EFFECT OF VALERIAN ROOT EXTRACTS (*VALERIANA OFFICINALIS*) ON ACETAMINOPHEN GLUCURONIDATION: *IN VITRO* AND *IN VIVO* STUDIES

by

Rama Sivasubramanian

B.Pharm, Department of Pharmaceutical Sciences, Nagpur University, India, 1991

M.Pharm, Department of Pharmaceutical Sciences, Nagpur University, India, 1993

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This dissertation was presented

by
Rama Sivasubramanian

It was defended on

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and approved by

Reginald F. Frye, Pharm D., PhD
(Committee Chair, Advisor)

Mary Margaret Folan PhD
(Committee member)

Marjorie Romkes PhD
(Committee member)

Paul L. Schiff PhD
(Committee member)

Raman Venkataramanan PhD
((Committee Co-chair, Advisor)

Effects of valerian root extracts (*Valeriana officinalis*) on acetaminophen

glucuronidation: *in vitro & in vivo* studies

Rama Sivasubramanian, M.Pharm

Herbal products have been shown to cause serious interactions when combined with conventional medications. A majority of these interactions are pharmacokinetic in nature and involve alteration in the activity of drug metabolizing enzymes. Valerian is a popular herbal product often used to treat insomnia and anxiety. Valerian extracts contain essential oils with sesquiterpenes such as valerenic acid and its derivatives. However, the drug interaction potential of valerian preparations is largely unknown. In human liver microsomes, valerenic acid forms a glucuronide conjugate suggesting that valerian extracts could interact with drugs that undergo glucuronidation. As glucuronidation is catalyzed by UDP- glucuronosyltransferase enzymes (UGT), the goal of this dissertation was to investigate the effect of valerian extracts on UGT activity. Acetaminophen was used as a probe substrate to measure UGT activity in these studies. A bioassay-guided fractionation approach was adopted to identify the major compounds in valerian extracts that are responsible for inhibition of UGT activity. The alcoholic extract of valerian was fractionated by liquid-liquid extractions followed by chromatographic methods. The organic extracts showed significant inhibitory activity compared to the aqueous extracts. Using various chromatographic and spectroscopic techniques, the major compounds present in the active fraction were identified as valerenic acid, acetoxyvalerenic acid and valerenal. The clinical implications of the inhibition of UGT enzymes by valerian extracts were investigated in a study in healthy human volunteers. Valerian

administration resulted in an increased acetaminophen maximum plasma concentration (C_{max}) and a decrease in time to reach the maximum plasma concentration (t_{max}), but did not affect the area under the plasma concentration-time curve (AUC) or half life. As these results were unexpected, human hepatocyte cultures were used to determine if enzyme induction potential of some components may offset the inhibition of UGT enzymes. We hypothesized that the inhibition observed in the microsomal study could be masked by an increase in enzyme activity due to induction of enzymes by chronic exposure to the extracts. In human hepatocyte cultures, valerian extracts inhibited UGT activity on acute exposure while chronic exposure increased UGT activity and mRNA levels. Our study indicates that there is no clinically significant interaction between acetaminophen and valereian. In vitro studies in human hepatocytes may better predict in vivo herb-drug interactions than studies in microsomes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I express my immense gratitude to my advisor, Dr. Reginald Frye for training me in clinical research, helping me in conceptualizing my project and providing invaluable guidance throughout the progress of this project. This project would not have been complete if Dr. Raman Venkataramanan had not adopted me as his student and taken on the role as my advisor. I am greatly indebted to him for his support, encouragement and guidance. I express my sincere gratitude to Dr. Paul Schiff for patiently and painstakingly guiding me through the fractionation experiments. I am grateful to Dr. Marjorie Romkes and Dr. Mary Margaret Folan (Maggie) for providing valuable insights and answers to my queries through out this project.

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ABBREVIATIONS

APAP	Acetaminophen
APAPG	Acetaminophen glucuronide
APAPS	Acetaminophen sulfate
CAR	Constitutive Androstane Receptor
COX	Cyclo-oxygenase
CYP	Cytochrome P450
GABA	Gamma-amino butyric acid
GSH	L-glutamyl-cysteinyl glycine
GST	Glutathione-S transferases
HPLC	High performance liquid chromatography
IS	Internal standard
LOQ	Limit of quantification
NAT	N-acetyltransferases
nm	Nanometer
NSAID	Non-steroidal anti-inflammatory drug
QC	Quality control
RSD	Relative standard deviation
ST	Sulfotransferase
UDP	Uridine-5´-diphosphoglucuronate
UGT	Uridine diphosphate Glucuronosyl transferase
UTP	Uridine-5´- triphosphate

1.0   INTRODUCTION

1.1   HERBAL PRODUCTS AND SAFETY

There is widespread use of botanicals in the U.S. population with an estimated one-third of adults admitting to the use of herbal products (Johnston 1997). The past decade has seen an unprecedented increase in the use of herbal remedies with sales exceeding $350 million in 1997 (Brevoort 1998; Blumenthal 2002). Herbal supplement use continues to grow significantly, with sales up by 50% from 1997 to 2002 (Tindle, Davis et al. 2005). Herbal remedies are often perceived as natural and therefore, inherently safe. Reports indicate that patients use herbs for a wide range of health problems, such as boosting the immune system, improving memory, and treating insomnia, depression, or diabetes (Kuo, Hawley et al. 2004). Some of the most commonly used herbs are St. John's wort, Chammomile, Gingko biloba, Echinacea, Ginseng, black cohosh and valerian (O'Hara, Kiefer et al. 1998). Prescription drug users may combine them with herbal remedies and approximately 70% of the individuals do not disclose herbal use to their physicians (Eisenberg, Kessler et al. 1993; Miller 1998; Kaye, Clarke et al. 2000; Kaufman, Kelly et al. 2002). In addition, herbal supplements are often complex mixtures of a multitude of pharmacologically active compounds. These factors increase the likelihood of herb-drug interactions and raise concerns about the safety of these products.

1.2   DRUG INTERACTIONS

A drug interaction is defined as the modulation of the pharmacological activity of a drug by the prior or concomitant administration of another drug (May 1997). The pharmacological activity of a drug may also be altered by other exogenous compounds of environmental or dietary origin. The pharmacological change can be mediated by two mechanisms: first, by

modulation of the pharmacodynamic properties of the drug and second by affecting the pharmacokinetics.

1.2.1 Pharmacodynamic interactions

A pharmacodynamic interaction occurs between two entities when they both bind to the same receptor or have the same pharmacological effect. These interactions can be additive or antagonistic. Pharmacodynamic interactions between herbs and narrow therapeutic index drugs, such as digoxin, phenobarbital, phenytoin and warfarin are well documented (1970; Miller 1998). Several herbal remedies have been associated with altered bleeding times in patients concurrently taking warfarin. Many of these products have a coumarin component such as dong quai (Page and Lawrence 1999) and fenugreek (Lambert and Cormier 2001)), while others contain different constituents with antiplatelet effects such as danshen ginko and ginger (Lo, Chan et al. 1992; Lo, Chan et al. 1995; Yu, Chan et al. 1997; Yuan, Wei et al. 2004). A pharmacodynamic mechanism has been suggested for the interaction of kava with benzodiazepines by interference with gamma amino butyric acid (GABA) receptors (Almeida and Grimsley 1996). Pharmacodynamic interactions are common with sedatives and hypnotics by receptor antagonist or agonist actions. St. John's wort has been shown to potentiate the effect of buspirone as well as serotonin re-uptake inhibitors possibly by inhibiting 4-hydroxytryptamine reuptake (Gordon 1998; Lantz, Buchalter et al. 1999; Prost, Tichadou et al. 2000; Dannawi 2002).

1.2.2 Pharmacokinetic interactions

A pharmacokinetic interaction is defined as the alteration in the absorption, distribution, metabolism or elimination of one drug by another exogenous compound, such as a drug, food or

herb. Of these mechanisms, a majority of pharmacokinetic interactions are attributed to those occurring through changes in metabolism. Drug metabolism has conventionally been categorized into two phases (Lin and Lu 1997). Phase I reactions, also called functionalization reactions, are mainly oxidative reactions catalyzed by cytochrome P450 (CYP) enzymes resulting in hydroxylation or demethylation. Phase II reactions involve the conjugative enzyme families such as the UDP-glucuronosyltransferases (UGTs), glutathione-S transferases (GSTs), sulfotransferases (STs), and N-acetyltransferases (NATs)(Parkinson 1996).

The focus of drug-drug and herb-drug interactions has remained on the CYP family of enzymes. Among herbal supplements, the best documented herb–drug interactions are that of St. John's wort (Hypericum perforatum) (Roby, Dryer et al. 2001; Karyekar, Eddington et al. 2002; Wang, Hamman et al. 2002; Markowitz, Donovan et al. 2003; Wenk, Todesco et al. 2004). *In vitro* studies have shown that hyperforin, considered an active constituent of St. John's wort, induces CYP3A4 and increases the activity of the transporter P-glycoprotein in the gut and intestine (Moore, Goodwin et al. 2000; Wang, Hamman et al. 2002; Komoroski, Zhang et al. 2004; Mannel 2004; Zhou, Lim et al. 2004). The first human study confirming this effect was published in 1997 (Kerb, Bauer et al. 1997). Subsequent studies have shown that administration of St. John's wort can substantially reduce the potency of a wide range of drugs including the immunosuppressants cyclosporine and tacrolimus (Ruschitzka, Meier et al. 2000; Ahmed, Banner et al. 2001; Beer and Ostermann 2001; Bauer, Stormer et al. 2003; Mai, Stormer et al. 2003), the HIV protease inhibitor indinavir (Piscitelli, Burstein et al. 2000) and the anti-cancer agent imatinib mesylate (Frye, Fitzgerald et al. 2004; Smith 2004). Recent studies suggest that Echinacea could interact with substrates of CYP3A4 and CYP1A2 enzymes (Gorski, Huang et al. 2004; Gurley, Gardner et al. 2004). *In vitro* studies indicate that Kava (piper methysticum)

could also cause drug interactions by inhibition of CYP2C9, 2C19, 2D6 and 3A4 enzymes (Mathews, Etheridge et al. 2002; Unger, Holzgrabe et al. 2002; Zou, Henderson et al. 2004).

While there is substantial evidence that interactions mediated by phase I enzymes are important, there is limited information on the role of phase II enzymes in drug interactions. Various compounds have been identified as inhibitors and inducers of UGT isozymes. Inhibitors of UGTs include NSAIDs, benzodiazepines, and tricyclic antidepressants. Immunosuppressants such as cyclosporine and tacrolimus are potent inhibitors of UGTs (Zucker, Rosen et al. 1997; Zucker, Tsaroucha et al. 1999). Some case reports suggest that a drug interaction occurs between sertraline and lamotrigine due to inhibition of glucuronidation by sertraline (Kaufman and Gerner 1998). Phenobarbital has been used as a UGT inducer to correct hyperbilirubinemia in patients with Crigler-Najjar syndrome. Other drugs known to induce UGTs include carbamezepine and phenytoin (Anderson 1998). Oral contraceptives have also been shown to induce UGTs leading to increased clearance of lamotrigine (Sabers, Buchholt et al. 2001). This interaction resulted in the re-occurrence of seizures or increased seizure frequency in epileptic patients. Induction of UGTs by dietary compounds such as flavonoids is well documented. The flavonoid chrysin is a potent inducer of UGT1A1. Other dietary inducers of UGTs are cruciferous vegetables such as brussels sprouts and broccoli (van der Logt, Roelofs et al. 2003; Walters, Young et al. 2004). Epidemiological studies in pursuit of anti-cancer strategies provide evidence of induction of phase II enzymes by various compounds of phytochemical and environmental origin such cigarette smoke (Paolini and Nestle 2003). Exposure to these compounds could potentially modulate the concentration of drugs undergoing metabolism via phase II pathways. Therefore, the role of UGT enzymes in xenobiotic metabolism and drug interactions requires further investigation.

1.3   VALERIAN

Valerian is frequently listed among the ten most widely used herbal supplements (Morris and Avorn 2003). Historically, valerian has been used as a sedative, anti-spasmodic, carminative and mild analgesic (Davidson and Connor 2000). The use of valerian to alleviate insomnia and nervous disorders has been reported (Kiesewetter and Muller 1958; Last 1969; Houghton 1999; LaFrance, Lauterbach et al. 2000; Krystal and Ressler 2001). It is often combined with hops (*humulus lupus*), lemon balm (*Melissa officinalis*), chamomile (*Chamaemelum nobile*), Kava kava (*Piper methysticum*) or St. John's wort (*Hypericum perforatum*)(Davidson and Connor 2000). Table 1 summarizes the various pathological conditions treated with valerian.

1.3.1   Formulations and dose

The herb is available as a capsule, tablet, tea or tincture. Valerian dosing varies and the recommendations range from 100-300 mg of extract once a day for anxiety and 300-900 mg for insomnia (Davidson and Connor 2000). In the case of fresh herb, the recommended dose is 2-3 g of the dried valerian root soaked in one cup of hot water for 10-15 minutes (Kush, Bleicher et al. 1998; Schulz, Hansel et al. 1998).

1.3.2   Pharmacognosy and chemistry

Valerian belongs to the family Valerianaceae and genus *Valeriana. Valeriana officinalis* is the species commonly used in the commercial preparations. Other species such as *V. wallichi* (Indian valerian), *V. edulis* (Mexican valerian) and *Nardostachys chinensis* (Chinese) are also used medicinally (Schultz and Eckstein 1962; Martinez 1969; Holzl and Jurcic 1975; Bagchi, Oshima et al. 1988; Herrera-Arellano, Luna-Villegas et al. 2001). Extracts from the roots and rhizomes

Table 1 Summary of pathophysiological conditions treated with valerian

Sedative effects	*Antispasmodic*	*Gastrointestinal*
Emotional Stress	Muscular pain	Digestive disturbances
Insomnia	Menstrual cramps	Stomach cramps
Nervousness	Bronchial spasms	Diarrhea
Anxiety	Coughs	Bloating
Nervous heart conditions		Colic
Children's anorexia		
Restlessness		
Trembling		
Tension headaches		
Graves disease		
Hypochondria		
Excitability		

are used in the preparation of phytomedicines. The presence of valerenic acid and its derivatives (acetoxyvalerenic acid and hydroxyvalerenic acid) are characteristic to *V. officinalis* and its subspecies (Bos, Woerdenbag et al. 1997; Letchamo, Ward et al. 2004). Other constituents in valerian include epoxy iridoid esters (valepotriates), their decomposition products such as baldrinal and homobaldrinal, amino acids (arginine, GABA, glutamine, tyrosine), essential oils (borneol, pinene, camphene) and alkaloids (Hansel and Schulz 1981). Recent chemical investigations have identified the presence of lignans and a bi-cyclic sesquiterpene acid (epoxy valerenic acid)(Dharmaratne, Nanayakkara et al. 2002; Schumacher, Scholle et al. 2002).
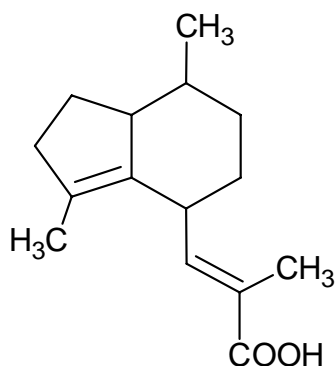
Figure 1   Valerenic acid

Valerian also contains small amounts of phenolic acids and flavonoids, valerosidatum, chlorogenic acid, caffeic acid, choline, β-sitosterol, fatty acids, and various minerals (2004). Commercial formulations are often standardized to valerenic acid content as it is believed to be primarily responsible for the sedative effect.

1.3.3   Pharmacology

The sedative effects of valerian have been evaluated in a number of clinical trials. Some studies indicate that valerian can improve sleep latency although no change was observed in the

electroencephalographs (Stevinson and Ernst 2000). The mechanism of the sedative effect remains elusive and several mechanisms have been proposed including GABA receptor agonist effects of valerenic acid and inhibition of GABA-transaminase, the enzyme system responsible for the central catabolism of GABA, by valerenic acids and valepotriates (Hansel and Schulz 1981; Koch 1982; Riedel, Hansel et al. 1982; Bodesheim and Holzl 1997). In addition, aqueous valerian extracts have been shown to cause the release of GABA as well as inhibit the reuptake of GABA into presynaptic terminals and surrounding glial cells in animal studies (Santos, Ferreira et al. 1994). However, recent studies postulate that the effects are also due to some of the other constituents like lignans and their binding to serotonin receptors and adenosine receptors (Schumacher, Scholle et al. 2002; Dietz, Mahady et al. 2005).

1.3.4   Valerian-drug Interactions

As opposed to some popular herbs such as St. John's Wort or Kava-kava, reports of drug interactions and pharmacokinetic studies with valerian or its constituents are scarce. Early animal studies investigated the interaction between valerian and barbiturates. These studies showed that valepotriates may prolong phenobarbital-induced sleep time. In mice, oral administration of valepotriates inhibited methacetin metabolism indicating possible interactions of valerian extracts with cytochrome P450 substrates (Braun, Dittmar et al. 1984; von der Hude, Scheutwinkel-Reich et al. 1986). Recent studies with expressed enzyme systems have shown that valerenic acids as well as alcoholic extracts inhibit CYP3A4 (Lefebvre, Foster et al. 2004; Strandell, Neil et al. 2004). However, valerian did not alter the pharmacokinetics of the CYP3A4 substrate alprazolam in healthy volunteers (Donovan, DeVane et al. 2004).

1.3.5 Preliminary studies

Previous work in our laboratory demonstrated that valerenic acid and alcoholic extracts of valerian inhibit the glucuronidation of acetaminophen and other UGT probe substrates(Alkharfy 2002). The magnitude of inhibition with the valerian extracts (approximately 70% inhibition) was greater than with valerenic acid alone (55%) suggesting other components of the extract contributed to the extent of interaction. The extract constituents that may contribute to the inhibition of acetaminophen glucuronidation and the clinical relevance have not been addressed. The formation of a glucuronide conjugate of valerenic acid further supported the theory that valerenic acid and other compounds present in valerian extracts could inhibit the glucuronide formation of drugs.

1.4   UDP-GLUCURONOSYL TRANSFERASE ENZYMES

Among the Phase II enzymes, UGTs are known to metabolize the largest number of xenobiotics, approximately 35% of all drugs metabolized by phase II detoxification enzymes (Evans and Relling 1999) (Figure 2) . UGTs catalyze the glucuronidation of a wide range of diverse endogenous and exogenous compounds. Although almost all classes of drugs are substrates of UGTs, a substantial number of psychoactive drugs and NSAIDs are known to be primarily metabolized to glucuronide conjugates. UGTs are also known to play a critical role in the homeostasis of numerous endogenous compounds. A well researched example is that of billirubin conjugation. Other endogenous compounds include fatty acids, retinoids, biles acids and steroids. Additionally, UGTs play a significant role in metabolism of compounds derived from diet as well as environmental sources such as tobacco smoke. Plant derived medicinal agents as well as dietary compounds are known to contain poly-phenolic compounds such as phenolic acid, flavonoids, stilbenes and lignans that serve as substrates for UGTs. Table 2

provides a representative listing of endogenous and exogenous compounds that undergo glucuronidation.
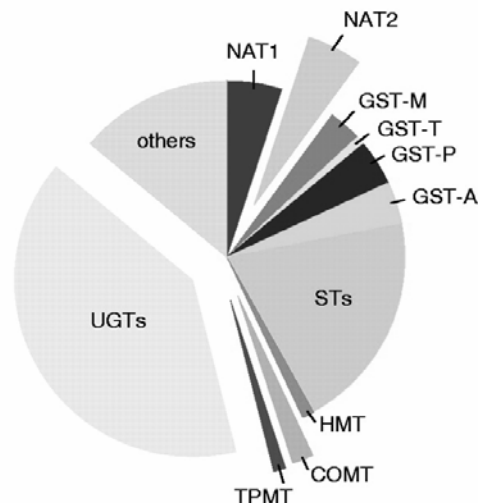
Figure 2   Enzymes involved in Phase II metabolism

The relative size of each section in the chart indicates the percentage of drugs metabolized by the enzyme. GST, glutathione S-transferase; HMT, histamine methyltransferase; NAT, N-acetyltransferase; STs, sulfotransferase; TPMT, thiopurine methyltransferase; UGTs, uridine 5′-triphosphate glucuronosyltransferase. (Reprinted with permission from Evans, W. E. and Relling, M. V. Science 1999, 286(5439): 487-490) Copyright 1999 AAAS. URL: http://www.sciencemag.org/

Table 2 Representative substrates of UGT enzymes.

	Xenobiotics		Endobiotics
	Drugs	**Dietary compounds**	

Drugs	**Dietary compounds**	**Endobiotics**
CNS		
Antidepressants	**Flavonoids**	**Steroids**
Amitriptyline	(-) –epicatechin	Androsterone
Imipramine	Quercetin	Estradiol
Doxepin	Chrysin	
Trazadone	Emodin	**Bile acids**
	Genistein	Lithocholic acid
Antipsychotics	Curcumin	Hyodeoxycholic acid
Olanzepine		
Chlorpromazine	**Terpenes**	**Retinoids**
Trifluoroperazine	Menthol	*t*-Retinoic acid
Haloperidol	Borneol	Hydroxyretinoic acid
	Limonene	
Benzodiazepines	Dihydroartemisinin	**Fatty acids**
Lorazepam	Farnesol	Arachidonic acid
Oxazepam		Linoleic acid
	Stilbene	
Anticonvulsant	Resveratrol	**Others**
Lamotrigine		Billirubin
Valproic acid		
NSAIDS		
Indenes		
Sulindac		
Indomethicin		
Profens		
Ibuprofen		
Flurbiprofen		
Ketoprofen		
Others		
Diclofenac		
Naproxen		

1.4.1 Classification and Nomenclature

UGTs are a superfamily of enzymes in humans. The enzymes are classified into two families that have been designated UGT1 and UGT2 based on their primary amino acid sequence predicted from cloned cDNAs (Burchell, Nebert et al. 1991; Mackenzie, Owens et al. 1997). Sequence homology between gene products of the UGT1 and UGT2 families is 38-48% while homology between members within each family is over 60%. The dendogram derived from this nomenclature is shown in Figure 3 (Burchell 2003).
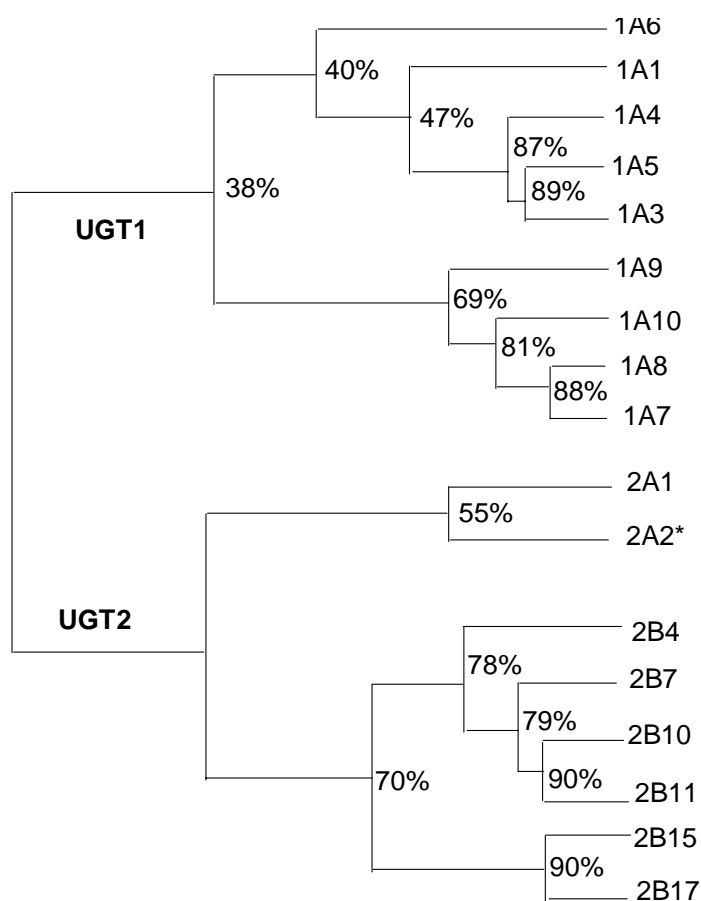
Figure 3   Phylogenetic tree of human UGT family

> Phylogenetic tree illustratrating the divergence of human UGT family based on amino acid sequence deduced from cDNA cloning experiments. * indicates cDNA and protein encoding UGT has not been identified in any human tissues (adapted from Tukey et. al Molecular Pharmacology 2001, 59(3): 405-414)
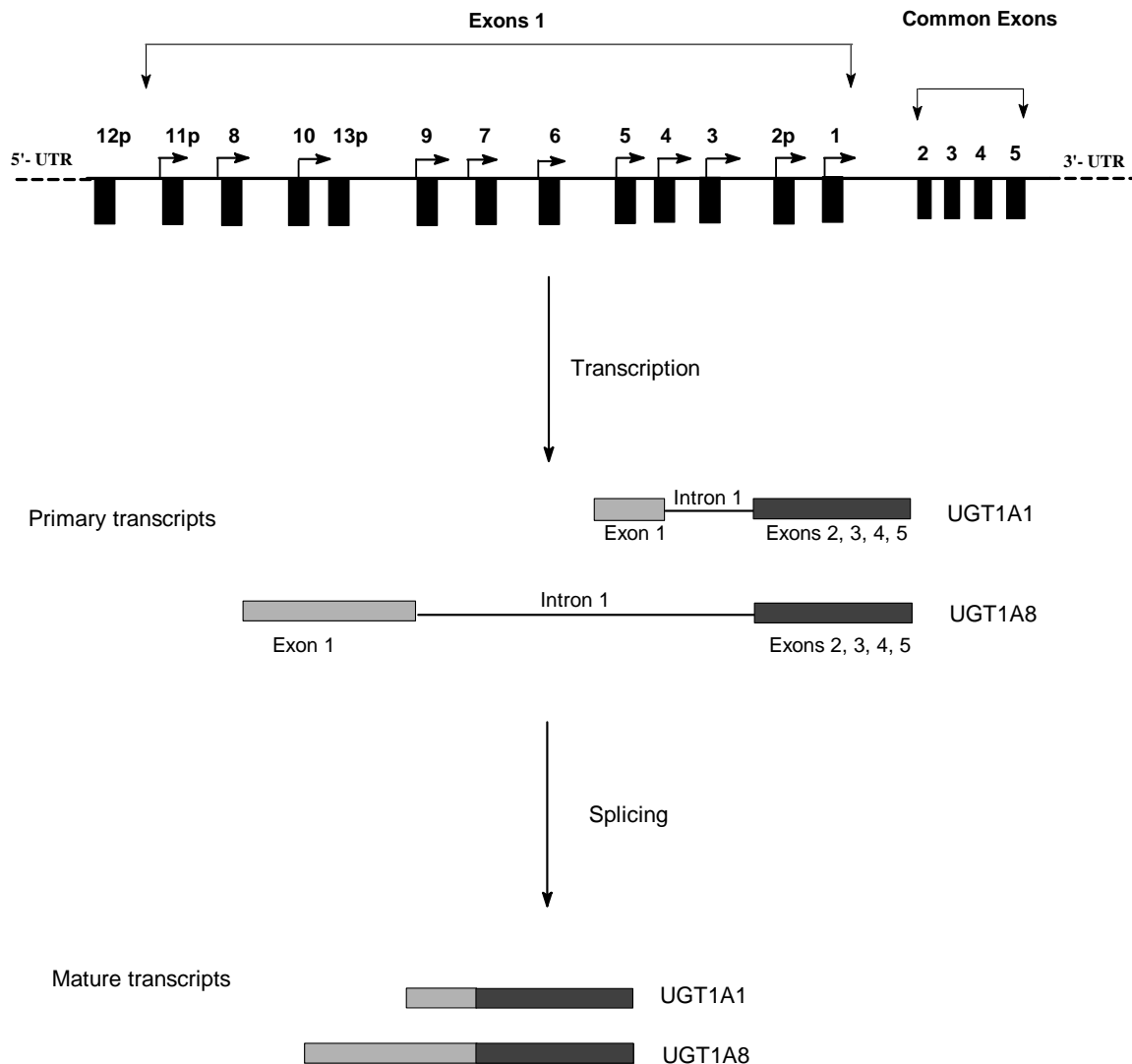
Figure 4   Gene structure and mechanism of transcription of UGT1A enzymes

The UGT1 gene locus is shown from 5´ to 3´ as an array of 13 linearly arranged exons 1 for UGT 1A genes and pseudogenes (designated as p) in combination with the common exons 2, 3, 4, 5. Each Exon 1 contains a proximal 5´ proximal TATA box that allows for independent initiation of transcription generating a series of overlapping primary transcripts. The exon 1 of primary transcripts are spliced to the common exons to form mature mRNAs (Figure adapted from Gong et. al, Pharmacogenetics 2001, 11:357-368)

1.4.2   Gene loci and transcription

The human *UGT1A* gene locus has been mapped to a 210 kb region with 17 exons on human chromosome 2 (2q37). The *UGT1A* gene contains 10 tandem variable first exons and a

14

common set of downstream constant exons and it has been predicted that at least nine functional proteins may be encoded. Transcription and RNA processing proceeds by a process of exon sharing, where the amino-terminal is encoded by a unique first exon   (A1, A3, A4, A5, A6, A7, A8, A9 and A10) and the carboxyl-terminal portion is encoded by the identical four down stream exons (2–5) (Owens and Ritter 1992). The gene structure and mechanism of transcription of UGT1A enzymes is represented in Figure 4. In contrast to UGT1 family, the human UGT2 family proteins are encoded by individual structural genes on chromosome 4 (Monaghan, Burchell et al. 1997; Burchell, Soars et al. 2000). The human UGT2 family is further subdivided into two subfamilies; UGT2A and UGT2B, which contain two (2A1, 2A2) and seven (2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28) members, respectively.

1.4.3   Cellular Localization of UGT enzymes

UGTs are located in the lumen of the endoplasmic reticulum and the nuclear envelope of cells (Radominska-Pandya, Czernik et al. 1999). Advances in molecular biology have led to the detection and characterization of 18 human UGTs to date. Human UGTs range from 529 to 534 amino acids in length, with the N-terminal portion of the protein located in the lumen of the endoplasmic reticulum and the C-terminal portion oriented towards the cytoplasm (Tephly and Burchell 1990). The aglycone-binding site is believed to be in the N-terminal portion of the UGT protein and this region shows the greatest variability in sequence among UGT isozymes. The C-terminal region of 15-20 amino acids is relatively conserved and is thought to be the UDPGA binding domain. Numerous studies with molecular probes support this model of the UGT enzyme topology. The hypothetical localization of the domains of the protein is shown in Figure 5. In addition to the structural characteristics, UGT activity is hypothesized to be influenced by its association with lipid, restricting access of aglycones to the active site as well as protein-

protein interactions derived from oligomer formation (Peters, Jansen et al. 1984; Zakim and

Dannenberg 1992; Ikushiro, Emi et al. 1997; Meech and Mackenzie 1997).
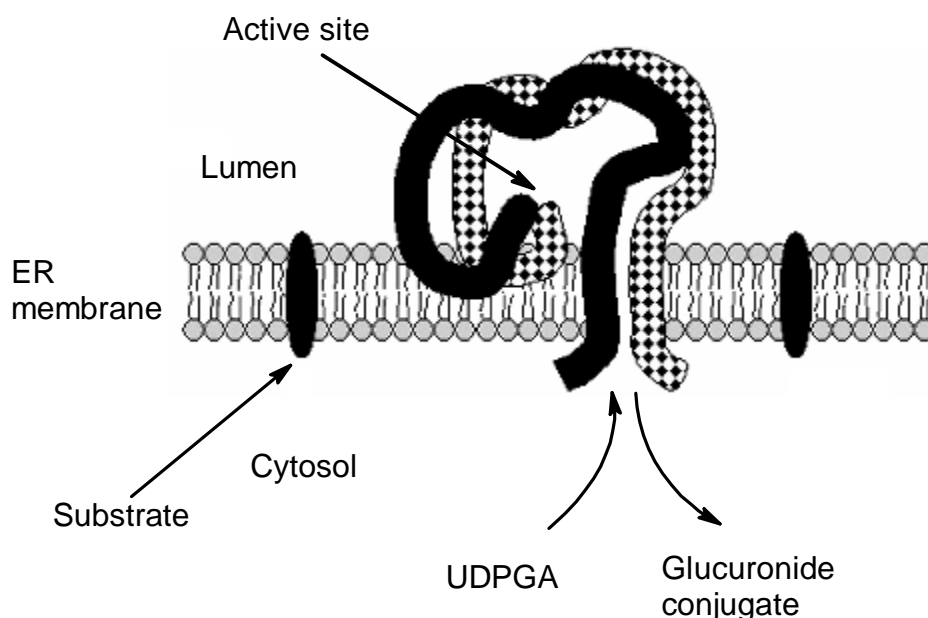
Figure 5   Hypothetical model of UGT topology depicting dimerization of UGT monomers.

Figure shows a hetero-dimer with the catalytic unit inside the lumen and formation of a proteinaceous channel to allow entry of UDPGA into the lumen. The substrate diffuses through the membrane bilayer and the glucuronide conjugate is released throught the channel into the cytosol. (Adapted from Radominska-Pandya A et. al. Drug Metab. Rev. 1999, 31:817-899 and Miners. J et. al. Annual Review of Pharmacology and Toxicology 2004, 44: 1-25)

1.4.4   Expression and distribution

Tissue specific expression of UGT isoforms has been identified with the help of modern

molecular techniques. The majority of UGTs including 1A1, 1A3, 1A4, 1A6, 1A9, 2B4 and 2B7

are expressed in the liver (Strassburg, Manns et al. 1997). In addition to the liver, gastrointestinal

tract and kidneys are known to express drug metabolizing enzymes. In addition to all the UGT

isoforms expressed in the liver, UGT 1A8 and 1A10 have been detected in the human colon and

designated as intestinal UGTs (Strassburg, Manns et al. 1998). The isoforms expressed in the

kidneys are UGT1A8, UGT1A9, UGT1A10 and UGT2B7 (McGurk, Brierley et al. 1998). Experiments with kidney microsomes suggest that the expression of 2B7 levels could approach that of the liver (Fisher, Vandenbranden et al. 2000). Other tissues where UGT expression has been detected are the ovary, prostate, olfactory tissue, brain and placenta (Turgeon, Carrier et al. 2001; De Leon 2003). It is apparent that UGT2B transcripts are abundantly expressed in steroid sensitive tissues such as the ovary and prostate suggesting a protective role against carcinogenic actions of steroids in these organs (Beaulieu, Levesque et al. 1998; Belanger, Hum et al. 1998)

1.4.5   Biochemical mechanism

UGT enzymes transfer the glucuronyl group from uridine-5´-diphosphoglucuronate (UDPG) to hydrophobic molecules (or aglycones) (Figure 6). The reaction is initiated by the coupling of D-glucose-1-phosphate to UTP to give UDP-glucose, followed by the oxidation of the primary alcohol to yield the co-substrate UDP-glucuronic acid (UDPGA). The conjugation occurs by an S_N2 substitution with both the UDPGA and the aglycone substrate bound to the active site of the enzyme (Dutton 1980). The conjugation reaction occurs at the nucleophilic acceptor group on the substrate with an electrophilic anomeric carbon (C-1) of glucuronic acid. The resulting product undergoes an inversion of configuration at the anomeric carbon (Jakoby 1980)
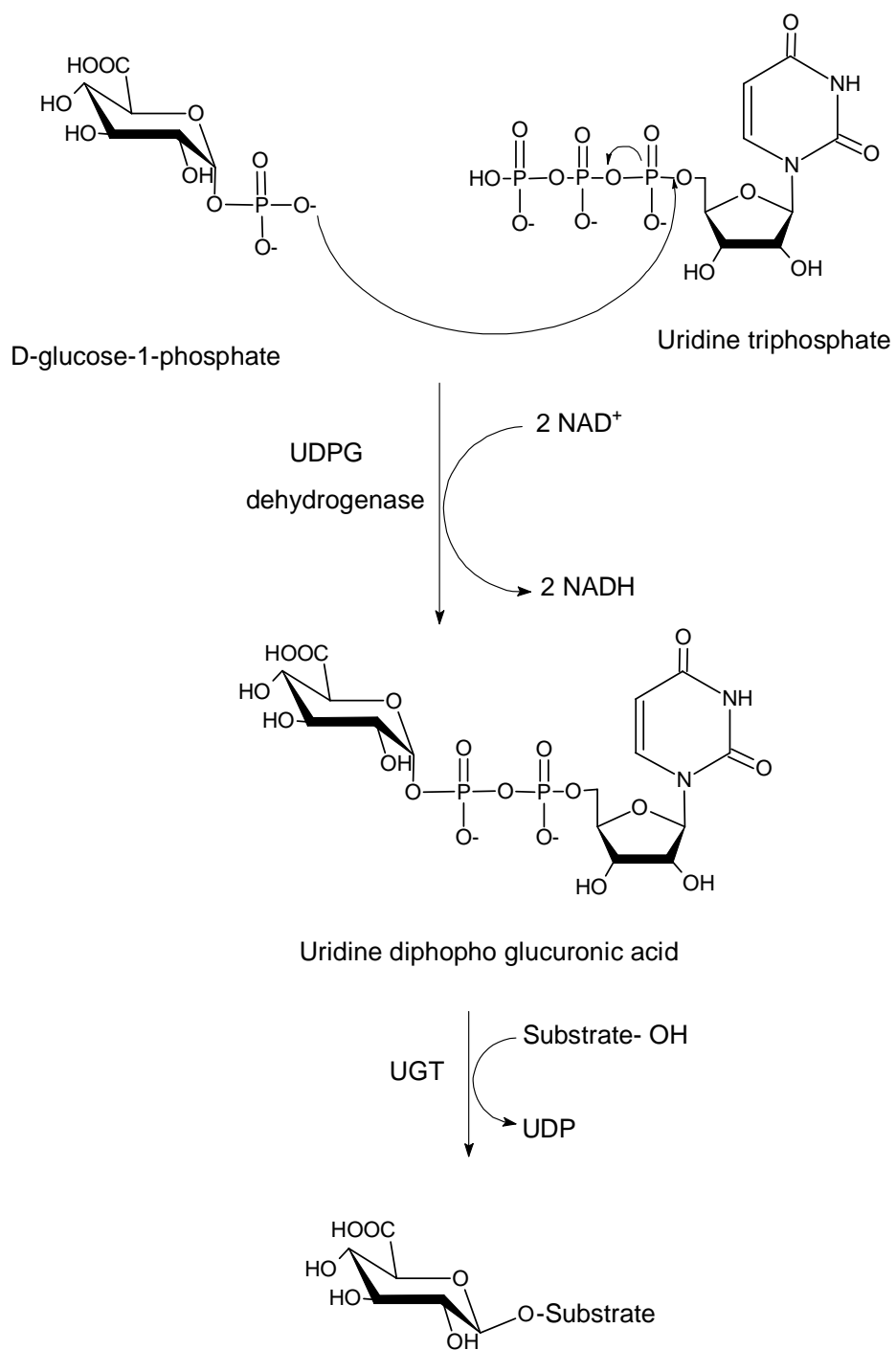
Figure 6 Biochemical pathway of glucuronidation

1.4.6   Substrate characteristics

Typically, glucuronidation involves compounds with nucleophilic functional groups of oxygen, nitrogen, sulfur, or carbon (Miners and Mackenzie 1991; Kroemer and Klotz 1992). The most common glucuronides are O-linked ethers from aliphatic alcohols and phenols or O-linked esters from carboxylic acid compounds. Although phase I metabolism often precedes glucuronidation, many compounds that contain functional groups such as hydroxyl, carboxy, amino or thiol, are directly conjugated without prior oxidation. (Sakaguchi, Green et al. 2004). Some common examples of drugs undergoing direct glucuronidation include morphine (Oguri, Ida et al. 1970; Yeh, Gorodetzky et al. 1977), acetaminophen, epirubicin (Weenen, van Maanen et al. 1984), ezetimibe (Ghosal, Hapangama et al. 2004), oxazepam (Sonne 1993), and valproic acid (Chapman, Keane et al. 1982). The four major chemical classes of compounds that undergo glucuronidation are shown in Figure 7.

Glucuronidation renders the compounds more hydrophilic, facilitating their excretion in bile or urine. In most cases, glucuronide conjugates are inactive. However, some exceptions have been documented. Morphine-6-O-glucuronide has been shown to be a more potent analgesic than morphine. Some carboxylic acid drugs such as valproic acid form acyl glucuronides that form protein adducts, which could result in carcinogenesis (Smith, Hasegawa et al. 1985; Williams, Worrall et al. 1992). Similarly, adverse drug events including nephritis and acute renal failure, associated with nonsteroidal drugs of the aryl-alkyl class have been postulated to be due to the electrophilic interactions of the acyl glucuronides with the sulfhydryl and hydroxyl groups of cell macromolecules (Spahn-Langguth and Benet 1992).

O-glucuronide

Propofol

Oxazepam

Estradiol

Coumarin

Benoxaprofen

S-glucuronide

2-Mercaptobenzothiazole

Disulfiram

N-glucuronide

Benzidine

Imipramine

C-glucuronide

Phenylbutazone
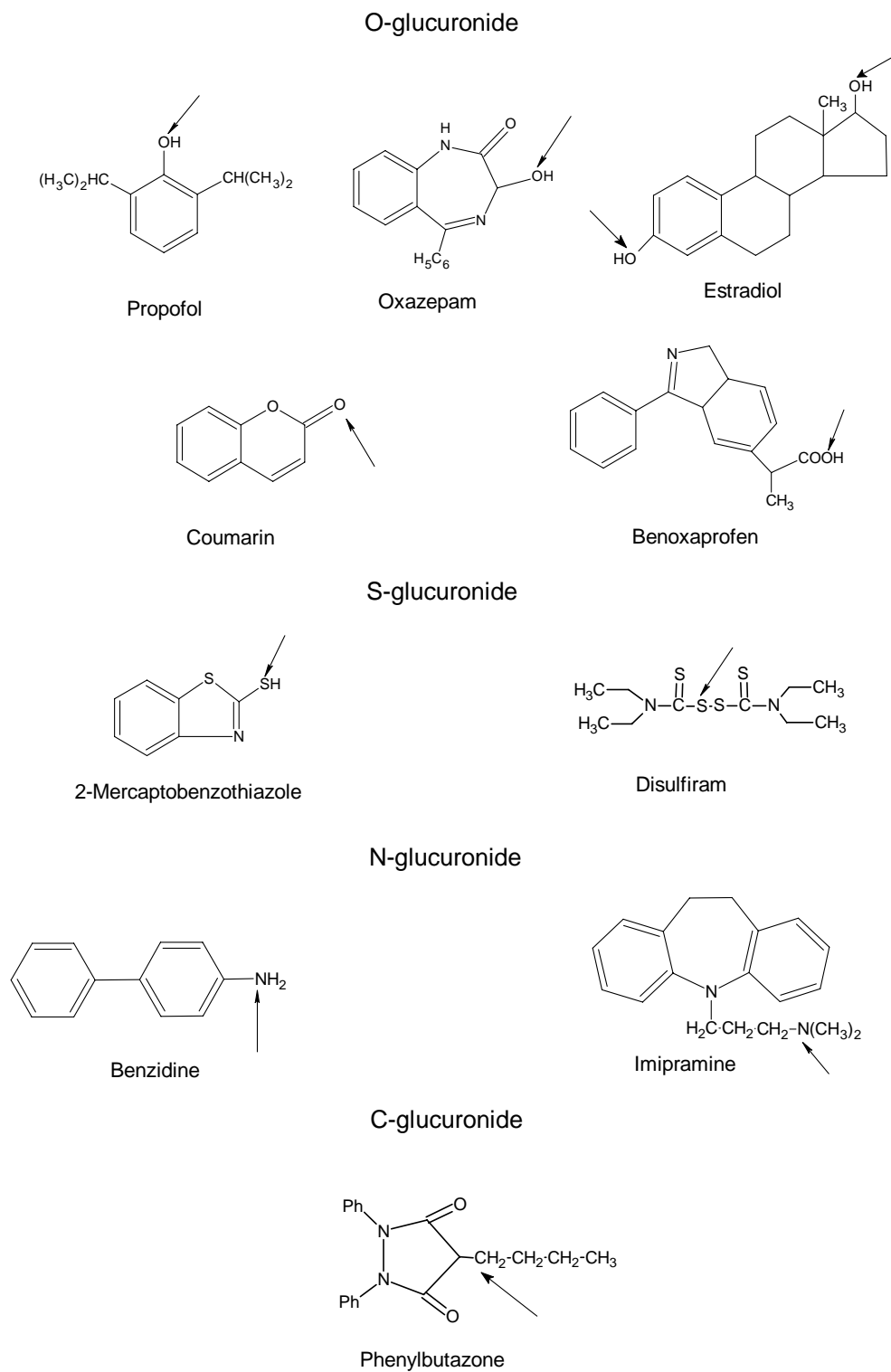
Figure 7 Four classes of glucuronide metabolites.
(The arrows indicate the position where glucuronidation occurs on the molecule)

The lack of substrate specificity exhibited by members of UGT families has been attributed to the characteristics of the substrate such as orientation of the groups and size of the chemical compound. Small and hydrophobic molecules are often glucuronidated by multiple UGTs (Lin and Wong 2002). Therefore, classification of the UGTs based on substrate specificity is limited. (Tephly and Burchell 1990) (Burchell 2003) In general, UGT1A enzymes conjugate a wider range of xenobiotics while UGT2B conjugate a substantial number of endogenous compounds (Radominska-Pandya, Little et al. 2001; McLean, Brandon et al. 2003). UGT1A6 is known to preferentially conjugate planar phenols while UGT1A9 conjugates bulky phenols (Tukey and Strassburg 2000). Isoforms in the UGT2 family metabolize a variety of endogenous steroid compounds (Coffman, King et al. 1998). Some isoforms do exhibit some substrate specificity. For example, UGT1A1 and UGT2B7 are primarily responsible for the glucuronidation of bilirubin and morphine, respectively (Coffman, Rios et al. 1997; Mackenzie, Miners et al. 2000).

1.4.7 Factors regulating expression

UGT enzyme expression in humans has been extensively studied and factors that determine levels of expression in the liver and extrahepatic tissues are beginning to be elucidated. These factors include age, diet, ethnicity, disease states, drug interactions, genetic polymorphisms and hormones (de Wildt, Kearns et al. 1999). Genetic polymorphisms have been reported for UGTs 1A1, 1A6, 1A7, 1A8, 2B7 and 2B15 (de Wildt, Kearns et al. 1999; Bhasker, McKinnon et al. 2000; Guillemette, Millikan et al. 2000; Tukey and Strassburg 2000; Huang, Galijatovic et al. 2002; Miners, McKinnon et al. 2002). Genetic polymorphisms in UGT1A1 lead to either inactive proteins or partially active proteins causing two familial congenital hyperbilirubinemia syndromes. The severe form of the syndrome is commonly known as Crigler-Najjar syndrome and a mild form is known as Gilbert's disease. The syndrome is characterized by elevated levels

of bilirubin, a breakdown product of heme. Recently, UGT1A1 promoter polymorphism was found to be associated with differences in SN-38 glucuronidation, indicating that genetic polymorphisms in UGT genes can alter pharmacokinetics of drugs and therefore alter their pharmacological effects and toxicities.

The mechanism of induction of UGT1A1 by xenobiotics is thought to be via activation of the orphan nuclear receptors constitutive androstane receptor (CAR) and Pregnane X receptor (PXR) (Sugatani, Kojima et al. 2001; Xie, Yeuh et al. 2003). Recently, UGT1A1 has been shown to be inducible by the aromatic hydrocarbon (Ah) receptor also (Yueh, Huang et al. 2003).

1.5   ACETAMINOPHEN: A PROBE FOR GLUCURONIDATION

The investigation of drug interactions can be facilitated by the use of phenotypic probes. For the probe approach to be successful, availability of selective substrates, inhibitors and inducers is essential. The broad substrate specificity is responsible for the lack of specific probe substrates, inhibitors and inducers. This has remained an obstacle in the phenotyping of UGT enzyme activity in humans. With increasing interest in phase II drug metabolism, some compounds have been identified as selective substrates for *in vitro* testing and to measure activity *in vivo*. For example serotonin in *in vitro* studies has been identified as a selective substrate for UGT1A6, and morphine is believed to be metabolized *in vivo* exclusively by UGT2B7. Some substrates commonly used as probes for specific UGT enzymes are highlighted in table 2. Historically, acetaminophen has been the suggested *in vivo* probe substrate to measure UGT1A6 activity (Bock, Forster et al. 1993; Burchell, Brierley et al. 1995; Court and Greenblatt 2000; King, Rios et al. 2000). Further, comparison of acetaminophen pharmacokinetics in cats, an animal species known to be deficient in UGT1A6 activity, and other species such as dogs and

humans suggests that UGT1A6 is the major enzyme catalyzing acetaminophen glucuronidation at therapeutic concentrations. However, recent studies have suggested that UGT1A9 to a large extent and UGT1A1 to a lesser extent contribute to acetaminophen glucuronidation (Court, Duan et al. 2001). UGT1A6 as well as UGT1A9 are expressed in a wide range of tissues including liver, intestine, lung, kidney and brain (Sutherland, Ebner et al. 1993; King, Rios et al. 2000) (Tukey and Strassburg 2000). The UGT1A6 gene is highly polymorphic, and variability in UGT1A6 activity in the liver has been attributed to three common single nucleotide polymorphisms (cSNPs), in the coding region as shown in Figure 8. Polymorphic expression and variability in UGT1A9 activity has also been reported. Several genetic polymorphisms have been identified, four in the coding region, Cys3Tyr, Met33Thy, Tyr242X, Asp256Asn, that lead to partial or complete inactivation of the protein (Jinno, Saeki et al. 2003; Villeneuve, Girard et al. 2003; Girard, Court et al. 2004).

Table 3 Partial list of substrates, inducers and inhibitors of UGT isoforms

Isoform	Substrates			Inducers	Inhibitors
	Endogenous	Drugs	Dietary compounds		
UGT1A1	Bilirubin Catechols Thyroid hormones	4-MU **Estradiol** Ezetimibe Etoposide SN-38	Chrysin Apigenin Fisetin Genistein Narigenin Galagin Alizarin Emodin	Phenobarbital Chrysin	Probenecid
UGT1A3	Catechol	Clozapine Amitriptyline	Apigenin Fisetin Genistein Naringenin Galagin		
UGT1A4	Pregnanediol	Posaconazole **Imipramine** Tamoxifen Promethazine	Menthol Borneol Carveol	Carbamazepine Phenytoin	Probenecid
UGT1A6	Serotonin	**APAP** 4-MU Valproic acid	Eugenol B-Naphthol	Phenobarbital Cruciferous vegetables	Probenecid Diclofenac
UGT1A9	Thyroid hormones Oestrogens	APAP **Propofol** 4-MU MPA R-Oxazepam Almokalant Valproic acid	Fisetin Naringenin Galagin Alizarin Emodin	Cruciferous vegetables, smoking	Probenecid
UGT2B7	Bile acids Androsterone Catechol Retinoids	**Morphine** Lorazepam Diclofenac AZT Rofecoxib R-Oxazepam Almokalant Valproic acid	Menthol	Cruciferous vegetables	Valproic acid

Substrates often used as probes are in bold
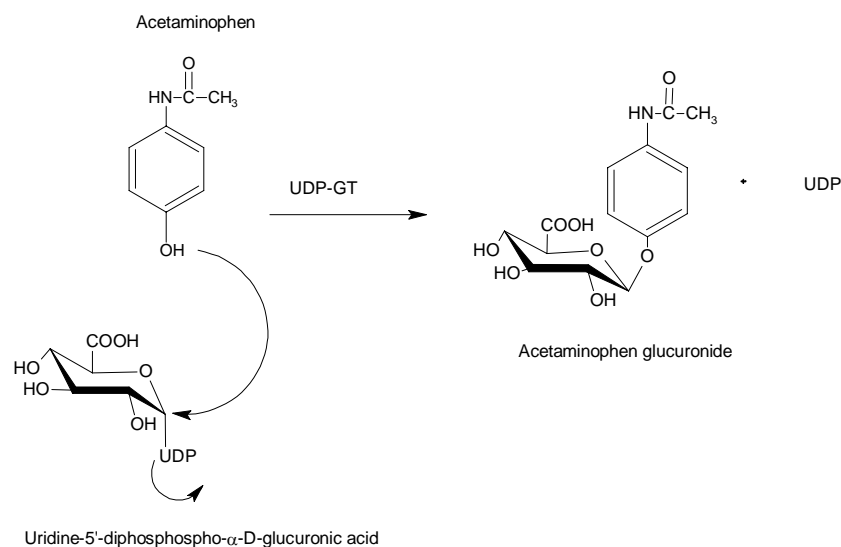
Figure 8   Acetaminophen glucuronidation
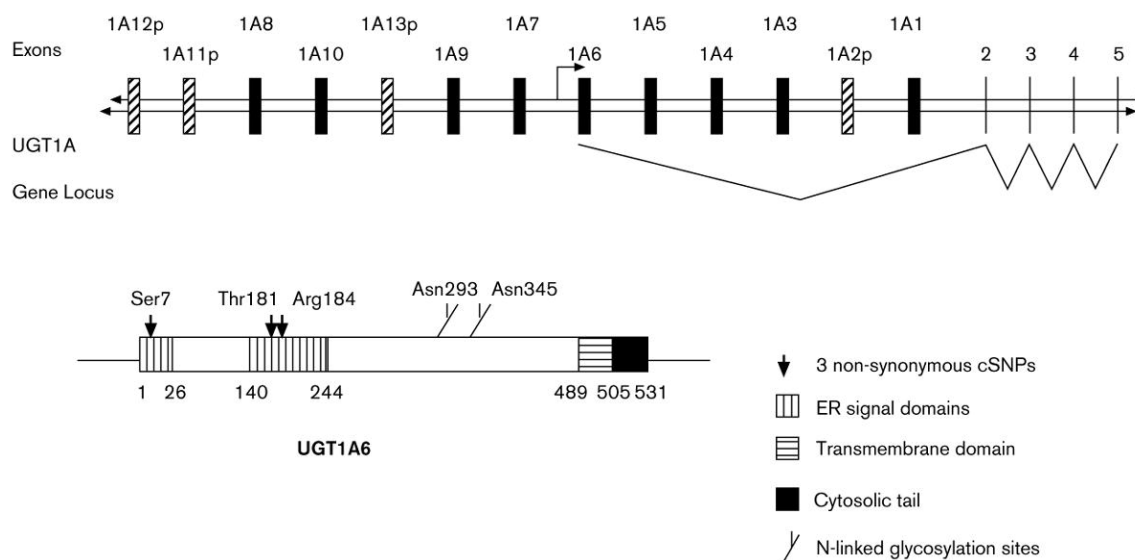
Figure 9   UGT1A6 gene
Figure showing the three coding polymorphism (Ref: Nagar. S et. al. Pharmacogenetics 2004, 14: 487-499)

1.6 SUMMARY AND OBJECTIVES OF RESEARCH

Valerian is classified as "generally recognized as safe" by the Food and Drug Administration. In spite of being one of the most popular herbs on the market, systematic pharmacokinetic studies investigating interactions between valerian and conventional medications are limited. Although a pharmacodynamic interaction has been suggested with barbiturates, no clinical study has been performed to confirm this interaction. Clearly, studies to investigate the interaction potential of valerian with conventional medications are warranted.

Preliminary experiments in our laboratory indicated that alcoholic extracts of valerian inhibited acetaminophen glucuronidation to a greater extent compared to valerenic acid alone. The first hypothesis based on these results was that multiple constituents of valerian extracts contributed to the inhibition of UGT enzymes in human liver microsomes. The first goal of this dissertation was to isolate and identify the major compounds in valerian extracts that inhibit glucuronidation. The novel bioassay-guided approach adopted to achieve this goal is described in Chapter 3. The second hypothesis, also based on the results of the microsomal experiments, was that administration of valerian will inhibit UGT enzymes *in vivo* in humans. This hypothesis was tested in a two-phase clinical study using acetaminophen as the phenotypic probe substrate in healthy human volunteers. The methods and results of the study are described in Chapter 4. In the first phase, acetaminophen was administered alone. Subjects were asked to self administer valerian at bed time for a week and return for the second phase, when acetaminophen was administered in combination with valerian. Blood and urine samples were obtained to measure acetaminophen and the glucuronide concentrations. The assay developed and validated to measure acetaminophen and acetaminophen glucuronide in human plasma is described in

Chapter 2. The pharmacokinetic parameters were calculated for the two phases and compared. The results showed that the administration of valerian did not significantly alter the pharmacokinetics of acetaminophen. The fact that the only significant observation was an increase in Cmax and reduction in tmax indicate that valerian increased the rate of absorption of acetaminophen but not the extent of absorption. These results were the basis of the third hypothesis, that valerian extracts induce UGT enzymes on chronic treatment and inhibit the enzymes on acute exposure, resulting in opposing effects that yield no net change in enzyme activity. As human liver microsomes lack cellular integrity, they cannot be used to evaluate enzyme induction. Hence, experiments were performed in human hepatocyte culture system to evaluate potential inductive effects. The design and results of these experiments is described in Chapter 5.

2.0 DEVELOPMENT AND VALIDATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR THE SIMULTANEOUS DETERMINATION OF ACETAMINOPHEN ACETAMINOPHEN GLUCURONIDE AND ACETAMINOPHEN SULFATE IN HUMAN PLASMA

2.1   INTRODUCTION

Acetaminophen (N-acetyl-p-aminophenol; APAP) was first synthesized in 1877 and was introduced into clinical practice as an analgesic in 1893 (Clissold 1986). Acetaminophen is a metabolite of phenacetin, which was developed as an analgesic drug in 1886 (Haas 1983). Phenacetin was withdrawn from the market after several reports of nephritis. Acetaminophen is now widely used alone and in combination with other drugs as an analgesic and antipyretic agent (Beaver 1981; Bannwarth, Latreyte et al. 1997; Renkes and Trechot 1998). The mechanism underlying the analgesic effect of acetaminophen is believed to be through the inhibition of peripheral cyclo-oxygenases (COX), central COX inhibition or indirect interaction with the serotonergic system (Brune 1988).

Acetaminophen pharmacokinetics has been extensively studied and is well characterized in humans. Modest inter-individual variability is observed in the formation of acetaminophen glucuronide as indicated by the seven fold variability in human liver microsomes (Fisher, Vandenbranden et al. 2000). After oral administration, acetaminophen is rapidly absorbed from the small intestine and peak plasma concentrations (Cmax) are attained 30–90 minutes post administration (Clements, Heading et al. 1978; Sahajwalla and Ayres 1991). The half life of acetaminophen is approximately 3 hours (Levy and Regardh 1971). In humans, acetaminophen is extensively metabolized into glucuronide (60%) and sulfate (35%) conjugates and only approximately 2% is excreted unchanged in the urine (Nelson 1982). About 3% of the drug is oxidized via the hepatic cytochrome P450 (CYP) system to a chemically reactive intermediate that combines with liver glutathione to form a non- toxic compound (Figure 10).
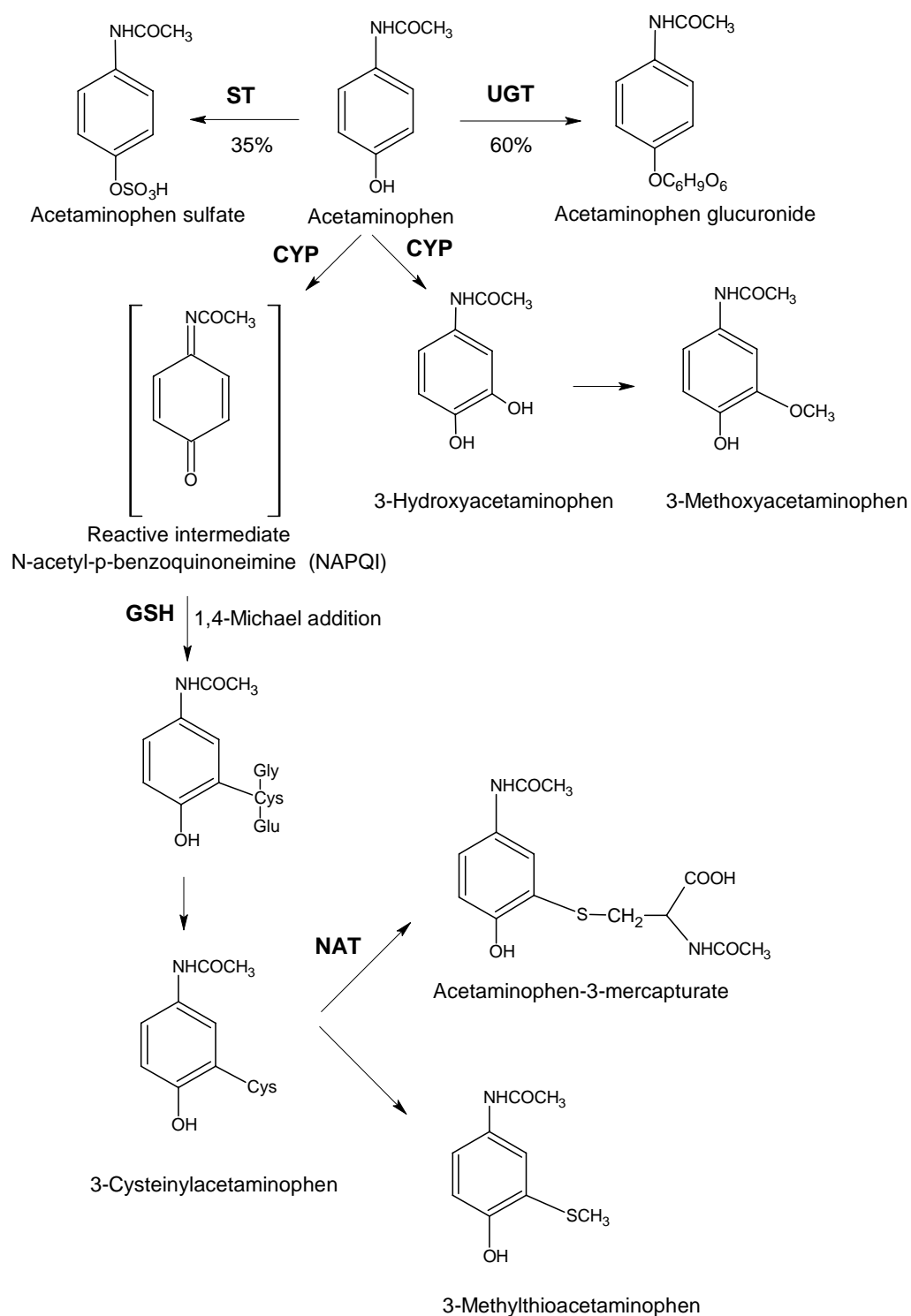
Figure 10 Pathways of biotransformation of acetaminophen in humans

Acetaminophen is not highly bound to plasma proteins at therapeutic concentrations and protein binding ranges from less than 5% to 24% (Milligan, Morris et al. 1994) (Rumack 2002). Because acetaminophen undergoes extensive glucuronidation, it is used as a probe substrate to measure UDP-glucuronosyltransferase (UGT) enzyme activity (Greenblatt, Abernethy et al. 1983; Studenberg and Brouwer 1993). UGT1A6 and UGT1A9 have been identified as the major isozymes catalyzing the formation of the glucuronide at low and therapeutic concentrations, respectively (Bock, Forster et al. 1993). UGT1A1 has also been shown to be involved in acetaminophen metabolism at high (toxic) concentrations (Court, Duan et al. 2001).

Acetaminophen and acetaminophen glucuronide concentrations have been measured in biological fluids (e.g., plasma, urine and saliva), for a variety of reasons including determination of dose related pharmacokinetics, drug interactions, effect of disease conditions and forensic purposes. Several assays have been published for the determination of acetaminophen along with the glucuronide, sulfate, cysteine and mercapturate metabolites. Older papers have used simple techniques such as thin-layer chromatography (Andrews, Bond et al. 1976). Although the technique is simple, it would take an enormous amount of time when applied to multiple plasma samples obtained from a clinical pharmacokinetic study. Recently, several HPLC methods have been described. However, most methods were unable to adequately separate acetaminophen and its glucuronide (al-Obaidy, McKiernan et al. 1996; Brunner and Bai 1999). Some assays did not use an internal standard or lacked details on reproducibility (Riggin, Schmidt et al. 1975; Wong, Solomonraj et al. 1976). Disadvantages of some of the other methods include a high lower limit of detection, complex sample preparation or long run times (Wong, Solomonraj et al. 1976; Blair and Rumack 1977; Howie, Adriaenssens et al. 1977; Lee, Ti et al. 1996; Brunner and Bai 1999).

31

As part of a study to investigate the effect of valerian extracts on acetaminophen glucuronidation in healthy human volunteers (Chapter 4), an HPLC method with UV detection was developed to measure acetaminophen and its glucuronide and sulfate metabolites in plasma. The dose of acetaminophen administered in the study was 500 mg and the estimated peak acetaminophen glucuronide concentrations from this dose ranges from 6-12 µg/ml. Earlier, an HPLC assay to measure acetaminophen and acetaminophen glucuronide in samples of human liver microsomes was developed and validated in our laboratory (Alkharfy and Frye 2001). This assay was modified for use with plasma and to increase the sensitivity.

2.2   EXPERIMENTAL

2.2.1   Reagents and chemicals

APAP, APAPG, APAPS and paraxanthine (1, 7–dimethylxanthine) were purchased from Sigma (St. Louis, MO, USA). Potassium phosphate, ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Acetic acid was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). All chemicals utilized were of the highest purity available for analytical research. Written informed consent was obtained from all the subjects and the protocol was approved by University of Pittsburgh Institutional Review Board (Protocol # 0312032). Blank human plasma was purchased from the Central Blood Bank of Pittsburgh (Pittsburgh, PA, USA). Plasma was separated from blood samples of the subjects in the study by centrifugation at 4°C. Plasma aliquots were stored at -80˚C until analyzed.

2.2.2   Instrumentation

The HPLC system consisted of Waters model 717 autosampler, model 501 HPLC pump and model 486 ultraviolet detector set at 254 nm (Waters corp., Milford, MA, USA). The mobile

phase consisted of 10% methanol, 1% acetic acid in 0.1M phosphate buffer. The mobile phase was degassed under vacuum and filtered prior to use. The mobile phase was delivered at a flow rate of 1 ml/min through a Waters Symmetry® C18 5µ, 3.9 × 150 mm column. Signal output was captured using Empower™ software (Waters Corp., Milford, MA, USA). The run time was 12 minutes.

2.2.3   Standard preparation

Stock solutions of APAP, APAPG (1 mg/ml) and APAPS were prepared in water and stored at -80˚C. Blank plasma was spiked with appropriate volumes of APAP stock to obtain concentrations of 0.5, 1, 2.5, 5, 10 and 20 µg/ml, which are concentrations that span the range expected for an acetaminophen 500 mg dose. For APAPG and APAPS, blank plasma was spiked to obtain final concentrations of 1, 2, 5, 10, 20 and 30 µg/ml. Aliquots (1 mL) were prepared and stored at -80˚C. Quality control standards were prepared in blank plasma at concentrations of 1.5, 7.5 , 15 µg/ml for APAP and 4, 12, 25 µg/ml for APAPG and APAPS.

2.2.4   Sample preparation

Aliquots of plasma (250 µL) were placed in microcentrifuge tubes. Plasma samples were deproteinized by addition of perchloric acid (25 µL, 6 %) containing 25 µg paraxanthine (internal standard). Samples were vortex-mixed briefly and then centrifuged at 14,000 g for 10 minutes. The supernatant was transferred to HPLC vials and a 150-µL aliquot was injected onto the HPLC system for analysis.

2.2.5   Calibration and linearity

Calibration curves were obtained daily for three days using six non-zero standard concentrations of APAP APAPG and APAPS in human plasma. Calibration curves were

constructed with triplicates of lowest concentration (0.5 µg/ml for APAP and 1 µg/ml for APAPG and APAPS) and duplicates of the higher concentrations. For each curve, the APAP to IS, APAPG to IS and APAPS to IS peak height ratios were calculated and plotted against the nominal APAP APAPG and APAPS concentrations. Calibration curves for APAP APAPG and APAPS were generated by weighted ($1/y^2$) linear regression analysis.

2.2.6   Precision and accuracy

The precision and accuracy of the assay was determined using quality control (QC) samples of a low concentration, a middle concentration, and a high concentration for APAP APAPG and APAPS. Intraday variation of the assay was assessed by injecting replicate samples of QC (n=12) at each concentration for APAP, APAPG and APAPS on the same day. Inter-day variation was assessed by injecting replicate QC (n=24) samples on Day 1 (n = 12), Day 2 (n = 6), and Day 3 (n = 6) for at each concentration for each compound. Mean, standard deviation and relative standard deviation (RSD) were calculated from the QC values and used in the estimation of intra- and inter-day precision. Accuracy (bias) is expressed as the percent deviation between the mean concentrations relative to the nominal concentration.

2.2.7   Selectivity and Stability

Selectivity was evaluated by processing and analyzing blank plasma obtained from six different sources. Blank plasma samples were processed in duplicate and compared to plasma spiked with the lowest (0.5µg/ml) APAP, (1 µg/ml) APAPG and (1 µg/ml) APAPS standards. Sample carryover was evaluated by inserting vials containing blank mobile phase in various positions throughout a validation run. Stability was not studied for this assay since several authors have reported that APAP, APAPG and APAPS are stable in plasma for at least 3 months

when stored at -80°C (Wilson, Slattery et al. 1982; Brunner and Bai 1999). The limit of quantitation (LOQ) was defined as the lowest standard value having a signal to noise (S:N) ratio of at least 10:1 and acceptable precision and accuracy (i.e., within 15%). The limit of detection (LOD) was defined as the smallest detectable peak having a S:N ratio of at least 3:1.

2.3   RESULTS

2.3.1   Chromatographic separation

Representative chromatograms for plasma samples are shown in Figure 11. The described method yielded sharp and well resolved peaks for APAP, APAPG, APAPS and the IS paraxanthine. There was no interference from endogenous compounds with any of the peaks. The figure depicts a blank plasma sample (no IS), plasma spiked with APAPG, APAPS, APAP and the IS, and a plasma sample from a subject at obtained two hours after acetaminophen administration. The retention times for APAPG, APAPS, APAP and IS were 3.5, 5.8, 6.7 and 9.8 minutes, respectively. The total time of analysis is 12 minutes.

2.3.2   Precision, Linearity, and Accuracy

A good linear relationship between the peak height ratio and concentration was observed over the entire range for APAP, APAPG and APAPS. Linear calibration curves were obtained for APAP over the concentration range of 0.5 to 20 µg/ml and for APAPG and APAPS over the range of 1.0 to 30 µg/ml. The mean correlation coefficient (r^2) for the standard curves was > 0.998. Response remained linear at all concentrations with no saturation of signal. Intra- and inter-day precision for APAP, APAPG and APAPS were within ± 3.6 %, ± 7.06 %, and ±8.1 respectively and the accuracy was within 1.1% 2.7% and 2.2 %for APAP, APAPG and APAPS respectively (Table 4).

A
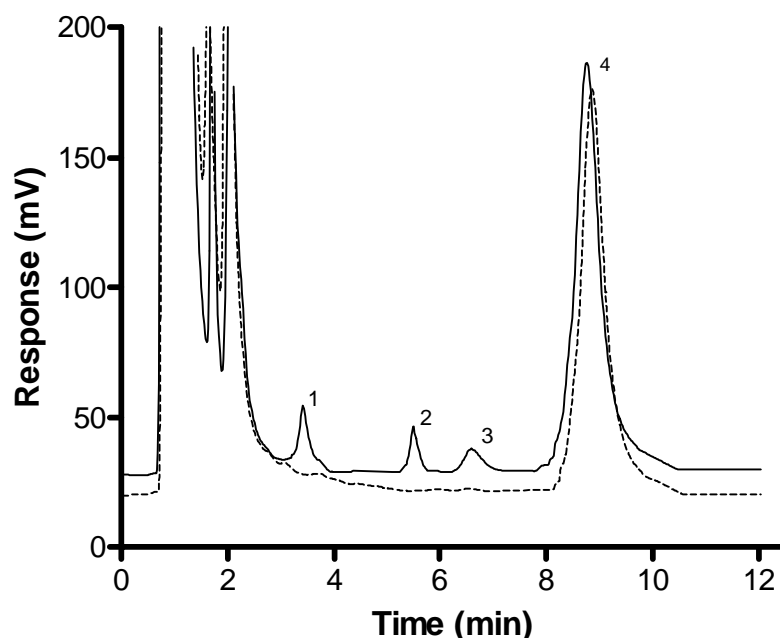
B
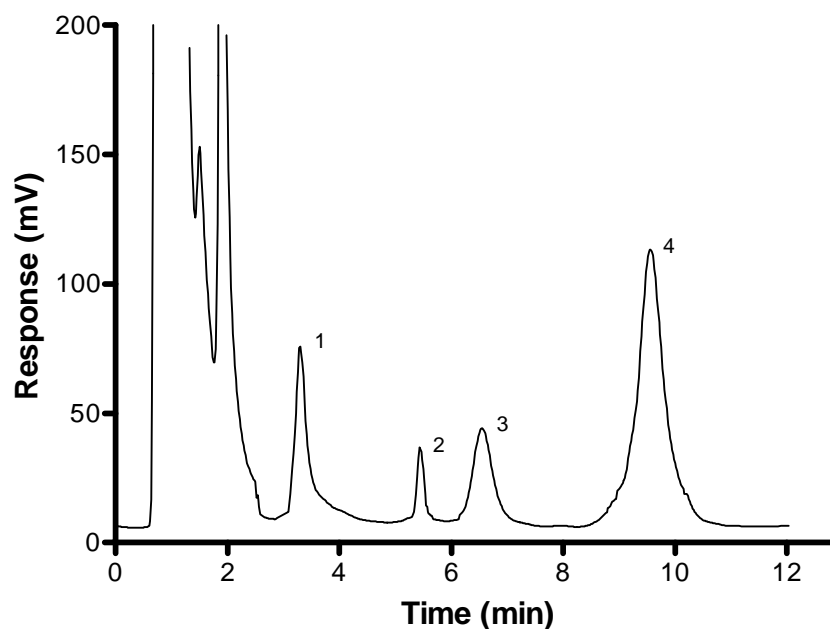
Figure 11 Representative chromatograms of acetaminophen, acetaminophen glucuronide, acetaminophen sulfate and paraxanthine in plasma

Panel A : blank plasma (----) and plasma spiked (—) at LOQ concentration of APAP (0.5μg/ml) and APAPG(1μg/ml) (offset = 10 mV); (B) acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in plasma at 2 hours after single 500 mg dose of acetaminophen ; Peaks (1) APAPG (2) APAPS (3) APAP and (4) I.S.

Table 4   Inter-day variability precision and accuracy of APAP APAPG and APAPS in calibration standards

	Concentration (µg/ml)		% RSD	% Bias
	Standards	Observed (mean ± SD)		
APAP	0.5	0.48 ± 0.02	4.06	-3.62
	1.0	0.99 ± 0.03	3.08	-1.08
	2.5	2.51 ± 0.02	0.66	0.59
	5.0	4.98 ± 0.09	1.83	-0.42
	10.0	10.11 ± 0.13	1.24	1.15
	20.0	19.96 ± 0.13	0.65	-0.22
APAPG	1	1.04 ± 0.06	6.03	3.55
	2	1.98 ± 0.05	2.53	-1.02
	5	5.01 ± 0.13	2.58	0.17
	10	10.12 ±0.18	1.79	1.21
	20	19.79 ± 0.20	1.03	-1.01
	30	30.10 ±0.15	0.49	0.35
APAPS	1	1.01 ± 0.07	7.1	1.2
	2	1.99 ± 0.03	1.95	-1.1
	5	4.99 ± 0.08	1.61	-1.0
	10	9.98 ± 0.21	2.08	-2.1
	20	19.96 ± 0.13	0.67	- 4.1
	30	30.03 ± 0.12	0.40	6.0

Table 5   Intra day and inter day variability precision and accuracy of APAP, APAPG and APAPS in plasma

		Intra-day (n= 12)			Inter-day (n= 24)		
	QC	Observed	% RSD	% Bias	Observed	% RSD	% Bias
APAP	1.5	1.5 ± 0.1	3.6	-0.4	1.5 ± 0.1	1.7	-0.2
	7.5	7.6 ± 0.2	2.3	1.1	7.5 ± 0.1	1.9	0.1
	15	15.0 ± 0.1	0.8	0.3	15.0 ± 0.1	0.7	0.3
APAPG	4	3.9 ± 0.3	7.06	-2.2	3.9 ± 0.2	5.6	-1.5
	12	11.7 ± 0.6	5.3	-2.7	11.8 ± 0.3	2.4	-1.5
	25	25.2 ± 0.5	1.9	0.8	25.1 ± 0.2	1.0	0.2
APAPS	4	4.1 ± 0.2	6.2	1.1	4.1 ± 0.3	8.1	2.2
	12	12.1 ± 0.2	1.7	1.8	12.2 ± 0.4	2.9	2.1
	25	24.9 ± 0.3	1.2	-1.0	25.2 ± 0.2	0.9	1.8

2.4   DISCUSSION

We report a simple and rapid HPLC method to separate and quantify acetaminophen and its glucuronide metabolite in plasma samples. This method does not require expensive or time consuming solid phase extraction techniques. Sample preparation was a simple perchloric acid deproteinization without requirement of further purification. The run time required for this method is short, minimizing the overall time required for assay. Since acetaminophen has been used for several years, a number of assays have been described to measure acetaminophen and its metabolites in various body fluids (Wagner, Wagner et al.). A 500 mg dose of acetaminophen was used in our study. In order to accurately estimate the pharmacokinetic parameters, a very sensitive reproducible analytical method was required. The present method is a simple, rapid assay with good resolution of acetaminophen and its glucuronide and sulfate metabolites. This method was used to support the pharmacokinetic study described in Chapter 4.

3.0 BIOASSAY GUIDED FRACTIONATION FOR ISOLATION AND IDENTIFICATION OF COMPOUNDS IN VALERIAN EXTRACTS THAT INHIBIT ACETAMINOPHEN GLUCURONIDATION

3.1 INTRODUCTION

3.1.1 Valerian

3.1.1.1 Taxonomy and Morphology

Valerian is a member of the family Valereianceae of the genus *Valeriana* L., which comprises of 200 species worldwide (Bailey, Bailey et al. 1976) (Hickey and King 1988). Valerian is indigenous to most parts of Europe and northern Asia (Flèuckiger and Hanbury 1879; Clapham, Tutin et al. 1987). Valerian is a perennial rhizomatous herb growing up to 5 feet and giving rise to new plants from horizontal runners. The most commonly used species is *Valeriana officinalis* L. *Valeriana officinalis* has pinnately divided basal leaves, stem leaves with 11-21 leaflets, a sparsely pubescent rachis and abaxial surfaces. The grooved stalk is usually simple with dentate-serrated leaves and with small fragrant pink or white flowers. The inflorescence is a terminal compound cyme. The genus exhibits polyploidy leading to diploid, tetraploid (central European) and octaploid (British origin) forms (Bos, Woerdenbag et al. 1994; Upton 1999; Wills, Bone et al. 2000).

The rhizomes, roots and stolons are used for medicinal purposes (Bos, Woerdenbag et al. 1994; Bos, Woerdenbag et al. 1996). When dried, the rhizomes are up to 50 mm long and up to 30 mm in diameter. The rhizome contains numerous thick, brown rootlets. The root is longitudinally wrinkled and approximately 100 mm long and 1-3 mm in diameter, almost cylindrical and almost the same color as the rhizome. The stolons are 20-50 mm long, pale yellowish-grey in color and possess prominent nodes separated by longitudinally striated internodes. The rootlets contain most of the essential oil. They are brittle and break in short, horny fractures.

3.1.1.2 Etymology of name

The origin of the name Valerian is uncertain, although the ancient Greek physicians Pliny and Dioscorides have used the term "Valeriane" to refer to *V. tuberose* (Pickering 1879). Some authors believed that the name was derived from Latin, in which valere means health or well-being and some claim that ancient Romans named it in honor of Valerius, the physician who used it first in medicine (Paxton and Hereman Samuel. 1868; Jaeger 1955). It seems to have first appeared in literature between the 9th and 10th centuries. Valerian has also been known as Nard and believed to originate from the Sanskrti *nalada* or Hebrew *nerd* (Kindåi, Levey et al. 1966). Valerian has been referred to as phu or fu as early as 1515, a name usually interpreted as an expression of disgust associated with the strong smell of long-dried valerian root (Thompson 1830; Flèuckiger and Hanbury 1879; Pickering 1879; Dioscorides, Goodyer et al. 1959). Valerian is known as *baldrian* in Germany and *valeriane* in France.

3.1.1.3 History of Valerian Use

The use of valerian in medicine dates back to the period of Hippocrates (460- 370 B.C.), when the ancient Greeks used valerian for its bitter and aromatic properties (Fuchs 1895-1908). The first century Greek physician Dioscorides and his contemporaries, Pliny (23-79 A.D.) and Galen (131-201 A.D.), are said to have recommended valerian for a myriad of disorders including heart palpitations, digestive problems, epilepsy, pain and urinary tract infections (LaFrance, Lauterbach et al. 2000). The Greeks are reported to have used valerian as an emmengogue, antiperspirant and antidote for poisons (Dioscorides, Goodyer et al. 1959). Historically, the Greek and Roman physicians mainly used valerian as a diuretic, analgesic and antispasmodic agent. In the Bible, valerian is mentioned as a flavoring agent for food and as a healing oil in a soothing salve for the head (Ainslie 1984; Sanyal 1984). From the 8th to the 13th centuries, valerian was a popular medicinal agent in the Arabian school, where it was

recommended to cure pustules of the mouth, to protect the gums from heat, to cure insanity and to strengthen breathing. In the 16th Century, the Italian botanist Fabius Columna reported that he was cured of epilepsy by valerian (Upton 2001). In medieval times, Valerian was used as a panacea and has been referred to as all heal. Valerian was regarded as the best treatment for epilepsy in the 18th and 19th centuries (Eadie 2004). During the 200-year period from 1733-1936, valerian was one of the six most prescribed medicines in European and American medicine (Hobbs 1990). It was in this period that valerian was established primarily as a nervine agent and since then, valerian has been used as a sedative and anxiolytic. Valerian was official in British pharmacopoeia in 1867, in the US pharmacopoeia from 1820 -1936 and in the US National Formulary until 1946 (Hobbs 1989). Valerian is currently listed under monographs for dietary supplements in a separate section of the USP. In 1967 it was official in the pharmacopoeias of Austria, Belgium, Brazil, Chile, Czechoslovakia, France, Germany, Hungary, Yugoslavia, Romania, Russia, Spain and Switzerland (Hobbs 1993). *V. officinalis* is included in the European Pharmacopoeia and was included in German commission E monographs since 1985 as a sleep aid. *V. wallichi* is official in the Indian Pharmacopoeia. In the ancient Indian medicine system, Ayurveda, *V. jatamamsi* is used as tonic, stimulant, antispasmodic, diuretic and as antidote for poisons (India. Dept. of Indian Systems of Medicine & Homoeopathy. 1999). In traditional Chinese medicine, *V. jatamamsi* has been known as an odorant. In the Chinese Pharmacopoeia called Pen Ts'ao, valerian is cited as being used as a deodorant, carminative, headache reliever, and treatment of malaria(Shin-Chen 1578; Perry 1980).Table 6 summarizes the uses of valerian over the centuries and its listing in official and unofficial compendia.

Table 6 Official and unofficial compendia

Herbal/ Compendia	Date	Uses
Pliny's *Natural History*	60 A.D.	Sedative
Galen & Pedanius Dioscorides's *De Materia Medica*	160 A.D.	Insomnia, antispasmodic , astringent, antiperspirant, digestive & urinary tract disorders
Leonhart Fuchs' *The Great Herbal*	1542	For colic, stomach disorders
Maththiolus	1544	Diuretic, anodyne, emmenagogic, carminative for coughs, asthma
Dodoens	1554	Gargle for throat infections
Turner	1568	Perfume
Phytobasanos of Columna	1592	Anticonvulsant
Gerard	1597	Diuretic, treatment of jaundice, cramps, convulsions ulcers of mouth and gum sores
Parkinson	1640	To treat coughs, plague, short-windedness
Culpeper's *Complete Herbal*	1649	Nervousness, trembling, headaches, and heart palpitations
James Alleyne's *A New English Dispensatory*	1733	Nervousness, hysteria
R. James' *Pharmacopoeia Universalis*	1745	Sudorific, diuretic, poor sight, asthma, cough, liver stagnancy, jaundice, epilepsy
Andrew Duncan's *Edingurgh New Dispensatory*	1791	Nervous system debility, epilepsy
William Cullen's *A Treatise of the Materia Medica*	1802	Antispasmodic, sedative for hysteria
James Thatcher's *The American New Dispensatory*	1813	Sedative, antispasmodic
Coxe's *American Dispensatory*	1830	Pain reliever, comforts stomach, induces sleep
London Dispensatory	1833	Hypochondriasis, flavoring agent for food
Jonathan Pereira's *The Elements of Materia Medica and Therapeutics*	1843	Nervine, antispasmodic
Trousseau's *Materia Medica*	1880	"vapors" in women
John Milton Scudder's *Specific Medications*	1903	Cerebral stimulant, analgesic, and sedative useful in nervous irritability
King's *American Dispensatory*	1898	Aromatic stimulant , rheumatism, hysteria, low grade fevers, as an aphrodisiac
Ellingwood's *Systematic Treatise on Materia Medica and Therapeutics*	1900	Nervine and sedative for the treatment of hysteria, epilepsy, and menopausal nervous anxiety
Grieve's *A Modern Herbal*	1931	Nervous overstrain, poor eye sight, insomnia, cardiac palpitations, cholera and as perfume
Torald Sollmann's *A Manual of Pharmacology*	1936	Antispasmodic, sedative

3.1.1.4 Chemical constituents

The constituents of major interest due to their sedative activity are the essential oil, sesquiterpenoids and epoxy iridoid esters (valepotriates) (Houghton 1999; Upton 1999). Apart from the iridoids and terpenoids, valerian is also known to contain small quantities of alkaloids, lignans and flavonoids (Upton 1999; Schumacher, Scholle et al. 2002; Marder, Viola et al. 2003). The primary constituents of *Valeriana officinalis* are summarized in Table 2. The roots and rhizomes are known to contain essential oils stored in the hypodermis. According to the *European Pharmacopoeia*, the crude drug, Valerianae radix contains not less than 0.5% (v/m) of essential oil(Bos, Woerdenbag et al. 1998). Typically, the essential oil content in roots harvested within 2 years ranges from 0.9-2.1%. The main constituents of the essential oil are valerenone (10-21%), cryptofauronol (5-12%), valerenal (2-5%), valerianol (1-6%) and bornyl acetate (1-3%). The total valerenic acid content (valerenic acid and acetoxy valerenic acid) of roots harvested within 2 years ranges from 0.1-0.9% and total valtrate content (valtrate and isovaltrate) ranges from 0.1-1.1%. During drying and storage, some valpotriates undergo hydrolysis and give rise to baldrinals (Dewick 2002). Structures of sesquiterpenoids, valepotriates and baldrinals are shown in Figure 12.

Table 7 Chemical constituents of *Valeriana officinalis*

1.Essential oil (0.2-1%)

- Monoterpenes

  (-)-Bornyl isovalerenate

  (-)-Bornyl isovalereneic acid

- Sesquiterpenes

  Valerenic acid (0.1- 0.9%)

  2-Hydroxy valerenic acid

  2-Acetoxyvalerenic acid

  Valerenal (3-16%)

  Valerenone (0-18%)

  Valerenol

  Pacifigogiol (0.7-8.6%)

2. Iridoids

- Monoene-Valepotriates

  Didrovaletrate

  Isovaleroxyhydroxydidrovaltrate(10-20%)

- Diene-Valepotriates

  Valtrate ((80-90%) Ref(Stahl and Schild 1971; Samuelsson 1992)

  Isovaltrate

  Acevaltarate

- Valepotriate Hydrines

- Desoxy monoene Valepotriates

  8, 11 Desoxididrovaltrate

  8,11 Desoxihomodidrovaltrate

- Other Valepotrites

  Patrinoside

  Valechlorine

  Valerosidate

- Breakdown products

  Baldrinal

  Homobaldrinal

3. Alkaloids

- Actinidine

- Isovaleramide

4. Caffeic acid derivatives

- Chologenic acid

5. Lignans

- 8-Hydroxypinoresinol

Sesquiterpenes
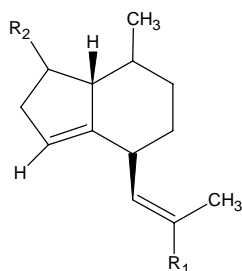
R1	R2	
CHO	H	Valerenal
COOH	H	Valerenic acid
COOH	OAc	Acetoxy valerenic acid
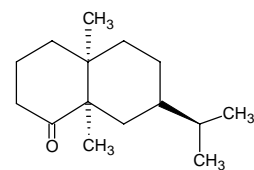
Valerenone

Valepotriates

Monoene type
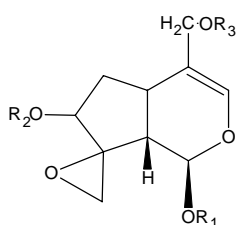
Diene Type

R1	R2	R3	
Iv	Ac	Iv	Didrovaltrate
Iv	Iv	Ac	Isodidrovaltrate
Miv	Ac	Iv	Homodidrovaltrate
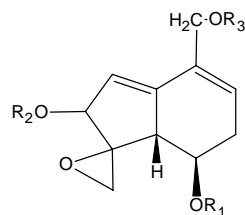
R1	R2	R3	
Iv	Iv	Ac	Valtrate
Iv	Ac	Iv	Isovaltrate
Aiv	Iv	Ac	Acevaltrate
Iv	Aiv	Ac	Homoacevaltrate

Baldrinals
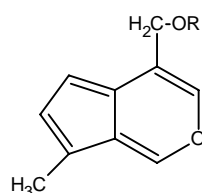
R		
Ac	Baldrinal
Iv	Homobaldrinal
H	Deacylbaldrinal

Side Chains

Iv Isovaleryl
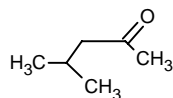
Miv β–Methylisovaleryl
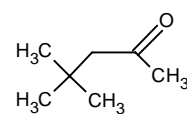
Aiv Acetoxyisovaleryl
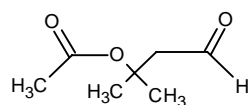
Figure 12 Structures of sesquiterpene derivatives and valepotriates in *Valeriana officinalis*

3.1.1.5 Cultivation and collection

Valerian is cultivated in several European countries including Britain, Belgium, France and Germany (Upton 1999). Valerian is also cultivated in the United States in Vermont, New Hampshire, New York and in the Pacific Northwest (St. Hilaire 2003). Harvest times vary geographically. The essential oil content varies with genotypes, harvest times, growing conditions, age of root, drying and extraction techniques. Essential oil content is reported to be higher in plants grown in higher elevations with dryer environment and in phosphate-rich soils. The essential oil content is higher in roots of one-year old plants compared to two year old plants. Valerenic acid content and valepotriate contents also follow the same pattern. Plants harvested in September are reported to have the maximum volatile oil content ranging from 1.2 % to 2.1 % while valepotriates are at a maximum in plants harvested in February-March. Root materials are typically dried at 50°C for 52 hours or at room temperature for 10 days. The valerenic acid content does not differ between the two drying methods. Volatile oil content of material collected in the Pacific Northwest ranges between 0.4% and 1.3% and material used for preparation of commercial products is required to contain at least 0.5% valerenic acid.

3.1.2 Bioassay guided fractionation

Plants have been a major source of medicinal compounds for the development of several pharmaceutical products. As natural products are often complex mixtures of chemical compounds, some of them lacking biological activity, isolation of the active compound is necessary for drug development. In the early 1950s, the strategy of bioassay-guided fractionation and isolation using chromatographic separation techniques revolutionized medicinal plant research. Bioassay-guided isolation is an integration of chemical separation of compounds in a mixture with biological testing (Figure 13). The first step in this process is testing an extract to

confirm its activity. The second step is a crude fractionation of the extract leading to separation of the compounds in the matrix and testing the crude fractions. Further fractionation is undertaken on the fractions that show biological activity, at a certain concentration threshold, while the inactive fractions are set aside or discarded. The process of fractionation and biological testing is repeated until pure compounds are obtained. The final step is structural identification of the pure compound. Although the bioactivity-guided fractionation methods are traditionally used in drug discovery, a similar approach is seldom adopted for drug metabolism studies, where either whole plant extracts or known constituents of medicinal plants are used. A well known example of studies performed with known constituents is hyperforin in St. Johns wort (Moore, Goodwin et al. 2000; Cantoni, Rozio et al. 2003). The bioassay guided approach was chosen for our studies since preliminary work in our laboratory comparing the effect of valerenic acid and the alcoholic extract of valerian on acetaminophen glucuronidation showed that the whole extract inhibited glucuronidation to a greater extent than valerenic acid alone. These results suggested that other compounds in the extract contributed to inhibition of acetaminophen glucuronidation. Being a systematic approach, this methodology precludes overlooking compounds that could be biologically active even if present in small quantities. The objective of the present study was isolation of compounds in ethanolic extracts of *Valeriana officinalis* capable of inhibiting acetaminophen glucuronidation by the bioassay guided approach.

```
                        Plant Material

                             │
                             ▼

                     Extraction (LLE/SPE)

            Bioassay │
                     ▼

        ┌────────────────────────────────┐
        │                                │
        ▼                                ▼

Activity    No                          Yes

                                         │ Fractionation
                                         ▼

        ┌──────┬──────┬──────┬──────┐
        │      │      │      │      │
   Bioassay│   │      │      │      │
        ▼      ▼      ▼      ▼      ▼

Activity    No     No     Yes     No     No

                            │ Isolation
                            ▼

                ┌───────────┼───────────┐
                │           │           │
                ▼           ▼           ▼

             Compd1      Compd 2     Compd 3

                │           │           │
                ▼           ▼           ▼

             Identify    Identify    Identify
```
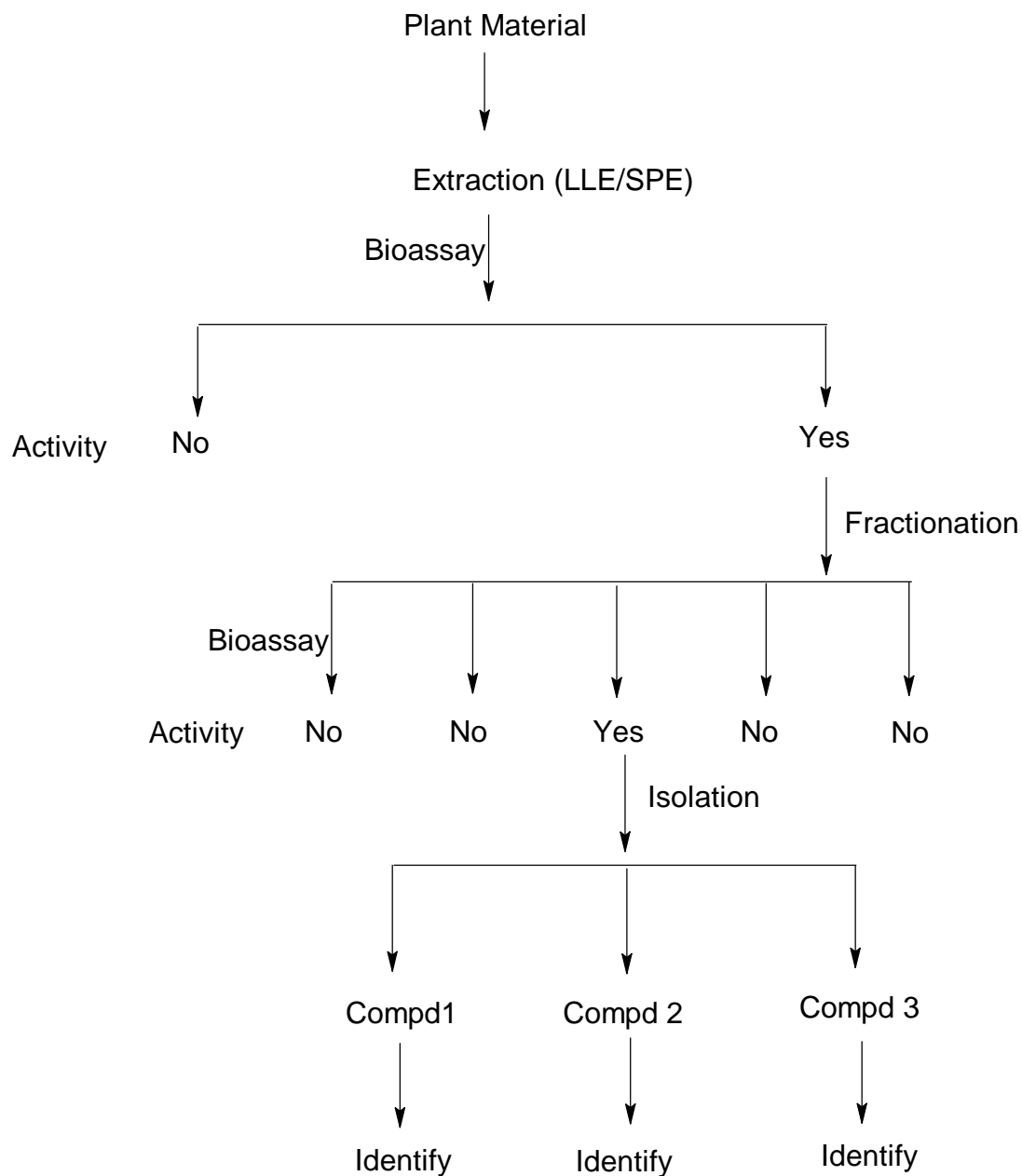
Figure 13 General scheme for bioassay guided isolation
          (Adapted from Rimando et. al Journal of Agronomy 2001 93 (1), 16-20)
          LLE- Liquid Liquid Extraction, SPE- Solid phase extraction

• EXPERIMENTAL

3.1.3 Materials and Methods

3.2.1.1 Plant material:

Herbal products are commonly marketed in two forms: the powdered herb, and the powdered herbal extract, the latter being incorporated into a suitable dosage form and standardized to contain a specific percent of a designated marker compound. Valerenic acid has been established as the marker compound for valerian products. Valerian extracts are available in the form of liquid or powder. The plant material for the experiments described in this project is found in a commercial product. The product was composed of a soft gel capsule containing a liquid alcoholic extract along with excipients. The label claim of containing 0.8% valerenic acid was confirmed in our laboratory by HPLC.

3.2.1.2 Chemicals

Valerian capsules (250mg; PharmAssure, Phoenix, AZ, USA) were obtained from a local retail pharmacy. Pure valerenic acid was purchased from Indofine chemical company (Sommerville, NJ, USA). Acetaminophen, acetaminophen glucuronide, alamethicin, uridine diphospho-glucuronic acid (UDPGA) and magnesium chloride were purchased from Sigma (St. Louis, MO, USA). Petroleum ether, ethyl acetate, methylene chloride, methanol and ethanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPTLC plates (5 x 7.5 cm) coated with silica gel 60 with fluorescence indicator (Merck, Darmstadt, Germany) were purchased from EM Science Gibbstown, NJ, USA.

3.2.1.3 Bioassay

The liver is the principal site of drug metabolism in the body and contains a wide spectrum of drug metabolizing enzymes. Therefore, in vitro studies on drug metabolism are usually performed using liver tissue. In vitro models of the liver commonly used to evaluate drug interactions are liver slices, hepatocytes, liver microsomes and cDNA-microsomes. Liver microsomes are advantageous as they are simple to use, can be preserved for long periods of time at -80°C and are relatively easy to prepare. This model system requires only a very small amount of compound in order to perform the tests. Therefore, liver microsomes were chosen as the model to evaluate the effect of the isolated fractions on UGT enzyme activity. Acetaminophen was used as the model substrate for determination of UGT activity as this drug is extensively glucuronidated. Incubations were performed as described by Alkharfy et. al (Alkharfy and Frye 2001). Human liver microsomes (0.5 mg/ml final protein concentration) and alamethicin (30μg/mg protein) were pre-incubated on ice for 5 min. The active site of the UGTs is located in the lumen of the ER resulting in latency in site activity. Alamethicin (an antifungal peptide) has been successfully used as a permeating agent to overcome the latency of UGT enzymes in previous studies in our laboratory. The incubation mixture consisted of magnesium chloride (5 mM), and acetaminophen (10 mM) in a final incubation volume of 250μl. The reaction was started by adding 4 mM of UDPGA, incubating the samples at 37°C for 1 hour, and terminating the reaction by addition of 25μl of 6% perchloric acid containing 25μg paraxanthine (internal standard), followed by vortex-mixing and cooling on ice. The supernatant was isolated by centrifuging the samples at 2000 g for 5 min. Acetaminophen glucuronide concentration in the microsomal incubates was determined by reverse phase HPLC, utilizing a symmetry C18 column and a mobile phase composed of methanol, acetic acid and water (10:1:89, v/v/v), with

UV detection at 250nm. Acetaminophen glucuronide formation was quantitated by comparing the peak height in the incubations to a standard curve containing known amounts of the metabolite in the range 0.1-25 nmol. The standard curve correlation coefficients (r^2) were 0.99. The formation rate of acetaminophen glucuronide in the incubations was 2.2nmol/min/mg protein in our laboratory and was simlar to that reported in the literature (Fisher, Campanale et al. 2000). Incubations were performed in the presence of extracts and fractions obtained by partitioning and chromatographic procedures and compared with incubations performed with vehicle only as a control to determine the effect of the extracts/fractions on acetaminophen glucuronide formation. Bioactivity is expressed as percent control.

3.2.1.4 Extraction and isolation

Mild fractionation and chromatographic methods were adopted in order to prevent destruction or alteration of potentially bioactive compounds. A simple solvent pair partitioning method was used to remove the maximal amount of inactive compounds. Capsules (250mg, n=10) of valerian root extracts were crushed and extracted three times with 80% aqueous EtOH (50 mL). The extracts were combined, and concentrated to dryness under reduced pressure at 40°C with a rotary evaporator. Approximately 1.2g of the crude extract was obtained from ten capsules, and this extract was stored at 4°C. This extract was subjected to liquid-liquid partitioning between H_2O (50 mL) and CH_2Cl_2 (2 × 250 mL). These fractions were separately evaporated *in vacuo* at 45°C and evaluated for bioactivity. The CH_2Cl_2 fraction showed strong inhibition potential of acetaminophen glucuronidation while the water fraction was inactive. The strongly active CH_2Cl_2 fraction (300 mg) was further partitioned between EtOAc and H_2O. After removing the solvents under vacuum, the residues from each partitioning [CH_2Cl_2 (300 mg)/water (800 mg); EtOAc (220mg)/H_2O (58.4mg)] were screened for bioactivity. The

53

sequence of extractions is shown in Figure 14. The bioactive EtOAc residue (223 mg) was subjected to normal-phase open column chromatography (Silica gel (6 g), 70-230 mesh column A: 10×100 mm). The column was slurry packed in petrol (30-60°C)- CH_2Cl_2 (1:4). The sample was dissolved in the same solvent, added to the column, and elution initiated with the identical solvent. Elution proceeded via a gradient CH_2Cl_2-MeOH system (CH_2Cl_2; CH_2Cl_2-MeOH mixtures; MeOH) to afford 10 fractions (F001-F010). Fraction 3 (F003), eluting with 99:1 CH_2Cl_2-MeOH, was bioactive and was obtained in substantial amounts (weight 22.3 mg) compared to the other fractions. Therefore, this fraction was further separated on a Si gel column (5g) (column B: 10×100 mm). The column was slurry packed in CH_2Cl_2, the sample dissolved in CH_2Cl_2 (3 mL) and added to the column. Elution proceeded via gradient mixtures of d-CH_2Cl_2-MeOH 100 %, (99.5:0.5) (99:1), (98:2), and (97:3) to yield 5 fractions. Only two adjacent fractions showed bioactivity, and these two active fractions were combined, and further chromatographed in a similar manner on a third Si gel column (Column C: 10 x 100mm). The combined fractions were dissolved in petrol-CH_2Cl_2 (1:4) and eluted with petrol- CH_2Cl_2 (1:4), CH_2Cl_2 and CH_2Cl_2-MeOH (19:1) to yield 3 fractions. Only fraction 1 was sufficiently bioactive and obtained in modest amounts.
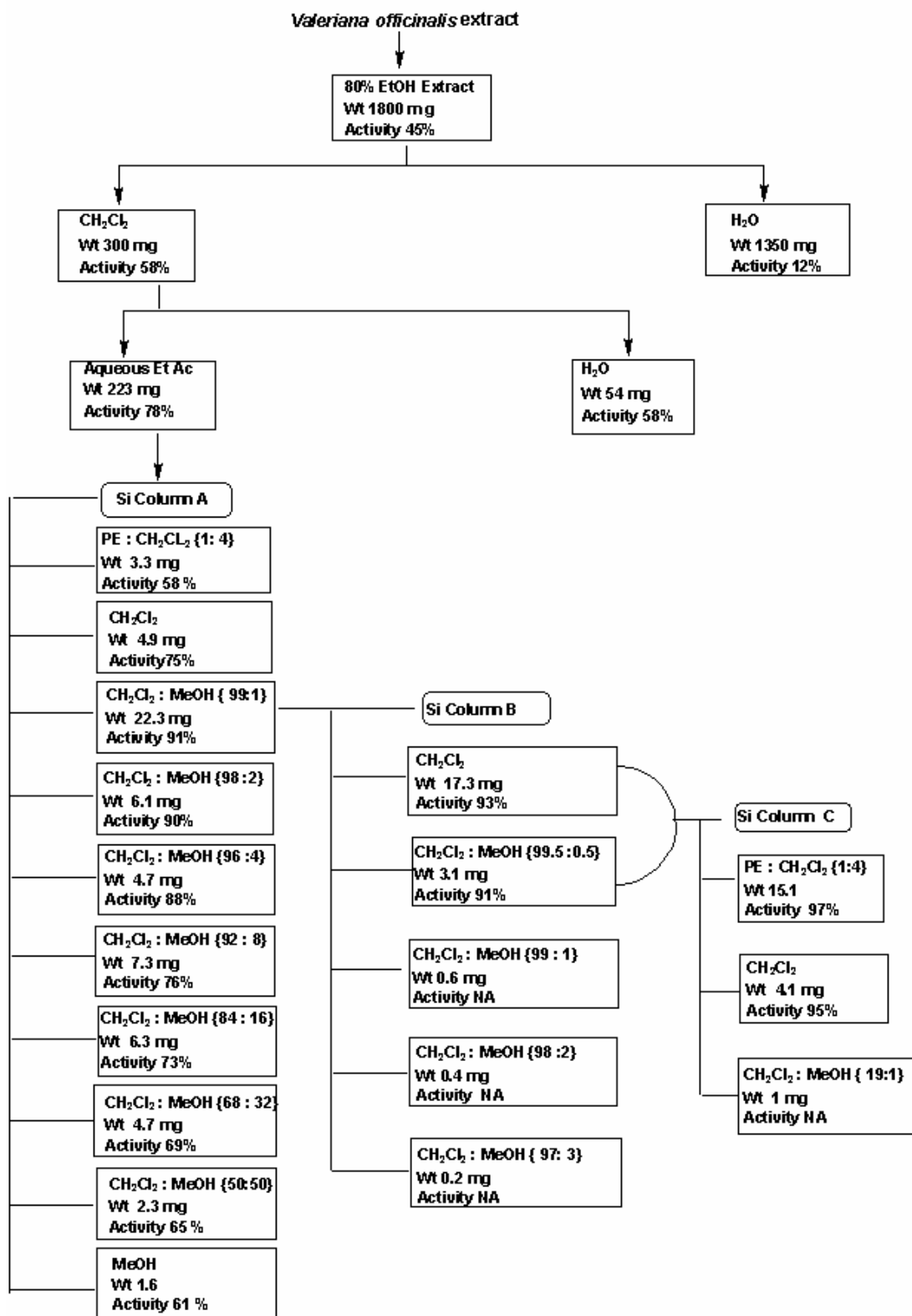
Figure 14 Scheme showing bioactivity - guided fractionation

3.2.1.5 Chemical Identification

Fraction 1 (15.1mg) of column C eluted with petrol-CH_2Cl_2 (1:4) and was analyzed by high performance thin-layer chromatography on HPTLC plates. Three μl volumes of sample solutions and standard (valerenic acid) were applied 6 mm apart and 8mm from the lower edge of the plates. Plates were subjected to the following visualization procedures:

a) UV (254 nm and 366nm)

b) Sprayed with anisaldehyde-sulfuric acid reagent and air dried.

The plates were then placed in the oven at 120°C for 2 minutes (or until color of standard developed).

Fractions were also analyzed by a gradient HPLC with diode array detection. Analyses were carried out on a μBondapack C18 column (Waters, Milford, MA) with a linear gradient elution of a mobile phase composed of MeOH (eluent A) and 0.5% H_3PO_4 (eluent B) . The gradient elution was programmed as follows: A was ramped up from 45% linearly to 80% from 0-40 min, then A maintained at 80% for another 20 min. After completion of the HPLC run, the column was washed with 100% MeOH for 5 min. The pump was programmed to return to initial conditions within 3 min. and equilibrated for 15 min. with the initial mobile phase for the next injection. The flow rate was constant at 1 ml/min. The photo-diode array (PDA) detector was set to scan from 200–250 nm. The wavelength selected for the target compounds was 215 nm (giving an average maximum absorbance for all the compounds).

Fractions corresponding to peaks were collected from the HPLC and further characterization of the fractions were performed by gas chromatography–mass spectrometry (GC-MS) (Hewlett Packard 5890 Series II gas chromatograph coupled to Hewlett Packard

5989A MS engine) operating in the EI mode at 70 eV, equipped with an HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 μm). GC oven initial temperature was 60°C and programmed to 280°C at a rate of 3°C/min. The carrier gas was helium at a flow rate 1 mL/min.

- **RESULTS**

3.1.4 Identification of isolated compounds

The capsules of valerian (Valeriana officinalis) were crushed and extracted with 80% alcohol. The ethanol extract was concentrated under reduced pressure at 40°C to a viscous residue that was dissolved in dichloromethane and partitioned between water and dichloromethane. The activity, as defined by inhibition of acetaminophen glucuronide formation, was retained in the organic phase (dichloromethane), with the aqueous phase being devoid of any inhibitory activity.

The dichloromethane extract was evaporated to a residue that was further partitioned between ethyl acetate and water. The activity was once again principally retained in the organic layer (ethyl acetate) while the aqueous layer fraction was only marginally active (Figure 15). The ethyl acetate extract was subjected to various chromatographic techniques including adsorption column chromatography (Figure 17), high-performance thin-layer chromatography (HPTLC) (Figures 16 & 18), and high pressure liquid chromatography (HPLC). Gas chromatography-mass spectrometry (GC-MS) was utilized to analyze fractions collected from HPLC and to aid in the identification of the three major sesquiterpene compounds: valerenic acid, acetoxyvalerenic acid and valerenal.
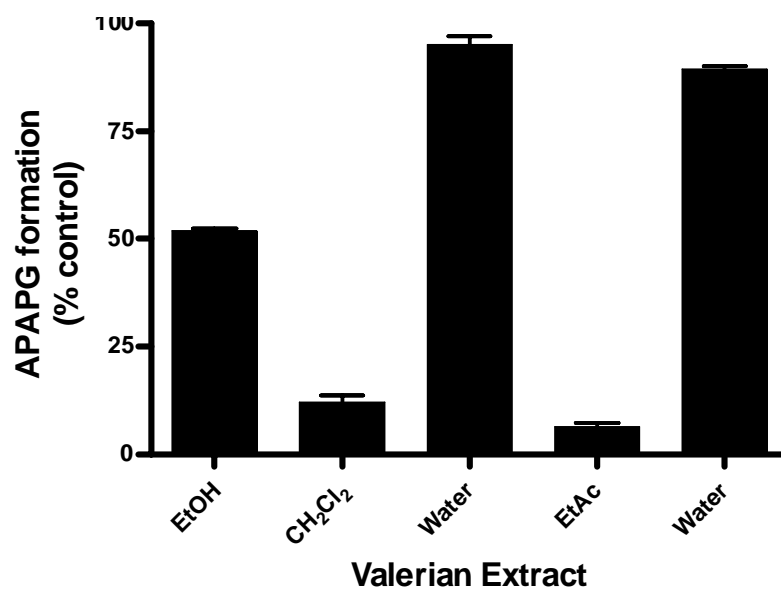
Figure 15   **Effect of the valerian extracts on UGT activity**
Acetaminophen glucuronide formation measured in pooled human liver microsomes after co-incubation with valerian extracts. Results are expressed as percent control. Data are means ± S.D. of duplicate values.
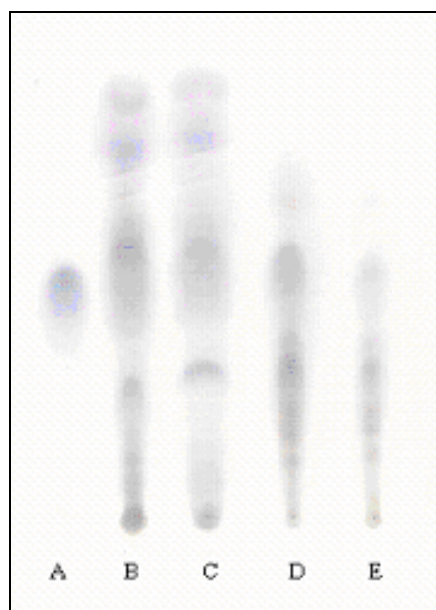
Figure 16 Thin Layer chromatography of valerian extracts
A: Valerenic acid standard, B: Dichloromethane extract, C: Ethyl acetate extract, D: Column C fraction1, E: Column C Fraction 2
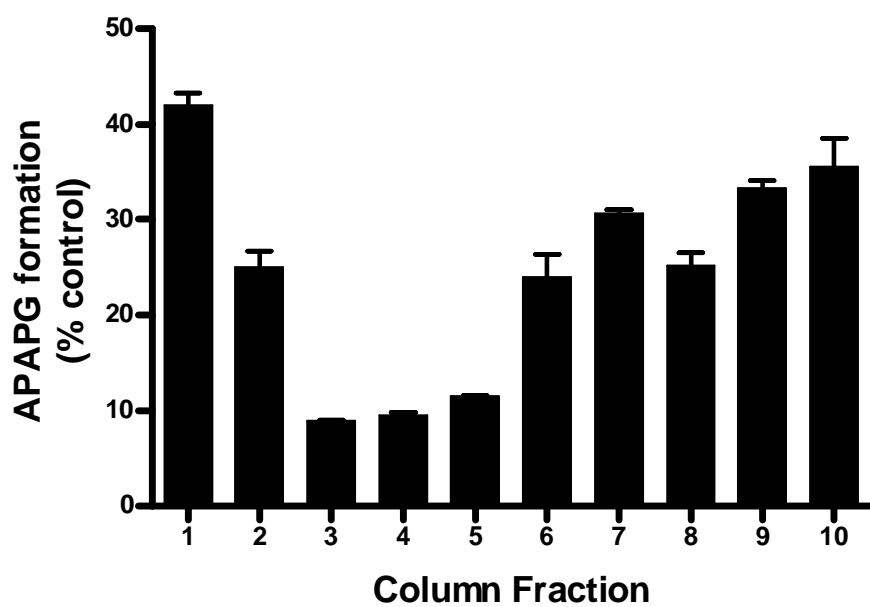
Figure 17   Effect of each fraction obtained from column A on UGT activity
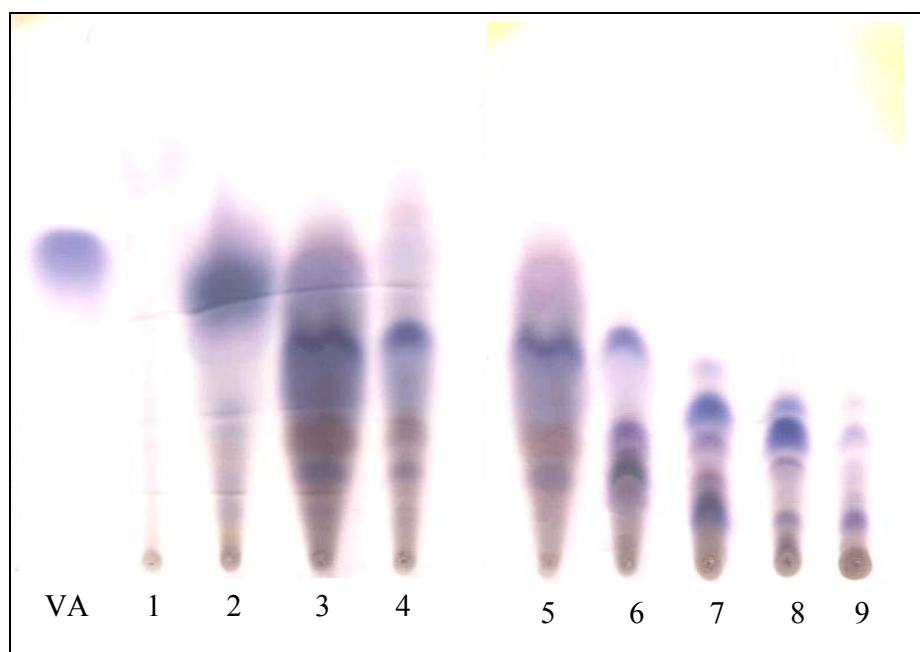
Figure 18 Thin layer chromatography of the corresponding column fractions

(fraction 10 not shown)

3.3.1.1 Valerenic acid (Compound 1)

Compound 1 was visible as a dark blue band on the thin-layer chromatogram after staining with Anisaldehyde reagent (R_f 0.48; hexane-ethyl acetate-acetic acid, [13:7:0.1]). The retention time for the acid compound by HPLC analysis (mobile phase - methanol: water: acetic acid [80:20:0.1, v/v/v]; Symmetry C_{18} column; UV detection at 230nm) was 5.5 minutes. The GC retention time was 10.1 min. The mass spectrum showed a molecular ion peak at m/z 234 (73%), with other significant fragment ions at m/z 161 (90%), 133 (73), 122 (75), 107 (88), 105 (88), 93 (51), 91 (100), 79 (66), and 77 (52). Compound 1 was identified as the sesquiterpene valerenic acid on the basis of a direct comparison (TLC R_f and color; HPLC and GC retention times; GC/EI/MS) with an authentic sample (Dalton Chemical Laboratories Inc., Ontario, Canada). The mass spectrum is shown in the figure 19.

Valerenic acid was first isolated from the roots of Valeriana officinalis in 1957 by A. Stoll and colleagues (A. Stoll 1957). The structure of this compound was first elucidated by a combination of spectrometric methods by G. Buchi and collegues(Buchi, Popper et al. 1960). Valerenic acid has subsequently been isolated from *Valeriana officinalis* as well as *Liqidambar styraciflua*(Tattje and Bos 1979; Culvenor, Edgar et al. 1986). In a recent study, dichloromethane and petrol extracts of valerian were screened for ligands of the melatonin, serotonin, benzodiazepine, glutamate, and GABA receptors, or serotonin transporter (Dietz, Mahady et al. 2005). Results indicated that these extracts exhibited significant binding at the 5-HT$_{5a}$ receptor. Valerenic acid and acetoxyvalerenic acid were identified as being present in the dichloromethane and petrol extracts, and is in agreement with our conclusions.

In mice studies, the essential oil of valerian and the isolated components valerenal, valerenic acid, valeranone, and isoeugenyl isovalerate showed sedative and muscle relaxant

activity(Hendriks, Bos et al. 1985). Valerenal and valerenic acid were shown to be the most potent compounds, with valerenic acid causing a decrease in rotorod and traction performance in mice at an intra-peritoneal dose of 100 mg/kg. In addition, valerenic acid was observed to produce a dose-related increase in pentobarbital-induced sleep at both 50 and 100 mg/kg ip dosage.

3.3.1.2 Acetoxyvalerenic acid (Compound 2)

Compound 2 was visible as a dark bluish-purple band on the thin-layer chromatogram after staining with anisaldehyde reagent (R_f 0.28; hexane-ethyl acetate-acetic acid, [13:7:0.1]). The retention time for the compound acid by HPLC analysis (mobile phase - methanol: water: acetic acid [80:20:0.1]; Symmetry C_{18} column; UV detection at 230nm) was 3.2 minutes. The GC retention time was 11.3 min. The mass spectrum exhibited important fragment ions at m/z 232 (85%)(M-60)(M-CH_3COOH), 187 (28), 159 (40), 145 (34), 131 (37), 120 (100), 105 (39), 91 (27), and 43 (32). The mass spectrum of this compound is shown in figure 20. Compound 2 was identified as the sesquiterpene acetoxyvalerenic acid on the basis of a direct comparison (TLC R_f and color; HPLC and GC retention times; GC/EI/MS) with an authentic sample (Dalton Chemical Laboratories Inc., Ontario, Canada). In addition to valerenic acid, acetoxyvalerenic acid has been identified as a characteristic constituent of *V. officinalis* and its subspecies (Riedel, Hansel et al. 1982). Acetoxyvalerenic acid was first isolated from the roots of *Valeriana officinalis* in 1960 and has subsequently been isolated from numerous Valeriana species spp. *collina, sanbucifolia*, and related taxa such as *V. celtica, V. angustifolia, V.montana, V.phu, and V. tripteris* (Buchi, Popper et al. 1960; Bos, Woerdenbag et al. 1997).

3.3.1.3 Valerenal (Compound 3)

The third major compound was identified as valerenal based on the GC/EI/MS fragmentation pattern shown in figure 21. The identification was established by comparing the fragmentation pattern of Compound 3 with that of literature values (Bos, Hendriks et al. 1986).The GC retention time of this compound was 6.8 min. Since a standard of this compound is not available, the R_f on thin layer chromatography was not determined. The mass spectrum showed a molecular ion peak at m/z 218, with other significant fragment ions at m/z 203(51), 189(26), 185(74), 175(82), 161(52), 148(23), 147(53), 145(29), 143(16), 135(15), 133(42), 131(23), 129 (15), 128(15), 121(30), 119(41), 117(29), 115(28), 109(20), 108(18), 107(49), 106(16), 105(74), 103(12, 95(24), 93(47), 91(100), 81(29), 79(57), 78(15), 77(56), 67(20), 65(24), 55(39), and 53(25). The spectrum is shown in figure 19.

Valerenal has been isolated from *V. Officinalis* and identified in previous studies(Bos, Woerdenbag et al. 1997). Recent studies have identified valerenal as one of the major constituents of *V .officinalis* and have established that the valerenal content in valerian essential oil increases on aging (Letchamo, Ward et al. 2004). However, in contrast to valerenic acid, no studies have been performed with valerenal to investigate its effects on central nervous system. In spite of being one of the major constituents of valerian, effects of valerenal remain unknown.
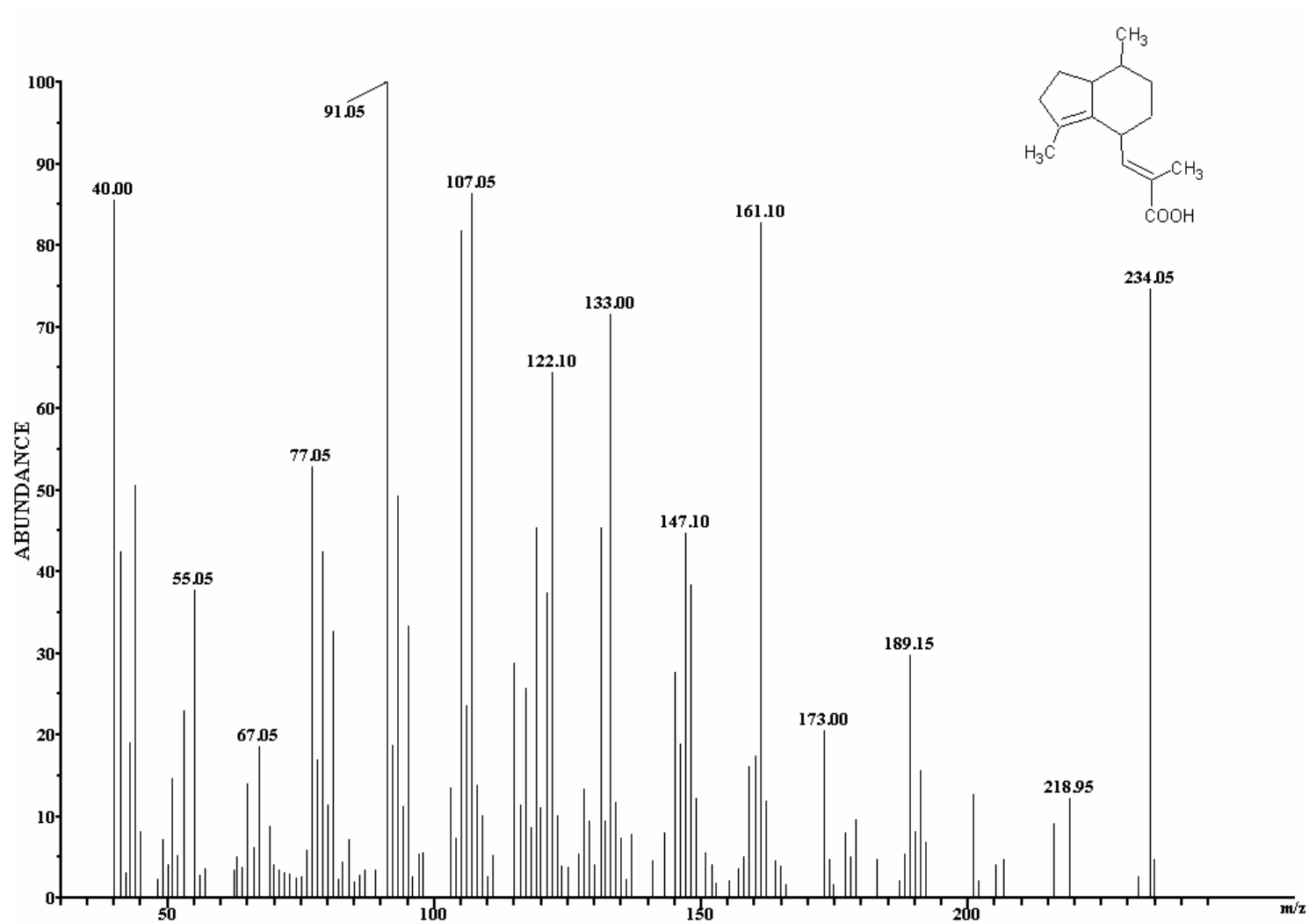
Figure 19 EI Mass spectrum of valerenic acid
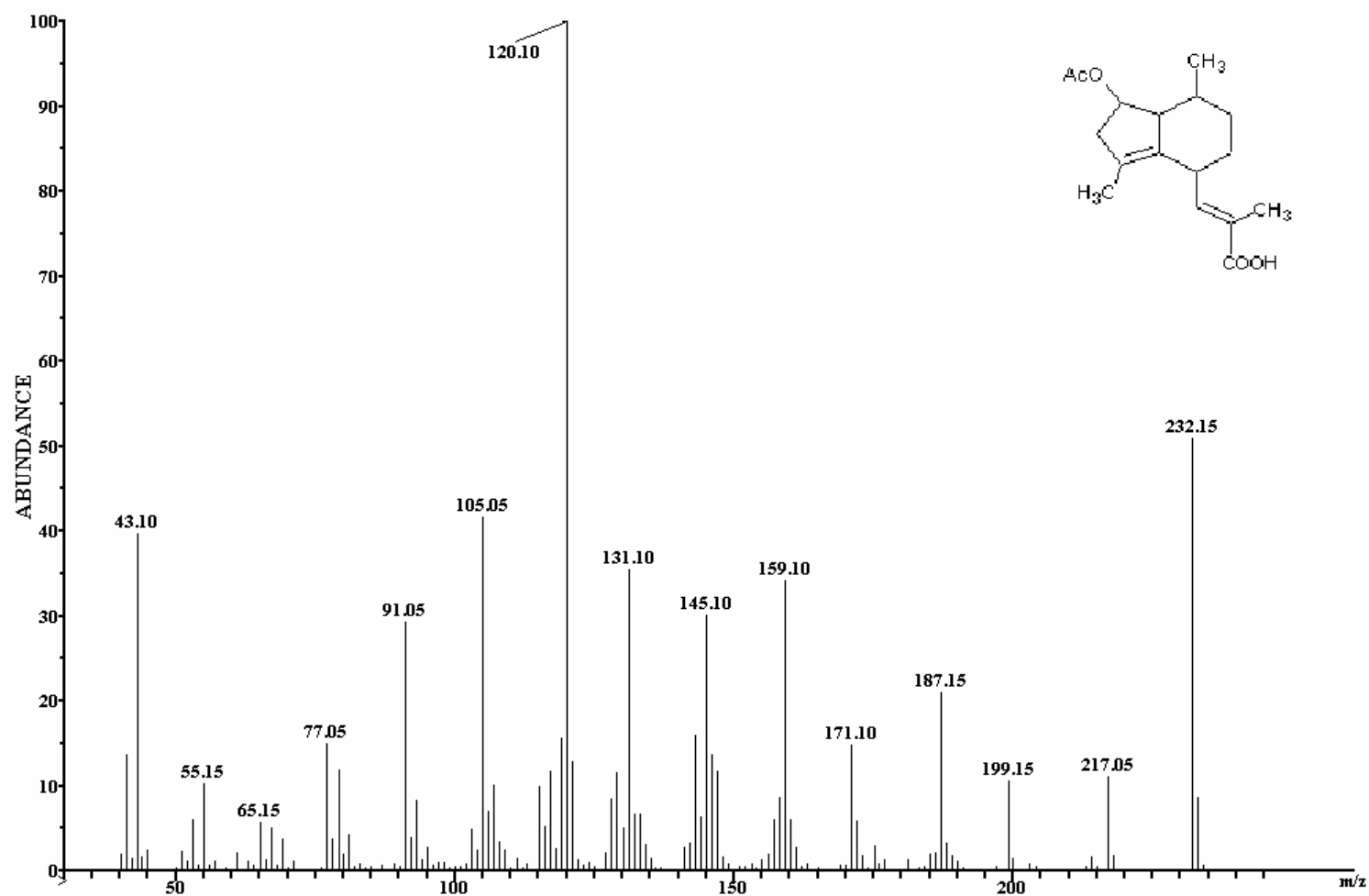
Figure 20 EI mass spectrum of acetoxyvalerenic acid
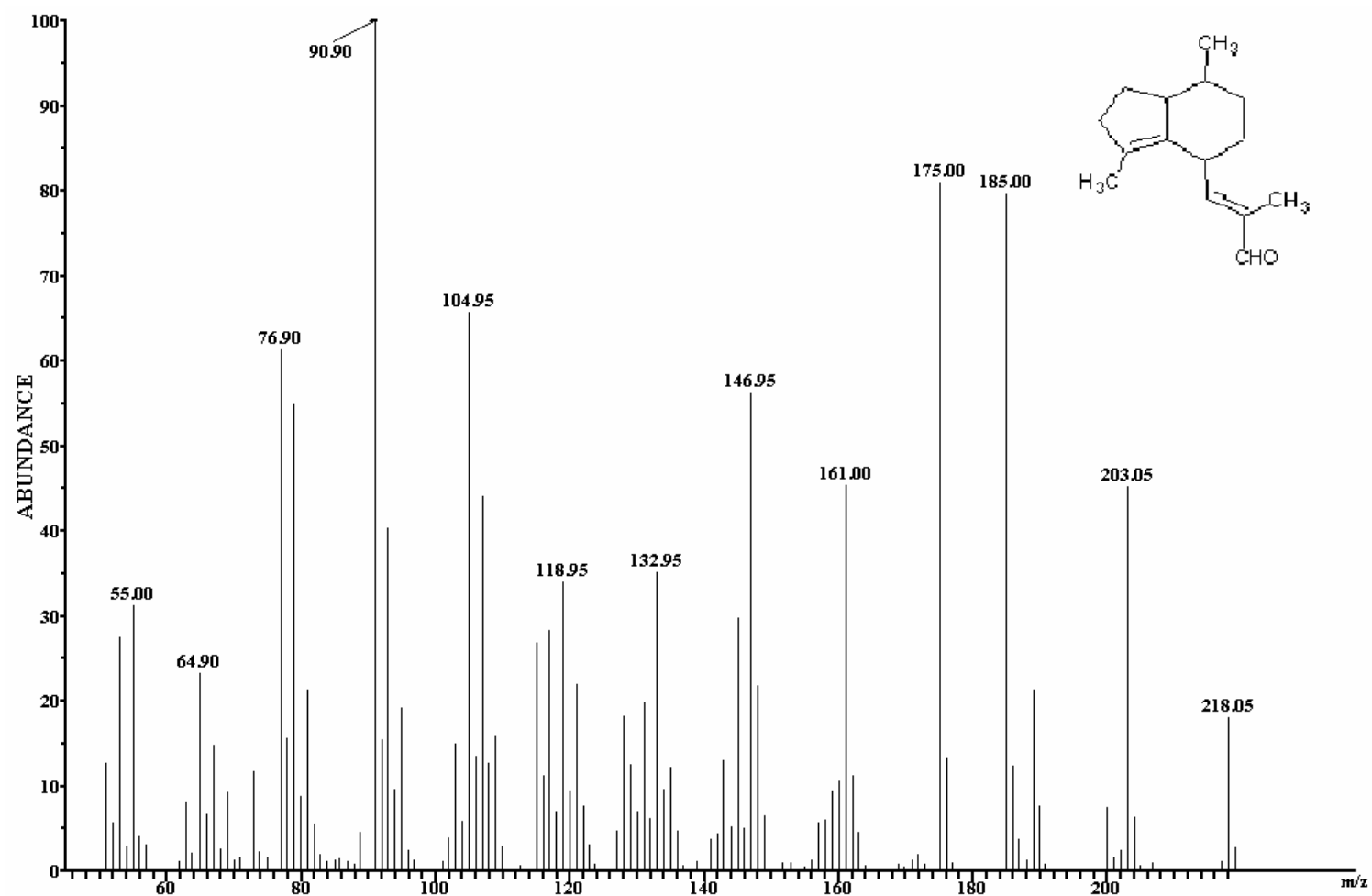
Figure 21 EI mass spectrum of valerenal

• DISCUSSION

One of the major constituents of all *Valeriana* species is the essential oils. Essential oils, also known as volatile oils, are generally complex mixtures of volatile organic compounds that render the characteristic odor and flavor to the plants. Essential oils can be extracted from various plant parts such as leaves, flowers, rhizomes, bark, resin, seeds, fruits or whole plant. In case of valerian, the essential oil is present in the roots and rhizomes of the plant. Previous reports on the chemical analysis of the composition of the volatile oil revealed that it was mainly composed of terpenoids.

The terpenoids, also called isoprenoids, are one of the largest groups of natural products found in nature. Terpenoids are built by the union of two or more five-carbon isoprene units (2-methylbuta-1, 3-diene) (Figure 22)(Ruzicka 1953; Ruzicka 1994). Most natural terpenoids have cyclic structures with one or more functional groups such as hydroxyl or carbonyl groups.
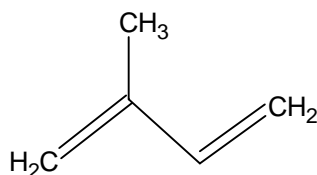
Figure 22 Isoprenoid unit

Previous studies have established the presence of both monoterpenoids and sesquiterpenoids in valerian. The co-occurrence of the three cyclopentane sesquiterpenoids, valerenic acid, acetoxy-valerenic acid, and valerenal, is a characteristic feature of V. officinalis

and distinguishes this species from V. edulis and V. wallichii (Riedel, Hansel et al. 1982). In several studies that have used the bioassay guided assay approach, the dichloromethane extract has been shown to possess biological activity (Dietz, Mahady et al. 2005). Consistent with our findings, these studies have also shown that this extract is mainly composed of valerenic acid, acetoxy valerenic acid and valerenal. These results support the hypothesis that in addition to valerenic acid, acetoxy valerenic acid and valerenal contribute to the inhibition of UGT enzymes. However, the potency of each of these compounds is still unknown as they were not available in the pure form when these experiments were conducted. The similarity in the structures of these compounds suggests that they could also undergo glucuronidation. Further, valerenal is the most abundant of the known sesquiterpenes and the data from this experiment suggests that it could be equally or more potent inhibitor of UGT enzymes. Therefore evaluation of the metabolism of this compound and identifying the enzymes responsible for its metabolism can provide further insight into the interaction potential of valerian preparations. Also, the enzyme catalyzing glucuronidation of vaelerenic acid need to be identified in order to determine if valerenic acid is capable of interacting with the substrates of some of the other UGT enzymes.

4.0   EFFECT OF VALERIAN ON ACETAMINOPHEN PHARMACOKINETICS IN HEALTHY HUMAN VOLUNTEERS

ABSTRACT

Objective: Valerian is a popular herbal product used as a sedative and anxiolytic agent. Most commercially available valerian products are standardized to valerenic acid content. Valerenic acid undergoes glucuronidation in vitro indicating that valerian extracts could impair conjugation of drugs that are metabolized by this pathway. As acetaminophen is known to be extensively glucuronidated, our study was designed to investigate the potential of metabolic interaction between valerian and acetaminophen.

Methods: The effect of valerian on acetaminophen glucuronidation was evaluated in eight healthy volunteers in an open label, two–session fixed sequence design study. The two study sessions were separated by one week. Subjects received 500 mg acetaminophen alone and in combination with 500mg valerian extract on study sessions 1 and 2 respectively. Subjects self administered 500 mg valerian extract at bedtime every night in the week separating the study sessions. Acetaminophen, acetaminophen glucuronide and acetaminophen sulfate were quantified in plasma and urine samples by HPLC with UV detection.

Results: Valerian administration increased the acetaminophen absorption rate constant (Ka) by 30% from 1.66hr-1 to 2.17 hr-1. Acetaminophen maximum plasma concentration (Cmax) was significantly increased (7.9 μg/ml versus 10.6 μg/ml) ($p < 0.05$) and time to reach maximum plasma concentration (tmax) was significantly reduced ($p < 0.05$). Valerian did not significantly alter acetaminophen, acetaminophen glucuronide or acetaminophen sulfate area under the concentration-time curve (AUC) or the terminal elimination half-life (t1/2) of acetaminophen.

Conclusions: These results indicate that valerian is capable of altering absorption rates of drugs. This effect is presumably due to an increase in gastrointestinal motility by the effect of valerian on gastric smooth muscle. However, significant interactions between valerian and drugs undergoing glucuronidation through UGT1A6/9 may not be anticipated.

4.1 INTRODUCTION

Valerian (Valeriana officinalis) has been used since ancient times as a panacea for epileptic seizures, gastrointestinal pain, rheumatism, dysmenorrhoea and low-grade fevers. Ancient Greek physicians Dioscorides and Galen are said to have made use of valerian as an aromatic and diuretic (Upton 1999). Currently, Valerian is among the ten most popular herbs used primarily for its sedative and tranquilizing effects (Morazzoni and Bombardelli 1995; Vickers, Zollman et al. 2001). Results of clinical trials support the assumption that valerian may be effective in treating insomnia (Stevinson and Ernst 2000).

In spite of its wide-spread use, specific scientific data regarding pharmacokinetics of its constituents and drug interaction potential is lacking. Although data from in vitro studies indicated that valerian could inhibit drug metabolizing enzymes CYP3A4 and CYP2C19 (Lefebvre, Foster et al. 2004; Strandell, Neil et al. 2004), results of an in vivo study in humans suggests that valerian does not alter CYP3A4 or CYP2D6 activities (Donovan, DeVane et al. 2004). The constituents of Valerian include valerenic acids, valepotriates, flavonoids and alkaloids(Dewick 2002). Most commercial products are standardized to valerenic acid content as it is believed to be the active constituent of valerian (Hendriks, Bos et al. 1981). As compounds containing carboxylic acid groups often undergo phase II metabolism by glucuronidation pathway, valerenic acid could be conjugated with glucuronic acid thereby interacting with substrates undergoing glucuronidation. Acetaminophen is known to undergo extensive metabolism by glucuronidation and has often been used both in vitro and in vivo as a probe substrate to determine the effects of drugs and endobiotics on glucuronidation. The objective of our investigation was to determine the effect of Valerian administration on the pharmacokinetics of acetaminophen and its glucuronide in healthy volunteers.

4.2   METHODS

4.2.1   Drugs and reagents

Valerian root extract capsules (Pharmassure, Phoenix AZ) and acetaminophen (Tylenol extra-strength, McNeil Consumer & Specilaity Pharmaceuticals, Fort Washington, PA) were purchased locally. Acetaminophen, acetaminophen glucuronide (APAPG) and acetaminophen sulfate (APAPS) for HPLC assay were purchased from Sigma (St Louis, MO). All chemicals were of analytical grade.

4.2.2   Subjects

Eight healthy volunteers (four male, four female) age: 22-40 years; 49-105kg were enrolled in the study. The study was approved by the University of Pittsburgh biomedical institutional review board and was conducted in the General Clinical Research Center of the University of Pittsburgh Medical center. Written informed consent was obtained from all subjects. All subjects underwent a screening history and physical examination to verify health status. Subjects were excluded if they were regular cigarette smokers, used any medications (except oral contraceptives) or herbal products, were pregnant or breastfeeding.

4.2.3   Study Design

This was an open-label, fixed-sequence interaction study. Intake of alcohol and caffeine was not permitted 24 hrs prior and grapefruit juice 48 hrs prior to the study sessions. During study session 1, subjects received 500 mg acetaminophen alone and during study session 2, subjects received 500 mg acetaminophen and 500 mg valerian extract together. Subjects were provided standardized meals during the study sessions. Blood was obtained for determination of concentrations of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate at 0

hours (30 minutes prior to dose) and at 0.25, 0.5, 1, 2, 3, 4, 6 and 8 hours after dose. Urine was collected in intervals of 0-8 hrs and 8-24 hrs. In the week separating the two study sessions, subjects were instructed to self-administer two valerian capsules daily within two hours of bedtime. Subjects were also instructed to keep a diary and note the times at which the dose was taken on each night in order to monitor adherence.

4.2.4 Sample collection and processing

Blood samples (3ml) were placed on ice immediately after collection and centrifuged within 15 minutes. The resultant plasma was stored at -80°C until analyzed. Urine samples were stored refrigerated until the end of the collection interval; after collection, the total volume was measured and aliquots were frozen at -80°C until analyzed.

4.2.5 Assays

Acetaminophen, acetaminophen glucuronide and acetaminophen sulfate were measured in plasma and urine using a modified HPLC method (Chapter 2) with UV detection described previously (Alkharfy and Frye 2001). Briefly, 250μl plasma was treated with 25μl 6% perchloric acid and centrifuged at 14,000g for 5 minutes. A 50μl aliquot of the supernatant was injected into the HPLC system. The HPLC system consisted of Waters model 717 autosampler, model 501 HPLC pump and model 486 ultraviolet detector set at 254 nm (Waters corp., Milford, MA, USA). Detection and quantification were performed using Empower 2 software (Waters, Corp., Milford, MA, USA). Separation was achieved with a Waters Symmetry® C18, 5μ, 150 mm × 3.9 column (Waters corp., Milford, MA, USA) and with isocratic flow of mobile phase composed of 10% methanol and 1% acetic acid in 100 μM phosphate buffer. Acetaminophen and its metabolites were detected at 254 nm. Acetaminophen concentration in plasma was quantitated in duplicate from a standard curve that was linear ($r^2 = 0.995$) over the range of 0.5 to 20 μg/mL,

73

with a intraday and interday and coefficients of variation <10%. Acetaminophen in urine was also assayed by the method described above. Acetaminophen, acetaminophen sulfate, and acetaminophen glucuronide were quantitated from unhydrolyzed urine. 50 µl of the supernatant was injected onto the HPLC column, and the glucuronide, sulfate, and acetaminophen were eluted with retention times of 3.5, 5.8 and 6.7, respectively.

4.2.6   Pharmacokinetic analysis

Acetaminophen concentration-time data for each individual data set was analyzed using WinNonlin® version 4.01 (Pharsight Corporation, Mountain View, CA, USA) and a one-compartment open model with first-order absorption and first-order elimination. This model was chosen based upon the Akaike Information Criterion (AIC) and a visual analysis of the curve fits and residual plots. The primary parameters calculated included the apparent volume of distribution (V_d/F), the absorption rate constant (K_a) and the elimination rate constant (K_{el}). The secondary parameters calculated were the area under the curve from time zero to infinity($AUC_{0-\infty}$), and the half-lives of absorption and elimination ($t_{1/2a}$ and $t_{1/2el}$) respectively. Individual area under the plasma concentration time curves from time 0-8 hrs (AUC_{0-8}) after dose was calculated by the linear-log trapezoidal method. The maximum plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) were determined directly from the observed plasma concentration-time profiles over the 8 hour sampling period. Apparent oral clearance (CL) was calculated as dose/ $AUC_{0-\infty}$. Formation clearance of the metabolites was calculated as

fm * CL_{APAP} where fm = Amount recovered in urine/dose

4.2.7   Statistical Analysis

Results are expressed as mean values ± SD in the text and tables and, for clarity, as mean values ± SEM in the figures. The differences in the pharmacokinetic variables were compared by

use of a paired t test or, in the case of tmax, by the Wilcoxon signed rank test. The differences in the pharmacokinetic parameters were assessed by 90% confidence intervals (CIs) for the geometric means ratio of valerian treatment over control. A lack of interaction was concluded if these 90% CI values fell within the range of 0.8 to 1.25. Statistical analyses were conducted with GraphPad InStat software (GraphPad Software for Science Inc, San Diego, Calif). A P value less than 0.05 was considered to be statistically significant.

4.3   RESULTS

The mean plasma concentration time profiles of acetaminophen, acetaminophen glucuronide and acetaminophen sulfate are shown in Figures 23 and 24. The effects of valerian extracts on peak plasma acetaminophen concentration, time to reach peak plasma concentration, clearance, and elimination $t_{1/2}$ for individual subjects are given in Table 8. Valerian treatment increased maximum plasma acetaminophen concentration by 47% ($P < 0.05$) as shown in Figure 25. This was accompanied by a 10% decrease in time to peak concentration ($P < 0.05$). There was no change in elimination $t_{1/2}$ or clearance caused by valerian.

Acetaminophen and its metabolites were measured in the urine and the formation clearance of the respective metabolites is shown in Table 9. The assumption was that recovery of the acetaminophen dose was complete and unaffected by valerian treatment. Glucuronidation and sulfation were the major routes of acetaminophen elimination.
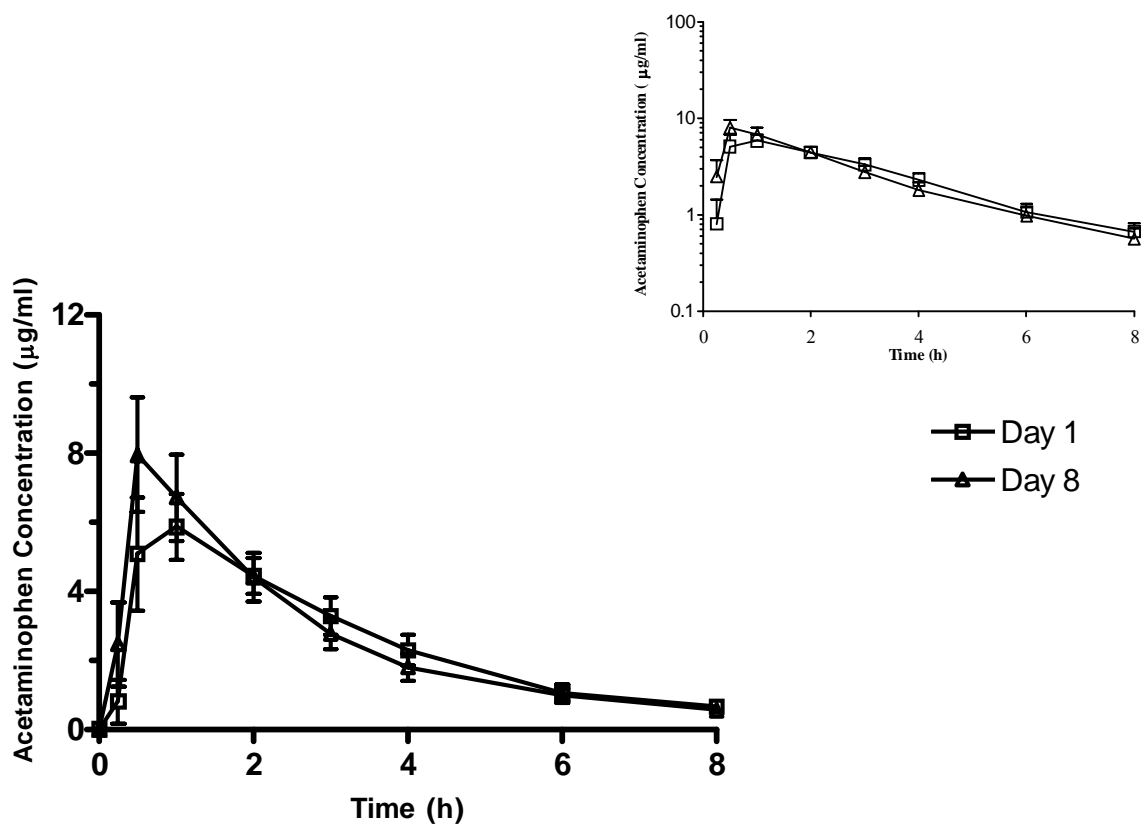
Figure 23 Mean (±SE) plasma acetaminophen concentration time profile with (—△—) and (—□—) without valerian
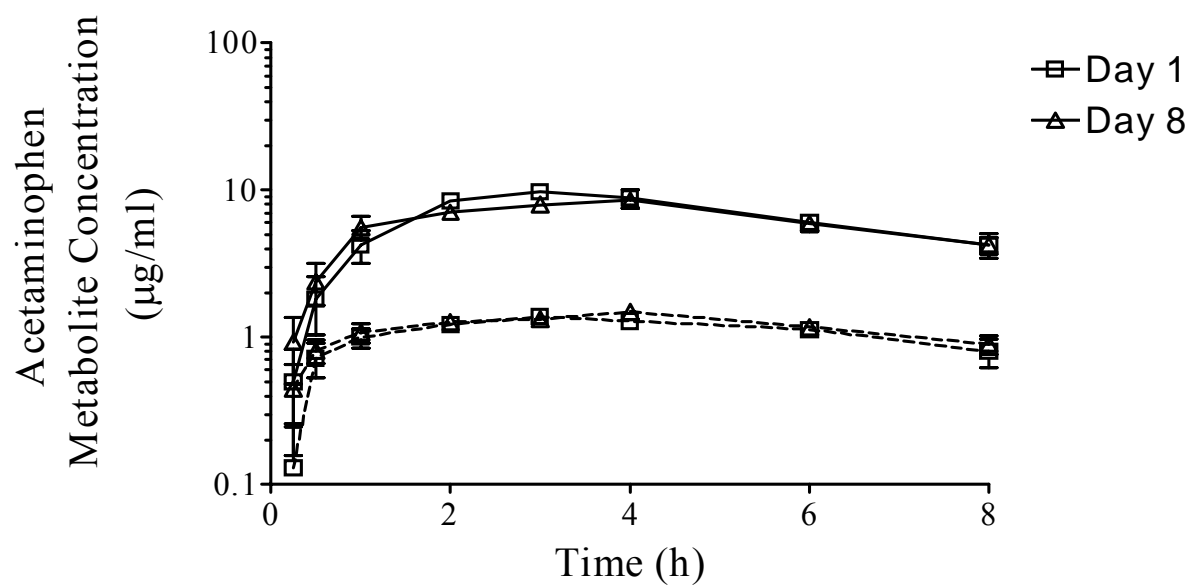
Figure 24 Concentration time profile with and without valerian for acetaminophen glucuronide and acetaminophen sulfate

Table 8 Plasma acetaminophen pharmacokinetic parameter estimates

Parameter	Control	Valerian	Geometric Mean ratio	90% CI	p value
Ka (hr^{-1})	1.66 + 0.807	2.17+ 0.810	1.73	1.62- 1.85	0.0202
Cmax (µg/l)	7.9 + 2.9	10.6 + 2.4	1.25	1.11-1.41	0.0235
Tmax (hr)	0.7 (0.5 - 0.9)*	0.6 (0.6 - 0.7) *	-	-	0.0208
AUC (hr*µg/l)	23.5+ 5.6	25.05 + 4.4	1.14	1.29- 1.00	0.2493
CL (ml/min)	372.8 + 19.9	341.0 + 54.9	0.88	0.77-0.99	0.2672

* tmax given as median and range

Table 9 Pharmacokinetic parameters of metabolites

Parameter	Control	Valerian
AU$_{APAPG}$	58.9 ± 4.9	55.3 ± 13.1
AU$_{APAPS}$	30.9 ± 3.4	32.3 ± 6.2
Cl$_{f\_Gluc}$	203.33 ± 51.5	193.5 ± 83
Cl$_{f\_Sulf}$	67.5 ± 24.7	55.17 ± 16.5

AU Amount recovered in the urine as percent of dose
Cl$_{f\ Gluc}$ Formation clearance of APAPG , Cl$_{f\ Sulf}$ Formation clearance of APAPS

A
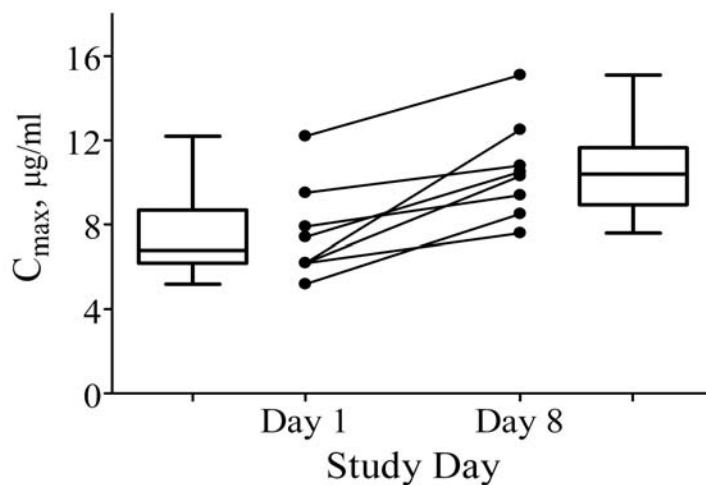
B
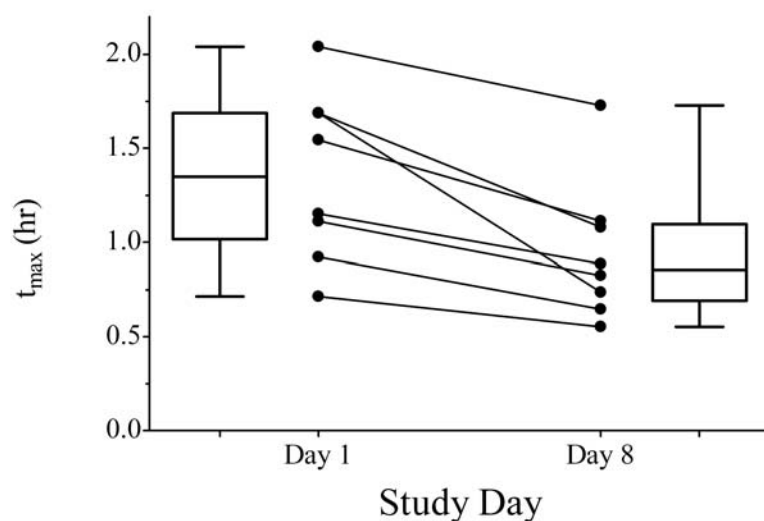
Figure 25   Effect of valerian administration on acetaminophen maximum plasma concentration (C_{max}) and time to reach C_{max} (t_{max})
Box and whisker plots for comparison of (A) C_{max} and (B) t_{max} without and with valerian administration The box represents lower to upper quartiles (25th to 75th percentiles). The line in the box represents the median and the whiskers indicate the extent of the data.

4.4   DISCUSSION

While a significant amount of data has emerged on the modulatory effects of botanicals such as St. John's wort, data on valerian products remain scarce in the medical literature. The few studies that have investigated Valerian are limited to cytochrome P450 enzymes. This is the first study investigating the interaction potential of Valerian by taking into consideration the chemical structure of valerenic acid and its capability to undergo glucuronidation. Our findings suggest that the generally recommended dosage of valerian is unlikely to significantly alter the disposition of coadministered medications primarily metabolized by UGT1A6/9, which are the enzymes responsible for acetaminophen glucuronidation. This conclusion is based on the finding that there were no significant differences in acetaminophen AUC or terminal elimination half-life.

An interesting finding of this study was the significant change in the maximum concentration in plasma and time to reach this maximum concentration. A similar effect was reported by the study investigating the effect of valerian administration on the CYP3A substrate alprazolam in humans. However, the cause of the increased absorption rate of alprazolam after valerian administration remains unknown. Among other effects, ancient medical texts suggest that valerian has a relaxing effect on the gut wall. In addition, valerian has been used in folk medicine to treat gastrointestinal pain and flatulence. An earlier study alluded to antispasmodic activity of valerian on gastric smooth muscle (Wagner and Jurcic 1979). This effect was confirmed in a later study using isolated pig-ileum (Hazelhoff, Malingre et al. 1982). In this study, valerenic acid, valtrate and valeranone were shown to exert papaverine–like spasmolytic effects on the smooth muscle. The use of acetaminophen as a substrate in our study had an added advantage that it has often been used to evaluate gastric emptying motor ability (Clements,

Heading et al. 1978; Harasawa, Tani et al. 1979; Mizuta, Kawazoe et al. 1990; Cavallo-Perin, Aimo et al. 1991; Feng, Li et al. 1996; Nakamura, Takebe et al. 1996; Hasebe and Harasawa 1997; Sanaka, Kuyama et al. 2002; Gandia, Bareille et al. 2003). The increased absorption rate of acetaminophen strongly suggests that valerian increases gastric emptying rate. This effect of valerian could also explain the increase in absorption rate observed with the alprazolam in the previous study. Although pharmacokinetic interactions with valerian can be ruled out based on this study, the change in absorption rate could have implications for effects of drugs acting centrally.

5.0 EVALUATION OF EFFECT OF VALERIAN EXTRACTS ON ACETAMINOPHEN GLUCURONIDATION IN HUMAN HEPATOCYTES

5.1   INTRODUCTION

Herbal products are used for a variety of reasons. Survey reports suggest that most often herbal products are used in treatment of chronic conditions such as insomnia, depression and post menopausal symptoms. A majority of herbal users use them on a daily basis and a significant number use them for a prolonged amount of time, some as long as three years. As with drugs, these patterns of use can affect the mechanism of drug interactions. While a single dose of the product may inhibit the enzyme, chronic use can result in induction of enzymes. A classic example of this phenomenon is HIV protease inhibitor, ritonavir which is both an inhibitor and inducer of CYP3A4 (Kageyama, Namiki et al. 2005). A similar phenomenon is reported for troleandomycin (Luo, Cunningham et al. 2002). Among herbs, hyperforin, a constituent of St. Johns wort is reported to have a similar effect on CYP3A4. Chronic exposure of hyperforin resulted in an increase in the CYP3A4 activity while acute exposure to hyperforin resulted in the inhibition of the enzyme.

Based on our preliminary studies with human liver microsomes, we hypothesized that the mechanism of interaction would be inhibition. However, the results of the clinical study did not support the hypothesis that the pharmacokinetics of acetaminophen would be altered by valerian administration. This could be because of the following reasons

a) The inhibition observed in the microsomal system was dose dependent and the concentration of the extracts was high enough to cause the inhibition, whereas in the clinical study these high concentrations were not achieved in vivo.

b) Administration of valerian for one week induced the UGT enzymes, which offset any potential inhibitory effects. Therefore, the enzyme levels did not change and the pharmacokinetic parameters of acetaminophen remained unaltered.

We hypothesized that compounds in valerian including valerenic acid will have a dual effect on UGT enzymes. Our hypothesis was further supported by the fact that valerenic acid (100 μM) was shown to activate PXR in reporter gene assays (Unpublished data from Dr. Wen Xie's lab through personal correspondence, Center for Pharmacogenetics, University of Pittsburgh, and School of Pharmacy). In order to investigate if this phenomenon occurs, based on the advantages and availability we selected the hepatocyte model. This model system is capable of measuring enzyme induction in addition to enzyme inhibition.

5.1.1 Models for metabolism studies

Various in vitro models have been used to study hepatic metabolism of drug molecules and other xenobiotics. These models have also been used to predict metabolic drug interactions (Ito and Houston 2004). The models are classified mainly into two types, cell-free systems/ homogenates and whole cell preparations. The cell–free systems include liver microsomes, S9 fractions and purified or heterologously expressed enzyme systems. Human liver microsomes are vesicles of endoplasmic reticulum obtained by differential centrifugation of cell homogenate. Owing to their ease of preparation, commercial availability, and long-term stability, these systems are most commonly used to generate preliminary information on the effect of drugs or xenobiotics on specific CYP or UGT enzymes and to screen for potential drug-drug interactions.

Moreover, their adaptability to high throughput techniques has led to their widespread use in metabolism studies. The cell free systems require supplementation of exogenous cofactors, such as a source of NADPH for CYP enzymes or conjugating moieties for phase II enzymes. These systems are not appropriate to study sequential metabolic reactions (i.e., the coupling of phase I and II reactions) due to the disruption of cellular integrity. The limitation of cell free systems is their suitability only for enzyme inhibition studies.

The whole cell systems used in drug metabolism studies are liver slices and intact hepatocytes. In these systems, gene expression, metabolic pathways, cofactors/enzymes and plasma membrane are largely preserved. Because of these characteristics, they serve as excellent models to test effects of drugs on enzyme induction and transport. Liver slices and hepatocytes are also suitable models to study sequential and/or parallel oxidative and conjugative biotransformation (Beamand, Price et al. 1993; Lake, Beamand et al. 1993). Although liver slices are relatively simple to use and their viability is longer than microsomes, they are not viable for enough time to study induction of enzymes (Berthou, Ratanasavanh et al. 1989). In this regard, hepatocyte cultures are viable for longer periods of time compared to liver slices. Hepatocyte cultures used in metabolism studies require the first 24 hours for adaptation of cells to culture conditions, thereafter remaining quite stable for a few days. In many respects they are the closest systems to in vivo models and have all the advantages of whole cell preparations.

5.1.2   Regulation of UGTs

One of the principal mechanisms of increased enzyme activity is by up regulation of gene expression. The expression of genes for drug metabolizing enzymes is controlled by several factors. Most of these factors regulate the gene expression at the mRNA transcription stage and to a smaller extent, mRNA splicing and translation stages. The genes contain specific regulatory

sequences called promoters that determine both the basal transcription of the gene and its response to specific stimuli. Regulatory proteins known as transcription factors bind to the gene promoter and activate or inhibit transcription. Transcription factors such as Hepatocyte Nuclear Factor 1 (HNF1), CAAT-Enhancer Binding Protein, Octamer transcription Factor 1 and Pre-B-cell leukemia transcription factor-2 (Pbx2), appear to control the constitutive levels of UGTs in tissues and organs (Mackenzie, Gregory et al. 2003). Activation of transcription factors occurs as a direct result of ligand binding or as an indirect result of phosphorylation or dephosphorylation of one or more transcription factors (Treisman 1996). Members of nuclear receptor family such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) represent the ligand-activated transcription factors (Mangelsdorf, Thummel et al. 1995). The nuclear receptors involved in regulation of phase I and phase II enzymes and their activators are shown in Table 10. Generally, the transcriptional regulation via CAR and PXR involves the formation of heterodimers between these receptors and retinoid X receptor (RXR) followed by binding of the heterodimer to response elements in the regulatory region of target genes, and transactivation of gene expression. This paradigm of the mechanism of induction by ligand-activated gene expression is represented in Figure 26. The example shows the activation of the aryl hydrocarbon receptor (AhR) on binding with an agonist, followed by dimerization of AhR with the Ah receptor nuclear translocator (Arnt) and translocation into the nucleus where the AhR / Arnt heterodimer binds to the dioxin-responsive enhancer (DRE) resulting in transcription of the gene (Ma 2001). Early studies showed that UGT enzymes were inducible by phenobarbital, and phenytoin (Brierley, Senafi et al. 1996). In the past several years, the nuclear receptor PXR has been implicated as a key mediator in regulation of cytochromes P450 as well as UGTs in many species (Bertilsson, Heidrich et al. 1998;

Blumberg and Evans 1998; Kliewer, Moore et al. 1998; Xie, Barwick et al. 2000). There is evidence from transgenic mouse studies that UGT1A1 and UGT1A6 are regulated by PXR and CAR (Xie, Yeuh et al. 2003). In the same study, treatment with prototypical inducers, rifampin and clotrimazole caused significant activation of the UGT reporter genes by the human PXR, further establishing the role of these nuclear receptors in the regulation of UGT enzymes. Recent studies have demonstrated that in addition to PXR and CAR, AhR also plays a regulatory role in the induction of UGT1A1 by characterization of a phenobarbital (PB) response enhancer module in the gene promoter region (Sugatani, Kojima et al. 2001).

Table 10   Nuclear receptors and their activators

	Nuclear Receptor	Activators
	AhR	Arochlor 1254 Dioxin 3-methylcholanthrene
ORPHAN RECEPTORS	PXR	Dexamethasone, Rifampin, Phenobarbital, Hyperforin, Bile acid
	CAR	Androgens, Rifampin, Phenobarbital
	PPAR γ	Fatty acids, Prostaglandins Leukotrienes
	FXR	Bile acids
	LXR	Oxysterols
ENDOCRINE RECEPTORS	VDR	Lithocholic acid
	GR	Cortisol
	RAR	All-*trans*-retinoic acid
	MR	Aldosterone
	ER	17 β estradiol
	PR	Progesterone
	AR	Oxysterols

Adpated from Carlberg C. Principles of gene regulation by bioactive lipids through members of the nuclear receptor superfamily. *Mechanisms of Signal Transduction and Inducible Gene Expression,* 2004: 77-95 (Key : AhR Aryl hydrocarbon receptor, PXR pregnane X receptor, CAR constitutive androstane receptor, PPAR γ Peroxisome proliferator-activated receptor Gamma,   FXR Farnesoid X-receptor, LXR Liver X receptor, VDR Vitamin D Receptor , GR Glucocorticoid Receptor, RAR retinoic acid receptor , MR Mineralcorticoid Receptor, ER Estrogen receptor, PR Progesterone receptor, AR Androgen Receptor)
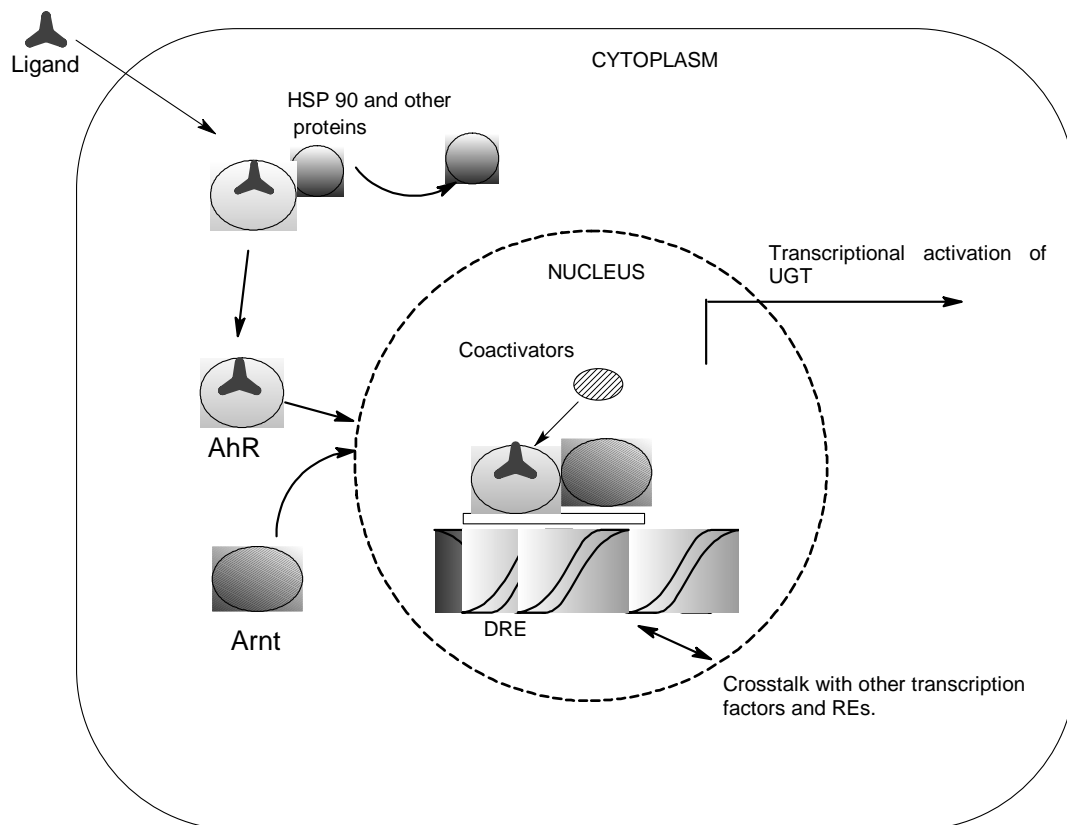
Figure 26 Mechanism of action of nuclear receptors.

In the absence of a ligand, the receptor is usually is in the cytoplasm due to their association with a large multiprotein complex of chaperones, such as Hsp90. Ligand binding induces dissociation of the complex and nuclear translocation. Once in the nucleus, the receptors regulate transcription by binding, generally as dimers, to drug response elements (DREs) normally located in regulatory regions of target genes.

5.1.3   Real-time PCR

Quantification of gene expression has been traditionally performed by measuring mRNA levels by northern blotting, in situ hybridization and RNAse protection assays, and reverse transcription polymerase chain reaction (RT-PCR) (Wang, Doyle et al. 1989; Hod 1992; Saccomanno, Bordonaro et al. 1992; Parker and Barnes 1999) (Weis, Tan et al. 1992). These methods are often laborious and time-consuming. Moreover, they require post-PCR processing such as running gels or transfer to membranes and results are often semi-quantitative (Sharp, Berk et al. 1980; Thomas 1980). The real-time PCR technique is simple, fast, precise, accurate and reproducible for quantitative determination of the mRNA (Higuchi, Fockler et al. 1993; Gibson, Heid et al. 1996; Heid, Stevens et al. 1996). The real-time PCR technique is based on fluorescence detection during the entire PCR amplification process. A dually labeled fluorescent probe hybridizes to the template in each cycle and an amplification plot of fluorescence signal versus cycle number is generated. The initial cycles of PCR, when there is little or no change in fluorescence signal is defined as the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold is set abovethe baseline and the parameter threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Instead of obtaining concentrations at the end of the reaction as is the case with most conventional methods, quantification of DNA in real time-PCR is based on measurements obtained during the early exponential phase. During this phase, the amount of the amplified product is proportional to the concentration of the template versus CTs for a set of standards. Concentrations of DNA of the unknown samples are determined from the standard curve using the CTs of the samples.

5.2 EXPERIMENTAL

5.2.1 Chemicals

Williams' E culture medium and medium supplements, dexamethasone and insulin, were obtained from Cambrex BioScience Walkersville, Inc. (Walkersville, MD). Penicillin G/streptomycin was obtained from Invitrogen (Carlsbad, CA). Phenobarbital (PB), dexamethasone (DEX), acetaminophen and acetaminophen glucuronide were obtained from Sigma (St. Louis, MO). Reagents for reverse transcription were purchased from Promega (Madison, WI). Forward and reverse primers for β-actin were synthesized by Applied Biosystems (Foster City, CA). Universal Taqman Master Mix® and TaqMan® Gene Expression Assay system (assay ID : Hs01592477_m1)for UGT1A6 and (assay ID: Hs01592475_m1)for UGT1A9 were purchased from Applied Biosystems. All solvents and other chemicals used were of HPLC grade or the highest purity available. Falcon culture dishes (100 mm and six well plates were purchased from Becton Labware (Franklin Lakes, NJ)

5.2.2 Isolation and culturing of primary human hepatocytes

Human hepatocytes are isolated by the three step collagenase perfusion technique. (Strom, Pisarov et al. 1996). Briefly, the liver was perfused with a buffered isotonic salt solution to eliminate blood cells and other components, then with collagen/dispase medium to digest the liver into individual cells or small clusters, and finally with a culture medium containing collagen type I to reorganize the multicellular architecture. The perfusion solutions and liver tissue were maintained at 37°C during perfusion. The digested tissue was collected and filtered. The hepatocytes were then purified by washing and the cell number estimated. Viability of the isolated cells was determined prior to plating by the trypan blue exclusion test. Acceptable

viability was 70% or higher. The hepatocytes (1.5×10^6) were plated on six-well culture plates previously coated with rat-tail collagen. The hepatocytes were plated in Williams E medium supplemented with 0.1 μM dexamethasone, 0.1 μM insulin, 0.05% gentamicin, and 10% bovine calf serum. Cells were allowed to attach for 4h. Culture medium was replaced with medium containing all the above mentioned supplements except bovine calf serum. The hepatocyte cultures were maintained at 37°C in a humid atmosphere containing 5% CO_2 in air until harvest. The cells were washed and medium replaced every 24 h. For the acute study, valerenic acid (50μM), ethanol (120μg), dichloromethane (40μg) and ethyl acetate (15μg) extracts in dimethyl sulfoxide (DMSO; 0.1% final concentration in medium) was added to the hepatocytes at 95 h of culture. For the chronic study, valerenic acid and the extracts were added to the culture at 48 and 72 h after plating. Donor information for the hepatocytes used in this experiment is described in Table 11

Table 11 Donor information for human hepatocyte preparations used

Donor HH #	Age	Sex [a]	Race [b]	Cause of death [c]	Drug History	Viability
1118	74y	F	C	HT/MVA	Hydrochlorothiazide, paxil, lasix, atenolol, imipramine, enalaprilat, pepcid, aspirin, calcium	76
1119	29y	F	C	-	None reported	75
1121	65y	F	C	ICH	None reported	78
1122	46y	F	C	HT	alprazolam, capoxone, verapamil, levothyroxine, modafanil, propranolol, triamterine,	73
1124	70	M	H	ICH	Amitriptyline, nifedipine	70
1215	36	M	AI	CA	None reported	82
1217	60	M	C	CA	None reported	81
1218	50	F	C	Anoxia	None reported	85

[a] M, male; F, female; [b] C, Caucasian; H, Hispanic, AI American Indian; [c] CA, cardiac arrest; HT, head trauma; ICH, intra cranial hemorrhage.

5.2.3 Evaluation of cytotoxicity

Hepatocytes were exposed to 50μM troglitazone (positive control), 50 and 100μM valerenic acid and 10μg/ml of valerian extracts for 48 h. Media was aspirated and 2ml of 0. 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to Williams' E medium at 96 h of culture and incubated for 30 min. At 30 min, the medium was aspirated and cells washed with Williams' E culture medium. Isopropanol (same volume as the medium) was then added to each well and shaken gently for 2 minutes. The isopropanol solution (200μl) was transferred to a 96-well plate, and the absorbance was measured at 490 nm.

5.2.4   Evaluation of activity

Two sets of experiments were performed, each set composed of hepatocyte cultures from three different human donors. One set of experiments were performed to evaluate the effect of acute exposure to valerenic acid and valerian extracts on UGT activity. For these experiments, the hepatocytes were cultured for 3 days with daily medium change. On day 4 (after 95 hours in culture), cells were exposed to HMM (negative control) or media containing PB (2mM; positive control), valerenic acid (50μM), ethanol (120μg), dichloromethane(40μg) or ethyl acetate(15μg) extracts in DMSO (0.1% final concentration) for 1 hour. At 96 hours, the hepatocytes were washed with fresh medium devoid of any supplements and incubated at 37°C with fresh medium for an hour. The hepatocytes were then exposed to a medium containing acetaminophen (5 mM) in water for 1 hr. This concentration of acetaminophen was based on previous studies where maximum formation of metabolite was observed at 5mM. The formation of APAPG was shown to be linear up to 120 min and for these experiments APAP was incubated for 60min. At the end of this time period the reaction was terminated by adding 125μl of 70% perchloric acid and the entire cellular components and medium were harvested. The sample was centrifuged at 2000 g for 5 min, and the supernatant was transferred into new vial and stored at -20°C until analysis. Phosphate buffer (250μl) was added to the residue containing the cellular components and stored at -80°C. Another set of experiments were performed to evaluate the effect of chronic exposure to valerenic acid and valerian extracts. For these experiments, cells were exposed to HMM (negative control) or media containing PB (2mM; positive control) in addition to valerenic acid and valerian extracts as described for acute exposure, for 3 days after plating, with media change daily. On day 4, cells were washed and incubated for 1 hour with media, followed by incubation

with APAP for 1 hour to evaluate UGT activity. Media and cells were harvested as described before.

5.2.5   Estimation of metabolite formation

The medium stored at -20°C was thawed and 75μl of the sample was injected onto Symmetry C18 column with a mobile phase of MeOH: Acetic acid (10:1) at a flow rate of 1 ml/min. APAPG was detected using UV absorbance at 254 nm. The concentration of the metabolite was quantitated by comparing the peak areas in samples to a standard curve containing known amount of the metabolite. Metabolite formation rate was calculated from the amount of APAPG in mg/ min.

5.2.6   Total protein estimation

The cells stored at -80°C were thawed and protein concentration was estimated by Lowry's method. Briefly the proteins were dissolved in 0.1M sodium hydroxide and mixed with 1ml 2 % sodium tartarate and 1ml 1% copper sulfate. Folin-Ciocalteu reagent was added to this mixture and the tubes were mixed gently. Color was allowed to develop for 60 min. At the end of 60 min. the solutions were transferred to 96 well plates and absorbance measured at 490 nm. The concentration of the protein was calculated from a standard curve prepared with bovine serum albumin (BSA) as a protein standard.

5.2.7   Evaluation of mRNA expression

Hepatocyte treatments were the same as for chronic exposure. After 96 hours in culture, total RNA was extracted from the cells using 1 mL Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The integrity of the isolated RNA was assessed by agarose gel electrophoresis and RNA concentration was measured by spectrophotometry.

Quantification of mRNA levels was performed using a quantitative RT-PCR technique. Reverse transcription of mRNA to cDNA was performed by a three step procedure. As total RNA isolated using routine methodologies are frequently contaminated with DNA, RNA was first treated with RNase-free DNase (Promega, Madison, WI) to remove genomic DNA contamination. Next, the synthesis of cDNA was primed by combining 2 µg of RNA with 0.5 µg of random hexamers (Promega) and incubating the samples at 70°C for 5 minutes. Samples were cooled to 4°C and the final step was the addition of reverse transcription mixture containing the reverse transcriptase, dNTPs and RNasin (Promega) to the previous mixture and incubation at 37°C for 60 minutes. The resulting cDNA was diluted 10-fold and stored at -20°C. Real time PCR was performed for quantification of UGT1A6 and UGT1A9 mRNA by Taqman® PCR reactions using the Perkin Elmer 7700 Sequence Detection System®. For an internal control, β-actin mRNA was measured in each sample using predeveloped TaqMan assay reagents for human β-actin (Applied Biosystems). Each 20 µl total volume PCR reaction contained 12.5 µl Universal Taqman Master Mix®, 1.25 µl of TaqMan® Gene Expression Assay system (Hs01592477_m1) for UGT1A6 and (Hs01592475_m1) for UGT1A9 and 5 µl of cDNA generated or standard. The following conditions were used for the Taqman® PCR reactions: 2 minute hold at 50°C, 10 minute hold at 95°C, 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The 50 cycles were followed by a 4°C hold. . A standard curve was generated from a cDNA dilution series ranging from 0.01 to 1ng. Values for mRNA levels of UGT 1A6 and UGT1A9 in each experimental sample were extrapolated from the standard curve, normalized to β-actin mRNA and expressed as fold change compared to DMSO control.

5.2.8   Data Analysis

Statistical analysis was performed using the PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Results are expressed as means ± standard deviation from at least three independent experiments. Statistical significance of the difference between the control and the test groups were analyzed using a one-way analysis of variance with a post hoc Dunnett's procedure. A p value of ≤ 0.05 was considered statistically significant.

5.3   RESULTS

5.3.1   Cytotoxicity of valerenic acid and extracts of valerian

Hepatocytes (HH118, HH1122, HH1124) were exposed to valerenic acid (50μM), ethanol (120μg), dichloromethane (40μg) and ethyl acetate (15μg) extracts of valerian dissolved in DMSO. Cytotoxicity was compared to untreated hepatocytes and troglitazone 50μM as positive control. The direct reduction of MTT tetrazolium salt as a measure of decreased mitochondrial activity, by the test agents was determined by spectrophotometry. Valerenic acid as well as the extracts at the concentrations used showed no reduction in mitochondrial activity as shown in the Figure 27 indicating that the compounds at the concentrations tested were devoid of cellular toxicity.

5.3.2   Effect of Acute exposure of valerenic acid and valerian extracts on UGT activity

The formation of acetaminophen glucuronide was determined after acute exposure to valerenic acid and valerian extracts in three cultures (HH1118, HH1121 and HH1122). For each treatment, the activity relative to the DMSO control was calculated. The acetaminophen

formation rate in control hepatocytes ranged from 112 to 182 pmol/min/mg protein. In contrast to results from microsomal studies, acute exposure to the alcoholic extract did not cause biologically significant inhibition of UGT activity. However, significant inhibition of UGT activity was observed with both concentrations of valerenic acid as well as the dichloromethane and ethyl acetate extracts tested. With both concentrations of valerenic acid 50μM and 100μM, APAP-G formation rates were inhibited to 64% ± 4.8 % and 46 % ± 6.7% of DMSO control, respectively. The Valerian alcoholic, dichloromethane and ethyl acetate extracts inhibited APAPG formation by 71 ± 16 %, 46± 4.2% and 38±5.4% respectively (Figure 28A).

5.3.3   Effect of Chronic treatment of valerenic acid and valerian extracts on UGT activity

The induction potential of valerenic acid and valerian extracts was tested by the chronic exposure experiments. Induction potential was compared to potent human UGT inducer, PB. PB was consistently a more potent inducer than valerenic acid and valerian extracts. In the three cultures tested (HH1119, HH1121 and HH1122), PB induced UGT activity to 2.8, 2.6 and 1.8 fold over DMSO control. All three valerian extracts also significantly induced UGT activity with the increased activity levels with the ethyl acetate extract approaching that of PB in all three cultures. The induction levels were 1.6, 2.0 and 2.2 fold that of DMSO control for the alcohol extract, dichloromethane extract and ethyl acetate extract respectively (Figure 28B).

5.3.4   Effect of valerenic acid and valerian extracts on UGT mRNA expression

Estimation of mRNA levels of UGT1A6 and UGT1A9 in three human hepatocytes cultures (HH1215, HH1217 and HH1218) was carried out by quantitative RT-PCR. Treatment with PB increased the mRNA levels of UGT1A6 by 4.3, 7.9and 8.9 folds over the DMSO control and UGT1A9 levels by 55, 47 and 85 fold over control in the three cultures, respectively. Valerenic acid (50 μM), alcoholic extract, the dichloromethane and the ethyl acetate extracts

increased UGT1A6 mRNA levels by 4.0 ± 0.8 , 2.4 ± 1.1, 5.1 ± 0.8 and 6.1 ± 1.3 (mean $\pm$ sd)

fold over control (Figure 29A). Valerenic acid (50 μM) and the alcoholic extract did not cause a

statistically significant increase in UGT1A9 mRNA. The valerian extracts increased UGT1A9

mRNA levels by 11 ± 7.3, 21 ± 12.7 and 34 ± 17.7 fold over control (Figure 29B).
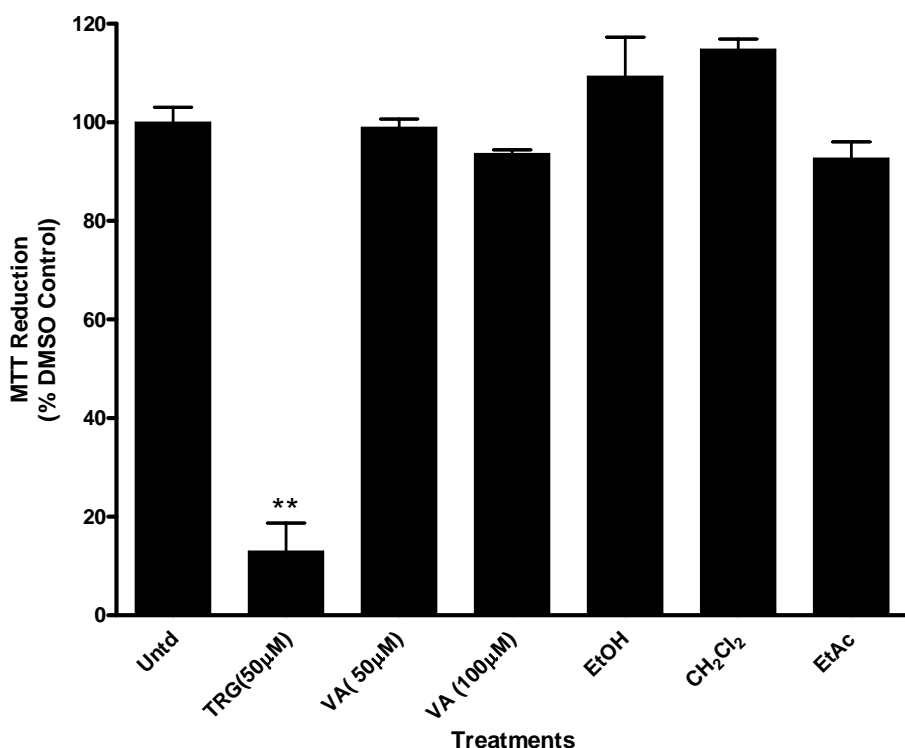
Figure 27 Effect of valerenic acid and valerian extracts on MTT reduction.

Hepatocytes were treated valerenic acid, and valerian extracts and compared with troglitazone treatment. MTT reduction was then measured. The figure shows the mean of triplicate treatments in three donors (HH118, HH1122, HH1124) and are expressed as a percentage of the value in DMSO treated cells, with the S.D. indicated by the vertical bars. ** significantly different from DMSO treated cells, $p \leq 0.01$.

A
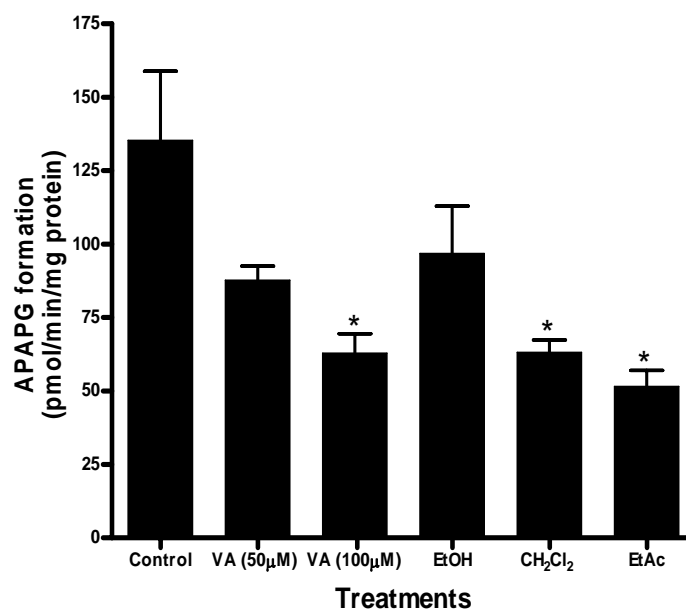
B
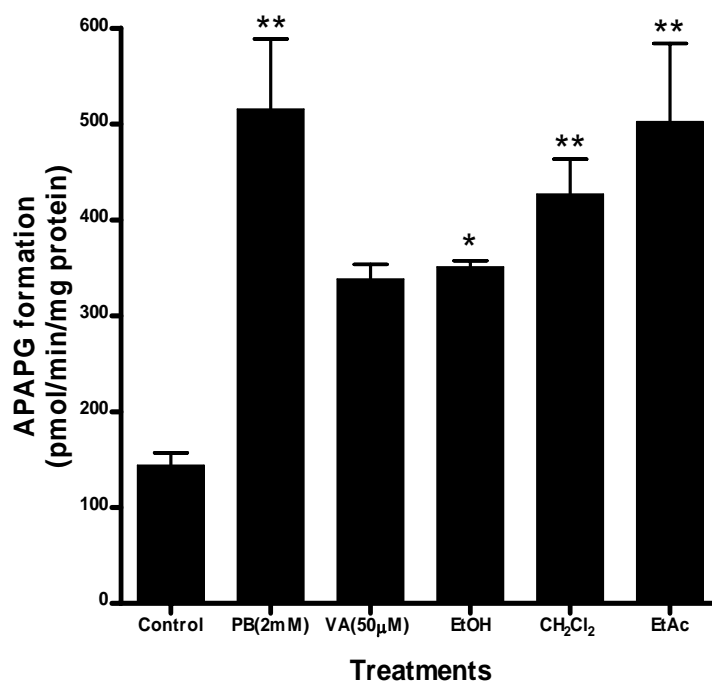
Figure 28   Effect of acute (A) and chronic (B) treatment of valerenic acid and valerian extracts on APAP glucuronidation

Hepatocytes were treated with valerenic acid and valerian extracts A. acute (1 hr exposure,) and B. chronic (72 hrs exposure, HH1118, HH1121 and HH1122). The figure shows the mean of triplicate treatments, with the S.D. indicated by the vertical bars. * indicates p≤0.05 ** indicates p≤0.01 compared to DMSO control.

101

A
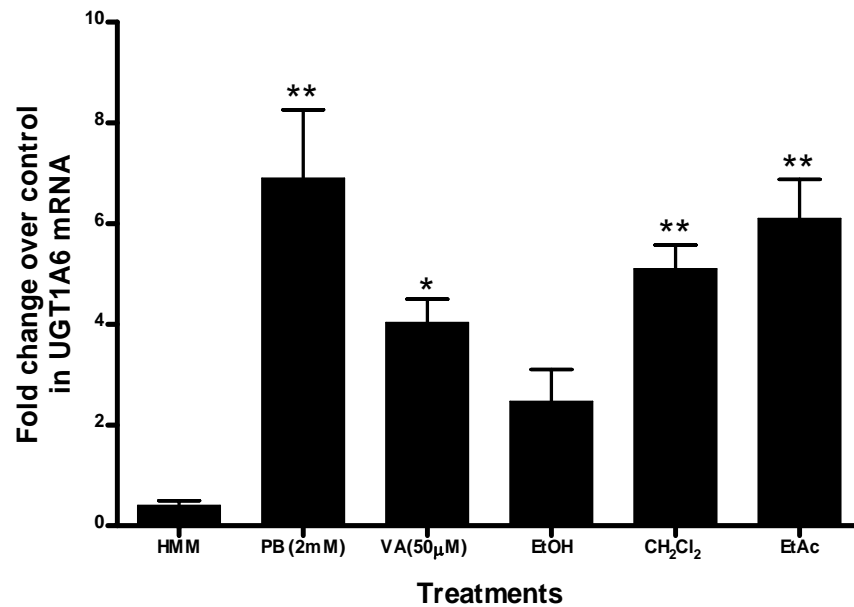
B
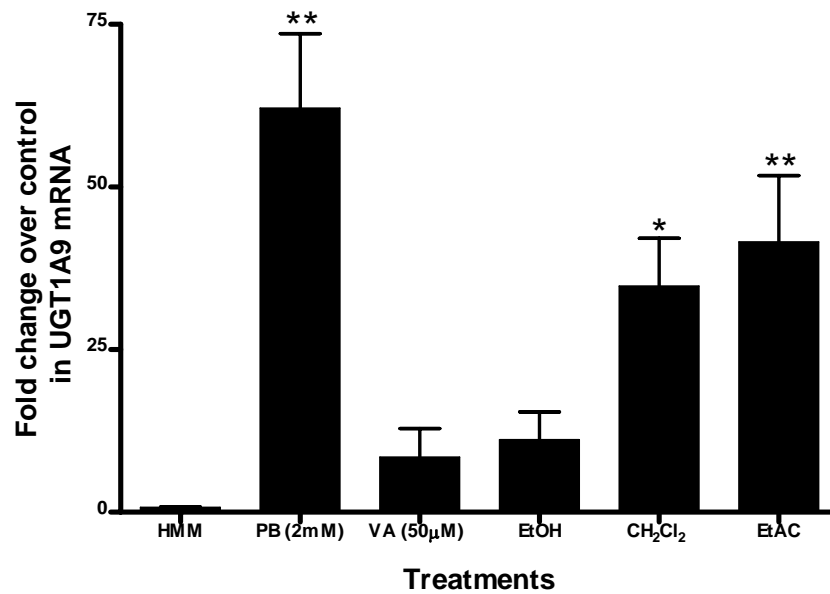
Figure 29 UGT1A6 (A) and UGT1A9 (B) mRNA expression after chronic treatment with valerenic acid and valerian extracts.
Hepatocytes were treated with valerenic acid and valerian extracts for 72 hrs
A) UGT1A6 mRNA expression, B) UGT1A9 mRNA expression. The figure shows the mean of triplicate treatments, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. * indicates p≤0.05 ** indicates p≤0.01

5.4 DISCUSSION

The prediction of interactions between herbs and drugs is extremely important, given the increasing popularity of alternative medicines and the likelihood of co-administration of conventional drugs and herbal products. *In vitro* studies are therefore important tools to determine the interaction potential of different herbal components with conventional drugs through the modulation of drug metabolizing enzymes. Most natural products are complex mixtures of several different chemical compounds and commercial products are usually extracts of herbs rather than single components. In view of the multiplicity of various herbal components in the herbal preparations, herb-drug interactions may result from any number of herbal components. Therefore, predicting interactions with herbs is complicated. In general, it has become standard practice to study the inhibition potential of herbal compounds on major drug-metabolizing enzymes. To date, all studies investigating the effects of valerian on drugs have been performed in cell-free systems such as expressed enzymes or human liver microsomes (Lefebvre, Foster et al. 2004; Strandell, Neil et al. 2004). Since these models lack cellular integrity, they are incapable of predicting effects of the herbs on gene expression.

Recent studies with natural products have clearly shown the utility of studying both inhibition and induction. For example studies in hepatocytes have shown the dual effect of St. John's Wort constituent hyperforin on CYP3A4 enzyme (Komoroski, Zhang et al. 2004). In that study, hyperforin was shown to induce CYP3A4 on chronic treatment and inhibit the enzyme on acute treatment. A similar study employed primary cultures of hepatocytes to study the effects of drugs as well as natural products on CYP3A expression (Raucy 2003). Another study utilized human hepatocyte cultures to show that bergamottin, a grapefruit juice component, causes inhibition of P450s activities while increasing P450 proteins and mRNA levels (Wen, Sahi et al.

2002). These studies clearly demonstrate that hepatocytes are excellent models to study enzyme induction as well as inhibition by both therapeutic agents and natural products.

Compounds in botanicals have been shown to alter the expression of drug metabolizing enzymes through various nuclear receptors. Terpenoids have been used as chemopreventive agents and monoterpenes such as limonene have been shown to induce drug metabolizing enzymes in animal studies (Maltzman, Christou et al. 1991). Other phenolic compounds such as flavonoids have also been shown to induce both phase I and phase II enzymes. Table 12 lists the various herbs with the respective chemical component and the nuclear receptors they are known to bind, suggesting that these herbs are capable of modulating the expression of genes regulated by the specific nuclear receptor.

In our study, treatments with valerenic acid at 50μM concentration did not cause significant inhibition on acute treatment or induction on chronic treatment. Similar effects were observed with the alcoholic extract on acute treatment. However, the dichloromethane extract and ethyl acetate extracts, which are devoid of the aqueous components, showed significant inhibition as well as induction effects.

Effects on gene expression after chronic treatments were evaluated by measuring mRNA levels of UGT1A6 and UGT1A9 in cells treated with valerian acid and valerian extracts. For UGT1A6, valerenic acid and dichloromethane and ethyl acetate extracts caused a significant increase in mRNA levels. Similarly, the dichloromethane and ethyl acetate extracts caused a significant increase in UGT1A9 mRNA levels, while valerenic acid and the alcoholic extract did not alter the mRNA levels.

Table 12 Compounds in herbal products and nuclear receptors they bind

Herb	Compound	Receptor
Rhei rhizoma	Lindleyin	ER
Ginseng	Ginsenoside-Rg1	ER
Grapeseed/red wine	Resveratrol	ER
Scutellaria baicalensis	Baicalein	AR
Dioscorea villosa	Diosgenin	PR
Longmu Zhuanggu Chongji	Vitamin D2	VDR
Xiao Chai Hu Tang	Retinoic acid	RAR
Guggul tree resin	Guggulsterone	FXR
Pseudolarix kaempferi	Pseudolaric acid B	PPARα
Hypericum perforatum	Hyperforin	PXR
Artemisia capillaris	Dimethylesculetin	CAR
Soy	Genistein	ER, AR, PR
Labiatae	Isoprenoids	PPARα/γ

Modified from Lazar, M A, East meets West: an herbal tea finds a receptor, *Journal of Clinical Investigation*, 2004, 113(1):23-25

These results indicate that the sesquiterpenes in the dichloromethane and ethyl acetate extracts of valerian are mild inducers of UGTs perhaps by activation of nuclear receptors PXR and CAR. The results of the hepatocyte experiments support our hypothesis that the induction of enzymes after chronic treatment is compensated by inhibition on acute treatment. These results provide the reason for our observations in the *in vivo* study demonstrating that hepatocytes are a better model system than human liver microsomes for investigating drug interactions.

6.0   CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this research was to investigate the potential of valerian to interact with drugs undergoing metabolism by phase II pathway via glucuronidation. In order to achieve this goal various approaches were utilized including isolated subcellular preparation, whole cell preparation and healthy human volunteers. Acetaminophen was used as a marker substrate to study glucuronidation since it undergoes extensive metabolism by this pathway. In order to measure the levels of acetaminophen and its metabolites in plasma an HPLC assay was developed and validated. A bioassay-guided fractionation method using human liver microsomes was adopted to isolate the potential constituents that could inhibit glucuronidation. This study was followed by an *in vivo* investigation of the effect of valerian preparations on acetaminophen pharmacokinetics in healthy human volunteers. In order to unravel the findings of the *in vivo* studies human hepatocyte cultures were used to evaluate the effects of acute and chronic exposure to valerian extracts on the expression and activity of UGT1A6/9 enzymes.

Given the increasing popularity of herbal products and the emerging evidence of herb-drug interactions, several *in vitro* studies have been performed to investigate the potential for herb drug interactions. Most studies are performed with whole herb preparations, making it difficult to conclusively attribute the interaction potential to any one component. In some studies, a single constituent believed to be the major constituent is investigated. As herbal products are often complex mixtures of multiple compounds, it is difficult to arrive at specific conclusions regarding their potential interactions based on theses studies. A bioassay guided approach aids the identification of the potential for interactions as wells as helps to identify the compound involved in this interaction process. This method was therefore adopted to isolate the fractions and identify the compounds in the active fractions that are responsible for alteration of

metabolism. Most plant derived compounds posses poly- phenol moieties capable of conjugation with glucuronides or sulfates. In our studies, the potential to alter glucuronidation was investigated because valerenic acid is conjugated with glucuronide in human liver microsomes and is therefore likely to alter metabolism of substrates by this pathway. As is the case in most preliminary studies, human liver microsomes were used as the model system because of its ease of preparation and availability. These experiments indicated that the organic fraction of the valerian extracts inhibit acetaminophen glucuronidation. The major compounds in the organic extracts were identified by means of various spectrometric and chromatographic methods as valerenic acid, acetoxyvalerenic acid and valerenal. These results are in agreement with previous studies that also identified the major compounds in the dichloromethane extracts to be valerenic acid, acetoxyvalerenic acid and valerenal.

Acetaminophen was used as a probe substrate to measure glucuronidation activity in our studies. In addition to being safe, predominantly metabolized by glucuronidation and having a short half life, the advantage of acetaminophen as a probe is that it can be evaluated in *in vitro* as well as *in vivo* studies. A previously developed HPLC method was utilized to measure acetaminophen and acetaminophen glucuronide in microsomal incubations. However, this method had to be modified in order to be able to measure acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in plasma. The modified method using ultraviolet detection was developed and validated. The advantages of this method are its simplicity and sensitivity, especially since a low dose of acetaminophen was administered in the clinical study.

Based on the results of our studies with human liver microsomes we anticipated an *in vivo* interaction with acetaminophen, a UGT1A6/9 substrate. Our hypothesis for this study was that simultaneous administration of valerian and acetaminophen would cause an increase in acetaminophen AUC and Cmax. Subjects were asked to self administer valerian capsules at bedtime for a week prior to the study day in order to evaluate the effect of chronic administration of valerian. Analysis of the pharmacokinetic parameter of acetaminophen indicated that valerian administration did not alter the AUC or half life of acetaminophen in human volunteers. However, valerian administration increased the peak plasma acetaminophen concentration (Cmax) and reduced the time to reach the peak concentration (tmax) indicating that valerian increased the rate of absorption of acetaminophen. A similar effect was reported with a study using alprozolam as a probe substrate for CYP3A4 and a similar effect was observed in our study investigating the effect of valerian on pharmacokinetics and pharmacodynamic of with lorazepam (unpublished). Collectively, these results indicate that valerian is capable of increasing gut motility resulting in increased rate of absorption of drugs from the gut.

Given the differences in results obtained from the microsomal study and the *in vivo* human study we further evaluated the interaction potential of valerian extracts in human hepatocyte cultures. Hepatocyte culture system is more reliable model in investigating the interaction potential of durgs. As a result of preservation of cellular integrity it is possible to investigate not only effects of enzyme inhibition but also enzyme induction. The hypothesis for our study was that valerian and its extracts would inhibit UGT1A6 and UGT1A9 on chronic and acute exposure. The alcoholic extracts of valerian did not cause significant inhibition and only a marginal induction of acetaminophen glucuronide, consistent with the results observed in the

109

clinical study. However, the organic extracts especially the ethyl acetate extracts showed significant inhibition of acetaminophen glucuronidation on acute exposure. However, on chronic exposure, these extracts caused a significant induction of acetaminophen glucuronide formation. Investigation of the mechanism of this induction was performed by quantification of mRNA levels of UGT1A6 and 1A9. The increase in mRNA levels was proportional to the observed increase in glucuronidation activity. This indicates that the induction of the enzymes is possibly through in-direct pathways, perhaps by the activation of nuclear receptors controlling the levels of these enzymes.

Based on our studies with human volunteers valerian does not appear to significantly alter glucuronidation. Therefore, it may be safe to administer valerian with drugs undergoing glucuronidation by UGT1A6/9. With regards to models for study of herb drug interactions, whole cell systems such as hepatocyte cultures are better than human liver microsomes to predict herb-drug interactions. The novel bioassay guided approach is advantageous compared to the whole herb extract or single compound investigations because of the variation in the herbal product preparations in the market. Identifying compounds in herbal formulations that have the potential to alter drug metabolizing enzymes can help evalutions of these products based on their composition.

Future research is required to identify the isozymes responsible for glucuronidation of valerenic acid. Also very little information is available regarding the pharmacokinetics and bioavailability of herbal products in general and valerian in particular. A study to determine the pharmacokinetics of the major compounds in valerian products is required. *In vitro* and *in vivo*

studies need to be performed in order to characterize the absorption of valerian compounds. In order to evaluate the effects of chronic exposure to valerian extracts followed by an acute exposure, an appropriately designed hepatocyte study is required. Although a pharmacokinetic interaction can be ruled out based on our findings, a pharmacodynamic interaction is possible with drugs with similar effects. Valerian being a sedative is anticipated to increase the potency of anesthetic agents such as midazolam or other sedatives such as lorazepam. A clinical study to confirm these potential interactions is warranted in the future.

EFFECT OF VALERIAN ON THE PHARMACOKINETICS OF ACETAMINOPHEN STUDY PROTOCOL, INFORMED CONSENT, ADVERTISEMENT, AND APPROVAL LETTER

A.   Names of Principal Investigator and Co-Investigators
Principal Investigator: Raman Venkataramanan, Ph.D.
Co-Investigators: Mordechai Rabinovitz, M.D., Reginald Frye, PharmD., Ph.D.
Rama Sivasubramanian, M.Pharm.

B.   Protocol Title: Effect of Valerian on the pharmacokinetics of Acetaminophen.

C.   Specific aim

The specific aim of this proposal is to evaluate the effect of valerian on the pharmacokinetics of acetaminophen in normal healthy subjects. The hypothesis is that the constituents in valerian extract will inhibit a glucuronide conjugating enzyme system (UGT1A6/9) that is responsible for conjugation of acetaminophen and will therefore reduce the clearance of acetaminophen from the body.

D.   Background Information and Significance

Background Information:

The prevalence of herbal product usage and alternative therapies has steadily increased in the recent years. Consumer spending on herbal products in the United States is estimated to be more than $5 billion/year (1). More than one third of Americans use herbal products and a majority of these are patients with psychiatric disorders. The most commonly purchased herbal products are St. John's wort, ginko biloba, ginseng, valerian root and chamomile. The use of an herbal product (or "natural product" as often advertised) as a sleep-aid and as an anxiolytic presents an attractive alternative remedy to some people. In fact, insomnia, depression and anxiety are the most common reasons for seeking alternative or complementary therapies. This raises concerns about unintended interactions between herbal medications and conventional therapies (2-4). Nearly 70% of the patients do not reveal their herbal use to physicians and pharmacists, making it difficult to identify herb-drug

interactions in patients (5). In addition, herbal products are not regulated by the FDA for quality or consistency. Identification of herb-drug interactions is essential in order to improve the safety of therapeutic agents.

Herbal products contain various chemical constituents. The elimination of exogenous chemicals including herbal components and several drugs is mediated through an array of drug and xenobiotic metabolizing enzymes. Glucuronidation is a major metabolic pathway that involves the transfer of glucuronic acid from uridine 5′-diphosphoglucuronic acid (UDPGA) to a multitude of endogenous and exogenous substrates including bilirubin, bile acids, steroid and thyroid hormones, drugs, pesticides, and carcinogens (6, 7). Uridine 5′-diphosphate [UDP]-glucuronosyltransferases (UGTs) enzymes are distributed throughout the body and are found in liver, kidney, intestine, stomach, brain, and skin. The liver and intestine are major sites of glucuronidation in the body due to the diverse and abundant expression of UGT enzymes. Drugs and herbal components can interact in the intestine and or at the liver and alter the pharmacokinetics of a drug. In this study we plan to evaluate the interaction between valerian, a commonly used herbal product and acetaminophen, a commonly used analgesic.

Valerian is one of the most commonly used herbal products to treat insomnia and anxiety. The products may contain the powdered plant material but more commonly a dried extract, which are sometimes standardized to a minimum level of particular constituents, usually valerenic acid. Consistency of valerian content will be maintained by using PharmAssure brand valerian capsules bearing the same batch number through out the study. This product has been analyzed and verified in our laboratory to contain 0.8% of valerenic acid, consistent with the amount stated on the label. Valerian extracts contain more than 100 different constituents including mixture of valepotriates and volatile oils. Substantial amounts of free amino acids; particularly gamma amino butyric acid GABA, tyrosine, arginine, and glutamine are also present in aqueous extract of the roots of valerian (8). Valerian has been shown to have sleep-inducing properties, anti-anxiolytic and tranquilizing effects both in animals and humans (9-12). In healthy human subjects, 400-900 mg daily of valerian extracts decreased sleep latency and nocturnal awakenings and improved the quality of sleep (12-14). Although the extracts produce significant effects, these are generally not seen until 2-3 weeks of therapy. The mechanism by which valerian exerts its tranquilizing or anxiolytic effect is not fully known, but in vitro receptor-binding studies have found valerian to increase neuronal release of GABA, decrease GABA reuptake, and decrease GABA degradation. Additionally, valerian has agonist activity on the GABA-receptor complex (15, 16). Valerenic acid itself has also been shown to increase pentobarbital-induced sleeping time in animals. Valerenic acid increases GABA levels by inhibiting its metabolism, which is associated with sedation and a decrease in central nervous system (CNS) activity (8). Additionally, kessyl alcohol, another volatile oil constituent of valerian, and valepotriates possess sleep-inducing and anxiolytic properties (8).

Valerenic acid is conjugated to a glucuronide metabolite in human liver microsomal system (personal observation). Acetaminophen is largely eliminated from the body by UGTs, including the UGT1A6 and 1A9. We have observed 80% reduction in UGT1A6/9 activity as measured by acetaminophen glucuronide formation in human liver microsomes treated with valerenic acid. These results indicate that both acetaminophen and valerian are eliminated by the same metabolic pathway, and therefore valerian could potentially modify acetaminophen pharmacokinetics *in vivo*. Acetaminophen was chosen because it is a commonly used analgesic that is extensively glucuronidated.

<u>Significance</u>: This is a pilot study to gather data on the feasibility and the potential for valerian extract to alter the pharmacokinetics of acetaminophen. The widespread availability and use of herbal remedies including valerian makes it imperative that the potential for interactions with conventional therapies be clearly defined.

Progress Report and Preliminary Studies

Methanolic extracts of valerian capsules, as well as pure valerenic acid, significantly inhibited the glucuronidation of estradiol (UGT1A1), acetaminophen (UGT1A6/9), morphine (UGT2B7), and testosterone (UGT2B15). The magnitude of inhibition ranged from 30% to 80% depending on the probe drug and the extract concentration. These in vitro observations strongly support the

need for further studies in vivo to determine whether the inhibition is of any clinical relevance

Figure 1
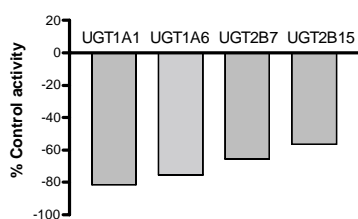
Research Design and Methods

Drug Information

The herbal product Valerian will be used in this investigation. We believe that an IND submission is not required for this study for the following reasons:

This product is available over-the counter in pharmacies and health food stores making it easily available to the public. These products do not require FDA approval.

This investigation is not intended to be reported to the FDA in support of a new indication for the use of Valerian and will not support any other significant change in the labeling of the product marketed.

This study will not support any significant changes in the advertising for this marketed product.

This study does not involve a route of administration or dosage level that significantly increases the risks associated with this marketed product.

Study Design and Methods

Screening Visit 1:

Informed consent will be obtained from all subjects prior to participation in this study. Each subject will have to pass a screening evaluation based on history, physical examination and the following biochemical and urinalysis tests: BUN, creatinine, serum albumin, SGOT, SGPT, bilirubin and normal hematocrit (males:40-52% females:35-47%). The screening visit will last approximately one (1) hour and 10 ml of blood will be drawn for biochemical measures to assess liver and kidney status. To participate, the subjects must not have any evidence of abnormal renal function (creatinine clearance estimated by Cockcroft-Gault method < 80 ml/min) or hepatic function (liver enzymes greater than 2.0-times the upper limit of normal or total bilirubin out of the normal range). In females, spot urine (spontaneous sample) sample will also be obtained for test for pregnancy. Age, height, and weight will be recorded in a demographic

record. Once subjects are determined eligible they will return to the GCRC for study visit 1 within one month of the screening visit.

Study Design overview
Healthy volunteers will be recruited to participate in this study such that at least eight subjects will complete the study. This study will require eight study days and five outpatient visits to the GCRC. The two visits will be separated by one week. Subjects will be instructed to abstain from taking any alcoholic beverages during the entire study. Subjects will be asked to abstain from taking caffeine containing beverages for 24 hours prior to and during the study visits and grapefruit or grapefruit juice 48 hours prior to and during the study visits.

Study Visit 2 (day 1): Subjects will report to the General Clinical Research Center by 7:00 AM on the day of the study. They will be instructed to fast from midnight, the night before. Subjects will be asked to empty their bladder; women will be given a urine pregnancy test at this time. An intravenous catheter will be inserted for the purposes of blood collection. A single oral dose of acetaminophen (500 mg) will be administered with 8 ounces of water at 8.00 AM. Subjects will be asked to comply with the instructions and study coordinator will ensure that subjects adhere to the study protocol. Nine venous blood samples (N= 9, 3 ml each) will be drawn at 0 (before the dose), at 15, 30min, 1, 2, 3, 4, 6, and 8 hr from an indwelling catheter into vacutainer tubes for measurement of acetaminophen, valerenic acid and metabolites of acetaminophen and valerenic acid. Total urine output will be collected in intervals from 0-8 and 8-24 hrs after acetaminophen administration. At approximately 10:00 AM subjects will be given a light snack, and after approximately 12:00 PM, subjects may eat per normal schedule. The subjects may drink water as needed. Subjects will be discharged from the GCRC after the 8-hour blood and urine collections are completed. Subjects will be given a urine collection container to collect all urine out put up to 24 hrs and asked to bring it to the GCRC the day after the study (visit 3). All urine samples will be tested for acetaminophen, acetaminophen glucuronide and valerenic acid levels.

Study days 2-7: Subjects will be provided with twelve (12) Valerian capsules (PharmAssure), 250 mg containing 2mg of Valerenic acid and will be instructed to self-administer two capsules daily within two hours of bedtime. Subjects will be instructed to keep a diary and note the times at which the dose was taken on each night in order to monitor compliance.

Study visit 4 and 5 (day 8 and 9): Subjects will report to the General Clinical Research Center by 7.00AM. Subjects will be given two capsules of Valerian 250mg containing 2mg valerenic acid at 8.00AM along with the dose of acetaminophen (500 mg) and subsequently undergo procedures as in visit 1. The additional dose of valerian is given in order to maximize the drug interaction potential.

Monitoring/Follow-up Procedures:
For this research study, the monitoring/follow-up procedures include measurement of blood pressure, temperature, and heart rate at 3 and 8 hours after the administration of acetaminophen. All blood samples will be placed on ice immediately after collection and centrifuged within 15 minutes. The resultant plasma will be stored at $-20^{o}C$ or lower until analyzed. Urine samples will be stored refrigerated until the end of the collection interval; after collection, the total volume will be measured and aliquots will be frozen at $-20^{o}C$ or lower until analyzed. A total of eighteen (18)

blood samples per subject will be obtained over the course of this study. The total amount of blood withdrawn for all acetaminophen study days, including the screening labs, will be 64ml (about 2.1 ounces or about 4 1/2 tablespoonfuls). After completion of the study the specimens of plasma and urine samples will be stored indefinitely in freezers in the pharmacokinetics laboratory under the control of the principal investigator of this research project. The samples will be assigned code numbers and the information linking these code numbers to the corresponding subjects will be kept in a separate, secure location. If a subject decides to withdraw or is eliminated from the study, the specimens obtained will be destroyed. Samples will not be given to secondary investigators. However, patients can give permission to be re-contacted to obtain consent to use their samples for other research projects.

Analytical Procedures

Drug analysis: Plasma and urine concentrations of acetaminophen, its glucuronide conjugate, its sulfate conjugate and valerenic acid will be determined using an HPLC method developed in our laboratory.

Data Analysis

The pharmacokinetic parameters of acetaminophen will be determined by non-compartmental methods. The elimination rate constant (k) will be obtained using nonlinear least-square regression of the terminal concentration-time data. The area under the concentration versus time curve (AUC) will be calculated by trapezoidal rule with extrapolation to infinity. Acetaminophen apparent oral clearance (CL/F) will be determined as dose/AUC and apparent volume of distribution will be determined for acetaminophen as dose/AUC x k. The urinary recovery of acetaminophen, acetaminophen glucuronide (AU_{APAPG}) and acetaminophen sulfate from 0-24 hours will also be determined. Fractional metabolic clearance for each pathway will also be calculated. Valerenic acid concentrations in plasma will be determined to allow for comparison to the in vitro inhibition data. The maximum observed plasma concentration (C max) and plasma AUC of valerenic acid will be calculated and correlated with % change in the clearance of acetaminophen.

G.   Biostatistical Design and Analysis

Sample Size and Power: This is a pilot study to evaluate the potential for an interaction between valerian and acetaminophen. Taking into account the low intraindividual coefficient of variation of acetaminophen pharmacokinetic parameters (usually below 20%), a sample size of 8 subjects attained a power of 0.8 and an alpha of 0.05 to determine a 25% change in acetaminophen clearance.

Statistical analysis: The pharmacokinetic data being calculated include $AUC_{0-\alpha}$, Cmax, apparent oral clearance, metabolic ratio (AUC of metabolite to parent drug concentration in the plasma), total urinary excretion of metabolites and fractional metabolic clearance. Paired t test will be used to evaluate the significance of the differences in the parameters measured at a $p \leq 0.05$.

H.   Recruitment Methods and Consent Procedures:

 Subject population

The study will be conducted in 8 healthy subjects (4 men/4 women), 18 – 65 years, and will involve the evaluation of the pharmacokinetics of orally administered acetaminophen before and during administration of Valerian. Based on past experience, we anticipate a screening failure rate of approximately 30%, thus the proposed number of subjects to be enrolled per IRB guidelines will be twelve, which allows for potential dropout of 4 subjects. Attempts will be made to recruit even numbers of men and women. In the event that we are unable to recruit equal number of both

genders with in 8 months, subjects will be recruited as received. All subjects enrolled will give written informed consent. As defined by NIH, this study will enroll children aged 18 to 21 years; children less than 18 years of age will be excluded from study participation.

Each subject will have to pass a screening evaluation based on history, physical examination and the following biochemical and urinalysis tests: BUN, creatinine, serum albumin, SGOT, SGPT, total bilirubin and hematocrit. Pregnant women, or women who are currently breast-feeding an infant, will not be allowed to take part in this study. Women of childbearing potential will be tested to exclude pregnancy using a urine pregnancy test. Use of estrogen and progestational hormone agents will be permitted.

Acetaminophen (Tylenol Extra strength) and Valerian (PharmAssure brand) will be provided at no cost to all participants for the study period by the investigator. Valerian is classified as a dietary supplement and its use is not approved by the FDA.

Inclusion Criteria:

Non-smokers (self-reported) between the ages of 18-65 years.

Signed Informed Consent.

Body Mass Index (BMI) < 31.

Normal hematocrit (males: 40-52% females: 35-47%)

> *Exclusion Criteria*

a.   Evidence of renal dysfunction (estimated creatinine clearance < 80ml/min).

b.   Impaired hepatic function (liver enzymes greater than two times the upper limit of normal or total bilirubin > 2.0 mg/dl).

Taking any medications other than oral contraceptives (for women)

Currently taking any herbal medicines

Women who are pregnant or are currently breastfeeding.

Blood donators within 2 months prior to the study

Patients with known G6PD deficiency

Subjects will be recruited through an advertisement (approved by the IRB) that will be placed in different locations in the University campus and in the Pitt News. We have requested and obtained a waiver of the requirement to obtain signed informed consent for the screening process, which will take place over the phone. We believe we meet the following criteria: The respective research procedures present no more than minimal risk of harm to the involved subjects and involve no procedures for which written consent is normally required outside of the research context. The information being obtained during the screening phone call is only to determine the eligibility of the subject for further participation in this study. Please refer to Appendix A for the screening script and screening tool that will be utilized. If the subject is not eligible for the study he/she will be so notified and the screening data will be destroyed. If the subject is eligible for study he/she will be so informed and scheduled for screening visit. Cold calling will not be used to recruit subjects**.** Informed Consent will be obtained prior to any screening procedures. Subjects considering enrollment in the study will be provided with an IRB approved consent form to read, and the study protocol will be explained to them. Any questions that the potential subjects have will be answered by a physician co-investigator. If interested in study participation, subjects will be asked to sign the consent form. A copy will be maintained in the subject's GCRC chart and a copy retained by the investigator. A physician co-investigator will obtain consent and sign the certification of informed consent. Eligible subjects will then undergo a screening assessment based on history, physical examination and laboratory tests to assess their suitability for the study.

Gender and Minority Inclusion Statement:
Women or men of all races will be eligible and recruited for this study. The racial and age mix of the study will be representative of the population of Western Pennsylvania. There will be no exclusion based on race, sex, or ethnicity.
Child Exclusion Statement:
Children below the age of 18 will be excluded from this study as Valerian is not commonly used to treat children and therefore this research topic is irrelevant to children.
2. Total Planned Enrollment: 8

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Sex/Gender**		
	Females	Males	Total
Hispanic or Latino			
Not Hispanic or Latino	4	4	8
Ethnic Category Total of All Subjects*	4	4	8
Racial Categories			
American Indian/Alaska Native			
Asian	0	0	0
Native Hawaiian or Other Pacific Islander			
Black or African American	1	1	2
White	3	3	6
Racial Categories: Total of All Subjects *	4	4	8

3.   Sources of Research Material
Subjects who participate in this study will provide medical information along with blood and urine samples. The blood and urine samples will be obtained specifically for research purposes in order to meet our specific aim.

4.   Recruitment Methods and Consent Procedures:
Subjects for this study will be recruited via advertisement. The study consent form will be presented by Mordechi Rabinovitz, M.D., and subjects will be asked to provide informed consent to participate in the pharmacokinetic study prior to any research procedures.

5.   Risk/Benefit Ratio
The risks involved with this study include the discomfort and inconvenience of having an intravenous catheter placed and blood samples collected with potential for pain, bruising, bleeding and infection. Side effects are listed below for each of the drugs used in this study. Side effects that are considered likely occur in more than 25 out of every 100 (25%) people who take the drug, common side effects occur in approximately 1 to 25% of people, and rare side effects occur in less than 1% of people. Additional information in the form of patient education leaflets is available in the appendices. These will be made available to study subjects.

Valerian capsules (PharmAssure) containing valerian root extract (250 mg) is an over-the-counter product used to treat insomnia and anxiety. Valerian products have been shown to be generally safe, even when fairly large doses as much as twenty times the daily-recommended dose was taken (6-20g)(17). Adverse reactions to valerian preparations are rare (5).
          Likely: None.
 Common: Valerian may cause headache, excitability, uneasiness, insomnia, mydriasis, and daytime sedation.

Rare: Paradoxical stimulation (including restlessness and palpitation) and hepatotoxicity have been experienced by a small percentage of consumers after long-term consumption

<u>Acetaminophen (Tylenol Extra Strength) containing 500 mg</u> is a common over-the -counter, FDA approved drug for treatment of pain and fever.

Likely: None
Common: None
Rare: **Acetaminophen may cause hematologic, allergic or other miscellaneous   adverse effects;**

Hematologic: Methemoglobinemia, hemolytic anemia neutropenia, thrombocytopenia, pancytopenia, and leukopenia. At very high doses (greater than 4g per day over a long term period), acetaminophen can cause liver damage.

Allergic: Urticarial and erythematous skin reactions, skin eruptions, fever.

Miscellaneous: CNS stimulation, rare blood disorders, hypoglycemic coma, jaundice, drowsiness, and glossitis. Possible liver damage in those who consume three or more alcoholic drinks daily.

No personal benefit will result from this study. Information obtained from this study, however, will provide increased knowledge about the potential effect of valerian on metabolism of drugs through UGT1A6/9 enzyme system. There is the inconvenience of participating in the study, collecting urine and having blood withdrawn. There is a minor risk of developing bruises associated with blood sampling**.** Pregnancy will not be permitted during the study due to unknown adverse events that could occur.

The research coordinator will monitor any adverse events in the subjects participating in this study and report occurrence of any events to the Institutional Review Board (IRB) immediately.

Any information obtained about the subjects for this research study will be kept confidential. Subject records will be indicated by a case number rather than by subject's name and the information linking these case numbers with the subject's identity will be kept separate from the research records**.**

6.   Risk Management Procedures: The risk of adverse experiences in this study will be minimized by utilizing only qualified individuals to conduct the study, the staff in GCRC in UPMC-MUH. Appropriate attention to detail in the experimental setting will be emphasized. Moreover, this study will use small, single doses of acetaminophen and short-term exposure to valerian and thus, the likelihood of dose-related adverse events should be minimized. In the event that a subject experiences an intolerable side effect, the subject will be withdrawn from the study and followed for resolution of the effect(s). A subject may also be removed from the study if in the opinion of the physician investigator; it is in the subject's best interest.

7.   Data Safety Monitoring Plan. This study involves a small number of subjects who will be closely monitored by the investigators and research personnel on the General Clinical Research Unit. The data and safety information obtained in each study subject will be reviewed at a weekly or biweekly meeting held by the investigators. As a part of these meetings, the research team will monitor data, confidentiality, and recruitment in addition to adverse events. A summary report from the meetings will be submitted to the IRB at the time of annual renewal.

We will comply with the IRB's policies for the reporting of serious and unexpected adverse events as detailed in Chapter 3.0, sections 3.4 and 3.5 of the IRB Reference Manual. If a serious life-threatening event occurs, the event will be reported immediately (i.e., within 24 hours) to both the FDA and the IRB. Unexpected reactions of moderate or greater severity will be reported to the IRB within 10 calendar days of the reaction. Minor events will be reported to the IRB at the time of annual review. All records related to the subjects in this research study will be stored in a locked file cabinet. Access to the research records will be limited to the researchers listed on the first page. Subjects will not be identified by name in any publication of the research results unless the subjects sign a separate release form.

8.   Costs and Payments

The subjects will not be charged for any studies related to this protocol. The subject's insurance will not be billed for this study. Subjects who participate will receive $ 50 for each part as reimbursement for expenses involved in participating in this study. The total compensation will be $ 100 for the two study periods. Subjects will be paid the entire amount when they bring the last urine sample on the day after the second visit. Funds from the pharmacokinetics laboratory will be used to cover these payments.

I.   Justification for Utilization of GCRC Resources

The GCRC use is being requested to utilize the facilities and expertise available to ensure proper execution of the study. It will ensure that drugs are given under medical supervision and samples are collected by qualified professionals with experience in the conduct of research studies. These factors are important to obtain results that are valid and interpretable.

J.   Study Size and GCRC Resources

1.   Number of Research Subjects: 12 (8 to complete)

2.   Total Number of Inpatient Days: 0

3.   Total Number of Outpatient Days: 12 screening + 16 outpatient visits

4.   Estimated Ancillary costs per IP day: none

5.   Estimated Ancillary costs per OP day: $ 226.08
    6.   Description of other GCRC Resources Requested: Yes, Nursing and nutrition support
                                                        requested.

K.   Research needs to be provided by Investigator's laboratory

Analytical assays required for the determination of concentrations of acetaminophen and its glucuronide will be conducted by Dr. Venkataramanan. Labeled storage containers for plasma/urine samples will be provided to GCRC.

L.   Funding Support

Funding from GCRC is requested for screening tests. Funds in the Clinical Pharmacokinetics Laboratory and Center for Pharmacodynamics will be utilized for the study.

M.   References Cited:
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N. **Qualifications of the Investigators**

Raman Venkataramanan Ph.D. is the director of the Clinical Pharmacokinetics Laboratory, and has conducted drug disposition studies in normal subjects, liver, heart, kidney and bone marrow transplant patients since 1982.

Reginald Frye, PharmD., PhD. is an assistant professor at the School of Pharmacy, University of Florida and has expertise in the evaluation of drug metabolism in humans.

Mordechi Rabinovitz, MD, is a gastroenterologist with extensive research experience in human subjects.

Rama Sivasubramanian is a Ph.D. student in the Department of Pharmaceutical Sciences in the School of Pharmacy. She will serve as the study coordinator.

PROTOCOL APPENDIX A

Healthy Volunteers Needed

Researchers at the University of Pittsburgh are seeking healthy volunteers between 18 & 65 years to take part in a study looking at how Valerian, an herbal product affects the elimination of Acetaminophen, a common pain reliever. A 1 hour screening visit to see if you are eligible and two one-day stays (1 or 2 weeks apart) in UPMC-Montefiore are required. Participants will be paid $100 upon study completion.

You may be eligible if you are
- Between 18 & 65 years old
- Healthy
- Non-smoking
- Not taking any medications or herbal products (except oral contraceptives)

For more information contact Rama Sivasubramanian, School of Pharmacy at 412-648-9436 or via email at rasst95@pitt.edu .

CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

TITLE: **Effect of valerian on the pharmacokinetics of acetaminophen**

PRINCIPAL INVESTIGATOR: Raman Venkataramanan, Ph.D.
                                               Professor, School of Pharmacy
                                               718 Salk Hall,
                                               Tel: 412-648-8547

CO-INVESTIGATORS: Mordechai Rabinovitz, M.D.
                                               Assoc. Professor of Gastroenterology
                                               3rd Floor Falk Clinic, School of Medicine
                                               Tel: 412-383-8687

                                               Reginald Frye, Pharm D., Ph. D.
                                               Assoc. Professor
                                               Univ. of Florida, College of Pharmacy
                                               Tel: 352-273-6238

                                               Rama Sivasubramanian, M.Pharm.
                                               Graduate Student, School of Pharmacy
                                               806 Salk Hall,
                                               412-648-9436

SOURCE OF SUPPORT: Clinical Pharmacokinetics laboratory funds

TO PERSONS WHO ARE INTERESTED IN PARTICIPATING IN THIS STUDY:
The following information is provided to inform you about the research project and your participation in it. Please read this form carefully. Please feel free to ask any questions that you may have about this study and/or about the information given below.

Why is this research being done?

The purpose of this study is to determine if the herbal product, valerian root extract (valerian) changes the way the body removes a drug, acetaminophen. Valerian is an herbal product sold over-the-counter and is used for sleep disorder and anxiety. Valerian is not approved by the Food and Drug Administration (FDA) as a drug, although it is sold in Health Food and Drug stores as a supplement and can be bought without a prescription. Acetaminophen (Tylenol®) is a common FDA approved over- the- counter pain reliever**.**

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                                                                    Subject's Initial
124

Who is being asked to take part in this research study?

You are being asked to participate in this research study because you are a healthy man or woman between 18-65 years of age. A total of eight healthy men and women will participate in this study. This study will require two (2) one day stays (each visit will last approximately 10 hours) in the General Clinical Research Center (GCRC) in UPMC-Montefiore hospital. The stays will begin the morning of study day 1 and study day 8.

What procedures will be performed for research purposes?

Screening Procedure:
If you decide to take part in this research study, you will first sign this informed consent. You will be required to spend approximately 1 hr initially to give your medical history and to give a blood sample (about 10 ml – two teaspoonfuls) to make sure that your red blood cell count is not low. You will also be required to give a spontaneous urine sample to assess your pregnancy status. You will take part in the experimental procedure if you meet the criteria for entry in to this study.

Experimental Procedure:
If you qualify to participate in this research study, you will have to come back to the General Clinical Research Center (GCRC) two more times. For both visits, you will arrive at the GCRC at 7.00 a.m. on the day of the study. You will be asked to abstain from taking any alcoholic beverages during the entire study. You will be asked to abstain from taking caffeine-containing beverages for 24 hours prior to and during the study visits and grapefruit or grapefruit juice 48 hours prior to and during the study visits. If you are a woman urine pregnancy test will be performed prior to any blood sample collection at both visits. Procedures performed to evaluate the effectiveness and safety of the experimental procedures are called "monitoring" or "follow-up" procedures. For this research study, the monitoring/follow-up procedures include measurement of your blood pressure, temperature, and heart rate at 3 and 8 hours after the administration of acetaminophen. The total length of your stay in the GCRC will be approximately 10 hours per visit.

Visit 1

If you agree to participate, you will be instructed to fast from midnight the night before the visit and arrive at the GCRC at 7:00 a.m. on the day of the study. You will have a small plastic tube inserted into a vein in your arm or hand to help with the collection of blood samples. You will also be required to give a spontaneous urine sample to assess your pregnancy status. A quarter teaspoon of blood will be taken from this tube in your vein. This is done to obtain baseline information of your blood in the absence of any drugs. At 8.00 AM you will receive one dose of acetaminophen (500 mg) by mouth. After taking the medicine, quarter of teaspoon of blood will be taken at

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Subject's Initial

125

various time points (8.15, 8.30, 9.00, 10.00, 11.00, 12.00, 2.00 & 4.00) from a tube in your vein during the day (total of about 2 tablespoons of blood) in order to determine the amount of acetaminophen, acetaminophen glucuronide and valerian products. Your urine will also be collected from the time at which the medicine was given to time of last blood sample collection (i.e. 8.00 AM to 4.00 PM). You will be provided a light snack at 10.00 AM and you may resume you normal eating schedule with lunch at 12.00 PM. A container will be provided to you to collect urine for another 16 hrs (8-24 hrs) and you will be asked to bring it to the GCRC the day after the study. The urine samples will be tested for acetaminophen and valerian levels. You will be given 12 valerian 250 mg capsules to take once a day until your next visit. You will take two valerian 250 mg capsules within two hours of bedtime every day for six days. You will be provided with a diary to record the time at which the valerian dose was taken each night and will be asked to return the diary to the study coordinator at the time of your second visit.

Visit 2

One week after the first visit, you will arrive in the GCRC at 7:00 a.m. on the day of the study after an over night fast. You will also be required to give a spontaneous urine sample to assess your pregnancy status. You will be given an additional dose of valerian (two 250 mg capsules) at 7:00 a.m. You will have a small plastic tube inserted into a vein in your arm or hand to help with the collection of blood samples. A quarter teaspoon of blood will be taken from this tube in your vein. This is done to obtain baseline information of your blood in the absence of any drugs. You will receive one dose of acetaminophen (500 mg) by mouth. Then, at various time points after taking the medicine, quarter of teaspoons of blood will be taken from a tube in your vein nine times during the day (total of about 2 tablespoon of blood). Your urine will also be collected from the time at which the medicine was given to time of last blood sample collection (i.e. 8.00 AM to 4.00 PM). **A** container will be provided to you to collect urine for another 16 hrs (8-24 hrs) and you will be asked to bring it to the GCRC the day after the study**.**

Monitoring/Follow-up Procedures:

Procedures performed to evaluate the effectiveness and safety of the experimental procedures are called "monitoring" or "follow-up" procedures. For this research study, the monitoring/follow-up procedures include measurement of your blood pressure, temperature, and heart rate at 3 and 8 hours after the administration of acetaminophen.

After completion of the study your blood and urine samples will be stored indefinitely in freezers in the pharmacokinetics laboratory under the control of the principal investigator of this research project. Your blood and urine samples will not bear your name for identification; instead they will be assigned code numbers. Information linking these code numbers to your name will be kept separate in a secure location. If you decide to withdraw or are eliminated from the study, your

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Subject's Initial

blood and urine samples will be destroyed. Your blood samples will not be given to secondary investigators. However, you can give permission to be re-contacted to obtain consent to use your samples for other research projects.

What are the possible risks, side effects, and discomforts of this research study?

You will not experience any personal benefits as a result of your participation in this study. The possible risks of this research may be due to the study drug and/or the blood tests. As with any investigational study, there may be adverse events or side effects that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious or life threatening.

Risks of the Study Drugs:
Acetaminophen is a common pain medication that has been safely used by large numbers of healthy adults but has not been given to subjects taking valerian in a research setting. The side effects listed for this drug are known primarily from the use of high doses rather than the two small doses that you will be taking. The side effects and their frequencies are listed below:

Side effects are listed below for each of the drugs used in this study. Side effects that are considered **likely** occur in more than 10 out of every 100 (10%) people who take the drug, **common** side effects occur in approximately 1 to 10% of people, and **rare** side effects occur in less than 1% of people.

Acetaminophen (Tylenol Extra Strength) containing 500 mg is a common over-the -counter, clinically proven analgesic agent.
Likely: None
Common: None
Rare: Acetaminophen may cause rare blood disorders (abnormal hemoglobin function, reduction in one or all blood cells), allergic reactions (such as rash, fever or hives) or other adverse effects such as excitement (central nervous system stimulation), tongue inflammation, drowsiness or jaundice (a condition where bilirubin builds up in the blood, causing a yellow color to the skin and whites of the eyes). Acetaminophen can also cause liver damage in those who consume three or more alcoholic drinks daily.
Valerian capsules (PharmAssure) containing valerian root extract (250 mg) is an over-the-counter product used to treat sleeplessness and anxiety. Adverse reactions to valerian preparations are rare.
Likely: None.
Common: Valerian may cause headache, excitability, uneasiness, insomnia(difficulty sleeping), mydriasis (dilated pupils), and daytime sedation.

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Subject's Initial

Rare: Unexpected stimulation including restlessness and palpitation (sensation of fast or irregular heartbeat) has been experienced by a small percentage of consumers after long-term consumption. Some liver problems in rare cases have also been associated with valerian use. While taking valerian, caution should be used when driving or operating machinery.

Animal studies to determine the effects of valerian on the fetus(unborn child) have not been done. To avoid risk to the fetus, it is important that you (for female participants) or your sexual partner (for male participants) do not become pregnant during the research study. Avoiding sexual activity is the only certain method to prevent pregnancy. However, if you choose to be sexually active, you must agree to use an appropriate double barrier method of birth control (such as female use of a diaphragm, intrauterine device, sponge and spermicide), in addition to the male use of condom or involve the female use of prescribed "birth control pills" or a prescribed birth control implant for at least two weeks after completion of the study. If you choose to be sexually active during the study you must accept the risk that pregnancy could still result, exposing you or your partner to potential loss of pregnancy as well as other unknown effects on the developing fetus.

Women who are capable of getting pregnant will have a negative pregnancy test prior to start of the study. If you become aware that you are pregnant during the course of this research study, you understand that you must stop taking valerian at once. You agree to do so and to contact the principal investigator listed on the first page and your physician, immediately.

If you are a man, it is recommended that you use an effective method of birth control while you are participating in the study and for 2 weeks following completion of the study. If you choose to be sexually active during this study you must accept the risk that pregnancy could still result, exposing you or your sexual partner to potential loss of pregnancy as well as other unknown effects on developing fetus.

Risks due to blood tests
The risks of participation in this study include the discomfort and inconvenience of having a plastic tube placed in your arm and possible lightheadedness from having some blood samples collected. Arm pain, bleeding, swelling, bruising and/or infection may result from having the plastic tube inserted in your arm and the blood withdrawal.

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Subject's Initial

What are the possible benefits from taking part in this study?

While you will not directly benefit from this study, all subjects who take valerian in the future may benefit from this study by our increased understanding of how the body handles drugs when used with valerian. This may help us to predict how to dose drugs that are important for their care.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?

You will be promptly notified if, during the conduct of this research study, any new information develops which may cause you to change your mind about continuing to participate.

Will my insurance provider or I be charged for the costs of any procedures performed as part of this research study?

Neither you, nor your insurance provider, will be charged for the costs of any of the procedures performed for the purpose of this research study. The study will cover the costs of the research services being provided.

Will I be paid if I take part in this research study?

For the study, valerian and acetaminophen will be provided to you by the investigators at no cost. Your parking will be provided to you at no cost at your screening visit. You will receive $50 payment for each part of the study to help cover the cost for travel, meals, or lodging associated with participating in this study. The total payment for participation in the entire study will be $ 100. You will receive this payment when you bring the last urine sample to the GCRC**.**

Who will pay if I am injured as a result of taking part in this study?

University of Pittsburgh investigators and their associates who provide services at the University of Pittsburgh Medical Center (UPMC) recognize the importance of your voluntary participation to their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research.

If you believe that you are injured as a result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first

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Subject's Initial

page of this form. Emergency medical treatment for injuries solely and directly relating to your participation in this research will be provided to you by the hospitals of UPMC. It is possible that the UPMC may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly to you. If your research related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. You will not receive monetary payment for, or associated with, any injury that you suffer in relation to this research**.**

Who will know about my participation in this research study?

Any information about you obtained from or for this research study will be kept as confidential (private) as possible. All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these case numbers with your identity will be kept separate from the research records. Access to your research records will be limited to the researchers listed on the first page of this form. You will not be identified by name in any publication of the research results unless you sign a separate form giving your permission (release).

Will this research study involve the use or disclosure of my identifiable medical information?

This research study will involve the recording of current and/or future identifiable medical information from your hospital and/or other (e.g., physician office) records. The information that will be recorded will be limited to information concerning your screening visit blood analysis and the levels of the two medications in your blood. This information will be used for the purpose of identifying if a drug-interaction exists between the two study medications in otherwise healthy subjects.

Who will have access to identifiable information related to my participation in this research study?

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study:

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Subject's Initial

130

Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information (which may include your identifiable medical information) for the purpose of monitoring the appropriate conduct of this research study.

In unusual cases, the investigators may be required to release identifiable information (which may include your identifiable medical information) related to your participation in this research study in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies**.**

Authorized representatives of the U.S. Food and Drug Administration may review and/or obtain identifiable information (which may include your identifiable medical information) related to your participation in this research study for the purpose of monitoring the accuracy of the research data. While the U.S. Food and Drug Administration understands the importance of maintaining the confidentiality of your identifiable research and medical information, the University of Pittsburgh and UPMC cannot guarantee the confidentiality of this information after it has been obtained by the U.S. Food and Drug Administration.

Authorized representatives of the UPMC hospitals or other affiliated health care providers may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study for the purpose of (1) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (2) addressing correct payment for tests and procedures ordered by the investigators; and/or (3) for internal hospital operations (i.e. quality assurance).

For how long will the investigators be permitted to use and disclose identifiable information related to my participation in this research study?

The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your identifiable medical information) related to your participation in this research study for a period of five years.

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Subject's Initial

131

Is my participation in this research study voluntary?

Your participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed, in general, to participate in the research study.) Whether or not you provide your consent for participation in this research study will have no affect on your current or future relationship with the University of Pittsburgh. Whether or not you provide your consent for participation in this research study will have no affect on your current or future medical care at a UPMC Health System hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

May I withdraw, at a future date, my consent for participation in this research study?

You may withdraw, at any time, your consent for participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above. (Note, however, that if you withdraw your consent for the use and disclosure of your identifiable information for the purposes described above, you will also be withdrawn, in general, from further participation in this research study.) Any identifiable research information recorded for, or resulting from, your participation in this research study prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above.

To formally withdraw your consent for participation in this research study you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

Your decision to withdraw your consent for participation in this research study will have no affect on your current or future relationship with the University of Pittsburgh. Your decision to withdraw your consent for participation in this research study will have no affect on your current or future medical care at a UPMC Health System hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

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Subject's Initial

If I agree to take part in this research study, can I be removed from the study without my consent?

It is possible that you may be removed from the research study by the researchers if, for example, your pregnancy test proves to be positive or you do not follow the instructions of the researchers.

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Subject's Initial

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VOLUNTARY CONSENT

All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by the researchers listed on the first page of this form. Any questions I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (412-578-8570). By signing this form I agree to participate in this research study.

I give my permission to be recontacted to obtain my consent if there a desire to use my de-identified biological sample in other research projects involving the study of different conditions other than those specified in the Description section of this consent form.

YES _____   NO_____

By signing this form, I agree to participate in this research study. A copy of this consent form will be given to me.

_____   _____
Participant's signature   Date and time
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CERTIFICATION OF INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise.

_____   _____
Printed Name of Person Obtaining Consent   Role in Research Study

_____   _____
Signature of Person Obtaining Consent   Date and Time

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