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Production of Inhibitory Polyclonal Antibodies against Cytochrome P450s

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Summary: Nine different antibodies against P450 isoforms were prepared using purified cytochrome P450s (P450) expressed in *E. coli*. Purified isozymes were injected into rabbits to raise specific antibody. The resulting antibodies were characterized for their specificity and sensitivity through each particular P450 enzyme-mediated probe reaction.

Anti-CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 antibodies proved to be strong immunoinhibitors with inhibitory effects specific to their corresponding antigen. Antiserum derived from the CYP2C19-immunized rabbits was reacted with CYP2C9 as well as CYP2C19 and immunoabsorbed with membrane-bound CYP2C9 expressed in *E. coli*. Antibody specific for CYP2C19 was obtained. Anti-CYP2C19 together with the anti-CYP2C8 and anti-CYP2C9 can be very useful for determining the contribution of a particular P450 in the metabolism of a drug. The developed inhibitory antibodies will serve as *in vitro*-specific tools for evaluating the quantitative contribution of individual P450 enzymes to drug metabolism.

Key words: human cytochrome P450; polyclonal antibody; inhibition

Introduction

The cytochrome P450 (P450 or CYP) superfamily contributes to the metabolism of xenobiotic chemicals and also endogenous compounds. In the process of drug development, it is important to determine which P450 metabolizes a drug of interest. This is a process referred to as reaction phenotyping. Four approaches *in vitro* (antibody inhibition, chemical inhibition, cDNA-expressed human P450 enzymes, and correlation analysis by measuring the rate of drug metabolism in several samples of human liver) have been developed for reaction phenotyping[1] and it is generally agreed that more than one approach should be chosen to identify which P450 enzyme is responsible for metabolizing a targeted drug.[2] Among these approaches, the use of specific antibodies to inhibit selected P450 enzymatic activity is most recommended. Due to the ability of antibodies to inhibit specifically and noncompetitively, this method alone can establish which P450 enzyme is responsible for biotransforming a drug of interest. Unfortunately, the utility of this method is limited by the availability of specific antibodies. The development of highly selective antibodies for such a purpose is always hampered by the extensive degree of sequence homology found between isozymes belonging to the P450 superfamily.[3]

A number of previous studies have reported the development of antibodies against P450 enzymes using different methods. Antibodies raised against synthetic peptides that represent small regions of an antigen proved to be particularly suitable for immunoblotting studies[4,5] but none were found to be potent inhibitors of P450 activity. Another modified attempt involved the production of inhibitory antibody using synthetic cyclic peptides as antigen.[6] However, this is not a general approach since the principal for determining an effective cyclic peptide, as antigen, still remains undetermined. On the other hand, monoclonal antibodies, which recognize a single epitope, have also been produced and characterized by enzyme-linked immunosorbent assay (ELISA), by immunoblotting, and also by their inhibitory potency toward cDNA-expressed P450 enzymes.[7–15] Shou *et al.*[16] has indicated that many monoclonal antibodies could not distinguish between closely related P450 subfamily members.

To determine the quantitative contribution of a

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particular P450 enzyme to drug metabolism (reaction phenotyping), it would be crucial to develop a panel of selective inhibitory antibodies for the major P450 enzymes, namely CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, involved in drug biotransformation. In the present study, human P450 enzymes were expressed in a bacterial heterologous expression system, purified to homogeneity, and injected into rabbits for production of inhibitory polyclonal antibodies.

Materials and Methods

Materials: 7-Ethoxyresorufin, resorufin, 7-hydroxycoumarin, and coumarin were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). Taxol, 6-hydroxytaxol, diclofenac, 4′-hydroxydiclofenac, (*S*)-mephenytoin, 4-hydroxymephentoin, bufuralol, 1′-hydroxybufuralol, chlorzoxazone, and 6-hydroxychlorzoxazone were purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Testosterone and 6β-hydroxytestosterone were purchased from Wako Pure Chemical (Osaka, Japan). Complete Freund's adjuvant was obtained from Gibco BRL Products (Rockville, Maryland). Emulgen 911 was a gift from Kao Chemical Co. (Tokyo, Japan). Cholic acid was obtained from Nacalai Tesque (Kyoto, Japan). Pooled human liver microsomes (H0610, lot no. 0010154) from 16 individuals were obtained from XenoTech LLC (Kansas City, Kansas). According to the provided datasheet, the P450 enzyme concentration was 409 pmol/mg and the protein concentration was 20 mg/mL. All other chemical and solvents were of the highest grade commercially available.

Expression of P450 enzymes in *Escherichia coli*: CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were each expressed in an *E. coli* expression system as described previously.[17] The expressed proteins from several lots were pooled after cell destruction and stored at -80°C until purification.

Purification of P450 enzymes: P450 enzymes expressed in *E. coli* were solubilized in buffer-A (0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol, 0.2 mM DTT, 0.2 mM EDTA, and 0.25 mM PMSF) containing 0.6% sodium cholate. The solubilization was performed at a concentration of 5 mg protein/mL, and 4°C facilitated by stirring for 30 min, and then the mixture was subjected to centrifugation at 30,000 g for 30 min. The supernatant was collected and applied to an octylamino-Sepharose column (2.0$\times$10 cm) pre-equilibrated with buffer-B (buffer-A containing 0.5% sodium cholate). The column was washed with 3 volumes of buffer-B and the bound protein was eluted with buffer-C (buffer-A containing 0.5% sodium cholate and 0.2% Emulgen 911). The eluted fraction was dialyzed overnight against 80 volumes of buffer-D (20 mM Tris-acetate, pH 7.2, containing 20% glycerol, and 0.2 mM DTT) at 4°C, and it was concentrated by ultrafiltration using Amicon 8050 fitted with a PM30-membrane (exclusion molecular weight, 30,000). The concentrated solution was applied to a DEAE-5PW column (0.75$\times$7.5 cm, Tosoh corp., Tokyo) pre-equilibrated with buffer-E (buffer-D containing 0.4% Emulgen 911). The column was then washed with buffer-E at a flow rate of 1 mL/min until the absorption at 417 nm reduced to the baseline level. Chromatography was performed at room temperature and at a flow rate of 1 mL/min with a linear gradient of sodium acetate from 0 to 0.2 M over 30 min in buffer-E. The elution of P450 enzyme was monitored at 417 nm. The main peak fraction was collected and subjected to further purification with a hydroxylapatite column (0.6$\times$10 cm, Koken, Tokyo) pre-equilibrated with buffer-F (10 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol, 0.2% sodium cholate and 0.2% Emulgen 911). Chromatography was performed at room temperature and at a flow rate of 0.7 mL/min with a linear gradient of sodium phosphate buffer (pH 7.2) from 10 to 350 mM over 50 min in the presence of 20% glycerol, 0.2% sodium cholate, and 0.2% Emulgen 911. To remove Emulgen 911 from each purified P450, the collected fraction was diluted with 4 volumes of 20% glycerol and applied to an open column packed with Bio-Gel HT (1.0$\times$3.0 cm, Bio-Rad Laboratories, Richmond) which had been pre-equilibrated with buffer-G (10 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol and 0.05% sodium cholate). The column was washed with buffer-G until the absorption of Emulgen 911 at 280 nm disappeared. Eventually, the bound P450 enzyme was eluted by buffer-H (350 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol and 0.05% sodium cholate). The purity of each P450 preparation during the purification process was checked by electrophoresis using 10% SDS-polyacrylamide gel and staining with 0.05% Coomassie Blue.

Preparation of antibody: Each purified P450 enzyme was diluted to a protein concentration of 10–50 μg/mL with saline and mixed with an equal volume of complete Freund's adjuvant to produce a homogeneous emulsion. Emulsified antigen was subcutaneously injected into female Japanese White rabbits (2–2.5 kg) obtained from Biotech (Saga, Japan) as described previously.[18] To raise and maintain the production of high titer antiserum, rabbits were then injected intravenously with 10–50 μg of the same P450 dissolved in saline at 2-week intervals after the first immunization. Four weeks after the first booster, we started drawing blood from an ear artery. Every lot of antiserum was verified for its inhibitory potency and specificity using pooled human liver microsomes and selected *E. coli*-expressed P450

enzyme. The specificity of resultant antibodies was also verified using an immunoblotting method with pooled human liver microsomes and purified P450s expressed in *E. coli*.

Purification of anti-CYP2C19 antibody by immunoabsorption: Due to the structural relatedness of CYP2C subfamily enzymes, the raised anti-CYP2C19 may show weak cross reactivity with CYP2C9, which is relatively abundant in human liver microsomes. To improve the inhibitory specificity of the anti-CYP2C19 antibody, we used an immunoabsorption approach as described herein. Every lot of antiserum derived from CYP2C19-immunized rabbits was analyzed for its potency to inhibit human liver microsomal (*S*)-mephenytoin 4′-hydroxylation. Antiserum, which strongly inhibited (*S*)-mephenytoin 4′-hydroxylation, was selected and subjected to immunoabsorption treatment. To minimize the cross reactivity of anti-CYP2C19 toward CYP2C9, one mL of anti-CYP2C19 serum was incubated with the membrane fraction containing 0–200 pmol of CYP2C9 expressed in *E. coli*. The incubation was performed at room temperature and facilitated with 60 min of shaking. After the incubation, the mixture was subjected to ultracentrifugation (105,000 g for 30 min). Nonspecific immunogloblins that bound to the membrane fraction could simply be removed as a pellet after ultracentrifugation. The inhibitory activity of anti-CYP2C19, which had been improved, was retrieved in the supernatant fraction. The specificity of this immunoabsorption-treated anti-CYP2C19 was verified by immunoblotting assay and probe reactions described in the following section.

Characterization of antibody: To characterize the inhibitory potency and specificity of resulting antibodies, 1–20 μL of each antiserum (or immunoabsorption-treated anti-CYP2C19) was added to 450 μL of 0.1 M potassium phosphate buffer, pH 7.4, containing 100 or 200 μg of pooled human liver microsomes or 10 pmol of *E. coli*-expressed CYP1A1 or CYP1A2. The mixture was preincubated for approximately 10 min at room temperature. Reactions were initiated by the addition of a targeted probe substrate followed by 400 μmol of NADPH and were performed at 37°C for 15 to 60 min depending on the probe substrate used. A control reaction was performed by replacing the antiserum with non-immune rabbit serum. Each particular P450 enzyme-mediated probe reaction was performed according to previous reports[19–26] with slight modifications. The inhibitory potency of the antibody was expressed in terms of the percentage of catalytic activity in the presence of immune serum against control activity in the presence of non-immune serum. All determinations were performed in duplicate and data are expressed as the mean.

Reactions were quenched by the addition of acid, alkali solution, ethyl acetate or dichloromethane. For 7-ethoxyresorufin *O*-dealkylation and coumarin 7-hydroxylation, the reactions were quenched by methanol and 4% perchloric acid respectively. The terminated mixtures were centrifuged and supernatant was measured directly using a fluorescence detector as described elsewhere.[19,20] Diclofenac 4′-hydroxylation and bufuralol 1′-hydroxylation are quenched with hydrochloric acid and sodium hydroxide solution respectively and then extracted with ethyl acetate (2–3 mL). Taxol hydroxylation, chlorzoxazone 6′-hydroxylation, and testosterone 6β-hyroxylation; were quenched and extracted with ethyl acetate (2–3 mL). The resulting organic phase was evaporated under reduced pressure at 40°C. The dry residue was then dissolved in mobile phase for HPLC. For (*S*)-mephenytoin 4′-hyroxylation, the quenched reaction mixture was treated with 3 mL of dichloromethane for extraction. The organic phase was evaporated under reduced pressure without heating. The dry residue was dissolved in mobile phase for HPLC.

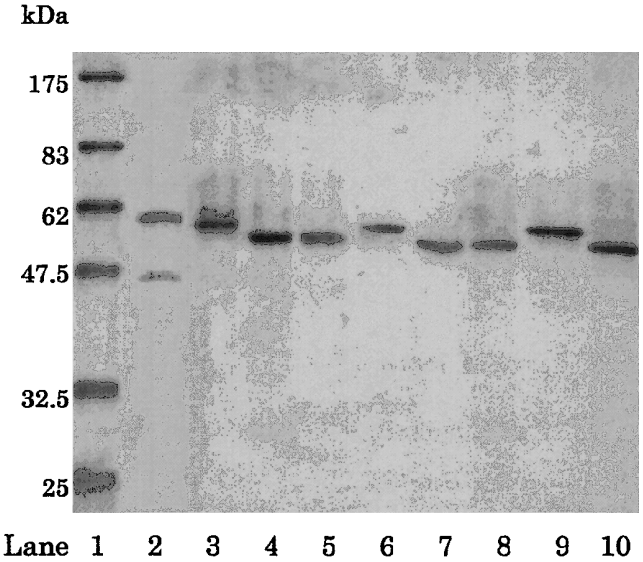

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified P450s expressed in *E. coli*.

Lane 1, protein marker. Lanes 2 to 10, 0.5 pmol of purified cytochrome P450. Lane 2, CYP1A1; lane 3, CYP1A2; lane 4, CYP2A6; lane 5, CYP2C8; lane 6, CYP2C9; lane 7, CYP2C19; lane 8, CYP2D6; lane 9, CYP2E1 and lane 10, CYP3A4.

Results

Purity of the antigen P450s: The cDNA-expressed P450s were purified to homogeneity, as judged by electrophoresis (**Fig. 1**). However, purified CYP1A1 degraded rapidly after purification and during storage. The degraded protein fraction has a lower molecular weight than the intact protein. We found that the degradation continued gradually during storage and the den-

Table 1. Purity of each purified P450

CYP	P450 content (nmol/mL)	Protein content (mg/mL)	Specific content (nmol/mg protein)
1A1	3.27	0.72	4.54
1A2	4.44	0.46	9.65
2A6	6.18	0.42	14.71
2C8	6.92	0.44	15.73
2C9	3.47	0.19	18.26
2C19	6.30	0.62	10.11
2D6	4.87	0.39	12.52
2E1	1.44	0.11	13.09
3A4	2.00	0.17	11.98

sity of the degraded protein band increased during the course of storage (data not shown). The specific content of the purified P450s ranged from 9.65 to 18.26 nmol/mg of protein, except that of CYP1A1 (**Table 1**). These highly purified P450s were used as antigen for antibody production.

Antibody production and characterization: In most cases, the rabbits started to produce high titer inhibitory antibody from 6 to 8 weeks after the primary immunization of antigen, as determined by inhibitory potency assay (**Fig. 2**). Titers of the antibody were well maintained throughout the production schedule if rabbits were boosted sufficiently (10–50 μg/dose) every two or three weeks. Rabbit 1 and rabbit 2 shown in **Fig. 2** immunized with the same amount of purified CYP2C9 as antigen prepared in a similar manner and treated with the same immunization schedule, produced antiserum having similar inhibitory titer. Rabbit 3, which had been immunized with double the amount, produced a relatively higher titer antiserum at week 6. Nevertheless, the inhibitory potency of the antiserum of these three rabbits reached a plateau at week 8. However, further study of the specificity of these antiserum lots showed that the rabbit 1 and rabbit 2 antiserum produced specific inhibition when incubated with pooled human microsomes (**Table 2**). Sera of rabbit 3 had an inhibitory effect not only on the corresponding antigen's reaction (CYP2C9 probe reaction) but also on human liver microsomal (*S*)-mephenytoin 4′-hydroxylation (CYP2C19 probe reaction) and to a lesser extent, taxol 6α-hydroxylation (CYP2C8 probe reaction, data not shown). A similar phenomenon occurred using rabbits selected for producing antibodies for each P450 enzyme. Thus, every lot of serum has been analyzed for its inhibitory potency and specificity.

Anti-CYP2C8, anti-CYP2C9, and anti-CYP2C19 antibodies: Rabbits immunized with CYP2C8 or CYP2C9 were found to produce specific antiserum, the inhibitory effect of which is antigen-specific despite the extensive sequence homology (78% at the amino acid level) between these two antigens. Anti-CYP2C8 potently inhibited human liver microsomal taxol 6α-hydroxylation but showed no inhibitory effect on other probe reactions being investigated (**Fig. 3A**). Anti-CYP2C9 inhibited human liver microsomal diclofenac 4′-hydroxylation, the CYP2C9 probe reaction, in a dose-respon-

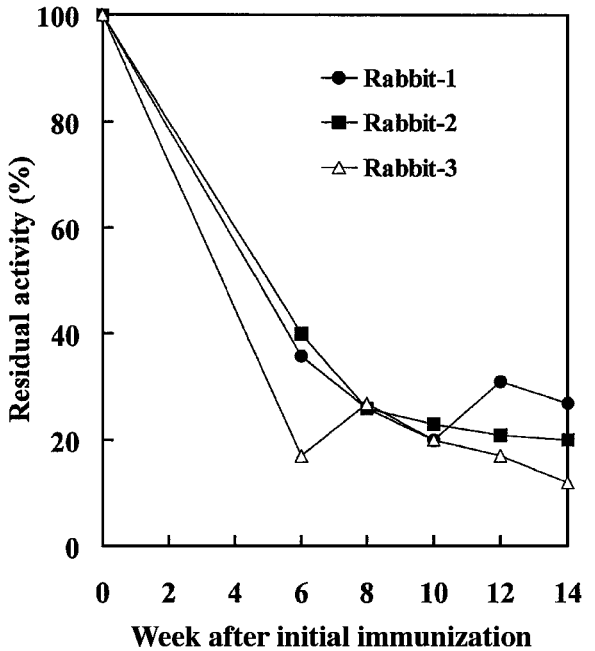

Fig. 2. Inhibitory studies using rabbits sera.

Rabbit 1 (●) and 2 (■) were treated with 10 μg of purified CYP2C9 and rabbit 3 (△) with 20 μg of the corresponding antigen every 2-weeks. For assay, 5 μL of each antiserum was added to 100 μg of pooled human liver microsomes. Incubation was performed in 0.1 M potassium phosphate buffer (pH 7.4), initiated by the addition of 400 μmol NADPH and 200 nmol diclofenac, and was performed for 15 minutes at 37°C. The inhibitory potency of antiserum was measured and expressed in terms of the percentage of catalytic activity in the presence of immune serum against control activity in the presence of non-immune serum.

Table 2. Inhibitory specificity of serum obtained from rabbits immunized with CYP2C9

Rabbit	% Residual activity*	
	Diclofenac 4′-hydroxylation	(*S*)-Mephenytoin 4′-hydroxylation
1	20	71
2	32	62
3	18	14

*: Ten μL of serum was incubated with 100 μg of pooled human liver microsomes and the potency of antiserum to inhibit diflofenac 4′-hydroxylation activity and (*S*)-mephenytoin 4′-hydroxylation activity was determined under the conditions described herein. Each probe reaction was initiated by addition of 400 μmol of NADPH and substrate (200 nmol of diclofenac (CYP2C9), 25 nmol of (*S*)-mephenytoin (CYP2C19)). The inhibitory potency of antiserum was measured and expressed as the percentage of catalytic activity in the presence of immune serum against control activity in the presence of non-immune serum.

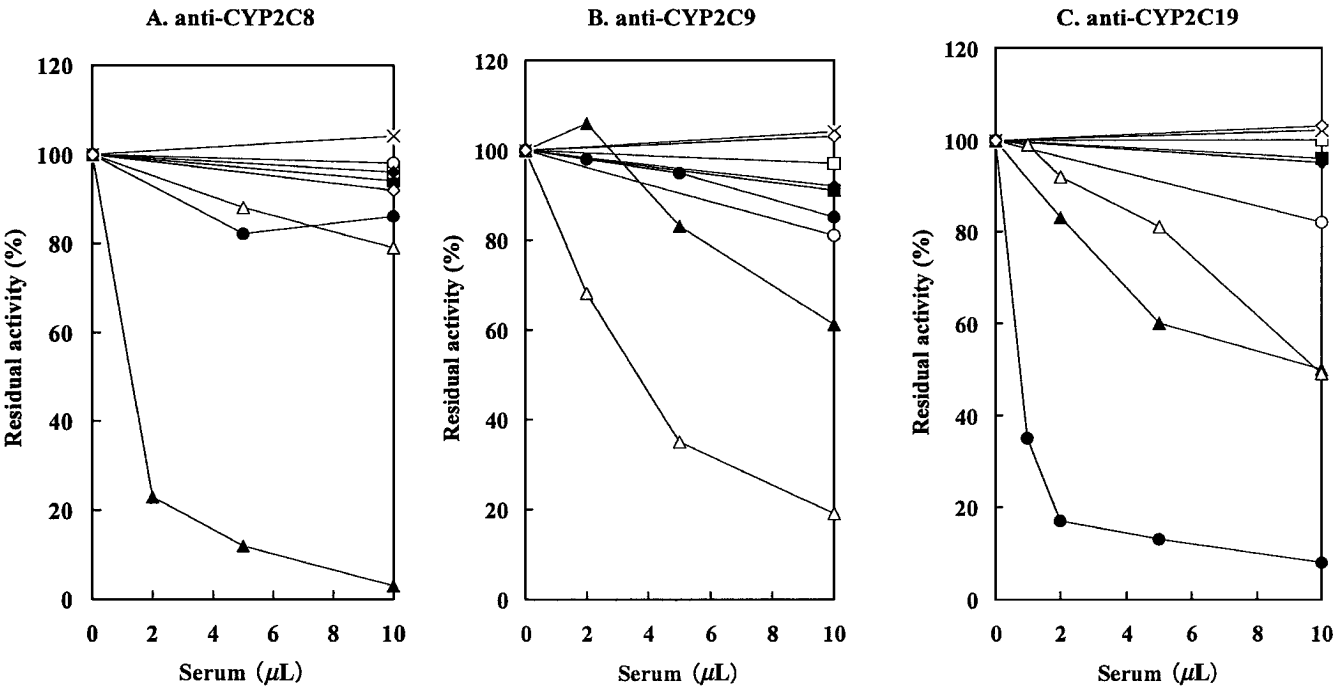

Fig. 3. Inhibitory studies of rabbits sera.

A: specificity of anti-CYP2C8 to inhibit human liver microsomal taxol 6α-hydroxylation. B: specificity of anti-CYP2C9 to inhibit human liver microsomal diclofenac 4′-hydroxylation. C: specificity of anti-CYP2C19 to inhibit human liver microsomal (*S*)-mephenytoin 4′-hydroxylation. Antibody was added to 100 μg of human liver microsomes or 10 pmol of expressed CYP1A1 and pre-incubated for 10 minutes at 37°C. Each probe reaction was initiated by addition of 400 μmol of NADPH and substrate [20 nmol of 7-ethoxyresorufin (CYP1A1, ■ and CYP1A2, □), 1 μmol of coumarin (CYP2A6, ×), 5 nmol of taxol (CYP2C8, ▲), 200 nmol of diclofenac (CYP2C9, △), 25 nmol of (*S*)-mephenytoin (CYP2C19, ●), 20 nmol of bufuralol (CYP2D6, ○), 250 nmol of chlorzoxazone (CYP2E1, ◆), 1 μmol of testosterone (CYP3A4, ◇)]. The inhibitory potency of antiserum was measured and expressed as the percentage of catalytic activity in the presence of immune serum against control activity in the presence of non-immune serum.

sive manner (**Fig. 3B**). When an over-dose of anti-CYP2C9 was added to human liver microsomes (>10 μL/100 μg microsomes), taxol 6α-hydroxylation was weakly inhibited, but the inhibitory level was low and the rest of the tested probe reactions were not unaffected.

CYP2C19-immunized rabbits could not provide antisera with a specific inhibitory effect if the antisera were not immunoabsorbed before use (**Table 3**). Antiserum derived from CYP2C19-immunized rabbits potently inhibited human liver microsomal (*S*)-mephenytoin 4′-hydroxylation (approximately 75% inhibition at 5 μL/100 μg pooled human liver microsomes). However, this antiserum also moderately inhibited human liver microsomal diclofenac 4′-hydroxylation when the serum volume added to human liver microsomes was doubled (10 μL/100 μg pooled human liver microsomes (**Fig. 3C**). A preliminary study was designed to determine the optimum conditions for immunoabsorption of the antiserum. The results showed that 100 pmol of cDNA-expressed CYP2C9 (membrane bound form) together with 1 mL of anti-CYP2C19; provided the best conditions in which most of the non-specific immunoglobulin could be easily removed from the serum. The resultant (post-treated) sera retained most of its inhibitory potency toward human liver microsomal (*S*)-mephenytoin 4′-hydroxylation and the non-specific inhibitory effect toward human liver microsomal diclofenac 4′-hydroxylation was reduced (**Table 3**).

Anti-CYP2C19 was pooled, immunoabsorbed and characterized. Inhibitory specificity of treated anti-CYP2C19 antibody was remarkably increased. Treated anti-CYP2C19 antibody inhibited human liver microsomal (*S*)-mephenytoin 4′-hydroxylation at the concentration of lower than 2 μL/100 μg microsomes (**Fig. 3C**).

Anti-CYP1A1 and anti-CYP1A2 antibodies: For the specificity of antisera collected from CYP1A1 or CYP1A2 immunized-rabbits, the inhibitory effect of antisera on 7-ethoxyresorufin *O*-dealkylation, were determined (**Fig. 4A** and **4B**). Both anti-CYP1A1 and anti-CYP1A2 inhibitory activity appeared to be specific to the corresponding antigen. Anti-CYP1A1 was found to inhibit strongly the 7-ethoxyresorufin *O*-dealkylation activity of cDNA-expressed the CYP1A1. This antibody

Table 3. The effect of immunoabsorption treatment for anti-CYP2C19

CYP2C9 (pmol/mL anti-CYP2C19)	Immunoabsorbed serum (μL)	(*S*)-Mephenytoin 4′-hydroxylation Residual activity (%)		Diclofenac 4′-hydroxylation Residual activity (%)	
		2.5	5	5	10
0		60	24	84	58
50		57	23	87	73
100		61	37	98	93
200		70	44	94	92

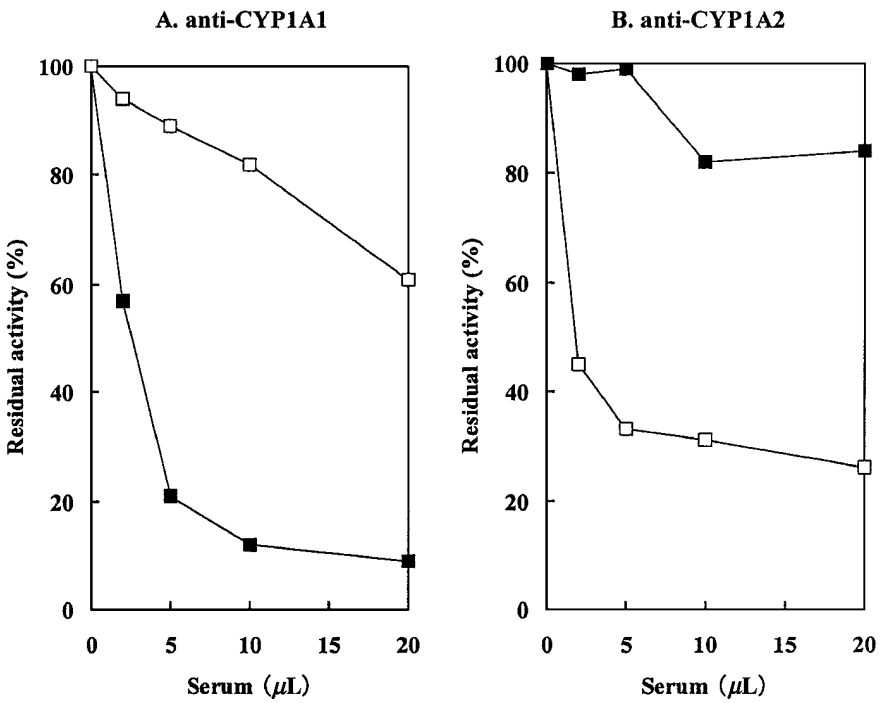

Fig. 4. Inhibitory studies using anti-CYP1A1 and anti-CYP1A2 antibodies.

A: specificity of anti-CYP1A1 verified with its inhibition of *E. coli*-expressed-CYP1A1 (■) and CYP1A2 (□) 7-ethoyresorufin *O*-dealkylation. B: specificity of anti-CYP1A2 verified by its inhibition of *E. coli* expressed-CYP1A1 (■) and CYP1A2 (□) 7-ethoyresorufin *O*-dealkylation. Antibody was added as indicated to 10 pmol of CYP1A1 or CYP1A2 and preincubated for 10 minutes at 37°C. Each probe reaction was initiated by addition of 400 μmol of NADPH and 20 nmol of 7-ethoxyresorufin. The inhibitory potency of antiserum was measured and expressed in terms of the percentage of catalytic activity in the presence of immune serum against control activity in the presence of non-immune serum.

was, if overdosed ($>10\,\mu$L serum/10 pmol of P450), acted to a much lesser extent to inhibit CYP1A2, which has the highest sequence homology with CYP1A1. Whereas, anti-CYP1A2 markedly inhibited the CYP1A2 activity expressed in *E. coli* but did not inhibit CYP1A1 enzymatic activity (**Fig. 4B**). These two antibodies were further characterized by analyzing the inhibitory specificity in pooled human liver microsomes (**Fig. 5A** and **5B**). The anti-CYP1A2 inhibitory profile was shown to be specific to antigen (**Fig. 5B**). Anti-CYP1A2 specifically inhibited human liver microsomal 7-ethoxyresorufin *O*-dealkylation activity and did not interfere with any other P450 probe reactions being investigated. Anti-CYP1A1, whose inhibitory effect appeared to be CYP1A1-specific, was found not only to inhibit CYP1A1-mediated activity but also to inhibit strongly human liver microsomal taxol 6α-hydroxylation (CYP2C8 probe reaction). Human liver microsomal 7-ethoxyresorufin *O*-dealkylation, which is mediated by hepatic available CYP1A2, was not inhibited by anti-CYP1A1.

Anti-CYP2A6, anti-CYP2D6, anti-CYP2E1, and anti-CYP3A4 antibody: Rabbits immunized with purified CYP2A6, CYP2D6, CYP2E1, and CYP3A4 were found to produce specific antibodies. Anti-CYP2A6 potently inhibited human liver microsomal coumarin 7-hydroxylation and showed no inhibitory effect toward other probe reactions (**Fig. 6A**). Anti-CYP2D6 specifically inhibited human liver microsomal bufuralol hydroxylation with no inhibitory effect on

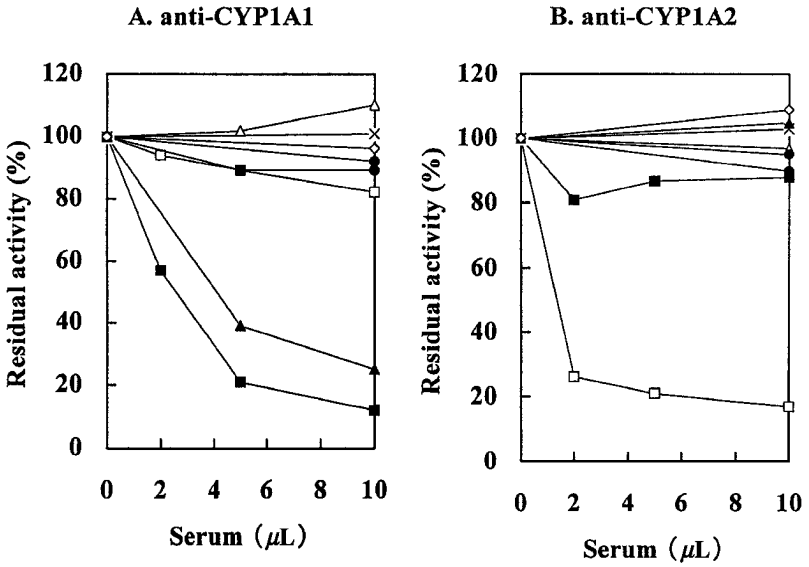

Fig. 5. Inhibitory studies using anti-CYP1A1 and anti-CYP1A2 antibodies.

A: specificity of anti-CYP1A1 to inhibit human liver microsomal 7-ethoyresorufin *O*-dealkylation. B: specificity of anti-CYP1A2 to inhibit human liver microsomal 7-ethoyresorufin *O*-dealkylation. Antibody was added to 100 μg of human liver microsomes or 10 pmol of expressed CYP1A1 and pre-incubated for 10 minutes at 37°C. Each probe reaction was initiated by addition of 400 μmol of NADPH and substrate [20 nmol of 7-ethoxyresorufin (CYP1A1, ■ and CYP1A2, □), 1 μmol of coumarin (CYP2A6, ×), 5 nmol of taxol (CYP2C8, ▲), 200 nmol of diclofenac (CYP2C9, △), 25 nmol of (*S*)-mephenytoin (CYP2C19, ●), 20 nmol of bufuralol (CYP2D6, ○), 250 nmol of chlorzoxazone (CYP2E1, ◆), 1 μmol of testosterone (CYP3A4, ◇)]. The inhibitory potency of antiserum was measured and expressed as the percentage of catalytic activity in the presence of immune serum against control activity in the presence of non-immune serum.

other probe reactions (**Fig. 6B**). The specificity of anti-CYP2E1 was also found to be high, and it inhibited human liver microsomal chlorzoxazone 6′-hydroxylation specifically (**Fig. 6C**). A relatively high percentage of uninhibited chlorzoxazone 6′-hydroxylation activity was observed, suggesting the contribution of other P450s. Anti-CYP3A4 showed a specific inhibitory effect toward human liver microsomal testosterone 6β-hydroxylation and no inhibition of other probe reactions investigated (**Fig. 6D**).

Discussion

From our previous study on antibody production, we realized that boosting rabbits with 100 μg/dose or more of purified antigen might result in a high titer but low specificity antibody (unpublished data). On the other hand, boosting rabbits with 50 μg/dose or less of purified antigen usually resulted in a sufficiently high titer and specific antibody. Our present study as shown in **Table 2** reconfirmed that rabbits boosted with lower dose (25 μg/dose) of antigen produced specificity antibody but rabbit boosted with higher dose (50 μg/dose) failed eventually. This result indicated that specificity of raised antibody is not solely depending on the purity of antigen but also the dosage of antigen applied during the production course.

By immunizing rabbits with purified antigens at concentrations from 10 to 50 μg/dose, we have successfully developed specific antibodies against major human P450s. We also learned that specific pathogen-free animals maintained in a clean environment during the course of antibodies production provided a better opportunity for obtaining specific antibodies, compared with those conventionally raised and maintained.

Using a heterologous expression system in *E. coli*, we expressed 9 major human P450 enzymes and purified them. We believe the preparation of a purified P450 enzyme, which maintains its stereo-conformation, is important for raising antibody that precisely recognizes the structural conformation and can inhibit the enzymatic activity of the corresponding antigen. In a previous attempt, in which denatured P450 was injected into rabbits, raised antibodies did not show potent inhibition (data not shown). Therefore, we believe that the injection of P450 enzymes that maintains their stereo-conformation is a crucial factor when raising inhibitory antibodies. Belloc *et al.* found that antibodies raised by purified P450s without typical CO-difference that recognized the respective antigens in immunoblotting but showed no inhibitory effect against the antigen's enzymatic activity in most cases.[27]

Except anti-CYP1A1 and anti-CYP2C19, all the resultant antibodies recognized a single protein band in human liver microsomes that showed the same mobility as the respective cDNA-expressed P450.

Nevertheless, the cross reactivity of anti-CYP1A1

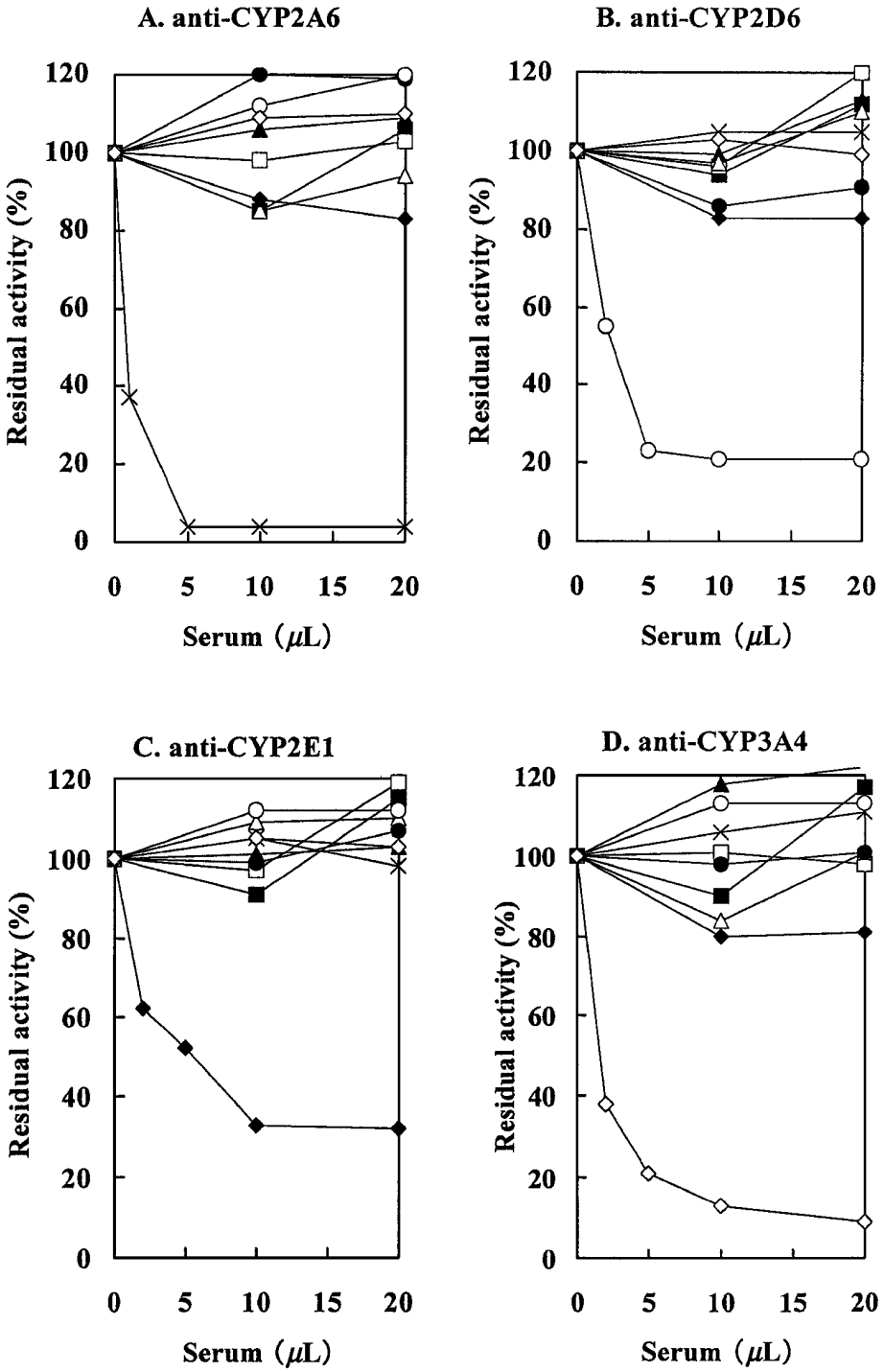

Fig. 6. Inhibitory studies of rabbit sera.

A: specificity of anti-CYP2A6 to inhibit human liver microsomal coumarin 7-hydroxylation. B: specificity of anti-CYP2D6 to inhibit human liver microsomal bufuralol 1′-hydroxylation. C: specificity of anti-CYP2E1 to inhibit human liver microsomal chlorzoxazone 6′-hydroxylation. D: specificity of anti-CYP3A4 to inhibit human liver microsomal testosterone 6β-hydroxylation. Antibody was added to 100 μg of human liver microsomes or 10 pmol of expressed CYP1A1 and pre-incubated for 10 minutes at 37°C. Each probe reaction was initiated by addition of 400 μmol of NADPH and substrate [20 nmol of 7-ethoxyresorufin (CYP1A1, ■ and CYP1A2, □), 1 μmol of coumarin (CYP2A6, ×), 5 nmol of taxol (CYP2C8, ▲), 200 nmol of diclofenac (CYP2C9, △), 25 nmol of (*S*)-mephenytoin (CYP2C19, ●), 20 nmol of bufuralol (CYP2D6, ○), 250 nmol of chlorzoxazone (CYP2E1, ◆), 1 μmol of testosterone (CYP3A4, ◇)]. The inhibitory potency of antiserum was measured and expressed as the percentage of catalytic activity in the presence of immune serum against control activity in the presence of non-immune serum.

Table 4. Inhibitory specificity of the developed antibodies

Antibodies	CYP1A1	CYP1A2	CYP2A6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4
anti-CYP1A1	***	—	—	***	—	—	—	—	—
anti-CYP1A2	—	***	—	—	—	—	—	—	—
anti-CYP2A6	—	—	***	—	—	—	—	—	—
anti-CYP2C8	—	—	—	***	—	—	—	—	—
anti-CYP2C9	—	—	—	*	***	—	—	—	—
anti-CYP2C19	—	—	—	*	*	***	—	—	—
anti-CYP2D6	—	—	—	—	—	—	***	—	—
anti-CYP2E1	—	—	—	—	—	—	—	***	—
anti-CYP3A4	—	—	—	—	—	—	—	—	***

*** Strong inhibition.
* Weak cross reaction observed when an overdose of antibody was applied.
— Inhibitory effect not detected.

toward CYP2C8 remains undefined. We assume that anti-CYP1A1 will be used as a tool for distinguishing CYP1A1 from CYP1A2 but not CYP2C8. If Anti-CYP1A1 applied for reaction phenotyping inhibited a tested reaction, we would have to use anti-CYP2C8 to set up a parallel inhibition test in order to find out which P450 is responsible for the reaction. Moreover, the cross reactivity of raised antibodies were not yet examined with other minor P450 isozymes, including CYP2B6, CYP3A5 and CYP3A7. To develop antibody for other P450 isozymes and to check the cross reactivity of the raised antibodies on other P450 are parts of our future studies.

In conclusion, these inhibitory antibodies will serve as useful tools for evaluating the contribution of major individual P450 enzymes to drug metabolism.

References

1) Wrighton, S. A., Vandenbranden, M., Stevens, J. C., Shipley, L. A., Ring, B. J., Rettie, A. E. and Cashman J. R.: *In vitro* methods for assessing human hepatic drug metabolism: their use in drug development. *Drug Metab. Rev.*, **25**: 453–484 (1993).
2) Tucker, G. T., Houston, J. B. and Huang, S. M.: Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential-toward a consensus. *Clin. Pharmacol. Ther.*, **70**: 103–114 (2001).
3) Romkes, M., Faletto, M. B., Blaisdell, J. A., Raucy, J. L. and Goldstein, J. A.: Cloning and expression of complementary DNAs for multiple members of the human cytochrome P450IIC subfamily. *Biochemistry*, **30**: 3247–3255 (1991).
4) Edwards, R. J., Adams, D. A., Watts, P. S., Davies, D. S. and Boobis, A. R.: Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. *Biochem. Pharmacol.*, **56**: 377–387 (1998).
5) Tang, Y. M., Chen, G. F., Thompson, P. A., Lin, D. X., Lang, N. P. and Kadlubar, F. F.: Development of an antipeptide antibody that binds to the C-terminal region of human CYP1B1. *Drug Metab. Dispos.*, **27**: 274–280 (1999).
6) Schulz-Utermoehl, T., Edwards, R. J. and Boobis, A. R.: Affinity and potency of proinhibitory antipeptide antibodies against CYP2D6 is enhanced using cyclic peptides as immunogens. *Drug Metab. Dispos.*, **28**: 544–551 (2000).
7) Fujino, T., Park, S. S., West, D. and Gelboin, H. V.: Phenotyping of cytochromes P-450 in human tissues with monoclonal antibodies. *Proc Natl. Acad. Sci. USA*, **79**: 3682–3686 (1982).
8) Gelboin, H. V., Goldfarb, I., Krausz, K. W., Grogan, J., Korzekwa, K. R., Gonzalez, F. J. and Shou, M.: Inhibitory and noninhibitory monoclonal antibodies to human cytochrome P450 2E1. *Chem. Res. Toxicol.*, **9**: 1023–1030 (1996).
9) Gelboin, H. V., Krausz, K. W., Goldfarb, I., Buters, J. T., Yang, S. K., Gonzalez, F. J., Korzekwa, K. R. and Shou, M.: Inhibitory and non-inhibitory monoclonal antibodies to human cytochrome P450 3A3/4. *Biochem. Pharmacol.*, **50**: 1841–1850 (1995).
10) Gelboin, H. V., Krausz, K. W., Shou, M., Gonzalez, F. J. and Yang, T. J.: A monoclonal antibody inhibitory to human P450 2D6: a paradigm for use in combinatorial determination of individual P450 role in specific drug tissue metabolism. *Pharmacogenetics*, **7**: 469–477 (1997).
11) Park, S. S., Waxman, D. J., Lapenson, D. P., Schenkman, J. B. and Gelboin, H. V.; Monoclonal antibodies to rat liver cytochrome P-450 2c/RLM5 that regiospecifically inhibit steroid metabolism. *Biochem. Pharmacol.*, **38**: 3067–3074 (1989).
12) Sai, Y., Yang, T. J., Krausz, K. W., Gonzalez, F. J. and Gelboin, H. V.: An inhibitory monoclonal antibody to human cytochrome P450 2A6 defines its role in the metabolism of coumarin, 7-ethoxycoumarin and 4-nitroanisole in human liver. *Pharmacogenetics*, **9**: 229–237 (1999).
13) Yang, T. J., Krausz, K. W., Shou, M., Yang, S. K., Buters, J. T., Gonzalez, F. J. and Gelboin, H. V.: Inhibitory monoclonal antibody to human cytochrome P450 2B6. *Biochem. Pharmacol.*, **55**: 1633–1640 (1998).
14) Yang, T. J., Sai, Y., Krausz, K. W., Gonzalez, F. J. and Gelboin, H. V.: Inhibitory monoclonal antibodies to human cytochrome P450 1A2: analysis of phenacetin *O*-deethylation in human liver. *Pharmacogenetics*, **8**:

375–382 (1998).
15) Krausz, K. W., Goldfarb, I., Buters, J. T., Yang, T. J., Gonzalez, F. J. and Gelboin, H. V.: Monoclonal antibodies specific and inhibitory to human cytochromes P450 2C8, 2C9, and 2C19. *Drug Metab. Dispos.*, **29**: 1410–1423 (2001).
16) Shou, M., Lu, T., Krausz, K. W., Sai, Y., Yang, T., Korzekwa, K. R., Gonzalez, F. J. and Gelboin, H. V.: Use of inhibitory monoclonal antibodies to assess the contribution of cytochromes P450 to human drug metabolism. *Eur. J. Pharmacol.*, **394**: 199–209 (2000).
17) Iwata, H., Fujita, K., Kushida, H., Suzuki, H. A., Konno, Y., Nakamura, K., Fujino, A. and Kamataki, T.: High catalytic activity of human cytochrome P450 co-expressed with human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Biochem. Pharmacol.*, **55**: 1315–1325 (1998).
18) Imaoka, S., Terano, Y. and Funae, Y.: Changes in the amount of cytochrome P450s in rat hepatic microsomes with starvation. *Arch. Biochem. Biophys.*, **278**: 168–178 (1990).
19) Burke, M. D. and Mayer, R. T.: Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450-binding of phenoxazone and a homologous series of its n-alkyl ethers (alkoxyresorufins). *Chem. Biol. Interact.*, **45**: 243–258 (1983).
20) Pearce, R., Greenway, D. and Parkinson, A.: Species differences and interindividual variation in liver microsomal cytochrome P450 2A enzymes: effects on coumarin, dicumarol, and testosterone oxidation. *Arch. Biochem. Biophys.*, **298**: 211–225 (1992).
21) Rahman, A., Korzekwa, K. R., Grogan, J., Gonzalez, F. J. and Harris, J. W.: Selective biotransformation of taxol to 6 alpha-hydroxytaxol by human cytochrome P450 2C8. *Cancer Res.*, **54**: 5543–5546 (1994).
22) Mancy, A., Antignac, M., Minoletti, C., Dijols, S., Mouries, V., Duong, N. T., Battioni, P., Dansette, P. M. and Mansuy, D.: Diclofenac and its derivatives as tools for studying human cytochromes P450 active sites: particular efficiency and regioselectivity of P450 2Cs. *Biochemistry*, **38**: 14264–14270 (1999).
23) Goldstein, J. A., Faletto, M. B., Romkes-Sparks, M., Sullivan, T., Kitareewan, S., Raucy, J. L., Lasker, J. M. and Ghanayem, B. I.: Evidence that CYP2C19 is the major (*S*)-mephenytoin 4′-hydroxylase in humans. *Biochemistry*, **33**: 1743–1752 (1994).
24) Kronbach, T., Mathys, D., Gut, J., Catin, T. and Meyer, U. A.: High-performance liquid chromatographic assays for bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan *O*-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. *Anal Biochem.*, **162**: 24–32 (1987).
25) Peter, R., Bocker, R., Beaune, P. H., Iwasaki, M., Guengerich, F. P. and Yang, C. S.: Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem. Res. Toxicol.*, **3**: 566–573 (1990).
26) Arlotto, M. P., Trant, J. M. and Estabrook, R. W.: Measurement of steroid hydroxylation reactions by high-performance liquid chromatography as indicator of P450 identity and function. *Methods Enzymol.*, **206**: 454–462 (1991).
27) Belloc, C., Baird, S., Cosme, J., Lecoeur, S., Gautier, J. C., Challine, D., de Waziers, I., Flinois, J. P. and Beaune, P. H.: Human cytochromes P450 expressed in Escherichia coli: production of specific antibodies. *Toxicology*, **106**: 207–219 (1996).