GLUCURONIDATION OF DIHYDROARTESMISININ IN VIVO AND BY HUMAN LIVER MICROSOMES AND EXPRESSED UDP-GLUCURONOSYLTRANSFERASES

KENNETH F. ILETT, BRIAN T. ETHELL, JAMES L. MAGGS, TIMOTHY M. E. DAVIS, KEVIN T. BATTY, BRIAN BURCHELL, TRAN QUANG BINH, LE THI ANH THU, NGUYEN CANH HUNG, MUNIR PIRMOHAMED, B. KEVIN PARK, AND GEOFFREY EDWARDS

Department of Pharmacology, University of Western Australia, Crawley, Western Australia (K.F.I.); Clinical Pharmacology and Toxicology Laboratory, Western Australian Centre for Pathology and Medical Research, Nedlands, Western Australia (K.F.I.); Department of Medicine, University of Western Australia, Fremantle Hospital, Fremantle, Western Australia (T.M.E.D.); School of Pharmacy, Curtin University, Perth, Western Australia (K.T.B.); Department of Biochemical Medicine, University of Dundee, Ninewells Hospital, Dundee, United Kingdom (B.B., B.E.); Tropical Diseases Research Centre, Cho Ray Hospital, Ho Chi Minh City, Vietnam (T.Q.B., L.T.A.T.); Bao Loc Hospital, Lam Dong Province, Vietnam (N.C.H.); Department of Pharmacology and Therapeutics, the University of Liverpool, Liverpool, United Kingdom (M.P., J.L.M., B.K.P., G.E.); and Division of Parasite and Vector Biology, Liverpool School of Tropical Medicine, Liverpool (G.E.)

(Received September 22, 2001; accepted June 4, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The aim of this study was to elucidate the metabolic pathways for dihydroartemisinin (DHA), the active metabolite of the artemisinin derivative artesunate (ARTS). Urine was collected from 17 Vietnamese adults with falciparum malaria who had received 120 mg of artesunate (ARTS) i.v., and metabolites were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). Human liver microsomes were incubated with [12-3H]DHA and cofactors for either glucuronidation or cytochrome P450-catalyzed oxidation. Human liver cytosol was incubated with cofactor for sulfation. Metabolites were detected by HPLC-MS and/or HPLC with radioactive detection. Metabolism of DHA by recombinant human UDP-glucuronosyltransferases (UGTs) was studied. HPLC-MS analysis of urine identified α-DHA-β-glucuronide (α-DHA-G) and a product characterized as the tetrahydrofuran isomer of α-DHA-G. DHA was present only in very small amounts. The ratio of the tetrahydrofuran isomer, α-DHA-G, was highly variable (median 0.75; range 0.09–0.4). Nevertheless, α-DHA-G was generally the major urinary product of DHA glucuronidation in patients. The tetrahydrofuran isomer appeared to be at least partly a product of nonenzymic reactions occurring in urine and was readily formed from α-DHA-G by iron-mediated isomerization. In human liver microsomal incubations, DHA-G (diastereomer unspecified) was the only metabolite found (Vmax 177 ± 47 pmol min⁻¹ mg⁻¹, Km 90 ± 16 μM). α-DHA-G was formed in incubations of DHA with expressed UGT1A9 (Km 32 μM, Vmax 8.9 pmol min⁻¹ mg⁻¹) or UGT2B7 (Km 438 μM, Vmax 10.9 pmol min⁻¹ mg⁻¹) but not with UGT1A1 or UGT1A6. There was no significant metabolism of DHA by cytochrome-P450 oxidation or by cytosolic sulfotransferases. We conclude that α-DHA-G is an important metabolite of DHA in humans and that its formation is catalyzed by UGT1A9 and UGT2B7.

Dihydroartemisinin (DHA¹; Fig. 1) is the lactol reduction product of artemisinin (qinghaosu) a sesquiterpene lactone endoperoxide that is the principal antimalarial constituent of Artemisia annua. DHA has potent antimalarial activity both in vitro and in vivo, but its low aqueous solubility restricts its administration to the enteral routes (Barradell and Fitton, 1995). DHA is also the common metabolite of its methyl (artemether) and ethyl (arteether) ethers, and its hemisuccinate ester (ARTS) (Lee and Hufford, 1990; Chi et al., 1991; De Vries and Dien, 1996), all three of which are used in the treatment of malaria (Barradell and Fitton, 1995; De Vries and Dien, 1996). Artemether and arteether are dealkylated relatively slowly (t1/2 = 8 and 24 h, respectively) (Lee and Hufford, 1990; Chi et al., 1991; De Vries and Dien, 1996), and antimalarial activity is most likely due to the parent compounds, rather than to DHA. However in vivo, ARTS is rapidly (t1/2 = 2–3 min) converted to DHA, which in turn is eliminated from the systemic circulation with a t1/2 of 40 to 50 min (Yang et al., 1985; Batty et al., 1998a,b; Zhao et al., 1988). Thus, most of the antimalarial activity resulting from ARTS administration is attributable to DHA.

When administered intravenously to male rats, [12-14C]DHA was converted principally to the biologically inactive (Ramu and Baker, 1995) α-DHA-β-glucuronide (α-DHA-G; Fig. 1) (Maggé et al., 1997, 2000). DHA is also eliminated in bile as minor glucuronides of nonendoperoxide isomers, namely a tetrahydrofurano acetate (Fig. 1),

¹ Abbreviations used are: DHA, dihydroartemisinin; ARTS, artesunate; UGT, UDP-glucuronosyltransferases; DHA-G, dihydroartemisinin glucuronide; [12-3H]DHA, [12-7H]DHA; HPLC, high-performance liquid chromatography; PAPS, adenosine 3'-phosphate 5'-phosphosulphate; UDPGA, UDP-glucuronic acid; LC-MS, liquid chromatography-mass spectrometry, tandem mass spectrometry.

Address correspondence to: K. F. Ilett, Ph.D., Department of Pharmacology, University of Western Australia, Queen Elizabeth II Medical Centre, Crawley, 6907, Western Australia. E-mail: kilett@receptor.pharm.uwa.edu.au
and a 3-hydroxydesoxy rearrangement product (Maggs et al., 1997). Endoperoxides readily undergo biomimetic iron (II)-mediated isomerization as a consequence of a one-electron reduction of the peroxide bridge, which produces carbon-centered radical intermediates (O’Neill et al., 2001a); the radical-rearrangement pathway producing furano acetate compounds in vitro has been confirmed (Butler et al., 1998). The mechanism(s) and site of isomerization in vivo remain unknown but the two rearrangement pathways for another DHA derivative, artelinic acid, are reported to be catalyzed by human liver microsomes (Idowu et al., 1997). However, Batty et al. (1998c) observed that isolated rat livers perfused with DHA in physiological buffer only very rarely excreted the furano acetate glucuronide in bile, notwithstanding the extensive biliary excretion of \( ^{3} \)H-DHA-G (Maggs et al., 2000). The rearrangements might occur partly in blood as artemether is actively isomerized by hemoglobin and blood in vitro (Blum et al., 1998). In the present study, we have investigated both the urinary metabolite profile of ARTS following its administration to patients with falciparum malaria as well as the in vitro metabolism of DHA by human liver microsomes and by purified UDP-glucuronosyltransferases (UGT).

**Materials and Methods**

**Chemicals.** DHA was synthesized as an epimeric mixture by a published method (Lin et al., 1987) and supplied by Professor R. Haynes, University of Science and Technology, Hong Kong, People’s Republic of China. \(^{3} \)H-DHA (\(^{3} \)H)DHA; 1.4 Ci mmol \(^{-1} \); Moravek Biochemicals Inc., Brea, CA) was also an epimeric mixture but was radiochromatographically homogeneous (>98.4%) by HPLC using the method employed for assaying glucuronidation of \(^{3} \)H-DHA in human liver microsomes. The sodium salts of \( \alpha \)-DHA \( \beta \)-glucuronide and \( \beta \)-DHA \( \beta \)-glucuronide were prepared by Dr. A. V. Stachulski, Ultrafine Chemicals (Manchester, UK) (O’Neill et al., 2001b). They were dissolved in deionized water (10 mM), and isomerized to their furano acetate and 3-hydroxydesoxy-DHA forms by reaction with iron (II) chloride as described previously (Maggs et al., 2000). HPLC grade solvents were obtained from Fisher Scientific (Loughborough, UK). Adenosine 3’-phosphate 5’-phosphosulphate (PAPS), 5a-androstene-3a,17β-diol, Brij 58, diazepam, diclofenac, ethinyl estradiol, ketoprofen, oxazepam, mycophenolic acid, 1-naphthol, naloxone, novobiocin, paracetamol, saccharolactone, and uridine diphosphate-\( \alpha \)-glucuronic acid (UDPGA; tri-sodium salt; catalogue no. U6751) were products of the Sigma-Aldrich Co., Ltd. (Gillingham, Dorset, UK). All other chemicals were of analytical reagent grade.

**Urinary Metabolites of Dihydroartemisinin following Intravenous Ad-**
ministration of ARTS. Vietnamese adults (n = 26) with uncomplicated falciparum malaria were given ARTS (120 mg i.v. bolus). The pharmacokinetics of ARTS and DHA in the plasma have been reported previously (Batty et al., 1998b, c). Urine samples (0–2, 2–4, and 4–6 h following the dose) from a subset of 17 of these patients were stored at −80°C for 12 to 15 months prior to being analyzed in the present study. ARTS was also administered orally (120 mg) to a healthy white male volunteer aged 46 years who was not taking concurrent medications and was a nonsmoker. This study was approved by the Ethics Committee of the Mersey Regional Health Authority. Urine was collected before dosing and as 2-h fractions. Aliquots were analyzed immediately by liquid chromatography-mass spectrometry (LC-MS).

Stability of DHA-G in Human Urine. Urine from the malaria patients (200 μl from 2- to 4-h collections) was transferred to capped glass tubes and maintained at 37°C. Aliquots were taken periodically for analysis by LC-MS. In addition, bile containing α-[3H]DHA-G (1.6 mM; 17.9 μCi ml⁻¹) produced by a rat liver perfused with [3H]DHA in a single-pass system (Batty et al., 1998c; Maggs et al., 2000) was diluted with either predosing bile from an anesthetized and cannulated rat (3:67, v/v) or urine freshly collected from a nonmedicated adult male volunteer (1:39, v/v). The mixtures were incubated in capped glass tubes at 37°C, and aliquots were analyzed by LC-MS.

Analysis of DHA Urinary Metabolites. Aliquots of urine (50 μl) and bile (2–20 μl) were eluted from an Ultracarb C5 5-μm column (Phenomenex, Macclesfield, UK) at room temperature with a gradient of acetonitrile in 0.1 M ammonium acetate, pH 6.9: 20 to 35% over 15 min, 35 to 70% over 10 min. The flow rate was 0.9 ml min⁻¹. Metabolites were characterized by positive-ion electrospray mass spectrometry using a Micromass Quattro II instrument (Micromass UK Ltd., Manchester, UK) as described previously (Maggs et al., 2000). Selected ion monitoring of six channels was performed with a dwell time of 200 ms and an interchannel delay of 20 ms. Data were processed via MassLynx 2.1 software (Micromass UK Ltd.). Proportions of metabolites were estimated from areas of peaks in the mass chromatograms for [M + NH₄]⁺.

Radiolabeled compounds were located with a Radiomatic Flo-One model A250 flow detector (Packard Bioscience, Pangbourne, UK) connected in parallel with the mass spectrometer. The eluate was mixed with Ultima Flo AP scintillant (Packard Bioscience B.V., Groningen, The Netherlands) at 1 ml min⁻¹.

Preparation of Human Liver Microsomes and Cytosols. Liver tissue was obtained from a human liver bank (renal transplant patients) maintained by the Department of Pharmacology and Therapeutics, University of Liverpool. Liver samples from eight patients (Table 1) were homogenized in buffer (0.05 M Tris, 1.15% KCl; pH 7.4), and microsomes were prepared by differential centrifugation and resuspended in 0.01 M phosphate buffer (pH 7.4). For three samples, the cytosolic fractions from the final microsomal centrifugation step were retained for evaluation of sulfotransferase activity. Approval for use of these human tissues was obtained from the Ethics Committee of the Mersey Regional Health Authority.

Glucuronidation of [12-3H]DHA Using Human Liver Microsomes. Preliminary experiments were performed to establish optimal reaction conditions and linearity of the assay. Microsomal protein concentration was determined colorimetrically (Bradford, 1976). For the determination of the kinetics of DHA glucuronidation without reference to the stereoselectivity of conjugation, the composition of the final reaction mixture (total volume 250 μl) was as follows: DHA (12.5–500 μM, containing 0.1 μCi [3H]DHA); UDPGA (3 mM); Brij 58 (0.1 mg mg⁻¹ of protein), tris-hydroxymethylaminomethane (0.05 M), MgCl₂ (5 mM), human liver microsomes (0.4–0.5 mg of protein). DHA was solubilized in ethanol, such that the final concentration in the reaction mixture did not exceed 2% (v/v). Reactions were incubated in duplicate at 37°C for 60 min and terminated by addition of 170-μl ice-cold methanol and vortexing for 10 s. Blank incubations contained no UDPGA. After centrifugation (2,500g, 5 min), 100 μl of the supernatant was injected to the HPLC column, and DHA-G was quantified as picomoles formed per minute per milligram of microsomal protein using radiochemical detection (see below).

The Michaelis-Menten equation was fitted to the primary data using the nonlinear regression analysis procedure in SigmaPlot version 6 (Jandel Scientific, San Rafael, CA) to yield estimates of Kₘ and Vₘₙₐₓ.

For the inhibitor studies, the reaction mixture was similar except that the DHA concentration was fixed at 250 μM, microsomal protein was 0.64 mg, and various other UGT substrates [ethyloestradioil, diclofenac, ketoprofen, oxazepam, diazepam, novobiocin, 5α-androstene-3α-17β-diol, 1-napthol, naloxone, lamotrigine, morphine, paracetamol, and mycophenolic acid; 4–6 concentrations (duplicates) ranging from 25–3000 μM] were added. Control incubations were carried out in triplicate and inhibitor incubations in duplicate at 37°C for 60 min. Inhibitors were solubilized in water or in dimethylsulphoxide as appropriate with the final concentration of the latter not exceeding 1% (v/v) in the reaction mixture. The percent inhibition at each inhibitor concentration was calculated and plotted against log₁₀ inhibitor concentration using the linear regression analysis procedure in SigmaPlot 6. The concentration causing 50% inhibition (IC₅₀) was then interpolated using the regression equation.

Analysis of [3H]DHA-G Produced by Human Liver Microsomes. Reversed-phase chromatography was performed on a Nucleosil 5 μ C₈, 25 cm ×
TABLE 2

Results of screening assays with expressed human UDP-glucuronosyltransferases.

Control activities indicate the activity of the expressed UGT preparation with an ideal substrate using a radiochemical HPLC assay.

<table>
<thead>
<tr>
<th>UGT Isoform</th>
<th>α-DHA-G Formation</th>
<th>Control Substrate</th>
<th>Control Activity pmol min⁻¹ mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td></td>
<td>Octylgallate</td>
<td>1.1</td>
</tr>
<tr>
<td>UGT1A6</td>
<td></td>
<td>1-Naphthol</td>
<td>3.2</td>
</tr>
<tr>
<td>UGT1A9</td>
<td></td>
<td>Propofol</td>
<td>1.2</td>
</tr>
<tr>
<td>UGT2B7</td>
<td></td>
<td>Hydroxyecdysoneic acid</td>
<td>0.75</td>
</tr>
</tbody>
</table>

4.6 mm i.d. column (Phenomenex) equipped with a Merck CN precolumn (BDH, Poole, Dorset, UK). The mobile phase consisted of acetonitrile: 0.1M ammonium chloride, 50:50 (v/v), pumped at a rate of 1 ml min⁻¹. Detection was achieved on-line using a Berthold Bioanalytical Instruments BetaFlow radiochemical detection system (Wallaic, Milton Keynes, Bucks, UK). Under these conditions, DHA-G and DHA chromatographed at approximate \( R_t \) values of 4.1 and 8.1 min, respectively. The epimers of DHA were partially resolved, but there was no indication at this short \( R_t \) of any corresponding resolution of the product peak, the stereochemistry of which was not specified. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**Glucuronidation of DHA by Recombinant UGTs.** DHA was incubated with recombinant human UDP-glucuronosyltransferases expressed in V79 (Chinese hamster lung fibroblasts) cells (Ethell et al., 2001). Cells expressing UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were resuspended in phosphate-buffered saline, pH 7.4, and were then disrupted by sonication (MSE Sonicator). UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were run in parallel with the DHA assays to ensure that the sonicated cell-protein in a volume of 100 μl was disrupted by sonication (MSE Sonicator) and collision energy, 25 eV using argon as the collision gas. The ion of interest was monitored by selected reaction monitoring (SRM) for DHA-G and DHA chromatographed at approximate \( R_t \) of 11.3 and 13.2 min, respectively. Authentic DHA-G and DHA eluted at 11.3 and 13.2 min, respectively. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**TABLE 2**

**Results of screening assays with expressed human UDP-glucuronosyltransferases.**

Control activities indicate the activity of the expressed UGT preparation with an ideal substrate using a radiochemical HPLC assay.

<table>
<thead>
<tr>
<th>UGT Isoform</th>
<th>α-DHA-G Formation</th>
<th>Control Substrate</th>
<th>Control Activity pmol min⁻¹ mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td></td>
<td>Octylgallate</td>
<td>1.1</td>
</tr>
<tr>
<td>UGT1A6</td>
<td></td>
<td>1-Naphthol</td>
<td>3.2</td>
</tr>
<tr>
<td>UGT1A9</td>
<td></td>
<td>Propofol</td>
<td>1.2</td>
</tr>
<tr>
<td>UGT2B7</td>
<td></td>
<td>Hydroxyecdysoneic acid</td>
<td>0.75</td>
</tr>
</tbody>
</table>

4.6 mm i.d. column (Phenomenex) equipped with a Merck CN precolumn (BDH, Poole, Dorset, UK). The mobile phase consisted of acetonitrile: 0.1M ammonium chloride, 50:50 (v/v), pumped at a rate of 1 ml min⁻¹. Detection was achieved on-line using a Berthold Bioanalytical Instruments BetaFlow radiochemical detection system (Wallaic, Milton Keynes, Bucks, UK). Under these conditions, DHA-G and DHA chromatographed at approximate \( R_t \) of 4.1 and 8.1 min, respectively. The epimers of DHA were partially resolved, but there was no indication at this short \( R_t \) of any corresponding resolution of the product peak, the stereochemistry of which was not specified. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**Glucuronidation of DHA by Recombinant UGTs.** DHA was incubated with recombinant human UDP-glucuronosyltransferases expressed in V79 (Chinese hamster lung fibroblasts) cells (Ethell et al., 2001). Cells expressing UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were resuspended in phosphate-buffered saline, pH 7.4, and were then disrupted by sonication (MSE Sonicator). UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were run in parallel with the DHA assays to ensure that the sonicated cell-protein in a volume of 100 μl was disrupted by sonication (MSE Sonicator) and collision energy, 25 eV using argon as the collision gas. The ion of interest was monitored by selected reaction monitoring (SRM) for DHA-G and DHA chromatographed at approximate \( R_t \) of 11.3 and 13.2 min, respectively. Authentic DHA-G and DHA eluted at 11.3 and 13.2 min, respectively. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**TABLE 2**

**Results of screening assays with expressed human UDP-glucuronosyltransferases.**

Control activities indicate the activity of the expressed UGT preparation with an ideal substrate using a radiochemical HPLC assay.

<table>
<thead>
<tr>
<th>UGT Isoform</th>
<th>α-DHA-G Formation</th>
<th>Control Substrate</th>
<th>Control Activity pmol min⁻¹ mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td></td>
<td>Octylgallate</td>
<td>1.1</td>
</tr>
<tr>
<td>UGT1A6</td>
<td></td>
<td>1-Naphthol</td>
<td>3.2</td>
</tr>
<tr>
<td>UGT1A9</td>
<td></td>
<td>Propofol</td>
<td>1.2</td>
</tr>
<tr>
<td>UGT2B7</td>
<td></td>
<td>Hydroxyecdysoneic acid</td>
<td>0.75</td>
</tr>
</tbody>
</table>

4.6 mm i.d. column (Phenomenex) equipped with a Merck CN precolumn (BDH, Poole, Dorset, UK). The mobile phase consisted of acetonitrile: 0.1M ammonium chloride, 50:50 (v/v), pumped at a rate of 1 ml min⁻¹. Detection was achieved on-line using a Berthold Bioanalytical Instruments BetaFlow radiochemical detection system (Wallaic, Milton Keynes, Bucks, UK). Under these conditions, DHA-G and DHA chromatographed at approximate \( R_t \) of 4.1 and 8.1 min, respectively. The epimers of DHA were partially resolved, but there was no indication at this short \( R_t \) of any corresponding resolution of the product peak, the stereochemistry of which was not specified. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**Glucuronidation of DHA by Recombinant UGTs.** DHA was incubated with recombinant human UDP-glucuronosyltransferases expressed in V79 (Chinese hamster lung fibroblasts) cells (Ethell et al., 2001). Cells expressing UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were resuspended in phosphate-buffered saline, pH 7.4, and were then disrupted by sonication (MSE Sonicator). UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were run in parallel with the DHA assays to ensure that the sonicated cell-protein in a volume of 100 μl was disrupted by sonication (MSE Sonicator) and collision energy, 25 eV using argon as the collision gas. The ion of interest was monitored by selected reaction monitoring (SRM) for DHA-G and DHA chromatographed at approximate \( R_t \) of 11.3 and 13.2 min, respectively. Authentic DHA-G and DHA eluted at 11.3 and 13.2 min, respectively. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**TABLE 2**

**Results of screening assays with expressed human UDP-glucuronosyltransferases.**

Control activities indicate the activity of the expressed UGT preparation with an ideal substrate using a radiochemical HPLC assay.

<table>
<thead>
<tr>
<th>UGT Isoform</th>
<th>α-DHA-G Formation</th>
<th>Control Substrate</th>
<th>Control Activity pmol min⁻¹ mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td></td>
<td>Octylgallate</td>
<td>1.1</td>
</tr>
<tr>
<td>UGT1A6</td>
<td></td>
<td>1-Naphthol</td>
<td>3.2</td>
</tr>
<tr>
<td>UGT1A9</td>
<td></td>
<td>Propofol</td>
<td>1.2</td>
</tr>
<tr>
<td>UGT2B7</td>
<td></td>
<td>Hydroxyecdysoneic acid</td>
<td>0.75</td>
</tr>
</tbody>
</table>

4.6 mm i.d. column (Phenomenex) equipped with a Merck CN precolumn (BDH, Poole, Dorset, UK). The mobile phase consisted of acetonitrile: 0.1M ammonium chloride, 50:50 (v/v), pumped at a rate of 1 ml min⁻¹. Detection was achieved on-line using a Berthold Bioanalytical Instruments BetaFlow radiochemical detection system (Wallaic, Milton Keynes, Bucks, UK). Under these conditions, DHA-G and DHA chromatographed at approximate \( R_t \) of 4.1 and 8.1 min, respectively. The epimers of DHA were partially resolved, but there was no indication at this short \( R_t \) of any corresponding resolution of the product peak, the stereochemistry of which was not specified. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**Glucuronidation of DHA by Recombinant UGTs.** DHA was incubated with recombinant human UDP-glucuronosyltransferases expressed in V79 (Chinese hamster lung fibroblasts) cells (Ethell et al., 2001). Cells expressing UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were resuspended in phosphate-buffered saline, pH 7.4, and were then disrupted by sonication (MSE Sonicator). UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were run in parallel with the DHA assays to ensure that the sonicated cell-protein in a volume of 100 μl was disrupted by sonication (MSE Sonicator) and collision energy, 25 eV using argon as the collision gas. The ion of interest was monitored by selected reaction monitoring (SRM) for DHA-G and DHA chromatographed at approximate \( R_t \) of 11.3 and 13.2 min, respectively. Authentic DHA-G and DHA eluted at 11.3 and 13.2 min, respectively. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).
FIG. 2. Selected-ion chromatogram (m/z 478 for [M + NH4]+) for A, α-DHA-G in urine from the white volunteer who took 120 mg of ARTS orally; and B, synthetic standards for β-DHA-G (Rt, 13.7 min) and α-DHA-G (Rt, 14.2 min).

See Materials and Methods for LC-MS conditions.
malaria patients (isomer/DHA-G ratio at \( t = 0 \), 0.23–0.91) underwent spontaneous isomerization (6–57%; mean = 26%) over 2 h at 37°C. No reaction occurred in the urine of five other patients (ratio at \( t = 0 \), 0.15–3.3). In contrast to the conjugate urine, the radiolabeled \( \alpha \)-DHA-G in rat bile was stable at 37°C for at least 2.5 h and at 28°C for 38 h. When bile was diluted 40-fold with freshly collected human urine and incubated at 37°C for 2 h, the glucuronide underwent 99 and 95% isomerization, as determined by LC-MS and radiometric analysis, respectively, but no hydrolysis. No rearrangement occurred when the bile was incubated in either acetate (100 mM, pH 5.2) or HEPES (50 mM, pH 7.4) buffer.

Isomerization of \( \alpha^{-}[3 \text{H}] \text{DHA-G} \) in bile-urine mixtures at 28°C was inhibited by disodium EDTA: 86% by 1 mM over 4 h and 60% by 2 mM over 16 h. Iron (II) chloride added to a patient’s urine at 37°C achieved 9, 16, and 26% conversion of \( \alpha \)-DHA-G over 0.5 h at 10, 50, and 100 mM, respectively. Coincubated EDTA (100 mM) inhibited 87% of the isomerization effected by 100 mM iron (II) chloride.

Metabolism of \( [\text{H}] \text{DHA} \) by Human Liver Microsomes and Cytosol. There was no observable turnover of \( [\text{H}] \text{DHA} \) incubated with either human liver microsomes (L17, L22, L34) and NADPH or human liver cytosols (L36, L37, L38, L39) and PAPS; appropriate control assays run in parallel showed substrate turnover indicating that the assay systems were functional. However, microsomes from all livers tested (L16, L17, L20, L22, L34, L38) could turnover between 5 and 12% of the \( [\text{H}] \text{DHA} \) during a 1-h incubation with UDPGA. The reaction product had an \( R_t \) of approximately 4.1 min on HPLC with radiochemical detection. Blank incubations without microsomes or cofactor produced no turnover of substrate. Preliminary experiments using microsomes from livers L17 and L22 established that 5 mM MgCl₂ and 0.1 mg of the detergent Brij 58 per milligram of protein concentration (0.5 mg per incubation) and duration of incubation (up to 2 h). The \( K_m \) and \( V_{max} \) for the formation of DHA-G by human liver microsomes were investigated in microsomes from four individual human livers, and these data are summarized in Table 3. The mean \( K_m \) was 90 \( \mu \)M (range 64–126 \( \mu \)M) while the mean \( V_{max} \) was 177 pmol min⁻¹ mg⁻¹ (range 64–253 pmol min⁻¹ mg⁻¹).

The effects of a variety of other UGT substrates on the glucuronidation of \( [\text{H}] \text{DHA} \) by human liver microsomes (L19, L20, L38) also were investigated. The IC₅₀ (\( \mu \)M) values for these “potential inhibitors” of DHA-G formation were ethynloestradiol (85), diclofenac (119), ketoprofen (284), oxazepam (321), diazepam (476), novobiocin (642), 5α-androstene-3α-17β-diol (1,020), 1-naphthol (1,057), naloxone (2141), lamotrigine (4861), morphine (>1,000), paracetamol (>1,000), and mycophenolic acid (>3,000).

Glucuronidation of DHA by Recombinant UGT. In preliminary experiments, disrupted cell pellets from V79 cells expressing the human UGT1A9 or UGT1A6 did not form DHA-G whereas preparations containing UGT1A9 and UGT2B7 resulted in significant metabolism to \( \alpha \)-DHA-G (Table 2). A detailed examination of the kinetics of DHA-G formation showed that the \( K_m \) was lower for UGT1A9 (32 \( \mu \)M) than for UGT2B7 (438 \( \mu \)M), whereas \( V_{max} \) was similar (8.9 and 10.9 pmol min⁻¹ mg⁻¹, respectively) (Fig. 3, A and B). Incubations with UGT1A9 appeared to generate only the glucuronide of \( \alpha \)-DHA (Fig. 4B), a finding that is consistent with the in vivo and other in vitro data, but selected reaction monitoring at least suggested the possibility of trace production of \( \beta \)-DHA-G by UGT2B7 (Fig. 4A).

### Table 3

<table>
<thead>
<tr>
<th>Liver Identification No.</th>
<th>( V_{max} ) (pmol min⁻¹ mg⁻¹)</th>
<th>( K_m ) (( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L16</td>
<td>253</td>
<td>107</td>
</tr>
<tr>
<td>L17</td>
<td>123</td>
<td>64</td>
</tr>
<tr>
<td>L22</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>L34</td>
<td>251</td>
<td>126</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>177 ± 47</td>
<td>90 ± 16</td>
</tr>
</tbody>
</table>

Discussion

Our study is the first to identify DHA-G as the primary urinary metabolite of DHA in humans. Using a combination of in vivo and in vitro approaches, DHA-G and its tetrahydrofuran isomer were the only significant metabolites found in the urine of patients with malaria who were treated with ARTS; thus, no deoxygenated or hydroxylated glucuronides analogous to known metabolites of other artemisinin derivatives (Lee and Hufford, 1990; Chi et al., 1991; Maggs et al., 1997; Maggs et al., 2000) were found by LC-MS analysis. DHA epimerizes freely in solution and both epimers have been found in the plasma of ARTS-treated patients (Batty et al., 1996). Irrespective of whether DHA is administered to volunteers and patients as ARTS or to rats as either the lactol (Maggs et al., 1997) or \( \alpha \)-methyl ether (Maggs et al., 2000), it is found as \( \alpha \)-DHA glucuronide by LC-MS.

Stereospecific glucuronidation of DHA also occurs in the isolated
perfusion of rat liver (Batty et al., 1998a). As might be expected from the 40 to 50 min t_{1/2} of DHA in patients (Batty et al., 1998b,c), these conjugates were in greatest concentration in the urine collected in the first 2 h after dose. The ratio of α-DHA-G to its tetrahydrofuran isomer in the urine varied widely between patients. Since the furano acetate isomer of DHA-G can be absent from freshly collected urine following administration of ARTS, and α-DHA-G isomerizes spontaneously in urine, the isomeric conjugate found in patients’ urine was likely to have been at least partly a product of reactions occurring ex vivo. The documented chemistry of reactions between iron (II) and endoperoxides (Maggs et al., 2000; O’Neill et al., 2001a) and the effect of EDTA observed in the present study suggest an action of iron in urine [urinary Fe^{2+} in healthy adult males is 0.73 ± 0.32 μM (Hjortso et al., 1990)]. The stability of α-DHA-G in bile might reflect the presence of iron-binding protein (Regoezci and Chindemi, 1995). Caution is required in the interpretation and quantification of the urinary metabolites of endoperoxide drugs. Thus, we conclude that between-patient variation in the ratio of α-DHA-G to its tetrahydrofuran isomer might be due primarily to varying levels of Fe^{2+} in the urine.

Studies using human liver microsomes showed that [3H]DHA-G was the only detectable metabolite of [3H]DHA. Other pathways such as cytosolic sulfation and microsomal CYP450 oxidation did not have a significant role in the disposition of DHA. The mean calculated unbound intrinsic clearance for the reaction to DHA-G was 3.1 × 10^{-6} 1 min^{-1} mg^{-1} of microsomal protein (Cl_{int} = V_{max}/K_{int}) corrected for a free fraction of DHA in human liver microsomal incubations of 0.64; P. Gibbons, personal communication). If it is assumed that the liver is approximately 2.2% of body weight in humans and that the yield of microsomal protein is around 25 mg g^{-1} of liver (G. T. Tucker, personal communication), in vivo hepatic plasma clearance calculated from the equation for the well stirred model is approximately 0.05 l h^{-1} kg^{-1}

\[ \text{CL}_{h} = \frac{\text{Q}_{h} \times f_{u} \times \text{Cl}_{int}}{\text{Q}_{h} + f_{u} \times \text{Cl}_{int}} \]

where Cl_{int} = total hepatic clearance, Q_{h} = hepatic plasma flow (0.78 l h^{-1} kg^{-1}), and f_{u} = DHA fraction unbound in plasma (0.56; Batty, 1999). This is 8.4-fold lower than the mean unbound plasma clearance for DHA after i.v. ARTS administration to patients (0.42 l h^{-1} kg^{-1}) (Batty et al., 1998a,b). Our finding is consistent with recent data for a range of glucuronidated drugs where CL_{h} calculated from in vitro human liver microsomal metabolism data as above, consistently underestimated in vivo clearance by around 10-fold (Soars et al., 2002).

Inhibitor studies of the glucuronidation of DHA in human liver microsomes showed low IC_{50} values (<500 μM) for substrates such as ethinyloestradiol, diclofenac, ketoprofen, oxazepam, and diazepam. Since they are substrates for UGT1A1, UGT1A8, UGT1A9, or UGT2B7 (Ebner et al., 1993; Sabolovic et al., 2000), we reasoned that these isoforms might be involved in the glucuronidation of DHA. Metabolism by UGT1A1, UGT1A6, and UGT1A10, or UGT2B15 can probably be excluded because high concentrations of the respective preferred substrates mycophenolic acid (Mojarrai and Mackenzie, 1997), lamotrigine (Magdalou et al., 1992), paracetamol (Bock et al., 1999), or 5α-androstene-3α,17β-diol (Belanger et al., 1995) were weak inhibitors of DHA glucuronidation. Using expressed human UGTs and a sensitive LC-MS-MS assay system, we were able to show a lack of DHA-G formation by UGT1A1 and UGT1A6 as well as significant, highly stereoselective α-DHA-G formation by UGT1A9 and UGT2B7. Such a high level of stereoselectivity for glucuronidation has been described only rarely (Aumont et al., 2001). These authors claimed stereospecific conjugation of cis-or trans-resveratrol...
by certain UGTs, but a stereoselective reaction at the stereogenic center of epimers does not appear to have been reported. While the $K_m$ for DHA glucuronidation by UGT1A9 (32 $\mu$M) was much lower than that for UGT2B7 (438 $\mu$M), these values were nevertheless comparable with $K_m$ values from the human liver microsomal experiments (90 $\mu$M) and suggest that UGT1A9 may be the dominant isoform for the metabolism of DHA. While the formation of DHA-G observed in human liver microsomes is consistent with metabolism by UGT1A9 in this tissue, it should be noted that this isoform is expressed in colon (Strassburg et al., 1998) and a variety of extrahepatic tissues (Albert et al., 1999), as well as in liver (Strassburg et al., 1998; Ren et al., 2000), and that other isoforms also may be involved.

In summary, our study has identified $\alpha$-DHA-$\beta$-G as the major metabolite, and UGT1A9 and UGT2B7 as the predominant isoforms involved in the clearance of DHA in humans. Apart from a role in the rapid clearance of DHA, the glucuronide pathway may have implications for drug interactions with DHA. There are examples of induction and of inhibition interactions arising from two substrates competing for UGT pathways in vivo. With induction, the oral clearance of propafenone is markedly increased during co-treatment with the inducer rifampicin, leading to a clinically significant interaction (Dilger et al., 2000). With inhibition, tacrolimus can significantly increase the area under the plasma concentration-time curve for mycophenolic acid in transplant patients and require dose reduction of the latter to avoid toxicity (Zucker et al., 1997). Thus, it is possible that mechanistically similar drug interactions may also occur with DHA. Inhibition or induction could, respectively, decrease or increase antimarial efficacy. Currently, the most common cotreatment used with ARTS or DHA is mefloquine, but there are no reports of any interactions. In vitro data from the present study also indicate that the antipyrine paracetamol is a very poor inhibitor of DHA glucuronidation and is therefore unlikely to result in a significant interaction in vivo.

Acknowledgments

We are indebted to Professor Trinh Kim Anh, Professor Nguyen Van Kim, and Vuong Van Chon, from Cho Ray Hospital and to Dr. Vo Thanh Chien, Dr. Vu Nam Bien, Dr. Huyhn Van Thien, Dang Thi Vinh Thuan, and staff of the Malaria and Biochemistry and Hematology Departments, at Bao Loc Hospital, for facilitating the conduct of this study.

References


Dilger K, Hofmann U, and Kiss T (2000) Enzyme induction in the elderly: effect of rifampin and of inhibition interactions arising from two substrates competing for drug interactions with DHA. There are examples of induction and of inhibition interactions arising from two substrates competing for UGT pathways in vivo. With induction, the oral clearance of propafenone is markedly increased during co-treatment with the inducer rifampicin, leading to a clinically significant interaction (Dilger et al., 2000). With inhibition, tacrolimus can significantly increase the area under the plasma concentration-time curve for mycophenolic acid in transplant patients and require dose reduction of the latter to avoid toxicity (Zucker et al., 1997). Thus, it is possible that mechanistically similar drug interactions may also occur with DHA. Inhibition or induction could, respectively, decrease or increase antimarial efficacy. Currently, the most common cotreatment used with ARTS or DHA is mefloquine, but there are no reports of any interaction. In vitro data from the present study also indicate that the antipyrine paracetamol is a very poor inhibitor of DHA glucuronidation and is therefore unlikely to result in a significant interaction in vivo.

Acknowledgments

We are indebted to Professor Trinh Kim Anh, Professor Nguyen Van Kim, and Vuong Van Chon, from Cho Ray Hospital and to Dr. Vo Thanh Chien, Dr. Vu Nam Bien, Dr. Huyhn Van Thien, Dang Thi Vinh Thuan and staff of the Malaria and Biochemistry and Hematology Departments, at Bao Loc Hospital, for facilitating the conduct of this study.

References


