THE EFFECT OF GINKGO BILOBA EXTRACT ON VALPROIC ACID METABOLISM BY HUMAN LIVER MICROSOMES FROM DONORS WITH THE CYP2C9*1/*1 GENOTYPE

by

ANDREW MASATO NUMA

B.Sc., The University of British Columbia, 1999

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE in

THE FACULTY OF GRADUATE STUDIES

(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

August 2005

© Andrew Masato Numa, 2005
ABSTRACT

_Ginkgo biloba_ extract (GBE) is a popular herbal preparation used primarily in the treatment of dementia, peripheral vascular diseases, and neurosensory problems. In this study, the effect of GBE on the oxidative metabolism of the anti-epileptic valproic acid (VPA) was investigated. Human liver microsomes (HLM) from donors with the CYP2C9*1/*1 genotype were incubated with VPA and GBE, and the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA were monitored by GC/MS in NICI mode. GBE inhibited the formation of all four metabolites in a dose-dependent manner. GBE from three different sources showed similar inhibition of metabolite formation.

Pre-incubation of HLM with a monoclonal antibody against CYP2C9 significantly reduced the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA, demonstrating that CYP2C9 is the major isoform responsible for their formation. Pre-incubation of HLM with monoclonal antibodies against CYP2B6 and CYP2A6 reduced their formation by a smaller amount, suggesting that they are minor isoforms involved in their formation. Pre-incubation of HLM with monoclonal antibodies against CYP2B6 and CYP2A6 followed by incubation with VPA and GBE dramatically reduced their formation. These results show that the inhibition of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA formation in HLM by GBE is due mostly to inhibition of CYP2C9.

These results were confirmed by incubations with recombinant enzymes. GBE inhibited 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA formation by recombinant CYP2C9; 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA formation by recombinant CYP2B6; and 3-OH-VPA formation by recombinant CYP2A6.

To elucidate which constituents of GBE are responsible for the inhibition seen with the whole extract, individual terpene trilactones (bilobalide and ginkgolides A, B, C, and J) and
flavonol glycosides (isorhamnetin-3-O-rutinoside, kaempferol-3-O-rutinoside, and quercetin-3-O-rutinoside) were incubated with VPA. However, at the concentrations present in GBE, these constituents failed to inhibit VPA metabolism. The aglycones of isorhamnetin, kaempferol, and quercetin inhibited VPA metabolism. Although the aglycones were not detected in GBE, they may be of importance \textit{in vivo}, as flavonol glycosides are hydrolyzed to their respective aglycones in the small intestine.

In conclusion, GBE inhibited the CYP2C9-, CYP2B6-, and CYP2A6-mediated metabolism of VPA. However, the effect could not be explained by the individual terpene trilactones or flavonol glycosides tested.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xv</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 The Cytochromes P450</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Biotransformation</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 CYP2C9</td>
<td>2</td>
</tr>
<tr>
<td>1.1.3 CYP2B6</td>
<td>3</td>
</tr>
<tr>
<td>1.1.4 CYP2A6</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Ginkgo biloba Extract</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 Background</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 Chemical composition</td>
<td>5</td>
</tr>
<tr>
<td>1.2.3 Pharmacological actions</td>
<td>8</td>
</tr>
<tr>
<td>1.2.4 Uses</td>
<td>8</td>
</tr>
<tr>
<td>1.2.5 Adverse effects</td>
<td>9</td>
</tr>
<tr>
<td>1.3 Valproic Acid</td>
<td>10</td>
</tr>
<tr>
<td>1.3.1 Therapeutic use</td>
<td>10</td>
</tr>
</tbody>
</table>
1.3.2. Mechanism of action ................................................................. 10
1.3.3. Chemistry and biotransformation ................................................. 11
1.4. Rationale and Hypothesis ............................................................... 14
1.5. Specific Objectives ..................................................................... 17

2. MATERIALS AND METHODS ............................................................. 19

2.1. Matériaux .................................................................................. 19

2.1.1. Acids, bases, salts, and buffers .................................................. 19
2.1.2. Organic solvents ..................................................................... 20
2.1.3. Gases .................................................................................... 20
2.1.4. GBE and its individual constituents ........................................... 20
2.1.5. VPA .................................................................................... 21
2.1.6. NADPH ................................................................................ 21
2.1.7. Derivatization agents .............................................................. 21
2.1.8. Metabolite standards ............................................................... 21
2.1.9. Internal standard .................................................................... 22
2.1.10. Microsomes and antibodies .................................................... 22

2.2. Standard Curves .......................................................................... 22

2.3. Microsomal Incubations ............................................................... 23

2.3.1. Optimization of total CYP content for incubations with HLM ... 23
2.3.2. Optimization of incubation time for incubations with HLM ....... 23
2.3.3. Intraday and interday variabilities for incubations with HLM .. 23
2.3.4. Effect of GBE from different lots and manufacturers on VPA metabolism by HLM ....................................................... 24
2.3.5. Concentration-dependent effect of GBE on VPA metabolism by HLM .... 25
2.3.6. Effect of GBE on CYP2C9-catalyzed VPA metabolism by HLM ......... 25
2.3.7. Effect of GBE on VPA metabolism by recombinant CYP2C9*1, CYP2B6, and CYP2A6 ......................................................... 26
2.3.8. Effect of individual constituents of GBE on VPA metabolism by HLM .... 26
2.4. Sample Preparation for VPA Metabolite Analysis ................................................. 27
  2.4.1. Extraction ......................................................................................... 30
  2.4.2. Derivatization .................................................................................. 30
2.5. GC/MS Assay .......................................................................................... 30
2.6. Data Analysis .......................................................................................... 33
  2.6.1. Calculation of PAR .......................................................................... 33
  2.6.2. Standard curves .............................................................................. 33
  2.6.3. Microsomal incubations ................................................................... 34
  2.6.4. Limit of quantitation ....................................................................... 34
  2.6.5. Intraday and interday variabilities ...................................................... 35
2.7. Statistical Analysis .................................................................................... 35
3. RESULTS ........................................................................................................... 36
  3.1. Validation of VPA GC/MS Assay ............................................................. 36
    3.1.1. Chromatograms ............................................................................ 36
    3.1.2. Standard curves ............................................................................ 37
    3.1.3. Limit of quantitation .................................................................... 37
  3.2. Optimization of VPA Metabolism Assay .................................................. 44
    3.2.1. Optimization of total CYP content for incubations with HLM ...... 44
3.2.2. Optimization of incubation time for incubations with HLM................. 44
3.2.3. Intraday and interday variabilities for incubations with HLM.............. 47

3.3. Effect of GBE on VPA Metabolism..................................................... 47
  3.3.1. Overview of experiments.......................................................... 47
  3.3.2. Effect of GBE from different lots and manufacturers on VPA metabolism by HLM................................................................. 52
  3.3.3. Concentration-dependent effect of GBE on VPA metabolism by HLM..... 52
  3.3.4. Effect of GBE on CYP2C9-catalyzed VPA metabolism by HLM .......... 55
  3.3.5. Effect of GBE on VPA metabolism by recombinant CYP2C9*1, CYP2B6, and CYP2A6 .................................................. 57
  3.3.6. Effect of individual constituents of GBE on VPA metabolism by HLM.... 61

4. DISCUSSION.......................................................................................... 66
5. LIMITATIONS AND FUTURE STUDIES .................................................. 76
6. SUMMARY AND CONCLUSIONS ........................................................... 79
7. REFERENCES ....................................................................................... 82
8. APPENDICES......................................................................................... 92
  8.1. Appendix 1. Amount of Terpene Trilactones and Flavonols in GBE (Indena, lot 1306A)................................................................. 92
  8.2. Appendix 2. HLM Activities ............................................................ 93
  8.3. Appendix 3. HLM Donor Profiles..................................................... 94
  8.4. Appendix 4. Information on Recombinant CYP.................................. 95
  8.5. Appendix 5. Information on Monoclonal Antibodies ......................... 96
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>GC oven temperature program</td>
<td>32</td>
</tr>
<tr>
<td>Table 2</td>
<td>Retention times of VPA and its metabolites on a representative day</td>
<td>41</td>
</tr>
<tr>
<td>Table 3</td>
<td>Coefficient of variation and bias of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA standards</td>
<td>43</td>
</tr>
<tr>
<td>Table 4</td>
<td>Intraday variabilities of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA formation by HLM</td>
<td>48</td>
</tr>
<tr>
<td>Table 5</td>
<td>Interday variabilities of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA formation by HLM</td>
<td>49</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Chemical structures of the terpene trilactones</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Chemical structures of the flavonols and flavonol rutinosides</td>
<td>7</td>
</tr>
<tr>
<td>Figure 3</td>
<td>VPA and selected oxidative metabolites</td>
<td>12</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Phase I metabolism of VPA</td>
<td>13</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Sample preparation scheme</td>
<td>28</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Representative total ion chromatogram</td>
<td>38</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Representative chromatograms for metabolite standards at m/z of 141 and 148</td>
<td>39</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Representative chromatograms for metabolite standards at m/z of 273 and 280</td>
<td>40</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Representative standard curves</td>
<td>42</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Optimization of total CYP content for incubations with HLM</td>
<td>45</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Optimization of incubation time for incubations with HLM</td>
<td>46</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Representative chromatograms for microsomal incubations at m/z of 141 and 148</td>
<td>50</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Representative chromatograms for microsomal incubations at m/z of 273 and 280</td>
<td>51</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Effect of different lots and brands of GBE on VPA metabolism by HLM</td>
<td>53</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Effect of GBE on VPA metabolism by HLM</td>
<td>54</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Effect of GBE on CYP2C9-mediated VPA metabolism by HLM</td>
<td>56</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Effect of GBE on VPA metabolism by recombinant CYP2C9*1</td>
<td>58</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Effect of GBE on VPA metabolism by recombinant CYP2B6</td>
<td>59</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Effect of GBE on VPA metabolism by recombinant CYP2A6</td>
<td>60</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Effect of terpene trilactones on VPA metabolism by HLM</td>
<td>62</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

3-OH-VPA 3-hydroxy-valproic acid; 2-\textit{n}-propyl-3-hydroxypentanoic acid
4-ene-VPA 4-ene-valproic acid; 2-\textit{n}-propyl-4-pentenoic acid
4-OH-VPA 4-hydroxy-valproic acid; 2-\textit{n}-propyl-4-hydroxypentanoic acid
5-OH-VPA 5-hydroxy-valproic acid; 2-\textit{n}-propyl-5-hydroxypentanoic acid
\(\mu\text{A}\) microampere
\(\mu\text{g}\) microgram
\(\mu\text{L}\) microlitre
\(\mu\text{m}\) micrometre
\(\mu\text{M}\) micromolar
BROD benzzyloxyresorufin \textit{O}-dealkylation
\(\degree\text{C}\) degrees Celsius
\(\text{CV}\) coefficient of variation
\(\text{CYP}\) cytochrome P450
\(\text{CYP2A6}\) cytochrome P450 2A6
\(\text{CYP2B6}\) cytochrome P450 2B6
\(\text{CYP2C9}\) cytochrome P450 2C9
\(\text{DIPEA}\) \textit{N},\textit{N}-diisopropylethylamine
\(\text{DMF}\) dimethylformamide
\(\text{DMSO}\) dimethylsulfoxide
\text{eV} electron volt
\text{g} gram
g  gravity
GABA  γ-aminobutyric acid
GBE  *Ginkgo biloba* extract
GC  gas chromatography
GC/MS  gas chromatography-mass spectrometry
h  hour
HCl  hydrochloric acid
HLM  human liver microsomes
IC\textsubscript{50}  inhibitory concentration 50
IgG  immunoglobulin G
LC/MS  liquid chromatography-mass spectrometry
LOD  limit of detection
LOQ  limit of quantitation
m  metre
mm  millimetre
MΩ  megaohm
MAb2A6  monoclonal antibody against CYP2A6
MAb2B6  monoclonal antibody against CYP2B6
MAb2C9  monoclonal antibody against CYP2C9
mg  milligram
MgCl\textsubscript{2}  magnesium chloride
min  minute
mL  millilitre
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTBSTFA</td>
<td>N-methyl-N-(\textit{tert}-butyldimethylsilyl)-trifluoroacetamide</td>
</tr>
<tr>
<td>mTorr</td>
<td>milliTorr</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>NADPH</td>
<td>(\beta)-nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NICI</td>
<td>negative ion chemical ionization</td>
</tr>
<tr>
<td>NMDA</td>
<td>(N)-methyl-D-aspartate</td>
</tr>
<tr>
<td>nmol</td>
<td>nanomole</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAR</td>
<td>peak area ratio</td>
</tr>
<tr>
<td>pmol</td>
<td>picomole</td>
</tr>
<tr>
<td>PROD</td>
<td>pentoxyresorufin (O)-dealkylase</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SIM</td>
<td>selected ion monitoring</td>
</tr>
<tr>
<td>TBDMSCl</td>
<td>\textit{tert}-butyldimethylsilyl chloride</td>
</tr>
<tr>
<td>Tris</td>
<td>tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>PFBBBr</td>
<td>pentafluorobenzyl bromide</td>
</tr>
<tr>
<td>VPA</td>
<td>valproic acid, 2-(n)-propylpentanoic acid; dipropylacetic acid</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight/weight</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Thomas Chang for his patience and guidance throughout my studies. I would also like to thank my committee members, Dr. Stelvio Bandiera, Dr. Ronald Reid, Dr. Wayne Riggs, and Dr. Zhaoming Xu; and external examiner, Dr. Emma Guns for taking time out of their busy schedules to provide invaluable feedback on this project. I would also like to thank fellow graduate students Vincent Tong, Tony Kiang, and Ted Lakowski; and laboratory members Xiaowei Teng and Jie Chen for their friendship.

A special word of gratitude is owed to Dr. Frank Abbott for allowing me to use the GC/MS and for providing the authentic VPA metabolite standards and heptadeuterated metabolites. I would also like to acknowledge Indena S.A. and Pharmaton S.A. for the provision of the Ginkgo biloba extracts. The National Cancer Institute at the National Institutes of Health provided the monoclonal antibodies.

I would also like to thank the following sources of financial support: the University of British Columbia for the Graduate Entrance Scholarship, the Rx&D Health Research Foundation and the Canadian Institutes of Health Research for the Graduate Research Scholarship in Pharmacy, and Merck Research Laboratories for the Partial M.Sc. Research Traineeship in Drug Metabolism. An operating grant from the Canadian Institutes of Health funded the research.

Finally, I would like to thank my family. Without their love and support, none of this would have been possible.
1. INTRODUCTION

1.1. The Cytochromes P450

1.1.1. Biotransformation

Humans are exposed to a large number of exogenous compounds each day through the diet, the environment, and pharmaceutical agents. Whereas hydrophilic xenobiotics are usually readily excreted, hydrophobic compounds must usually undergo metabolism into more polar metabolites for excretion. This is achieved via Phase I and Phase II metabolism. Phase I reactions introduce a new functional group into the substrate molecule or modify an existing functional group by oxidation, hydroxylation, reduction, or hydrolysis. The cytochrome P450 (CYP) enzymes are the major mediators of Phase I metabolism. CYP-mediated reactions generally result in the mono-oxygenation of substrates and require molecular oxygen and a supply of reducing equivalents from β-nicotinamide adenine dinucleotide phosphate (NADPH) (Omura, 1999). Phase II metabolism generally involves the conjugation of a hydrophilic moiety such as glucuronic acid or glutathione to the substrate to make it more water-soluble (Meyer, 1996).
CYPs comprise a superfamily of heme-thiolate proteins responsible for the biotransformation of a vast array of xenobiotics as well as mostly steroidal endogenous compounds. The CYP enzymes are grouped into families (indicated by Arabic numerals), in which members share at least 40% homology in their amino acid sequences. They are further divided into subfamilies (indicated by letters), whose members share at least 55% homology in their amino acid sequences (Nelson et al., 1996). CYP enzymes have broad but overlapping substrate specificity. Thus, a single CYP may be involved in the metabolism of a large number of different chemicals, and a single chemical may be metabolized by several different CYP isoforms.

The majority of xenobiotic metabolizing CYP enzymes are found in the liver. However, certain CYP isoforms are expressed in extrahepatic tissues such as the gastrointestinal tract, lungs, and kidneys (Smith et al., 1998). The major CYP enzymes expressed in the liver are members of the CYP3A subfamily (34% of total hepatic CYP content), members of the CYP2C subfamily (19%), CYP1A2 (13%), and CYP2D6 (3%). CYP3A4 and CYP3A5 metabolize approximately 36% of clinically important drugs. CYP2C8 and CYP2C9 are involved in the metabolism of approximately 17%. CYP2C18 and CYP2C19 metabolize approximately 8%. CYP1A2 is involved in the metabolism of approximately 8%. CYP2D6, though making up only a small proportion of total hepatic CYP, metabolizes 21% of clinically important drugs. Minor isoforms include CYP2E1, CYP2A6, and CYP2B6, which make up 7%, 4%, and 1%, respectively, of total CYP isoforms expressed in the liver (Rendic and Di Carlo, 1997).

1.1.2. CYP2C9

In humans, there are at least four members of the CYP2C subfamily: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. Despite sharing over 82% similarity in amino acid identity, they
exhibit relatively little overlap in substrate specificity. The CYP2C isoforms are expressed in the liver, the most abundant form being CYP2C9 (Goldstein and de Morais, 1994; Miners and Birkett, 1998).

Probes for CYP2C9 activity include (S)-warfarin 7-hydroxylation (Rettie et al., 1992), and diclofenac 4'-hydroxylation (Leemann et al., 1993), tolbutamide methylhydroxylation, and phenytoin 4-hydroxylation (Veronese et al., 1991). Other examples of substrates of CYP2C9 are chloramphenicol, flurbiprofen, ibuprofen, and naproxen (Omiecinski et al., 1999). Specific inhibitors of CYP2C9 include sulphaphenazole (Mancy et al., 1996) and sulfamethoxazole (Wen et al., 2002). Rifampin is an inducer of members of the CYP2C subfamily, but it also induces other isoforms (Rae et al., 2001).

Polymorphisms in the CYP2C9 gene result in variant alleles. The wild type CYP2C9*1 protein has relatively high enzyme activity, with the mutant CYP2C9*2 and CYP2C9*3 proteins exhibiting lower enzyme activities (Goldstein, 2001). CYP2C9 polymorphisms has been found to influence the metabolism of warfarin (Furuya et al., 1995), tolbutamide (Sullivan-Klose et al., 1996), and phenytoin (Hashimoto et al., 1996). Furthermore, the effect of an inhibitor may be genotype-dependent, as is the case for benzbromarone, which is an inhibitor of metabolism by CYP2C9*1 but an activator of metabolism by CYP2C9*3 (Hummel et al., 2005).

1.1.3. CYP2B6

In humans, CYP2B6 is expressed at low levels in the liver (Mimura et al., 1993). CYP2B6 is a polymorphic enzyme (Ariyoshi et al., 2001). CYP2B6 has been considered an enzyme of relatively little importance, but has been receiving more attention due to its role in the activation of pro-drugs such as cyclophosphamide (Chang et al., 1993). (S)-Mephenytoin N-demethylation has been suggested to be a specific marker for CYP2B6 (Ekins et al., 1998).
Bupropion hydroxylation also appears to be a fairly selective reaction for CYP2B6 (Faucette et al., 2000). Pentoxyresorufin O-dealkylation (PROD) and benzyloxyresorufin O-dealkylation (BROD) are often utilized to measure CYP2B activity in vitro (Lubet et al., 1985; Nerurkar et al., 1993). Orphenadrine is a mechanism-based inhibitor of CYP2B6, but also inhibits other isoforms (Guo et al., 1997). Phenobarbital and cyclophosphamide are inducers of CYP2B6 (Gervot et al., 1999).

1.1.4. CYP2A6

In humans, CYP2A6 is predominately hepatic, with a limited range of known substrates (Fernandez-Salguero and Gonzalez, 1995). CYP2A6 is a polymorphic enzyme (Fernandez-Salguero et al., 1995). Nicotine is perhaps its most well known substrate (Nakajima et al., 1996). Nitrosamines found in tobacco smoke are activated by CYP2A6 (Yamazaki et al., 1992). Coumarin 7-hydroxylation is commonly used experimentally as a marker for CYP2A6 activity (Pelkonen et al., 1985). Tranylcypromine and diethyldithiocarbamate inhibit CYP2A6, but are not specific for this isoform (Draper et al., 1997). Phenobarbital and pyrazole induce CYP2A6 (Donato et al., 2000).

1.2. Ginkgo biloba Extract

1.2.1. Background

Ginkgo biloba, also known as the maidenhair tree, is the sole surviving species of the family Ginkgoaceae. It is the oldest living tree known, and has thus earned the name "living fossil" (Joshi and Kaul, 2001). Fruits and seeds from Ginkgo biloba have been used for millennia in traditional Chinese medicine, mainly in the treatment of asthma (Ernst, 2002). Today, extracts made from the leaves of Ginkgo biloba are used for medicinal purposes. It is now one of the
most popular medicinal plants with annual worldwide sales in 1998 estimated to be greater than $1 billion US (van Beek, 2002).

1.2.2. Chemical composition

_Ginkgo biloba_ extract (GBE) is a complex mixture containing many chemical constituents. Extracts of the leaves are subjected to a multi-step procedure in which unwanted components are removed and desirable compounds are enriched. Commercially available GBE is typically standardized to contain 6% terpene trilactones and 24% flavonol glycosides (Yoshikawa et al., 1999). The terpene trilactones, which have received the most attention due to their chemical uniqueness, include bilobalide and ginkgolides A, B, C, and J (van Beek, 2005). Their structures are shown in Figure 1. The major flavonols are mostly derivatives of kaempferol, quercetin, and isorhamnetin. The flavonols occur mostly as glycosides, with the aglycones occurring at relatively low concentrations (van Beek, 2002). Chemical structures of the aglycones and rutinosides of these flavonols are shown in Figure 2.

Other constituents include proanthocyanidins, carboxylic acids, biflavones, and allkylphenols. Proanthocyanidins make up approximately 7% of standardized extracts. Carboxylic acids, which include ascorbic acid, quinic acid, and shikimic acid, comprise approximately 13% of standardized extracts. Biflavones such as amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin are present in leaves, but do not occur in standardized extracts (van Beek, 2002). Allkylphenols include the ginkgolic acids, ginkgols, and bilobols. Standardized extracts now contain little allkylphenols, which can cause contact dermatitis and may be mutagenic (Fuzzati et al., 2003).
Figure 1. Chemical structures of the terpene trilactones. Structures for (A) the ginkgolides and (B) bilobalide are shown.
Figure 2. Chemical structures of the flavonols and flavonol rutinosides.
1.2.3. Pharmacological actions

GBE possesses anti-oxidant activity. GBE shows the ability to scavenge free radicals such as the peroxyl (Maitra et al., 1995), hydroxyl, and superoxide radicals (Noda et al., 1997). GBE also inhibits lipid peroxidation (Dumont et al., 1995) and suppresses the production of active oxygen (Rong et al., 1996).

Ginkgolide B is a potent inhibitor of platelet activating factor (PAF), a potent inflammatory autacoid (Chung et al., 1987). PAF plays an important role in pathologies such as asthma, shock, ischemia, anaphylaxis, graft rejection, renal disease, CNS disorders and numerous inflammatory conditions. (Braquet and Hosford, 1991). However, it is questionable whether after oral consumption of *Ginkgo* preparations, enough ginkgolides are present in the bloodstream to cause PAF antagonism in humans (Braquet, 1993).

1.2.4. Uses

Since GBE possesses many biological actions, it is not surprising that GBE is used in the treatment of a wide range of conditions. In clinical practice, GBE is primarily utilized in the treatment of memory impairment, dementia, peripheral vascular diseases, and neurosensory problems (De Smet, 2002; Ernst, 2002).

GBE is marketed for its purported effect on the improvement of memory. A review of 40 clinical trials investigating the efficacy of GBE on memory found 8 trials of good methodological quality. Of these 8 studies, 7 showed positive effects of GBE on cognitive function compared to placebo. However, the authors warned of a possible publication bias (Kleijnen and Knipschild, 1992). Another meta-analysis of 11 clinical studies concluded that GBE is superior to placebo (Hopfenmuller, 1994).
Another major use of GBE is in the treatment of dementia. Dementia is defined as an acquired global impairment of cognitive capacities. Approximately 5% of people over 65 years of age are affected by dementia, of which 70% of cases are thought to be due primarily to Alzheimer's disease (Evans et al., 2004).

GBE is also used for intermittent claudication, which is characterized by symptom relief comparable to pentoxifylline, one of the two drugs for the management of intermittent claudication approved by the US FDA (Jacoby and Mohler, 2004).

There are a number of reports in the literature suggesting that GBE may be effective in the management of tinnitus, a condition characterized by the perception of sound in the absence of external acoustic stimulation. However, there also appears to be a strong placebo effect in tinnitus management. The limited evidence does not demonstrate that GBE is effective for tinnitus (Hilton and Stuart, 2004).

GBE may be useful in the treatment of glaucoma, which can be caused by either increased intraocular pressure or poor circulation resulting in damage to the optic nerve. GBE may be useful in glaucoma caused by the latter, as it derives its effect not through the lowering of intraocular pressure, but by improving blood flow to the optic nerve (Head, 2001; Rhee et al., 2001; Evans et al., 2004).

1.2.5. Adverse effects

Common adverse effects of Ginkgo are mild, transient, and reversible, such as gastrointestinal symptoms, headache, nausea, and vomiting. Serious effects include bleeding and seizures (Ernst, 2002).
1.3. Valproic Acid

1.3.1. Therapeutic use

Valproic acid (VPA), otherwise known as 2-n-propylpentanoic acid or dipropylacetic acid, is used in the treatment of epilepsy (Pinder et al., 1977). Several clinical trials have demonstrated its safety and efficacy in adults and children for the treatment of generalized seizures (absence, tonic-clonic, and myoclonic), partial seizures (simple, complex, and secondarily generalized), and compound or combination seizures (Davis et al., 1994). More recently, VPA has found its way into the treatment of bipolar disorder and migraines. A meta-analysis of clinical trials showed that for bipolar disorder, VPA was more effective than placebo, equally effective as lithium and carbamazepine, but less effective than olanzapine (Macritchie et al., 2003). According to a recent review of clinical trials, VPA was an efficacious and well-tolerated agent for the preventive treatment of migraine, chronic daily headache, and cluster headache, as well as for the treatment of acute migraine attacks (Freitag, 2003).

1.3.2. Mechanism of action

The mechanism of the anti-epileptic action of VPA is not well understood. Several mechanisms of action have been proposed. VPA potentiates the effect of γ-aminobutyric acid (GABA), but does not directly interact with postsynaptic GABA receptors (Owens and Nemeroff, 2003). Instead, VPA increases regional neuronal concentrations of GABA by inhibiting its degradation and increasing its synthesis (Loscher, 2002). VPA also inhibits N-methyl-D-aspartate (NMDA) receptor-mediated excitation (Zeise et al., 1991). Another possible mechanism is a non-specific membrane stabilizing effect by blockade of voltage-dependent sodium currents (McLean and Macdonald, 1986).
1.3.3. Chemistry and biotransformation

VPA is a C-8 branched fatty acid, as shown in Figure 3. The pure acid, which is a colourless liquid with a molecular weight of 144.2 g/mol, is highly soluble in organic solvents. The sodium salt is very soluble in water (Kuo et al., 2004).

In contrast with its simple chemical structure, the biotransformation of VPA is highly complex. Phase I metabolism of VPA, shown in Figure 4, utilizes pathways such as β-oxidation, ω-hydroxylation, and (ω-1)-hydroxylation, which are normally reserved for the biotransformation of endogenous fatty acids (Abbott and Anari, 1999). Mitochondrial β-oxidation is involved in the formation of (E)-2-ene-VPA, 3-keto-VPA, and 3-OH-VPA (Schafer and Luhrs, 1978). Another pathway of 3-OH-VPA formation is CYP-dependent (ω-2)-hydroxylation. The (ω-1)-hydroxylation of VPA to 4-OH-VPA and ω-hydroxylation to 5-OH-VPA are also CYP-dependent (Prickett and Baillie, 1984). The 3-ene-VPA metabolite is formed by CYP-dependent desaturation of VPA (Bjorge and Baillie, 1991). Further β-oxidation of 3-ene-VPA leads to (E,E)-2,3′-diene-VPA (Rettenmeier et al., 1987).

Of particular interest is the CYP-dependent desaturation of VPA to 4-ene-VPA (Rettie et al., 1992). The 4-ene-VPA metabolite has been shown to be the most toxic metabolite of VPA in isolated hepatocytes (Kingsley et al., 1983), and is teratogenic in mice (Nau and Loscher, 1986). Subsequent β-oxidation of 4-ene-VPA leads to the production of another hepatotoxic metabolite, (E)-2,4-diene-VPA (Kassahun et al., 1991).
Figure 3. VPA and selected oxidative metabolites. Structures for (A) VPA, (B) 4-ene-VPA, (C) 4-OH-VPA, (D) 5-OH-VPA, and (E) 3-OH-VPA are shown. *Indicates a chiral centre.
Figure 4. Phase I metabolism of VPA. Enzymatic pathways include (A) β-oxidation, (B) CYP-dependent desaturation, (C) CYP-dependent ω-hydroxylation, (D) CYP-dependent (ω-1)-hydroxylation, and (E) CYP-dependent (ω-2)-hydroxylation. Adapted from Abbott and Anari (1999).
VPA also undergoes phase II conjugation. The major VPA metabolite in humans is VPA glucuronide (Dickinson et al., 1989). The conjugation of VPA with D-glucuronic acid is catalyzed by UDP-glucuronosyltransferase (UGT) enzymes UGT1A3, UGT1A6, UGT1A9, and UGT2B7 (Ethell et al., 2003; Sakaguchi et al., 2004). Phase I metabolites of VPA have also been found to undergo glucuronidation (Granneman et al., 1984; Tatsuhara et al., 1987).

1.4. Rationale and Hypothesis

One case report has suggested a possible interaction between GBE and warfarin. A 78-year-old woman stabilized on warfarin for 5 years experienced an intracerebral hemorrhage after 2 months of chronic Ginkgo biloba use (Matthews, 1998). It is possible that this interaction may be either pharmacodynamic or pharmacokinetic in nature.

The pharmacodynamic explanation rests on the fact that both Ginkgo biloba and warfarin possess anti-coagulant properties. In vitro tests have shown that ginkgolide B is a potent inhibitor of PAF (Chung et al., 1987). Warfarin derives its anticoagulant effect through antagonism of Vitamin K, a cofactor in the bioactivation of coagulation factors II, VII, IX, and X (Hirsh et al., 2001). Thus, GBE may have had an additive effect on the anticoagulant effect of warfarin. This theory is supported by another case report in which after one week of ingesting 80 mg of Ginkgo biloba daily, a 70-year-old man taking 325 mg of aspirin daily developed hyphema, a spontaneous bleeding from the iris into the anterior chamber of the eye. Aspirin is an inhibitor of platelet aggregation. The patient continued to take aspirin, but the bleeding stopped upon discontinuation of Ginkgo biloba. (Rosenblatt and Mindel, 1997).

There have also been a number of case reports in which patients taking Ginkgo biloba have experienced bleeding without the concomitant use of anticoagulant drugs. A 61-year-old man developed subarachnoid hemorrhage after consuming 40 mg tablets of Ginkgo biloba 3 or 4
times daily for more than six months. Bleeding time was increased, but returned to normal after discontinuation of *Ginkgo biloba* (Vale, 1998). Right parietal hematoma was detected in a man taking 120 mg of GBE daily for the previous 18 months (Benjamin et al., 2001). In another case report, an otherwise-healthy 33-year-old woman taking 120 mg of *Ginkgo biloba* daily for 2 years presented with headaches, nausea, and vomiting. An MRI of the brain revealed bilateral subdural hematomas. Bleeding times were prolonged but returned to normal after cessation of *Ginkgo biloba* ingestion (Rowin and Lewis, 1996). However, some doubt was raised as to whether the hematomas can be attributed to GBE (Odawara et al., 1997). In another case report, a 72-year-old woman who had been taking 50 mg of *Ginkgo biloba* daily for 6 to 7 months complained of memory impairment and dizziness. A brain CT showed left frontal subdural hematoma (Gilbert, 1997). However, caution was advised in interpreting whether this was due to coincidence or if there was a causal relationship between the use of *Ginkgo* and the patient's hematoma (Lewis and Rowin, 1997). Indeed, there is debate over whether enough ginkgolides are present in GBE and whether enough ginkgolides are absorbed after oral ingestion to cause clinically relevant PAF antagonism in humans (Chung et al., 1987; Braquet, 1993). In one clinical trial of GBE for dementia involving 309 patients, there was one case of subdural hematoma, and that case occurred in the placebo group. (Le Bars et al., 1997).

Since there are some doubts as to whether the interaction between GBE and warfarin is a pharmacodynamic one, an alternative explanation was explored. It is also possible that the interaction of GBE with warfarin is a pharmacokinetic one involving the CYP enzymes. It has been shown that CYP2C9 is the major isoform responsible for the metabolism of (S)-warfarin (Rettie et al., 1992). In addition, several constituents of GBE have been shown to inhibit CYP2C9 (Zou et al., 2002). This led to the research hypothesis:
GBE reduces CYP2C9-mediated drug clearance in humans. The effect by GBE may be due to one or more of its constituents such as kaempferol and its glycosides, quercetin and its glycosides, isorhamnetin and its glycosides, the ginkgolides, or bilobalide.

To probe the effect of GBE on CYP2C9, a substrate probe was needed. For this project, VPA, which is metabolized by CYP2C9 (Sadeque et al., 1997; Anari et al., 2000; Ho et al., 2003), was chosen. Thus, the experimental hypothesis was:

GBE and some of its constituents such as kaempferol and its glycosides, quercetin and its glycosides, isorhamnetin and its glycosides, the ginkgolides, and bilobalide inhibit CYP-mediated oxidative metabolism of VPA in vitro.

In addition to CYP2C9, CYP2B6 and CYP2A6 also appear to be involved in the oxidative metabolism of VPA (Sadeque et al., 1997; Anari et al., 2000). In the present study, the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA was monitored. The formation of the four metabolites were measured using a slightly modified version of the GC/MS assay utilized by Ho et al. (2003).

The in vitro system used to study VPA metabolism was human liver microsomes (HLM). All HLM were from donors with the CYP2C9*1/*1 genotype. This one genotype was chosen since it is the most prevalent, and in order to reduce variability in the results, since CYP2C9 allelic variants have been shown to exhibit different enzyme kinetics towards VPA (Ho et al., 2003).
1.5. Specific Objectives

To validate the VPA GC/MS assay by:

- ensuring that VPA metabolites and internal standards are adequately resolved in chromatograms;
- determining the dynamic range of the standard curves of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA; and
- determining accuracy and precision for limits of quantitation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA.

To optimize the VPA enzyme assay by:

- optimizing total CYP content of HLM for incubations with VPA;
- optimizing incubation time of HLM for incubations with VPA; and
- determining intraday and interday variabilities for incubations of HLM with VPA using optimized conditions.

To investigate the effect of GBE on VPA metabolism by:

- comparing the effect of GBE from different lots and manufacturers on VPA metabolism by HLM;
- determining the effect of GBE concentration on VPA metabolism by HLM;
- determining the effect of GBE on CYP2C9-catalyzed VPA metabolism by HLM;
- determining the effect of GBE on VPA metabolism by recombinant CYP2C9*1, CYP2B6, and CYP2A6; and
determining the effect of some of the individual constituents of GBE on VPA metabolism by HLM.
2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Acids, bases, salts, and buffers

Tris(hydroxymethyl)aminomethane (Tris) and magnesium chloride (MgCl₂) were purchased from Sigma (St. Louis, MO). Anhydrous sodium sulfate and solid sodium hydroxide (NaOH) were acquired from EM Science (Darmstadt, Germany). Monobasic potassium phosphate, phosphoric acid and hydrochloric acid (HCl) were purchased from Fisher (Fair Lawn, NJ).

A 1 M solution of NaOH was made by dissolving solid NaOH in water. Phosphoric acid was diluted to 0.1 M with water. Potassium phosphate buffer of 1 M concentration was prepared by adding monobasic potassium phosphate to water, and adjusting the pH to 3.0 with phosphoric acid. Tris buffer of 0.2 M concentration containing 6 mM MgCl₂ was made by adding Tris and MgCl₂ to water, and adjusting the pH to 7.4 by drop-wise addition of HCl. These stock buffers were stored at 4°C until use. All water used was of 18 MΩ quality.
2.1.2. **Organic solvents**

Ethyl acetate and hexane were purchased from Fisher. Acetone was acquired from Caldeon Laboratories (Georgetown, ON). Dimethylsulfoxide (DMSO) was from Sigma. Ethyl acetate and DMSO were of HPLC grade. Hexane and acetone were of GC grade.

2.1.3. **Gases**

Nitrogen, helium, and methane were purchased from Praxair (Mississauga, ON). Helium and methane were of Ultra High Purity 5.0 quality.

2.1.4. **GBE and its individual constituents**

Two different lots of GBE (1306A and 302831) were obtained from Indena S.A. (Milan, Italy). Another lot (63964, GK501™) was provided by Pharmaton S.A. (Bioggio, Switzerland). The chemical composition of GBE of lot 1306A is shown in Appendix 1. GBE was prepared fresh daily in 0.1 M Tris containing 3 mM MgCl₂. For experiments with individual GBE constituents, GBE was prepared in 0.1 M Tris buffer containing 3 mM MgCl₂ and 0.05% DMSO (v/v).

Bilobalide and ginkgolides A, B, and C were purchased from LKT (St. Paul, MN). Ginkgolide J was acquired from ChromaDex (Santa Ana, CA). Quercetin was obtained from Sigma. Kaempferol, isorhamnetin, kaempferol-3-O-rutinoside, isorhamnetin-3-O-rutinoside, and quercetin-3-O-rutinoside were purchased from Indofine Chemical Company (Hillsborough, NJ). Individual constituents were prepared fresh each day in 0.1 M Tris buffer containing 3 mM MgCl₂ and 0.05% DMSO (v/v).
2.1.5. **VPA**

The free base form of VPA was purchased from Acros Organics (New Jersey, NJ). The free base was hydrolyzed by the addition of an equimolar amount of 1 M NaOH. After one day at 4°C, the solution was diluted to 10 mM by the addition of water. The stock solution was aliquoted and stored at -20°C until use.

2.1.6. **NADPH**

NADPH was purchased from Sigma. It was prepared fresh each day in water and placed on ice, shielded from exposure to light, until use.

2.1.7. **Derivatization agents**

*N,N*-Diisopropylethylamine (DIPEA) and *tert*-butyldimethylsilyl chloride (TBDMSCl) were purchased from Sigma. Pentafluorobenzyl bromide (PFBBR), dimethylformamide (DMF), and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) were acquired from Pierce (Rockford, IL). MTBSTFA containing 2% TBDMSCl (w/v) was prepared by adding 0.1 g of TBDMSCl to a 5 g bottle of MTBSTFA.

2.1.8. **Metabolite standards**

The stock solution of metabolite standards used in the preparation of standard curves contained 0.5 µg/mL each of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. These VPA metabolites were synthesized in the laboratory of Dr. Frank Abbott (Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, BC). This solution was aliquoted and stored at -20°C.
2.1.9. Internal standard

The internal standard stock solution contained 2 µg/mL each of heptadeuterated VPA metabolites 4-ene-[2H7]-VPA, 2-ene-[2H7]-VPA, 4-OH-[2H7]-VPA, 5-OH-[2H7]-VPA, and 3-OH-[2H7]-VPA. These heptadeuterated metabolites were synthesized in the laboratory of Dr. Frank Abbott (Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, BC). This solution was aliquoted and stored at -20°C.

2.1.10. Microsomes and antibodies

HLM from individuals with the CYP2C9*1/*1 genotype (donors HH18, HG30, HH47, HH91, and HG95), control insect cell microsomes, and recombinant CYP2C9*1, CYP2B6, and CYP2A6 were purchased from Gentest (Woburn, MA). HLM activities are shown in Appendix 2. Donor history is provided in Appendix 3. Information in recombinant CYP is provided in Appendix 4. Monoclonal antibodies against CYP2C9 (MAb2C9) (Krausz et al., 2001), CYP2B6 (MAb2B6) (Yang et al., 1998), and CYP2A6 (MAb2A6) (Sai et al., 1999), and control IgG were provided by the National Cancer Institute at the National Institutes of Health (Bethesda, MD). Information about the antibodies is shown in Appendix 5.

2.2. Standard Curves

Standard curves were prepared on each experimental day. Metabolite standards (0-200 µL) were added to borosilicate glass screw-top vials. The volume of each tube was topped up to 1 mL with water. To each tube, 75 µL of 0.1 M phosphoric acid and a 200 µL mixture containing 1 mM VPA in 0.1 M Tris buffer 3 mM MgCl$_2$ (pH 7.4) were added. To each tube, 50 µL of internal standard was added.
2.3. Microsomal Incubations

2.3.1. Optimization of total CYP content for incubations with HLM

In a polypropylene eppendorf tube, a mixture containing 1 mM VPA, 1 mM NADPH, and 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) was pre-warmed at 37°C for 2 min in a shaking water bath. The reaction was initiated with the addition of HLM (donor HH47, 3 determinations) containing 20, 40, 60, 80, or 100 pmol of total CYP. The final incubation volume was 200 µL. The blank did not contain any microsomes. After 20 min, the incubation was terminated with the addition of 75 µL of ice-cold 0.1 M phosphoric acid. To each tube, 50 µL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water.

2.3.2. Optimization of incubation time for incubations with HLM

In a polypropylene eppendorf tube, a mixture containing 1 mM VPA, 1 mM NADPH, and 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) was pre-warmed at 37°C for 2 min in a shaking water bath. The reaction was initiated with the addition of HLM (donor HH47, 3 determinations) containing 60 pmol of total CYP. The final incubation volume was 200 µL. After 10, 20, 30, 40, or 50 min, the incubation was terminated with the addition of 75 µL of ice-cold 0.1 M phosphoric acid. For the blank, the reaction was terminated immediately after the addition of microsomes. To each tube, 50 µL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water.

2.3.3. Intraday and interday variabilities for incubations with HLM

In a polypropylene eppendorf tube, a mixture containing 1 mM VPA, 1 mM NADPH, and 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) was pre-warmed at 37°C for 2 min in a
shaking water bath. The reaction was initiated with the addition of HLM (donor HG30) containing 60 pmol of total CYP. An equivalent amount of inactivated microsomes, which were prepared by heating HLM at 65°C for 20 min, was used for the blank. The final incubation volume was 200 μL. After 20 min, the incubation was terminated with the addition of 75 μL of ice-cold 0.1 M phosphoric acid. To each tube, 50 μL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water. This procedure was repeated on four separate days. On each day, four determinations were performed.

2.3.4. Effect of GBE from different lots and manufacturers on VPA metabolism by HLM

In a polypropylene eppendorf tube, a mixture containing 1 mM VPA, 1 mM NADPH, and 0.3 mg/mL of GBE (Indena, lot 1306A), GBE from a different lot (Indena, lot 302831), or GBE from a different manufacturer (Pharmaton, lot 63964) in 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) was pre-warmed at 37°C for 2 min in a shaking water bath. For the control, 0.1 M Tris buffer containing no GBE was used. The reaction was initiated with the addition of HLM (donors HH18, HH47, HH91, and HG95; two determinations each) containing 60 pmol of total CYP. An equivalent amount of inactivated microsomes was used for the blank. The final incubation volume was 200 μL. After 20 min, the incubation was terminated with the addition of 75 μL of ice-cold 0.1 M phosphoric acid. To each tube, 50 μL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water.
2.3.5. **Concentration-dependent effect of GBE on VPA metabolism by HLM**

In a polypropylene eppendorf tube, a mixture containing 1 mM VPA, 1 mM NADPH, and 0.01, 0.03, 0.1, 0.3, 0.6, or 1 mg/mL of GBE (Indena, lot 1306A) in 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) was pre-warmed at 37°C for 2 min in a shaking water bath. For the control, 0.1 M Tris buffer containing no GBE was used. The reaction was initiated with the addition of HLM (donors HH18, HH47, HH91, and HG95; two determinations each) containing 60 pmol of total CYP. An equivalent amount of inactivated microsomes was used for the blank. The final incubation volume was 200 µL. After 20 min, the incubation was terminated with the addition of 75 µL of ice-cold 0.1 M phosphoric acid. To each tube, 50 µL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water.

2.3.6. **Effect of GBE on CYP2C9-catalyzed VPA metabolism by HLM**

For incubations involving antibodies, the protocol was changed slightly. HLM (donors HH18, HG30, and HH47; two determinations each) containing 60 pmol of total CYP were pre-incubated at 37°C with 3 µL of MAb2C9 or 3 µL of MAb2B6 and 3 µL of MAb2A6 in 0.1 M Tris buffer containing 3 mM MgCl₂ (100 µL incubation volume) for 5 min in a shaking water bath. A previous study determined that near maximal inhibition of VPA metabolism by MAb2C9, MAb2B6, or MAb2A6 alone was seen in HLM using 3 µL of antibody (Kiang et al., 2005). Product formation for incubations with antibodies was normalized to product formation for pre-incubations with an equivalent volume of control IgG (3 or 6 µL). Inactivated HLM containing 60 pmol of total CYP were used for the blank. The reaction was initiated with the addition of 100 µL of a pre-warmed mixture containing 2 mM VPA, 2 mM NADPH, and 0.1 M Tris buffer or 2 mg/mL GBE (Indena, lot 1306A) in 0.1 M Tris buffer. The final concentrations were the same as...
in other experiments (1 mM VPA, 1 mM NADPH, and 1 mg/mL GBE in 200 μL of 0.1 M Tris buffer). After 20 min, the incubation was terminated with the addition of 75 μL of ice-cold 0.1 M phosphoric acid. To each tube, 50 μL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water.

2.3.7. **Effect of GBE on VPA metabolism by recombinant CYP2C9*1, CYP2B6, and CYP2A6**

In a polypropylene eppendorf tube, a mixture containing 1 mM VPA, 1 mM NADPH, and 0.3 or 1 mg/mL GBE (Indena, lot 1306A) in 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) was pre-warmed at 37°C for 2 min in a shaking water bath. For the control, 0.1 M Tris buffer containing no GBE was used. The reaction was initiated with the addition of microsomes containing 40 pmol of cDNA-expressed CYP2C9*1, CYP2B6, or CYP2A6 (3 determinations each). Control insect cell microsomes were used for the blank. The final incubation volume was 200 μL. After 30 min, the incubation was terminated with the addition of 75 μL of ice-cold 0.1 M phosphoric acid. To each tube, 50 μL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water.

2.3.8. **Effect of individual constituents of GBE on VPA metabolism by HLM**

In a polypropylene eppendorf tube, a mixture containing 1 mM VPA, 1 mM NADPH, and 500 μg/mL of GBE (Indena, lot 1306A) or its individual constituents in 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) and 0.05% DMSO (v/v) was pre-warmed at 37°C for 2 min in a shaking water bath. For the vehicle control, 0.1 M Tris buffer containing 3 mM MgCl₂ (pH 7.4) and 0.05% DMSO (v/v) was used. The reaction was initiated with the addition of HLM (donors HH18, HG30, HH47, and HH91; two determinations each) containing 60 pmol of total CYP. Inactivated HLM containing 60 pmol of total CYP were used for the blank. The final incubation
volume was 200 μL. After 20 min, the incubation was terminated with the addition of 75 μL of ice-cold 0.1 M phosphoric acid. To each tube, 50 μL of internal standard was added. The contents were then transferred to a borosilicate glass screw-top vial containing 1 mL of water.

The concentrations of the terpene trilactones and isorhamnetin-3-\(O\)-rutinoside were the concentrations present in 500 μg/mL of GBE (bilobalide; ginkgolides A, B, C, and J; and isorhamnetin-3-\(O\)-rutinoside in Appendix 1). The concentrations of kaempferol-3-\(O\)-rutinoside and quercetin-3-\(O\)-rutinoside were the concentrations of their more abundant diglycoside present in 500 μg/mL of GBE (kaempferol diglycoside 1 and quercetin diglycoside 2 in Appendix 1). The concentrations of the flavonol aglycones were the concentrations that would be present in 500 μg/mL of GBE if all the flavonol glycosides were converted to their respective aglycones (sum under isorhamnetin, kaempferol, and quercetin in Appendix 1). The individual constituents tested were 14 μg/mL (43 μM) of bilobalide, 5.5 μg/mL (13 μM) of ginkgolide A, 1.5 μg/mL (4 μM) of ginkgolide B, 7 μg/mL (16 μM) of ginkgolide C, 3 μg/mL (7 μM) of ginkgolide J, 3 μg/mL (5 μM) of isorhamnetin-3-\(O\)-rutinoside, 5 μg/mL (8 μM) of kaempferol-3-\(O\)-rutinoside, 12 μg/mL (20 μM) of quercetin-3-\(O\)-rutinoside, 20.5 μg/mL (65 μM) of isorhamnetin aglycone, 31.5 μg/mL (110 μM) of kaempferol aglycone, and 53 μg/mL (175 μM) of quercetin aglycone.

2.4. Sample Preparation for VPA Metabolite Analysis

Samples from microsomal incubations and standards were processed according to the scheme shown in Figure 5. Extraction and derivatization are described in detail below.
Incubations

100 µL of 0.2 M Tris (pH 7.4) +
20 µL of 10 mM VPA +
20 µL of 10 mM NADPH +
(60 – x) µL of water
↓
Pre-warmed for 2 min
↓
Initiated reaction with
x µL of microsomes
↓
Incubated for 30 min
↓
Terminated reaction with
75 µL of 0.1 M phosphoric acid
↓
Added 1000 µL of water
↓
Added 50 µL of internal standard

Standards

(1000 – y) µL of water +
y µL of metabolite standards
↓
Added 100 µL of 0.2 M Tris (pH 7.4) +
20 µL of 10 mM VPA +
80 µL of water
↓
Added 75 µL of 0.1 M phosphoric acid
↓
Added 50 µL of internal standard

Extraction

Added 1 mL of 1 M potassium phosphate buffer (pH 3.0) +
8 mL of ethyl acetate
↓
Capped then gently rotated for 30 min
↓
Centrifuged at 1600 x g for 10 min
↓
Transferred organic layer to new vial
↓
Added 1 g of anhydrous sodium sulfate
↓
Capped then gently rotated for 15 min
↓
Centrifuged at 1600 x g for 10 min
↓
Decanted into new vial
↓
Dried under stream of 2.5 psi nitrogen gas until volume reduced to 100-200 µL
↓
(continued on next page)

Figure 5. Sample preparation scheme.
Derivatization

Added 30 μL of DIPEA +
10 μL of PFBB
↓
Capped then vortexed
↓
Incubated for 1 h at 45°C
↓
Added 20 μL of DMF +
40 μL of MTBSTFA containing 2% TBDMS
↓
Capped then vortexed
↓
Incubated for 2 h at 65°C
↓
Dried under stream of 2.5 psi nitrogen gas
↓
Added 200 μL of hexane
↓
Capped then vortex for 1 min
↓
Centrifuged at 1600 x g for 10 min
↓
Transferred hexane layer to glass insert

Figure 5 (continued).
2.4.1. Extraction

The pH was adjusted to 3.0 with 1 mL of 1 M potassium phosphate buffer. Extraction was performed by adding 8 mL of ethyl acetate to tubes containing incubation samples or standards which were then gently rotated for 30 min. Following centrifugation at 1600 x g for 10 min in a Beckman GP centrifuge (Fullerton, CA), the organic layer was transferred to a new vial. The organic phase was dried over approximately 1 g of anhydrous sodium sulfate for 15 min. Following centrifugation at 1600 x g for 10 min, the organic phase was decanted into another new vial. The organic phase was evaporated under a stream of 2.5 psi nitrogen gas for 2-3 h until the volume was reduced to 100-200 µL in a Zymark TurboVap LV evaporator (Hopkinton, MA), with the bath temperature set at 25°C.

2.4.2. Derivatization

Samples were incubated for 1 h at 45°C with 30 µL of DIPEA and 10 µL of PFBBBr. Samples were then incubated for 2 h at 65°C with 20 µL of DMF and 40 µL of MTBSTFA containing 2% TBDMSCl. The samples were dried under a stream of 2.5 psi nitrogen for 30 min. Following the addition of 200 µL of hexane, each tube was vortexted for 1 min, then centrifuged at 1600 x g for 10 min. The hexane layer was transferred to a 250 µL glass insert, placed in an autosampler vial, and capped with a Teflon-silicone septum (all from Chromatographic Specialties, Brockville, ON).

2.5. GC/MS Assay

The GC/MS system consisted of an HP 7683 Series autosampler, an HP 7683 Series injector, and an HP 6890 Series GC system interfaced with an HP 5973 Mass Selective detector (Avondale, PA). The front inlet, GC/MS interface, and ion source temperatures were set at
250°C, 270°C, and 200°C, respectively. The septum purge flow rate of the carrier gas, helium, was 20 mL/min for 2 min. The nominal initial flow was set at 19.18 psi and 0.5 mL/min. All instruments were controlled by HP Enhanced ChemStation (version B.01.00).

Autosampler vials were loaded onto the autosampler, and 1 μL was injected in splitless mode. The oven was held steady at 40°C for 0.5 min. Next, the temperature was rapidly increased to 140°C at a rate of 75°C/min. The temperature was then raised at a rate of 1°C/min to 160°C. Finally, the temperature was increased to 270°C at a rate of 10°C/min. All temperatures were raised using a linear gradient. The oven temperature program is shown in Table 1.

Separation was achieved using a SolGel-1ms GC capillary column (60 m x 0.25 mm id, 0.25 μm film thickness) from SGE (Austin, TX), which was connected via a fused silica union to a Z guard column (5 m x 0.25 mm id) from Phenomenex (Torrance, CA). The MS was operated in negative ion chemical ionization mode (NICI) with selected ion monitoring (SIM). The fixed element emission current was 50 μA and the electron energy was 150 eV. The reagent gas was methane, and its pressure was set at 0.18 mTorr. Ions at m/z of 141 (4-ene-VPA), 148 (2-ene-[\text{H}]_{7}-VPA), 273 (4-OH-VPA, 5-OH-VPA, and 3-OH-VPA), and 280 (4-OH-[\text{H}]_{7}-VPA, 5-OH-[\text{H}]_{7}-VPA, and 3-OH-[\text{H}]_{7}-VPA) were monitored.
Table 1. GC oven temperature program.

<table>
<thead>
<tr>
<th>Start Time (min)</th>
<th>End Time (min)</th>
<th>Start Temp (°C)</th>
<th>Final Temp (°C)</th>
<th>Ramp (°C/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>40</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>1.83</td>
<td>40</td>
<td>140</td>
<td>75</td>
</tr>
<tr>
<td>1.83</td>
<td>21.83</td>
<td>140</td>
<td>160</td>
<td>1</td>
</tr>
<tr>
<td>21.83</td>
<td>37.83</td>
<td>160</td>
<td>270</td>
<td>10</td>
</tr>
</tbody>
</table>
2.6. Data Analysis

2.6.1. Calculation of PAR

Chromatograms were integrated using ChemStation Integrator. Peaks were manually identified and peak areas were entered into Microsoft Excel (Redmond, WA). Peak area ratio (PAR) was calculated by dividing the peak area of a metabolite by the peak area of its heptadeuterated analog. For 4-ene-VPA, 2-ene-[2H7]-VPA was used as the internal standard due to poor resolution of 4-ene-[2H7]-VPA. To calculate the PAR for 4-OH-VPA, the sum of the peak areas of the two diastereomers of 4-OH-VPA was divided by the sum of the peak areas of the two diastereomers of 4-OH-[2H7]-VPA.

\[
\text{PAR of 4-ene-VPA} = \frac{\text{Peak area of 4-ene-VPA}}{\text{Peak area of 2-ene-[2H7]-VPA}}
\]

\[
\text{PAR of 4-OH-VPA} = \frac{\text{Peak areas of 4-OH-VPA (1) + 4-OH-VPA (2)}}{\text{Peak areas of 4-OH-[2H7]-VPA (1) + 4-OH-[2H7]-VPA (2)}}
\]

\[
\text{PAR of 5-OH-VPA} = \frac{\text{Peak area of 5-OH-VPA}}{\text{Peak area of 5-OH-[2H7]-VPA}}
\]

\[
\text{PAR of 3-OH-VPA} = \frac{\text{Peak area of 3-OH-VPA}}{\text{Peak area of 3-OH-[2H7]-VPA}}
\]

2.6.2. Standard curves

PAR values for the metabolite standards were calculated. Standard curves for 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA were constructed by plotting the PAR values against the amount of standard in grams. A best fit line was drawn using the least squares method. A relationship in the form \( y = mx + b \), where \( y \) is the PAR, \( m \) is the slope, \( x \) is the amount of standard in grams, and \( b \) is the \( y \)-intercept, was determined for each of the four metabolites.
2.6.3. *Microsomal incubations*

PAR values for enzyme incubations and blanks were calculated. The PAR values for enzyme incubations were corrected by subtracting from them the PAR value for the blank. The amount of metabolite in a particular sample was determined by calculating the values of $x$ from the standard curve equation, based on the corrected PAR values of that particular sample. For incubations with HLM, product formation is expressed as pmol/min/nmol total CYP; and for incubations with recombinant CYP, as pmol/min/nmol CYP.

2.6.4. *Limit of quantitation*

The limit of quantitation (LOQ) was determined for 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. LOQ is defined as the minimum amount of metabolite that can be detected with acceptable precision and accuracy. According to convention, coefficient of variation (CV), a measure of precision, of less than 20% is considered acceptable. Bias, a measure of accuracy, of less than 20% is acceptable (Shah et al., 1991).

Standard curves were constructed and 0.5, 1, 1.5 and 2 ng of metabolite standards were spiked into the same matrix as that of the standard curve. Six replicates were performed for each amount of standard. The measured amounts of standard were calculated from the standard curves, and CV and bias were determined.

\[
CV = \frac{\text{Standard Deviation of Measured Amount}}{\text{Mean of Measured Amount}} \times 100\%
\]

\[
\text{Bias} = \frac{\text{Mean of Measured Amount} - \text{Nominal Amount}}{\text{Nominal Amount}} \times 100\%
\]
2.6.5. *Intraday and interday variabilities*

The intraday and interday variabilities of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA formation were determined. Standard curves were constructed. Microsomal incubations were performed on four different days. Four determinations were carried out on each day. Intraday variability, as measured by the CV of product formation of four replicates on one day, of less than 20% was considered acceptable. Interday variability, as measured by the CV of the average product formation over four days, of less than 20% was considered acceptable (Shah et al., 1991).

2.7. *Statistical Analysis*

In experiments involving recombinant CYP or HLM from only one donor, data are expressed as mean ± standard deviation (SD). In experiments where HLM from more than one donor were used, data are expressed as mean ± standard error of the mean (SEM). In cases where product formation was less than LOQ, a value midway between limit of detection (LOD) and LOQ is used in the calculation of the mean. Since LOD was not determined in this study, in such cases the midpoint of nought and LOQ (i.e. half of LOQ) was used. Statistical significance for differences between treatment groups was determined by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test using SigmaStat for Windows (version 1.0) from Jandel (San Rafael, CA). Statistical significance for differences between treatment groups and control was determined using one-way ANOVA followed by Dunnett's test. Differences were considered statistically significant if \( p < 0.05 \).
3. RESULTS

3.1. Validation of VPA GC/MS Assay

3.1.1. Chromatograms

The MS recorded the total ion current. A representative total ion chromatogram is shown in Figure 6. The MS also performed SIM.

The molecular ion of 4-ene-VPA, visible at $m/z = 141$, was clearly resolved as shown in Figure 7A. Due to the relatively large amount of VPA ($m/z = 143$), some carry-over was seen at $m/z = 141$. As shown in Figure 7B, at $m/z = 148$, 4-ene-[\textsuperscript{2}H\textsubscript{7}]-VPA was not adequately resolved. Therefore, 2-ene-[\textsuperscript{2}H\textsubscript{7}]-VPA was used as the internal standard for 4-ene-VPA. On a representative day, the retention time for 4-ene-VPA was 21.16 min. Its heptadeuterated analog, 4-ene-[\textsuperscript{2}H\textsubscript{7}]-VPA, eluted 0.25 min earlier, at 20.91 min. Elution of 2-ene-[\textsuperscript{2}H\textsubscript{7}]-VPA occurred at 24.34 min.

As shown in Figure 8A, at $m/z = 273$, the molecular ions of 3-OH-VPA, 5-OH-VPA, and the two diastereomers of 4-OH-VPA were resolved. At $m/z = 280$, 3-OH-[\textsuperscript{2}H\textsubscript{7}]-VPA, 5-OH-[\textsuperscript{2}H\textsubscript{7}]-VPA and the two diastereomers of 4-OH-[\textsuperscript{2}H\textsubscript{7}]-VPA were resolved, as shown in Figure 8B.
The two diastereomers of 3-OH-[\textsuperscript{2}H\textsubscript{7}]-VPA were not separated and appeared as one peak. On a representative day, the retention times for the diastereomers of 4-OH-VPA were 32.13 and 32.39 min. Elution of 3-OH-VPA occurred at 32.24 min. The retention time of 5-OH-VPA was 33.45 min. Their heptadeuterated analogs eluted approximately 0.05 min earlier, at 32.08, 32.34, 32.19, and 33.40 min, respectively. Retention times are listed in Table 2.

3.1.2. Standard curves

Standard curves were constructed for 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. For the 4-ene-VPA standard curve, 0.5-10 ng of 4-ene-VPA standard was used. For 4-OH-VPA, 2-100 ng of 4-OH-VPA standard was used to construct the standard curve. For the 5-OH-VPA standard curve, 2-100 ng of 5-OH-VPA standard was used. For 3-OH-VPA, 0.5-5 ng of 3-OH-VPA standard was used to construct the standard curve. The standard curves were linear in their dynamic ranges. Representative standard curves from one experiment are shown in Figure 9.

3.1.3. Limit of quantitation

The LOQs of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA were determined. Their values were 0.5 ng (3.5 pmol), 2 ng (12.5 pmol), 2 ng (12.5 pmol), and 0.5 ng (3.1 pmol), respectively, as shown in Error! Reference source not found.. At the LOQ, accuracy (bias) and precision (CV) were both less than 20% for all four metabolites.
Figure 6. Representative total ion chromatogram. This sample contained 100 ng of each metabolite and internal standard. Peaks for (I) 4-ene-VPA and 4-ene-[\(^2\)H\(_7\)]-VPA; (II) 2-ene-[\(^2\)H\(_7\)]-VPA; (III) 3-OH-VPA, 3-OH-[\(^2\)H\(_7\)]-VPA, 4-OH-VPA, and 4-OH-[\(^2\)H\(_7\)]-VPA; and (IV) 5-OH-VPA and 5-OH-[\(^2\)H\(_7\)]-VPA can be seen.
Figure 7. Representative chromatograms for metabolite standards at $m/z$ of 141 and 148. In this sample, the metabolite standard contained 100 ng each of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. The internal standard contained 100 ng each of 2-ene-[${^2}$H$_7$]-VPA, 4-ene-[${^2}$H$_7$]-VPA, 4-OH-[${^2}$H$_7$]-VPA, 5-OH-[${^2}$H$_7$]-VPA, and 3-OH-[${^2}$H$_7$]-VPA. At (A) $m/z = 141$, (I) 4-ene-VPA and (II) VPA are visible. At (B) $m/z = 148$, (III) 4-ene-[${^2}$H$_7$]-VPA and (IV) 2-ene-[${^2}$H$_7$]-VPA are visible.
Figure 8. Representative chromatograms for metabolite standards at $m/z$ of 273 and 280. In this sample, the metabolite standard contained 100 ng each of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. The internal standard contained 100 ng each of 2-ene-$[^{2}H_{7}]$-VPA, 4-ene-$[^{2}H_{7}]$-VPA, 4-OH-$[^{2}H_{7}]$-VPA, 5-OH-$[^{2}H_{7}]$-VPA, and 3-OH-$[^{2}H_{7}]$-VPA. At (A) $m/z = 273$, (I) 4-OH-VPA, (II) 5-OH-VPA, and (III) 3-OH-VPA are visible. At (B) $m/z = 280$, (IV) 4-OH-$[^{2}H_{7}]$-VPA, (V) 5-OH-$[^{2}H_{7}]$-VPA, and (VI) 3-OH-$[^{2}H_{7}]$-VPA are visible.
Table 2. Retention times of VPA and its metabolites on a representative day.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/z</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-ene-VPA</td>
<td>141</td>
<td>21.16</td>
</tr>
<tr>
<td>VPA</td>
<td>141</td>
<td>21.51</td>
</tr>
<tr>
<td>4-ene-[2H7]-VPA $^a$</td>
<td>148</td>
<td>20.91</td>
</tr>
<tr>
<td>2-ene-[2H7]-VPA</td>
<td>148</td>
<td>24.34</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>273</td>
<td>32.13</td>
</tr>
<tr>
<td>3-OH-VPA</td>
<td>273</td>
<td>32.24</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>273</td>
<td>32.39</td>
</tr>
<tr>
<td>5-OH-VPA</td>
<td>273</td>
<td>33.45</td>
</tr>
<tr>
<td>4-OH-[2H7]-VPA</td>
<td>280</td>
<td>32.08</td>
</tr>
<tr>
<td>3-OH-[2H7]-VPA</td>
<td>280</td>
<td>32.19</td>
</tr>
<tr>
<td>4-OH-[2H7]-VPA</td>
<td>280</td>
<td>32.34</td>
</tr>
<tr>
<td>5-OH-[2H7]-VPA</td>
<td>280</td>
<td>33.40</td>
</tr>
</tbody>
</table>

$^a$The peak for 4-ene-[2H7]-VPA was not adequately resolved. $^b$VPA has m/z = 143, but due to its relatively high concentration, some carry-over is seen at m/z = 141.
Figure 9. Representative standard curves. For (A) 4-ene-VPA, 0.5-10 ng of standard 4-ene-VPA was used. For (B) 4-OH-VPA, 2-100 ng of 4-OH-VPA was used. For (C) 5-OH-VPA, 2-100 ng of 5-OH-VPA was used. For (D) 3-OH-VPA, 0.5-5 ng of 5-OH-VPA was used. The internal standard contained 100 ng each of 2-ene-[2H7]-VPA, 4-OH-[2H7]-VPA, 5-OH-[2H7]-VPA, and 3-OH-[2H7]-VPA. PAR values were calculated by dividing the peak areas of the metabolite standards by the peak areas of their respective internal standards.
Table 3. Coefficient of variation and bias of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA standards.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CV (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 pmol 4-ene-VPA</td>
<td>15</td>
<td>-7</td>
</tr>
<tr>
<td>12.5 pmol 4-OH-VPA</td>
<td>11</td>
<td>-20</td>
</tr>
<tr>
<td>12.5 pmol 5-OH-VPA</td>
<td>14</td>
<td>-3</td>
</tr>
<tr>
<td>3.1 pmol 3-OH-VPA</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

Samples containing nominal amounts of standards were assayed. Amounts of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA were calculated from standard curves. The CV of 6 replicates was calculated for measured amounts of standard. Bias comparing measured versus nominal amounts of spiked standard were determined.
3.2. Optimization of VPA Metabolism Assay

3.2.1. Optimization of total CYP content for incubations with HLM

HLM containing 20, 40, 60, 80, or 100 pmol of total CYP were incubated for 20 min with 1 mM VPA. The formation of 4-ene-VPA and was linear up to 60 pmol of total CYP (Figure 10A). The formation of 4-OH-VPA was linear up to at least 100 pmol of total CYP (Figure 10B). The formation of 5-OH-VPA was linear up to at least 100 pmol of total CYP (Figure 10C). The formation of 3-OH-VPA was linear up to 60 pmol of total CYP (Figure 10D). Thus, 60 pmol of total CYP was chosen as the amount to use in all further experiments with HLM.

3.2.2. Optimization of incubation time for incubations with HLM

HLM containing 60 pmol of total CYP were incubated for 10, 20, 30, 40, or 50 min with 1 mM VPA. The formation of 4-ene-VPA was linear up to 20 min (Figure 11A). The formation of 4-OH-VPA was linear up to 30 min (Figure 11B). The formation of 5-OH-VPA was linear up to 30 min (Figure 11C). The formation of 3-OH-VPA was linear up to 20 min (Figure 11D). Thus, an incubation time of 20 min was used in all further experiments with HLM.
Figure 10. Optimization of total CYP content for incubations with HLM. HLM containing 0, 20, 40, 60, 80, or 100 pmol of total CYP were incubated with 1 mM VPA for 20 min. The formation of (A) 4-ene-VPA, (B) 4-OH-VPA, (C) 5-OH-VPA, and (D) 3-OH-VPA was monitored. Error bars represent SD (3 determinations).
Figure 11. Optimization of incubation time for incubations with HLM. HLM containing 60 pmol of total CYP were incubated with 1 mM VPA for 0, 10, 20, 30, 40 or 50 min. The formation of (A) 4-ene-VPA, (B) 4-OH-VPA, (C) 5-OH-VPA, and (D) 3-OH-VPA was monitored. Error bars represent SD (3 determinations).
3.2.3. **Intraday and interday variabilities for incubations with HLM**

HLM containing 60 pmol of total CYP were incubated for 20 min with 1 mM VPA. Mean formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA over the four days were 30±4, 436±49, 303±20, and 11±3 pmol/min/nmol total CYP, respectively. As shown in Table 4, the intraday variabilities of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA were less than 20%. As shown in Table 5, although the interday variabilities of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA were less than 20%, the interday variability was not less than 20% for 3-OH-VPA. Therefore, for samples from the same donor, all treatments in one experiment were performed on the same day to avoid any potential issues with interday variability.

3.3. **Effect of GBE on VPA Metabolism**

3.3.1. **Overview of experiments**

The effect of GBE on VPA metabolism was investigated in a series of experiments. First, the effect of different brands and lots of GBE on VPA metabolism by HLM was examined (Section 3.3.2). Then, using one lot of one brand of GBE, the concentration of GBE was varied to observe the dose-dependence of inhibition and to determine the IC$_{50}$, the concentration at which 50% inhibition is observed (Section 3.3.3). Next, immunoinhibition studies were employed to ascertain which CYP isoform in HLM GBE inhibits (Section 3.3.5). Recombinant CYPs were then used to confirm which CYP isoforms are inhibited by GBE (Section 3.3.4). Finally, individual constituents of GBE were tested to determine if they are responsible for the inhibitory effect of GBE (Section 3.3.5).
Table 4. Intraday variabilities of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA formation by HLM.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-ene-VPA</td>
<td>7</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>5</td>
</tr>
<tr>
<td>5-OH-VPA</td>
<td>6</td>
</tr>
<tr>
<td>3-OH-VPA</td>
<td>16</td>
</tr>
</tbody>
</table>

HLM from donor HG30 containing 60 pmol of total CYP were incubated with 1 mM VPA for 20 min. The formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA was monitored. CV is that of 4 determinations performed on one day.
Table 5. Interday variabilities of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA formation by HLM.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-ene-VPA</td>
<td>14</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>11</td>
</tr>
<tr>
<td>5-OH-VPA</td>
<td>7</td>
</tr>
<tr>
<td>3-OH-VPA</td>
<td>30</td>
</tr>
</tbody>
</table>

HLM from donor HG30 containing 60 pmol of total CYP were incubated with 1 mM VPA for 20 min. The formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA was monitored. CV is that of 4 determinations performed on 4 separate days.
Representative chromatograms for microsomal incubations at m/z of 141 and 148. HLM were incubated with 1 mM VPA. At (A) m/z = 141, the formation of (I) 4-ene-VPA can be seen, and at (B) m/z = 148, the internal standard (II) 2-ene-[^2H_2]-VPA is visible. HLM were incubated with 1 mM VPA and 0.3 mg/mL GBE (Indena, lot 1306A). At (C) m/z = 141, a decrease in the formation of 4-ene-VPA can be seen, and at (D) m/z = 148, the internal standard 2-ene-[^2H_2]-VPA is visible.
Figure 13. Representative chromatograms for microsomal incubations at m/z of 273 and 280. HLM were incubated with 1 mM VPA. At (A) m/z = 273, the formation of (I) 4-OH-VPA, (II) 5-OH-VPA, and (III) 3-OH-VPA can be seen, and at (B) m/z = 280, the internal standards (IV) 4-OH-$[^2]H_7$-VPA, (V) 5-OH-$[^2]H_7$-VPA, and (VI) 3-OH-$[^2]H_7$-VPA are visible. HLM were incubated with 1 mM VPA and 0.3 mg/mL GBE (Indena, lot 1306A). At (C) m/z = 273, the formation of 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA can be seen, and at (B) an m/z = 280, the internal standards 4-OH-$[^2]H_7$-VPA, 5-OH-$[^2]H_7$-VPA, and 3-OH-$[^2]H_7$-VPA are visible.
The formation of 4-ene-VPA (Figure 12A) and of 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA (Figure 13A) can be seen in chromatograms. The inhibition of the formation of 4-ene-VPA (Figure 12C) and of 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA (Figure 13C) by GBE can also be seen visually in chromatograms.

3.3.2. Effect of GBE from different lots and manufacturers on VPA metabolism by HLM

HLM containing 60 pmol of total CYP were incubated for 20 min with 1 mM VPA and 0.3 mg/mL of GBE (Indena, lot 1306A), GBE from a different lot (Indena, lot 302831), or GBE from a different manufacturer (Pharmaton, lot 63964). Control values for the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA were 19±3, 311±39, 206±28, and 9±2 pmol/min/nmol total CYP, respectively. The three GBE preparations inhibited the formation of all four metabolites. Formation of all four metabolites was significantly different from control. As shown in Figure 14, all three extracts inhibited 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA formation to a similar extent. Inhibition of 4-ene-VPA formation, however, varied among the extracts. For all further experiments, the extract which was the most characterized chemically (Indena, lot 1306A, Appendix 1) was used.

3.3.3. Concentration-dependent effect of GBE on VPA metabolism by HLM

HLM containing 60 pmol of total CYP were incubated for 20 min with 1 mM VPA and 0.01, 0.03, 0.1, 0.3, 0.6, or 1 mg/mL of GBE (Indena, lot 1306A). Control values for the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA were 18±5, 256±40, 173±22, and 9±2 pmol/min/nmol total CYP, respectively. As shown in Figure 15, GBE inhibited the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA in a dose-dependent manner with IC\textsubscript{50} values of 0.34±0.04, 0.18±0.03, 0.21±0.02, and 0.37±0.10 mg/mL, respectively.
Figure 1A. Effect of different lots and brands of GBE on VPA metabolism by HTM. HLM were incubated with 1 mM VPA and 0.3 mg/ml of GBE (lot 1306A). GBE from a different lot (labeled 10.3023) or GBE from a different manufacturer were incubated with 1 mM VPA. Product formation is relative to control (water). The formation of (A) 4-ene-VPA, (B) 4-OH-VPA, and (C) 3-OH-VPA was monitored. Error bars represent SEM (n = 4). * Significantly different from Indena (lot 3023) (p < 0.05).

(Figure 1B) Product Formation (Percentage of Control)

(Figure 1C) Product Formation (Percentage of Control)

(Figure 1D) Product Formation (Percentage of Control)
Figure 15. Effect of GBE on VPA metabolism by HLM. HLM were incubated with 1 mM VPA and 0.01, 0.03, 0.1, 0.3, 0.6, or 1 mg/mL of GBE (Indena, lot 1306A). Product formation is relative to control (water). The formation of (A) 4-ene-VPA, (B) 4-OH-VPA, (C) 5-OH-VPA, and (D) 3-OH-VPA was monitored. Error bars represent SEM (n = 4).
3.3.4. Effect of GBE on CYP2C9-catalyzed VPA metabolism by HLM

HLM containing 60 pmol of total CYP were pre-incubated with 3 μL of MAb2C9 then incubated for 20 min with 1 mM VPA, pre-incubated with 3 μL of MAb2B6 and 3 μL of MAb2A6 then incubated for 20 min with 1 mM VPA, or pre-incubated with 3 μL of MAb2B6 and 3 μL of MAb2A6 then incubated for 20 min with 1 mM VPA and 1 mg/mL of GBE (Indena, lot 1306A). Mean values for the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by microsomes in the absence of antibodies and control IgG were 22±2, 333±42, and 230±26 pmol/min/nmol total CYP, respectively. All inhibition by treatment groups were compared to control incubations, in which an equivalent volume of control IgG was pre-incubated with HLM. The formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by HLM was reduced by 73±1%, 75±0%, and 77±2%, respectively, when microsomes were pre-incubated with MAb2C9. The formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA was reduced slightly after pre-incubation with MAb2B6 and MAb2A6. Inhibition was 19±2%, 20±10%, and 17±5%, respectively. The formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by HLM pre-incubated with MAb2B6 and MAb2A6 was strongly inhibited by GBE. GBE reduced the formation of 4-ene-VPA to levels below LOQ. The formation of 4-OH-VPA and 5-OH-VPA was reduced by 89±1% and 86±1%, respectively. All inhibition seen was statistically different from control.
Figure 16. Effect of GBE on CYP2C9-mediated VPA metabolism by HLM. HLM were pre-incubated with MAb2C9 then incubated with 1 mM VPA, pre-incubated with MAb2B6 and MAb2A6 then incubated with 1 mM VPA, or pre-incubated with MAb2B6 and MAb2A6 then incubated with 1 mM VPA and 1 mg/mL of GBE (Indena, lot 1306A). The formation of (A) 4-ene-VPA, (B) 4-OH-VPA, and (C) 5-OH-VPA is shown. Values are relative to control activities (equivalent volume of control IgG), shown in panels (D), (E), and (F). Error bars represent SEM (n = 3). *Significantly different from control (p < 0.05).
3.3.5. **Effect of GBE on VPA metabolism by recombinant CYP2C9*1, CYP2B6, and CYP2A6**

Recombinant CYP2C9*1, CYP2B6, and CYP2A6 containing 40 pmol of CYP were incubated for 30 min with 1 mM VPA and 0.3 or 1 mg/mL GBE (Indena, lot 1306A). Control values for the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by cDNA-expressed CYP2C9*1 were 18±4, 252±66, and 143±46 pmol/min/nmol CYP, respectively. No 3-OH-VPA was detected. As shown in Figure 17, GBE at both concentrations significantly inhibited the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by cDNA-expressed CYP2C9*1.

Control values for the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by cDNA-expressed CYP2B6 were 12±1, 224±17, and 229±40 pmol/min/nmol CYP, respectively. No 3-OH-VPA was detected. GBE at both concentrations significantly inhibited the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by cDNA-expressed CYP2B6, as shown in Figure 18. The inhibitory effect of GBE was not as strong as for recombinant CYP2C9*1.

Control values for the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA by cDNA-expressed CYP2A6 were 6±1, 82±2, 31±1, and 16±1 pmol/min/nmol CYP, respectively. As shown in Figure 19, 1 mg/mL of GBE significantly inhibited the formation of 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA by cDNA-expressed CYP2A6. At 1 mg/mL of GBE, the formation of 4-ene-VPA was below LOQ. The inhibitory effect of GBE on recombinant CYP2A6 was weak compared to the effect on recombinant CYP2C9*1 and CYP2B6.
Figure 17. Effect of GBE on VPA metabolism by recombinant CYP2C9*1. Recombinant CYP2C9*1 microsomes were incubated with 1 mM VPA and 0.3 or 1 mg/mL of GBE (Indena, lot 1306A). The formation of (A) 4-ene-VPA, (B) 4-OH-VPA, and (C) 5-OH-VPA is shown. Error bars represent SD (3 determinations). *Significantly different from control ($p < 0.05$).
Figure 18. Effect of GBE on VPA metabolism by recombinant CYP2B6. Recombinant CYP2B6 microsomes were incubated with 1 mM VPA and 0.3 or 1 mg/mL of GBE (Indena, lot 1306A). The formation of (A) 4-ene-VPA, (B) 4-OH-VPA, and (C) 5-OH-VPA is shown. Error bars represent SD (3 determinations). *Significantly different from control (p < 0.05).
Figure 19. Effect of GBE on VPA metabolism by recombinant CYP2A6. Recombinant CYP2A6 microsomes were incubated with 1 mM VPA and 0.3 or 1 mg/mL of GBE (Indena, lot 1306A). The formation of 3-OH-VPA is shown. Error bars represent SD (3 determinations). *Significantly different from control ($p < 0.05$).
3.3.6. Effect of individual constituents of GBE on VPA metabolism by HLM

HLM were incubated for 20 min with 1 mM VPA and 500 μg/mL of GBE (Indena, lot 1306A, Appendix 1), 14 μg/mL of bilobalide, 5.5 μg/mL of ginkgolide A, 1.5 μg/mL of ginkgolide B, 7 μg/mL of ginkgolide C, 3 μg/mL of ginkgolide J, 3 μg/mL of isorhamnetin-3-O-rutinoside, 5 μg/mL of kaempferol-3-O-rutinoside, 12 μg/mL of quercetin-3-O-rutinoside, 20.5 μg/mL of isorhamnetin aglycone, 31.5 μg/mL of kaempferol aglycone, or 53 μg/mL of quercetin aglycone. The concentrations of the terpene trilactones and isorhamnetin-3-O-rutinoside were the concentrations present in 500 μg/mL of GBE. The concentrations of kaempferol-3-O-rutinoside and quercetin-3-O-rutinoside were the concentrations of their more abundant unidentified diglycoside (kaempferol diglycoside 1 and quercetin diglycoside 2 in Appendix 1). The concentrations of the flavonol aglycones were the concentrations that would be present in 500 μg/mL of GBE if all the flavonol glycosides were converted to their respective aglycones (sum under isorhamnetin, kaempferol, and quercetin in Appendix 1). Control values (no DMSO) for the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA by HLM were 23±5, 325±31, 213±17, and 12±1 pmol/min/nmol total CYP, respectively. For vehicle control (0.05% DMSO, v/v), formation was 21-27% lower.

As shown in Figure 20, bilobalide and ginkgolides A, B, C, and J did not inhibit VPA metabolism. GBE appeared to inhibit the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and perhaps 3-OH-VPA; however, due to the large variability seen on this day, GBE was not significantly different from control by one-way ANOVA.
Inhibition was not statistically different from control (p 0.05).

Ivermectin SEM (n = 4). Ginkgo biloba (GB) inhibited the formation of 4-ene-VP4, 4-ene-VP4, 4-ene-VP4, 4-ene-VP4, and Ginkgolide B. Inhibition of Ginkgolide B, C, or J at the concentrations found in 0.5 mg/ml of Ginkgo biloba vehicle control was 0.05% DMSO.

Figure 2. Effect of terpene fractions on VP4 metabolism by HLM. HLM were incubated with 1 mM VP4 and Ginkgo biloba fractions of 4-ene-VP4, 4-ene-VP4, 4-ene-VP4, and Ginkgolide B. Inhibition of Ginkgolide B, C, or J at the concentrations found in 0.5 mg/ml of Ginkgo biloba vehicle control was 0.05% DMSO.
Shown, Error bars represent SEM (n = 4). *Significantly different from control (p < 0.05).

Figure 2. Effect of flavonol glycosides on VPA metabolism by HLM. HLM were incubated with 1 mM VPA and

<table>
<thead>
<tr>
<th>Compound</th>
<th>Product Formation (pmol/min/nmol total CYP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBE</td>
<td></td>
</tr>
<tr>
<td>Isorhamnetin-3-O-rutinoside</td>
<td></td>
</tr>
<tr>
<td>Kaempferol-3-O-rutinoside</td>
<td></td>
</tr>
<tr>
<td>Quercetin-3-O-rutinoside</td>
<td></td>
</tr>
</tbody>
</table>

### Notes
- GBE, isorhamnetin-3-O-rutinoside at the concentration found in 0.5 µg/mL GBE. 
- For kaempferol-3-O-rutinoside or quercetin-3-O-rutinoside or 4-OH-VPA, Vehicle control was 0.5% DMSO.

(1) 3-OH-VPA

(2) 4-OH-VPA

(3) 4-ene-VPA
Figure 22. Effect of flavonoid aglycones on VPA metabolism by HLM. HLM were incubated with 1 mM VPA and GBE or GB1. Significant difference from control (p > 0.05).

Product Formation (pmol/min/nmol total CYP)

(a) 3-OH-VPA

(b) 4-OH-VPA

(c) 5-OH-VPA

(d) 4-ene-VPA

Error bars represent SEM (n = 4).

Glyphosates were detected in their respective aglycones. Vehicle control was 0.05% DMSO. Their effect on the formation of the aglycones ofisorhamnetin, kaempferol, and quercetin at the concentrations that would be found in 0.5 mg/ml of GBE is all.

Control GBE

Isorhamnetin

Kaempferol

Quercetin

Product Formation (pmol/min/nmol total CYP)

Control GBE

Isorhamnetin

Kaempferol

Quercetin

Product Formation (pmol/min/nmol total CYP)

Control GBE

Isorhamnetin

Kaempferol

Quercetin

Product Formation (pmol/min/nmol total CYP)

Control GBE

Isorhamnetin

Kaempferol

Quercetin

Product Formation (pmol/min/nmol total CYP)

Control GBE

Isorhamnetin

Kaempferol

Quercetin

Product Formation (pmol/min/nmol total CYP)

Control GBE

Isorhamnetin

Kaempferol

Quercetin

Product Formation (pmol/min/nmol total CYP)

Control GBE

Isorhamnetin

Kaempferol

Quercetin
As shown in Figure 21, GBE significantly reduced the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. Formation of all four metabolites in incubations with the rutinosides of isorhamnetin, kaempferol, and quercetin was not significantly different from vehicle control.

GBE and the aglycones of kaempferol and quercetin significantly inhibited the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA, as shown in Figure 22. The aglycone of isorhamnetin inhibited 4-OH-VPA, and 5-OH-VPA formation. Quercetin exhibited the strongest effect, followed by kaempferol and isorhamnetin. No significant reduction of 3-OH-VPA formation was observed in incubations with any of the flavonol aglycones or GBE.
4. DISCUSSION

The VPA assay has been shown to be a sensitive assay for quantitating the VPA metabolites 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA (Abbott et al., 1986; Kassahun et al., 1989; Kassahun et al., 1990; Anari et al., 2000; Ho et al., 2003). In this study, the assay was used to measure the inhibition of their formation by GBE.

This assay proved especially suitable for the measurement of the inhibition of 4-OH-VPA and 5-OH-VPA formation. The amounts of 4-OH-VPA and 5-OH-VPA formation by HLM in the absence of GBE were quite high, averaging at about 64 and 42 ng (333 and 219 pmol/min/nmol total CYP), respectively. With the LOQ for both 4-OH-VPA and 5-OH-VPA being 2 ng, it was possible to quantify inhibition of up to 97 and 95 % on average, respectively.

The assay presented more challenges in the measurement of the inhibition of 4-ene-VPA and 3-OH-VPA formation. The amounts of 4-ene-VPA and 3-OH-VPA formation by HLM in the absence of GBE were only slightly greater than their LOQ of 0.5 ng, with production averaging about 3.7 and 2.2 ng (22 and 11 pmol/min/nmol total CYP), respectively. Thus, for 4-ene-VPA and 3-OH-VPA, it was possible to measure inhibition of up to 86 and 77 % on average, respectively.
In this study, the ratio of formation rates of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by HLM was 1:15:10. This is comparable to the ratio of 1:18:9 observed in HLM by Sadeque et al. (1997). The ratio of formation rates of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by recombinant CYP2C9*1 was 1:14:8. This is roughly comparable to the 1:10:11 ratio obtained by Ho et al. (2003). The relatively lower proportion of 4-OH-VPA obtained by Ho et al. may be due to slight differences in methodology. Ho et al. froze their incubation mixtures at -20°C until analysis. However, 4-OH-VPA has been shown to readily form a γ-lactone (Schafer and Luhrs, 1978), and the lower proportion seen may have been due to a loss of 4-OH-VPA between the time of incubation and derivatization. In this study, incubation mixtures immediately underwent extraction and derivatization, with 4-OH-VPA undergoing tert-butyldimethylsilylation to form a more stable molecule. The ratio of formation rates of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by recombinant CYP2B6 was 1:18:19. For recombinant CYP2A6, the ratio of formation rates of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA was 1:15:6.

Since natural products are complex mixtures of many different constituents and may differ from lot to lot and between manufacturers, to ensure that the inhibition seen with the GBE used in this project is representative of commercially available GBE products, it was compared to GBE from another lot from the same company and GBE from another manufacturer. The three preparations yielded similar inhibition of 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA formation. This is not surprising since they had been standardized to contain 24% flavonols and 6% terpene trilactones. There was, however, statistically different difference for 4-ene-VPA inhibition by the three GBE preparations, but this may be due to chance.

GBE inhibited the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA in a dose-dependent manner. In order to determine which isoform GBE inhibits to cause a reduction
in the formation of these metabolites, immunoinhibition studies were carried out. Key to determining which specific CYP isoforms are inhibited by GBE is an understanding of which isoforms are involved in the formation of these four VPA metabolites.

In the present study, MAb2C9 decreased the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by HLM from all three donors tested by approximately 75%. Similar inhibition was seen in another study, where MAb2C9 inhibited the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by HLM by at least 75%, with inhibition of at least 80% for recombinant CYP2C9*1 (Kiang et al., 2005). MAb2C9 has been shown to be highly specific against recombinant human CYP2C9*1, CYP2C9*2, and CYP2C9*3, while showing no cross-reactivity towards recombinant human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5, as measured by ELISA. Tolbutamide methylhydroxylation by recombinant CYP2C9*1 was inhibited by greater than 90% with MAb2C9. MAb2C9 inhibited diclofenac 4'-hydroxylation by 80-90% in recombinant CYP2C9*1, CYP2C9*2, and CYP2C9*3. In HLM, MAb2C9 inhibited diclofenac activity by 85-90% (Krausz et al., 2001). These results suggest that MAb2C9 inhibits CYP2C9*1 under these experimental conditions, and that CYP2C9 is the major isoform in HLM responsible for the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA. The residual activity is likely due to CYP2B6 and CYP2A6.

The present study showed that in HLM from all three donors tested, pre-incubation with MAb2B6 and MAb2A6 decreased the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by approximately 20%. Another study showed that pre-incubation of HLM with MAb2B6 or MAb2A6 alone had little or no effect on the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA. Pre-incubation with MAb2B6 alone resulted in at least 90% inhibition of the formation of
these three metabolites by CYP2B6. Pre-incubation with MAb2A6 alone resulted in at least 90% inhibition of the formation of these three metabolites by CYP2A6 (Kiang et al., 2005). MAb2B6 has been shown to exhibit strong binding to recombinant human CYP2B6, but without significant cross-reactivity to recombinant human CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, and CYP3A5, as tested by ELISA. MAb2B6 inhibited the metabolism of phenanthrene, diazepam, 7-ethoxycoumarin, and testosterone by cDNA-expressed CYP2B6 by 90-91% (Yang et al., 2003). ELISA and immunoblot analyses showed strong binding of MAb2A6 to recombinant human CYP2A6, while showing no significant cross-reactivity to CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5. MAb2A6 inhibited CYP2A6-catalyzed metabolism of coumarin by at least 94% in both cDNA-expressed CYP2A6 and HLM (Sai et al., 1999). Therefore, MAb2B6 and MAb2A6 inhibit CYP2B6 and CYP2A6 under these experimental conditions. With CYP2B6 and CYP2A6 inhibited, the approximately 80% of remaining activity is likely due to CYP2C9.

In HLM pre-incubated with MAb2B6 and MAb2A6, GBE decreased the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA to approximately 10% of control IgG activity. With the CYP2B6 and CYP2A6 being blocked by antibodies, the inhibition seen was due to the inhibition of CYP2C9 by GBE. In other words, the inhibition by GBE of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA formation by HLM is due mostly to the inhibition of CYP2C9 by GBE. In another study performed using HLM, an extract of Ginkgo biloba Special herbal tea inhibited CYP2C9 (Foster et al., 2003). However, this blended tea contained other herbal ingredients such as lemongrass, licorice root, spearmint leaf, peppermint leaf, cinnamon bark, ginger root, and sage leaf in addition to standardized GBE. Since some of these other components are known to inhibit
CYP (Paolini et al., 1998; Unger and Frank, 2004), the inhibition seen cannot be attributed solely to *Ginkgo biloba*.

To confirm the results seen in immunoinhibition studies with HLM, the effect of GBE on VPA metabolism by recombinant CYP was examined. GBE inhibited the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by cDNA-expressed CYP2C9*1 in a dose-dependent manner. GBE strongly inhibited the formation of these three metabolites. Gaudineau et al. (2004) also observed inhibition of cDNA-expressed CYP2C9 by GBE, with a $K_i$ of 14±4 μg/mL. The formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by cDNA-expressed CYP2B6 was also inhibited by GBE in a dose-dependent manner, although the inhibition seen was not as strong as for CYP2C9*1. GBE inhibited the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA, and 3-OH-VPA by cDNA-expressed CYP2A6 in a dose-dependent manner. Inhibition of CYP2A6 was relatively weak.

Experiments with recombinant CYP confirmed the results observed with the immunoinhibition studies. Namely, that the major CYP isoforms involved in the oxidative metabolism of VPA to 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA are CYP2C9, CYP2B6, and CYP2A6; and that GBE inhibits all three isoforms. CYP2A6 is the major isoform responsible for 3-OH-VPA formation. These results are in agreement with those of Kiang et al. (2005), who used a panel of human cDNA-expressed microsomes to investigate the metabolism of VPA to 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. The desaturation of VPA to 4-ene-VPA was catalyzed by CYP2C9*1, CYP2B6, and CYP2A6, with CYP2C9*1 exhibiting the highest activity. Formation of 4-OH-VPA by (ω-1)-hydroxylation was catalyzed by CYP2C9*1, CYP2B6, and CYP2A6 with CYP2C9*1 being the most active. CYP2C9*1, CYP2B6, and CYP2A6 catalyzed ω-hydroxylation of VPA to 5-OH-VPA. CYP2A6 was the most active in the
(ω-2)-hydroxylation of VPA to 3-OH-VPA, with CYP1A1, CYP2B6 CYP4F3B, and CYP4F2 exhibiting lower activity. CYP1A2, CYP1B1, CYP2B8, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A11, and CYP4F3A did not display any significant activity in the formation of these four metabolites.

GBE is a complex mixture of many constituents. In an effort to discover which constituent of GBE is responsible for its inhibitory effect seen on VPA metabolism, the effect of individually quantified and commercially available constituents on VPA metabolism by HLM was investigated.

None of the individual terpene trilactones at the concentrations found in the extract, namely 14 μg/mL (43 μM) of bilobalide, 5.5 μg/mL (13 μM) of ginkgolide A, 1.5 μg/mL (4 μM) of ginkgolide B, 7 μg/mL (16 μM) of ginkgolide C, and 3 μg/mL (7 μM) of ginkgolide J, inhibited VPA metabolism. This is in agreement with another study which showed that 153 pM bilobalide, 200 pM ginkgolide A, 200 μM ginkgolide B, and 200 μM ginkgolide C did not inhibit cDNA-expressed CYP2C9 (Zou et al., 2002). Similarly, bilobalide and ginkgolides A, B, C, and J had either weak or negligible inhibitory capacity (defined as less than 50% inhibition at a concentration of 100 μg/mL) in recombinant CYP2C9 (von Moltke et al., 2004). In contrast, He and Edeki (2004) reported that ginkgolides A and B inhibited CYP2C9 in HLM with IC_{50} values of 113 and 168 μM, respectively. However, the concentrations at which they saw inhibition were approximately 10-fold greater than the concentrations used in this study, and thus would not explain the inhibition by GBE of CYP2C9-mediated VPA metabolism. Gaudineau et al. (2004) observed that the terpenoid fraction of GBE strongly inhibited recombinant CYP2C9, with a K_i of 15±6 μg/mL. However, such this crude terpenoid fraction likely contained compounds other than the terpene trilactones tested in this project. Thus, individually, the
terpene trilactones were not responsible for the inhibitory effect of GBE on CYP2C9. No studies pertaining to the effect of terpene trilactones on CYP2B6 or CYP2A6 have been published to date.

The flavonol glycosides tested did not inhibit VPA metabolism. Isorhamnetin-3-O-rutinoside was identified in the extract. The identities of the two diglycosides of kaempferol and the two diglycosides quercetin were not determined. It was assumed that one of kaempferol diglycosides was kaempferol-3-O-rutinoside and one of quercetin diglycosides was quercetin-3-O-rutinoside. This was most likely a safe assumption, since it is known that the major diglycosides of kaempferol and quercetin contain biloside or rutinose as sugar moieties (Bedir et al., 2002). Therefore, individually, these three flavonol diglycosides do not explain the inhibitory effect of GBE. This is in agreement with a study by von Moltke et al. (2004), in which four kaempferol glycosides and five quercetin glycosides extracted from Ginkgo biloba leaves showed either weak or negligible (less than 50%) inhibition of CYP2C9 at a much higher concentration of 100 µg/mL. The concentrations of kaempferol-3-O-rutinoside and quercetin-3-O-rutinoside used for this project were 5 µg/mL and 12 µg/mL, respectively. However, there were other flavonol glycosides present in GBE that were not identified and thus not tested. A flavonoid fraction isolated from GBE by Gaudineau et al. (2004) inhibited CYP2C9. However, it is possible that chemical constituents other than that flavonol glycosides eluted in this fraction. Thus, it cannot be concluded that flavonol glycosides are responsible for the inhibition of VPA metabolism by GBE.

Although the terpene trilactones and flavonol glycosides tested did not inhibit VPA metabolism, there are many other constituents in GBE which could explain the inhibition seen with the extract. Other constituents known to be inhibitory towards CYP2C9 in HLM include
amentoflavone, sesamin, (Z,Z)-4,4'-(1,4-pentadiene-1,5-diyl)diphenol and 3-nonadec-8-enyl-
benzene-1,2-diol. In particular, amentoflavone exhibited strong inhibition of CYP2C9, with an
IC\textsubscript{50} of 0.035 \textmu M (von Moltke et al., 2004). Another candidate is tamarixetin, which is
metabolized to quercetin by HLM and cDNA-expressed CYP2C9 (Breinholt et al., 2002).
However, whether these constituents were present in the extract used in this study is not known.
Ginkgolic acids I and II significantly inhibited cDNA-expressed CYP2C9, with IC\textsubscript{50} values of
2.41 and 1.94 \textmu M, respectively (Zou et al., 2002). However, the ginkgolic acids are usually
removed from standardized extracts due to their toxicity (van Beek, 2002).

The identities of the constituents responsible for the inhibitory effect of GBE \textit{in vitro}
were not ascertained in this project. Although the flavonol glycosides did not inhibit VPA
metabolism \textit{in vitro}, flavonols may be of importance \textit{in vivo} since flavonol glycosides are
deglycosylated to their respective aglycones in human small intestine mucosa (Rasmussen and
Breinholt, 2003). Lactase-phlorizin hydrolase, which is localized to the apical membrane of
small intestinal epithelial cells, and cytosolic \beta-glucosidase have exhibited activity towards
flavonoid glycosides (Nemeth et al., 2003; Jiang et al., 2005). In humans, both flavonol
glycosides and aglycones are absorbed (Hollman and Katan, 1997).

The aglycones of kaempferol and quercetin inhibited the \textit{in vitro} formation of 4-ene-VPA,
4-OH-VPA, and 5-OH-VPA at the concentrations that would be present in 500 \textmu g/mL of GBE if
all the flavonol glycosides were converted to their respective aglycones. The over 50% inhibition
of formation of the three CYP2C9-catalyzed VPA metabolites by 175 \textmu M quercetin is consistent
with other studies. Zou et al. Values of IC\textsubscript{50} for inhibition of CYP2C9 in HLM range from 25.8
\textmu M (von Moltke et al., 2004) to 35 \textmu M (He and Edeki, 2004). Zou et al. (2002) reported an IC\textsubscript{50}
for recombinant CYP2C9 of 3.14 \textmu M, but warned that this may be an underestimate due to the
fluorescence of quercetin which may have interfered with their assay. In the present study, 65 μM isorhamnetin inhibited the formation of 4-OH-VPA and 5-OH-VPA. However, Zou et al. (2002) reported that isorhamnetin at 100 μM did not inhibit cDNA-expressed CYP2C9. Overall, these results point to inhibition of CYP2C9, the major isoform responsible for the formation of these metabolites, and perhaps CYP2B6 by the aglycones of isorhamnetin, kaempferol and quercetin. Since the formation of 3-OH-VPA was not inhibited by any of the flavonol aglycones, they are unlikely to inhibit CYP2A6 in vivo.

One possible reason that the aglycones inhibited VPA metabolism whereas the rutinosides did not is that rutinoside is a large sugar moiety. In rat hepatic microsomes, the aglycones of isorhamnetin, kaempferol, and quercetin inhibited EROD activity in rat hepatic microsomes, with inhibition decreasing as the size of the sugar moiety increased. Monoglycosides (isorhamnetin-3-O-glucoside, kaempferol-3-O-glucoside, and quercetin-3-O-glucoside) exhibited less inhibition than aglycones, and diglycosides (isorhamnetin-3-O-rutinoside, kaempferol-3-O-rutinoside, and quercetin-3-O-rutinoside) exhibited less inhibition than monoglycosides (Kuo et al., 2004). Thus, it is possible that the presence of bulky sugar moieties may decrease the inhibition by flavonols of human CYP2C9, CYP2B6, and CYP2A6 as they did in rat CYP1A.

While it is difficult to predict what will happen in vivo based on the results from in vitro studies, the inhibitory effect of GBE on CYP2C9, CYP2B6, and CYP2A6 may have several consequences. The inhibition of CYP2C9 may lead to higher plasma levels of drugs metabolized by CYP2C9 such as tolbutamide (Leemann et al., 1993), as well as drugs with a narrow therapeutic window, such as phenytoin (Veronese et al., 1991) and warfarin (Rettie et al., 1992). GBE may be involved in a pharmacokinetic interaction with warfarin through inhibition of
CYP2C9. However, it would be difficult to discern whether an interaction between GBE and warfarin is pharmacokinetic or pharmacodynamic, since the ginkgolides possess anti-platelet activity (Chung et al., 1987). Inhibition of CYP2B6 by GBE could lead to prodrugs such as cyclophosphamide not being activated (Chang et al., 1993). Inhibition of CYP2A6 by GBE could have a protective effect, since many nitrosamines in tobacco smoke are activated by CYP2A6 (Yamazaki et al., 1992).
5. LIMITATIONS AND FUTURE STUDIES

One advantage of the VPA assay is that it allows the study of multiple CYP isoforms by using only one substrate, VPA. However, since each VPA metabolite is formed by more than one CYP isoform, it is difficult to determine which isoform is being affected by a particular inhibitor. Also, since in this study VPA was used as the substrate, with the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA being monitored, the effect of GBE on only those isoforms involved in their production (CYP2C9, CYP2B6, and CYP2A6) could be ascertained. Other studies have shown the effect of GBE on other isoforms of CYP. For example, in addition to CYP2C9, GBE inhibits cDNA-expressed human CYP1A2, CYP2E1, and CYP3A4 (Budzinski et al., 2000; Gaudineau et al., 2004). In rat hepatic microsomes, GBE inhibits CYP1A (Kuo et al., 2004). In future studies, to gain a more complete profile of the effect of GBE on different CYP isoforms, specific probe substrates could be used to measure the activity of each isoform in HLM as well as in cDNA-expressed CYP.

This study demonstrated that GBE inhibits CYP2C9, CYP2B6, and CYP2A6 in HLM and cDNA-expressed microsomes. However, the results of experiments performed in vitro cannot always be extrapolated to the in vivo situation. It may be worthwhile to perform a study...
comparing \textit{in vitro} and \textit{in vivo} inhibition of CYP2C in an animal model. For example, Ohnishi et al. (2003) found that GBE inhibited rat CYP3A \textit{in vitro} in small intestine and liver microsomes, \textit{ex vivo} in small intestine and liver microsomes after a single oral administration of GBE, and \textit{in vivo} after a single oral administration of GBE using diltiazem as a probe for CYP3A. A similar design could be utilized, except using substrate probes for CYP2C, CYP2B, and CYP2A. Likewise, in humans, clearance of CYP2C9, CYP2B6, and CYP2A6 substrates after a single oral dose of GBE versus placebo could be investigated.

Another possible experiment would be to investigate induction by GBE. When GBE and tolbutamide were co-administered in rats, the hypoglycemic activity of tolbutamide was potentiated, pointing to inhibition of CYP2C by GBE. However, the hypoglycemic action of tolbutamide in rats pre-treated with GBE for 5 days was significantly attenuated compared to rats receiving no GBE, suggesting induction of CYP2C. In rat liver microsomes, GBE competitively inhibited (S)-warfarin 7-hydroxylase (Sugiyama et al., 2004a), although it is not known which enzyme in rats is responsible for the metabolism of (S)-warfarin. Likewise, liver microsomes from rats treated with GBE for 5 days showed an increase in (S)-warfarin 7-hydroxylase and PROD, pointing to the possible induction of CYP2C and CYP2B (Umegaki et al., 2002; Yang et al., 2003). After discontinuation of GBE treatment, activities recovered to normal levels (Sugiyama et al., 2004b). Treatment of rats with GBE for 4 weeks increased the levels of CYP2B mRNA (Shinozuka et al., 2002). Another possible experiment would be to look at the effect of GBE on induction of CYP2C9, CYP2B6, and CYP2A6 in humans by measuring the clearance of CYP2C9, CYP2B6, and CYP2A6 substrates after chronic administration of GBE versus placebo.

In this study, individual terpene trilactones and flavonol glycosides did not inhibit the formation of VPA metabolites. It is possible that other chemicals not tested in this study were
responsible for the inhibitory effect of GBE. Further studies could focus on identifying and quantifying such compounds, and investigating their effect on VPA metabolism using microsomes. Another possibility is that while individually, the compounds tested did not inhibit CYP2C9, in combination they may exhibit synergy, as often occurs with phytomedicines (Williamson, 2001). If so, then in future experiments, combinations of different constituents could be tested to see if they have inhibitory potential.
6. SUMMARY AND CONCLUSIONS

- The VPA assay is a versatile assay, allowing the simultaneous measurement of CYP2C9, CYP2B6, and CYP2A6 activity. The desaturation of VPA to 4-ene-VPA is catalyzed by CYP2C9, CYP2B6, and CYP2A6. The formation of 4-OH-VPA by (ω-1)-hydroxylation is mainly catalyzed by CYP2C9, with CYP2B6 and CYP2A6 playing minor roles. The Ω-hydroxylation of VPA to 5-OH-VPA is catalyzed mainly by CYP2C9, with CYP2B6 and CYP2A6 playing minor roles. CYP2A6 is the major isoform responsible for the (ω-2)-hydroxylation of VPA to 3-OH-VPA.

- GBE inhibits VPA metabolism \textit{in vitro}. GBE decreased the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA in HLM. GBE may interact with VPA \textit{in vivo}.

- GBE inhibits CYP2C9 \textit{in vitro}. GBE decreased the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA in HLM by cDNA-expressed CYP2C9*1. GBE may interact with drugs metabolized by CYP2C9. The interaction seen with warfarin \textit{in vivo} is more difficult to explain as the ginkgolides have been shown to possess anti-platelet activity (Chung et al., 1987). Thus, it could be a pharmacodynamic interaction between the two
blood thinning agents, a pharmacokinetic interaction in which GBE inhibits CYP2C9-mediated metabolism of (S)-warfarin, or a combination of both.

- **GBE inhibits CYP2B6 *in vitro*.** GBE decreased the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by HLM and cDNA-expressed CYP2B6. GBE may be contraindicated for patients on drugs metabolized by CYP2B6.

- **GBE inhibits CYP2A6 *in vitro*.** GBE decreased the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA by HLM and cDNA-expressed CYP2A6. GBE may be contraindicated for patients on drugs metabolized by CYP2A6.

- The inhibitory effect of GBE seen in HLM is due mostly to inhibition of CYP2C9. CYP2C9 is responsible for approximately 75% of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA formation in HLM. In HLM pre-incubated with MAb2B6 and MAb2A6, GBE dramatically reduced the formation of these three largely CYP2C9-mediated metabolites.

- The terpene trilactones at the levels present in the extract are not responsible for inhibition of VPA metabolism by GBE *in vitro*. Bilobalide and ginkgolides A, B, C, and J did not inhibit VPA metabolism at the concentrations found in GBE.

- The flavonol rutinosides at the levels present in the extract are not responsible for inhibition of VPA metabolism by GBE *in vitro*. Isorhamnetin-3-0-rutinoside, kaempferol-3-0-rutinoside, and quercetin-3-0-rutinoside did not inhibit VPA metabolism at the concentrations thought to be in GBE.

- The flavonol aglycones inhibit VPA metabolism *in vitro*. Although not present in the extract, the aglycones of isorhamnetin, kaempferol, and quercetin, inhibited VPA
metabolism. However, they may of importance *in vivo* since flavonol glycosides are converted to their respective aglycones (Rasmussen and Breinholt, 2003).
7. REFERENCES


## 8. APPENDICES

### 8.1. Appendix 1. Amount of Terpene Trilactones and Flavonols in GBE (Indena, lot 1306A)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (% w/w)</th>
<th>Constituent</th>
<th>Amount (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol</td>
<td></td>
<td>Quercetin</td>
<td></td>
</tr>
<tr>
<td>Aglycone</td>
<td>not detected</td>
<td>Aglycone</td>
<td>not detected</td>
</tr>
<tr>
<td>Diglycoside 1</td>
<td>2.4</td>
<td>Diglycoside 1</td>
<td>0.9</td>
</tr>
<tr>
<td>Diglycoside 2</td>
<td>1.6</td>
<td>Diglycoside 2</td>
<td>1.0</td>
</tr>
<tr>
<td>Other glycosides</td>
<td>6.6</td>
<td>Other Glycosides</td>
<td>4.4</td>
</tr>
<tr>
<td>Sum</td>
<td>10.6</td>
<td>Sum</td>
<td>6.3</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td></td>
<td>Terpene trilactones</td>
<td></td>
</tr>
<tr>
<td>Aglycone</td>
<td>not detected</td>
<td>Bilobalide</td>
<td>2.8</td>
</tr>
<tr>
<td>3-O-Rutinoside</td>
<td>0.6</td>
<td>Ginkgolide A</td>
<td>1.1</td>
</tr>
<tr>
<td>Other Glycosides</td>
<td>3.5</td>
<td>Ginkgolide B</td>
<td>0.3</td>
</tr>
<tr>
<td>Sum</td>
<td>4.1</td>
<td>Ginkgolide C</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ginkgolide J</td>
<td>0.6</td>
</tr>
</tbody>
</table>

GBE (Indena, lot 1306A) was standardized to contain 6% terpene trilactones and 24% flavonol glycosides. The terpene trilactones were quantified using LC/MS by ChomaDex (Santa Ana, CA). The flavonols were quantified using GC by Indena (Milan, Italy).
<table>
<thead>
<tr>
<th>Donor</th>
<th>Lot</th>
<th>Protein Content (mg/mL)</th>
<th>Total CYP Content (pmol total CYP/mg protein)</th>
<th>Rate of Product Formation (pmol/min/mg protein)</th>
<th>Rate of Product Formation (pmol/min/pmol total CYP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP2C9</td>
<td>CYP2B6</td>
<td>CYP2A6</td>
</tr>
<tr>
<td>HH18</td>
<td>1</td>
<td>20</td>
<td>370</td>
<td>3900</td>
<td>32</td>
</tr>
<tr>
<td>HG30</td>
<td>2</td>
<td>20</td>
<td>535</td>
<td>6190</td>
<td>48</td>
</tr>
<tr>
<td>HH47</td>
<td>1</td>
<td>20</td>
<td>260</td>
<td>2900</td>
<td>6.5</td>
</tr>
<tr>
<td>HH91</td>
<td>1</td>
<td>20</td>
<td>340</td>
<td>3400</td>
<td>28</td>
</tr>
<tr>
<td>HG95</td>
<td>1</td>
<td>20</td>
<td>230</td>
<td>2100</td>
<td>12</td>
</tr>
</tbody>
</table>

Total CYP content and rate of product formation (pmol/min/mg protein) were drawn from information sheets provided by Gentest (Woburn, MA). Total CYP content was measured by the method of Omura and Sato. CYP2C9 activity was measured by the diclofenac 4'-hydroxylase assay. CYP2B6 activity was measured by the (S)-mephenytoin N-demethylation assay. CYP2A6 activity was measured by the coumarin 7-hydroxylase assay. All assays were conducted using 0.8 mg/mL of protein incubated with an NADPH generating system (1.3 mM NADP+, 3.3 mM glucose 6-phosphate, and 0.4 U/mL of glucose 6-phosphate dehydrogenase), and 3.3 mM MgCl₂. The incubation time was 20 min for CYP2B6 and CYP2A6, and 10 min for CYP2C9. For CYP2B6, 0.05 M potassium phosphate buffer (pH 7.4) was used, and for CYP2C9 and CYP2A6, 0.1 M Tris (pH 7.4) was used. The rate of product formation (pmol/min/pmol total CYP) was calculated by dividing the rate of product formation (pmol/min/mg protein) by the total CYP content.
<table>
<thead>
<tr>
<th>Donor</th>
<th>HH18</th>
<th>HG30</th>
<th>HH47</th>
<th>HH91</th>
<th>HG95</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9 Genotype</td>
<td>*1/*1</td>
<td>*1/*1</td>
<td>*1/*1</td>
<td>*1/*1</td>
<td>*1/*1</td>
</tr>
<tr>
<td>CYP2C19 Genotype</td>
<td>*1/*1</td>
<td>*1/*1</td>
<td>*1/*1</td>
<td>*1/*2</td>
<td>*1/*1</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Age</td>
<td>78</td>
<td>28</td>
<td>53</td>
<td>55</td>
<td>47</td>
</tr>
<tr>
<td>Race</td>
<td>African American</td>
<td>Caucasian</td>
<td>African American</td>
<td>Caucasian</td>
<td>Hispanic</td>
</tr>
<tr>
<td>Cause of Death</td>
<td>CVA (^a)</td>
<td>Cardio-pulmonary arrest</td>
<td>CVA</td>
<td>Cerebrovascular</td>
<td>Closed head trauma</td>
</tr>
<tr>
<td>Social History</td>
<td>Tobacco use</td>
<td>No tobacco use</td>
<td>No tobacco use Alcohol use</td>
<td>Not available</td>
<td>Tobacco use</td>
</tr>
<tr>
<td>Medical History</td>
<td>Hypertension NIDDM (^b)</td>
<td>Asthma</td>
<td>Arthritis</td>
<td>Rocephin</td>
<td>Zantac</td>
</tr>
<tr>
<td>Medication Given During Hospitalization</td>
<td>Insulin</td>
<td>Zantac</td>
<td>Not available</td>
<td>Dopamine</td>
<td>Fortaz</td>
</tr>
</tbody>
</table>

Donor history was provided by Gentest (Woburn, MA). \(^a\)CVA = cerebrovascular accident. \(^b\)NIDDM = non-insulin dependent diabetes mellitus.
<table>
<thead>
<tr>
<th>Product Name</th>
<th>Lot</th>
<th>Protein Content (mg/mL)</th>
<th>CYP Content (pmol/mL)</th>
<th>Rate of Product Formation (pmol/min/pmol CYP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CYP2C9*1 (Arg_{144}) + P450 Reductase + Cytochrome b\textsubscript{5} Supersomes™</td>
<td>20</td>
<td>3.6</td>
<td>2000</td>
<td>21 \textsuperscript{a}</td>
</tr>
<tr>
<td>Human CYP2B6 + P450 Reductase + Cytochrome b\textsubscript{5} Supersomes™</td>
<td>13</td>
<td>11</td>
<td>2000</td>
<td>13 \textsuperscript{b}</td>
</tr>
<tr>
<td>Human CYP2A6 + P450 Reductase + Cytochrome b\textsubscript{5} Supersomes™</td>
<td>5</td>
<td>13.8</td>
<td>2000</td>
<td>21 \textsuperscript{c}</td>
</tr>
<tr>
<td>Insect Cell Control Supersomes™</td>
<td>31</td>
<td>5.0</td>
<td>N/A \textsuperscript{d}</td>
<td>N/A</td>
</tr>
</tbody>
</table>

All information was drawn from information sheets provided by Gentest (Woburn, MA). Insect cells (BTI-TN-5B1-4) infected with wild type baculovirus (*Autographa californica*) were used to prepare all microsomes. \textsuperscript{a}CYP2C9 activity was measured by the diclofenac 4'-hydroxylase assay. \textsuperscript{b}CYP2B6 activity was measured by the 7-ethoxy-4-trifluoromethylcoumarin assay. \textsuperscript{c}CYP2A6 activity was measured by the coumarin 7-hydroxylase assay. \textsuperscript{d}Not applicable. All assays were conducted using 0.8 mg/mL of protein incubated with an NADPH generating system (1.3 mM NADP\textsuperscript{+}, 3.3 mM glucose 6-phosphate, and 0.4 U/mL of glucose 6-phosphate dehydrogenase), and 3.3 mM MgCl\textsubscript{2}. For CYP2B6, 0.05 M potassium phosphate buffer (pH 7.4) was used, and for CYP2C9 and CYP2A6, 0.1 M Tris (pH 7.4) was used. The incubation time was 15 min for CYP2B6, and 10 min for CYP2C9 and CYP2A6. For CYP2C9, 20 pmol of CYP was used. For CYP2B6 and CYP2A6, 10 pmol was used.
8.5. Appendix 5. Information on Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb2C9</td>
<td>763-15-5</td>
<td>IgG1</td>
</tr>
<tr>
<td>MAb2B6</td>
<td>49-10-20</td>
<td>IgG2b</td>
</tr>
<tr>
<td>MAb2A6</td>
<td>151-45-4</td>
<td>IgG1</td>
</tr>
<tr>
<td>Control (Egg Lysozyme)</td>
<td>Hy-Hel-9</td>
<td>IgG1</td>
</tr>
</tbody>
</table>

Information on antibodies was provided by the National Cancer Institute at the National Institutes of Health (Bethesda, MD).