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 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 0.09–0.64). 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 (V_max 177 ± 47 pmol min⁻¹ mg⁻¹, K_m 90 ± 16 μM). α-DHA-G was formed in incubations of DHA with expressed UGT1A9 (K_m 32 μM, V_max 8.9 pmol min⁻¹ mg⁻¹) or UGT2B7 (K_m 438 μM, V_max 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 artesinin (qinghaosu) a sesquiterpene lactone endoperoxide that is the principal antimalarial constituent of Artemisia annua. DHA has potent antimalarial activity 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 artesunate (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 (t_1/2 = 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 (t_1/2 = 2–3 min) converted to DHA, which in turn is eliminated from the systemic circulation with a t_1/2 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) (Maggs et al., 1997, 2000). DHA is also eliminated in bile as minor glucuronides of nonendoperoxide isomers, namely a tetrahydrofurano acetate (Fig. 1), and a tetrahydrofuran isomer, α-DHA-G, was highly variable (median 0.75; range 0.09–0.64). 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 (V_max 177 ± 47 pmol min⁻¹ mg⁻¹, K_m 90 ± 16 μM). α-DHA-G was formed in incubations of DHA with expressed UGT1A9 (K_m 32 μM, V_max 8.9 pmol min⁻¹ mg⁻¹) or UGT2B7 (K_m 438 μM, V_max 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.
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. [12-$^{3}$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), 5α-andostene-3α,17β-diol, Brij 58, diazepam, diclofenac, ethinyloestradiol, ethylenedioctadiol, ketoprofen, oxazepam, mycophenolic acid, 1-naphthol, naloxone, novobiocin, paracetamol, saccharolactone, and uridine dinucleoside $\alpha$-D-glucuronic acid (UDP-DA; 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 among those affected (Batty et al., 1998c). The pharmacokinetics of ARTS and DHAs in the plasma of these patients have been reported previously (Batty et al., 1998c). 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 Ci/ml) 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 C8 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-Oneβ 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.

**TABLE 1**

<table>
<thead>
<tr>
<th>Liver</th>
<th>Gender</th>
<th>Age y</th>
<th>Cause of Death</th>
<th>Medications</th>
<th>Histology</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>L16</td>
<td>M</td>
<td>57</td>
<td>Respiratory arrest</td>
<td>Not available</td>
<td>Normal</td>
<td>Ex-smoker</td>
</tr>
<tr>
<td>L17</td>
<td>F</td>
<td>46</td>
<td>Cardiac arrest</td>
<td>Paracetamol, dobutamine, gelafusine</td>
<td>Normal</td>
<td>Nil</td>
</tr>
<tr>
<td>L20</td>
<td>F</td>
<td>49</td>
<td>RTA*</td>
<td>Not available</td>
<td>Normal</td>
<td>Nil</td>
</tr>
<tr>
<td>L22</td>
<td>M</td>
<td>32</td>
<td>RTA*</td>
<td>Normal</td>
<td>Normal</td>
<td>Nil</td>
</tr>
<tr>
<td>L34</td>
<td>M</td>
<td>31</td>
<td>RTA*</td>
<td>Not available</td>
<td>Normal</td>
<td>Nil</td>
</tr>
<tr>
<td>L36</td>
<td>M</td>
<td>24</td>
<td>RTA*</td>
<td>Dexamethasone, phenytoin</td>
<td>Normal</td>
<td>Nil</td>
</tr>
<tr>
<td>L37</td>
<td>M</td>
<td>41</td>
<td>Subarachnoid haemorrhage</td>
<td>Captopril, ranitidine</td>
<td>Normal</td>
<td>Smoker, hypertension, alcoholism</td>
</tr>
<tr>
<td>L38</td>
<td>F</td>
<td>36</td>
<td>Asphyxia</td>
<td>Not available</td>
<td>Normal</td>
<td>Nil</td>
</tr>
<tr>
<td>L39</td>
<td>F</td>
<td>10</td>
<td>post asthma attack</td>
<td>Normal</td>
<td>Normal</td>
<td>Nil</td>
</tr>
</tbody>
</table>

*RTA, road traffic accident.

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 ml⁻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 Vₘₐₙ.

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 [ethinylestradiol, diclofenac, ketoprofen, oxazepam, diazepam, novobiocin, 5α-androstene-3α,17β-diol, 1-naphthol, 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 dimethylsulfoxide 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 5µ C8, 25 cm ×
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 (Wallac, 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 epimerized. The area under the DHA-G peak (in disintegrations per minute) was expressed as a percentage of the total radioactivity in the chromatogram (% turnover).

**Gluconidation 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). Cells expressing the total radioactivity in the chromatogram (% turnover).

**Control activities indicate the activity of the expressed UGT preparation with an ideal substrate using a published method with minor modifications and albendazole as a positive control substrate (Rawden et al., 2000).** Briefly, the final reaction mixture (total volume 250 \( \mu \)l) contained NADP (4 mM), glucose 6-phosphate (40 mM), MgCl\(_2\) (20 mM), glucose-6-phosphate dehydrogenase (10 units), K\(_2\)HPO\(_4\) (20 mM, pH 7.4), \([\text{H}]\)DHA (100 \( \mu \)M), and human liver microsomes (2.5 mg of protein). Incubations were carried out at 37°C for 30 min and terminated by the addition of 170-\( \mu \)l ice-cold methanol and vortexing for 10 s. After centrifugation (12,500g, 5 min), supernatant (100 \( \mu \)l) was subjected to HPLC analysis with radiochemical detection as above.

**Sulfation of \([\text{H}]\)DHA by Human Liver Cytoxon.** Assays for sulfotransferase activity were carried out essentially as described previously (Foldes and Meek, 1973); sulfation of paracetamol was included as a positive control. Briefly, the reaction mixture (total volume 250 \( \mu \)l) contained \([\text{H}]\)DHA (100 \( \mu \)M) or paracetamol (1 mM), PAPS (0.4 mM), Na\(_2\)HPO\(_4\) (10 mM, pH 7.4), and human liver cytosol (0.4–1 mg of protein). Blank incubations contained no PAPS. Incubations were carried out at 37°C for 60 min and terminated by the addition of 170-\( \mu \)l ice-cold methanol and vortexing for 10 s. After centrifugation (12,500g, 5 min), supernatant (100 \( \mu \)l) was subjected to HPLC analysis with radiochemical detection as above.

### Results

#### Patient Characteristics.

The characteristics of the subset of 17 patients in the present study were similar to those of the larger group of 26 from which it was drawn (Batty et al., 1998c), male/female 16:1, age (mean \( \pm \) S.D.) 28 \( \pm \) 8 years, weight 50 \( \pm \) 5 kg, hematocrit 37 \( \pm \) 7%, hemoglobin 126 \( \pm \) 21 g l\(^{-1}\), plasma creatinine 102 \( \pm \) 27 \( \mu \)M, bilirubin 17 \( \pm \) 11 \( \mu \)M, alanine aminotransferase 36 \( \pm \) 20 units l\(^{-1}\), parasite count on admission (geometric mean) 9,616 parasites/\( \mu \)l.

#### Urinary Metabolites of DHA Following Administration of ARTS.

When the urine of a healthy volunteer administered artesunate orally was analyzed by LC-MS on the day of collection, it was found to contain \( \alpha \)-DHA-G (\( R_t \), 14.1 min) but neither \( \beta \)-DHA-G (\( R_t \), 13.7 min) nor the glucuronides' furano acetate isomers (Fig. 2). The metabolite was identified by chromatographic (Fig. 2) and mass spectrometric (Maggs et al., 2000) comparisons with the two synthetic glucuronides. Although a very small peak in the mass chromatogram for \( m/z \) 478 ([M + NH\(_4\)]\(^+\)), which was also found in the urine of patients, coeluted with synthetic \( \beta \)-DHA-G, it did not yield the diagnostic fragments of DHA glucuronide (Maggs et al., 2000).

\( \alpha \)-DHA-G and its furano acetate isomer (\( R_t \), 9.5 min) were found in stored urine from the malaria patients at diminishing concentrations—estimated from peak areas of the ion at \( m/z \) 478 ([M + NH\(_4\)]\(^+\))—in the consecutive 2-h collections; \( \beta \)-DHA-G was not found. The furano acetate conjugate was identified by chromatographic and mass spectrometric (Maggs et al., 2000) comparisons with the major product of iron (II)-catalyzed rearrangement of authentic \( \alpha \)-DHA-G. A minor isomeric product (\( R_t \), 5.5 min), characterized previously as the glucuronide of 3-hydroxydesoxyDHA, and excreted in bile by rats administered \( \alpha \)-DHA-G i.v. (Maggs et al., 1997), was not detected in human urine.

In urine samples from three patients, the peak area for \( \alpha \)-DHA-G in 2- to 4-h and 4- to 6-h collections was 22 to 50% and 1 to 20%, respectively, of that in the 0- to 2-h urine and for the isomeric furano acetate glucuronide, 30 to 41% and 1 to 33%, respectively. The 2- to 4-h patient urine samples were taken for interindividual comparison. They contained the furano acetate and DHA glucuronides in highly variable proportions, expressed as a ratio of peak areas for \( m/z \) 478: 0.09 to 64.4 (mean = 5.7, median = 0.75; \( n \) = 17). The estimates of the ratio are only approximate because the electrospray signal response of the furano acetate conjugate is known from comparisons of mass and radioactivity chromatograms to be greater than that of DHA glucuronide (Maggs et al., 1997). The ratio appeared unrelated to urinary pH (5.4–6.8). DHA was found by LC-MS in about 50% of the human urine samples but usually only in the first 2-h collection.

#### Oxidative Metabolism of \([\text{H}]\)DHA by Human Liver Microsomes.

The possibility of cytochrome P450-dependent metabolism of DHA was explored using a published method with minor modifications and albendazole as a control.
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.
achieved 9, 16, and 26% conversion of mM over 16 h. Iron (II) chloride added to a patient inhibited by disodium EDTA: 86% by 1 mM over 4 h and 60% by 2 and these data are summarized in Table 3. The mean were investigated in microsomes from four individual human livers, protein gave maximal DHA-G production. Addition of the /H9252 5 and 12% of the [3 H]DHA during a 1-h incubation with UDPGA. livers tested (L16, L17, L20, L22, L34, L38) could turnover between the assay systems were functional. However, microsomes from all human liver cytosols (L36, L37, L38, L39) and PAPS; appropriate with either human liver microsomes (L17, L22, L34) and NADPH or 87% of the isomerization effected by 100 mM iron (II) chloride.

Metabolism of [3 H]DHA by Human Liver Microsomes and Cytosol. There was no observable turnover of [3 H]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 [3 H]DHA during a 1-h incubation with UDPGA. The reaction product had an Rf 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 1 mg per incubation) and duration of incubation (up to 2 h). The Kn and Vmax for the formation of DHA-G were investigated in microsomes from four individual human livers, and these data are summarized in Table 3. The mean Kn was 90 μM (range 64–126 μM) while the mean Vmax 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 [3 H]DHA by pooled human liver microsomes (L19, L20, L38) also were investigated. The IC50 (μM) values for these “potential inhibitors” of DHA-G formation were ethinylestradiol (85), diclofenac (119), ketoprofen (284), oxazepam (321), diazepam (476), novobiocin (642), 5α-androstene-3α-17β-diol (1,020), 1-naphthol (1057), nalofoxone (2141), lamotrigin (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 V77 cells expressing the human UGT1A1 or UGT1A6 did not form DHA-G whereas preparations containing UGT1A9 and UGT2B7 resulted in significant metabolism to α-DHA-G (Table 2). A detailed examination of the kinetics of DHA-G formation showed that the Kn was lower for UGT1A9 (32 μM) than for UGT2B7 (438 μM), whereas Vmax 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 α-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 β-DHA-G by UGT2B7 (Fig. 4A).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Vmax and Kn for the glucuronidation of [3 H]DHA by human liver microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Identification No.</td>
<td>Vmax (pmol min−1 mg−1)</td>
</tr>
<tr>
<td>L16</td>
<td>255</td>
</tr>
<tr>
<td>L17</td>
<td>123</td>
</tr>
<tr>
<td>L22</td>
<td>64</td>
</tr>
<tr>
<td>L34</td>
<td>251</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>177 ± 47</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 Hufferd, 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 O-methyl ether (Maggs et al., 2000), it is found as α-DHA glucuronide by LC-MS. Stereospecific glucuronidation of DHA also occurs in the isolated 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 α-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 α-[3 H]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 α-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 [3 H]DHA by Human Liver Microsomes and Cytosol. There was no observable turnover of [3 H]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 [3 H]DHA during a 1-h incubation with UDPGA. The reaction product had an Rf 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 gave maximal DHA-G production. Addition of the β-glucuronidase inhibitor saccharolactone (2 mM) did not increase the reaction yield. Linearity of the assay was demonstrated for micromolar protein concentration (0.5–1 mg per incubation) and duration of incubation (up to 2 h). The Kn and Vmax for the formation of DHA-G were investigated in microsomes from four individual human livers, and these data are summarized in Table 3. The mean Kn was 90 μM (range 64–126 μM) while the mean Vmax 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 [3 H]DHA by pooled human liver microsomes (L19, L20, L38) also were investigated. The IC50 (μM) values for these “potential inhibitors” of DHA-G formation were ethinylestradiol (85), diclofenac (119), ketoprofen (284), oxazepam (321), diazepam (476), novobiocin (642), 5α-androstene-3α-17β-diol (1,020), 1-naphthol (1057), nalofoxone (2141), lamotrigin (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 V77 cells expressing the human UGT1A1 or UGT1A6 did not form DHA-G whereas preparations containing UGT1A9 and UGT2B7 resulted in significant metabolism to α-DHA-G (Table 2). A detailed examination of the kinetics of DHA-G formation showed that the Kn was lower for UGT1A9 (32 μM) than for UGT2B7 (438 μM), whereas Vmax 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 α-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 β-DHA-G by UGT2B7 (Fig. 4A).
perfused 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 $\alpha$-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 $\alpha$-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 $0.73 \pm 0.32$ $\mu$M (Hjortso et al., 1990)]. The stability of $\alpha$-DHA-G in bile might reflect the presence of iron-binding protein (Regoezzi 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 $\alpha$-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 $[\text{H}]$DHA-G was the only detectable metabolite of $[\text{H}]$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 $3.1 \times 10^{-6}$ $\text{mg}^{-1}$ of microsomal protein (CL$_{int}$ = $V_{max}$/K$_{m}$; 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$^{-1}$

$$\text{CL}_{h} = \frac{Q_{h} \times f_{u} \times \text{CL}_{int}}{Q_{h} + f_{u} \times \text{CL}_{int}}$$

where CL$_{h}$ = 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 $\mu$M) for substrates such as ethynloestradiol, 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 UGT1A4, UGT1A6, UGT1A10, or UGT2B15 can probably be excluded because high concentrations of the respective preferred substrates mycophenolic acid (Mojarrabi and Mackenzie, 1997), lamotrigine (Magdalou et al., 1992), paracetamol (Bock et al., 1999), resveratrol (Batt 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 $\alpha$-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

**Fig. 4.** LC-MS-MS selected-ion reaction monitoring of the transition of parent ion (m/z 460.17) to daughter ion (m/z 113) of $\alpha$-DHA-G and $\beta$-DHA-G.

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**Fig. 4.** LC-MS-MS selected-ion reaction monitoring of the transition of parent ion (m/z 460.17) to daughter ion (m/z 113) of $\alpha$-DHA-G and $\beta$-DHA-G.

A, supernatant from in vitro incubation of DHA with UGT2B7 and appropriate cofactors; B, supernatant from in vitro incubation of DHA with UGT1A9 and appropriate cofactors; and C, reference standards for $\beta$-DHA-G (R, 11.3 min) and $\alpha$-DHA-G (R, 13.2 min). See Materials and Methods for LC-MS-MS conditions.
by certain UGTs, but a stereoselective reaction at the stereogenic center of epimers does not appear to have been reported. While the Km of DHA glucuronidation by UGT1A9 (32 μM) was much lower than that for UGT2B7 (438 μM), these values were nevertheless comparable with Km values from the human liver microsomal experiments (90 μM) and suggest that UGT1A9 may be the dominant isozyme 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 isozyme is expressed in colon (Strassburg et al., 1998), and that other isoforms also may be involved.

In summary, our study has identified α-DHA-β-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 cotreatment with the inductor 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 also might occur with DHA. Induction or inhibition could, respectively, decrease or increase antimalarial 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 antipyretic paracetamol is a very poor inhibitor of DHA glucuronidation and is therefore unlikely to result in a significant interaction in vivo.

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