Genetic Variability of an Uridine-diphosphoglucuronosyltransferase: UGT2B7

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy. Graduate Department of Pharmacology. University of Toronto

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Genetic Variability of an Uridine-diphosphoglucuronosyltransferase: UGT2B7
Mayank Patel
Ph.D. thesis (1997), Pharmacology, University of Toronto

Abstract

Reports of genetically based variations in drug glucuronidation have essentially focused on their relation to Gilbert's Syndrome and Crigler-Najjar type I and II syndromes, which have been linked to abnormalities in the UGT1 gene complex. To date, information about the genetic variability of other UGT isozymes has been limited. Oxazepam is one of a number of C-3 hydroxylated benzodiazepines for which glucuronide conjugation is the predominant pathway of biotransformation. The drug is normally formulated as a racemic mixture of inactive (R), and active (S), enantiomers.

1. Inhibition studies suggested that the two diastereomeric glucuronidations are catalysed by different UGT isozymes. The inhibition profile of (S)oxazepam glucuronidation suggests that UGT2B7 is the catalysing enzyme.

2. a) In vivo, the (S) glucuronide was preferentially formed and excreted in nine of eleven subjects. The ratios of (S) to (R) glucuronide metabolites (S/R ratios) were 3.87 ± 0.79 (mean ± s.d.) and 3.52 ± 0.60 in urine and plasma, respectively. However, both ratios were significantly lower in two subjects (p < 0.01). In these two atypical subjects, the half-life of (R,S) oxazepam was also markedly longer (14.7 and 15.9 h) than in the other subjects (8.1 ± 3.2 h). In 66 additional subjects, the S/R glucuronide ratio was found to be bimodally distributed, with ~10% of all subjects possessing ratios below an apparent antimode of 2.0.

b) In vitro formation of oxazepam glucuronides by microsomes from four of 37
Caucasian livers were characterized by low S/R glucuronide ratios, and abnormally high $K_m$ values for the formation of the (S) glucuronide, but not of the (R) glucuronide.

3. a) First-strand cDNA was synthesized from the mRNA of each liver by reverse-transcription. RFLV analyses and nucleotide sequencing showed that three of the four atypical livers were positive for a point mutation at position 802 (T for C) of the UGT2B7 cDNA coding region. The mutation results in the substitution of histidine for tyrosine at residue 268 of the UGT2B7 enzyme. The three individuals were heterozygous for the mutation. Full-length sequence analysis for the fourth atypical liver, showed no deviations from the wild type sequence of UGT2B7.

b) Expression studies in COS-1 cells showed that the $V_{max}$ of glucuronidation of (S)oxazepam was greatly diminished with the variant form of UGT2B7. Glucuronidation efficiencies of estriol and 2-OH-estriol were also significantly reduced, whereas the glucuronidation of ketoprofen, (S)naproxen, and hyodeoxycholic acid were unaffected.
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<th>Description</th>
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<tbody>
<tr>
<td>4-OH-P</td>
<td>4-hydroxypropranolol</td>
</tr>
<tr>
<td>AGP</td>
<td>alpha 1-acid glycoprotein</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CN 1, CN 2</td>
<td>Crigler-Najjar Syndromes 1 and 2</td>
</tr>
<tr>
<td>CPQ-carboxylic acid</td>
<td>6-chloro-4-phenyl-2(1H)-quinazolinecarboxylic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>dideoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>dideoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>dideoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dTTP</td>
<td>dideoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis-Menten hyperbolic rate constant</td>
</tr>
<tr>
<td>MuLV</td>
<td>Murine Leukemia Virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>naphtho oxylactic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotine adenine diphosphonucleotide, reduced</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RFLV</td>
<td>restriction fragment length variation</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
</tbody>
</table>
TLC  thin layer chromatography
UDPGA  uridine diphospho-D-glucuronic acid
UGT  uridine-diphospho-glucuronosyltransferases
UV  ultraviolet
V_{max}  maximal Michaelis-Menten velocity estimate
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1. INTRODUCTION
1. UDP-GLUCURONOSYLTRANSFERASES

Background

Glucuronidation is quantitatively one of the most important of the so-called Phase II (conjugative) biotransformation pathways, since it has profound effects on the disposition of a large number of xenobiotics as well as that of numerous endogenous compounds (Mulder et al., 1989; Miners and Mackenzie, 1991). Bilirubin, a product of heme catabolism, is the most extensively studied UGT substrate. At high concentrations, it has been shown to elicit cellular damage, making its elimination from the body critical. Glucuronidation represents the major pathway of clearance for a number of drugs, including morphine, oxazepam, lorazepam, propofol, naloxone, and zidovudine. Xenobiotic carcinogens, environmental pollutants, and plant constituents are also known substrates for glucuronidation. Uridine-diphospho-glucuronosyltransferases (UGTs), are located in the endoplasmic reticulum and nuclear membrane of hepatocytes, and also in extrahepatic tissues such as kidney and intestine. In general, they convert a large number of hydrophobic endo- and xenobiotics into inactive water-soluble products. However, this is not the sole activity of UGTs.

Recently, several odorants (eg. borneal, 2-ethyl-1-hexanol, guaicol, eugenol) have been determined to be substrates of rodent and bovine olfactory-specific UGTs (Lazard et al, 1990; Burchell, 1991). Glucuronidation of such odorants prevents them from stimulating adenylate cyclase activity, thus ceasing an olfactory response (Lazard et al, 1991, Burchell, 1991). In contrast, 3α,17α-dihydroxy-5β-pregn-20-one-3-glucuronide, a male piscine pheromone, has
been demonstrated to be an extremely potent odorant (Clark et al, 1991; Lambert and Resink, 1991). Glucuronides appear to be involved in both the termination and initiation of olfactory responses.

Extract from embryonic chicken brain has been shown to contain a UGT which transfers glucuronic acid from UDP-glucuronic acid (UDPGA) to glycolipid (neolactotetraosyl ceramide) and glycoprotein acceptors (Oka et al., 1992). Chou et al, (1991) have suggested that the carbohydrate moieties of these lipids are involved in cell-cell interactions and cellular adhesion in the cerebellum, and in myelinogenesis.

Biochemistry

The glucuronidation reaction itself involves the enzyme-catalysed addition of D-glucuronic acid, supplied by the essential cofactor uridine diphospho-D-glucuronic acid (UDPGA), to a suitable atom (often oxygen, nitrogen, or less commonly, carbon) on an acceptor substrate to produce a beta-D-glucuronic conjugate. Studies indicate that the reaction most likely occurs via a random order sequential mechanism. It was found that electron donating groups increased the rate of glucuronide conjugation. This result is consistent with nucleophilic attack of the C-1 carbon of the UDPGA by an SN2 mechanism (Yin et al., 1994).

The enzymes capable of mediating this reaction are membrane-bound (microsomal) proteins termed uridine-diphosphoglucuronosyltransferases (UGTs). The hepatic endoplasmic reticulum (ER) contains several well characterized UGTs, membrane-bound proteins of 50-54 kDa, and also less well identified UGTs, with nucleotide binding sites located on the luminal surface. There is evidence that the substrates for these enzymes, UDPGA, biosynthesized in the
cytosol, are transported into the lumen of the ER via unknown mechanisms. Data suggest the presence, in rat liver microsomal vesicles, of a specific, carrier-mediated transport process for UDPGA (Radomsinska et al., 1994).

Drake et al. (1992), determined the active site orientation of microsomal glucuronosyltransferases with the use of the photoaffinity analog [32P]5-azido-UDPGA. Photolabeled UDP-glucuronosyltransferases were only susceptible to trypsin digestion in disrupted vesicles, indicating a luminal orientation of UDP-glucuronosyltransferases. Cytosylglucuronic acid synthase, a prokaryotic UGT, differs from eukaryotic UGTs in being a soluble protein with no apparent phospholipid requirement (Gould and Guo, 1994).

Primary sequence analysis has demonstrated the presence of a single stretch of 17 amino acids, located near the carboxy-terminus, which is capable of crossing a lipid bilayer (Sabatini et al, 1982). This transmembrane domain is present in all eukaryotic UGTs.

A sufficient supply of UDPGA is required for glucuronidation. In rats, UDPGA levels have been measured to be close to the $K_m$ values of UDPGA for UGTs (Ullrich and Bock, 1984). Therefore, glucuronidation is affected by decreased nucleotide levels after treatment with D-galactosamine (Otani et al, 1976) or ethanol (Moldeus et al, 1978).

Phylogeny

The UGT enzymes comprise a large family of structurally related isozymes with distinct but often overlapping acceptor substrate selectivities. Initial classification of UGT multiplicity had been based upon differential developmental regulation, response to enzyme inducers, or
Figure 1.1. UGT phylogeny (adapted from Burchell et al, 1990). Human forms are noted in black, and the corresponding trivial names are listed to the right. Additional members of the human UGT1 family have been identified (see Figure 1.2). Evolutionary distance is noted in arbitrary units.
tissue-selective expression (Burchell & Coughtrie, 1989). Antisera to rat bilirubin/phenol UGT and testosterone/phenol UGT isoforms have been shown to cross-react strongly with partially purified piscine UGT isoforms with molecular masses of 52, 53 and 57 kDa (Clarke et al., 1992). Thus, fish and mammalian UDPGTs apparently possess a high degree of evolutionary conservation.

Molecular techniques have led to the cloning and expression of a large number of distinct UGT gene products from several species, which has led to a systematic classification system based upon amino acid sequence homology (Figure 1.1; Burchell et al., 1991). The UGTs have thus been divided into two major gene families, UGT1 and UGT2. In the UGT1 family, alternative exon splicing has been proposed to produce multiple products from a single gene (Figure 1.2). These isoforms have identical 246 amino acid 3' carboxyl-terminal domains that are encoded by four consecutive exons which are shared by all UGT1 isoforms. The amino-terminal domains of the UGT1 isoforms are unique, each being encoded by a different 5' exon (Bosma et al., 1994). Several of the UGT1 isoforms are efficient catalysts of bilirubin glucuronidation.

Within the UGT2 family, the UGT2A subfamily contains enzymes that are expressed specifically in olfactory epithelium, while the UGT2B subfamily contains a large number of orthologues (among several species investigated) that can conjugate a great variety of drugs as well as endogenous substrates (Burchell et al., 1994). They are often termed steroid UGTs although they are clearly involved in the conjugation of a diverse array of substrates.

To date, eleven human UGT isozymes have been identified and characterized by transient expression in COS cells, or stable expression in V79 and HEK 293 fibroblasts. The human UGT1 gene complex has been localized to position 2q37 (on the long arm of chromosome 2; Brierly and
Burchell, 1993). Several human UGT2B genes have been assigned to chromosome 4 (Monaghan et al., 1994). Murine genes, encoding the UGT1 complex and a UGT2B ortholog, are present on chromosomes 1 and 5 respectively (Miles et al., 1991). These murine chromosomes are known to contain syntenic regions with the aforementioned human chromosomes (Nadeau and Reiner, 1989).

Specificity

Due to the misconception that UGTs are non-specific, the potential significance of a given UGT is often overlooked. According to Burchell and Coughtrie (1992; pp. 219), "The efficiency of drug glucuronidation and overlapping specificity of UGTs catalysing glucuronidation has probably 'masked' the non-deleterious existence of polymorphic variation of these enzymes in humans". A list of substrates glucuronidated by each of 12 different cDNA-expressed human UGTs has been tabulated (Table 1.1). This list does not permit many conclusions (e.g. 4-hydroxyestosterone is listed as a substrate of four different UGTs, ethynylestradiol of two UGTs). Acetaminophen and ketoprofen are each listed as a substrate of one UGT. It is likely that the number of UGTs participating in the glucuronidation of a given substrate varies with the substrate.

Species-differences in UGT specificity only make the picture murkier. Morphine-3-glucuronidation has been shown to be catalyzed by UGT2B (Pritchard et al., 1994) and UGT1 orthologues in rats (Ishii et al., 1994), but only by UGT2B orthologues in dogs (Oguri et al., 1996). El Mouelhi et al., (1993) reported that rat UGT1A1 conjugates (R) naproxen at a much higher rate (> 17-fold) than its (S) enantiomer. In contrast, the human orthologue conjugated
Figure 1.2. Structure of the UGT1 gene locus, located on human chromosome 2. Exons 2 to 5 are shared between UGT1 isoenzymes.

The Human UGT1 Gene Complex (150 kb)
Table 1.1. Substrate specificity of cDNA expressed human UGT isozymes. Known inducers of particular isozymes are also noted.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Substrates</th>
<th>Inducers</th>
</tr>
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<tr>
<td>UGT1.1</td>
<td>bilirubin, ethinylestradiol, octylgallate</td>
<td>phenobarbital</td>
</tr>
<tr>
<td>UGT1.4</td>
<td>bilirubin</td>
<td>phenobarbital</td>
</tr>
<tr>
<td>UGT1.6</td>
<td>1-naphthol, BP-3,6 quinol, acetaminophen, vanilin</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>UGT1.02</td>
<td>propofol, 4-tertiary butylphenol, carvacrol, galangin</td>
<td></td>
</tr>
<tr>
<td>UGT2B4</td>
<td>estriol, hydroxycholic acid, 4-hydroxyestrone, 17-epiestriol</td>
<td></td>
</tr>
<tr>
<td>UGT2B7</td>
<td>hydroxycholic acid, 4-hydroxyestrone, 17-epiestriol, estriol, 2-OH-estriol</td>
<td>prolactin, NSAIDs</td>
</tr>
<tr>
<td>UGT2B8</td>
<td>1-nitrophenol, 1-naphthylamine, 4-hydroxybiphenyl, eugenol</td>
<td></td>
</tr>
<tr>
<td>UGT2B9</td>
<td>4-hydroxyestrone</td>
<td></td>
</tr>
<tr>
<td>UGT2B10</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>UGT2B11</td>
<td>4-hydroxyestrone, 4-methylumbelliferone</td>
<td></td>
</tr>
<tr>
<td>UGT2B17</td>
<td>testosterone, dihydrotestosterone, androsterone</td>
<td></td>
</tr>
</tbody>
</table>
both enantiomers at equal rates. In line with the high constitutive expression of UGT1A1 in extrahepatic tissues, a high \((R)/(S)\) ratio of naproxen glucuronidation was found in rat testes, intestine, lung and kidney. These observations demonstrated that \((R)\) naproxen represents a stereoselective substrate of rat UGT1A1, but not of the human ortholog.

Even within a species, gender-specific differences have been reported. Zhu et al., (1996) evaluated the effects of pH, \textit{in vitro} inhibitors, \textit{in vivo} enzyme inducers, age, and sex on the glucuronidation of estradiol and estrone by rat liver microsomes. The pH dependence curves for these estrogens by liver microsomes from adult male rats were very different from those by liver microsomes from adult female rats. These results suggest that liver microsomes from adult male and female rats have different estrogen glucuronosyltransferases. Female rats catalyzed the glucuronidation of estrone and estradiol more rapidly than age-matched male rats.

Recombinant DNA techniques are being used with increasing frequency to elucidate xenobiotic and endogenous substrates of particular UGTs. In one study, five human hepatic UDP-glucuronosyltransferase (UGT) cDNAs were stably expressed in tissue culture cell lines (Burchell \textit{et al.}, 1994). More than 100 drugs and xenobiotics were used as substrates for glucuronidation catalyzed by the cloned human transferases to determine the chemical structures accepted as substrates. UGT1.6 exhibited a limited substrate specificity for planar phenolic compounds, whereas UGT1.02 was more accepting of nonplanar phenols, anthraquinones, flavones, aliphatic alcohols, aromatic carboxylic acids, steroids and many drugs of varied structure. UGT1.0 (bilirubin UGT) catalyzed the glucuronidation of ethinylestradiol. UGT2B9 and UGT2B4 (steroid/bile acid UGTs) also catalyzed the glucuronidation of some xenobiotics. Levels of UGT1.02 activity towards some substrates were sufficient to allow determination of kinetic
parameters for the enzyme reaction. More recently, cDNA expression in stable cell lines has shown that the human isozyme UGT2B17 (Beaulieu et al., 1997) metabolizes the C₁₉ steroids testosterone, dihydrotestosterone, and androsterone. UGT2B17 is closely related to UGT2B15 (95% amino acid identity; Chen et al., 1994).

Known substrates of UGTs are increasing as cDNA-expression studies have made it possible to test a larger number of compounds for potential glucuronidation with specific isoforms of UGT. Glucuronidation of the two enantiomeric dopamine D₁ antagonists, NNC 0756 ([(+)-8-chloro-7-hydroxy-5-(2,3-dihydrobenzofuran-7-yl)-3-methyl-2,3,4,5-tetrahydro-1H-3-benzazepine, acetate]), and NNC 0772 [(-)-8-chloro-7-hydroxy-5-(2,3-dihydrobenzofuran-7-yl)-3-methyl-2,3,4,5- tetrahydro-1H-3-benzazepine, HCl] showed a high degree of stereoselectivity (Hansen et al., 1992). V79 (Chinese hamster lung fibroblast) cell lines expressing a functional recombinant phenobarbital-inducible rat liver UGT2B1, showed a degree of stereo- and regiospecificity, preferring (S)ibuprofen to the (R) enantiomer (Pritchard et al., 1994). UGT2B1 was also shown to glucuronidate lithocholic acid but not hyodeoxycholic acid, which differs by only a single hydroxyl group, thus indicating that functional groups are important determiners of the substrate specificity of UGTs.

In addition to the eleven human UGTs currently characterized, it should be noted that human isoforms capable of glucuronidating many drugs, such as morphine and tertiary amine drugs, have yet to be identified. Thus, it is possible that the human UGT gene family may be of a size similar to that of the cytochrome P450 gene family, which has over 30 human isoforms (Gonzalez, 1992).
In addition to the eleven human UGTs currently characterized, it should be noted that human isoforms capable of glucuronidating many drugs, such as morphine and tertiary amine drugs, have yet to be identified. Thus, it is possible that the human UGT gene family may be of a size similar to that of the cytochrome P450 gene family, which has over 30 human isoforms (Gonzalez, 1992). Finally, the functional role of each UGT in vivo has yet to be clearly determined.
Regulation of UGT Activity

Xenobiotics. The regulation of UGTs is poorly understood, particularly with respect to family 2 isoenzymes. Family 1 UGT isoenzymes appear to be differentially regulated. It is known that bilirubin glucuronidation is induced by phenobarbital-type inducers (Arias et al., 1969; Schmid, 1972). Bilirubin UGTs such as UGT1.1 (Sutherland et al., 1993) and UGT1.4 (Owens and Ritter, 1992) have been specifically induced by phenobarbital. Glucuronidation of some drugs is enhanced in cigarette smokers, who are presumably exposed to 3-methylcholanthrene-type inducers. These drugs include acetaminophen (Bock et al., 1987, 1994; Mucklow et al., 1980), oxazepam (Greenblatt et al., 1980), propranolol and 1-naphthol (Fleischmann et al., 1986). Increased acetaminophen glucuronidation was also observed after eating brussels sprouts and cabbage, which contain 3-methylcholanthrene-type inducers (Pantuck et al., 1984). However, evidence of increased drug glucuronidation in smokers is still controversial, probably due to the small effects observed, and the large interindividual variation. Indirect evidence points to UGT1.6 as a 3-methylcholanthrene-inducible UGT isozyme because this isozyme conjugates acetaminophen with relatively higher affinity than other UGTs (Bock et al., 1993). These findings in humans are similar to those obtained with the orthologous rat UGT1.6 (Iyanagi et al., 1986).

On the other hand, there is also evidence that acetaminophen glucuronidation is increased by phenobarbital-type inducers (Perucca and Richens, 1979; Prescott et al., 1981) and by oral contraceptive steroids (Miners et al., 1983; Mitchell et al., 1983). The effects of different types of inducers on acetaminophen glucuronidation are probably due to the fact that acetaminophen represents an overlapping substrate for different UGTs.
The UGT1 gene structure is shown in Figure 1.2. Alternative splicing of a common precursor RNA has been proposed to produce different isozymes of the UGT1 family (Owens and Ritter, 1992). Since each of the variable exons has also been found to be associated with its own TATA box and transcription initiation site, it is likely that each UGT1 isozyme is under the control of its own promoter, allowing for independent regulation at the transcriptional level. A similar type of transcriptional regulation has been shown for the angiotensin converting enzyme gene (Kumar et al, 1991). Ritter et al (1993) have shown that a correctly oriented 3kb fragment, encompassing the proposed UGT1.6 5'-flanking/promoter region, can successfully drive the expression of a downstream chloramphenicol acetyltransferase gene in HepG2 cells. However, regulation of expression by xenobiotics was not observed with this 3kb 5'-flanking region.

All UGT genes characterized to date have transcription initiation sites within 120 bp of their ATG translation start codons. In addition, UGT2B genes contain consensus binding sites for a trans-acting regulatory protein, hepatocyte specific factor 1, within 70 bp of their respective transcriptional initiation sites. The UGT2B1 gene, in particular, has several other DNA promoter elements, including a consensus binding site for the transcriptional factor AP-1 (Mackenzie and Rodburn, 1990). The promoter activity of this particular 5'-flanking region is species and tissue specific.

Ontogeny. The developmental expression of human UGT activity has also been investigated. Only one isoform, glucuronidating 5-hydroxytryptamine, appears to be present in the fetus at levels comparable to the adult (Leakey et al, 1987; Coughtrie et al, 1988). Other UGTs were shown to be present at less than 20% of adult levels. Chloramphenicol glucuronidation is markedly lower in neonates, leading to "gray baby syndrome" (Weiss et al, 1960). Acetaminophen
clearance has also been reported to be lower in neonates due to glucuronidation deficiencies (Lederman et al., 1983). Ontogenic regulation of UGT expression is poorly understood. Corticosteroids (Dutton, 1980), prolactin and thyroid hormones (Labrune et al., 1992) have been implicated in the surge of phenol and bilirubin UGTs after birth.

**Genetic Multiplicity**

Multiplicity of UGTs has been suggested historically by allelic variants such as the Crigler-Najjar syndrome types I and 2 (CN 1 and 2) (Arias et al., 1969). They are characterized by inherited unconjugated hyperbilirubinemia due to deficient bilirubin UGTs. However, in these patients, glucuronidation of other substrates was found to be unaffected. Four human isozymes of the UGT1 gene complex have been characterized: UGT1.1 and UGT1.4, conjugating bilirubin; UGT1.6, conjugating planar phenols and UGT1.7, conjugating planar and bulky phenols (Wooster et al., 1991). Various mutations in this gene complex have been shown to be responsible for the CN 1 and CN 2 (Aono et al., 1993). A similar mutation has been identified in the Gunn rat (Iyanagi, 1991).

Polymorphic glucuronidation of drugs has not been extensively studied in humans. This has largely been due to the lack of suitable drugs for screening. The overall effectiveness of the glucuronidation system, in addition to interaction with other conjugation pathways, such as sulfation, has made it difficult to elucidate the specificity of individual UGTs and determine the existence of polymorphic variation of these enzymes. Genetic variation of androsterone glucuronidation has been observed in rats (Matsui and Hokazaki, 1979). Homma et al. (1992) determined the cause of this particular polymorphic variation to be the loss of a single UGT.
isozyme, due to a gene deletion. Cloning and expression of human UGTs is furthering the characterization of individual isoymes and their potential variation in humans.

To date, the known defects of glucuronidation in man have been restricted to hereditary hyperbilirubinemias.

**Gilbert's Syndrome.** Thought to affect approximately 1 in 20 Caucasian individuals, Gilbert's syndrome is a mild hereditary hyperbilirubinemia characterized by a decreased formation of bilirubin diglucuronide and increased levels of bilirubin monoglucoronide, and associated with a generally decreased hepatic UGT activity (Gilbert and Lereboulet, 1901; Odell and Childs, 1980). The genetic basis of Gilbert's syndrome is ill-defined. Variation in the gene encoding the UGT1.1 enzyme and serum bilirubin levels have been studied in a Scottish population (Monaghan et al, 1996). Sequence variation of the promoter upstream of the UGT1.1 exon I was observed, and genotypes were assigned as follows: 6/6 (homozygous for a common allele bearing the sequence [TA]6TAA), 7/7 (homozygous for a rarer allele with the sequence [TA]7TAA), and 6/7 (heterozygous with one of each allele). Individuals in the population with the 7/7 genotype had significantly higher bilirubin concentrations than those who had the 6/7 or 6/6 genotype. All individuals who were phenotypically positive for Gilbert's syndrome had the 7/7 genotype. The frequency of the 7/7 genotype in this particular Scottish population was 10-13%. A more severe form of Gilbert's syndrome, where the gene defect lies in the coding sequence of the gene, has been described by Koiwai et al (1995).

Gilbert's syndrome has been associated with a decreased clearance of several drugs such as tolbutamide, rifamycin, josamycin, and acetaminophen (Ullrich et al, 1987; de Morais et al, 1992). However, the study of drug glucuronidation associated with this disease has not provided
conclusive answers, only controversial ones. This is probably due to the heterogenous nature of the biochemical lesion, and a lack of knowledge about the substrate specificity of the UGT isozyme involved. Further studies require the availability of a drug known to be a substrate of UGT1.1.

**Crigler-Najjar Syndrome.** Crigler-Najjar syndrome is a hereditary form of severe unconjugated hyperbilirubinemia caused by a dysfunction of bilirubin glucuronidation in humans (Crigler and Najjar, 1952; Roy Chowdhury et al, 1989). Unless treated with plasmapheresis or liver transplantation, infants often develop severe neurological damage from bilirubin encephalopathy (kernicterus). Two forms of the syndrome have been identified: Crigler-Najjar syndrome Type 1 (CN 1), and Crigler-Najjar syndrome Type 2 (CN 2). CN 1 patients have unconjugated plasma bilirubin levels greater than 340 μM, and CN 2 patients have levels between 60-340 μM. The two types also respond differently to barbiturate therapy, with only CN 2 patients showing a 20-70% decrease in unconjugated plasma bilirubin levels (Roy Chowdhury et al, 1989). Analysis of bile aspirates has also been used to diagnose the disease type (Sinaasappel and Jansen, 1991).

Enzymatic, immunochemical and molecular genetic analyses have been used to characterize the molecular basis of these syndromes. Enzyme analysis of CN 1 liver samples has revealed that in addition to a complete absence of bilirubin glucuronidation, there is also a lack of glucuronidation of phenols, 5-hydroxytryptamine, ethinylestradiol and propofol (Moghrabi et al, 1993).

Accumulating evidence indicates that mutations in the human UGT1 gene locus abolish hepatic bilirubin UDP-glucuronosyltransferase activity and cause the subsequent accumulation of
bilirubin to toxic levels in patients with CN 1, inherited as an autosomal recessive trait. A critical mutation in a common exon of the UGT1 gene complex should inactivate the entire locus. A deleterious mutation in an exon 1 of the gene should affect the amino terminus of a single isoform. Analysis of mutations at the UGT1 locus were performed in three individuals that were clinically diagnosed with CN 1 (Erps et al, 1994). Each patient carried a single base substitution that altered conserved residues in the transferase enzyme molecule, serine to phenylalanine at codon 376 and glycine to glutamic acid at codon 309. Each was homozygous for the defect as demonstrated by sequencing and RFLPs. Mutant cDNAs, constructed by site-directed mutagenesis, inserted into expression vectors, and transfected into COS-I cells, supported the synthesis of the bilirubin transferase protein but only cells transfected with the wild-type cDNA expressed bilirubin UDP-glucuronosyltransferase activity. Thus, alterations at Gly 309 and Ser 376 were the genetic basis for CN 1 in these individuals. These results suggest that the two codons are part of the active site of the enzyme.

Aono et al (1994) have reported a defect of UGT1.1, resulting from a point mutation, in a patient with CN 1. This mutation was a single nucleotide substitution: C was changed to A at base position 840 in UGT1.1 cDNA, and this change resulted in a stop codon. The patient was homozygous for the defect, and his parents and brother, who were clinically normal, were heterozygous for the defective allele.

Ritter et al (1993) have reported that in an individual with CN 1, the cDNA for UGT1.1 was missing the phenylalanine codon at position 170 in exon 1. At pH 7.6, routinely used for bilirubin glucuronidation studies, normal UGT1.1 protein has approximately one-third the activity seen at the major pH optimum of 6.4. The altered isozyme had nearly normal activity at
pH 7.6 and was inactive at pH 6.4, a result consistent with the definition of a pH-sensitive mutant. The Km value for bilirubin using the wild-type protein was approximately 2.5 μM at both pH 6.4 and 7.6 and that for the mutant was 5.0 μM at pH 7.6. The structure of the wild-type enzyme compared to that of the mutant indicated that hydrophobic properties at the active center may be critical for metabolizing the lipophilic substrate. The low ion/pH requirements for bilirubin glucuronidation may signal the basis for the distribution of these isozymes to an organelle (endoplasmic reticulum) that can establish compatible conditions/compartments for each catalysis.

CN 2 is characterized by chronic nonhemolytic unconjugated hyperbilirubinemia due to reduced hepatic bilirubin UGT activity. The two human bilirubin UGT isozymes, UGT1.1 and UGT1.4, were analyzed in a 5-year-old Japanese male patient with CN 2 (Aono et al, 1993). Point mutations were found on exons 1 of the UGT1.1 and UGT1.4 genes: single nucleotide substitutions of G by A, and of T by C, at position 211 of UGT1.1 cDNA and at position 395 of the UGT1.4, respectively. Another point mutation (T by G) was discovered on common exon 5, at position 1456 of the UGT1.1 cDNA, corresponding to position 1459 of the UGT1.4 cDNA. These three mutations result in changes of glycine to arginine and of tyrosine to aspartic acid at amino acid positions 71 and 486 of the UGT1.1 protein, and of leucine to proline and of tyrosine to aspartic acid at amino acid positions 132 and 487 of the UGT1.4 protein, respectively. The patient was homozygous for all defects and his parents and brother were heterozygous for all defective alleles. The findings suggest that the CN 2 is inherited as an autosomal recessive trait.

A reported case of CN 2 (responsive to phenobarbital treatment; Gollan et al, 1975) where the mutation was located in exon 2, not the expected exon 1.1, had caused an amino acid change from Gln to Arg and a considerable reduction in the levels of UGT proteins (Moghrabi et
The level of bilirubin UGT was inducible by phenobarbital therapy, leading to higher levels of the corresponding UGT catalyst. This case demonstrates that similar phenotypes can arise from different genotypes. Indeed 20 different genetic lesions have been demonstrated to be associated with the severe hyperbilirubinaemia of CN 1 and CN 2 (Burchell et al., 1994).

Interindividual Variation of Drug Glucuronidation.

Large interindividual variations of hepatic microsomal drug glucuronidation have been determined in vitro (Burchell et al., 1989), but even 10-fold differences in extremes might be expected as normal within a population. Also, any variation in UGT activities may well be related to age, disease state, or exposure to xenobiotics, as well as genetic background.

Choosing suitable probes for the study of interindividual variability of drug glucuronidation has been hampered by several confounding factors. Drugs which are largely excreted as glucuronides in humans may well be oxidized to their active metabolite prior to glucuronidation. Propranolol is metabolized by side chain oxidation to naphtho-oxylactic acid (NL A), a major metabolite, and by ring hydroxylation to 4-hydroxypropranolol (4-OH-P), which is then conjugated to glucuronide or sulfate (Walle et al., 1985). The formation of NLA was 55% less in poor metabolizers of mephenytoin and formation of 4-OH-P was 75% less in poor metabolizers of debrisoquine (Ward et al., 1989). Therefore apparent polymorphic variation in the formation of propranolol glucuronides would be considerably influenced by polymorphism of drug oxidation.

Many hydroxylated drug metabolites are potential substrates for sulfotransferases and UGTs. Sulfation is classically considered the high affinity, low capacity system, whereas
glucuronidation is believed to be a low affinity high capacity system (Mulder et al., 1989). Although the structure of the substrate will to a large extent determine the affinity of the interaction with the isozyme, the dose of drug administered may also determine whether sulfation or glucuronidation is the primary system responsible for its clearance. Thus, a drug which is a preferred substrate for one isoenzyme within a family would be a more useful tool in an investigation of polymorphisms of drug glucuronidation, than one which has an affinity for multiple isozymes. Caldwell et al (1982) studied the variation of acetaminophen and salicylamide conjugation in normal human volunteers. The glucuronide:sulfate (G:S) ratio for conjugation with acetaminophen and salicylamide can vary up to 16-fold in the population studied. Four of the volunteers were remarkable for their relative inability to form acetaminophen sulfate and therefore the G:S ratio may merely be indicative of individual limitations in sulfate conjugation, and not variability of glucuronidation.

Enzymatic and Molecular Analysis of UGTs

Substrate Probes for UGT Isoforms. All human UGT isoforms so far characterized have been demonstrated to glucuronidate more than one substrate (Table 1.1) and much substrate specificity overlap occurs between different isoforms. Assessment of the substrate specificity of human UGT isoforms characterized to date has revealed that certain substrates appear to be specific for different UGT isoforms (as shown in bold in Table 1.1). It should be noted that the assumption that these are the best probe substrates is only restricted by our current knowledge.

UGT Enzyme Assays. There are numerous different enzyme assays for UGTs, some substrate specific and others more universal (Dutton et al., 1981). Both spectrophotometric and
radiometric methods are utilized in the measurement of UGT activities. Generally most radiometric UGT assays rely on the difference in solubilities or polarities of the acceptor substrate, glucuronide or UDPGA. The most reliable and accurate assays involve the use of radioactively labelled substrate or UDPGA. Assays used for the measurement of 1-naphthol and steroid glucuronidation use $^{14}$C-labelled aglycone and are based on the separation of the enzymatically formed water soluble glucuronides from the free aglycone substrate which partitions into an organic phase such as chloroform or dichloroethane. One popular method that is suited to assaying the glucuronidation capacity of UGTs towards most substrates is the universal thin layer chromatographic system described by Bansal & Gessner (1980), utilising UDPGA labelled with $^{14}$C at the glucuronide acid moiety. This system, used in conjunction with a digital autoradiography signal detector, is often used for the assessment of the substrate specificity of cloned expressed human UGTs. A variation of this solid phase separation system is the utilization of solid phase extraction columns to separate radiolabelled aglycones such as morphine (Puig & Tephly, 1986) from glucuronides of the parent compound. Recently, this universal method has been adapted for HPLC (Pritchard et al., 1993). Numerous methods for the HPLC measurement of UGT activity have been described. One advantage of HPLC is that it can separate glucuronide isomers of compounds that may be glucuronidated at two possible positions, such as bilirubin (Odell et al., 1990), morphine (Rane et al., 1986) and β-estradiol (Senafi et al., 1994).

**UGT Latency.** The localization of UGT active sites within the lumen of the ER results in much of the activity being latent. This has important implications when assaying UGT activities and is often responsible for the variations in results obtained by different investigators. The
latent UGT activity may be revealed by a number of methods that disrupt microsomal membrane vesicles of homogenised cells such as detergents and sonication. The incorrect assumption that a simple, standard addition of detergent can be used to liberate all the latent UGT activity leads to major errors, as latency can vary depending on the species, tissue, ontogeny and the substrate used. Preparation to preparation variation also occurs. To ensure all the latent UGT activity is measured, samples should therefore be carefully titrated with detergent or optimised with whatever membrane disruption method is used. Sonication is often overlooked as an effective means of removing latency. It has been shown to be effective in the study of recombinant human UGT expressed in hamster lung fibroblasts (Senafi et al., 1994).

**cDNA Expression in Mammalian Cell Lines.** As substrate specificity cannot be determined from DNA sequence alone, newly identified and sequenced UGT clones require functional analysis. In order to characterize the clone, a cDNA insert is recloned in a mammalian expression vector, which should contain an eukaryotic promoter/enhancer, capable of driving transcription of the cDNA insert when harboured by a variety of cell types. The vector also contains a selectable marker, such as the neomycin resistance gene. cDNA clones constructed in such a vector are suitable for either transient or stable transfection of cells.

Transient transfection methods have been used extensively to express the UGTs in COS cells. COS cells are cultured in media supplemented with 10% fetal calf serum, 2mM glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin. When the cells reach 70-80% confluence they are transfected either using DEAE-dextran followed by chloroquine treatment (Mackenzie, 1986), or by lipofection (Ritter et al, 1990). Lipofection has the advantage of greater
transfection efficiency, but is more expensive. The transfected cells are cultured for 42-72 hours prior to harvesting and assayed for UDPGT activity.

Transient transfection produces modest amounts of UGT protein, and hence any enzyme assays to determine substrate range must be prolonged and sensitive. A higher level of clone expression can be achieved by using a stably transfected cell line which once established can be used to perform extensive substrate specificity studies. V79 cells (Chinese hamster lung fibroblast) or HEK293 cells (human embryonic kidney) are cultured to 70% confluence and transfected (Sutherland et al, 1992). Stably transfected colonies (resistant to geneticin) are isolated and propagated.
2. OXAZEPAM

Metabolism

Oxazepam is a 1,4-benzodiazepine anxiolytic with a hydroxyl group at the asymmetric C3 position (Figure 1.3). Prescribed clinically as a racemate, its therapeutic activity stems from the 3S enantiomer, which has an approximately 200-fold higher affinity for central nervous system binding sites than the 3R enantiomer (Corbella et al., 1973; Mohler et al., 1977). Oxazepam is also an active metabolite of 1,4-benzodiazepine anxiolytics such as diazepam and halazepam.

These drugs undergo microsomal cytochrome P450-mediated N-dealkylation and C3-hydroxylation to form oxazepam (Figure 1.3). In vivo, C3-hydroxylation has been shown to be stereoselective, with a higher formation rate of the clinically active 3(S)oxazepam (Corbella et al., 1973; Mohler et al., 1977). D-glucuronic acid couples with the C3 hydroxyl group of oxazepam to form pharmacologically inactive glucuronide conjugates (Ruelius et al., 1979; Greenblatt et al., 1983). The asymmetry of the C3 position is not affected by this coupling and the resulting glucuronides are diastereomeric (Ruelius et al., 1979; Mascher et al., 1984). In vivo, the appearance of glucuronide conjugates in plasma has been reported to be stereoselective (Vree et al., 1991). It is not clear whether these observations reflect stereoselectivity in the glucuronidation of oxazepam, conversion, in vivo, of 3R oxazepam to 3S oxazepam, prior to glucuronidation, or the stereoselectivity of intestinal glucuronidases towards 3(R)oxazepam glucuronide. With respect to the potential stereoselectivity of glucuronidases, it has been reported that 3-acetyl conjugates of oxazepam are stereoselectively hydrolysed by endogenous esterases (Yang et al., 1990).
Figure 1.3. Major in vivo pathways involved in the formation and metabolism of oxazepam.
Oxidative metabolites of oxazepam have been reported to be produced by human, B6C3F1 mouse and F344 rat microsomes (Griffin et al, 1995). The major metabolite in all three species was 6-chloro-4-phenyl-2(1H)-quinazolinecarboxylic acid (CPQ-carboxylic acid). In addition, rat microsomes produced 4'-hydroxyoxazepam and oxazepam-dihydrodiol in NADPH-containing incubations.

Protein Binding

Extensive accumulation of benzodiazepines is an important factor in the induction of physical dependence. The mechanistic basis for accumulation of nordiazepam and its metabolite, oxazepam, have been examined in crossover studies in drug-naive, and in dependent dogs (Wala et al, 1993). Steady-state plasma levels of neither drug can be predicted from single-dose pharmacokinetics. The benzodiazepine antagonist, flumazenil, significantly reduced steady-state plasma levels of total oxazepam, presumably by competing for plasma protein binding sites. The binding of diazepines to human serum albumin (HSA) and to alpha 1-acid glycoprotein (AGP) has been measured by means of fluorescence and circular dichroism spectroscopies (Maruyama et al, 1992). Diazepines have one tight binding site on both HSA and AGP. The binding parameters of the diazepine:HSA systems were slightly higher than those of diazepine:AGP systems. The driving force for the binding of diazepines to both proteins was deemed to be hydrophobic interaction. Steric effects and electrostatic interactions were also observed.
Disposition

Oxazepam concentrations in vivo, have been determined with use of a fluorescence polarization immunoassay. The mean pharmacokinetic parameters determined for a single 30 mg dose of oxazepam for oral clearance, apparent volume of distribution, and plasma half-life were 9.8 L/h, 65.7 L, and 5.1 h, respectively (Mole et al, 1993). Christensen et al (1992) examined the sedative and cortisol suppressing properties of oxazepam. Oxazepam showed maximal sedation after 2 h, and maximal plasma levels, reaction time prolongation and cortisol suppression after 3 h. The t_{max} of sedation was 1 h earlier than the peak blood concentration.

Adverse Effects

Central. The effects of oxazepam on reaction time and event-related potentials have been investigated in a visual search paradigm using double-blind designs (Heinze et al, 1994). Significant effects of oxazepam were observed in a number of psychometric tests including increased search time and impairment of short-term memory and word-recognition. In a placebo-controlled, double blind, crossover experimental design, ingestion of oxazepam (40 mg) was shown to impair vigilance and oculomotor activity, reducing the efficiency of tasks requiring frequent saccadic eye movements, such as reading (van Leeuwen et al, 1994). Generally, these adverse effects of oxazepam are due to an overmanifestation of its therapeutic actions.

In half the patients seeking advice for anxiety, panic and phobias at London Hospital (UK), the cause was alcohol or benzodiazepines (Cohen, 1995). When symptoms are persistent following a distressing event, alcohol or benzodiazepines are preferentially abused. There was a
large variation in individual vulnerability and the mechanism responsible for the listed symptoms is thought to be rebound arousal.

Stijnen et al (1992) have reported that changes in the pharmacodynamics of anticonvulsant effect of oxazepam can be explained by the disappearance of the tolerance/withdrawal phenomenon with age. This is compatible with generally decreased efficiency of homeostatic control mechanisms in the elderly.

**Prenatal Toxicity.** Growth and neurodevelopment have been studied in children born to mothers who used benzodiazepines in therapeutic doses as their only psychotropic drug throughout pregnancy. When compared with children born to mothers without any known use of psychotropic drug, the benzodiazepine-exposed children had a pattern of craniofacial anomalies. Gross motor development was retarded up to 18 months. Impaired fine motor functions were also prevalent (Laegreid et al, 1992).

Prenatal exposure to oxazepam in mice has been linked to a decrease in GABA(a) agonist-induced depression, and rebound hyperactivity (Laviola et al, 1992).

**Cellular Toxicity.** Oxazepam has been determined to be a potential hepatocarcinogen in mice. It is a phenobarbital-type enzyme inducer in rodents. An association between its enzyme induction capabilities and carcinogenesis has been observed by Griffin et al (1995). In another study, hepatocellular adenomas and carcinomas were increased in exposed mice. Survival of B6C3F1 mice receiving 2500 and 5000 ppm oxazepam was also lower than that of controls. Early deaths were due to increased incidences of hepatoblastoma and hepatocellular carcinoma, and nearly all mice receiving 2500 or 5000 ppm developed hepatocellular neoplasia. An increase in follicular cell hyperplasia of the thyroid gland occurred in all exposed groups of B6C3F1 mice,
and thyroid gland follicular cell adenoma was increased in exposed females. Further studies of the capacity of oxazepam to induce liver cell mitogenesis and an evaluation of the frequency of activated H- and K-ras oncogenes in the liver tumors of B6C3F1 mice has shown that many of the neoplastic and nonneoplastic responses of mice to oxazepam resemble those observed with phenobarbital (Bucher et al, 1994).

Cunningham et al (1994) have shown that oxazepam induces replicative DNA synthesis much like phenobarbital. Oxazepam-induced formation of micronuclei has been observed in Syrian Hamster embryo fibroblast cells, human amniotic fluid fibroblast-like cells and L5178Y mouse cells. The increase in micronucleus fractions was found to be dose-dependent in all three cell lines. The time course of micronucleus induction in L5178Y cells showed a maximum at 5 h after treatment, suggesting that the micronuclei were formed in the first mitosis after treatment. Kinetochore staining revealed the presence of kinetochores in approximately 50% of the micronuclei in all three cell types (Stopper et al, 1993).
Conjugation with glucuronic acid is catalysed by the microsomal UDP-glucuronosyltransferase (UGT) enzyme system (Dutton, 1980; Burchell et al., 1991). The majority of human UGTs whose cDNA have been cloned, preferentially conjugate endogenous compounds such as bilirubin, catechol estrogens, hyodeoxycholic acid and estriol (Ritter et al., 1990; Miners and Mackenzie, 1991). Glucuronidation is also an important pathway for the elimination of many clinically used drugs and their metabolites (Miners and Mackenzie, 1991). An undetermined number of isozymes can catalyze the transfer of glucuronic acid to any of a large number of xenobiotics or endogenous compounds, forming water-soluble conjugates with increased rates of excretion. Through inhibition and kinetic studies in human liver microsomes, enzyme purification, and the expression of human UGT cDNAs in cell lines, overwhelming evidence has been provided for the presence of UGT heterogeneity in humans (Burchell et al., 1991; Miners and MacKenzie, 1991).

UGT-catalysed conjugation of xenobiotic compounds is manifest with distinct but overlapping substrate specificities. In addition, most xenobiotics are also potential substrates of other metabolic pathways. Thus, the identification of a suitable xenobiotic probe, which undergoes glucuronidation without significant interaction from other pathways, could provide substantial information about UGT heterogeneity in humans.

In addition to being a therapeutic agent on its own, (S)oxazepam is also an active metabolite of 1,4-benzodiazepine anxiolytics such as diazepam and halazepam. These drugs undergo microsomal cytochrome P-450-mediated N-dealkylation and C3-hydroxylation to
preferentially form (S)oxazepam (Corbella et al, 1973). Thus, (S)oxazepam glucuronide is the predominant excreted metabolite of a widely prescribed group of drugs, and any interindividual differences in (R,S) oxazepam glucuronidation may have both toxicological and pharmacogenetic implications.

To date, information about the specificity of individual UGT isozymes towards clinically used drugs is limited. Any emerging knowledge of drug-metabolizing UGT isozymes, with respect to substrate specificity and regulation by xenobiotics, will help to predict pharmacokinetic interactions in individual patients.

In vivo, the appearance of oxazepam glucuronide conjugates in plasma has been reported to be stereoselective. In humans, Vree, et al (1991), have reported that two and a half times more 3(S) oxazepam glucuronide is excreted in urine than 3(R)oxazepam glucuronide after a racemic dose of oxazepam. It is not clear to what degree these observations reflect stereoselectivity in the glucuronidation of oxazepam, in vivo conversion of 3(R)oxazepam to 3(S)oxazepam prior to the glucuronidation process, or stereoselectivity of intestinal glucuronidases towards 3(R) oxazepam glucuronide. Yang et al (1990) have reported that 3-acetyl conjugates of oxazepam are stereoselectively hydrolyzed by endogenous esterases.

Estriol and 3,4-catechol estrogens have been identified as primary substrates for UGT2B7 (trivial name: UDPGTh-2), a cDNA-cloned human UGT isozyme expressed in COS-1 cells (Ritter et al., 1990). Recently, Jin et al., (1993) reported that a single amino acid variant of UGT2B7, expressed in COS-7 cells, was capable of glucuronidating non-steroidal anti-inflammatory drugs (NSAIDS) and certain other carboxylic acid-containing drugs.
In order to determine the particular UGT isoform(s) involved in the glucuronidation of oxazepam, competition experiments were carried out in human liver microsomal preparations (Patel, et al 1995a). Competitive inhibition of (S)oxazepam glucuronide formation by endogenous compounds and xenobiotics containing carboxylic acid functional groups, including NSAIDS, indicated that it was a substrate of UGT2B7. Formation of the (R) glucuronide was not competitively inhibited by known substrates of UGT2B7.

As reported during the course of this work, graphical analyses (Endrenyi and Patel, 1991a; 1991b) and maximum likelihood analyses indicated that the clearance, in vivo, of (S)oxazepam was bimodally distributed in a Caucasian population after the administration of (R,S)oxazepam (Patel et al., 1995b). The clearance of (R)oxazepam did not exhibit any bimodality. We also found that the in vitro \( K_m \) of (S)oxazepam glucuronidation was bimodally distributed in a group of 37 human donor livers, whereas the \( K_m \) of (R)oxazepam was normally distributed (Patel et al., 1995b). Analyses indicated that 10% of individuals glucuronidated the therapeutically active (S)oxazepam poorly. The in vitro data, in particular, strongly indicate that the glucuronidation of (S)oxazepam is polymorphic, and that the basis of this is genetic.

Inhibition of (S)oxazepam glucuronidation was well-described by a one-site logistic model and the inhibition profile suggests that UGT2B7 is the catalyzing enzyme. These observations indicated that irregularities in the sequence or expression of UGT2B7 are likely involved in the polymorphic glucuronidation of (S)oxazepam.

Reverse-transcriptase polymerase chain reaction (RT-PCR) and restriction fragment length variation (RFLV) techniques were used to study the sequence of UGT2B7 cDNA from individual livers in our liver bank. Two different UGT2B7 cDNA sequences differing at
nucleotide 802, were isolated. The coding difference leads to an amino acid change at residue 268. Both forms were expressed in COS-1 monkey kidney cells to determine their respective glucuronidation kinetics with oxazepam and other substrates.

The effect of polymorphic variants of UGTs on drug metabolism has important implications, but specific conclusions have been difficult to make. This is particularly true of isozymes belonging to the UGT2 family. As more molecular genetic information becomes available, simpler models, such as cDNA expressed systems, can be used to complement data obtained in vivo.
IL OBJECTIVES
1. INHIBITION STUDIES OF (S)OXAZEPAM GLUCURONIDATION

Although 1,4-benzodiazepines are ultimately cleared as oxazepam glucuronide, little is known about the particular UGT isozyme(s) responsible for the conjugation at the C3 position of these molecules. Reported interactions with other xenobiotics have been limited. Sawe et al. (1982) observed some inhibition of morphine glucuronidation by oxazepam in human liver microsomal preparations. However, this was only observed at very high non-physiological pH and no corroborative reports have since been published.

An inhibition profile, using known substrates of specific UGT isozymes, was assembled to clarify the contribution of those specific isozymes to (R) and (S)oxazepam glucuronidation.
2. INTERINDIVIDUAL VARIABILITY OF (S)OXAZEPAM GLUCURONIDATION

Genetic factors are known to be significant contributors to interindividual variability in the disposition of drugs (Kalow, 1989). Therefore, inter-individual differences in (S)oxazepam glucuronidation would have pharmacogenetic as well as toxicological implications. A series of studies were carried out to determine whether such differences can be observed in the glucuronidation of oxazepam between subjects \textit{in vivo} and \textit{in vitro}. Having shown that the glucuronidation of (S)oxazepam is inhibited by substrates of UGT2B7 (Patel \textit{et al}, 1995a), these studies should be particularly informative about this specific isozyme.
3. RFLV ANALYSES AND cDNA EXPRESSION OF VARIANT AND WILD TYPE UGT2B7

In the results of the two previous sections, graphical and maximum likelihood analyses have indicated that the clearance in vivo of (S)oxazepam was bimodally distributed in a Caucasian population after the administration of (R,S)oxazepam (Patel et al., 1995b). The glucuronidation of (S)oxazepam, but not (R)oxazepam, was competitively inhibited by substrates of UGT2B7 (Patel et al., 1995a). The rank order of inhibition was identical to the relative potency of UGT2B7 substrates (Ritter et al., 1991; Jin et al., 1993). Inhibition of (S)oxazepam glucuronidation by these compounds was well-described by a one-site logistic model and the inhibition profile suggests that UGT2B7 is the catalyzing enzyme. Together, these observations indicate that irregularities in the sequence or expression of UGT2B7 are likely involved in the polymorphic glucuronidation of (S)oxazepam.

Reverse-transcriptase polymerase chain reaction (RT-PCR) and restriction fragment length variation (RFLV) techniques were used to study the sequence of UGT2B7 cDNA from individual livers from our liver bank. Two different UGT2B7 cDNA sequences, differing at nucleotide 802, were isolated. The coding difference leads to an amino acid change at residue 268. Both forms were expressed in COS-1 monkey kidney cells to determine their respective glucuronidation kinetics with oxazepam and other substrates.
III. METHODS AND MATERIALS
1. INHIBITION STUDIES OF (S)OXAZEPAM GLUCURONIDATION

Chemicals

Racemic oxazepam was supplied by Wyeth Pharmaceuticals. Morphine and codeine were generously provided by Dr Victoria Otton of the Addiction Research Foundation, Toronto. Ketoprofen, ibuprofen, and fenoprofen, all in racemic form, as well as (S)naproxen, were obtained from our colleague Dr. Ted Inaba (Department of Pharmacology, University of Toronto). Hyodeoxycholic acid, estriol, p-nitrophenol, and acetaminophen were purchased from the Sigma Chemical Co. (St Louis, MO), as was the metabolic cofactor UDP-glucuronic acid. Chemicals used for chromatography were of analytical grade and also procured from Sigma. The BCA protein concentration assay reagents were purchased from Pierce (Rockford, IL).

Human liver microsomes

Human liver tissue was obtained from three renal transplant donors (K-27, K-28, and K-29) with consent of the next-of-kin, and stored at -70° C. A portion was cut, weighed, and thawed at room temperature, after which it was minced and an equivalent volume of 1.15% cold KCl was added. The tissue was then homogenized for 30 s with a Brinkman polytron (setting 4). Further homogenization followed with 10 strokes of a Potter-Elvehjem glass tissue grinder at 3000 rpm. The homogenized tissue was then centrifuged at 9000 x g in a Sorvall RC2-B centrifuge for 20 min at 4° C, and the pellet discarded. The supernatant from the initial
Centrifugation was centrifuged at 100000 g for 1 h at 4 °C with a Beckman L2-65B ultracentrifuge. The pellet was then washed with 6 vol. cold 1.5% KCl and centrifuged at 100000 g for 1 h at 4 °C with a Beckman L2-6 5B ultracentrifuge. The pellet was resuspended in 50 mm Tris-HCl (pH 7.4) and stored at -70 °C until needed. Microsomal protein concentration was measured using the BCA protein concentration assay (Pierce, Rockford, IL).

**Enzymatic glucuronidation**

Glucuronidation of oxazepam was assayed using human liver microsomes from three different donors. Final incubation concentrations were 2.5 mM UDPGA, 1.5 mg/mL microsomal protein, 0 to 0.5 mM (S,R)oxazepam, and 0.5 mM MgCl2, in 50 mM Tris-HCl (pH 7.4), adjusted to a final volume of 200 uL in 1.5 mL capped incubation tubes (Eppendorf). All incubations were performed under linear conditions, with respect to time (under 40 min) and protein concentration. All mixtures were preincubated on ice for 1 min prior to the addition of UDPGA, after which they were placed in a self-stirring water bath at 37 °C. The reactions were terminated by the addition of 25 uL 0.1 N HCl, lowering the pH of the mixture to 3.3. This value was determined to be optimal to stop enzymatic glucuronidation without hydrolyzing the metabolic product. The incubation tubes were then centrifuged for 6 min at 10000 g and 100 uL of the supernatant was injected directly onto the chromatographic column.

**Inhibition studies**

Inhibition of oxazepam glucuronidation was observed in the presence of a number of known xenobiotic substrates of UGT: ketoprofen, (S)naproxen, ibuprofen, fenoprofen, clofibric...
acid, p-nitrophenol, acetaminophen, codeine, and morphine. Incubation conditions were the same as noted above, with the following exceptions: the concentration of \((R,S)\text{oxazepam}\) was fixed at 0.3 mM, and the incubation time was limited to 30 min. The concentration range of the inhibitors was 0 to 10 mM. In the case of morphine and codeine, the incubation concentration of \((R,S)\text{oxazepam}\) was lowered to 0.1 mM in order to increase the probability of observing any low affinity interaction between the glucuronidation of \((R,S)\text{oxazepam}\) and that of morphine and codeine, as previously reported (Sawe et al., 1982). Hyodeoxycholic acid and estriol, known endogenous substrates of UGT2B7 in humans, were also incubated with \((R,S)\text{oxazepam}\), albeit at lower concentrations (0-5 mM) than the xenobiotic inhibitors listed above. The enzymatic reaction was terminated by decreasing the pH of the mixture to 3.3, as previously described, and the incubation tubes were centrifuged for 6 min at 10000 g and 100 uL of the supernatant were injected directly onto the chromatographic column.

**Chromatographic analysis**

The samples were injected using a WISP automated programmable injector (Waters), and the mobile phase was delivered via a Waters 650 solvent delivery system at 1.0 ml/min, onto a 25 cm C18 reverse-phase ODS column. The mobile phase consisted of 19% acetonitrile, and 7.5% isopropanol, in 0.3% phosphoric acid. Glucuronides of 3(R)- and 3(S)oxazepam were detected using a Waters programmable multiwavelength detector with the detection wavelength set at 230 nm, and AUFS set at 0.01. The extinction coefficient of oxazepam (in methanol) is 34.2 cm\(^{-1}\) mM\(^{-1}\) at 230 nm (Yang et al., 1990). By comparing the relative absorbances of pre-B-glucuronidase-digested and post-digested oxazepam glucuronide, this was determined to
accurately approximate the molar extinction coefficient of the oxazepam glucuronide. Therefore, oxazepam standards were used to calculate the amounts of oxazepam glucuronide detected. The detector was attached to a Shimadzu C4 Chromatopac analyser and recorder set at an attenuation of 2 and a chart speed of 5.0 mm/min.

**Quantitative analysis**

Kinetic and statistical analyses of the data were achieved with the use of Sigmaplot 6.0 (Jandel Scientific), and SAS (SAS Institute, Cary, NC). Inhibition data were fit to a logistic model, with the use of a proportional weighting scheme ($\omega = 1/[\text{Inhibitor}]$).
2. INTERINDIVIDUAL VARIABILITY OF (S)OXAZEPAM GLUCURONIDATION

Chemicals

Racemic oxazepam, in pure form and as an oral preparation (Serax®), was supplied by Wyeth Pharmaceuticals. Chemicals used for chromatography were of analytical grade and obtained from the Sigma Chemical Co. (St. Louis, MO), as were the metabolic cofactor UDP-glucuronic acid (UDPGA) and the deconjugating enzyme β-glucuronidase (E. coli, type VII-A). The BCA protein concentration assay reagents were purchased from Pierce (Rockford, IL). The Swiss liver samples were kindly provided by Dr. U.A. Meyer of the University of Basel, Switzerland.

In Vivo Glucuronidation in Human Subjects

Study 1:

Eleven healthy student volunteers (6 male, 5 female) between 23 and 31 years of age were given 15 mg (R,S) oxazepam p.o. after an overnight fast. Urine samples were collected upon spontaneous voiding for 32 h and pooled into predetermined time intervals: 0-8, 8-16, 16-24, and 24-32 h. After noting collection volumes, 10 mL samples were stored for analysis. Blood samples (3 mL) were collected over the same 32 h time period in heparinized tubes. Collection times of blood samples were: 0, 4, 8, 12, 24, and 32 h after drug administration. All urine and blood samples were stored at -20°C for a maximum of 10 days until processed.
Study 2:

Additionally, 8 h overnight urine samples were obtained from a further 19 student volunteers (11 male, 8 female), each of whom had also been given 15 mg (R,S) oxazepam p.o. after a 6h fast. These samples were stored in the same manner as the above samples.

Environmental factors have been shown to significantly influence the glucuronidation of xenobiotics. Enzyme induction as a result of cigarette smoking, chronic intake of anticonvulsants such as phenytoin and carbamazepine (Miners, et al., 1986), oral contraceptive steroids (Miners, et al., 1986) and ethanol (Critchley, et al., 1982) have been reported to change the metabolic profile of glucuronidated drugs in vivo. Such confounding factors should be controlled when the influence of genetic factors on glucuronidation are being considered. Therefore, all 30 student subjects were screened for the use of potential UGT inducers prior to their participation (Table 3.1).

Study 3:

Eight hour urine samples from 42 patients who had been given 15 mg (R,S) oxazepam p.o. were obtained. Among these 42 patients, there were 11 schizophrenics, 16 Alzheimer's patients, and 15 geriatric controls, all under some form of drug therapy (Fisman, et al., 1988). Unlike the student samples, these samples had been stored over a long term at -20°C.
Sample Preparation

After separation from red blood cells, human plasma samples (150 µL) were deproteinized with 0.5 mL 0.33 M perchloric acid, and centrifuged at 12000 g for 5 min. The supernatant (100 µL) was injected onto the chromatographic column.

Urine samples (50 µL) were diluted in 1.95 mL of mobile phase, and 50 µL of this mixture was injected onto the column.
Table 3.1. Summarized characteristics of subjects and kidney donors tested for oxazepam glucuronidation capability. All subjects were Caucasian.

<table>
<thead>
<tr>
<th>Study</th>
<th>n Subjects</th>
<th>Age (mean ± s.d.)</th>
<th>Prescription drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>1</td>
<td>11 university students</td>
<td>26.1±2.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19 university students</td>
<td>23.1±3.7</td>
</tr>
<tr>
<td></td>
<td>3 a</td>
<td>11 schizophrenic patients</td>
<td>73.5±6.9</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>16 Alzheimer’s patients</td>
<td>78.1±6.8</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>15 geriatric controls</td>
<td>73.6±6.9</td>
</tr>
<tr>
<td>In vitro</td>
<td>4 a</td>
<td>17 Canadian kidney-donor livers</td>
<td>32.6±13.3</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>20 Swiss kidney-donor livers</td>
<td></td>
</tr>
</tbody>
</table>
**Human Liver Microsomes**

Human liver tissue was obtained from 17 Canadian and 20 Swiss renal transplant donors, with consent of next of kin, and stored at -70°C (Table 3.1). A portion was weighed and thawed at room temperature, after which it was minced and one volume of cold 1.15% KCl was added. The tissue was then homogenized for 30s with a Brinkman polytron (setting 4). Further homogenization followed with 10 strokes of a Potter-Elvehjem glass tissue grinder at 3000 rpm. The homogenized tissue was then centrifuged at 9000 g in a Sorvall RC2-B centrifuge for 20 min at 4°C, and the pellet was discarded. The supernatant from this first centrifugation was further centrifuged at 100000 g for 1 h at 4°C with a Beckman L2-65B ultracentrifuge. The pellet was then washed with 6 vol. cold 1.15% KCl and recentrifuged at 100000 g for 1 h at 4°C. The pellet was resuspended in 50 mM Tris-HCl (pH 7.4) and stored at -70°C until needed.

**In Vitro Microsomal Glucuronidation**

**Study 4:**

Microsomal protein concentration was measured using the BCA protein concentration assay (Pierce, Rockford, IL).
Glucuronidation of oxazepam was assayed in human liver microsomes from 37 individual donors. All incubations were performed under linear rate conditions, with respect to incubation time and protein concentration. Final incubation concentrations were 2.5 mM UDPGA, 1.5 mg/mL microsomal protein, 0.5 mM MgCl₂, and 0 to 0.5 mM (R,S) oxazepam, in 50 mM Tris-HCl (pH 7.4), adjusted to a final volume of 200 μL in 1.5 mL capped incubation tubes (Eppendorf). Detergent was not used to solubilize the microsomes as it was determined not to be beneficial in this case (Patel, et al., 1995a). All mixtures were preincubated on ice for 1 min prior to the addition of UDPGA, after which they were placed in a self-stirring water bath at 37°C for 30 to 60 min. The incubations were terminated with the addition of 25 μL of 0.1 N HCl, effectively lowering the pH of the mixture to 3.3, a value low enough to stop enzymatic glucuronidation without hydrolysis of the metabolic product (Patel, et al., 1995a). The incubation tubes were then centrifuged for 6 min at 10000 g and 100 μL of the supernatant was injected directly onto the chromatographic column.

Chromatographic Analysis

The samples were injected using a WISP automated programmable injector (Waters), and the mobile phase was delivered by a Waters 650 solvent delivery system at 1.0 mL/min onto a 5 μ, 4.6 mm x 25 cm reverse-phase ODS column (Beckman). The mobile phase consisted of 19% acetonitrile, and 7.5% isopropanol, in 0.3% phosphoric acid. Glucuronides of 3R and 3S oxazepam were detected using a Waters programmable multiwavelength detector with the detection wavelength set at 230 nm. The molar extinction coefficient of oxazepam (in methanol) is 34.2 cm⁻¹mM⁻¹ at 230 nm (Yang et al., 1990). By measuring the relative absorbances of equal
molar amounts of oxazepam glucuronide and oxazepam (after deconjugation by β-glucuronidase), this value was also confirmed to be the molar extinction coefficient of oxazepam glucuronide.

Thus, we were able to use oxazepam standards to calculate the amounts of oxazepam glucuronide detected. Absorbances were analyzed and recorded by a Shimadzu C-R4A Chromatopac analyzer.

Quantitative Analysis

Kinetic and statistical analyses were performed by the application of appropriate one and two component hyperbolic models to the data, with the use of Sigmaplot 6.0 (Jandel Scientific) and SAS 6.3 (SAS Institute Inc., Cary, NC). The data were not weighted.
3. RFLV ANALYSES AND cDNA EXPRESSION OF VARIANT AND WILD TYPE UGT2B7

RNA Extraction

Human liver total RNA was isolated from 37 individual post-mortem samples (see previous section, Table 3.1). RNA was isolated according to the acid guanidium thiocyanate - phenol chloroform extraction method, described by Chomczynski and Sacchi (1987). Liver tissue was homogenized in RNeasy B (Gibco/BRL) (100 mg of liver tissue per 2 mL). Chloroform was added to the homogenate (1:10, v:v), and the mixture was sealed, shaken, and left on ice for 5 min. This was followed by centrifugation at 12000g for 15 min at 4°C, which separated the mixture into an aqueous phase, containing RNA, and an interphase and phenolic phase, containing DNA and cellular proteins. The aqueous phase was aspirated and transferred to a fresh tube, mixed with an equal volume of isopropanol, left on ice for 20 min to precipitate RNA, and centrifuged at 12000g for a further 15 min at 4°C to form an RNA pellet. Following the second centrifugation, the supernatant was discarded, and the pellet was resuspended in 75% ethanol, made with DEPC-treated RNase-free H₂O. After a final centrifugation at 7500g for 8 min at 4°C, the aqueous phase was discarded, and the pellet was dried under vacuum for 10 min and resuspended in 25-50 μL of 1mM EDTA, made with DEPC-treated H₂O. Optical density was measured at 260 and 280 nm to determine extraction efficiency and total RNA concentration. As a qualitative test, gel electrophoresis was performed on a 1% agarose gel with 1-2 μg of total RNA to detect the presence of 28S and 18S ribosomal RNA. Individual RNA samples were treated with RNase-free DNase and stored at -70°C until needed.
First-Strand Synthesis of cDNA by RT-PCR

First-strand cDNA was synthesized from human liver total RNA by reverse-transcription, using a GeneAmp® RNA PCR Kit (Perkin Elmer/Cetus). Total RNA (2-3 μg) was diluted in DEPC-treated RNase-free H₂O to a final volume of 15 μL, and incubated at 65°C for 10 min and left on ice for 5 min. In a fresh PCR tube, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4.5 mM MgCl₂, and 1 mM each of dATP, dCTP, dGTP, and dTTP, were mixed with 100 pmol of oligo-dT reverse-transcription primer, and 10 units of RNase inhibitor. This mixture was incubated at 42°C for 2 min, and 60 units of Murine Leukemia Virus (MuLV) reverse-transcriptase was added, followed by the addition of RNase-free total RNA (2-3 μg in 15 μL). The final volume of 27 μL was incubated at 42°C for a further 30 min to allow for first-strand cDNA synthesis. Reverse-transcription was then heat-inactivated for 5 min at 95°C, followed by 5 min at 4°C.

Synthesis of first-strand cDNA was also carried out with random hexamer reverse-transcription primers. These were used in the place of oligo-dT primers in the protocol described above (Figure 3.1).

PCR Amplification of Actin cDNA

RNA from post-mortem tissue is often highly degraded, and may not yield first-strand cDNA of sufficient quality. In order to gauge the success of the first-strand syntheses described above, a 300 bp segment of actin cDNA was amplified using first-strand cDNA as template. Actin is a protein with ubiquitous expression. An amplification mixture of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.8 mM MgCl₂, 1 mM each of dATP, dCTP, dGTP, and dTTP, 50 pmol each of
Figure 3.1. Schematic of first-strand cDNA synthesis and subsequent PCR amplifications of UGT2B7 cDNA. Segments amplified by P_5P_6 and P_3P_4 were subsequently used for RFLV analyses, and expression studies, respectively.
forward and backward actin-specific oligonucleotide primers and 2.5 units of AmpliTaq® polymerase was pre-incubated at 94°C for 5 min. At this point, 4.5 μL of the previous reaction mixture containing first-strand cDNA was added in a hot start. The amplification mixture had a final volume of 50 μL and was subject to 25 cycles of the following: 45s of denaturation at 94°C, 45s of annealing at 50°C, and 45 s of polymerase extension at 72°C, using a GeneAmp® PCR System 9600 Thermal Cycler (Perkin Elmer/Cetus). A 4.5 μL aliquot of each sample was reamplified under the identical conditions, and 10 μL of the final amplification product was electrophoresed in a 1% agarose gel containing 0.01% ethidium bromide.

PCR Amplification of UGT2B7 cDNA

Amplification of first-strand cDNA was carried out with different combinations of UGT2B7-specific forward and backward oligonucleotide primers (Figure 3.1; Table 3.2). The forward primers, P₁ (5'-AACTCCTGGAATTTTCAG-3'), P₃ (5'-TGCATTGCACCAGGATGTC-3'), and P₅ (5'-TGACATGAAGAACiTGGGATC-3'), were complementary to bases 778 to 795, 14 to 5, and 681 to 700 of the UGT2B7 coding region, respectively. The backward primers, P₂ (5'-GTCTTCCATTTCCCTAGGC-3'), P₄ (5'-AACTGAAGTAGTCTCACC-3'), and P₆ (5'-TCAACATTTGGTAAGAGTGG-3'), were complementary to bases 882 to 864, 1642 to 1625, and 824 to 805, respectively. The primers were designed to minimize cross-hybridization with the cDNA of homologous UGT isozymes. Each reaction contained a mixture of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 1 mM each of dATP, dCTP, dGTP, and dTTP, 100 pmol each of forward and backward UGT2B7-specific oligonucleotide primers and 2.5 units of AmpliTaq® polymerase. This mixture was pre-incubated at 94°C for 3 min, prior to the addition of 4.5 μL of
Table 3.2. Oligonucleotide primers used to amplify UGT2B7 cDNA segments by PCR.

<table>
<thead>
<tr>
<th>Forward primers</th>
<th>Primers</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P_1$ (5'-AACTCCTGGAAATTTTCAG-3')</td>
<td>778 to 795</td>
</tr>
<tr>
<td></td>
<td>$P_3$ (5'-TGCATTGCACCAGGATGTC-3')</td>
<td>-14 to 5</td>
</tr>
<tr>
<td></td>
<td>$P_5$ (5'-TGACATGAAGAAGTGGGATC-3')</td>
<td>681 to 700</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Backward primers</th>
<th>Primers</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P_2$ (5'-GTCTTCCATTTCCCTTAGGC-3')</td>
<td>882 to 864</td>
</tr>
<tr>
<td></td>
<td>$P_4$ (5'-AACTGAAGTAGTCTCACC-3')</td>
<td>1642 to 1625</td>
</tr>
<tr>
<td></td>
<td>$P_6$ (5'-TCAAACATTGGTGAGTGCGG-3')</td>
<td>824 to 805</td>
</tr>
</tbody>
</table>
first-strand cDNA template, producing a final amplification volume of 50 μL. Typically, each amplification mixture underwent 35 cycles of denaturation at 94°C for 45s, annealing at 53°C for 45s, and polymerase extension at 72°C for 90s. (Annealing temperatures and polymerase extension times were optimized for each primer combination.). These amplification cycles were followed by 7 min at 72°C, and a final hold at 4°C. All reactions were carried out with a GeneAmp® PCR System 9600 Thermal Cycler (Perkin Elmer/Cetus). A 10μL aliquot of each amplification was loaded onto a 0.7% agarose gel containing 0.01% ethidium bromide.

Amplification products were cut from the gel, and reamplified using the same primers as in the first round of PCR amplifications, or with nested primers (Figure 3.1). Again, the size of the amplification products was determined by electrophoresis in a 0.7 or 1% agarose gel containing 0.01% ethidium bromide.

UGT2B7 cDNA segments amplified by P₁ and P₃ (105 bp), and by P₃ and P₆ (144 bp), were ligated into Sma I-digested pBluescript® II SK⁺ phagemid, in an overnight reaction with T4 DNA ligase (Stratagene). The ligation mixture was used to transform XL1-Blue cells, and subclones were grown overnight. Plasmid was purified according to the method of Mierendorf and Pfeffer (1989), and direct plasmid sequencing was carried out according to the dideoxy chain-termination method (Sanger et al., 1977; Carlson and Messing, 1984) with the Sequenase® 2.0 7-deaza dGTP sequencing kit (US Biochemical). When larger cDNA segments required sequencing, a series of subclones with overlapping nested deletions was created with exonuclease III and mung bean nuclease, using the Exo/Mung Kit (Stratagene), and the supplier’s protocol.
Restriction Fragment Length Variation Analysis of UGT2B7 cDNA

Each of the UGT2B7 cDNA amplifications described by the schematic in Figure 3.1 includes position 802 of the coding region. This position provides a recognition site for the restriction endonuclease Mnl I. Jin et al. (1993) had isolated a UGT2B7 variant from a λ gt11 cDNA library constructed from mRNA obtained from the liver of a single human donor. This variant differed from previously reported UGT2B7 cDNA at only one nucleotide; C was substituted by T at position 802. This point mutation in the variant UGT2B7 cDNA results in the substitution of tyrosine for histidine at residue 268, and a loss of the Mnl I recognition site. UGT2B7 cDNA, representing 37 individuals, was restriction-digested by Mnl I (New England Biolabs) to test for the presence of this point mutation. All cDNA segments from the second round of PCR amplifications in Figure 3.1 were subjected to the digestion. In a final volume of 20 μL, less than 1 μg of cDNA was mixed with 200 mM potassium glutamate, 50 mM Tris-acetate, 20 mM magnesium acetate, 2 μg bovine serum albumin (Fraction V, Sigma), and 1 mM β-mercaptoethanol. Following the addition of Mnl I (2 units), the mixture was incubated at 37°C for 2 h, and the digestion was stopped by 10 mM EDTA (pH 8). Digested cDNA fragments were either electrophoresed in an agarose gel (0.7%, with 0.01% ethydium bromide), or amplified, by PCR, using the same oligonucleotide primers which had been used to generate the pre-digestion segments (Figure 3.2).

Due to the proximity of the mutation site to another Mnl I restriction site at nucleotide position 826, allele-specific amplification of the digested fragments was deemed necessary. P7 and P8 encompass a region of UGT2B7 cDNA which includes the mutation site but excludes any other Mnl I restriction site (Figure 3.2). Since the reported mutation at position 802 leads to the loss of
Figure 3.2. Schematic of RFLV analysis of UGT2B7 cDNA.

UGT2B7 cDNA

5'       802  UGT2B7 coding

3'

variant  144 bp segment containing position 802
wt

Restriction endonuclease digestion with Mnl I

Allele-specific amplifications with P5 and P6

wt                       variant
a recognition site for Mnl I, alleles with the mutation should yield digestion products which can be amplified by P$_2$ and P$_5$. Amplification mixtures of 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 3 mM MgCl$_2$, 1 mM each of dATP, dCTP, dGTP, and dTTP, 100 pmol each of forward (P$_2$) and backward (P$_5$) UGT2B7-specific oligonucleotide primers, and 2.5 units of AmpliTaq® polymerase were pre-incubated at 94°C for 3 min. Mnl I-digested fragments (4.5 μL) were added as a template for amplification, to a final reaction volume of 50 μL. Each amplification mixture underwent 35 cycles of denaturation at 94°C for 45s, annealing at 53°C for 45s, and polymerase extension at 72°C for 90s. The size of the amplification products was determined by electrophoresis in a 0.7 or 1% agarose gel containing 0.01% ethidium bromide, or in an 8% polyacrylamide gel.

Expression of UGT2B7 cDNAs in COS-1 Cells

Sequence and restriction fragment length variation (RFLV) analyses detected the presence or absence of the variant UGT2B7 mRNA in individual livers. Full-length cDNA of the variant UGT2B7, as well as cDNA containing the full coding region of wild-type UGT2B7, were isolated from 0.7% agarose gels following the digestion of appropriate pBluescript® II SK$^-$ subclones with Eco RI. Both inserts were ligated, separately, into the Eco RI site of the 4.1 kb eukaryotic expression vector pSG5 (Stratagene; Figure 3.3). Plasmids were transfected into E. coli NM522 cells and purified by polyethylene glycol precipitation (Maniatis et al., 1990). Proper orientation of the cDNA inserts with respect to the SV40 promoter of pSG5 was determined by restriction enzyme analysis and DNA sequence analysis. Monkey kidney COS-1 cells (80% confluent) were
Figure 3.3. Schematic of UGT2B7 cDNA insertion into mammalian pSG5 expression vector.
plated and grown overnight in medium containing 10% fetal bovine serum (Gibco/BRL). Purified plasmids were transiently transfected into washed COS-1 cells for 5 h with the carrier Lipofectamine® (Gibco/BRL), according to the manufacturer’s protocol. (Control cells were transfected with the UGT2B7 cDNA inserted in the reverse direction with respect to the SV40 promoter of pSG5 (Figure 3.3).) The COS-1 cell line expresses the T-antigen, which is capable of enhancing transcription via the SV40 promoter. Transfection was blocked by washing with phosphate-buffered saline and the cells were maintained in appropriate media, containing 10% fetal bovine serum, at 37°C in 10% CO₂. After an incubation period of 72 h, the cells were harvested and homogenized in 50 mM Tris-HCl (pH 7.4). Protein concentration was measured with the BCA® protein concentration analysis (Pierce, Rockford, IL), and the homogenate was stored at 70°C, until assayed for glucuronidation activity.

Glucuronidation Assays using cDNA-Expressed UGT2B7 and Human Liver Microsomes

COS-1 cells transfected with variant UGT2B7 cDNA, and those transfected with wild-type UGT2B7 cDNA, were analysed for glucuronidating activity, along with the appropriate controls. Incubations conditions were modified from Patel et al. (1995a). In a final volume of 200 μL, COS-1 cell homogenate protein (200 μg), was incubated with up to 0.5 mM aglycone, 4.5mM MgCl₂ and 50 μM radiolabeled [³¹C]UDP-glucuronic acid (UDPGA), in 50mM Tris-HCl (ph 7.4). The cofactor [³¹C]UDPGA consisted of 4 μM [³¹C]UDPGA (0.2 μCi), plus 46 μM unlabeled UDPGA. Incubations were performed under linear conditions, with respect to time (under 90 min), and COS-1 homogenate protein concentration. Mixtures were preincubated on
ice for 1 min prior to the addition of \([^{14}C]UDPGA\), after which they were incubated at 37°C for 1 h. Glucuronosyltransferase activity was terminated by the addition of 25 \(\mu\)L 0.1 N HCl, which lowered the incubation pH to 3.3 without hydrolysis of the conjugated glucuronide. Low pH also stabilizes acyl glucuronides formed by aglycons with carboxylic acid functional groups. Ethanol (300 \(\mu\)L) was added, and the incubation tubes were micro-centrifuged at 10000 g for 6 min. The supernatant (200 \(\mu\)L) was aspirated and analysed by thin layer chromatography (TLC), according to a protocol modified from Bansal and Gressner (1980). The TLC solvent was acetone, 30% ammonia solution, butan-1-ol, glacial acetic acid, and water (25:0.75:35:9:30.25). In order to confirm the presence of glucuronides, \(\beta\)-glucuronidase-treated (5.5 units, pH 4.6) aliquots were co-chromatographed with untreated samples on silica gel plates. Glucuronide-containing regions of the TLC plates were detected by autoradiography, removed and quantitated by a Beckman LS5000 liquid scintillation counter.

Microsomal preparations from human liver samples (Patel et al., 1995b) were also assayed for glucuronidating activity with a protocol similar to that used for COS-1 cell homogenate. Only the microsomal protein concentration was adjusted (50 \(\mu\)g/incubation).
IV. RESULTS
1. INHIBITION STUDIES OF (S) OXAZEPAM GLUCURONIDATION

Assay optimization

Figures 4.1a and b show that the rate of total glucuronide formed was linear up to incubation times of 40 min, and microsomal protein concentrations of 1.0 mg/ml. In order to reduce experimental variability due to "latency", microsomal enzymes are often maximally activated by the addition of a detergent to the incubation mixture. However, in this particular case, the addition of a detergent (digitonin) was found not to be beneficial (Figure 4.2). (Unlike rat or mouse UGT activity, human UGT activity has recently been reported not to undergo detergent activation in vitro (Hansen and Stentoft, 1995).) Therefore, to minimize the incubation volume, its addition was omitted. Terminating the reaction by decreasing the pH to 3.3, via the addition of 0.1 N HCl, was found to effectively stop further formation of oxazepam glucuronide. The stability of the glucuronides was not compromised over a period of 6 h at this low pH.

A chromatogram of the (R) and (S)oxazepam glucuronide diastereomers is shown in Figure 4.3. Under these chromatographic conditions, the parent compound (R,S)oxazepam is observed after 60 min. Significantly higher amounts of (S)oxazepam glucuronide were observed in vitro than (R)oxazepam glucuronide. This observation is consistent with previously reported observations in vivo in plasma and urine (Ruelius et al., 1979; Vree et al., 1991).
Figure 4.1. In vitro assay optimization. At 37°C, the formation rate of S(·) and R(·)oxazepam glucuronide remained linear up to enzymic incubation times of 40 min (A), and microsomal protein concentrations of 1.0 mg/mL (B). Each point represents the average of five replicates.
Figure 4.2. The effect of detergent activation on the formation rate of \( S(\phi) \) and \( R(\phi) \)oxazepam glucuronide. Increasing concentrations of digitonin only decreased the rate of glucuronidation.
Figure 4.3. HPLC analysis of (S) and (R) oxazepam glucuronide. Under these conditions, the parent compound was observed after 60 min.
Enzymatic glucuronidation

The rate of appearance of (R) and (S)oxazepam glucuronides, with increasing incubation concentrations of (R,S)oxazepam, is graphically depicted in Figure 4.4 for the three livers.

For each of the three livers, enzymatic glucuronidation rates of oxazepam were fitted with a simple hyperbolic (Michaelis-Menten) model (Figure 4.4). In each case, the single-site models were determined to provide significantly better fits (p < 0.01) than alternative models (straight line, complex hyperbola). Kinetic parameters (Km, Vmax) were estimated, along with their asymptotic errors (Table 4.1).

Both Vmax, and Km, values differed significantly between the two diastereoisomers (p < 0.01), strongly suggesting that (S)oxazepam was a better substrate for conjugation by UGT than (R)oxazepam. However, confounding factors such as the possible conversion of (R) to (S)oxazepam prior to conjugation, or the stereoselective hydrolysis in vitro of (R)oxazepam glucuronide should not be dismissed.

Inhibition studies

Ketoprofen inhibited the formation of (S)oxazepam glucuronide and - at very high concentrations - (R)oxazepam glucuronide (Figure 4.5a). Inhibition of the formation of (S)oxazepam glucuronide was well-described by a logistic fit indicating one-site competitive interaction between (S)oxazepam and ketoprofen (p < 0.01). This was not the case with respect to the formation of (R)oxazepam glucuronide (p < 0.68). These observations were consistent with those made when fenoprofen, ibuprofen, (S)naproxen, clofibric acid, and the endogenous compounds hyodeoxycholic acid and estriol were incubated with racemic oxazepam. All of these
Table 4.1. Estimated kinetic parameters (with asymptotic errors) for the formation of $S(\alpha)$ and $R(\alpha)$ oxazepam glucuronide by microsomes obtained from three human livers. The parameters characterize a simple hyperbolic fit of the data.

<table>
<thead>
<tr>
<th>Liver</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol min$^{-1}$ per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)OX-G</td>
<td>0.17 ± 0.02</td>
<td>207.4 ± 11.3</td>
</tr>
<tr>
<td>(R)OX-G</td>
<td>0.21 ± 0.02</td>
<td>68.4 ± 3.3</td>
</tr>
<tr>
<td>K-28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)OX-G</td>
<td>0.18 ± 0.02</td>
<td>197.8 ± 11.9</td>
</tr>
<tr>
<td>(R)OX-G</td>
<td>0.23 ± 0.03</td>
<td>42.5 ± 2.6</td>
</tr>
<tr>
<td>K-29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)OX-G</td>
<td>0.12 ± 0.01</td>
<td>277.3 ± 18.6</td>
</tr>
<tr>
<td>(R)OX-G</td>
<td>0.18 ± 0.02</td>
<td>73.2 ± 3.6</td>
</tr>
</tbody>
</table>
Figure 4.4 In vitro kinetics of S(\(\ast\)) and R(\(\ast\)) oxazepam glucuronidation by microsomes from three human livers. Each point represents five replicate observations. Simple hyperbolas were fitted to the data to obtain kinetic parameters for the formation of each diastereomer in each liver.
Figure 4.5 (A) Differential inhibition of $S(\bullet)$ and $R(\circ)$ oxazepam glucuronide formation by ketoprofen. (B) Inhibition profile of $(S)$ oxazepam glucuronide formation by known substrates of UGT2B7 and by morphine. [ketoprofen (x), S naproxen (A), ibuprofen (o), fenoprofen (-), clofibric acid (i), morphine (o), estriol (+), hyodeoxycholic acid (+)]
compounds are known to be substrates for UGT2B7 and a single amino acid variant of UGT2B7 (Ritter et al., 1990; Jin et al., 1993). Figure 4.5b shows the inhibition profile of (S)oxazepam glucuronide formation by the physiological substrates estriol and hyodeoxycholic acid, several NSAIDS, and clofibric acid. The rank order is consistent with that observed by Jin et al. (1993) who expressed a single amino acid variant of UGT2B7 in COS-7 cells.

Like p-nitrophenol (Figure 4.5b), neither morphine nor codeine appeared to competitively inhibit the formation of (R) or (S)oxazepam glucuronide at physiological pH (7.4). The non-interaction between morphine and oxazepam glucuronidation appears to be inconsistent with results reported by Sawe et al. (1982). It should be noted that these previous interactions were observed at a nonphysiological pH (> 8.7) and may not be indicative of the in vivo situation.

(S)oxazepam glucuronide was inhibited by xenobiotics and endogenous compounds with the same rank order as reported for a single amino acid variant of UGT2B7 (tyrosine for histidine at residue 268) expressed in COS-7 cells Jin et al., (1993). However, in that particular study it was reported that oxazepam was only negligibly glucuronidated by this variant of UGT2B7. Thus it may be the case that substitution of tyrosine for histidine at residue 268 of UGT2B7 may alter substrate specificity with respect to (S)oxazepam. Unlike the NSAIDS, oxazepam does not contain a carboxylic acid functional group. However, its site of glucuronidation is adjacent to a cyclic ketone (Figure 1.3), and this may facilitate its conjugation by UGT2B7.
2. INTERINDIVIDUAL VARIABILITY OF (S)OXAZEPAM GLUCURONIDATION

In Vivo Glucuronidation in Human Subjects

Study 1:

Full kinetic analyses were performed on the data for those 11 subjects who had undergone an extensive schedule of blood sampling and urine collection. The observation of relatively high ratios of (S) to (R) glucuronides in urine and plasma is consistent with that previously reported in a single human subject by Vree et al. (1991).

In nine of the 11 subjects, the (S) glucuronide was observed at significantly higher levels than the (R) glucuronide in both plasma and urine. Urinary and plasma ratios of (S) to (R) oxazepam glucuronide in the 9 "typical" subjects were 3.87±0.79 (mean±s.d.) and 3.52±0.60 respectively. Within subjects, these ratios were consistent between 8 and 24 h after drug administration. Estimated pharmacokinetic parameters are tabulated in Table 4.2. In two of the 11 subjects, the urinary and plasma ratios of (S) to (R) oxazepam glucuronides were significantly lower than those observed in the other nine subjects (a difference of more than 2 s.d. in each case; Table 4.2). Figure 4.6 shows urinary and plasma dispositions of (S) to (R) oxazepam glucuronides in both a "typical" and an "atypical" subject.

Up to 24 h after drug administration, a good correlation (Spearman's rank order correlation $r_s = 0.92$, p<0.05) was shown to exist between the mean observed urinary ratios of (S) to (R) glucuronide and the plasma clearance of (R,S) oxazepam in all 11 subjects. Since the urinary ratio of (S) to (R) glucuronide was apparently consistent between t=4 and 24 h, this observation implies that the ratio at t=8 h may be a good, single-point, indicator of plasma (R,S)
Table 4.2. Estimated kinetic parameters (with asymptotic errors) for the disposition of \((R)\) and \((S)\) oxazepam glucuronide in 11 subjects. The parameters characterize a single compartment open model with simple Michaelis-Menten kinetics. Two of the subjects had an abnormally low plasma clearance and were not grouped with the others.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters (Study 1: 11 subjects)</th>
<th>Typical subjects (n = 9) (mean ± sd)</th>
<th>Atypical subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((R,S)) oxazepam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearance (ml min(^{-1}) kg(^{-1}))</td>
<td>1.21 ± 0.18</td>
<td>0.42</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>8.1 ± 3.2</td>
<td>14.7</td>
</tr>
<tr>
<td>Volume of distribution (l kg(^{-1}))</td>
<td>0.78 ± 0.19</td>
<td>0.69</td>
</tr>
<tr>
<td>((R): (S)) glucuronide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio ((\text{S})/(\text{R}); t = 8–24 h)</td>
<td>3.52 ± 0.60</td>
<td>1.15</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((R), (S)) glucuronide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery ((S)) gluc. (%dose: t = 32 h)</td>
<td>42.3 ± 10.1</td>
<td>21.9</td>
</tr>
<tr>
<td>Recovery ((R)) gluc. (%dose: t = 32 h)</td>
<td>12.1 ± 3.9</td>
<td>16.7</td>
</tr>
<tr>
<td>Ratio ((\text{S})/(\text{R}); t = 8–24 h)</td>
<td>3.87 ± 0.79</td>
<td>1.41</td>
</tr>
</tbody>
</table>
Figure 4.6. Plasma and urine levels of $S$ and $R$ oxazepam glucuronide after oral administration of 15 mg $(R, S)$ oxazepam in human subjects. The upper graph (A) is data from a "typical glucuronidator, and the lower graph (B) is data from a slow or "atypical" glucuronidator.
oxazepam clearance. Figure 4.7 shows a correlation of $r = 0.90 \ (p<0.05)$ between the observed urinary ratios of ($S$) to ($R$) glucuronide at $t=8$ h and the plasma clearance of ($R,S$) oxazepam for all 11 subjects. The two individuals with low estimates of plasma clearance also displayed low urinary ($S$) to ($R$) glucuronide ratios.

Studies 2 and 3:

Eight hour urinary ratios were obtained from a further 19 student volunteers and 47 patients. A distribution of these ratios is shown in Figure 4.8. Due to differences in age, health status, drug intake, and the duration of sample storage, the student data were demarcated from the patient data. Maximum likelihood estimates of parameters from a probit model indicated that neither set of subjects were normally distributed ($\chi^2$ test with $p<0.05$). The total distribution was skewed positively (skewness=3.01). Probit (Bliss, 1934) and NTV (Endrenyi and Patel, 1991) analyses of the log-transformed ratios showed evidence of an antimode at a cumulative frequency (F) of 0.104, corresponding to a urinary ($S$) to ($R$) diastereomeric ratio of 1.9.

When the individuals with low ($<1.9$) urinary ($S$) to ($R$) ratios were removed, the student distribution approximated normality under the maximum likelihood model, whereas the patient distribution continued to display positive skewness. The prevalence of higher ($S$) to ($R$) glucuronide ratios in the patient population may be indicative of induced liver enzyme activity due to a long-term exposure to anxiolytics or other drugs. Age-related factors, as well as the possible degradation of ($R$)oxazepam glucuronide during storage, may have been responsible for the larger ($S$) to ($R$) glucuronide ratios observed in the patients. Notably, low urinary ($S$) to ($R$) ratios were observed in both student and patient populations.
Figure 4.7. Correlation between the ratio of $(S)/(R)$ oxazepam glucuronide in urine at $t=8$ h and the estimated plasma clearance of $(R,S)$ oxazepam in the 11 subjects for whom plasma kinetics were estimated.

![Graph showing the correlation between S/R ratio and plasma clearance of oxazepam]

$r_5 = 0.90$
Figure 4.8. The distribution of urinary ratios of $(S)$ to $(R)$ oxazepam glucuronide in 30 student volunteers (○) and 47 patients (□). A histogram representation of the data (A), shows that the data is skewed positively (skewness=3.01), and has an apparent antimode at $(S)/(R)=2$. Probit (B), and NTV (C) analyses of log transformed ratios also show evidence of an antimode at $F=0.104$, corresponding to $(S)/(R)=1.9$. 

[Histogram A] 

[Probit B] 

[NTV C]
The two students with low urinary diastereomeric ratios for whom full kinetics were available also had markedly low plasma clearance of (R,S) oxazepam (Figure 4.7; Table 4.2). This implicates enzymatic glucuronidation by liver UGT(S), rather than renal function, as the major source of the observed variability between typical and atypical individuals. However, confounding factors such as differential sequestration, the possible interconversion of (R) and (S) oxazepam, or the stereoselective hydrolysis of either (R) or (S) oxazepam glucuronide cannot be dismissed.

In Vitro Microsomal Glucuronidation

Study 4:

In vitro glucuronidation of (R,S) oxazepam was measured in microsomes prepared from 37 individual kidney donor livers. Figure 4.9 shows the formation rate of (R) and (S) glucuronide diastereomers in relation to the concentration of parent compound in two of the livers tested. With the use of non-linear curve-fitting, it was determined that the in vitro formation of (R) and (S) oxazepam glucuronide diastereomers by human liver microsomes was best described by single-site Michaelis-Menten kinetics in all 37 livers.

Under the assumption of normality, two-way analysis of variance indicated differences in the \( V_{\text{max}} \), but not in the \( K_m \) values, between Canadian and Swiss livers in the formation of both (R) and (S) oxazepam glucuronide. The difference in \( V_{\text{max}} \) was indicated by an approximation of Student’s t statistic for unequal sample sizes (p<0.05). All 37 livers were obtained from Caucasian donors. Thus the observed difference in \( V_{\text{max}} \) values between Canadian and Swiss livers may reflect a number of factors including inconsistencies in the initial handling and storage processes, as well as the relative duration for which they had been stored. Differential cultural and
Figure 4.9 *In vitro* kinetics of *(S)* and *(R)* oxazepam glucuronidation by microsomes from two human livers: K-27 (A), and K-16 (B). Liver K-27 is representative of typical oxazepam glucuronidators, whereas K-16 is atypical. Each point represents five replicate observations. Simple hyperbolas were fitted to the data to obtain kinetic parameters for the formation of each diastereomer in each liver (Table 4.3). Scatchard representations of the data have been included.
Table 4.3. Estimated kinetic parameters (with asymptotic errors) for the formation of \((R)\) and \((S)\) oxazepam glucuronide by microsomes obtained from the livers of 37 healthy kidney donors. The parameters characterize a simple hyperbolic fit of the data and the livers have been classified as either typical or atypical (poor) glucuronidators of \((S)\)oxazepam.

<table>
<thead>
<tr>
<th></th>
<th>Typical livers ((n = 33))</th>
<th>Atypical livers ((n = 4))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((\text{Mean} \pm \text{SD}))</td>
<td>((\text{Mean} \pm \text{SD}))</td>
</tr>
<tr>
<td>(K_{m} ) (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>((R))</td>
<td>0.25 \pm 0.08</td>
<td>0.23 \pm 0.15</td>
</tr>
<tr>
<td>((S))</td>
<td>0.21 \pm 0.07</td>
<td>0.57 \pm 0.05</td>
</tr>
<tr>
<td>(V_{\text{max}}) (nmol min(^{-1}) per mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>((R))</td>
<td>69.8 \pm 19.1</td>
<td>75.9 \pm 21.1</td>
</tr>
<tr>
<td>((S))</td>
<td>248.7 \pm 89.30</td>
<td>199.8 \pm 58.70</td>
</tr>
</tbody>
</table>
environmental pressures may also have been factors in the relatively higher \( V_{\text{max}} \) values observed in the Swiss livers.

In both groups of livers, the pooled estimates of \( K_m \) and \( V_{\text{max}} \) for the formation of \((R)\) oxazepam glucuronide differed significantly from those estimated for \((S)\) oxazepam glucuronide formation \((p<0.001; \text{ Table } 4.3)\). The latter observation confirms prior work \((\text{Patel et al., } 1995a)\) which indicated the involvement of different UGT isozymes in the formation of \((R)\) and \((S)\) oxazepam glucuronides through differential inhibition of \((R)\) and \((S)\) oxazepam glucuronidation by NSAIDs and other carboxylic acid-containing compounds.

The \( V_{\text{max}} \) estimates of \((R)\) and \((S)\) oxazepam glucuronidation in individual livers were deemed to be lognormally distributed by maximum likelihood analysis \((p<0.05)\), and by probit and NTV analyses. Pooled maximum likelihood analysis of the individual \( K_m \) estimates indicated that they were lognormally distributed with respect to the formation of the \((R)\) glucuronide, but not the \((S)\) glucuronide \((p<0.05)\). This was confirmed by probit and NTV analyses of the respective distributions which indicated an antimode at \( F=0.891 \), corresponding to a \( K_m(S) \) of 0.46 mM \((\text{ Figure } 4.10)\). Of the 37 livers tested, 4 (10.8%) were observed to have abnormally high estimates of \( K_m \) for the formation of \((S)\) oxazepam glucuronide, and none were observed for the formation of \((R)\) oxazepam glucuronide \((\text{ Figure } 4.10)\).
Figure 4.10 Distributions of the formation $K_m$'s of (S) and (R) oxazepam glucuronide in vitro by livers from 20 Swiss (a) and 17 Canadian (b) individuals. Histograms of the two sets of data (A,D) show that both distributions are skewed in the positive direction. However, only the formation $K_m$ of the (S) glucuronide has an apparent antimode (at $K_m=0.45$ mM). is confirmed by probit (B,E), and NTV (C,F) analyses of the log transformed values of $K_m$. 

![Histograms](image)

![Probit analyses](image)

![NTV analyses](image)
3. RFLP ANALYSES AND cDNA EXPRESSION OF VARIANT AND WILD TYPE UGT2B7

Synthesis of UGT2B7 cDNA by RT-PCR of Human Liver mRNA

Total RNA was isolated from 37 post-mortem human liver samples, and first-strand cDNA was synthesized from the mRNA of each liver by reverse-transcription. RNA extracted from post-mortem liver tissue is often highly degraded by RNase activity. Synthesis of suitable quality first-strand cDNA is unlikely under such conditions. Using first-strand cDNA as a template, a 300 bp segment of actin cDNA was amplified to test the quality of RNA extracted from individual livers, and the subsequent first-strand cDNA synthesis. Actin is an ubiquitous protein, with high levels of expression in most tissues.

In order to determine whether inter-individual differences in (S)oxazepam glucuronidation (Patel et al., 1995a) were associated with a point mutation at position 802 of the UGT2B7 coding region, (Jin et al., 1993) it was necessary to successfully amplify at least one of the cDNA segments described in Figure 3.1. Each combination of oligonucleotide primers was designed to amplify a segment of double-stranded UGT2B7 cDNA containing the mutation site. Following multiple rounds of UGT2B7-specific PCR amplification and gel purification, each of the cDNA segments was successfully amplified from all 37 livers (Figure 4.1). Oligonucleotide primers P5 and P6 amplified a 144 bp segment which contained no additional Mnl I restriction sites (other than the one affected by the point mutation at position 802), a feature which made it particularly useful for RFLV analyses (Figure 3.2).
Figure 4.11. PCR amplification of UGT2B7 cDNA segments following first-strand cDNA synthesis. The 1.6 kb full-length clone was used to express UGT2B7 in COS-1 cells, and the smaller segment was used in RFLV analysis.
Oligonucleotide primers P₁ and P₂, amplified the entire UGT2B7 coding region. Functional assays, measuring the glucuronidating activity of cDNA-expressed UGT2B7, required the entire coding region for insertion into an expression vector and subsequent transfection into cells.

**Restriction Fragment Length Variation Analysis of UGT2B7 cDNA**

RFLV analyses were designed to test for the presence, or absence, of a point mutation at position 802 of the UGT2B7 open reading frame. Substitution of T for C at nucleotide 802 of the UGT2B7 cDNA results in the loss of a recognition site for the restriction endonuclease Mnl I, allowing for analysis by RFLV. Unfortunately, Mnl I has a relatively short, nonpalindromic recognition sequence (5'-CCTC-3'), properties which increase the probability of additional recognition sites for Mnl I occurring on any given segment of double-stranded cDNA. (Restriction endonucleases with nonpalindromic recognition sequences can recognize additional sites on the antisense strand of double-stranded cDNA.) The 1.6 kb coding region of UGT2B7 cDNA contains a total of nine sites recognized by Mnl I (Figure 3.2). Three recognition sites are on the sense strand, and six are on the antisense strand. One of the Mnl I cleavage sites (position 826) is particularly close to the cleavage site lost due to the point mutation (position 809; Mnl I cleaves 7 nucleotides downstream from the recognition sequence). Direct analysis of Mnl I-digested UGT2B7 cDNA fragments would require the resolution, by gel electrophoresis, of fragments differing in length by only 17 nucleotides. This was not practical. Therefore, post-digestion, allele-specific, amplification was performed using P₁ and P₂. These primers amplify a 144 bp segment of UGT2B7 cDNA which contains only one Mnl I cleavage site, at position 809 (Figure 3.2). This
site is not cleaved when T is substituted for C at position 802. Therefore, only intact “fragments” containing the point mutation were amplified after digestion with Mnl I. Of the 37 livers tested, three livers showed amplification of a 144 bp cDNA fragment after digestion with Mnl I (Figure 4.12). These three individuals were deemed to be positive for at least one copy of the variant UGT2B7 allele. All three livers were previously phenotyped as being atypical glucuronidators of oxazepam (Patel et al., 1995a). Of the four livers which had been phenotyped as atypical glucuronidators, one did not contain a copy of the variant allele.

**Sequence Analysis of UGT2B7 cDNA**

Fragments of UGT2B7 cDNA successfully amplified by P5 and P6 after digestion with Mnl I were sequenced to confirm the presence of the point mutation. Amplification products of P1 and P2 (105 bp), not subject to digestion by Mnl I, were also sequenced for all 37 livers. As predicted by the RFLV analyses, only the three livers which which showed the variant allele-specific amplification contained the point mutation at position 802 (Figure 4.13). However, pre-digestion cDNA from all three livers also produced subclones which did not contain the mutation. Therefore, these three livers could not have been homozygous for the variant UGT2B7 allele.

Of the four livers which had been phenotyped as atypical glucuronidators of (S)oxazepam, one was determined not to be positive for the point mutation at position 802 of UGT2B7 cDNA. Full-length cDNA was sequenced for this particular individual. The cDNA was found to encode a 529 amino acid protein which was identical to a previously published UGT2B7 clone, with consensus sequences for three potential asparagine-linked glycosylation sites at residues 67, 68,
Figure 4.12. Allele-specific PCR amplification of UGT2B7 cDNA fragments following digestion by Mnl I. Three out of four livers phenotyped as being atypical were positive for a point mutation at position 802 of UGT2B7 cDNA. (All three were confirmed to be heterozygous for mutation.) None of the 33 livers phenotyped as being typical had the mutation.
Figure 4.13. Sequence analysis of wild-type and variant UGT2B7 cDNA showing point mutation at position 802.
Figure 4.14. Transcribed sequence of UGT2B7. The signal peptide is underlined, and three potential asparagine-linked glycosylation sites are marked with arrows. A dashed box outlines the putative transmembrane region. The mutation site at position 802 is boxed. (Adapted from Ritter et al, 1990)
and 315 (Figure 4.14; Ritter et al., 1991). (Full-length variant UGT2B7 cDNA was also sequenced for subsequent expression studies in COS-1 cells.)

Thus, allele-specific amplification of RFLV fragments, and subsequent nucleotide sequencing, showed that three of four livers, which had previously been phenotyped as atypical glucuronidators (Patel et al., 1995b), were positive for a point mutation at position 802 (T for C) of the UGT2B7 cDNA coding region (Figure 4.12). The mutation results in the substitution of histidine for tyrosine at residue 268 of the UGT2B7 enzyme (Figure 4.15). All three individuals were confirmed to be heterozygous for the mutation. According to Hardy-Weinberg monogenic prediction, if the frequency of heterozygotes is 10%, homozygotes should comprise 0.3% of the population.

Full-length UGT2B7 cDNA sequence analysis for the fourth liver, which was phenotyped as an atypical glucuronidator, but did not test positive for the point mutation at position 802, showed no coding differences when compared to the previously published sequence of UGT2B7 (Ritter et al., 1991). The atypical phenotype in this individual may have been due to reduced levels of overall UGT expression, or specifically reduced levels of UGT2B7 expression.

Glucuronidation by cDNA-Expressed Variant and Wild-Type UGT2B7 in COS-1 Cells

UGT2B7 cDNA studies were undertaken, in part, to determine whether variation in (S)-oxazepam glucuronidation is due to genetic irregularities in the sequence or expression of UGT2B7. The studies were based on three observations: (S)-oxazepam glucuronidation rates are bimodally distributed, with approximately 10% of Caucasian individuals having lower rates than
the general population (Patel et al., 1995b); an inhibition profile of (S)oxazepam glucuronidation indicates that UGT2B7 is the glucuronidating enzyme (Patel et al., 1995 a); a single amino acid variant of UGT2B7, isolated from the cDNA library of a single human liver, appeared to glucuronidate oxazepam with negligible efficiency (Jin et al., 1993).

Using radiolabeled cofactor, [³¹C]UDPGA, the glucuronidating activity of COS-1 cells transfected with variant and wild-type UGT2B7 cDNA was quantitated. Control cells, transfected with cDNA in reverse orientation with respect to the SV40 promoter of the eukaryotic expression vector pSG5, showed negligible native glucuronidating activity towards the tested substrates (Figure 4.15; Table 4.4). Kinetic parameters, and corresponding asymptotic errors, of both cDNA-expressed transferases were estimated for the glucuronidation of oxazepam, ketoprofen, (S)naproxen, hyodeoxycholic acid, and also for the endogenous steroids estriol, 2-OH-estriol (Figure 4.15; Table 4.4). There were no appreciable differences in either the maximal rate ($V_{\text{max}}$), or the metabolic rate constant ($K_m$) of variant and wild-type UGT2B7 in the glucuronidation of the nonsteroidal anti-inflammatory drugs ketoprofen, and (S)naproxen. Hyodeoxycholic acid glucuronidation was similarly unaffected by the single amino acid difference between the two enzymes. Estriol, and 2-OH-estriol, both show a significant decrease in $V_{\text{max}}$ ($p<0.01$) and an increase in $K_m$ ($p<0.05$) when glucuronidated by the variant form of UGT2B7. Most striking, was the nearly complete decrease in the ability of the variant UGT2B7 to glucuronidate oxazepam.
Figure 4.15. Glucuronidating activity of UGT2B7 cDNA expressed in COS-1 cells. The ability of wild-type (●) and variant (○) UGT2B7 to glucuronidate various drugs and endogenous substrates and drugs was compared. Kinetic parameters were estimated for each compound. The polymorphism appears to be substrate-specific. Each point represents an average of three separate transfections. UGT2B7 cDNA inserted with reverse-orientation relative to the pSG5 initiation site provided negative controls (△).
Table 4.4. Estimated kinetic parameters (with asymptotic errors) for the formation of oxazepam glucuronide by cDNA expressed variant and wild-type UGT2B7 in COS-1 cells. The parameters characterize a simple hyperbolic fit of the data, shown in Figure 4.15. Differences in the $V_{\text{max}}$ of glucuronidation were noted between variant and wild-type UGT2B7 for three of the six substrates: oxazepam, 2-OH-estriol, and estriol ($p<0.01$). Differences in $K_m$ were less marked for these substrates ($p<0.05$).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxazepam</td>
<td>wild type 211±43 183±52 151±37 0.18±0.02 0.16±0.02 0.07±0.01</td>
<td>1.22±0.37 1.26±0.34 2.51±0.41 0.18±0.02 23.1±2.11 27.3±2.1</td>
</tr>
<tr>
<td>ketoprofen</td>
<td>variant 451±223 197±65 142±42 0.23±0.05 0.19±0.02 0.07±0.01</td>
<td>0.02±0.02 1.19±0.29 2.39±0.47 0.23±0.05 12.1±2.71 29.7±2.8</td>
</tr>
<tr>
<td>(S)naproxen</td>
<td>wild type 211±43 183±52 151±37 0.18±0.02 0.16±0.02 0.07±0.01</td>
<td>1.22±0.37 1.26±0.34 2.51±0.41 0.18±0.02 23.1±2.11 27.3±2.1</td>
</tr>
<tr>
<td>ketoprofen</td>
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<td>0.02±0.02 1.19±0.29 2.39±0.47 0.23±0.05 12.1±2.71 29.7±2.8</td>
</tr>
<tr>
<td>2-OH-estriol</td>
<td>wild type 211±43 183±52 151±37 0.18±0.02 0.16±0.02 0.07±0.01</td>
<td>1.22±0.37 1.26±0.34 2.51±0.41 0.18±0.02 23.1±2.11 27.3±2.1</td>
</tr>
<tr>
<td>estriol</td>
<td>variant 451±223 197±65 142±42 0.23±0.05 0.19±0.02 0.07±0.01</td>
<td>0.02±0.02 1.19±0.29 2.39±0.47 0.23±0.05 12.1±2.71 29.7±2.8</td>
</tr>
<tr>
<td>hyodeoxycholic acid</td>
<td>wild type 211±43 183±52 151±37 0.18±0.02 0.16±0.02 0.07±0.01</td>
<td>1.22±0.37 1.26±0.34 2.51±0.41 0.18±0.02 23.1±2.11 27.3±2.1</td>
</tr>
<tr>
<td>hyodeoxycholic acid</td>
<td>variant 451±223 197±65 142±42 0.23±0.05 0.19±0.02 0.07±0.01</td>
<td>0.02±0.02 1.19±0.29 2.39±0.47 0.23±0.05 12.1±2.71 29.7±2.8</td>
</tr>
</tbody>
</table>
V. DISCUSSION
Stereospecific Glucuronidation of (S)Oxazepam by UGT2B7

UGT2B7 substrates inhibited the formation of (S)oxazepam glucuronide and - at very high concentrations - (R)oxazepam glucuronide (Figure 4.5). Inhibition of the formation of (S)oxazepam glucuronide was well-described by a logistic fit indicating one-site competitive interaction between (S)oxazepam and UGT2B7 substrates (p < 0.01 in each case). This was not the case with respect to the formation of (R)oxazepam glucuronide (p < 0.68 for ketoprofen, with similar results for other UGT2B7 substrates). This is the best evidence to date that the glucuronidation of (R) and (S)oxazepam is mediated by separate UGT isozymes. Dissimilar distributions of $K_m$ for (R) and (S)oxazepam glucuronide formation in 37 livers are corroborative of their differential inhibition (Figure 4.10). However, product formation by cDNA-expressed UGT2B7 in COS-1 cells was not sufficiently high to enable analysis by a stereoisomer-sensitive HPLC assay. Radiometric analysis following TLC separation of product from unutilized cofactor ([14C]UDPGA) is not capable of discriminating between the formation of (R) or (S)oxazepam glucuronide. Therefore, due to the availability of oxazepam only as a racemate, we were unable to test the enantiomers separately for product formation by cDNA-expressed UGT2B7. It may be possible to separate racemic (R,S)oxazepam into its individual enantiomers with a chiral chromatographic column. This was not attempted, but may be necessary in any future studies which aim to discover which UGT isozyme(s) are specifically involved in the glucuronidation of the therapeutically inactive (R) enantiomer of oxazepam.

Glucuronidation of enantiomers by separate UGTs has been reported for other drugs. cDNA-expressed rat phenol UDP-glucuronosyltransferase (UGT1A1) conjugates (R) naproxen, but conjugation of the (S) enantiomer is negligible (el Mouelhi et al., 1993). In line with the high
constitutive expression of UGT1A1 in extrahepatic tissues, a high R/S ratio of naproxen glucuronidation was found in rat testes, intestine, lung and kidney.

In lieu of relevant immunohistochemical or protein purification data, the inhibition profile of (S)oxazepam glucuronidation (Figure 4.5) was the most compelling evidence that UGT2B7 was involved until the cDNA-expressed enzyme was shown to glucuronidate oxazepam (Figure 4.15; Table 4.4). Of the UGT2B subfamily, the isoforms which have the highest protein sequence identity (>75.6%) to UGT2B7 are UGT2B9, UGT2B10, UGT2B4, UGT2B11, and UGT2B8. All but UGT2B10 (Jin et al., 1993) are known to share at least one substrate with UGT2B7, but only UGT2B4 glucuronidates hyodeoxycholic acid, a known substrate of UGT2B7 (Jackson et al., 1987). However, the relative potencies of hyodeoxycholic acid and estriol, as substrates of UGT2B7 and UGT2B4, are reversed (Ritter et al., 1992). In V79 (Chinese hamster lung fibroblast) cell lines expressing a functional rat UGT2B1, the substrate specificity toward more than 100 compounds was determined (Pritchard et al., 1994). Phenolic and alcoholic substrates included a range of carboxylic acids of both endogenous and exogenous (NSAIDS, fibrate hypolipidemic agents, and sodium valproate) origin. Determination of apparent kinetic constants for the glucuronidation by UGT2B1 of selected aglycones revealed a high maximal velocity toward the 3-position of morphine, but not hyodeoxycholic acid or oxazepam.

Interindividual Variation in the Glucuronidation of (S)Oxazepam

Clear bimodalities were observed in the distributions of $K_m$ of (S) oxazepam glucuronidation in vitro, and were also inferred in the plasma clearance of (R,S) oxazepam by the plasma and urinary ratios of (S) to (R) oxazepam in vivo. The consistencies between the in vitro
and in vivo data strongly suggest genetic variability between individuals with respect to the glucuronidation of (S) oxazepam.

Due to high water-solubility, the renal excretion of glucuronide conjugates, including oxazepam glucuronide diastereomers, is primarily a function of plasma concentration, as implied by the data in Figure 4.6 (Vree et al., 1991; Greenblatt et al., 1983). The appearance rate of the glucuronides in plasma is theoretically a function of hepatic conjugation. Assuming this simple model, urinary ratios of (S) to (R) oxazepam glucuronide should reflect the relative hepatic formation rates of each diastereomer. Figure 5.1 shows the distribution of the ratios of in vitro formation rates of (S) to (R) glucuronides in the 37 livers tested. Probit and NTV analyses of the in vitro ratios demonstrate an antimode at F=0.108, corresponding to a (S)/(R) ratio of 2.12. This is consistent with observations made in urine, which show an antimode at F=0.104, corresponding to a (S)/(R) ratio of 1.91 (Figure 4.8). This consistency between the hepatic formation and urinary appearance of (R) and (S) oxazepam glucuronide suggests that other dispositional factors were not significantly reflected in the urinary (S) to (R) glucuronide ratio. Potentially, these could have included the stereospecific enzymatic hydrolysis of the (R) glucuronide (Ruelius et al., 1979), in vivo conversion of 3(R) oxazepam to 3(S) oxazepam prior to the glucuronidation process, separate enterohepatic recirculation, or dissimilar renal handling of the two diastereomers. It follows that the observed ratio in vivo was mainly a reflection of the individual hepatic conjugation rate constants for (R) and (S) oxazepam.

Low (S) to (R) glucuronide ratios in urine 8 h after oral administration were shown to correlate well ($r_s=0.90$) with decreased plasma clearances of (R,S) oxazepam in the 11 subjects for whom pharmacokinetic parameters were determined (Figure 4.7). Since urinary (S) to (R)

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Figure 5.1 The distribution of the formation ratios of (S) to (R) oxazepam glucuronide in vitro by livers from 20 Swiss (s) and 17 Canadian (c) individuals, at a (R,S) oxazepam concentration of 0.25 mM. A histogram representation of the data (A), shows an apparent antimode at (S)/(R)=2. Probit (B), and NTV (C) analyses of log transformed ratios also show evidence of an antimode at F=0.108, corresponding to (S)/(R)=2.1.
ratios are likely a function of hepatic conjugation rates, an abnormally high $K_m$ for the hepatic glucuronidation of $(S)$ oxazepam may be responsible for the decreased plasma clearance of $(R,S)$ oxazepam in vivo in a significant percentage of individuals. The proportion of livers (10.8%) with high estimates of $K_m$ for $(S)$ oxazepam glucuronidation in vitro is consistent with the 8 out of 77 individuals (10.4%) who had low urinary ratios of $(S)$ to $(R)$ glucuronides in vivo.

Genetic factors involved in the glucuronidation of endo- and xenobiotics have been inferred by others to be significant contributors to interindividual variability in the disposition of drugs undergoing glucuronidation. Yue et al. (1989) have found that, upon administration of codeine, codeine-6-glucuronide (C6G) excretion accounted for 45% of the codeine dose in Oriental subjects and 62% of the dose in Caucasians. It was not clear, in this particular study, whether the lower formation of C6G in Orientals was independent of their capacity for the parallel dispositional pathways of O- and N-demethylation. However, in a more recent report, a significantly lower partial clearance via glucuronidation was shown to be responsible for the decreased overall clearance of codeine in Orientals (Yue et al., 1991).

Consistently bimodal distributions of three distinct groups of subjects - students, patients, and tissue donors - suggests that genetic, rather than environmental, factors were responsible for the decreased individual plasma clearances of $(R,S)$ oxazepam and the increased $K_m$ of $(S)$ oxazepam glucuronidation reported here. A single UGT isozyme catalysed the glucuronidation of $(S)$ oxazepam in vitro (Figure 4.9). When a single enzyme is involved in the catalysis, a significant elevation of the $K_m$ in some individuals characteristically indicates structural differences in that enzyme for those individuals. Interindividual differences in enzymic structure implicate differences at the genetic level. Preliminary data appear to indicate that similarly low $(S)/(R)$
A high degree of correlation ($r_s = 0.90$) was observed between the ratio of $(S)/(R)$ oxazepam glucuronide excretion rates in urine, and the plasma clearance of oxazepam (Figure 4.7). In vitro studies, using microsome preparations from post-mortem human livers, yielded $(S)/(R)$ glucuronide ratios similar to those observed in vivo, confirming that selective renal excretion or bacterial glucuronidase activity in the intestine were not involved (Figure 4.8; 4.9). Approximately 10% of the individuals studied were classified as atypical glucuronidators of oxazepam, including four of 37 livers. Jin et al. (1993) had isolated a UGT2B7 variant from a λ gt11 cDNA library constructed from mRNA obtained from the liver of a single human donor. This variant differed from previously reported UGT2B7 cDNA at only one nucleotide; C was substituted by T at position 802. This point mutation in the variant UGT2B7 cDNA results in the substitution of tyrosine for histidine at residue 268, and the loss of a Mnl I recognition site. RNA extracted from all 37 livers was subject to reverse transcription and reamplified with UGT2B7-specific primers (Figure 4.11). RFLV analyses, designed to test for the presence of the point mutation, were performed on cDNA amplified from each liver. Three of the four livers
Figure 5.2. Phenotypes of oxazepam glucuronidation in members of five families. (S)/(R) glucuronide ratios are shown for each individual. Atypical glucuronidators (♂, ● (male, female)) are differentiated from typical glucuronidators (□, ○ (male, female)). From these limited number of families, the mode of inheritance is not clear. The atypical phenotype may be inherited as an autosomal recessive trait.
phenotyped as being atypical were shown to contain the point mutation (Figure 4.12). Nucleotide sequencing confirmed the presence of the point mutation in the three livers (Figure 4.13). Sequencing of predigestion cDNA showed that all three livers were heterozygous for the variant UGT2B7 allele. Of the 33 livers which were phenotyped as being normal, none tested positive for the mutant allele. Two obvious questions arise from these observations. How would homozygosity for the variant allele be manifested phenotypically? (Assuming simple monogenic transmission, 1 or 2 individuals out of 400 is predicted to be homozygous for the variant.) What was the underlying factor causing the atypical phenotype in the individual who did not have a variant allele of UGT2B7 (KDC 19; Figure 4.12)? Full-length sequence analysis of this individual showed no variance from the reported sequence of UGT2B7. The atypical phenotype in this individual may have been due to reduced levels of overall UGT expression, or specifically reduced levels of UGT2B7 expression. Lamb et al (1994) have recently reported the differential induction of mouse Ugt1.6 and rabbit UGT1.6 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an Ah receptor agonist. UGT2B7 induction in humans via the Ah receptor complex is not known. However, its regulation, whether by the Ah receptor or other means warrants investigation.

Wild type and variant UGT2B7 cDNAs were expressed in COS-1 cells to test for glucuronidating activity. Estimated $K_m$ for the formation of oxazepam glucuronide by wild type UGT2B7 approximated the $K_m$ for (S)oxazepam glucuronide formation in liver microsomal preparations (Table 4.3; 4.4). There was a decrease in $V_{max}$ estimates expected in cell-expressed systems. The $V_{max}$ for the formation of oxazepam was greatly reduced for the variant form of UGT2B7, with an increase in estimated $K_m$. Since the atypical livers were confirmed to be heterozygous for the variant allele, the cDNA expression studies predict a decrease in $V_{max}$, but
little change in the $K_m$ of $(S)$oxazepam glucuronidation between normal and atypical livers. This was not the case. If $K_m$ estimates from the liver microsomes of atypical individuals are accurate, then it is likely that UGT2B7 is not the sole enzyme glucuronidating $(S)$oxazepam, or that the functional properties of the variant form in this particular cell-expression system differs from its properties in humans. *In vivo*, glucuronidation of $(S)$ oxazepam by atypical individuals was consistent with heterozygosity, as evidenced by partial, but not complete, decreases in the formation of $(S)$ oxazepam glucuronide.

**DNA Sequence Variation of UGTs**

cDNA-expression studies showed that a point mutation in UGT2B7 at position 802 (residue 268) leads to substrate-specific variations in function. These observations imply that the point mutation is related to substrate recognition, as opposed to catalytic activity. Perhaps the amino acid difference at residue 268 has greater impact on substrates of UGT2B7 with hydroxyl functional groups as opposed to carboxyl functional groups. More substrates require testing to confirm this hypothesis.

Photoaffinity labeling of UGT2B4 has implicated residues 299-446 as being involved in the binding of cofactor (Pillot *et al.*, 1993). Meech *et al.*, (1996) studied the roles of the membrane-spanning and membrane-proximal cytoplasmic domains in UGT activity by site-directed and deletional mutagenesis techniques. Truncated forms of the enzyme, forms with altered residues, or forms with heterologous tails appended to the carboxyl terminus were generated. The presence of the transmembrane domain was a critical requirement for UGT activity whereas the cytoplasmic domain seemed to be a modulator of activity but was not essential.
UGT2B Gene Structure

At this point relatively little is known about the gene structure of the UGT2 family, as compared to the UGT1 family. Determination of the gene structure of the UGT2B gene family has been performed primarily in the rat. Mackenzie and Rodbourn (1990) screened a λEMBL3 rat genomic DNA library by Southern hybridization to $^{32}$P-labelled cDNA clone UDPGTr-2 (UGT2B1). A full length clone was mapped by restriction enzyme digestion, and the Eco RI fragments were used to make plasmid subclones. The sequence of the genomic clone was compared to the known UDPGTr-2 cDNA sequence, revealing that the 1.9kb cDNA is encoded by a 12 kb gene consisting of 6 exons. Haque et al. (1991) cloned and sequenced much of the rat UGT2B2 gene in the same manner. This clearly demonstrated that unlike the UGT1 isoforms, at least 2 of the UGT2B enzymes in rat are encoded by separate genes, with no shared (alternately spliced) exons.

To determine the human UGT2B gene structures, Monaghan et al. (1994) compared the structures of rat genes UGT2B1 and UGT2B2, and found that although the sizes of the 5'- introns varied, the intron/exon boundaries of the two genes were in the same relative position within the coding region. This information together with the splice site consensus sequences was used to predict the likely position of introns within the human UGT2B cDNAs.

Analysis of Human UGT Polymorphisms and Mutations

Deficiency in the UGT1 gene product bilirubin UGT causes a severe and potentially fatal unconjugated hyperbilirubinemia as described in section I. There is obviously a requirement for mutational analysis, carrier detection and prenatal diagnosis. Several defects in the UGT1.1 gene
have already been reported but the diverse nature of the disease means that each new family presenting with the disease requires a lengthy genetic analysis. Informative intragenic polymorphic probes for CN 1 have recently been described, and can be used to offer prenatal screening to some affected families even when the underlying mutation is unknown (Moghrabi et al, 1993). Blood samples are taken from family members and used to prepare genomic Southern blots after restriction enzyme digestion with Msp 1. Two cDNA probes, one from the unique region of UGTL4 and the other from the UGTL constant region, are hybridized to the genomic blot and the haplotype which cosegregates with the disease is determined. This information can then be used in the prenatal diagnosis during future pregnancies when fetal DNA is similarly analyzed.

During the isolation and sequencing of the UGTI gene, sequences flanking the exons were also determined. These sequences can be used to generate oligonucleotide PCR primers to amplify each exon of the UGT1.1 gene, including the splice sites, from the genomic DNA of CN patients. The PCR products can be sequenced and compared to the known sequence. Furthermore, the mutation must be shown to be absent in the general control population, to ensure that it cosegregates with the defect and is not simply a functional variant present in the population. No spontaneous mutations resulting in CN have been reported to date. Several point mutations and small deletions in the UGT1.1 gene have been identified, and presumed to be the cause of CN syndrome, however in only in very few cases has the enzyme activity of the cloned expressed "mutant" enzyme been measured directly (Erps et al, 1994), as in the case of the UGT2B7 variant.
VI. CONCLUSIONS
Logistic analyses of the inhibitions by known substrates of UGT2B7 on the formation of (S)oxazepam glucuronide in microsomal preparations from human liver tissue indicated competitive interaction at a single-site (p<0.01; Figure 4.5). The formation of the (R) diastereoisomer was not competitively inhibited. Distributions of $K_m$ for the formation of the (R) and (S) glucuronides were dissimilar in 37 livers (Figure 4.10). Oxazepam was shown to undergo glucuronidation by cDNA-expressed UGT2B7 in COS-1 cells. These cells were shown to have no native oxazepam glucuronidating capacity (Figure 4.15; Table 4.4). Taken together, these data indicate that UGT2B7 glucuronidates (S)oxazepam, but not (R)oxazepam. The involvement of other UGT isozymes cannot be ruled out, even though the inhibition profile (Figure 4.5) is inconsistent with the relative substrate potencies of known UGTs.

Approximately 10% of individuals were phenotyped to be atypically poor glucuronidators of (S)oxazepam. Three of four atypical livers were found to be heterozygous for a variant allele of UGT2B7 containing a point mutation (T for C) at position 802 of the coding region. Expression studies in COS-1 cells clearly showed that changes in glucuronidation due to the variant allele are substrate-specific. Glucuronidation efficiencies of estriol and 2-OH-estriol were significantly reduced, whereas the glucuronidation of ketoprofen, (S)naproxen, and hyodeoxycholic acid were apparently unaffected. The concept that genetic variants of UGT isozymes are significant contributors to interindividual variability in the glucuronidation of drugs has remained controversial. This is the first direct finding that drug glucuronidation is affected by a polymorphic variant of the UGT2B family.
VII. FUTURE WORK
Recently, propofol glucuronidation and the effect of concomitantly administered drugs were evaluated in microsomal fractions from rat, rabbit, and human livers (Le Guellec et al., 1995). Inhibitions obtained with chemicals or drugs glucuronidated by either UGT1 or UGT2 families (1-naphthol, 4-hydroxybiphenyl, carvacrol, n-propylgallate, ketoprofen, chloramphenicol, acetylsalicylic acid) indicated that at least two UGT isoforms are involved in propofol glucuronidation. Inhibition was observed with several potentially coadministered drugs including acetylsalicylic acid, ketoprofen, oxazepam, and fentanyl. Thus, UGT2B7 may be one of the isoforms involved in propofol metabolism.

Immunohistochemical studies involving the development of an antibody specific to UGT2B7 should help to determine the possible involvement of other UGT isoforms in the metabolism of (S)oxazepam by the specific inhibition or immunoprecipitation of UGT2B7.

More generally, future studies will focus on the structure and function of UGTs. Knowledge of the enzyme activity sites will lead to definitions of substrate specificity which are important for prediction of drug metabolism. Li et al (1997) have recently reported that cDNA expressed chimeric constructs have made it possible to deduce the functional domains of rabbit liver UGT2B16 and 2B13. To characterize the structural domains of UGT2B16 and UGT2B13, a series of chimeric cDNAs were constructed that contained portions of both UGT2B16 and UGT2B13. Chimeric 2B16_{300-2B13_{531}}, which contained the amino-terminal UGT2B16 amino acids 1-300 followed by amino acids 301-531 of UGT2B13, as well as chimeric 2B16_{334-2B13_{531}} and 2B16_{434-2B13_{531}} proteins, catalyzed the glucuronidation of 4-hydroxyestrone, indicating that the carboxyl terminus of UGT2B13 could substitute for those same regions on UGT2B16. However, the replacement of the carboxyl end of UGT2B13 with 2B16_{300-531} or 2B16_{34-531}
dramatically impaired the catalytic function of the chimeric proteins. These results indicate that the carboxyl end of UGT2B13 plays an important role in the functional and possible conformational state of the protein.

Certain important human UGTs have not yet been cloned. Transferases responsible for human olfactory glucuronidation have not been isolated, although rat and bovine olfactory UGT2A homologues have been cloned. Should a human UGT2A clone be isolated, it's chromosomal localization will be of great interest. UGT2A may be a neighbour of the UGT2B group on chromosome 4, or be encoded elsewhere in the genome, potentially part of a third gene cluster in the highly divergent UGT gene family.
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IX. APPENDICES
## Pharmacokinetic parameter estimates after ingestion of 15 mg oxazepam in 11 student volunteers
(atypical individuals are listed first)

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<th>% recovery</th>
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<td>t(1/2) (h)</td>
<td>Vd (L/kg)</td>
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Plasma cozapecm glucuronide concentrations and renal excretion rates in 11 student volunteers (* denotes atypical individuals).

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### Appendix C

Estimates of Km (mM) for the formation of (R) and (S) oxazepam glucuronide in 20 Swiss and 17 Canadian kidney donor livers (atypical livers are listed first)

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