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CYTOCHROME P450 2C9 IS RESPONSIBLE FOR HYDROXYLATION OF THE NAPHTHOQUINONE ANTIMALARIAL DRUG 58C80 IN HUMAN LIVER

RICHARD J. WEAVER, MAURICE DICKINS* and M. DANNY BURKE†

Department of Biomedical Sciences, University of Aberdeen, Marischal College, Aberdeen AB9 1AS; and *Department of Bioanalysis and Drug Metabolism, The Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, U.K.

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Abstract—2-(4-*t*-Butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone (58C80) is an experimental naphthoquinone antimalarial drug which undergoes extensive alky hydroxylation in man. By means of purification, N-terminal amino acid sequencing and inhibition by antibodies and sulfaphenazole, we have identified the form of cytochrome P450 primarily responsible for 58C80 hydroxylation in human liver, P450hB$_{20-27}$, to be a member of the P450 2C9 subfamily. P450hB$_{20-27}$ is a low-spin haemoprotein with molecular mass 54 kDa. 58C80 hydroxylation in human liver microsomes was dependent on either NADPH or NADH, with the activity supported by NADH being 35% of that supported by NADPH. With purified P450hB$_{20-27}$ cytochrome b_5 stimulated the NADH-dependent activity 8-fold but inhibited the NADPH-dependent activity by 30%. 58C80 is a novel substrate structure for human P450 2C and these results significantly broaden the range of drugs which have been directly shown (i.e. using a purified enzyme as opposed to expressed cDNA) to be metabolized by human P450 2C forms that are incontrovertibly expressed in human liver *in vivo*.

2-(4-*t*-Butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone (codenamed 58C80;‡ Fig. 1) is a novel antimalarial compound which is extensively metabolized in man by hydroxylation of the *t*-butyl sidechain. Approximately 85% of a 58C80 dose is excreted as the *t*-butyl hydroxylated metabolite (codenamed 298C80) and its glucuronide in urine over 24 hr [1]. Initial experiments (unpublished) indicated that 58C80 hydroxylation is catalysed by cytochrome P450, which is a large gene superfamily of haemoproteins comprising many different isoenzymic forms [2, 3].§ Individual P450 forms differ in substrate specificity, susceptibility to inhibitors and genetic regulation, with certain forms exhibiting inducibility or polymorphism in man. Since these P450 characteristics can have a major influence on drug interactions and adverse drug reactions, it was deemed important to identify and characterize the form of human P450 which is responsible for 58C80 hydroxylation in man.

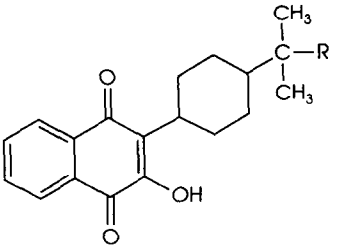

Fig. 1. Chemical structures of 58C80 and metabolite 298C80. R = CH_3 in 58C80; R = CH_2OH in 298C80.

MATERIALS AND METHODS

Materials. Emulgen 911 was generously supplied by the Kao Atlas Corporation (Tokyo, Japan). Alkoxyresorufins were synthesized as described previously [4]. The drug 58C80, authentic *t*-butyl hydroxylated metabolite (298C80) and [^{14}C]58C80 were synthesized in The Wellcome Research Laboratories (Beckenham, U.K.). *S*-Mephenytoin and 4′-hydroxy-*S*-mephenytoin were kindly provided by Dr M. Lennard, Sheffield University. Sulfaphenazole was a generous gift from Ciba-Geigy AG (Basel, Switzerland). All other materials were obtained from the usual commercial biochemical suppliers.

Animals and pretreatments. Adult, 200–250 g male and female Sprague–Dawley rats, bred in the

† Corresponding author.

‡ Abbreviations: AROD, alkoxyresorufin *O*-dealkylase; BROD, benzyloxyresorufin *O*-dealkylase; BSA, bovine serum albumin; CsA-met, cyclosporin A metabolism; CHP, cumene hydroperoxide; COH, coumarin 7-hydroxylase; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; EROD, ethoxyresorufin *O*-deethylase; HAP, hydroxylapatite; MPOH, *S*-mephenytoin 4′-hydroxylation; MROD, methoxyresorufin *O*-dealkylase; PB, phenobarbital; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonylfluoride; PROD, pentoxyresorufin *O*-dealkylase; TBOH, tolbutamide hydroxylation; 58C80, 2-(4-*t*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone; 58OH, 58C80 hydroxylase; 3MC, 3-methylcholanthrene.

§ The nomenclature used for cytochrome P450 is one of those suggested by Nelson DR *et al.*, The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. *DNA Cell Biol* **12**: 1–51, 1993.

University of Aberdeen and fed Oxoid breeding diet and water *ad lib.*, were acclimatized for 1 week prior to death or treatment with either phenobarbitone (PB, 0.1% w/v in drinking water for 5 consecutive days) or 3-methylcholanthrene (3MC, 1% w/v in olive oil, 80 mg/kg i.p. once). Rats were killed 24 hr after the final treatment with PB or 72 hr after the single dose of 3MC.

Liver microsome preparation. Human livers were removed with permission from renal transplant donors at Aberdeen Royal Infirmary within 30 min of circulatory arrest into ice-cold isotonic saline, chopped into approximately 1–2 cm cubes, frozen at −80° within 1 hr and stored at −80° until use. Rat livers were excised immediately upon death of the animal from cervical dislocation into ice-cold 10 mM sodium–potassium phosphate buffer (NaKPi), pH 7.6, containing 1.15% KCl, while human livers were first thawed at room temperature and then placed in this buffer. All subsequent procedures for preparing microsomes were carried out on ice or at 4°. Livers were weighed in the above buffer and microsomes prepared essentially as described previously [5] with the following modifications: livers were homogenized using an Ultra-Turrax homogenizer (type TP18/2, Janke and Kunkel AG, Staufen Breisgau, Germany), instead of a teflon-glass Potter–Elvejhem homogenizer due to the fibrous nature of human liver, for a maximum of 25–30 sec at medium speed (to minimize enzyme denaturation); the solutions used to wash and homogenize the liver and to wash the initial microsomal pellet were buffered with 0.1 M Tris (since haemoglobin contamination of human microsomes is much lower using Tris than Pi) and contained 20% glycerol (which specifically prevents denaturation of cytochrome P450 to P420); the final microsomal pellet was resuspended at high protein concentration, approximately 20 mg protein/mL (0.5 mL 10 mM NaKPi, pH 7.6, containing 20% glycerol/g liver weight), to improve enzyme stability during storage. When preparing microsomes to be used for purifying P450 or other enzymes, 0.1 mM phenylmethylsulphonylfluoride (PMSF) was added freshly to the liver washing and homogenization buffers to minimize proteolysis.

Purification of human cytochrome P450hB$_{20-27}$. Human P450 was purified by column chromatography at 4° by a modification of a method used previously to purify human P450 3A [6]. Large and small (the latter less than 8 mL packed stationary phase) columns were loaded and eluted at 55 and 30 mL/hr, respectively. Column effluents were monitored continuously for absorbance at 280 nm (for protein) and 405 nm (for haem), whilst individual and/or pooled eluted fractions were analysed for protein, total P450, 58C80 hydroxylase activity, methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin *O*-dealkylase (MROD, EROD, PROD and BROD) activities, by ELISA using antibodies against rat P450 1A1, rat P450 2B1 and human P450 3A, respectively (to detect the presence of these P450 subfamilies), and by SDS–PAGE (for assessing the presence and relative purity of proteins with molecular mass in the range 45–60 kDa, which is characteristic of P450), as detailed below. Individual contiguous fractions showing similar attributes on analysis as above were pooled. From the second stage of the purification onward, only fractions showing a combination of high 405 nm absorbance, the presence of P450, high 58C80 hydroxylase activity and increased purity of 45–60 kDa proteins on SDS–PAGE were carried forward for further purification. Fractions were concentrated and dialysed as necessary using Amicon stirred cells with PM10 membranes. All percentage concentrations were w/v unless shown otherwise.

Microsomal suspensions from liver Nos 20 and 27 were combined, solubilized at approximately 20 mg protein/mL by the addition of a 20% aqueous solution of CHAPS detergent to a final concentration of 0.6% and slow end-over-end rotation in a sealed tube at 4° for 20 min, then centrifuged at 180,000 *g* for 50 min. The supernatant, containing 2414 nmol P450 at 0.37 nmol P450/mg protein, was diluted to 20 mg protein/mL in buffer A [100 mM KPi, pH 7.25, containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.1 mM PMSF] and loaded onto an 88 × 4.5 cm column of *n*-octylamine-Sepharose CL4B equilibrated with buffer B (buffer A containing in addition 0.35% cholate). The total column loading was 6 mg sample protein/mL Sepharose, ensuring that the bound sample occupied no more than the upper third of the column. The column was washed with buffer B, then in three consecutive steps with buffer B containing 0.08%, 0.16% and 0.5% Emulgen, and finally with buffer B containing 0.15% deoxycholate.

The fractions eluting in 0.16% Emulgen, which were combined as pool e (Fig. 3), were dialysed against buffer C (10 mM Tris, pH 7.8, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT and 0.05% CHAPS), concentrated from 1.7 L to 60 mL and loaded onto a 47 × 2.5 cm column of Q-Sepharose pre-equilibrated with buffer D (buffer C containing in addition 0.1% Emulgen). The column was washed with buffer D then with a linear 0–400 mM KCl gradient in buffer D.

The fractions eluting in the column wash, which were combined as pool b (Fig. 4), were dialysed against buffer E (10 mM KPi, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.05% CHAPS and 0.1% Emulgen), concentrated from 110 to 8 mL and loaded onto an 8 × 1.25 cm column of hydroxylapatite (HAP, Bio-gel HTP) pre-equilibrated with buffer E. The column was washed with buffer E, then with a stepped 0–200 mM KPi gradient in buffer E.

The fractions eluting in 200 mM KPi, which were combined as pool d (Fig. 5), were dialysed against buffer F (10 mM KPi, pH 7.0, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1% Emulgen and 0.05% CHAPS), concentrated from 30 to 8 mL and loaded onto a 2 × 1 cm column of S-Sepharose pre-equilibrated with buffer F. The column was washed with buffer F then with a linear 0–250 mM KCl gradient in buffer F.

The fractions eluting in 30–45 mM KCl were pooled, dialysed against buffer G (5 mM KPi, pH 7.7, containing 20% glycerol, 0.1 mM EDTA and 0.1 mM DTT), concentrated from 18 to 4 mL and loaded onto a 3 × 1.25 cm column of HAP (Bio-

gel HTP) pre-equilibrated with buffer G. The column was washed with buffer G containing 0.1% cholate, to remove Emulgen from the sample (monitored by A_{280}), then the purified P450 ($P450hB_{20-27}$) was eluted as a tight band with 400 mM KPi buffer, pH 7.7, containing 20% glycerol, 0.1 mM EDTA and 0.1 mM DTT, dialysed against 10 mM KPi buffer, pH 7.7, containing 20% glycerol and stored at −80°.

Purification of human NADPH-cytochrome c reductase and cytochrome b_5. NADPH-cytochrome *c* reductase was purified from human liver microsomes by a modification of the method of Yasukochi and Masters [7]. The same procedure as for the purification of $P450hB_{20-27}$ was followed through the octylamine-Sepharose column stages and the bulk of NADPH-cytochrome *c* reductase activity and cytochrome b_5 were eluted in Buffer B containing 0.15% deoxycholate. 2′,5,-ADP Sepharose was thoroughly washed on a filter with, successively, distilled water and six times the suspension volume of 6 M urea containing 2 M NaCl and 1 mM EDTA, packed into a 10 × 1 cm column and equilibrated with buffer H (10 mM KPi, pH 7.7, containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 0.4 mM PMSF and 2 μM FMN). The fractions recovered in 0.15% deoxycholate from the octylamine-Sepharose column were pooled, dialysed against buffer I (10 mM KPi, pH 7.7, containing 20% glycerol, 2 μM FMN and 0.1% Emulgen) and loaded onto the 2′,5,-ADP Sepharose column. The column was washed with buffer I, then cytochrome b_5 was eluted with buffer J (30 mM KPi, pH 7.7, containing 20% glycerol, 2 μM FMN and 0.15% deoxycholate) as a single protein with molecular mass = 17.5 kDa on SDS–PAGE and no residual P450 or NADPH-cytochrome *c* reductase activity. Finally NADPH-cytochrome *c* reductase was eluted in buffer K (30 mM KPi, pH 7.7, containing 5 mM 2′-ADP and 2 μM FMN) as a single protein with molecular mass = 78 kDa on SDS–PAGE, a specific activity (following dialysis as below) of 68 μmol cytochrome *c* reduced/min/mg protein and containing no residual P450 or cytochrome b_5. The purified NADPH-cytochrome *c* reductase and cytochrome b_5 were each extensively dialysed against 10 mM KPi, pH 7.6, containing 20% glycerol, 0.1 mM EDTA and 0.1 mM DTT to remove detergent and stored at −80°.

Purification of rat P450. Cytochromes P450 1A1 and P450 1A2 were each purified from 3MC-treated rat liver microsomes, while P450 2B1, P450 2C6 and P450 3A1/2 (an approximately equimolar mixture of the similar forms, P450 3A1 and P450 3A2, as judged from N-terminal sequence analysis), were each purified from PB-treated rat liver microsomes, using essentially the method used to purify human $P450hB_{20-27}$ (above). During purification P450 1A1, P450 1A2 and P450 2B1 were monitored by their characteristic profiles of MROD, EROD, PROD and BROD activities [8], while P450 1A1, P450 1A2 and P450 3A1/2 were also monitored immunochemically using monoclonal antibodies raised, respectively, as described elsewhere [9], to the purified P450 1A1 (antibody RM1) and to a previously purified human P450 3A form, P450hA7 [6] (antibody HL3). The identities of the purified P450s were confirmed by N-terminal amino acid sequence analysis as described previously [6], with the modification that individual protein bands, separated by SDS–PAGE and electroblotted onto Immobilon membranes, were cut out and individually sequenced in an Applied Biosystems 470A protein sequencer, as described elsewhere [10]. Purified P450 1A1, P450 1A2, P450 2B1, P450 2C6 and P450 3A1/2 each gave single protein bands on SDS–PAGE, with molecular mass = 56, 54, 53, 51 and 52 kDa, respectively, and specific contents of 16.6–19.3 nmol/mg protein.

Analytical procedures. Microsomal protein was measured according to Lowry *et al.* [11], but the protein concentration of column fractions and purified enzymes was measured by the method of Shaffner and Wiseman [12], which we have found to be unaffected by glycerol, detergents and other components of solubilization buffers [6]. Cytochromes P450 and b_5 were measured according to Omura and Sato [13] and Strittmatter *et al.* [14], respectively. NADPH-cytochrome *c* reductase activity at 37° was measured as described by Phillips and Langdon [15] (1 U of activity reduces 1 μmol cytochrome *c*/min).

Microsomal NADPH-dependent alkoxyresorufin *O*-dealkylase (NADPH-AROD) activities were measured at 37° using the kinetic fluorimetric method of Burke *et al.* [8]. Cumene hydroperoxide-dependent AROD (CHP-AROD) activities were measured in the same way, except that 250 μM NADPH was replaced by 75 μM CHP [10 μL 15 mM CHP in dimethyl sulphoxide (DMSO)/2 mL reaction] and the substrate concentration was 12.5 μM (25 μL 1 mM substrate in DMSO) instead of 5 μM. NADPH-AROD activities of purified P450 were measured using reconstituted monooxygenase system comprising: 1.25 mg lecithin (Cambridge Bioscience, Cambridge, U.K.)/nmol P450 (10 μL 2.5 mg/mL lecithin in chloroform, evaporated to dryness under nitrogen on the bottom of a small glass test tube), followed by the addition of 11.5 U of purified human NADPH-cytochrome *c* reductase/nmol P450 and between 0.01–0.14 nmol purified P450. These components were allowed to mix by standing on ice for 5 min, then 10 μL AROD substrate (1 mM in DMSO) was added and 125 mM KPi buffer, pH 7.6, to a total reaction volume of 1 mL, and finally 10 μL NADPH (50 mM in water) to start the reaction. CHP-AROD activities of column fractions and purified P450 were measured as for microsomes, but using either 50–100 μL of fraction containing 0.5–1.0 nmol P450 or 0.1–1.0 nmol purified P450 and omitting lecithin and NADPH-cytochrome *c* reductase.

Microsomal 58C80 hydroxylase (58OH) activity was measured for 30 min at 37° using a 1 mL reaction comprising: 1 mg microsomal protein, 96 μM [^{14}C]-58C80 (10 μL of a 3 mg/mL solution in *N*,*N*-dimethylformamide, sp. act. 518 nCi/μmol), 0.6 U isocitric dehydrogenase, 2.5 mM DL-isocitrate, 250 μM $NADP^+$, 5 mM $MgCl_2$ and 125 mM KPi buffer, pH 7.5, containing 0.1 mM EDTA to 1 mL. Reaction blanks substituted water for isocitrate. The reaction mixture minus isocitrate was pre-incubated at 37° for 5 min, then the reaction was started by the addition of isocitrate and eventually stopped after

30 min by the addition of 800 μL 1 M phosphoric acid (pH 2.5) and vortex mixing. The reaction dependency on NADPH and NADPH was compared by omitting the regeneration system and starting the reaction with 10 μL 100 mM NADPH or NADH. 58OH activity of column fractions and purified P450 was measured similarly, but using a reconstituted monooxygenase system as described for NADPH-AROD except that 30 U of purified NADPH-cytochrome *c* reductase/nmol P450 were used, the reaction contained 20 pmol P450 in a total volume of 1 mL in 125 mM KPi buffer, pH 7.5, and 10 μL 100 mM NADPH were added to start the reaction. The arrested reaction mixture was extracted twice with 5 mL ethyl acetate by intermittent vortexing for 5 min, separation of the organic and aqueous phases by centrifugation for 15 min at 3000 rpm in a bench centrifuge, pipetting off of the upper ethyl acetate layer, re-extraction of the remaining aqueous layer, and evaporation of the combined organic extracts to dryness under nitrogen. The dried extract was redissolved in 50 μL methanol and 20 μL were spotted onto a fluorescent, aluminium-backed silica gel TLC plate (Kieselgel 60 F_{254}, E. Merck, Darmstadt, Germany), which was developed using ethyl acetate–methanol (95:5). 58C80 ($R_f = 0.8$) and the sole metabolite (298C80, $R_f = 0.62$) were identified as fluorescence-quenching spots under short wavelength UV light, scraped from the plate into scintillation vials, the scrapings mixed with 0.5 mL methanol and allowed to stand for 20 min, and the ^{14}C measured by scintillation counting using xylene–Lumax (Rhone-Poulenc Chemicals Ltd, Manchester, U.K.) (3:1) scintillation cocktail.

Microsomal *S*-mephenytoin-4′-hydroxylation (MPOH) was measured at 37° using a modification of the method of Shimada *et al.* [16]. Incubations (1 mL total volume) contained 200 μM *S*-mephenytoin, a standard NADPH generating system and 1–1.5 mg microsomal protein. Incubation was for 45 min to ensure initial velocity reaction conditions. The incubation was stopped by mixing a 450 μL aliquot with 100 μL 6% perchloric acid, followed by the internal standard, phenobarbitone (160 μL of a 0.2% solution), then the incubate was deproteinized by centrifugation and the supernatant pH adjusted with 50 μL 0.5 N NaOH. The deproteinized supernatant was extracted with 11 mL methyl *tert*-butyl ether for 40 min, then evaporated to dryness under vacuum. The extract residue was redissolved in 250 μL 25% aqueous acetonitrile and 100 μL aliquots analysed by HPLC, using a Waters Novapack C_{18} radial compression cartridge column and a linear mobile phase gradient of 18–30% acetonitrile in water over 12 min, with detection at 224 nm. Baseline separations were achieved, with retention times of 5.0, 7.5, 8.75 and 11.56 min for 4′-hydroxymephenytoin, nirvanol (*N*-desmethyl mephenytoin), phenobarbitone (internal standard) and *S*-mephenytoin, respectively. Coumarin 7-hydroxylation was measured fluorimetrically as described by Miles *et al.* [17]. Cyclosporin A metabolism was measured using HPLC with on-line radioisotope detection as described previously [6].

Detergent removal from column fractions. Because Emulgen and possibly other solubilization detergents used inhibit P450 activities, they were removed from column fractions prior to AROD and 58OH measurement, using a modification of the method of Horigome and Sugano [18]. Glass wool was washed with ether, dried, siliconized by soaking for 5 min in Sigmacote (Sigma Chemical Co., Poole, U.K.) solution, dried and placed as a small pad in the bottom of a 1.5 mL eppendorf tube with a small hole cut in the conical end. Bio-heads SM2 (Bio-Rad Laboratories, Hemel Hempstead, U.K.) were washed in ether and dried. All drying was at room temperature in air. Up to 0.5 mL of column fraction was mixed with 1 mL (packed volume) of washed Bio-beads in the tube by gentle stirring with a thin glass rod for 60 min on ice, then recovered by centrifugation at 4° for 5 min at approximately 200 *g* in a bench centrifuge. This procedure returned AROD activities to 80–90% of their original value after 50–70% inhibition by 0.32% Emulgen.

SDS–PAGE and immunoblotting. Microsomal, column fraction and purified P450 proteins were resolved by discontinuous SDS–PAGE [19] using a 10% acrylamide resolving gel as described previously [20] and electroblotted onto nitrocellulose by the method of Towbin *et al.* [21] and immunostained as described elsewhere [6].

Immunoinhibition and immunoquantitation. Polyclonal antibodies were raised in rabbits to purified rat P450 1A1 and P450 2B1 and human P450 3A P450hA7) and P450hB$_{20-27}$, respectively, and stored at −80° as 50% ammonium sulphate fraction lyophilisates as described previously [6], then reconstituted for use at 20 mg protein/mL in phosphate-buffered saline (PBS, Oxoid). Pre-immune rabbit serum globulin was prepared likewise. For immunoinhibition studies, microsomal protein at approximately 20 mg protein/mL and antibody or pre-immune globulin were incubated in 50 μL reaction buffer on ice for 20 min, with occasional swirling, prior to adding the rest of the reaction components. P450 was immunoquantified by ELISA as follows: 50 μL aliquots of microsomes, column fraction or purified P450 were dispensed in triplicate into a 96-well Micro-Titre plate and evaporated to dryness at 37° overnight. The wells were washed thrice for 15 min with 1% bovine serum albumin (BSA) in PBS, drained, 50 μL of anti-P450 antibody (diluted 1:500–1:3000 in PBS, depending on the P450) was pipetted into each well, the plate covered, incubated at 37° for 60 min, washed thrice for 15 min with 0.5% BSA in PBS containing 0.1% Triton X-100, then drained. A 50 μL aliquot of alkaline phosphatase-linked goat anti-rabbit second antibody (1:1000 dilution in PBS) was dispensed into each well, the plate covered, incubated at 37° for 60 min, washed thrice for 15 min with 0.5% BSA in PBS containing 0.1% Triton X-100, then thoroughly drained. A 50 μL aliquot of *p*-nitrophenolphosphate (1 mg/mL in 0.1 M glycine, pH 10.4, containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$) was pipetted into each well, the plate covered, incubated at 37° for 30 min, colour development arrested by the addition of 50 μL 4 N NaOH and the absorbance measured at 620 nm using a Titretek Multiskan MC. A standard curve of purified P450 was constructed for each assay using 50 μL aliquots containing 0.1–1.0 μg purified

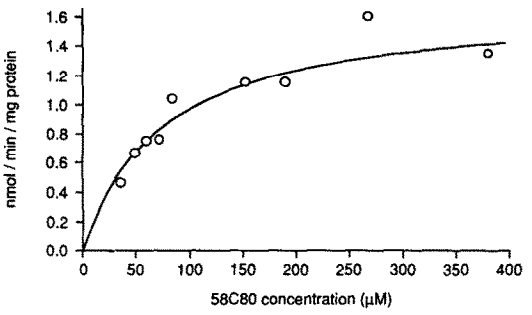

Fig. 2. Effect of substrate concentration on the rate of 58C80 hydroxylation to 298C80 by human liver microsomes. [^{14}C]58C80 was incubated for 20 min with liver microsomes from individual No. 29 (1 mg microsomal protein/mL incubate) and analysed as described in Results. Values are means of duplicate measurements. The curve was fitted by non-linear regression to the Michaelis–Menten equation.

P450 protein serially diluted in 10 mM KPi buffer, pH 7.5. Microsomal samples (approximately 20 mg protein/mL) were assayed as 50 μL aliquots of serial dilutions (20–300-fold) in 10 mM KPi buffer, pH 7.6. Column fractions were assayed undiluted after prior removal of detergent as described above.

RESULTS

Microsomal 58OH *activity*

A TLC assay for NADPH-dependent 58OH activity *in vitro* was developed for this study, as described in Materials and Methods. Human liver microsome preparations from three different individuals each converted 58C80 (Fig. 1, R = CH_3) to a single metabolite, which was deduced in each case to be the *t*-butyl-hydroxy-58C80 metabolite previously observed *in vivo* [2-(4-*t*-hydroxybutyl-cyclohexyl)-3-hydroxy-1,4-naphthoquinone; 298C80; Fig. 1, R = CH_2OH], since it had the same R_f (0.62–0.64) on TLC as the authentic compound. At 96 μM substrate concentration 58OH activity was linearly related to microsomal protein concentration up to 1.25 mg protein/mL (during a 20 min reaction) and to incubation time up to 80 min (using 1 mg microsomal protein) (data not shown). Non-linear regression (Ultrafit program, Biosoft, Cambridge) to fit the data obtained with increasing substrate concentrations to the Michaelis–Menten equation (Fig. 2) gave K_m (apparent) = 93.8 ± 11.7 μM and V_{max} = 2.40 ± 0.38 nmol/min/mg protein (mean ± SEM for liver Nos 19, 29 and 31, from a male aged 62, a female aged 63 and a female aged 45, respectively). Microsomes from two further human livers (liver Nos 20 and 27) with similar 58OH activities (1.2 and 0.95 nmol/min/mg protein, respectively) were combined for the purification of P450. Both livers were from females, aged 62 and 42, respectively, neither of whom had been receiving treatment with drugs believed to be inducers of P450. Individual No. 20 was a non-smoker and did not drink alcohol, but this information was not known for individual No. 27. The 58OH activity and P450 content of the combined microsomes were 1.1 nmol/min/mg protein and 0.37 nmol P450/mg protein, respectively.

Purification of human P450hB$_{20-27}$

A detailed description of the purification is given in Materials and Methods, while the column elution profiles are shown in Figs 3–5. Human liver microsomes solubilized with CHAPS detergent were loaded onto an octylamine-Sepharose column. A peak of fractions absorbing at 405 nm (i.e. A_{405} fractions), presumed therefore to contain haem, was eluted in the column wash. The subsequent inclusion of 0.08% Emulgen in the buffer failed to elute any A_{405} fractions, but three peaks of A_{405} fractions were eluted in consecutive detergent gradient steps of 0.16%, 0.5% Emulgen and 0.15% deoxycholate (Fig. 3). Of the P450 applied to the column 81% was recovered. The A_{405} fractions which contained P450 were combined into seven pools (a–g), on the basis of similarities and differences in SDS–PAGE protein banding patterns. The highest 58C80 hydroxylase activity (2.9 nmol/min/mg protein) was found in pool e, which incorporated the first 84% of the peak that eluted in 0.16% Emulgen; this pool comprised 44% of the total P450 and 70% of the total 58OH activity recovered from the column, was recognised by antibodies to P450 1A, P450 2B and P450 3A, and possessed MROD, EROD and BROD activities.

Dialysed pool e was loaded onto a Q-Sepharose column and five peaks of A_{450} fractions (all containing P450 on the evidence of their reduced CO-complexed absorption spectra) were eluted: one peak in the column wash and four peaks in a linear 0–400 mM KCl gradient (Fig. 4). The A_{405} fractions were combined into seven pools (a–g), using SDS–PAGE criteria as above. The highest 58C80 hydroxylase activity (34 nmol/min/mg protein) was in pool b, which constituted the last 55% of the peak eluting in the column wash; this pool comprised 24% of the total P450 (at a specific content of 7.5 nmol P450/mg protein) and 79% of the total 58OH activity recovered from the column, showed a considerable purification of four protein bands in the 45–60 kDa molecular mass range on SDS–PAGE, was recognised by antibodies to P450 1A, P450 2B and P450 3A, and had EROD and BROD activities but lacked MROD activity.

Dialysed pool b from Q-Sepharose was loaded onto a HAP column and three peaks of A_{405} fractions (all containing P450) were eluted in a stepped 0–200 mM KPi gradient (Fig. 5). The A_{405} fractions were combined into four pools (a–d), using SDS–PAGE criteria as above. The highest 58C80 hydroxylase activity (94 nmol/min/mg protein) was in pool d, which formed the last 37% of a peak of A_{405} fractions eluting in 200 mM KPi; this pool comprised 35% of the total P450 (at a specific content of 15.2 nmol P450/mg protein) and 54% of the total 58OH activity recovered from the column, showed a single major protein band on SDS–PAGE at 54 kDa, was not recognised by antibodies to P450 1A, P450 2B or P450 3A, and had EROD activity but neither BROD nor MROD activities. The

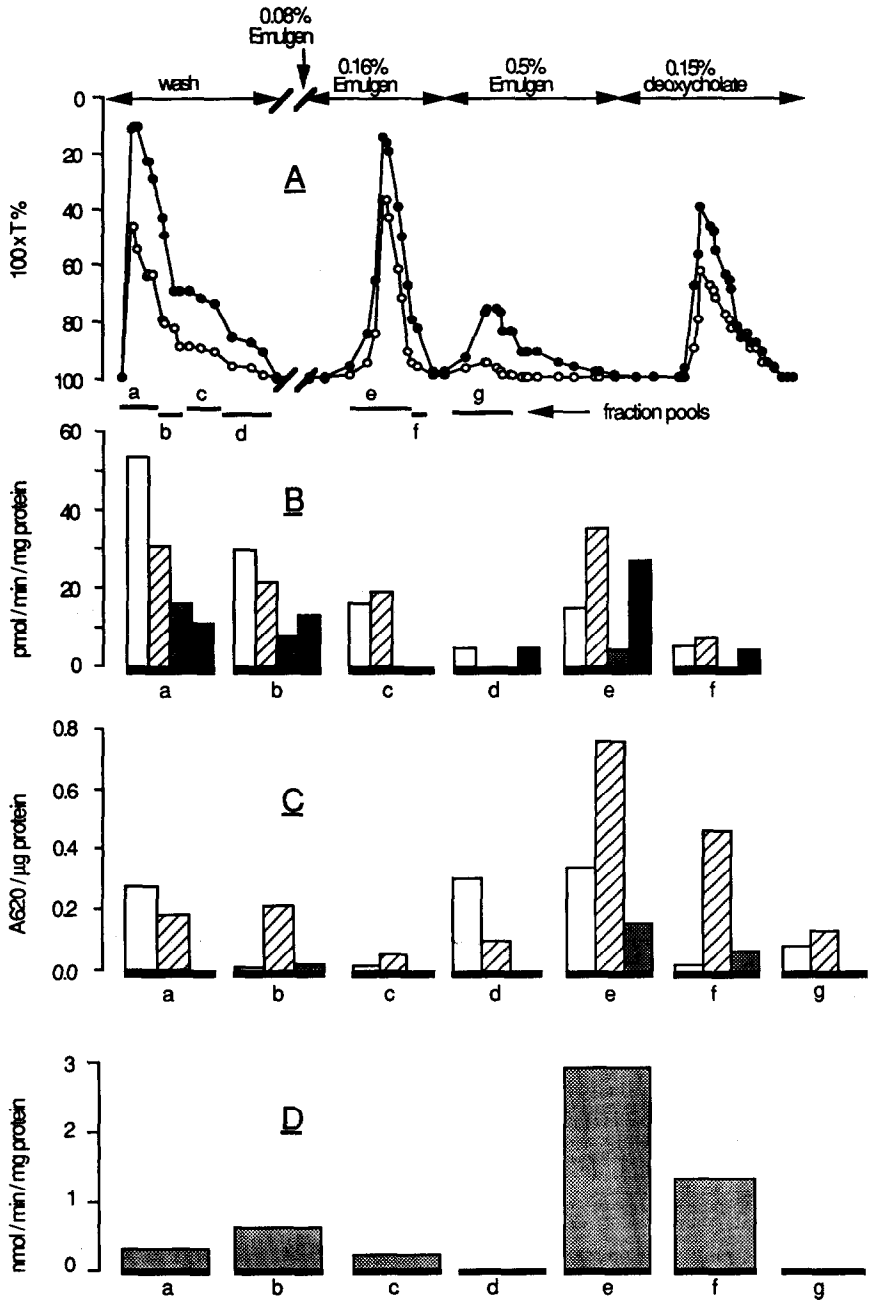

Fig. 3. Elution of P450hB$_{20-27}$ from octylamine-Sepharose. Human liver microsomes were solubilized, passed down an octylamine-Sepharose 4B column and analysed as described in Materials and Methods. (A) The % transmittance at 280 (●) and 405 (○) nm is shown for fractions eluted by a stepwise gradient of Emulgen 911 and deoxycholate. The eluted fractions were combined into seven pools, marked a–g as shown by bars below the elution profile. (B) The MROD (□), EROD (▨), PROD (▩) and BROD (■) activities of each of the fraction pools a–f. (C) The relative levels of P450 forms recognised by antibodies against rat P450 2B1 (□), rat P450 1A1 (▨) and human P450 3A (▩) in each of the fraction pools a–g are shown as the absorbance at 620 nm obtained in the ELISA assay used. (D) The 58C80 hydroxylase activities of each of the fraction pools a–g.

percentage of 58OH activity recovered in pool d was relatively low because only the very core of the peak of fractions was incorporated into this pool.

Dialysed pool d from hydroxylapatite was loaded onto an S-Sepharose column and two pools of A_{405} fractions (both containing P450) were eluted, in the column wash and in a linear 0–250 mM KCl gradient. Virtually all the 58C80 hydroxylase activity

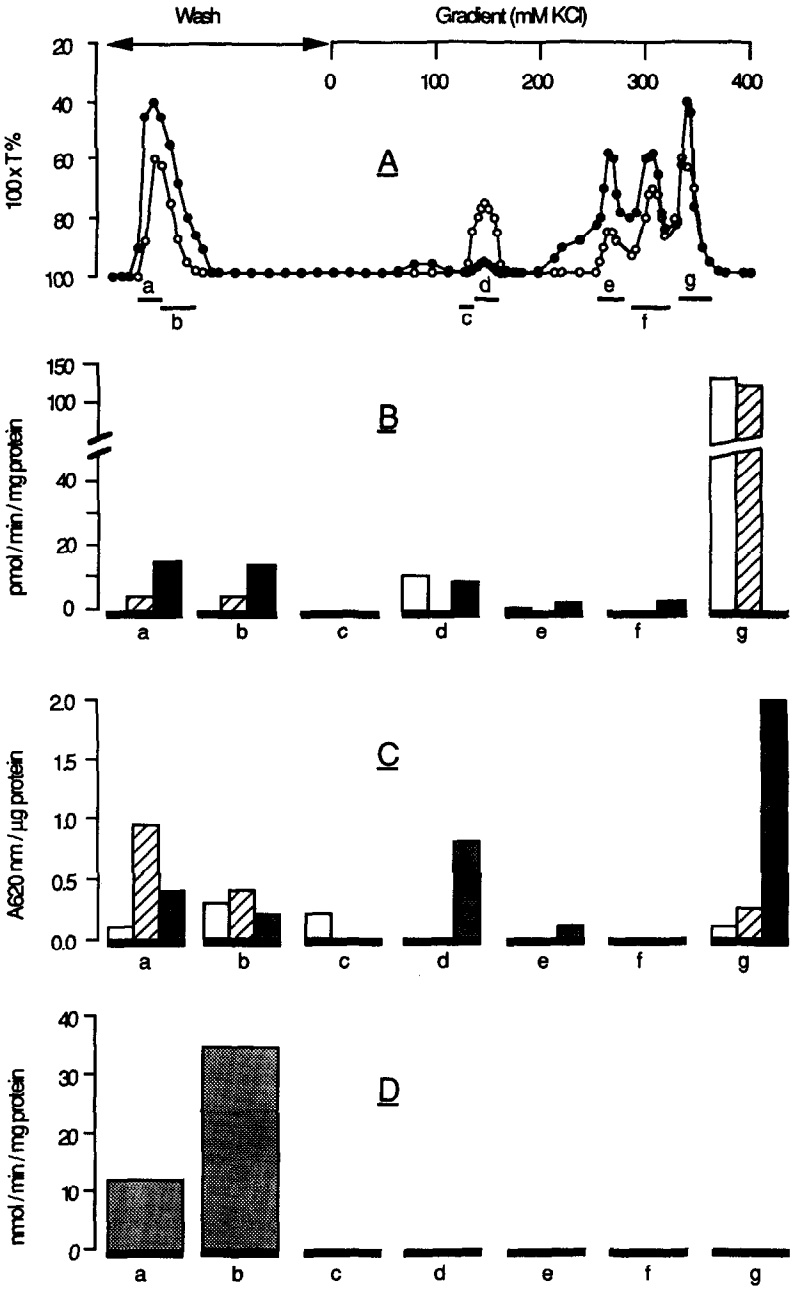

Fig. 4. Elution of P450hB$_{20-27}$ from Q-Sepharose. Fraction pool e from the octylamine-Sepharose was dialysed, passed down a Q-Sepharose column and analysed as described in Materials and Methods. (A) The % transmittance at 280 (●) and 405 (○) nm is shown for fractions eluted in the column wash and by a linear gradient of KCl. The eluted fractions were combined into seven pools, marked a–g as shown by bars below the elution profile. (B) The MROD (□), EROD (▩) and BROD (■) activities of each of the fraction pools a–g. (C) The relative levels of P450 forms recognised by antibodies against rat P450 2B1 (□), rat P450 1A1 (▩) and human P450 3A (▩) in each of the fraction pools a–g are shown as the absorbance at 620 nm obtained in the ELISA assay used. (D) The 58C80 hydroxylase activities of each of the fraction pools a–g.

(124 nmol/min/mg protein) was in the pool eluting in 30–45 mM KCl, which comprised 84% of the total P450 recovered from the column. Emulgen was removed from this pool on a small HAP column and the purified P450 was designated P450hB$_{20-27}$ (h to indicate a human P450, B to distinguish it from the P450hA7 previously purified by us and 20–27 to identify the two livers from which it was purified). P450hB$_{20-27}$ constituted the principal pool of P450 fractions showing 58C80 hydroxylase activity (sp.

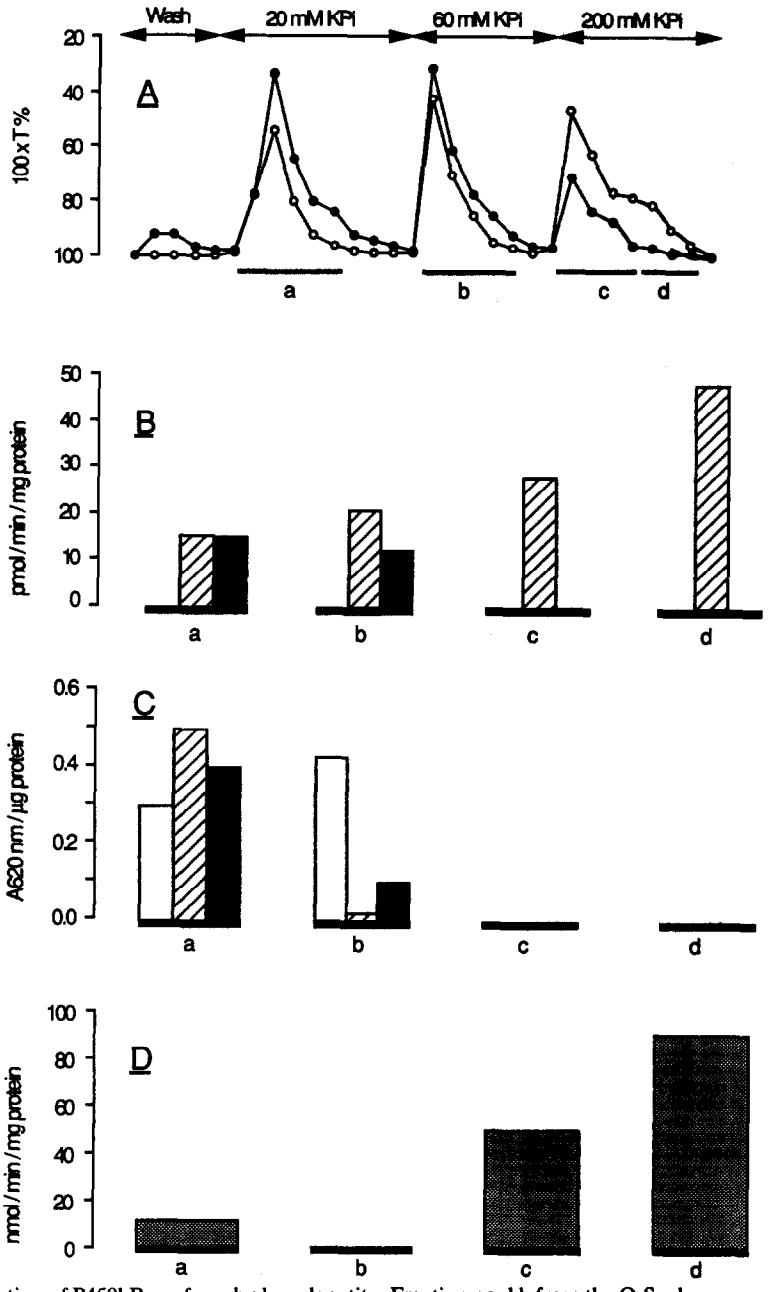

Fig. 5. Elution of P450hB$_{20-27}$ from hydroxylapatite. Fraction pool b from the Q-Sepharose was dialysed, passed down a hydroxylapatite column and analysed as described in Materials and Methods. (A) The % transmittance at 280 (●) and 405 (○) nm is shown for fractions eluted in the column wash and by a stepped gradient of KPi. The eluted fractions were combined into four pools, marked a–d as shown by bars below the elution profile. (B) The MROD (□), EROD (▨) and BROD (■) activities of each of the fraction pools a–d. (C) The relative levels of P450 forms recognised by antibodies against rat P450 2B1 (□), rat P450 1A1 (▨) and human P450 3A (■) in each of the fraction pools a–d are shown as the absorbance at 620 nm obtained in the ELISA assay used. (D) The 58C80 hydroxylase activities of each of the fraction pools a–d.

act. = 124 nmol/min/mg protein, representing a purification of 113-fold over liver microsomes and a yield of 1.1%), comprised approximately 1% of the total microsomal P450 with a specific content of 17.2 nmol P450/mg protein (almost the theoretical maximum specific content for a haemoprotein of molecular mass 54 kDa and representing a P450 purification of 46-fold over liver microsomes) and had no residual AROD activity (Table 1).

Physical characteristics of P450hB$_{20-27}$

Purified P450hB$_{20-27}$ was a single protein band on

Table 1. Purification of P450hB$_{20-27}$

Stage	Protein (mg)	P450 Total (nmol/protein)	P450 Specific content (nmol/mg)	P450 Purification (fold)	P450 Yield (%)	58OH Total (nmol/min)	58OH Specific activity (nmol/min/mg protein)	58OH Purification (fold)	58OH Yield (%)
Microsomes	6524	2414	0.37	1	100	7176	1.1	1	100
Octylamine-Sepharose (pool e)	652	861	1.3	3.5	36	1891	3.0	2.7	26
Q-Sepharose (pool b)	11.3	88.0	7.5	20.3	3.6	390	34.0	30.9	5.4
Hydroxylapatite (pool d)	1.9	28.8	15.2	41.1	1.2	172	92.0	83.6	2.4
S-Sepharose (30–45 mM KCl)	0.6	16.0	17.2	46.5	0.7	77	124.0	112.7	1.1

P450hB$_{20-27}$ was purified from human liver microsomes by successive stages of column chromatography and analysed as described in Materials and Methods. The pools refer to combined fractions as shown in Figs 4–6.

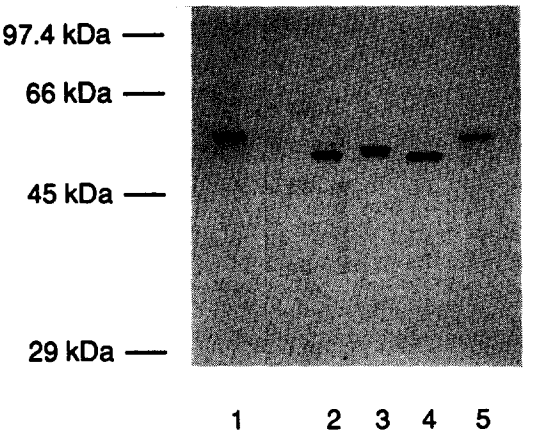

Fig. 6. SDS–PAGE analysis of purified P450hB$_{20-27}$. Human P450hB$_{20-27}$ and P450 3A (P450hA7), and rat P450 1A1, P450 2B1 and P450 3A1/2 were purified from human liver microsomes and analysed by SDS–PAGE (1 μg protein loading) as described in Materials and Methods. Molecular masses were obtained using the Sigma SDS-6H molecular mass marker standard mixture, including carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and phosphorylase b (97.4 kDa). The P450s and their molecular masses are: lane 1, P450 1A1 (56 kDa); lane 2, P450 3A1/2 (52 kDa); lane 3, P450 2B1 (53 kDa); lane 4, P450hA7 (51.5 kDa) and lane 5, P450hB$_{20-27}$ (54 kDa).

SDS–PAGE with a molecular mass of 54 kDa (Fig. 6). The N-terminal sequence of P450hB$_{20-27}$ was obtained for the first 18 amino acids as: MDSLVVLVLXLSXLLLLS. Residues 10 and 13 (X) could not be identified and were possibly cysteine residues lost during sequencing. The absolute absorption spectra of oxidized, dithionite-reduced and dithionite-reduced CO-complexed P450hB$_{20-27}$ are shown in Fig. 7, where the absence of a 648 nm absorption peak in the oxidized spectrum indicates that P450hB$_{20-27}$ was in the low-spin state.

Immunochemical characteristics of P450hB$_{20-27}$

A polyclonal antibody raised against purified P450hB$_{20-27}$ recognised the purified P450hB$_{20-27}$ strongly as a single stained band of molecular mass 54 kDa on immunoblots following SDS–PAGE, but gave only weak cross-recognition of purified rat P450 2C6 and did not recognise purified rat P450 1A1, P450 1A2, P450 2B1 or P450 3A1/2 or a purified human P450 3A form (P450hA7 [6]), even when these were present at a 4-fold higher concentration than P450hB$_{20-27}$ (data not shown). Conversely, purified P450hB$_{20-27}$ was not recognised on immunoblots by polyclonal antibodies raised against purified rat P450 1A1, P450 2B1, P450 2C6 or human P450 3A (P450hA7), which recognised their respective antigens strongly (data not shown). The anti-P450hB$_{20-27}$ antibody strongly recognised a band at molecular mass 54 kDa on immunoblots of human liver microsomes resolved by SDS–PAGE and in some livers also recognised more weakly a second band at molecular mass 50 kDa (Fig. 8).

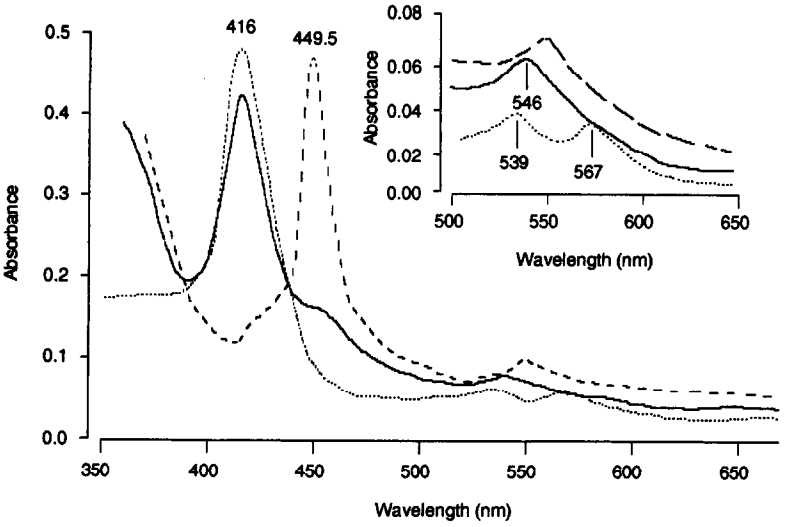

Fig. 7. Absolute optical absorption spectra of purified P450hB$_{20-27}$. The spectra of buffer solutions of oxidized (---), dithionite-reduced (——) and dithionite-reduced CO-complexed (·····) purified P450hB$_{20-27}$ were measured against a reference solution of buffer, using a Cary 219 double beam scanning spectrophotometer.

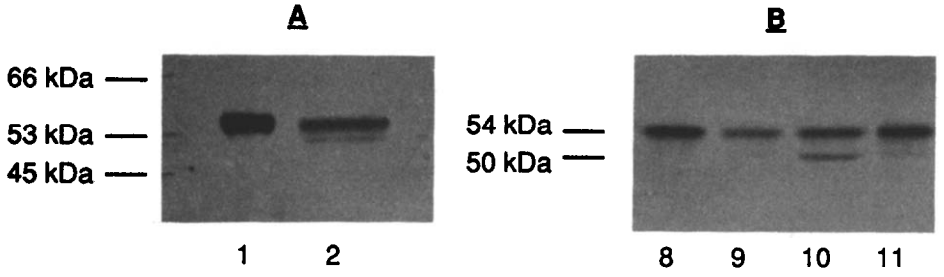

Fig. 8. Immunoblot of purified P450hB$_{20-27}$ and human liver microsomes with antibody against P450hB$_{20-27}$. Purified P450hB$_{20-27}$ (2 μg) and human liver microsomes (15 μg) were electrophoresed by SDS–PAGE, immunoblotted with polyclonal antibody against purified P450hB$_{20-27}$ (40 μg/mL), then stained with diaminobenzidine as described in Materials and Methods. Molecular mass markers (Sigma SDS-6H mixture, plus Sigma bovine liver L-glutamic dehydrogenase = 53 kDa) were transiently visualized on the electroblot with Ponceau red dye and their positions marked with a scalpel blade. (Blot A) lane 1, P450hB$_{20-27}$; lane 2, microsomes combined from the livers (Nos 20 and 27) used to purify P450hB$_{20-27}$. (Blot B) Microsomes from liver Nos 8, 9, 10 and 11 (lanes are numbered correspondingly). In both blots the upper band is at molecular mass 54 kDa and the lower band is at 50 kDa. The difference between the two blots in the degree of vertical separation between the bands is due to a difference in the gel running times.

Metabolic characteristics of P450hB$_{20-27}$

A monooxygenase system reconstituted from purified P450hB$_{20-27}$ and purified NADPH-cytochrome *c* reductase as described in Materials and Methods catalysed the *t*-butyl-hydroxylation of 58C80, with no other metabolite being formed, as determined by TLC analysis. Both the microsomal and reconstituted 58OH activities were NADPH dependent, although NADH was 35% as efficient as NADPH in supporting the microsomal activity. In supporting the reconstituted reaction, NADH was only 12% as efficient as NADPH in the absence of cytochrome b_5 but was 91% as efficient as NADPH in the presence of cytochrome b_5 (Table 2). NADH-supported, reconstituted 58OH activity increased with the concentration of cytochrome b_5 added and was maximal when cytochrome b_5 was equimolar with P450hB$_{20-27}$ (data not shown). However, whereas reconstituted NADH-dependent 58OH activity was greatly stimulated by cytochrome b_5, the reconstituted NADPH-dependent activity was 30% inhibited by cytochrome b_5. Reconstituted, NADPH-supported 58OH activity increased with the concentration of NADPH-cytochrome *c* reductase added up to a ratio of 0.6 nmol reductase/nmol P450hB$_{20-27}$, but then decreased from the maximum

Table 2. NADPH and NADH dependence of 58C80 hydroxylation by human liver microsomes and purified $P450hB_{20-27}$

Nucleotide cofactor present	58C80 hydroxylation: Microsomes (nmol/min/mg protein)	58C80 hydroxylation: $P450hB_{20-27}$ (nmol/min/mg protein)	Cytochrome b_5 incorporated with $P450hB_{20-27}$
None	0.0	0.0	
NADPH	1.1	124.0	–
		86.8	+
NADH	0.39	14.8	–
		113.0	+

58C80 was incubated ±NADPH or ±NADH with either purified $P450hB_{20-27}$ (20 pmol) in a reconstituted monooxygenase system of microsomes (1 mg protein) from liver No. 20, as described in Materials and Methods. When cytochrome b_5 was incorporated in the reconstituted system it was in an equimolar ratio with $P450hB_{20-27}$. The concentrations of NADPH and NADH were 1 mM.

The results are means of duplicate determinations.

at higher ratios, declining by 50% at 1.5 nmol reductase/nmol $P450hB_{20-27}$ (data not shown). The reconstituted, NADPH-supported 58OH activity was linearly related to incubation time up to 60 min (using 96 μM substrate and 20 pmol $P450hB_{20-27}$ per reaction) and increased with substrate concentration up to 240 μM (data not shown), with the Michaelis–Menten parameters for the reconstituted system being K_m (apparent) = 141.5 μM and V_{max} = 53.8 nmol/min/mmol $P450hB_{20-27}$.

The functional specificity of $P450hB_{20-27}$ was assessed by measuring the inhibitory effects of antibodies raised against $P450hB_{20-27}$ and other P450 forms on 58OH and other monooxygenase activities in human liver microsomes. EROD, coumarin 7-hydroxylation (COH), MPOH and cyclosporin A metabolism (CsA-met) were measured as probes for the activities of the human P450 1A, P450 2A, P450 2C and P450 3A subfamilies, respectively [6, 16, 17, 22]. Human liver microsomal 58OH activity was strongly inhibited by the anti-$P450hB_{20-27}$ antibody but not by antibodies raised against purified human P450 3A (P450hA7, which totally inhibited CsA-met in human liver microsomes), purified rat P450 1A1 (which completely inhibited EROD in human liver microsomes) or purified rat P450 2B1 [which almost totally inhibited PROD in PB-induced rat liver microsomes (data not shown) and COH in human liver microsomes] (Fig. 9; Table 3). (The inhibition of COH by anti-P450 2B1 antibody was probably due to a strong cross-reaction with human P450 2A6, confirmed on immunoblots of P450 2A6 expressed from cDNA in lymphoblastoma cells, kindly provided by Dr C. L. Crespi, Gentest Corporation, Woburn, MA 01801, U.S.A.; data not shown.) Conversely, the anti-$P450hB_{20-27}$ antibody also almost totally inhibited MPOH but did not strongly inhibit human liver microsomal EROD or CsA-met, although it did inhibit COH by 33%.

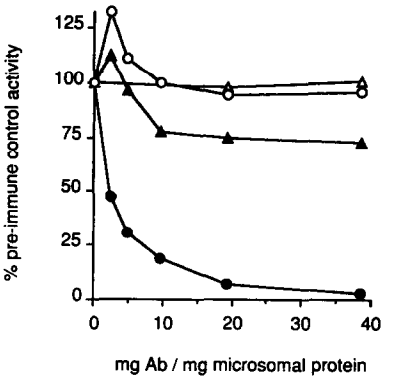

Fig. 9. Effect of anti-P450 antibodies on human liver microsomal 58C80 hydroxylation. 58C80 was metabolized by microsomes from liver No. 20 in the presence of varying amounts of antibodies against purified $P450hB_{20-27}$ (●), human P450 3A (P450hA7) (○), rat P450 1A1 (▲) or rat P450 2B1 (△) as described in Materials and Methods. In each incubation the total amount of immunoglobulin was kept constant at a ratio of 40 mg globulin/mg microsomal protein by mixing antibody with appropriate amounts of a pre-immune serum globulin preparation. The 100% control activity value was the specific activity of 58C80 hydroxylation measured in the presence of pre-immune serum globulin (40 mg/mg microsomal protein) without antibody. The presence of pre-immune serum globulin alone caused less than 10% change in 58C80 hydroxylation activity (data not shown). Results are means of duplicate determinations.

Table 3. Effects of anti-P450 antibodies on human liver microsomal monooxygenase activities

Activity*	Antibody: P450 1A1 (% inhibition by antibody)	P450 2B1	P450 3A	$P450hB_{20-27}$
EROD	98	6	0	10
COH	0	91	0	33
CsA-met	0	6	100	4
MPOH	4	10	0	96
58OH	27	2	6	97

* 100% control activities (nmol/min/mg microsomal protein):
EROD (liver No. 30) 0.041
COH (liver No. 52) 1.022
CsA-met (liver No. 25) 0.030
MPOH (liver No. 32) 0.027
58OH (liver No. 19) 1.480

Monooxygenase reactions were carried out with microsomes from human liver Nos 19, 25, 30, 32 and 52 in the presence of either antibody to an individual P450 or pre-immune globulin preparation at a ratio of 20 mg antibody or globulin/mg microsomal protein, or neither, as described in Materials and Methods. The 100% control activity values (activity without either pre-immune globulin or antibody) and the livers used are listed as a footnote to the table. Livers were chosen to show high activity for the reaction being measured, in order to facilitate the measurement of inhibition by antibody. The presence of pre-immune serum globulin alone caused less than 10% change in any activity (data not shown).

Results are means of duplicate determinations.

DISCUSSION

It is possible to identify the forms of human hepatic cytochrome P450 involved in the metabolism of a particular drug by inhibiting the reaction with anti-P450 antibodies or isozyme-selective compounds, correlating the reaction in a bank of livers with other reactions acting as characteristic markers for individual forms of P450, or metabolizing the drug by expressed cDNA. Arguably the most definitive method of identification, however, is to isolate the P450 form involved, which is the approach we have taken here. At each stage of the purification described here, the pool of fractions containing the form, P450hB$_{20-27}$, was the single pool containing both the highest specific activity for hydroxylation of the drug, 58C80, and the majority of the 58C80 hydroxylation activity eluted from the column; this indicates that P450hB$_{20-27}$ is the main P450 form responsible for the hydroxylation of 58C80 in human liver microsomes. Further evidence for the primacy of P450hB$_{20-27}$ in 58C80 hydroxylation is the complete inhibition of this activity in human liver microsomes by a polyclonal antibody raised against purified P450hB$_{20-27}$ whilst antibodies against other forms of P450 were either non- or much less strongly inhibitory.

The N-terminal amino acid sequence of P450hB$_{20-27}$ indicates it to be a member of the P450 2C family. To date, at least 10 human P450 2C proteins have been purified and at least 15 partial or full-length cDNAs for human P450 2C have been isolated, although several of these proteins and cDNAs appear to be the same entities. Based on amino acid and nucleotide sequences, six human members of the P450 2C family are recognised: 2C8, 2C9, 2C10, 2C17, 2C18 and 2C19 (Table 4). The purified proteins P450HLx [23], P450-B [24] and P450$_{MP-3}$ [25], together with the cDNAs IIC2 [26], Hp1-1 and Hp1-2 [27], MP-12 and MP-20 [25], and clones 7b and 35g [28], are all designated as variants of P450 2C8. The purified proteins P450$_{MP-1}$ and P450$_{MP-2}$ [16], P450meph [29], P450human-2 [30, 31], P450-C [24] and P450$_{MP}$ [32], together with the cDNAs IIC1 [26], MP-4 [25] and clones 186, 25 and 65 [28], are all designated as variants of P450 2C9. The individual 2C9 cDNAs differ mainly at deduced amino acid Nos 144, 159 and 276. The cDNA, MP-8 [33], is designated 2C10, which differs consistently from all the 2C9 forms in only two amino acids (positions 358 and 417, which are, respectively, C and D in 2C10 but Y and G in 2C9), while cDNA clone 254c is 2C17, clones 6b and 29c are 2C18 and clone 11a is 2C19 [28]. Purified P450$_{TB}$, for which no N-terminal sequence is available, was tentatively assigned to subfamily 2C10 on the basis of partial peptide sequences which include D at amino acid position 417 [32]. Since the distinction between the subfamilies 2C9 and 2C10 relies on differences in amino acids far removed from the N-terminal sequence, plus the fact that the only definitive 2C10 cDNA sequence reported (MP-8) lacks the codons specifying the first five N-terminal amino acids [34], it seems prudent to designate a 2C9/2C10 P450 protein for which the only sequence information available is the N-terminus as being 2C9 rather than 2C10. Hence, we consider P450hB$_{20-27}$ to be a member of the P450 2C9 family according to its N-terminal amino acid sequence.

The designation of P450hB$_{20-27}$ as a member of the P450 2C9 subfamily is supported by the observation that 58OH activity in human liver microsomes was strongly inhibited (66%) by sulfaphenazole (1 μM), which is a selective inhibitor of P450 2C9-dependent tolbutamide hydroxylation in man [35, 36] (data not shown).

P450hB$_{20-27}$ apparently has the highest purity of any P450 2C protein purified from human tissue. Its specific content (17.2 nmol/mg protein) is close to the theoretical maximum for a protein of molecular mass 54 kDa [29] and this and the degree of purification (46-fold based on P450 content) compare favourably with the purified P450 2C proteins cited above (up to 15 nmol/mg protein and 23-fold purification). The degree of purification of the characteristic 58OH activity of P450hB$_{20-27}$ (113-fold) was also very high compared with the values given for other purified human P450 2C forms: 6-fold purification of tolbutamide hydroxylation (TBOH) by P450$_{TB}$ [32], 7-fold for tetrahydrocannabinol 7-hydroxylation by P450-C [37], 13-fold for hexobarbitone hydroxylation by P450$_{MP-1}$ [25] and 12–14-fold for MPOH by P450$_{MP-1}$ and P450$_{MP-2}$ [16].

The elution characteristics of P450hB$_{20-27}$ from chromatography columns during purification (i.e. the detergent and salt concentrations needed for elution) resembled those of the other purified human P450 2C forms, P450$_{MP-1}$, P450$_{MP}$, P450$_{TB}$, P450human-2 and P450meph (see above for citations), except that P450human-2 and P450meph eluted from octylamine-Sepharose at a much higher concentration (0.5%) of non-ionic detergent (Emulgen 911 and Lubrol PX, respectively). The absorption spectra and low-spin characteristics of P450hB$_{20-27}$ were also similar to those of the other purified human P450 2C forms cited.

The minimum molecular mass of P450hB$_{20-27}$ on SDS–PAGE is 54 kDa. The molecular masses of other purified and expressed P450 2C proteins vary widely between laboratories. However, the majority of publications show P450 2C8 proteins as having lower molecular masses (48–51 kDa) than P450 2C9 proteins (54–56 kDa) (Table 4). This diversity of molecular mass is supported by reports of anti-human P450 2C antibodies recognising two or three protein bands on immunoblots of human liver microsomes, at approximately 48, 50 and 55 kDa [16, 29, 31].

P450hB$_{20-27}$, at 54 kDa, follows this molecular mass trend, while the anti-P450hB$_{20-27}$ antibody recognised a weak band at 50 kDa in addition to the strong band at 54 kDa on human liver microsome immunoblots. It thus appears that there are three groups of human P450 2C forms, with high, low and intermediate molecular mass, and that P450hB$_{20-27}$ ranks with the high molecular mass forms.

NADH was surprisingly effective compared to NADPH in supporting human liver microsomal 58OH activity (the NADH-supported 58OH activity was 35% of the NADPH-supported activity, whereas a figure of 10% is more common). A large stimulation

Table 4. N-terminal amino acid sequences of human P450 2C forms

Subfamily and trivial name	Amino acid sequence	kDa of purified or expressed protein	Ref.
2C8			
P450HLx	MEPFVVLVL–LSFMLLFSLW	50.5	[23]
P450-B	MEPFVVLVL–LSSMLLS	50	[24]
IIC2*	MEPFVVLVLCLSFMLLFSLWRQSCRRRKL	51	[26]
Hp1-1*†	MEPFVVLVLCLSFMLLFSLWRQSCRRRKL		[27, 36]
MP-20*	----------SFMLLFSLWRQSCRRRKL		[25]
$P450_{MP-3}$	MEPFVVLVL–LSFMLLFSL	48	[25]
2C9			
$P450_{MP-1}$	MDSLVVLVL–LS–LLLLSLWRQSSG	48	[16]
$P450_{MP-2}$	MDSLVVLVL–LS–LLLLSLWRQSSG–G–L	50	[16]
P450meph	MDSLVVLV	55 (or 52)	[29] [41]
IIC1*	MDSIVSLVLCLSCLLLLSLWRQSSGRGKL	54	[26]
human-2	MDSLVVLVLCLSCLLLLSLWRQSSGR	56	[31]
P450-C	M–SLVVLVLLLS–LLL	54	[24]
2C9*	MDSLVVLVLCLSCLLLLSLWRQSSGR	55	[42]
$P450_{MP}$‡	MD–LVVLVL–L		[32]
25 and 65*	MDSLVVLVLCLSCLLLLSLWRQSSGRGKL		[28]
$P450hB_{20-27}$	MDSLVVLVL–LS–LLLLS	54	This paper
2C10			
MP-8*	------VLVLCLSCLLLLSLWRQSSGRGKL	48	[33, 34]
2C18	MVPAVALVLCLSCLFLISL		
29c and 6b*			[28]
2C19	MDPFVVLVLCLSCLLLLS		
11a*			[28]

– Missing codon or amino acid within sequence or towards N-terminus.

* cDNA, showing deduced amino acid sequence and molecular mass of expressed protein where given.

† Same N-terminal sequence for cDNAs Hp1–2 [27] and clones 7b and 35g [28].

‡ Assigned here to subfamily 2C9 on the basis of partial peptide sequences which include Y at amino acid position 358.

No N-terminal sequence is given for purified $P450_{TB}$ [32], which is assigned to subfamily 2C10 on the basis of partial peptide sequences that include D at amino acid position 417, while the 2C9 cDNAs MP-4 [25] and clone 186 [28] and the 2C17 cDNA clone 254c [28] lack the 5′-nucleotides coding for the N-terminal amino acid sequence.

(almost 8-fold) by cytochrome b_5 of the NADH-supported 58OH activity of purified $P450hB_{20-27}$ in a reconstituted monoxygenase system contrasted with a sizeable (30%) cytochrome b_5 inhibition of the NADPH-supported 58OH activity of purified $P450hB_{20-27}$, suggesting that in liver microsomes the NADH-dependent 58OH activity, but not the NADPH-dependent activity, involves cytochrome b_5. $P450hB_{20-27}$ differed from other human P450 2C activities in respect to the effects of cytochrome b_5, which stimulated some NADPH-supported activities of purified $P450_{MP-1}$ and $P450_{MP-2}$ (MPOH [16]) and P450-C (nitrosodimethylamine N-demethylation [24]), although not TBOH by purified $P450_{MP-1}$ [38]. The NADH dependency of these reactions with human liver microsomes and purified P450 appears not to have been examined, however. The similarity of apparent K_m values between microsomal and reconstituted 58OH activities (94 and 141 μM, respectively) indicates that there was little alteration in the active site biochemistry of $P450hB_{20-27}$ during purification. The mean apparent K_m (94 μM) and V_{max} (2.4 nmol/min/mg protein) values for microsomal 58OH were, respectively, similar to and higher than the mean apparent K_m and V_{max} values for MPOH in eight polymorphically extensive metabolizes of mephenytoin (37.8 μM and 4.85 nmol/hr/mg protein [39]) and for TBOH in eight unclassified individuals (103 μM and 0.24 nmol/min/mg protein [35, 40]), although it was not tested whether the individuals from whom $P450hB_{20-27}$ was purified were extensive or poor metabolizers for mephenytoin. The 58OH activity of purified $P450hB_{20-27}$ in a reconstituted system, measured using 96 μM substrate, was 7.2 nmol/min/nmol P450, which is greater than any other activity reported for purified human P450 2C except for tetrahydrocannabinol 7-hydroxylation by P450-C (16.2 nmol/min/nmol P450 [35]). This high activity accords with the rapid hydroxylation of 58C80 in man *in vivo* [1].

The inhibition, albeit weak, of 58OH by anti-P450 1A antibody and of EROD and COH by anti-$P450hB_{20-27}$ antibody requires explanation. Immunoblots indicated there to be no cross-reactivity between the two antibodies and the different forms

of P450 potentially involved. The results might indicate minor contributions by P450 1A to 58OH activity and by $P450hB_{20-27}$ to EROD and COH in human liver microsomes. Since, however, pre-immune serum globulin can cause up to 30% non-specific inhibition or stimulation of P450 activities, with batch-to-batch variation occurring between these limits, then, notwithstanding that the degree of antibody inhibition was calculated relative to a pre-immune serum globulin control, we prefer to discount any antibody inhibition of less than 30% as being possibly due to batchwise variations in non-specific effects.

$P450hB_{20-27}$ is the first human P450 2C to have been purified using as the selection criterion a metabolic activity other than MPOH or TBOH that was subsequently shown to be highly selective for P450 2C. Furthermore, the hydroxylations of hexobarbitone, phenytoin and tetrahydrocannabinol were previously the only activities other than MPOH and TBOH to have been shown to be both metabolized by a purified human P450 2C ($P450_{MP-1}$, $P450_{MP-2}$ and P450-C, respectively) and selective for this P450 subfamily [16, 25, 37], although for phenytoin hydroxylation the activity of the purified P450 was not even twice that of microsomes. The structure of 58C80 is very different from previously known selective substrates of the human P450 2C subfamily. This identification of $P450hB_{20-27}$ as the human 58C80 hydroxylase therefore significantly broadens the range of drugs that have been directly shown (i.e. using purified enzymes as opposed to expressed cDNA) to be metabolized by a P450 2C protein that is incontrovertibly expressed *in vivo* in human liver.

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REFERENCES

1. Hudson AT, Dickins M, Ginger CD, Gutteridge WE, Holdich T, Hutchinson DBA, Pudney M, Randall AW and Latter VS, 566C80: a potent broad spectrum anti-infective agent with activity against maleria and opportunistic infections in AIDS patients. *Drugs Exp Clin Res* **17**: 427–435, 1991.
2. Gonzalez FJ, Human cytochromes P450: problems and prospects. *Trends Pharmacol Sci* **13**: 346–352, 1992.
3. Wrighton SA and Stevens JC, The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22**: 1–21, 1992.
4. Burke MD and Mayer RT, 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.
5. Burke MD, Bridges JW and Park DV, The effects of the non-ionic detergent Tween 80 on hepatic microsomal hydroxylation. *Xenobiotica* **5**: 261–277, 1975.
6. Shaw PM, Barnes TS, Cameron D, Engeset J, Melvin WT, Omar G, Petrie JC, Rush WR, Snyder CP, Whiting PH, Wolf CR and Burke MD, Purification and characterization of an anticonvulsant-induced human cytochrome P-450 catalysing cyclosporin metabolism. *Biochem J* **263**: 653–663, 1989.
7. Yasukochi Y and Masters BSS, Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reduction purified by biospecific affinity chromatography. *J Biol Chem* **251**: 5337–5344, 1976.
8. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34**: 3337–3345, 1985.
9. Barnes TS, Shaw PM, Burke MD and Melvin WT, Monoclonal antibodies against cytochrome P-450 recognizing different pregnenolone 16α-carbonitrile-inducible rat cytochromes P-450. *Biochem J* **248**: 301–304, 1987.
10. Bagby S, Barker PD, Hill HAO, Sanghera GS, Dunbar B, Ashby GA, Eady RR and Thorneley RNF, Direct electrochemistry of two genetically distinct flavodoxins isolated from *Azotobacter chroococcum* grown under nitrogen-fixing conditions. *Biochem J* **277**: 313–319, 1991.
11. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
12. Schaffner W and Wiseman C, A rapid and sensitive, and specific method for the determination of protein in dilute solutions. *Anal Biochem* **56**: 502–514, 1973.
13. Omura T and Sato R, The carbon monooxide-binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
14. Strittmatter P, Enoch HG and Fleming P, Incorporation of microsomal electron-transfer components into liposomes: considerations for diffusion-limited reactions. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 52, pp. 206–211. Academic Press, New York, 1975.
15. Phillips AH and Langdon RG, Hepatic triphosphoryridine nucleotide-cytochrome *c* reductase: isolation, characterization, and kinetic studies. *J Biol Chem* **237**: 2652–2659, 1962.
16. Shimada T, Misono KS and Guengerich FP, Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism. *J Biol Chem* **261**: 909–921, 1986.
17. Miles JS, McLaren AW, Forrester LM, Glancey MJ, Lang MA and Wolf CR, Identification of the human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity. *Biochem J* **267**: 365–371, 1990.
18. Horigome T and Sugano H, A rapid method for removal of detergents from protein solution. *Anal Biochem* **130**: 393–396, 1983.
19. Laemmli UK, Cleavage of structural protein during the assembly of the head of bacteriophage. *Nature* **227**: 680–685, 1970.
20. Burke MD, Falzon M and Milton AS, Decreased hepatic microsomal cytochrome P450 due to indomethacin: protective roles for 16, 16-dimethylprostaglandin $F_{2\alpha}$ and inducing agents. *Biochem Pharmacol* **32**: 389–397, 1983.
21. Towbin H, Staehelin T and Gordon JO, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
22. Forrester LM, Henderson CJ, Glancey MJ, Back DJ,

Park BK, Ball SE, Kitteringham NR, McLaren AW, Miles JS, Skett P and Wolf CR, Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Biochem J* **281**: 359–368, 1992.

23. Wrighton SA, Thomas PE, Willis P, Maines SL, Watkins PB, Levin W and Guzelian PS, Purification of a human liver cytochrome P-450 immunochemically related to several cytochromes P-450 purified from untreated rats. *J Clin Invest* **80**: 1017–1022, 1987.
24. Lasker JM, Raucy J, Kubota S, Bloswick BP, Black M and Lieber CS, Purification and characterization of human liver cytochrome P-450-ALC. *Biochem Biophys Res Commun* **148**: 232–238, 1987.
25. Ged C, Umbenhauer DR, Bellew TM, Bork RW, Srivastava PK, Shinriki N, Lloyd RS and Guengerich FP, Characterization of cDNAs, mRNAs, and proteins related to human liver microsomal cytochrome P-450 (*S*)-mephenytoin 4′-hydroxylase. *Biochemistry* **27**: 6929–6940, 1988.
26. Kimura S, Pastewka J, Gelboin HV and Gonzalez FJ, cDNA and amino acid sequences of two members of the human P450IIC gene subfamily. *Nucleic Acids Res* **15**: 10053–10054, 1987.
27. Okino ST, Quattrochi LC, Pendurthi UR, McBride OW and Tukey RH, Characterization of multiple human cytochrome P-450 1 cDNAs. *J Biol Chem* **262**: 16072–16079, 1987.
28. Romkes M, Faletto MB, Blaisdell JA, Raucy JL and Goldstein JA, Cloning and expression of complementary DNAs for multiple members of the human cytochrome P450IIC subfamily. *Biochemistry* **30**: 3247–3255, 1991.
29. Gut J, Meier UT, Catin T and Meyer UA, Mephenytoin-type polymorphism of drug oxidation: purification and characterization of a human liver cytochrome P-450 isozyme catalyzing microsomal mephenytoin hydroxylation. *Biochim Biophys Acta* **884**: 435–447, 1986.
30. Kawano S, Kamataki T, Yasumori T, Yamazoe Y and Kato R, Purification of human liver cytochrome P-450 catalyzing testosterone 6β-hydroxylation. *J Biochem* **102**: 493–501, 1987.
31. Yasumori T, Kawano S, Nagata K, Shimada M, Yamazoe Y and Kato R, Nucleotide sequence of a human liver cytochrome P-450 related to the rat male specific form. *J Biochem* **102**: 1075–1082, 1987.
32. Srivastava PK, Yun C-H, Beaune PH, Ged C and Guengerich FP, Separation of human liver microsomal tolbutamide hydroxylase and (*S*)-mephenytoin 4′-hydroxylase cytochrome P-450 enzymes. *Mol Pharmacol* **40**: 69–79, 1991.
33. Umbenhauer DR, Martin MV, Lloyd RS and Guengerich FP, Cloning and sequence determination of a complementary DNA related to human liver microsomal cytochrome P-450 *S*-mephenytoin 4-hydroxylase. *Biochemistry* **26**: 1094–1099, 1987.
34. Brian WR, Srivastava PK, Umbenhauer DR, Lloyd RS and Guengerich FP, Expression of a human liver cytochrome P-450 protein with tolbutamide hydroxylase activity in *Saccharomyces cerevisiae*. *Biochemistry* **28**: 4993–4999, 1989.
35. Doecke CJ, Veronese ME, Pond SM, Miners JO, Birkett DJ, Sansom LN and McManus ME, Relationship between phenytoin and tolbutamide hydroxylations in human liver microsomes. *Br J Clin Pharmacol* **31**: 125–130, 1991.
36. Relling MV, Aoyama T, Gonzalez FJ and Meyer UA, Tolbutamide and mephenytoin hydroxylation by human cytochrome P450s in the CYP2C subfamily. *J Pharmacol Exp Ther* **252**: 442–447, 1990.
37. Bornheim LM, Lasker JM and Raucy JL, Human hepatic microsomal metabolism of Δ^1-tetrahydrocannabinol. *Drug Metab Dispos* **20**: 241–246, 1992.
38. Knodell RG, Hall SD, Wilkinson GR and Guengerich FP, Hepatic metabolism of tolbutamide: characterization of the form of cytochrome P-450 involved in methyl hydroxylation and relationship to *in vivo* disposition. *J Pharmacol Exp Ther* **241**: 1112–1119, 1987.
39. Meier UT, Dayer P, Male P-J, Kronbach T and Meyer UA, Mephenytoin hydroxylation polymorphism: characterization of the enzymatic deficiency in liver microsomes of poor metabolizers phenotyped *in vivo*. *Clin Pharmacol Ther* **38**: 488–494, 1985.
40. Miners JO, Smith KJ, Robson RA, McManus ME, Veronese ME and Birkett DJ, Tolbutamide hydroxylation by human liver microsomes. *Biochem Pharmacol* **37**: 1137–1144, 1988.
41. Meier UT and Meyer UA, Genetic polymorphism of human cytochrome P-450 (*S*)-mephenytoin 4-hydroxylase. Studies with human autoantibodies suggest a functionally altered cytochrome P-450 isozyme as cause of the genetic deficiency. *Biochemistry* **26**: 8466–8474, 1987.
42. Veronese ME, Mackenzie PI, Doecke CJ, McManus ME, Miners JO and Birkett DJ. Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. *Biochem Biophys Res Commun* **175**: 1112–1118, 1991.