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QUANTIFICATION OF TWO CYTOCHROME P-450 ISOENZYMES BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) using monoclonal and polyclonal antibodies has been developed to quantify individual cytochrome P-450 isoenzymes in microsomal preparations, namely UT-A and PB-B. This very sensitive method can be used for the rapid processing of large quantities of determinations and requires only limited amounts of antibodies.

INTRODUCTION: Cytochrome P-450 exists as several molecular forms which differ in their primary structure, substrate specificity and inducibility (1-6). Studies on the multiplicity and regulation of cytochrome P-450 require a rapid, sensitive, and specific method for quantifying these isoenzymes. Several immunochemical assays have been developed for this purpose. Radial immunodiffusion techniques require large amounts of antibodies and are not very sensitive (6). Rocket immunoelectrophoresis (7) presents the same drawbacks as the aforementioned method. SDS-PAGE crossed immunoelectrophoresis (5) is more sensitive, specific, and requires lower amounts of antibodies than the two other methods. Nevertheless, this method is not rapid enough for processing large numbers of samples. ELISA or RIA tests might fulfill all of our requirements, i.e. speed, application to large numbers of samples, sensitivity and minimal need for amounts of antibodies. Microsomal cytochrome b5 and NADPH cytochrome c reductase were recently measured by and ELISA test (8), while two cytochrome P-450 isozymes were quantified by an RIA test using monoclonal antibodies (9). Having produced

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three monoclonal antibodies against a phenobarbital-inducible isoenzyme of P-450 (P-450 PB-B) (14) and a polyclonal antibody against the main constitutive P-450 isoenzyme (P-450 UT-A) (5), we developed an ELISA test for measuring the two isoenzymes in different preparations. The results are compared to those obtained by immunoelectrophoresis, the most specific method currently available.

MATERIALS AND METHODS: All of the chemicals were of the highest grade commercially available. Immunoglobulins were obtained from Dakopaths (Copenhagen, Denmark). Male Sprague-Dawley rats (150-200 g) and female Balb/c mice (3 weeks to 12 months old) were purchased from Iffa Credo (Les Oncins, France). β-Naphthoflavone (20 mg/kg) dissolved in corn oil and phenobarbital (80 mg/kg in isotonic NaCl) were injected i.p. daily for three days. The livers were used for the preparation of microsomes (10). Cytochrome P-450 was measured according to Omura and Sato (11) and proteins according to Lowry et al. (12).

The purification of PB-B and UT-A homogeneous in SDS PAGE has been described elsewhere (5). Polyclonal antibodies were raised in female New Zealand rabbits as previously described (5). Monoclonal antibodies towards P-450 PB-B were produced according to the method of de Fazekas et al. (13). Three hybridomas were selected, namely 2 A_2, 2 C_2 and 4 D_6. They were used for the quantification of cytochrome P-450. Their biochemical properties are described elsewhere (14). Quantification of cytochrome P-450 was performed by immunoelectrophoresis as previously described (5). All ELISA determinations were performed in 96-well plates obtained from NUNC Plastics (Roskilde, Denmark). The coating buffer was made of Na_2CO_3 (14.7 mM), $NaHCO_3$ (27.4 mM); NaN_3 (3 mM) at pH 9.6. The substrate contained 2.2' azino-di-(3-ethylbenzthiazoline) sulfonic acid (750 mg/l) (Boehringer); Na_2HPO_4 (15.5 mM); citric acid (4,2 mM); H_2O_2 (6.10^{-4}%) (v/v).

Cytochrome P-450 (20-1000 fmol) diluted in coating buffer containing 2% (w/v) cholate were coated in each well overnight at 4°C. Wells were washed three times with tween 20 (0.05%, v/v) in PBS (in 10 mM phosphate buffer (pH 7.4) containing NaCl (0.9% w/v), and hereafter called PBS) and dried; free sites were saturated for 2 hours at 37°C with 200 µl fetal calf serum 5% (v/v) in PBS. Wells were washed three times with tween 20 (0.05%, v/v) in PBS and dried. Plates can be stored at this stage for several weeks at 4°C. The antibody (100 µl per well) at the appropriate dilution was then allowed to incubate with the antigen for 3 hours at 37°C or overnight at 4°C. The wells were then washed three times as above prior to incubation with horseradish peroxidase conjugated rabbit antimouse immunoglobins (diluted 1/250) for 1 hour at 37°C. The plates were again washed with tween 20 (0.05%, v/v) in PBS. Peroxidase substrate solution (100 µl) was finally added to each well. After incubation for 1 hr at 37°C, the reaction was stopped by the addition of H_2SO_4 1N (100 µl per well). The absorbance of the green color was measured at 405 nm with an automatic colorimeter (Titertek)..

RESULTS AND DISCUSSION:

Antibody specificity: With immunoelectrophoresis, the three monoclonal antibodies recognized PB-B as a single band. The antibodies did not exhibit any reaction with the major P-450s isolated from liver microsomes of untreated, isosafrole or β-naphthoflavone-treated animals, or with

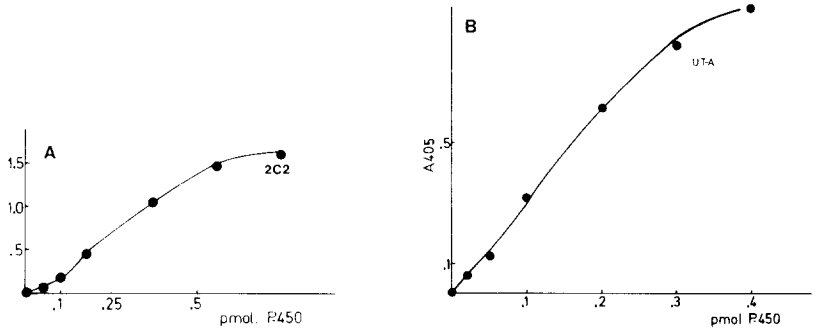


Fig. 1: ELISA - reaction of antibodies with cytochrome P450 as a function of the amount of cytochrome P-450 coated on the plate.

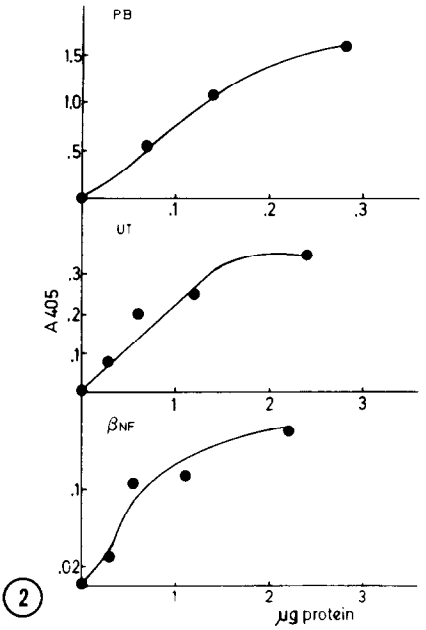
A. Monoclonal antibody 2 C_2 (anti-P-450 PB-B) was diluted ascitic liquid (1/5000). Identical curves were obtained with the two other monoclonal antibodies, namely 2 A_2 and 4 D_6.

B. Reaction of the anti-P-450 UT-A (1/10,000) polyclonal antibody.

P 450 PB-C, an isoenzyme induced by phenobarbital (5,14). On the contrary, these monoclonal antibodies, as the corresponding polyclonal antibody (anti P-450 PB-B (5)), react with P-450 PB-D, another isoenzyme induced by phenobarbital. The isoenzymes P-450 PB-B and P-450 PB-D are identical to P-450 forms b and e of Ryan and co-workers; both are induced by PB and are immunologically indistinguishable (5,6). Therefore, the monoclonal antibodies measured the sum of P-450 PB-B and P-450 PB-D (i.e. b + e) and were not more specific than the polyclonal antibodies. They recognized a common epitope present on the two isoenzymes. The specificity of the polyclonal antibody towards P-450 UT-A was tested by immunoelectrophoresis; it was specific for P-450 UT-A and did not react with any of the isoenzymes mentioned above (5).

Optimization of conditions: A dilution of the antibody used was selected to yield the highest sensitivity and the greatest range of linearity, which ranges from 1/1000 to 1/10,000 as a function of the antibody. These conditions enabled us to use very minute amounts of antibodies. A 1/5000 dilution was used for verifying the linearity of the 405 nm absorbance as a function of the quantity of coated cytochrome P-450. Figure 1 shows that the absorbance is linear from .02 to 0.5 pmol of coated cytochrome P-450 PB-B for monoclonal antibodies, and to 0.3 pmol of coated cytochrome P-450 UT-A.

Quantification of P-450s in microsomes: Figures 2 and 3 show that the 405 nm absorbance was linear as a function of the quantity of coated microsomal proteins. In the case of monoclonal antibodies, the linearity was verified up to 0.2 µg protein for PB-induced microsomes and up to 1



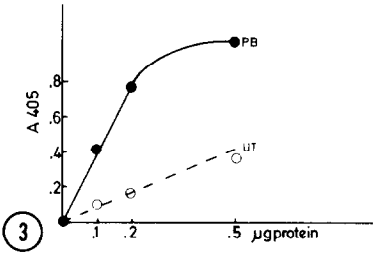


Fig. 2: Reaction of the monoclonal antibody 2A2 (1/5000) with liver microsomal proteins from untreated (UT), PB- or BNF-induced rats.

Fig. 3: Reaction of the anti-P-450 UT-A (1/10,000) antibody with liver microsomal proteins of untreated (UT) and PB-treated (PB) rats.

μg for uninduced and β-naphthoflavone-induced microsomes. When anti-P-450 UT-A was used, the 405 nm absorbance was linear up to 0.5 and 0.3 μg for control and phenobarbital-induced microsomes, respectively. The proportion of coated cytochrome P-450 is identical for the purified isoenzymes and the microsomal fractions because 1) coating was performed under identical conditions in both cases (the 0.2% cholate added to the coating buffer to solubilize the microsomes was also used for purified isoenzymes); 2) efficiency of coating is due mainly to the nature of the proteins; and 3) subsaturating amounts of protein were always used even when expressed in μg of total protein per cm^2 of coated polystyrene area (15).

Table 1 shows the quantification of P-450 PB-B by the three monoclonal antibodies, and the comparison of the results with those obtained by immunoelectrophoresis. The two tests yielded very similar values. Immunoelectrophoresis allows for the verification of the specificty of the results, and hence one can consider that our ELISA results are valuable because they were not significantly different from those obtained by immunoelectrophoresis. Moreover, our results are very close to those previously obtained with phenobarbital, β naphthoflavone or uninduced microsomes (5). These results confirm the validity of our test

Table 1: Quantification of P-450 PB-B by ELISA and immunoelectrophoresis in rat liver microsomes

Induction	Assay	Antibody		
		2 A_2	2 C_2	4 D_6
None	ELISA	0.15±0.12 (11)	0.05±0.02 (10)	0.05±0.03 (10)
	Immunoelectrophoresis	0.1 (2)	0.1 (2)	-
Phenobarbital	ELISA	2.5 ± .6 (9)	1.6 ± .4 (10)	2.4 ± .4 (7)
	Immunoelectrophoresis	2.1 (1)	1.8-2.6 (2)	1.9 (1)
β-Naphthoflavone	ELISA	0.15±0.15 (11)	0.08±0.05 (12)	0.04±0.03 (10)
	Immunoelectrophoresis	0.03-0.05 (2)	0.03 (1)	-

Results are expressed in nmoles P-450 mg protein as means ± S.D. (n).

and the reproducibility of the coating system (7 to 12 experiments were performed)

Polyclonal antibodies can also be used. Figure 3 shows the results obtained with anti-P 450 UT-A. The reaction is linear up to 0.2 (PB-induced) or 0.5 (control) µg of total microsomal proteins. The amount of UT-A is higher in PB (1.1 ± 0.2 nmol x mg prot^{-1}) than in UT A (0.3 ± 0.05 nmol x mg prot^{-1}) microsomes. This fact has been confirmed by immunoelectrophoresis (data not shown). These results contrast with the effect of phenobarbital treatment on levels of P 450 UT-A in other studies (5,16) and can only be attributed to differences in the animals used for these studies.

The limit of detection was 0.03 nmols cytochrome P-450 per mg microsomal protein for both P-450 UT-A and P-450 PB-B. The necessary quantity of microsomal protein ranged from 2 to 15 µg, depending upon the type of microsomes used. Routinely, we used three different concentrations of microsomal proteins and four wells for each assay.

CONCLUSIONS: We have developed and adapted a method for quantifying two P-450 isoenzymes in microsomes. This method is very sensitive and

gave similar results to those obtained with immunoelectrophoresis. It allows for the rapid processing of a large number of determinations. It should be useful for the analysis of small samples such as cultured cells, biopsies, etc. It might also be used for kinetic studies of induction in vivo or with cultured cells. Such studies are now in progress in our laboratory, in parallel with the development of other monoclonal or polyclonal antibodies for the assay of other cytochrome P-450 isoenzymes.

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