.

Preparation of the enzymes

The animals were sacrificed by cervical dislocation and a small liver sample (10–15 mg), obtained by a fine-needle aspiration biopsy method [9], was rapidly excised and washed three times in 1 ml of 0.25 M cold sucrose. After this procedure, the samples were diluted with a four-fold volume of 0.25 M sucrose and homogenized in an ice bath with a Potter homogenizer equipped with a Teflon pestle.

Enzymatic assay

The incubation mixture was prepared at 0°C in stoppered glass tubes containing 0.1 M Na_2HPO_4–KH_2PO_4 buffer (pH = 7.4, 860 μl), glucose-6-phosphate (75 μl of stock solution 0.11 M), NADP (5 μl of stock solution 0.11 M), glucose-6-phosphate dehydrogenase (5 μl of stock solution 700 U ml^{-1}), microsome homogenate (40 μl) and aldrin (40 μl of stock solution 0.01 M in methanol), just prior to incubation. After 20 min of incubation with gentle shaking in a water bath at 37°C, the reaction was stopped by transferring the tubes into ice. The synthesized dieldrin and unreacted aldrin were then extracted with 2.5 ml hexane and the two phases separated by centrifugation at 1500*g* for 10 min. The organic phase was transferred into a second tube. The same extraction procedure was repeated twice, the organic phases collected from the extractions of the same sample, were pooled and evaporated to dryness under a stream of nitrogen. The residues were dissolved in 100 μl of methanol, 20 μl aliquots of which were used for chemical analysis.

Chromatographic system and conditions

The Gilson HPLC system (Gilson SA, France) employed consisted of a model 302 pump, a model 7215 Rheodyne injector (Cotati, CA, USA), and a model Holochrome H-MD variable-wavelength UV detector (215 nm) connected to an Apple II integrator.

Chromatographic separation was performed on an analytical 100 × 4.6 mm, i.d., reversed phase Spherisorb ODS-2 (3 μm, particle size) column protected by a 3 cm disposable Spherisorb ODS-2 column (10 μm, PhaseSEP Ltd, UK).

The separations were performed at ambient temperature and the detector set at 0.05 absorbance units, full scale. The mobile phase consisted of a mixture of acetonitrile–water (66:34, v/v). Water (HPLC grade) was obtained by double distillation from glass and was purified by means of a "Milli-Q Waters Purification System" (Millipore, Bedford, MA, USA). The acetonitrile was filtered through an FA 0.5 μm filter (Millipore, Bedford, MA, USA). The mobile phase was prepared daily and delivered at a flow rate 1.5 ml min^{-1}.

Measurement of standard curves

A standard solution of dieldrin was prepared in methanol at a concentration of 100 μg ml^{-1}. This solution was further diluted with methanol to concentrations of 4, 2, 1, 0.75, 0.5 and 0.25 μg ml^{-1}, respectively.

The standard solution of aldrin, was prepared similarly at a concentration of 100 μg ml^{-1}. The standard curves for aldrin were linear between 0.25–4 μg ml^{-1} and gave rise the following equations $y = 0.021x - 0.682$ ($r = 0.997$) for dieldrin, and $y = 0.026x - 0.901$ ($r = 0.998$) for aldrin.

A limit of detection of 0.1 μg was observed for dieldrin (at a signal to noise ratio of 5), when an injection volume of 10 μl was used.

Precision data were obtained by replicate analyses (seven determinations each at six different concentrations) and resulted in RSD values ranging from 3.1 to 5.3%. Protein concentration was measured by the method of Bradford [10], using bovine serum albumin as a standard. Individual points were compared using the Student non-paired *t*-test. Differences were deemed statistically significant if $P < 0.05$.

Results and Discussion

Under the described chromatographic conditions, synthesized dieldrin and the remaining aldrin gave well resolved peaks with retention times of 4.18 and 8.30 min respectively, as shown in Fig. 1. Dieldrin was found not to be further metabolized during the period of the reaction. This demonstrates one of the main advantages of the epoxidation reaction insofar as it enables a relatively long period of incubation to be employed. Because of the heterogeneous distribution of the enzyme in various liver lobes, as reported by Sumner [11] and Matsubara [12], there is an asymmetrical cytochrome P-450 distribution both in control animals and in animals treated with PB. PB induction of isoenzymes enhances the asymmetry with respect to total cytochrome P-450 within the lobes. In fact a direct comparison with the data reported in ref. 11, showed that the specific cytochrome P-450 content of the right and median lobes is higher than those from the other lobes and a significant asymmetry distribution was apparent. A small portion of median lobe was removed in which the cytochrome P-450 monooxygenase activity was found to be considerably higher.

PB and Aroclor 1254 administration increased liver weight in relation to doses used. Table 1 shows the cytochrome P-450 monooxygenase activity in rats treated with PB and Aroclor 1254. PB treatment caused a significant induction of cytochrome P-450 at dosages of 80 mg kg^{-1} (i.p. for 4 days, $P < 0.001$) and 12.5 mg kg^{-1} (o.s. for 7 days, $P < 0.01$). The Aroclor 1254 was also significant for the three used doses (10 mg kg^{-1}, $P < 0.05$; 20 mg kg^{-1}, $P < 0.01$; 40 mg kg^{-1}, $P < 0.001$).

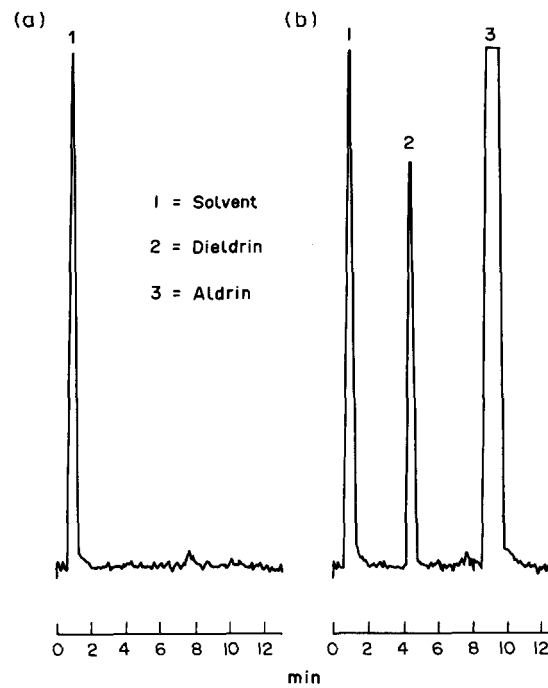

Figure 1
(a) Chromatogram of a blank sample. (b) Typical chromatogram of a sample containing aldrin and synthesized dieldrin.

Table 1
Effects of PB and Aroclor 1254 on liver weight and cytochrome P-450 aldrin epoxidase activity in male rats

Treatment	Doses	Liver weight % of body weight (mean ± SD)	μM dieldrin mg protein × min (mean ± SD)
Control		4.12 ± 0.09	0.857 ± 0.033
Aroclor 1254	10 mg kg^{-1}	4.22 ± 0.10	1.277 ± 0.136†
Aroclor 1254	20 mg kg^{-1}	4.39 ± 0.13*	1.930 ± 0.117‡
Aroclor 1254	40 mg kg^{-1}	4.97 ± 0.21*	2.995 ± 0.091*
PB (o.s.)	12.5 mg kg^{-1}	4.92 ± 0.31*	1.942 ± 0.283†
PB (i.p.)	80 mg kg^{-1}	5.30 ± 0.27*	5.090 ± 0.650*

Comparison between control and treatment groups.
† = $P < 0.05$.
‡ = $P < 0.01$.
* = $P < 0.001$.

At lower doses, a moderate increase of microsomes induced by Aroclor 1254 was found; in fact PB caused a stronger and more specific induction of the monooxygenases aldrin epoxidases system, whilst Aroclor 1254, being a mixed inducer, produced a lower increase of the enzyme concentration. In former studies [13, 14] conducted on the induction by Aroclor 1254 and 1260, it was demonstrated that these mixtures induced cytochrome P-450 and P-448 forms, which were indistinguishable from those induced by PB and 3-MC. It is important to emphasize that Aroclor 1254 is a mixture of isomers with a predominance of tetrachlorobiphenyl (TCB). TCB induces two different forms of cytochrome P-450, which can activate two isomers of TCB, namely 2,4,2′,4′-TCB and 2,4,2′,5′-TCB; with P-448, the cytochrome induced by 3-MC, activating the 3,4,3′,4′-TCB isomers.

Many reports [15–17] have indicated that isomers of Aroclor 1254 chlorinated in the *ortho* and *para* positions (2,2′ and 4,4′) of both phenyl rings are PB type inducers of microsomal mixed-function oxidase activity, whilst those compounds chlorinated in *meta* and *para* positions (3,3′ and 4,4′) of both phenyl rings are 3-MC-type inducers of cytochrome P-448 containing monooxygenase activity.

Studies on hepatic cytochrome P-450 by Funae [18] revealed that the HPLC profile of PCB-treated microsomes, showed distinctly that the PCB manner of induction was PB and 3-MC mixed-type. According to HPLC profiles, induction of rat hepatic microsomal cytochrome P-450 could be classified into three types: PB-type, 3-MC-type, and their mixed-type. A typical example for the mixed-type is the induction mode observed with PCB treatment. These three types also were confirmed by the value of co-reduced maxima of cytochrome P-450 selectively induced by treatment with PB, 3-MC and PCB, namely 450, 447 and 448 nm, respectively.

Ryan [13] compared the SDS–polyacrylamide gel profiles, cross-reactivity with antibodies and spectral maxima of co-reduced form of cytochrome P-450 from PB-, 3-MC- and PCB-treated rats. They reported that the properties of PB-treated cytochrome P-450 differed from those of 3-MC-treated rats and that the properties of PCB-treated cytochrome P-450 belonged to the mixture of PB- and 3-MC-treated animals.

The new HPLC technique is shown to be a specific, rapid and useful method for evaluation of cytochrome P-450 induction. As the method used very small amounts of hepatic tissue (10–15 mg), it may be employed clinically using one or two biopsy punches. In the experimental studies the aim of the method was to recognize in living

animals the development of enzymatic induction, which can be caused by chemical substances. Using the fine-needle aspiration biopsy method [9] it should be possible to monitor individual animals by serial punctures over a long period of time.

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