XENOBIOTICA, 2001, VOL. 31, NO. 6, 321-343

Carbamazepine: a 'blind' assessment of CYP-associated metabolism and interactions in human liver-derived *in vitro* systems

O. PELKONEN†*, P. MYLLYNEN†, P. TAAVITSAINEN†, A. R. BOOBIS‡, P. WATTS‡, B. G. LAKE§, R. J. PRICE§, A. B. RENWICK§, M.-J. GÓMEZ-LECHÓN¶, J. V. CASTELL¶, M. INGELMAN-SUNDBERG||, M. HIDESTRAND||, A. GUILLOUZO††, L. CORCOS††, P. S. GOLDFARB‡‡ and D. F. V. LEWIS‡‡

† University of Oulu, Department of Pharmacology and Toxicology, PO Box 5000, FIN-90014 Oulu, Finland
‡ Imperial College School of Medicine, Department of Medicine, Section of Clinical Pharmacology, London, UK
§ TNO BIBRA International Ltd, Carshalton, UK
¶ Unidad Hepatología Experimental, Centro de Investigación, Hospital Universitario La Fe. Valencia, Spain
|| Karolinska Institutet, Institute of Environmental Medicine, Division of Molecular Toxicology, Stockholm, Sweden
†† University of Rennes, Faculty of Pharmacy, INSERM U456, Rennes, France
‡‡ University of Surrey School of Biological Sciences, Guildford, UK

Received 11 December 2000

1. The ability of various *in vitro* systems for CYP enzymes (computer modelling, human liver microsomes, precision-cut liver slices, hepatocytes in culture, recombinant enzymes) to predict various aspects of *in vivo* metabolism and kinetics of carbamazepine (CBZ) was investigated.

2. The study was part of the EUROCYP project that aimed to evaluate relevant human *in vitro* systems to study drug metabolism.

3. CBZ was given to the participating laboratories without disclosing its chemical nature.

4. The most important enzyme (CYP3A4) and metabolic route (10,11-epoxidation) were predicted by all the systems studied.

5. Minor enzymes and routes were predicted to a different extent by various systems.

6. Prediction of a clearance class, i.e. slow clearance, was correctly predicted by microsomes, slices, hepatocytes and recombinant enzymes (CYP3A4).

7. The 10,11-epoxidation of CBZ by the recombinant CYP3A4 was enhanced by the addition of exogenous cytochrome-b_5, leading to a considerable over-prediction.

8. Induction potency of CBZ was predicted in cultured hepatocytes in which 7-ethoxycoumarin *O*-deethylase was used as an index activity.

9. It seems that for a principally CYP-metabolized substance such as CBZ, all liver-derived systems provide useful information for prediction of metabolic routes, rates and interactions.

* Author for correspondence; e-mail: olavi.pelkonen@oulu.fi

Xenobiotica ISSN 0049-8254 print/ISSN 1366-5928 online
http://www.tandf.co.uk/journals
DOI: 10.1080/00498250110055479

Introduction

Unsatisfactory pharmacokinetic properties have been pointed out as a major reason for the failure of new chemical entities (NCEs) in drug development in man (Prentis *et al.* 1988, Kennedy 1997). Prediction of human pharmacokinetic properties of NCEs at an early phase of drug discovery and development is therefore of outmost importance for the success rate later on. The pharmaceutical industry has therefore directed great efforts to develop fast and reliable *in vitro* methods for this purpose (Rodrigues 1994).

Prediction of hepatic clearance is especially important for drugs eliminated by hepatic metabolism. The enzymes responsible for drug metabolism in humans have been intensively studied during recent years and the knowledge has increased considerably (e.g. Pelkonen *et al.* 1998). It is now possible to determine which cytochrome P450 (CYP) enzyme is involved in the metabolism of a certain drug by using cDNA-expressed enzymes (Rodriques 1999). The metabolism in liver preparations like microsomes and S9 fractions can reflect the metabolic profile and be the basis for clearance and bioavailability predictions. More complex systems like isolated hepatocytes and tissue slices give a more complete picture of the metabolism. Isolated human hepatocytes have been proposed to be the best model for *in vivo* metabolic clearance predictions (Houston and Carlile 1997).

This study was a part of the EUROCYP project of the EU Biomed 2 program that aimed to evaluate relevant human *in vitro* systems to study drug metabolism and compare the results with the situation *in vivo*. A substance, carbamazepine, was given to participants without disclosing its chemical nature. The *in vitro* assay systems used were cDNA-expressed CYP enzymes, liver microsomes, isolated hepatocytes and precision-cut liver slices. Furthermore, a panel of human hepatocytes and CYP-selective chemical inhibitors was used to elucidate the metabolism of carbamazepine. All assays were run at participating laboratories specialized in the specific *in vitro* systems and the samples were sent to University of Oulu for analysis.

Carbamazepine (CBZ; 5-carbamoyl-5*H*-dibenz-[*b,f*]azepine) is a widely used anti-epileptic drug, which is nowadays the first-line drug of choice in most types of epilepsy. CBZ was selected for this blind assessment of *in vitro* liver-derived systems because there is a lot of knowledge about its kinetic and metabolic characteristics in humans. Furthermore, a reliable assay for measuring the metabolic profile of CBZ was available in the University of Oulu.

CBZ is metabolized to over 30 metabolites both in rat and humans (Lertratanangkoon and Horning 1982). In humans it is mainly oxidized to its major metabolite carbamazepine-10,11-epoxide (CBZ-E; figure 1). The epoxide is mainly hydrolyzed to the 10,11-*trans*-dihydrodiol metabolite (10,11-D) prior to excretion in urine (Faigle and Feldman 1995). Several minor metabolites are also formed including metabolites analysed in this study: 9-hydroxymethyl-10-carbamoyl acridan (9-AC), 3-hydroxy-carbamazepine (3-OH-CBZ) and 2-hydroxy-carbamazepine (2-OH-CBZ; figure 1). A summary of *in vivo* kinetics of CBZ is presented in table 1. CBZ is a known inducer of CYP systems (Faigle and Feldman 1995). It induces its own metabolism and its metabolism is also inducible by many other drugs (Levy and Wurden 1995). Clinically significant interactions are due to induction by CBZ (also autoinduction, Levy and Wurden 1995).

In order to perform predictions of *in vivo* characteristics of CBZ in a 'blind' fashion, specific goals of the study were: (1) determination of enzymes responsible

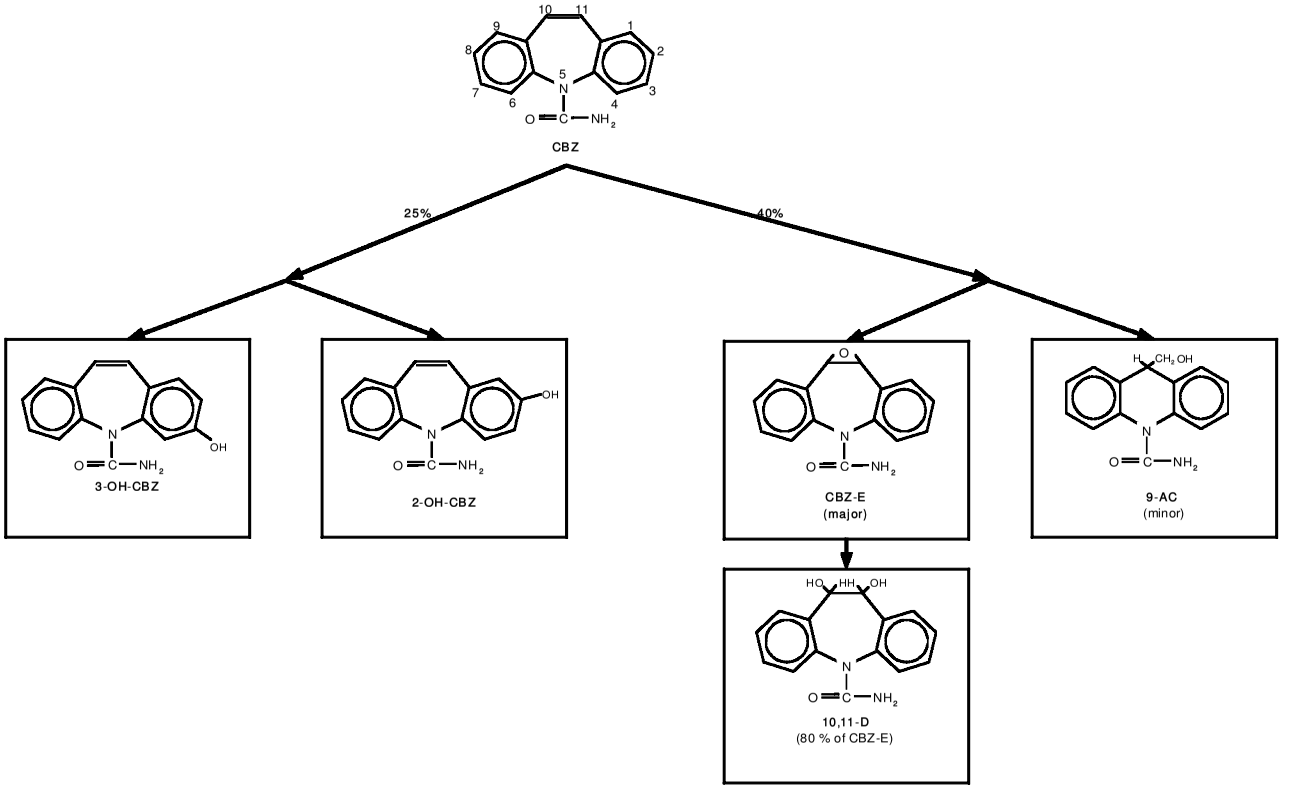

Figure 1. Principal metabolic pathways of CBZ in humans. Carbamazepine is metabolized to over 30 metabolites (Lertranangoon and Horning 1982) and only the metabolites detected in this study are presented. In humans, metabolism of CBZ proceeds down four major pathways: oxidation of 10,11 double bond (40%), hydroxylation of the six-membered aromatic rings (25%), direct *N*-glucuronidation (15%) and substitution of the six-membered rings with sulphur-containing groups (5%; Faigle and Feldman 1995). 10-OH-CBZ represents < 0.1% of CBZ dose and has only been detected in the haemofiltrate of one uraemic patient *in vivo* (Beyer and Spiteller 1992) and hence it is not presented. About 15% of the dose is directly *N*-glucuronidated (Faigle and Feldman 1995). About 1% is excreted unchanged in urine (e.g. Morselli 1995).

Table 1. Summary of the *in vivo* kinetics of carbamazepine (Tomson 1983, Dollery 1991, Morselli 1995).

Oral bioavailability	75-85%
Clearance	25-35 ml min^{-1}
Half-life of CBZ	18-55 h
Half-life of CBZ (after induction)	5-26 h
Half-life of CBZ-E	6 h
Volume of distribution	0.8-2.0 Lkg^{-1}

for metabolism (molecular modelling, microsomes, recombinant enzymes), (2) elucidation of metabolism and kinetics (microsomes, recombinant enzymes, liver slices, hepatocytes), (3) determination of induction potential (hepatocytes), and (4) comparison of findings and prediction with *in vivo* literature data on CBZ in order to find a system(s) that best predicts the *in vivo* situation.

Material and methods

Materials

Carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZ-E), 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (10,11-D), 2-hydroxy-carbamazepine (2-OH-CBZ), 3-hydroxy-carbamazepine (3-OH-CBZ), 9-hydroxymethyl-10-carbamoyl acridan (9-AC) and 10-hydroxy-10,11-dihydro-carbamazepine (10-OH-CBZ) were gifts from Novartis (Basel, Switzerland). According to Novartis, the chemical purity of CBZ and OCBZ was > 99%. HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Triethylamine was purchased from Fluka AG Chemische Fabrik (Buchs, Switzerland).

Determination of CBZ and its metabolites

HPLC analysis was done as described by Pienimäki *et al.* (1995). The HPLC system consisted of a Merck-Hitachi L-6200 gradient pump and L-4250 UV-VIS Detector set at 212 nm. The data were processed with a personal computer using Merck Hitachi D-6000 HPLC software. Chromatographic separation was achieved at room temperature using a mobile phase consisting of acetonitrile (20%) and buffer (80%, 20 mM KH_2PO_4, pH 6.3, with 0.05% triethylamine) at a flow rate 1.0 ml min^{-1} in conjunction with a LiChrospher 100 RP-18 (4 × 4 mm i.d., 5 μm) precolumn and Superspher 60 RP-select B (125 × 4 mm i.d., 4 μm) column from Merck.

Samples were extracted twice with 5 ml diethyl ether. Cells were dissolved in 500 μl 0.1 M Na-K phosphate buffer, pH 5, and sonicated prior to extractions. The evaporated samples were dissolved in 80 μl methanol : H_2O (5 : 2 v/v). A 20-μl aliquot was injected into HPLC. If necessary (high CBZ concentration) samples were further diluted. In these cases, both samples were analysed to maximize the accuracy in the detection of different concentrations of substrates and metabolites.

The extraction ratios were determined for hepatocytes (medium and cells) and liver slices (medium only). Generally, extraction ratios were very close to 100% except for 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (10,11-D). The extraction ratio for it was quite inefficient varying from 29 to 61%. This may have caused false-negative results in the analysis of this metabolite in the experiments where the amount of 10,11-D produced was low.

Candidate in vitro *assay systems*

Human liver microsomes

Origin of livers and preparation of microsomes. Human liver samples used were obtained from kidney transplantation donors. Appropriate Ethics Committees approved the collection of surplus tissue in the University of Oulu and in the Imperial College School of Medicine in London. For studies with CYP specific inhibitors microsomes from three livers were used. All patients died of intracerebral haemathoma (two male, one female) and they had no known liver pathology. Patients' ages ranged from 40 to 54. For other studies pool of microsomes from 10 livers were used. Microsomes were

prepared according to standard procedures. The final microsomal pellet was suspended in 0.1 M phosphate buffer, pH 7.4, to achieve a concentration of ~20 mg protein ml^{-1}. Protein content was measured by the method of Bradford (1976). The livers were thoroughly characterized for their CYP-specific model activities and showed to contain all expected activities, which could be inhibited by CYP-selective chemical inhibitors (data not shown). These were considered a proof of existence of specified CYP enzymes.

Inhibition by CBZ of CYP-specific enzyme activities. The following enzyme assays, which display at least some CYP-isoform specificity, were employed: ethoxyresorufin *O*-deethylation (CYP1A1/2) (Burke *et al*. 1977), coumarin 7-hydroxylation (CYP2A6) (Aitio 1978), with the slight modifications of Raunio *et al*. (1988, 1990), tolbutamide methylhydroxylation (CYP2C9) (modified from Knodell *et al*. 1987, Sullivan-Klose *et al*. 1996), mephenytoin 4′-hydroxylation (CYP2C19) (Wrighton *et al*. 1993), dextromethorphan *O*-demethylation (CYP2D6) (modified from Park *et al*. 1984, Kronbach *et al*. 1987), chlorzoxazone 6-hydroxylation (CYP2E1) (Peter *et al*. 1990), and testosterone 6β-hydroxylation (CYP3A4/5) (Waxman *et al*. 1983). The incubation conditions were basically similar in different reactions. If not otherwise stated below, the analytical method was applied according to the stated references.

In the primary determination of IC_{50}, CBZ was added in different concentrations (final concentrations in the incubation mixture: 0.1, 1, 10, 100 and 1000 μM) into the incubation mixture in a small volume of DMSO and the activity was compared with the control incubates into which only solvent was added. IC_{50} (concentration of inhibitor to cause 50% inhibition of the original enzyme activity) was determined graphically from the plot of the logarithm of inhibitor concentration versus per cent of activity remaining after inhibition using Microcal™ Origin™, v.4.10 (Microcal Software, Inc., MA, USA). The reference inhibitors used were furafylline for CYP1A2, methoxsalen for CYP2A6, sulphaphenazole for CYP2C9, omeprazole for CYP2C19, quinidine for CYP2D6, pyridine for CYP2E1 and ketoconazole for CYP3A4. Methoxsalen and omeprazole are known to inhibit also other CYPs than their targets, but there are no sufficiently selective reference inhibitors for CYP2A6 and CYP2C19 (Pelkonen *et al*. 1998).

In vitro *incubation system for CBZ metabolism*. CBZ was incubated up to 2 h at 37°C with human liver microsomes in an incubation system, which included human liver microsomes (pooled sample from 10 livers; 1 mg), NADPH (1.2 mM), NADPH-regenerating system (glucose 6-phosphate dehydrogenase; 4 mM NADP), phosphate buffer, pH 7.4, and 1 mM CBZ.

Inhibition of microsomal metabolism of CBZ by CYP-selective inhibitors. Inhibition of CBZ metabolism in human liver microsomes by various CYP-specific inhibitors was studied in the incubation system described above (CBZ concentration 1 mM, incubation time 60 min). The following inhibitors were used: CYP1A2: furafylline (concentrations used: 1, 2, 5 μM); CYP2A6: coumarin (10, 20, 50 μM); CYP2C8: quercetin (3, 10, 30 μM); CYP2C9: sulphaphenazole (3, 10, 30 μM); CYP2C19: S-mephenytoin (100, 200, 500 μM); CYP2D6: quinidine (1, 5, 20 μM); CYP2E1: pyridine (1, 5, 20 μM); CYP3A4: ketoconazole (1, 5, 20 μM). All inhibitors were added in methanol (1%), except pyridine, which was added in water. The formation of 10,11-epoxide, 3-hydroxy-CBZ and 10,11-diol was estimated as described below.

Correlation study. CBZ was incubated in an above-described incubation system and metabolism was determined in nine different human liver samples, which had previously been characterized for their content and activity of all of the major drug metabolizing forms of P450 (CYP1A2, CY1B1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A11). The formation of 10,11-epoxide, 3-hydroxy-CBZ and 10,11-diol were correlated with CYP-specific activities and the amounts of CYP proteins quantitated by antipeptide antibodies in Western immunoblots as described by Edwards *et al*. (1998).

Precision-cut human liver slices

Preparation and culture of liver slices. The sources of the tissue culture materials were as described by Beamand *et al*. (1993) and Lake *et al*. (1998). Samples of human liver (surplus to transplant requirements) were collected and transported to TNO BIBRA on ice. Samples from two different livers were used. Tissue cylinders from liver samples were prepared using a 10-mm diameter motor-driven tissue-coring tool. From the cylinders, liver slices (200-300 μm) were prepared in oxygenated (95% O_2/5% CO_2) Earle's balanced salt solution containing 25 mM D-glucose, 50 μg ml^{-1} gentamicin and 2.5 μg ml^{-1} fungizone using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munford, AL, USA). The liver slices were floated onto Vitron, Inc. (Tucson, AZ, USA) type C titanium roller inserts (two slices per insert) and cultured in glass vials containing 1.7 ml culture medium in a Vitron dynamic organ incubator. The culture medium consisted of Williams' medium E

containing 2 mM L-glutamine, 0.1 μM insulin, 0.1 μM dexamethasone, 50 μg ml^{-1} gentamicin and 2.5 μg ml^{-1} fungizone. Liver slice cultures were maintained at 37°C in an atmosphere of 95% O_2/5% CO_2. After 60 min, the medium was changed to fresh medium containing 2 mM L-glutamine, 0.1 μM insulin, 0.1 μM dexamethasone and 0-2500 μM CBZ (added in 10 μl dimethyl sulphoxide ml^{-1} medium). Liver slices were incubated for 40-160 min and the incubations terminated by removing the vials from the incubator and plunging them into ice. Appropriate blank incubations (i.e. liver slices in medium without any CBZ and CBZ in medium without any liver slices) were also performed. In order to achieve a good recovery of parent compound and metabolites (Worboys *et al.* 1995), the liver slices were removed from the mesh of the roller inserts and homogenized in the culture medium by sonication (Beamand *et al.* 1993). Liver slice/medium homogenates were assayed for total protein content by the method of Lowry *et al.* (1951) employing bovine serum albumin as standard. Total protein content was determined in order to allow for any differences in liver slice thickness between vials and to provide a scaling factor for intrinsic clearance calculations. The liver slice/medium homogenates stored at −80°C prior to despatch to the laboratory undertaking the analysis of CBZ metabolites.

Studies on CBZ metabolism and kinetics. Slices were incubated for various periods ranging from 0.5 to 6 h with CBZ concentrations ranging from 0.5 to 5000 μM. CBZ was prepared as stock solutions in dimethyl sulphoxide (DMSO) and then added to the culture media at a final DMSO concentration of 10 μl ml^{-1} medium. For all incubations, serum free culture media were employed. The media comprised either RPMI 1640 medium with hormones (see above), Williams' medium E with L-glutamine and hormones (see above) or phenol red free RPMI 1640 medium containing 0.1 μM insulin and 0.1 μM dexamethasone. Because of the short-term nature of these incubations, it was not considered necessary to supplement the media with either gentamicin or fungizone. The CBZ concentrations employed were based on solubility and toxicity considerations, together with other data (e.g. concentrations employed in incubations with liver microsomal fractions). The CBZ concentrations and incubation periods were modified as a result of initial experiments. In all experiments, the incubations were terminated by removing the vials from the incubator and placing them in ice. The liver slices were then removed from the mesh of the roller inserts and homogenized in the culture medium by sonication. It was considered necessary to prepare liver slice/medium homogenates, rather than just harvest the media, as other studies have demonstrated that xenobiotic metabolites may be retained inside the liver slices (Worboys *et al.* 1995). Appropriate blank incubations (e.g. liver slices in medium without any CBZ and CBZ in medium without any liver slices) were also performed. Samples of the CBZ dilutions in tissue culture medium and culture medium alone were also provided for analysis. Liver slice/medium homogenates were assayed for total protein content to allow for any differences in liver slice thickness between vials and to provide a scaling factor for kinetic calculations (see below). The liver slice/medium homogenates were stored at −80°C prior to dispatch to the appropriate laboratory undertaking the analysis of CBZ and its metabolites.

Human cultured hepatocytes

Procedures in Laboratory A

Isolation and culture of human hepatocytes. Surgical liver biopsies (weighting 1-3 g) were obtained from patients undergoing cholecystectomy after informed consent was obtained. Patients had no known liver pathology nor did they receive medication during the weeks prior to surgery. None of the patients were habitual consumers of alcohol or other drugs. A total of 6 liver biopsies (two males, four females) were used. Patients' ages ranged from 35 to 77 years. Two liver biopsies were used for metabolic studies because the concentration of CBZ was too low in four of the incubations and only incubations from two biopsies could be used for metabolite analysis and calculations for hepatic clearances. Hepatocytes were isolated using a two-step tissue microperfusion technique as described elsewhere (Gómez-Lechón *et al.* 1997). Cellular viability, estimated after isolation by the dye exclusion test with 0.4% trypan blue in saline, was > 90%. Hepatocytes were seeded on fibronectin-coated plastic dishes (3.5 μg cm^{-2}) at a density of 8×104 viable cells cm^{-2} and cultured in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% new-born calf serum, 10 mM glucose, 50 mU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 0.2% bovine serum albumin and 10 nM insulin. One hour later the medium was changed, and after 24 h, cells were shifted to serum-free, hormone-supplemented medium (10 nM insulin, 10 nM dexamethasone). The medium was changed daily. Under these culture conditions, cells are metabolically competent (Donato *et al.* 1995, Gómez-Lechón *et al.* 1997).

Preparation of CBZ for incubations. A 50 mM stock solution of the compound was prepared in DMSO and then diluted in culture medium. The final concentration of the solvent in culture medium did not exceed 0.5% (v/v). Control cultures were treated with the same amount of solvent.

Cytotoxicity assays. Hepatocytes were seeded on 96-well microtitre plates, and treatment with increasing concentrations of the CBZ started after 24 h of culture. Cells were exposed to the compound during the following 48 h, and the compound was added daily at the time of medium renewal. Cellular

viability was assessed at the end of the treatments by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test as described by Borenfreund *et al.* (1988).

IC calculations for cytotoxicity. IC_{50} and IC_{10} represent the concentration of the compound that reduces viability by 50 and 10% with respect to the controls and were determined from the dose-response curves obtained. In five individual hepatocyte preparations, IC_{10} was usually > 250 μM except in one culture, in which it was 50 mM. The IC_{50} value was 1 mM > IC_{50} > 0.75 mM.

7-Ethoxycoumarin O-deethylation activity assay. This reaction was catalysed by several P450 isozymes including CYP1A2, CYP2A6, CYP2B6, CYP2C8-9, CYP2E1, CYP3A4/5 (Waxman *et al.* 1991). Activity was measured in intact cells (Gómez-Lechón *et al.* 1997). Monolayers were washed twice with warm PBS and the assay was initiated by adding 800 μM 7-ethoxycoumarin to the culture medium. Cells were incubated for ~30 min at 37°C, and the reaction was stopped by aspirating the incubation medium from plates. To hydrolyze any 3-hydroxycoumarin conjugates formed, 1 ml of the medium sample was incubated with β-glucuronidase/arylsulphatase (200 Fishman units/1200 Roy units) for 2 h at 37°C. Hydrolysis was stopped by adding 125 μl TCA (15%) and 2 ml chloroform and then shaking the mixture for 5-10 min at 37°C. After centrifugation (2000g for 10 min), the organic phase was extracted with 1 N NaOH and the fluorescence of the aqueous phase was measured (368 nm excitation, 456 nm emission) in a microplate fluorimeter. Activity is expressed as pmol 3-hydroxycoumarin formed min^{-1} and mg^{-1} cellular protein assessed by the Lowry method (Lowry *et al.* 1951).

Incubation of human hepatocytes with CBZ. For metabolic studies hepatocytes were incubated with various concentration of the compound (75, 125, 250 μM). Treatment was started at 24 h of culture and incubation medium and cells were subsequently frozen after 10, 24 and 48 h of continuous incubation with the compound, to be further analysed for the metabolic profile. Parallel plates without cells, incubated with medium containing the compound, were collected and also frozen after the same incubation periods.

Procedures in Laboratory B

The tissue sample was obtained from a liver that could not be used for transplantation. The French National Ethics Committee has approved collection of human liver tissue. Hepatocytes were isolated by the two-step collagenase perfusion method as previously described (Guguen-Guillouzo *et al.* 1982) then seeded at a density of 10^6 cells per dish in 1 ml Williams' medium supplemented with 0.2% bovine insulin, 3.2% bovine serum albumin, 1% glutamine, 0.1% penicillin/streptomycin, 0.2% gentamycin and 10% foetal calf serum. This medium supplemented with 7×10^{-5} hydrocortisone hemisuccinate but lacking foetal calf serum was renewed daily.

Cell viability estimated by trypan blue exclusion was 85%. CBZ was dissolved in DMSO before addition to the culture medium at the concentrations of 0.02, 0.1, 0.5, 1 and 2.5 mM (in 0.25% DMSO v/v). CBZ was first added after 24 h and then with each medium renewal. Media and cells were harvested after 16, 24, 48 and 72 h of exposure to CBZ and stored at −80°C before analysis. EROD activity in hepatocytes was quite low: 0.23 pmol min^{-1} mg^{-1} cellular proteins (min-max in our hands: 0.2-8.0; mean 3.0 with 19 cell populations). Nifedipine oxidation for CYP3A4 was 6.24 nmol h^{-1} mg^{-1} cellular protein.

cDNA-expressed enzymes

Yeasts were produced that expressed one of the following human cytochrome P450 enzymes: CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, and the expression level was relatively high (30-300 pmol mg^{-1} microsomal protein). All microsomes produce good difference spectra showing a homogenous peak at ~450 nm in the reduced carbon monoxide bound state of the haemoprotein with no cytochrome P420 present. In order to achieve good expression of the CYP2C19 and CYP2D6 enzymes, the plasmid cDNA:s were modified to include a triplet of adenine bases just prior to the initiating ATG codon (Krynetski *et al.* 1995).

The expression was carried out with the *Saccharomyces cerevisiae* strain W(R), which had been genetically modified also to over express the yeast reductase (Truan *et al.* 1993). A galactose-inducible promoter in the plasmid and in the yeast genome, respectively, was used to control expression. After transformation with a lithium acetate method of the plasmid into the W(R) yeast strain, selection of clones was achieved by growing the yeast in adenine and uracil deficient media. To achieve higher expression levels, the yeasts were first grown to a high density with glucose as main energy source, thereafter the expression of the plasmid was initiated by the addition of galactose. Preparation of microsomes was done as described by Oscarson *et al.* (1997) with the exception that the microsomes were collected in the last step by ultracentrifugation. Cytochrome P450 and NADPH-cytochrome P450 reductase were measured as described (Oscarson *et al.* 1997). Control microsomes were prepared from yeast transformed with the V60 plasmid without insert. The CYP2C19 and CYP2B6 pV60 plasmids were generous gifts from Charlotta Otter (Astra Zeneca, Mölndal, Sweden). The catalytic properties of

the yeast microsomes were evaluated using probe substrates for different P450 isoforms and found to appropriate (Anderson *et al*. 2001).

Incubations with yeast microsomes, corresponding to 400 μg protein, were carried out for 30 min with 1 mM CBZ in 0.5 ml phosphate buffer, pH 7.4, and 1 mM NADPH. For further determination of K_m and V_{max}, 250 μg CYP3A4 containing microsomes was used and the time was shortened to 8-10 min to ensure linearity. All incubations were done at 37°C and the samples were pre-incubated for 2 min prior to the addition of NADPH. In order to investigate the influence of NADPH cytochrome P450 reductase and cytochrome-b5 on CYP3A4-mediated CBZ metabolism, human or rat NADPH cytochrome P450 reductase and rat cytochrome-b5 were mixed with the CYP3A4 containing microsomes and left on ice for 10 min prior to the incubation procedure. Human reductase was produced recombinantly in *Escherichia coli* and purified on an 2′, 5′-ADP Sepharose column essentially as described by Shen *et al*. (1989). Rat NADPH cytochrome P450 reductase and rat cytochrome-b_5 were purified as described by Ingelman-Sundberg *et al*. (1980) to a specific content of 13 nmol flavin mg^{-1} and 30 nmol mg^{-1}, respectively.

Modelling

Molecular modelling of mammalian microsomal P450s from the CYP102 bacterial crystal structure template has been carried out for all of the major P450 families associated with the Phase 1 metabolism of drugs and other foreign compounds (Lewis 1999). Three-dimensional models of human P450s have been constructed from the CYP102 haemoprotein domain, for which the X-ray coordinates are known, both in the substrate-bound (Li and Poulos 1997) and substrate-free states (Ravichandran *et al*. 1993). The methodologies employed in homology modelling of P450 isoforms from CYP102 are described in detail elsewhere, including a general reference to the rationale for using the CYP102 structure as a preferred modelling template (Lewis 1996). The model of CYP2C8 was constructed as part of the EUROCYP collaboration and details have not been published elsewhere, although the related enzymes CYP2C9 and CYP2C19 have been modelled previously and the current CYP2C8 model is based on these former CYP2C subfamily models, being closer to CYP2C9 in both sequence and active site structure. A template of known CYP2C8 substrates fits the enzyme's active site closely, and this shows how hydrogen bonds and π–π stacking of aromatic rings cooperately assist in orientating substrates for metabolism in the experimentally observed positions.

A number of potential probe substrates (25 in total) for various human P450 isozymes have been identified and tested in models for CYPlA1, CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Lewis 1999). After this preliminary work, CBZ was tested in these models.

Calculations

Scaling from *in vitro* candidate systems to the whole liver. Intrinsic clearance (CL_{int}) is defined as the proportional constant between initial rate (v_0) and the drug concentration (C). According to the Michaelis-Menten equation:

$$V_o/C = V_{max}/K_m + C = CL_{int}.$$

At low substrate concentrations $CL_{int} = V_{max}/K_m$. At low substrate concentrations CL_{int} could be calculated from initial rates and from V_{max}/K_m (Ito *et al*. 1998).

In all *in vitro* systems, either the disappearance of the parent compound or the appearance of metabolites was used to determine K_m and V_{max} using either Michaelis-Menten or Eadie-Hofstee plots. It was not always possible strictly to use the initial linear conditions, but the deviations were probably not too large. Furthermore, not all data/time points were necessarily used for all calculations and also no allowance was made for either loss or recovery of CBZ metabolites in the incubations. Consequently, one should bear in mind that calculated K_m and V_{max} are approximate because of experimental imprecisions due to the inbuilt nature of experiments (very little advanced knowledge about the compound and optimal experimental set-up, i.e. a situation typical at the screening phase of NCEs) and because of interindividual biological variability (liver samples are from a limited number of individuals). In all systems, appropriate scaling factors are required to convert the primary kinetic data (V_{max}/K_m or intrinsic clearance) to whole human liver data (hepatic intrinsic clearance). The scaling of the results from system intrinsic clearance was somewhat different in each *in vitro* system.

In the recombinant system, velocity was calculated as pmol metabolite formed $pmol^{-1}$ spectrally determined P450 min^{-1}. Subsequent scaling to human liver microsomes was achieved using V_{max}/K_m. In order to extrapolate the data from the recombinant system into the situation in human liver microsomes, we used the concentrations of the various cytochromes P450 as provided by Gentest Co. (http://www.gentest.com/prod_inserts/H161_2.htm#immunoquantitation). Further scaling was carried out as described below.

In liver microsomes, the microsomal intrinsic clearance has been converted to the whole liver clearance by assuming that 1 g liver contains 50 mg microsomal protein and that the liver mass is 1500 g.

In liver slices scaling was performed on the basis of liver slice whole homogenate protein content compared to human liver total protein content (B. Houston, University of Manchester, personal

communication). Human liver whole homogenate protein has been determined by homogenizing samples of liver used to prepare the liver slices and determining the protein content by the method of Lowry *et al*. (1951). For seven separate human liver samples total protein content was determined to be 128.4 ± 6.5 mg g^{-1} wet weight liver (mean ± SEM, $n = 7$, range 102-153 mg g^{-1} liver). Also higher values, e.g. 200 mg protein g^{-1} liver wet weight, have been used in the literature.

In hepatocytes, the value for calculating whole liver clearance was estimated according to the following assumptions: average hepatocyte volume 11 000 μm^3, density of liver tissue (1.1), percentage of volume that hepatocytes represent within the liver tissue (80%). Based on these assumptions, 60-70 million hepatocytes g^{-1} liver tissue were obtained. These values are much closer to the yields obtained after gentle digestion of liver tissue with collagenase ($30\text{-}40 \times 10^6$ cells g^{-1}). Consequently, the average of 65×10^6 cells g^{-1} liver was used for calculations of hepatic intrinsic clearance. Also much higher values, e.g. 120×10^6 cells g^{-1} liver, have been used; consequently the activity values presented here should be doubled.

Scaling from liver to in vivo

To calculate the hepatic organ clearance the well-stirred model including or excluding protein binding was applied with the following equation:

$$Cl_H = Q_H * Cl_{int} * F_u / Q_H + Cl_{int} * F_u,$$

where $Q_H = 1450$ ml min^{-1} and F_u is the unbound fraction; or

$$\text{Organ clearance } (CL_H) = Q_H * CL_{int}/(Q_H + CL_{int}).$$

The prediction of presystemic elimination was calculated on the basis of equation:

$$F_H = Q_H/(Q_H + CL_{int}),$$

where F_H is bioavailability. For the calculation of total body clearance and half-life, renal clearance was assumed to be negligible, human body weight was assumed as 70 kg and distribution volume V_D was taken from literature (= L kg^{-1} body weight). The equations used were as follows:

$$K_{el} = CL_{total}/V_D \text{ and } t_{1/2} = 0.693/K_{el}.$$

Results

Metabolite patterns and metabolic rates of CBZ in various in vitro *systems*

Metabolites found in the different in vitro *systems*. The formation of CBZ metabolites was studied in various *in vitro* systems and the results are summarized in table 2. CBZ 10,11-epoxide was the major metabolite in all *in vitro* systems. The corresponding diol metabolite was detected in

Table 2. Carbamazepine metabolites detected in the different *in vitro* systems. Percentages are only approximate and one must also take into consideration the interindividual variability in activities.

	Carbamazepine metabolites (% of total)				
System	CBZ-E	3-OH-CBZ	10,11-DIOL	9-AC	2-OH-CBZ
Liver microsomes	major (> 90%)	minor (< 10%)	trace	n.d.	n.d.
Liver slices	major (70-80%)	minor (1-3%)	minor (2%)	medium (15-25%)	minor (1-2%)
Hepatocytes	major (> 80%)	minor (1%)	minor (1-2%)	medium (5-15%)	trace
Recombinant enzymes	major (100%)	n.d.	n.d.	n.d.	n.d.
*In vivo**	major	minor	about 80% of epoxide	minor	minor

n.d., Not detected.
* Data from Faigle and Feldman (1995).

microsomes, slices and hepatocytes. In the recombinant enzyme system, only the epoxide metabolite was detectable. The lack of diol metabolite in recombinant enzyme system was presumably due to a lack of epoxide hydrolase, which is responsible of the formation of 10,11-diol (Kerr and Levy 1995) in this system. It is also possible that the formation rate of the minor metabolites may be too slow to produce detectable amounts in some *in vitro* systems. Hepatocytes produced one unidentified metabolite. More detailed results as well as some quantitative data are given below for each *in vitro* system (table 3).

Human liver microsomes. Metabolites were detected at a substrate concentration of ≥ 50 μM. 10,11-Epoxide was the major metabolite, 3-hydroxycarbamazepine was quantifiable and 10,11-dihydrodihydroxy-carbamazepine was present in trace amounts at ≥ 500 μM. Average formation rates were for CBZ 10,11-epoxide: 460 pmol mg^{-1} min^{-1}; for 3-hydroxy-carbamazepine: 10 pmol mg^{-1} min^{-1}, and for 10,11-dihydrodihydroxycarbamazepine: 5 pmol mg^{-1} min^{-1}. Assuming that the average formation of all metabolites is 500 pmol mg^{-1} min^{-1} and $K_m = 400$ μM, Cl_{int} for microsomes is 1.2 μl min^{-1} mg, Cl_{int} g^{-1} liver (assuming 50 mg microsomal protein g^{-1} liver) 60 μl g^{-1} min, and for the whole liver (assuming 1500 g) 90 ml min^{-1}.

Precision-cut human liver slices. CBZ metabolism was time-dependent and metabolite formation was linear with respect to incubation time up to 160 min. It was assumed that metabolite production was linear with respect to liver slice protein concentration. The following metabolites were detected (table 2 and figure 2): the 10,11-epoxide was the major metabolite at all time points measured (~70-80% of all the detectable metabolites at 1000 μM concentration and after 2.5 h of incubation). The amount of the 9-acridane metabolite was about a one-sixth to one-quarter (15-25%) and other metabolites, 10,11-diol, 3-hydroxy and 2-hydroxy (between 1 and 3%), were barely detectable at only longer incubation times and at higher concentrations of CBZ.

The kinetics of CBZ metabolism were estimated by first calculating the total (i.e. sum of all detected) metabolites and then performing kinetic calculations by use of Michaelis-Menten and Eadie-Hofstee plots. The estimated $K_m = \sim 360$ μM and $V_{max} = \sim 17$ pmol min^{-1} mg^{-1} liver slice whole homogenate protein. In the two individual studies, the values were similar. Assuming that 1 g liver contains 200 mg homogenate protein, CL_{int} was calculated to be 9.4 μl min^{-1} g^{-1} liver and ~ 14 ml min^{-1} per whole liver.

Human hepatocytes

Laboratory A. The 10,11-epoxide was the major metabolite in all cultures studied (table 2), representing > 75% of all the detectable metabolites. Other metabolites, including 3-hydroxy, 10,11-diol and 9-acridane, were formed in much lower quantities. Traces of 2-hydroxycarbamazepine were found in one experiment. Also an unidentified metabolite was present. CBZ has several metabolites

Table 3. Hepatic intrinsic clearances (based on disappearance of carbamazepine and/or appearance of metabolites) in various *in vitro* systems. K_m of S in parentheses are estimates. Carbamazepine has a relatively low plasma clearance *in vivo* in man (~ 27.5 ml min^{-1}) (Dollery 1991).

Candidate system	K_m or S^+ (μM)	V^+_{max} (averages)	Assumptions in extrapolations	Cli^+ (ml min^{-1})
Liver microsomes: disappearance	(500)	224 pmol min^{-1} mg^{-1} microsomal protein	50 mg microsomal protein g^{-1} liver	42
Metabolite formation	(400)	500 pmol min^{-1} mg^{-1} microsomal protein	50 mg microsomal protein g^{-1} liver	90
Liver slices: metabolite formation	300	17 pmol min^{-1} mg^{-1} slice whole homogenate protein	200 mg protein g^{-1} liver	15.8
Hepatocyles (laboratory A): disappearance	(170)	2.2 pmol min^{-1} mg^{-1} cell protein	67 million hepatocytes g^{-1} liver	18
Metabolite formation	(170)	0.4 pmol min^{-1} mg^{-1} cell protein	67 million hepatocytes g^{-1} liver	4
Hepatocytes (laboratory B): metabolite formation	362	457 pmol min^{-1} 10^6 cells	65 million hepatocytes g^{-1} liver	0.5
Recombinant CYP3A4: metabolite formation	250	1.3 pmol min^{-1} $pmol^{-1}$ P450	127 pmol CYP3A4 mg^{-1} microsomes	40
Recombinant CYP3A4+ b_5: metabolite formation	250	4.7 pmol min^{-1} $pmol^{-1}$ P450		160

$^+$Values are calculated on the basis of the following number of individual livers: microsomes, a pool of 10 livers; slices, two livers; hepatocytes, A: two livers; B, one liver; recombinants enzymes, one set of experiments.

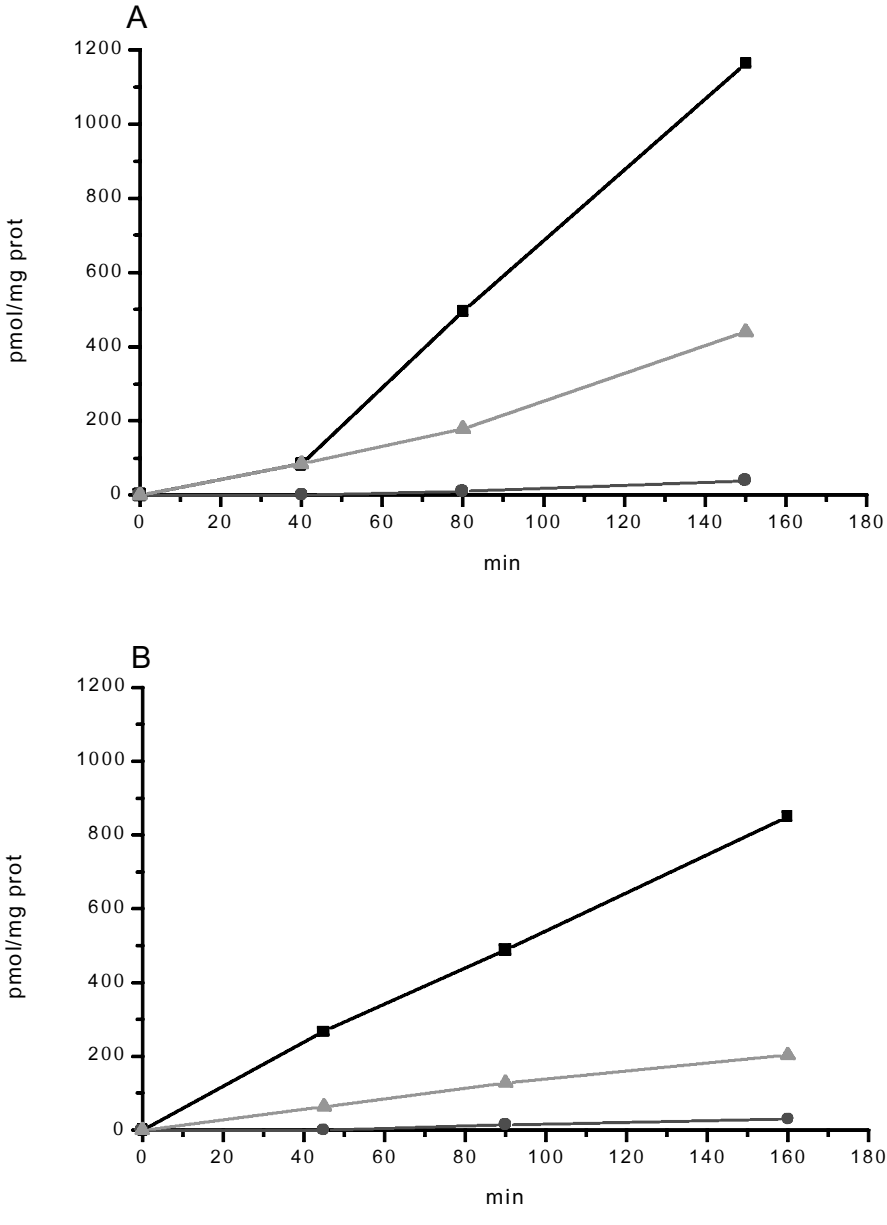

Figure 2. Formation of CBZ-E (■), 9-AC (▲) and 10,11-D (●) by human liver slices incubated with 500 μM CBZ. Slices from two livers were used and incubations are shown separately.

that were not analysed in this study and this unidentified peak most likely presents one of those metabolites.

Studies of control incubations indicated that CBZ does not extensively bind to plastic (data not shown). The amount of drug metabolized by human hepatocytes was fairly linear for 48 h. The appearance of metabolites was also linear (figure 3), but for unknown reasons the amount of metabolites produced was only ~ 20-25% of CBZ disappearance from the incubation. Therefore, the data for both drug

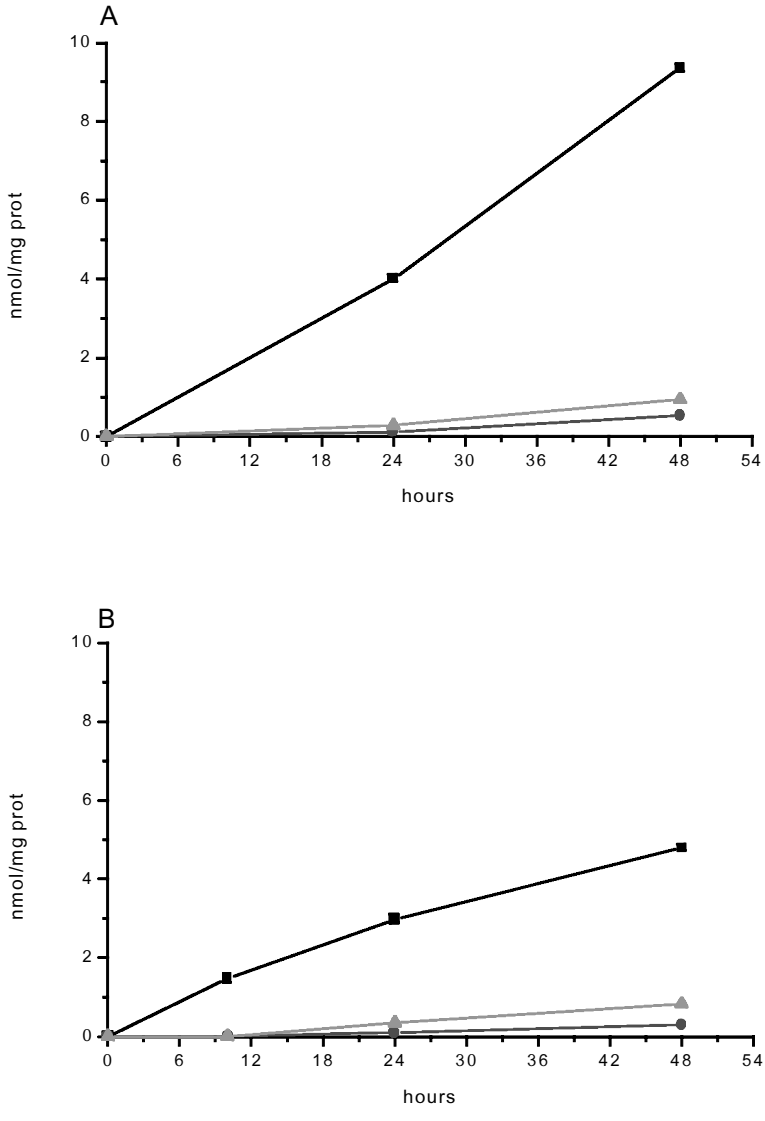

Figure 3. Formation of CBZ-E (■), 9-AC (▲) and 10,11-D (●) by hepatocytes incubated with 100 μM CBZ. Hepatocytes originating from two livers were used and they are presented separately.

disappearance and metabolite appearance were used for the determination of intrinsic clearances (table 3). Assuming that 1 g liver contains 65 million hepatocytes, hepatic intrinsic clearance was ~ 18 ml min^{-1} for disappearance and 4 ml min^{-1} for metabolite production.

Laboratory B. The 10,11-epoxide was the major metabolite in all cultures studied (table 2), representing > 80% of all the detectable metabolites. Other metabolites, 3-hydroxy, 10,11-diol and 9-acridane, were formed in much lower quantities. Traces of 2-hydroxycarbamazepine were found in a few of the experiments.

Incubations without cells were not done and intrinsic clearances were calculated only using the data for metabolite formation. Assuming that 1 g liver contains 65 million hepatocytes, hepatic intrinsic clearance was ~ 0.5 ml min^{-1} for metabolite production.

Recombinant expressed human CYP enzymes. Yeast microsomes were incubated with CBZ initially in the blind study. The conversion of the compound under the standard conditions used was, however, very low. No significant conversion was seen using standard conditions and the different recombinant microsomes. Addition of cytochrome-b_5 to the different microsomes revealed a significant, but low, conversion to the 10,11 epoxide in CYP3A4 containing microsomes where the reaction exhibited an apparent $K_m > 1000$ μM and $V_{max} = 0.6$-0.9 pmol 10,11-oxide min^{-1} and pmol P450 yielding a clearance = 8 ml min^{-1}.

At this point the identity of the substance was revealed and we tried to get conditions suitable for obtaining significant metabolism. It was hypothesized that the use of NADPH-cytochrome yeast reductase was not optimal for the reaction and yeast reductase was therefore replaced with recombinant human reductase. This had a substantial effect on catalysis and a velocity of 1.2 pmol pmol min^{-1} was obtained in the absence of cytochrome-b_5 and upon addition of cytochrome-b_5 V_{max} was calculated to be 4.7 pmol pmol min^{-1} and the apparent K_m was lower, namely 250 μM.

Comparison of clearance estimates in various in vitro *systems.* Intrinsic clearances, based on the disappearance of CBZ and/or appearance of metabolites, in various *in vitro* systems, as detailed above, were compared (table 3). Although values for microsomes, liver slices and hepatocytes differ quite considerably from each other, they nevertheless place CBZ pharmacokinetically into the class of low-clearance drugs. An interesting finding was the dependence of the metabolic activity of the recombinant CYP3A4 on the presence of cytochrome-b_5 and human versus yeast reductase: the activity was about four times higher in the presence of cytochrome-b_5 and a 20-fold increase in activity was seen when using human reductase instead of yeast reductase.

Identification of CYP enzymes with affinity and/or metabolic capacity in various in vitro *systems*

Molecular modelling: computer prediction of affinity of CBZ for CYP enzymes. CBZ was tested in models for CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. On the basis of the homology model calculations, CBZ is probably a substrate (or at least has affinity to) for CYP3A4 (figure 4B), CYP2C8 (figure 4C), CYP2C9 and, possibly, CYP1A2, in this order of preference with positions of metabolism as indicated in figure 4A. In each case, the substrate molecule is orientated for metabolism via a combination of hydrogen bond and π-stacking interactions.

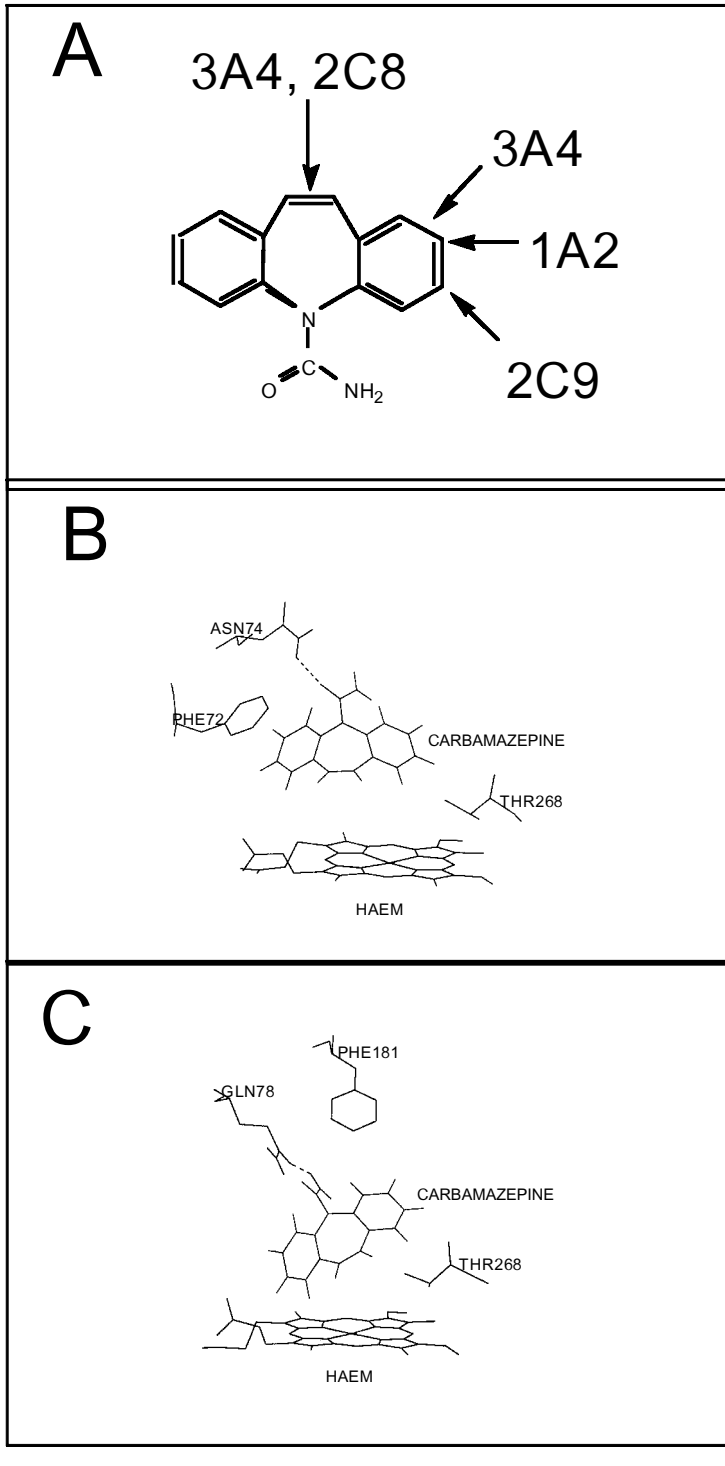

Figure 4. Computer modelling. (A) Positions of metabolisms for CBZ indicated by computer modelling. (B) Active site region of CYP3A4 containing the bound substrate, CBZ. (C) Active site region of CYP2C8 containing the bound substrate, CBZ.

Inhibition of CYP-selective model activities in human liver microsomes

The inhibitory effect of CBZ on CYP-specific model activities was screened by determining approximate IC_{50} (table 4). CBZ inhibited CYP2C19 most potently, with $IC_{50} = 37$ μM, and less potently with CYP2C9 and CYP3A4. CBZ had little or no effect on CYP1A2, CYP2A6, CYP2D6, and CYP2E1 activities. CYP2C8 was not studied because of the unavailability of a suitable marker enzyme assay.

Inhibition of microsomal metabolism of CBZ by CYP-specific inhibitors. The effect of various CYP-specific inhibitors on CBZ metabolism in human liver microsomes was studied (table 5). It has to be noted that the inhibitors might not be absolutely specific. First, a wide range of concentrations were employed for the determination of approximate IC_{50} and thereafter concentrations near known or slightly higher than the K_i for CYP-specific model activities were selected to measure the percentage of inhibition. The main enzyme involved in the formation of CBZ-10,11-epoxide appears to be CYP3A4, with possible minor contributions from CYP1A2, CYP2A6 and

Table 4. Effect of carbamazepine on CYP-model activities in human liver microsomes.

		IC_{50}/K_i (μM)		
CYP	Reference inhibitor	IC_{50} ($n = 2$)	K_i	Reference inhibitor IC_{50}
1A2	furafylline	> 1000		0.045
2A6	methoxsalen	> 1000		0.7
2C9	sulphaphenazole	226	90	4.1
2C19	omeprazole	37	8	67
2D6	quinidine	> 1000		0.41
2E1	pyridine	> 1000		4.7
3A4/5	ketoconazole	327	109	0.34

Table 5. Inhibition of microsomal metabolish of carbamazepine by CYP-specific inhibitors.

Target P450	Inhibitor	Highest concentrations tested (μM)	CBZ-E formation (% inhibition)	3-OH-CBZ formation (% inhibition)	10,11-D formation (% inhibition)
1A2	furafylline	5	26	41	5-30
2A6	coumarin	50	33	69	*
2C8	quercetin	30	42	36	n.q.
2C19	*S*-mephenytoin	500	negligible	negligible	n.q.
2D6	quinidine	20	35	44	n.q.
2E1	pyridine	20	negligible	negligible	n.q.
3A4	ketoconazole	20	94	27	n.q.

All inhibitors were added in methanol (1%), except pyridine, which was added in water.
n.q., quantification not possible.
* Stimulation.
Data are the average of six determinations.

CYP2C8. The main enzyme involved in the formation of 3-hydroxycarbamazepine appears to be CYP2A6, with smaller contributions from CYP1A2, CYP2C8, CYP2D6 and CYP3A4. Levels of 10,11-dihydrodiol were such that quantification was difficult, and no conclusions could be reached regarding specificity of inhibition.

Correlation study. The following correlations between CBZ metabolite formation and amount/activity of various CYPs in nine human liver samples are presented in table 6. It seems evident that only CYP3A4 is involved in the formation of CBZ-E. There is evidence for the involvement of CYP2A6 and to a lesser extent CYP2D6 in the formation of 3-OH-CBZ. The formation of 10,11-D, a secondary metabolite, was detected with only three samples and there was some evidence for CYP2C8 and CYP2C19 in its formation.

The overall conclusion with respect to specificity from the microsomal studies is that CBZ 10,11-epoxide is produced by CYP3A4, with possible minor contributions from CYP1A2, CYP2A6 and CYP2C8. 3-Hydroxycarbamazepine appears to be produced by CYP2A6 and CYP2D6, with smaller contributions from CYP1A2, CYP2C8 and CYP3A4. 10,11-Dihydrodiol appears to be produced by CYP2C8 and CYP2C19.

Recombinant expressed human CYP enzymes. All recombinantly expressed isoforms were incubated with 1 mM CBZ. Only CYP3A4 formed appreciable amounts of any metabolite as described above (table 2).

Induction potential of CBZ in human hepatocytes

The potential effects of CBZ on CYP induction were examined after 48-72 h incubation, by measuring 7-ethoxycoumarin *O*-deethylase activity, after incubating hepatocytes for 48-72 h with increasing concentrations of CBZ up to 250 μM. CBZ produced a concentration-dependent induction (table 7). The concentration causing maximal induction, ~2-fold, in these conditions appeared to be between 25 and 75 μM.

Table 6. Significant correlations between carbamazepine metabolite formation and amount/activity of various CYPs in nine human liver samples.

Metabolite	Correlation with enzyme	Corresponding enzyme (CYP)	Correlation with immunohistochemically determined CYP content
CBZ-E	midazolam 1′-hydroxylase activity ($r = 0.915, p < 0.001$)	3A4	3A4 ($r = 0.736, p < 0.01$)
10,11-D	*S*-mephenytoin 4-hydroxylase activity ($r = 0.843, p < 0.02$)	2C19	total P450 ($r = 0.807, p < 0.01$) 2C8 ($r = 0.755, p < 0.05$)
3-OH-CBZ	coumarin 17-hydroxylase activity ($r = 0.765, p < 0.02$)	2A6	2A6 ($r = 0.839, p < 0.01$) 2D6 ($r = 0.695, p < 0.05$)

Table 7. Effect of carbamazepine on 7-ethoxycoumarin *O*-deethylase activity in human cultured hepatocytes. Control activities in four individual cultures studied ranged from 2.4 ± 0.05 to 4.41 ± 0.8 pmol min^{-1} mg^{-1}.

Carbamazepine concentration (μM)	*n*	7-Ethoxycoumarin *O*-deethylase activity (% control culture)
5	1	159
10	2	135
25	3	167
50	1	207
75	3	205
125	2	193
250	3	207

Discussion

Elucidation of metabolism and kinetics (microsomes, liver slices, hepatocytes, recombinant enzymes)

Detection and formation rates of metabolites. In plasma and urine of patients, CBZ-10,11-epoxide is the major metabolite. All the systems studied produced the major *in vivo* metabolite, and in this respect well predicted the *in vivo* situation. Some other minor metabolites were detectable in liver microsomes, liver slices and hepatocytes, roughly in the proportions found *in vivo*. In recombinant enzymes, only the epoxide metabolite could be produced (by CYP3A4). In hindsight, it seems probable the inability of the recombinant system to detect other minor metabolites might be due to a slow metabolic turnover of CBZ.

Metabolic rate in various in vitro systems. CBZ is typically a slow-clearance drug with a half-life of 18-55 hours. Hepatic intrinsic clearances calculated were in most systems clearly < 100 ml min^{-1}, predicting low extraction and slow clearance. The only exception was the recombinant CYP3A4 enzyme fortified with cytochrome-b_5, which produced a clearance $= \sim 160$ ml min^{-1}. However, the omission of cytochrome-b_5 led to a value of one-quarter of the above value, i.e. 40 ml min^{-1}.

Substrate disappearance corresponded to the formation of metabolites in various systems, but not in every case. In hepatocytes, the disappearance of CBZ itself was much larger than the appearance of the metabolites. The reason for this discrepancy is not known, but it could have been due to the binding of CBZ to the incubation vessels, but this was not apparent in the liver slice system (or in microsomes).

Regarding, prediction ability, microsomes, slices and hepatocytes were reasonably accurate in the prediction of the *in vivo* situation. The recombinant system, although predicting the major metabolite, gave predictions which were higher than with the other systems and clearly needs further refinement.

Determination of enzymes responsible for substrate affinity and metabolism (molecular modelling, microsomes, recombinant enzymes)

On the basis of extensive *in vivo* studies in association of various *in vitro* investigations, the major metabolizing CYP enzyme is CYP3A4, which catalyses the major 10,11-epoxidation of CBZ (Kerr *et al.* 1994). This enzyme and reaction were correctly identified by all the systems studied. It is of interest that while microsomes and computer modelling predicted possible minor participants for this and other (less important) metabolic routes, the recombinant system identified only CYP3A4. A possible explanation for this 'insensitivity' (if it is indeed so, because it is difficult to prove at the present moment, whether the predictions of other systems as to minor participants and metabolic routes are actually true or only apparent or artificial) is that CBZ is a slowly metabolized substance and would need some refinement of the system. From the *in vivo* point of view, it is assuring that all the *in vitro* systems predicted the major enzyme catalysing the most important pathway.

CYP2C8 as a minor contributor to the 10,11-epoxidation was implicated by computer modelling and by microsomal inhibitor and correlation studies, but not by the use of recombinant enzymes. The study of Kerr *et al.* (1994) has convincingly demonstrated the minor role of CYP2C8. The inability of recombinant CYP2C8 to catalyse 10,11-epoxidation might be due to the low formation rate, but may also be related to the artificial nature of the recombinant system.

Inhibitor and correlation studies in liver microsomes indicated CYP2A6 as a main enzyme for the formation of 3-hydroxycarbamazepine, with a minor contribution from the other four CYPs. However, CBZ at the maximum concentration of 1 mM did not inhibit microsomal coumarin 7-hydroxylation and the recombinant CYP2A6 did not catalyse the formation of this metabolite. Again, it is probable that the slow metabolic rate is responsible for this discrepancy between different systems.

Prediction of induction potential

CBZ is one of the 'classical' *in vivo* inducers of several CYP-related activities. In this study, isolated hepatocytes was the only system in which induction potential was studied. A 2-day exposure of cells to CBZ led to a 2-3-fold induction of the marker activity 7-ethoxycoumarin *O*-deethylase, thus correctly predicting the *in vivo* induction potential of this substance (Levy and Wurden 1995). It has recently been demonstrated that long-term culturing of liver slices in the presence of potential inducers would lead to an increase of CYP-associated activities (Lake *et al.* 1996, 1997, 1998). However, in this survey we did not employ the liver slice system in this manner.

The detection of induction potential of CBZ by cultured hepatocytes would certainly have led to a more detailed scrutiny of this property of the compound (if it were an NCE) and revelation of the CYP3A4 induction. Induction is involved in many important *in vivo* interactions caused by CBZ (Levy and Wurden 1995), and consequently, cultured hepatocytes correctly predicted this phenomenon. It is also probable that liver slices would have been predicted the induction potential of CBZ (see above).

Prediction of potential inhibitory interactions

All systems demonstrated that CYP3A4 is the principal CBZ metabolizing enzyme in human liver systems. From the drug interaction point of view, it seems unlikely that CBZ itself would cause problems because its K_m for CYP3A4, $\geq$ 400 μM, is much higher than the highest total plasma concentrations achieved, $\sim$ 40-80 μM (Morselli 1995). A high degree of accumulation in the liver should be assumed if significant interactions caused by CBZ would be implicated. On the other hand, the interference by potent CYP3A4 inhibitors of CBZ metabolism is much more likely and has actually been demonstrated *in vivo* in a number of instances for known CYP3A4 substrates such as calcium-channel blocking agents and macrolide antibiotics (Levy and Wurden 1995).

It is of interest that CBZ inhibited CYP2C19 and CYP2C9 activities at lower concentrations than CYP3A4. These enzyme were not implicated, at least prominently, by any other candidate system. However, a high affinity for CYP2C19, if indeed true, would possibly lead to CBZ associated interactions with compounds metabolized by CYP2C19 such as omeprazole and imipramine (Pelkonen *et al.* 1998). The effect of CBZ on omeprazole metabolism has also been studied *in vivo*. CBZ induced CYP3A4 catalysed sulphoxidation of omeprazole but had no or less effect on CYP2C19 catalysed hydroxylation (Bertilsson *et al.* 1997).

As stated above, specific aspects of *in vivo* pharmacokinetics are often reasons for drug failure and there is a need for *in vitro* systems to be better able to predict the *in vivo* data. With respect to CBZ, hepatocytes predicted the induction potential of CBZ. All systems predicted the slow clearance of the compound, although there was quite a large variability (which, incidentally, is also true for the *in vivo* situation). All systems predicted the major enzyme, CYP3A4, was responsible for catalysing the principal metabolic pathway, the 10,11-epoxidation of CBZ. This finding would certainly have led to studies on the reactivity of this epoxide metabolite, if CBZ had really been an NCE. Minor metabolites and less important enzymes catalysing both the principal and less important pathways were variously predicted by different systems, which may related to the relatively low formation of these metabolites resulting in sensitivity problems. In summary, whatever the system, important qualitative and quantitative aspects of CBZ pharmacokinetics were predicted, and the next task should be the refinement of various systems as to their predictive power.

Acknowledgements

The authors acknowledge the contributions of the other members of the EUROCYP project: Dieter Paul, Rainer Klocke (Fraunhofer Institute, Hannover, Germany), Tommy B. Andersson (AstraZeneca, Mölndal, Sweden), Jarmo S. Salonen (Orion Pharma, Turku, Finland), and Gisela Backfisch (Boehringer-Mannheim, Germany). They also thank Novartis for providing carbamazepine, oxcarbazepine and their metabolites.

References

Aitio, A., 1978, A simple and sensitive assay of 7-hydroxycoumarin deethylation. *Annals of Biochemistry*, **85**, 488-491.

Andersson, T. B., Sjöberg, H., Hoffman, K.-J., Boobis, A. R., Watts, P., Edwards, R. J., Lake, B. G., Price, R. J., Renwic, A. B., Gómez-Lechón, M. J., Castell, J. V., Ingelman-Sundberg, M., Hidestrand, M., Goldfarb, P., Lewis, D., Corcos, L., Guillouzo, A., Taavitsainen, P. and Pelkonen, O., 2001, An assessment of human liver-derived *in vitro* systems to predict the *in vivo* metabolism and clearance of Almokalant, *Drug Metabolism and Disposition*, **29**, 712-720.

Beamand, J. A., Price, R. J., Cunnigham, M. E. and Lake, B. G., 1993, Culture of precision-cut liver slices: effect of some peroxisome proliferators. *Food and Chemical Toxicology*, **31**, 137-147.

Bertilsson, L., Tybring, G., Widen, J., Chang, M. and Tomson, T., 1997, Carbamazepine treatment induces the CYP3A4 catalysed sulphoxidation of omepratzole, but has no or less effect on hydroxylation via CYP2C19. *British Journal of Clinical Pharmacology*, **44**, 186-189.

Beyer, C. and Spiteller, G., 1992, A new carbamazepine metabolite in uraemic filtrate. *Xenobiotica*, **22**, 1029-1035.

Borenfreund, H., Babich, H. and Martin-Alguacil, N., 1988, Comparisons of two *in vitro* cytotoxicity assays — the neutral red (NR) and tetrazolium MTT tests. *Toxicology In Vitro*, **2**, 1-6.

Bradford, M. M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry*, **72**, 248-254.

Burke, M. D., Prough, R. A. and Mayer, R. T., 1977, Characteristics of a microsomal cytochrome P-448-mediated reaction ethoxyresorufin O-deethylation. *Drug Metabolism and Disposition*, **5**, 1-8.

Dollery, C., 1991, Carbamazepine. In C. Dollery (ed.), *Therapeutic Drugs* (Edinburgh: Churchill Livingstone), pp. C49-53.

Donato, M. T., Castell, J. V. and Gómez-Lechón, M. J., 1995, Effect of model inducers on cytochrome P450 activities of human hepatocytes in primary cultures. *Drug Metabolism and Disposition*, **23**, 553-558.

Edwards, R. J., Adams, D. A., Watts, P. S., Davies, D. S. and Boobis, A. R., 1998, Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. *Biochemical Pharmacology*, **56**, 377-387.

Faigle, J. W. and Feldman, K. F., 1995, Carbamazepine. Chemistry and biotransformation. In R. H. Levy, R. H. Mattson and B. S. Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 499-513.

Gómez-Lechón, M. J., Donato, M. T., Ponsoda, X., Fabra, R., Trullenque, R. and Castell, J. V., 1997, Isolation, culture and use of human hepatocytes in drug research. In J. V. Castell and M. J. Gómez-Lechóen (eds), *In Vitro Methods in Pharmaceutical Research* (London: Academic Press), pp. 129-154.

Guguen-Guillouzo, C., Campion, J. P., Brissot, P., Glaise, D., Launois, B., Bourel, M. and Guillouzo, A., 1982, High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver. *Cell Biology and International Reports*, **6**, 625-628.

Houston, J. B. and Carlile, D. J., 1997, Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metabolism Reviews*, **129**, 891-922.

Ingelman-Sundberg, M. and Johansson, I., 1980, Cytochrome b_5 as electron donor to rabbit liver cytochrome P-450 in reconstituted phospholipid vesicles. *Biochemistry and Biophysical Research Communications*, **97**, 582-589.

Ito, K., Iwatsubo, T., Kanamitsu, S, Nakajima, Y. and Sugiyama, Y., 1998, Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annual Reviews in Pharmacology and Toxicology*, **38**, 461-499.

Kennedy, T., 1997, Managing the drug discovery/development interface. *Drug Discovery Today*, **2**, 436-444.

Kerr, B. M. and Levy, R. H., 1995, Carbamazepine. Carbamazepine epoxide. In R. H. Levy, R. H. Mattson and B. S Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 529-541.

Kerr, B. M., Thummel, K. E., Wurden, C. J., Klein, S. M., Kroetz, D. L., Gonzalez, F. J. and Levy, R. H., 1994, Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. *Biochemical Pharmacology*, **47**, 1969-1070.

Knodell, R. G., Hall, S. D., Wilkinson, G. R. and Guengerich, F. P., 1987, Hepatic metabolism of tolbutamide: Characterization of the form of cytochrome P-450 involved in methyl hydroxylation and relationship to *in vivo* disposition. *Journal of Pharmacology and Experimental Therapeutics*, **241**, 1112-1119.

Kronbach, T., Mathys, D., Gut, J., Catin, T. and Meyer, U. A., 1987, 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. *Annals of Biochemistry*, **162**, 24-32.

Krumdieck, C. L., dos Santos, J. E. and Ho, K. J., 1980, A new instrument for the rapid preparation of tissue slices. *Annals of Biochemistry*, **104**, 118-123.

Krynetski, E. Y., Drutsa, V. L., Kovaleva, I. E. and Luzikov, V. N., 1995, High yield expression of functionally active human liver CYP2D6 in yeast cells. *Pharmacogenetics*, **5**, 103-109.

Lake, B. G., Ball, S. E., Renwick, A. B., Tredger, J. M., Kao, J., Beamand, J. A. and Price, R. J., 1997, Induction of CYP 3A isoforms in cultured precision-cut human liver slices. *Xenobiotica*, **27**, 1165-1173.

Lake, B. G., Tredger, J. M., Renwick, A. B., Barton, P. B. and Price, R. J., 1998, 3,3′-Diindoloylmethane induces CYP1A2 in cultured precision-cut human liver slices. *Xenobiotica*, **28**, 803-811.

Lertratanangkoon, K. and Horning, M. G., 1982, Metabolism of carbamazepine. *Drug Metabolism and Disposition*, **10**, 1-10.

Levy, R. H. and Wurden, C. J., 1995, Carbamazepine. Interactions with other drugs. In R. H. Levy, R. H. Mattson and B. S. Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 543-554.

Lewis, D. F. V., 1996, *Cytochromes P450: Structure, Function and Mechanism* (London: Taylor & Francis).

Lewis, D. F. V., Dickins, M., Eddershaw, P. J., Tarbit, M. H. and Goldfarb, P. G., 1999, Cytochrome P450 substrate specificities, substrate structural templates and enzyme active site geometries. *Drug Metabolism and Drug Interactions*, **15**, 1-49.

Li, H. and Poulos, T. L., 1997, The structure of the cytochrome p450BM-3 haem domain complexed with the fatty acid substrate, palmitoleic acid. *Nature Structural Biology*, **4**, 140-146.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.

Morselli, P. L., 1995, Carbamazepine. Absorption, distribution and excretion. In R. H. Levy, R. H. Mattson and B. S. Meldrum (eds), *Antiepileptic Drugs*, 4th edn (New York: Raven), pp. 515-528.

Oscarson, M., Hidestrand, M., Johansson, I. and Ingelman-Sundberg, M., 1997, A combination of mutations in the CYP2D6*17 (CYP2D6Z) allele causes alterations in enzyme function. *Molecular Pharmacology*, **52**, 1034-1040.

Park, Y. H., Kullberg, M. P. and Hinsvark, O. N., 1984, Quantitative determination of dextromethorphan and three metabolites in urine by reverse-phase high-performance liquid chromatography. *Journal of Pharmacological Science*, **73**, 24-29.

Pelkonen, O., Mäenpää, J., Taavitsainen, P., Rautio, A. and Raunio, H., 1998, Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, **28**, 1203-1253.

Peter, R., Bocker, R., Beaune, P. H., Iwasaki, M., Guengerich, F. P. and Yang, C. S., 1990, Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chemical Research in Toxicology*, **3**, 566-573.

Pienimäki, P., Fuchs, S., Isojärvi, J. and Vähäkangas, K., 1995, Improved detection and quantitation of carbamazepine and oxcarbazepine and their metabolites by high-performance liquid chromatography. *Journal of Chromatography B*, **673**, 97-105.

Prentis, R. A., Lis, Y. and Walker, S. R., 1988, Pharmaceutical innovation by the seven UK-owned pharmaceutical companies 1964-1985. *British Journal of Clinical Pharmacology*, **25**, 387-396.

Raunio, H., Syngelmä, T., Pasanen, M., Juvonen, R., Honkakoski, P., Kairaluoma, M. A., Sotaniemi, E., Lang, M. A. and Pelkonen, O., 1988, Immunochemical and catalytical studies on hepatic coumarin 7-hydroxylase in man, rat and mouse. *Biochemical Pharmacology*, **37**, 3889-3895.

Raunio, H., Valtonen, J., Honkakoski, P., Lang, M. A., Stahlberg, M., Kairaluoma, M. A., Rautio, A., Pasanen, M. and Pelkonen, O., 1990, Immunochemical detection of human liver cytochrome P450 forms related to phenobarbital-inducible forms in the mouse. *Biochemical Pharmacology*, **40**, 2503-2509.

Ravichandran, K. G., Boddupalli, S. S., Hasermann, C. A., Peterson, J. A. and Deisenhofer, J., 1993, Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science*, **261**, 731-736.

Rodrigues, A. D., 1994, Use of *in vitro* human metabolism studies in drug development. An industrial perspective. *Biochemical Pharmacology*, **48**, 2147-2156.

Rodrigues, A. D., 1999, Integrated cytochrome P450 reaction phenotyping. Attempting to bridge the gap between cDNA-expressed cytochromes p450 and native human liver microsomes. *Biochemical Pharmacology*, **57**, 465-480.

Shen, A. L., Porter, T. D., Wilson, T. E. and Kasper, C. B., 1989 Structural analysis of the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase by site-directed mutagenesis. *Journal of Biological Chemistry*, **264**, 7584-7589.

Sullivanklose, T. H., Ghanayem, B. I., Bell, D. A., Zhang, Z.Y., Kaminsky, L. S., Shenfield, G. M., Miners, J. O., Birkett, D. J. and Goldstein, J. S., 1996, The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics*, **6**, 341-349.

Tomson, T., Tybring, G. and Bertilsson, L., 1983, Single-dose kinetics and metabolism of carbamazepine-10,11-epoxide. *Clinical Pharmacology and Therapy*, **33**, 58-65.

Truan, G., Cullin, C., Reisdorf, P., Urban, P. and Pompon, D., 1993, Enhanced *in vivo* monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b_5. *Gene*, **125**, 49-55.

Waxman, D. J., Lapenson, D. P., Aoyama, T., Gelboin, H. V., Gonzales, F. J. and Korzekwa, K., 1991, Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Archives in Biochemistry and Biophysics*, **290**, 160-166.

Worboys, P. D., Bradbury, A. and Houston, J. B., 1995, Kinetics of drug metabolism in rat liver slices. Rates of oxidation of ethoxycoumarin and tolbutamide, examples of high- and low-clearance compounds. *Drug Metabolism and Disposition*, **23**, 393-397.

Wrighton, S. A., Stevens, J. C., Becker, G. W. and VandenBranden, M., 1993, Isolation and characterization of human liver cytochrome P450 2C19: correlation between 2C19 and S-mephenytoin 4′-hydroxylation. *Archives of Biochemistry and Biophysics*, **306**, 240-245.