Assessing Cytochrome P450 and UDP-Glucuronosyltransferase Contributions to Warfarin Metabolism in Humans

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · MAY 2009

Impact Factor: 3.53 · DOI: 10.1021/tx900031z · Source: PubMed

8 AUTHORS, INCLUDING:

Grover P Miller
University of Arkansas for Medical Sciences
53 PUBLICATIONS **1,117** CITATIONS
SEE PROFILE

Drew Jones
St. Jude Children's Research Hospital
14 PUBLICATIONS **182** CITATIONS
SEE PROFILE

Suzanne N Owen
University of Arkansas at Little Rock
5 PUBLICATIONS **138** CITATIONS
SEE PROFILE

Anna Radominska-Pandya
University of Arkansas for Medical Sciences
187 PUBLICATIONS **5,530** CITATIONS
SEE PROFILE

Published in final edited form as:
Chem Res Toxicol. 2009 July ; 22(7): 1239. doi:10.1021/tx900031z.

Assessing Cytochrome P450 and UDP-Glucuronosyltransferase Contributions to Warfarin Metabolism in Humans

Grover P. Miller[†], **Drew R. Jones**[†], **Shane Z. Sullivan**[†], **Anna Mazur**[†], **Suzanne N. Owen**[‡], **Neil C. Mitchell**[‡], **Anna Radominska-Pandya**[†], and **Jeffery H. Moran**[*,‡]
Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, 4301 West Markham Street, Slot 516, and Arkansas Public Health Laboratory, Arkansas Department of Health, 201 South Monroe Street, Little Rock, Arkansas 72205

Abstract

As a step toward exploring a targeted metabolomics approach to personalized warfarin (Coumadin) therapy, we developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method capable of quantifying specific enantiomeric (*R* and *S*) contributions of warfarin (WAR) and the corresponding hydroxywarfarins (OH-WAR) and glucuronides (-GLUC) generated by cytochrome P450s (CYP) and UDP-glucuronosyltransferases (UGTs), respectively. Evaluation of quality control samples and three commercially available human samples showed that our analytical approach has the ability to measure 24 unique WAR metabolites in human urine. Evaluation of the human data also provides new insights for evaluating WAR toxicity and begins characterizing important UGT metabolic pathways responsible for WAR detoxification. Data revealed the significance of specific metabolites among patients and the corresponding enzymatic capacity to generate these compounds, including the first report of direct WAR glucuronidation. On the basis of total OH-WAR levels, (*S*)-7-OH-WAR was the predominant metabolite indicating the significance of CYP2C9 in WAR metabolism, although other CYP2C enzymes also contributed to clearance of this isomer. (*R*)-WAR hydroxylation to OH-WARs was more diverse among the patients as reflected in varying contributions of CYP1A2 and multiple CYP2C enzymes. There was wide variation in the glucuronidation of WAR and the OH-WARs with respect to the compounds and patients. 6- and 7-OH-WAR were primarily (>70%) excreted as glucuronides unlike 4′-OH-WAR and 8-OH-WAR. For all patients, UGT1A1 is likely responsible for 6-*O*-GLUC production, although UGT1A10 may also contribute in one patient. 7-*O*-GLUC levels reflected contributions from potentially five different UGT1A enzymes. In all cases, WAR, 4′-OH-WAR, 8-OH-WAR, and the corresponding glucuronides were minor metabolites with respect to the others. Taken together, these data suggest that both P450 and UGT reactions contribute to the generation of excretable products in human urine, thereby generating complex metabolic networks.

Introduction

Despite potential complications during treatment, warfarin (Coumadin) remains one of the most widely prescribed oral anticoagulants in the Western world. Patients receive warfarin as a racemic mixture consisting of both (*R*)- and (*S*)-forms, which differ in potency and metabolic

*To whom correspondence should be addressed. Jeffery.Moran@arkansas.gov..
[†]University of Arkansas for Medical Sciences.
[‡]Arkansas Department of Health.

Supporting Information Available: Positive mode multiple reaction monitoring (MRM) experimental conditions (Table 1) and negative mode MRM experimental conditions (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

processing. Initial dosages are typically determined by considering age and underlying conditions and, more recently, by identifying genetic factors (1). Approximately 92% of the administered dose is thought to be excreted through urinary pathways (2), and (*S*)-warfarin is about 3–5 times more potent than (*R*)-warfarin (3). Medical management of this drug is further complicated by warfarin having a very narrow therapeutic window and wide interindividual variability. Underdosing leads to thrombosis, while overdosing causes dangerous bleeding episodes.

Warfarin metabolism alters its structure and consequently impacts anticoagulant activity and bioavailability; hence, an understanding of these processes is critical for developing better anticoagulant treatment strategies. Cytochrome P450s (P450 or CYP for a particular isoform) introduce a hydroxyl group at one of five positions on warfarin to yield 6-, 7-, 8-, 10-, and 4′-hydroxywarfarins (OH-WAR) (Figure 1). The formation of each of these compounds is dependent on the particular P450, and typically, each P450 generates multiple products at defined ratios. (*S*)-Warfarin is primarily hydroxylated by CYP2C9 with minor contributions from CYP2C8, CYP2C18, and CYP2C19 (4–6). By contrast, (*R*)-warfarin undergoes hydroxylation by CYP1A2 and CYP3A4 (4,5,7) and may serve as a substrate for CYP1A1, CYP2C8, CYP2C18, CYP2C19, and CYP3A4 (4,5,7).

Unlike P450 reactions, phase II metabolism of warfarin has been studied very little. This is surprising because warfarin and especially the hydroxylated products are attractive substrates for phase II glucuronidation by UDP-glucuronosyltransferases (UGTs). Conjugation of xenobiotic compounds with glucuronic acid (GLUC) by UGTs is generally thought to be important for the transport and excretion of xenobiotic compounds through urine and/or bile (8). GLUC conjugation of warfarin metabolites is known to occur in rodents (9), and Kaminsky and Zhang (5) suggest that glucuronide metabolites of warfarin are excreted in human urine. Recent studies have begun characterizing the overall importance of this pathway by using human recombinant UGT expression systems (10,11). Those studies demonstrate that 6-, 7-, and 8-OH-WARs are glucuronidated by multiple UGTs including hepatic (UGT1A1, 1A3, and 1A9) and extra-hepatic (UGT1A8 and 1A10) enzymes. Only UGT1A10 conjugates 4′-OH-WAR, and no known UGTs recognize WAR or 10-OH-WAR as substrates.

The development of “omic” approaches reflects the desire to broaden the scope of efforts correlating biomarkers (genes, transcripts, proteins, or molecules) to pharmacological outcomes and thus improve our understanding of drug targeting and processing. Pharmacogenomics utilizes genomic information to understand individual variations in drug response. While early reports linked genes and warfarin treatment through familial resistance to drug treatment, recent multigene and genome-wide analyses indicate that CYP2C9 and vitamin K epoxide reductase (VKER) polymorphisms account for approximately 10 and 25%, respectively, of the population variance observed in warfarin dosage (12). Other genes are also implicated in these studies, although further work is necessary to confirm their significance. Only recently has a study broadened the scope of pharmacogenomic analysis to include potential phase II enzymes, which may clarify their significance in warfarin metabolism after refinement of the statistical algorithm (13).

Metabolomics is a comprehensive and quantitative analysis of metabolites in biological systems and thus more closely reflects the phenotype of the individual (14). Specifically, metabolic profiles of drugs reflect processing by multiple enzymes, whose activities are modulated by genetic polymorphisms, age, sex, diet, and the presence of other drugs and confounding factors. For warfarin, many of these factors are known to contribute to complex drug inactivation and clearance pathways for a particular individual. By utilizing the end products of metabolism for determining treatment strategy, metabolomics potentially offers a higher degree of personalization.

As an initial step toward developing a metabolomic approach for warfarin treatment, we developed a targeted method to identify and quantify phase I P450 and phase II UGT warfarin metabolites. The chiral liquid chromatography–tandem mass spectrometry (LC-MS/MS) method developed has the unique ability to quantitatively measure warfarin, OH-WARs, and corresponding glucuronides in human urine while accounting for specific enantiomeric contributions. Three commercially available urine samples from patients undergoing warfarin therapy were used to test this new system and to begin simultaneously assessing the importance of multiple metabolic pathways involved in warfarin detoxification. Supporting evidence is provided, which shows that both phase I P450 and phase II UGT reactions are important for efficient warfarin detoxification and excretion. These analyses confirm the importance of CYP2C9 metabolism and reveals for the first time that glucuronides of warfarin and OH-WARs are formed in vivo at high levels.

Materials and Methods

Materials

All chemicals used in this study were of at least reagent grade. Unless otherwise specified, all chemicals and reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). Racemic warfarin, 4′-OH-WAR, 6-OH-WAR, 7-OH-WAR, 8-OH-WAR, 10-OH-WAR, 7-hydroxycoumarin, as well as (*R*)-warfarin and (*S*)-warfarin were used as primary standards. Ethyl alcohol (100%) was purchased from AAPER (Shelbyville, KY). Recombinant CYP2C9*1, CYP3A4, CYP1A2, and CYP2C19 were expressed in baculovirus-infected insect cells and purchased from BD Biosciences (San Jose, CA). Commercially available urine specimens from three females (F84, F85, and F87) were provided by the Emergency Response and Air Toxicants Branch, Division of Laboratory Science National Center for Environmental Health Centers for Disease Control and Prevention (DeKalb County, GA). Patient studies were conducted under IRB-approved protocols, and all patients signed informed consent documentation. Other than age, gender, and that each was receiving warfarin, no specific patient histories were provided.

LC-MS/MS Analysis

An Agilent 1100 HPLC system coupled to an API4000 Triple Quadrupole (MS/MS) mass spectrometer (Applied Biosystems) was used to analyze and quantify WAR, OH-WARS, and the corresponding glucuronides (GLUCs). Initial attempts to resolve all enantiomeric contributions in a single analysis proved unsuccessful. The (*R*)-isomers of 6-, 7-, and 8-hydroxywarfarin could not be separated; therefore, measurements of these metabolites were accomplished in two analytical phases. The first analytical phase fully resolved warfarin and the various hydroxywarfarins but did not account for specific enantiomers. This analytical phase was accomplished by reverse-phase HPLC using a 4.6 mm × 150 mm C18 Zorbax Eclipse 5 μm XDB-C18 column provided by Agilent (Santa Clara, CA). The operating temperature was 40 °C, and the sample injection volume was 5.0 μL. HPLC mobile phases consisted H_2O (0.1% acetic acid v/v) (A) and methanol (0.1% acetic acid v/v) (B). The flow rate was 1 mL/min. A gradient was used to elute warfarin species as follows: 50% A and 50% B for the first 30 s, followed by a linear gradient from 50 to 90% B (0.5–5.5 min), 90 to 100% B (5.5–5.6 min), 100% B maintained for 2.3 min, and 100 to 50% B (7.9–8.0 min) with 50% B maintained for 2 min. The total run time per sample was 10 min. Specific MS/MS experimental conditions used in this study are noted in the Supporting Information, Table 1.

To determine the relative abundance of the (*R*)- and (*S*)-enantiomers of each metabolite, a 4.6 mm × 25 cm Chirobiotic V 5 μm column supplied by Supelco (Bellefonte, PA) was incorporated during the second analytical phase. Mobile phases consisted of H_2O (0.1% acetic acid v/v) (A) and acetonitrile (0.1% acetic acid v/v) (B) with a flow rate of 800 μL/min.

Enantiomeric separations were accomplished under isocratic conditions (80% A and 20% B). The sample injection volume was 15.0 μL. Enantiomeric MS/MS analyses were performed in negative ion mode by electrospray ionization (ESI) using a Turbo IonSpray source. A total run time of 20 min was required for adequate resolution. Specific MS/MS experimental conditions used in this study are noted in the Supporting Information, Table 2.

Quantitative Measurements

An internal standard method was used to quantitatively measure warfarin and OH-WARs by comparing relative MRM responses associated with authentic standards (0.5–100 nM) made in human base urine known to be free of warfarin and warfarin metabolites. Each response was normalized to that of the internal standard, 7-hydroxycoumarin (10 μM). When urine was treated with an excess of β- glucuronidase (GUS), GLUC-conjugated warfarin metabolites were liberated, allowing quantitative measurements of the corresponding OH-WARs and/or warfarin. Enantiomeric determinations of (*R*)-WAR, (*R*)-4′-OH-WAR, (*R*)-10-OH-WAR, as well as (*S*)-WAR and all of the (*S*)-OH-WAR metabolites were directly derived from standard curves generated with authentic standards. However, (*R*)-6-, (*R*)-7-, and (*R*)-8-OH-WAR coeluted and could not be measured directly. Measurements of these three metabolites were calculated by taking the difference of the amount measured in the first analytical phase, which contained both (*R*)- and (*S*)-isomers from the amount of the (*S*)-isomer measured in the second analytical phase (eq 3).

Glucuronidated metabolites were measured by incubating 250 μL of urine with 2000 units of GUS in 100 mM sodium acetate buffer, pH 5.0. The final volume for each of these reactions was 1 mL. Control reactions omitting GUS were included with each sample set. Reactions were incubated overnight at 37 °C, after which they were stored at −80 °C. Immediately before analysis, both samples were diluted with an equal volume of ethanol containing 7-hydroxycoumarin (10 μM final concentration). The difference between GUS-treated samples and controls represented the amount of glucuronidated metabolites present. Example calculations are as follows:

Example Calculation of Glucuronides

$$[7-\mathrm{OH}-\mathrm{WAR}]_{\mathrm{GUS}}=[7-\mathrm{OH}-\mathrm{WAR}]_{\mathrm{control}}=[7-O-\mathrm{GLUC}-\mathrm{WAR}] \quad (1)$$

Example Calculation of Glucuronide Enantiomers

$$[(S)-7-\mathrm{OH}-\mathrm{WAR}]_{\mathrm{GUS}}-[(S)-7-\mathrm{OH}-\mathrm{WAR}]_{\mathrm{control}}=[(S)-7-O-\mathrm{GLUC}-\mathrm{WAR}] \quad (2)$$

Example Calculation of (*R*)-Glucuronide Enantiomers

$$[7-O-\mathrm{GLUC}-\mathrm{WAR}]_{\text{measured in analytical phase I}}-[(S)-7-O-\mathrm{GLUC}-\mathrm{WAR}]_{\text{measured in analytical phase II}}=[(R)-7-O-\mathrm{GLUC}-\mathrm{WAR}] \quad (3)$$

Precision, Accuracy, and Method Detection Limit Studies

Quality control high (100 nM) and quality control low (10 nM) solutions were made from base human urine. Quality control samples were spiked with a mixture of WAR, 4′-OH-WAR, 6-

OH-WAR, 7-OH-WAR, 8-OH-WAR, and 10-OH-WAR. Because WAR and OH-WARs exist as racemic mixtures, the same quality control high and quality control low solutions were used for enantiomeric measurements, but concentrations of each enantiomer were 50 and 5 nM, respectively. Accuracy and precision were evaluated over nonconsecutive days by taking the mean of seven independent analyses and calculating the % RSD. The lower limits of quantitation for each analytical phase were determined by multiplying the standard deviation of the quality control low sample by 3.

Recombinant Cytochrome P450 Incubations and GUS Reactions

Commercially available enantiomers of WAR were used to make enantiomeric assignments. Enantiomeric pure OH-WARs were generated through specific chemoenzymatic reactions. CYP1A2, CYP2C9*1, CYP2C19, and CYP3A4 were each incubated individually with (*R*)-WAR or (*S*)-WAR. The enzyme was at 25 nM in 50 μM potassium phosphate buffer. The substrate was at 1 mM final concentration with EDTA at a final concentration of 0.1 mM. A glucose-6-phosphate NADPH regeneration system was used with $NADP^+$, glucose-6-phosphate, and glucose 6-phosphate dehydrogenase in excess. Each reaction was incubated for 30 min at 37 °C and quenched with an equal volume of ethanol containing 7-hydroxycoumarin for a final concentration of 10 μM. These reactions generated stereospecific OH-WARs, which could be used for OH-WARs enantiomeric retention time assignments.

Results

Analytical Method Development and Validation

Separation was optimized for the measurement of warfarin and monohydroxylated warfarin derivatives during the first analytical phase. Internal standard (7-hydroxycoumarin), WAR, 4′-, 6-, 7-, 8-, and 10-OH-WAR achieved baseline separation. Retention times for 7-hydroxycoumarin, WAR, 4′-, 6-, 7-, 8-, and 10-OH-WAR were 2.7, 6.6, 5.0, 5.6, 6.0, 6.2, and 5.4 min, respectively (Figure 2A). Standard curves (0.5–100 nM) provided a wide linear working range ($r^2 \geq 0.999$), and recovery of quality control specimens at 10 and 100 nM showed a high degree of precision and accuracy (Table 1). Similar results were also observed during the second analytical phase. A chiral LC-MS/MS method was optimized for separation and measurement of specific warfarin enantiomers. Internal standard (7-hydroxycoumarin), (*S*)-WAR, (*S*)-4′-, (*S*)-6-, (*S*)-7-, (*S*)-8-, and (*S*)-10-OH-WAR achieved baseline separation (Figure 2B). Retention times for 7-hydroxycoumarin, (*S*)-WAR, (*S*)-4′-, (*S*)-6-, (*S*)-7-, (*S*)-8-, and (*S*)-10-OH-WAR were 6.8, 16.5, 13.9, 13.5, 14.5, 12, and 11.2 min, respectively. (*R*)-WAR, (*R*)-4′-OH-WAR, and (*R*)-10-OH-WAR eluted at approximately 12, 9.5, and 8 min, respectively, while three enantiomers, (*R*)-6-, (*R*)-7-, and (*R*)-8-OH-WAR, coeluted around 10 min. Standard curves (0.25–50 nM) provided a wide linear working range ($r^2 \geq 0.991$). Recovery of quality control specimens at 5 and 50 nM showed a high degree of precision and accuracy (Table 1). For both analytical phases, minimum detection limit (MDL) and lower limit of quantification (LLQ) are provided for each analyte (Table 1). MRLs were 8-fold higher than the LLQ to incorporate dilution factors associated with GUS treatment. All metabolites were quantitatively measured in each analytical phase as described in the Materials and Methods.

Urine Sample Analysis

In an effort to assess the applicability of this new method and to begin assessing the toxicological significance of metabolic pathways now being recognized as important determinants for warfarin toxicity (10,11), WAR and OH-WARs were measured in three purchased human urine samples received from female donors. Chromatographs obtained for F84 are provided as representative examples (Figures 2B, C, E, F). Retention times and specific mass transitions of urinary metabolites corresponded well with authentic standards (Figure 2A,

D). Optimization parameters previously established for OH-WAR-GLUCs (10) were included during the first analytical phase to ensure that GUS treatment was complete. The complete disappearance of glucuronides and a concomitant increase in WAR and OH-WAR responses post-GUS treatment shows complete GLUC hydrolysis (Figure 2B, C). Relative abundances of all of the WAR metabolites measured were highly variable among the specimens (Figure 3). Both native WAR and 4-O-GLUC-WAR (glucuronide of native warfarin) were identified in each of the three urine samples, but direct WAR glucuronidation seemed to be primarily associated with the (*R*)-enantiomer (Figure 3). The most predominant metabolites observed in all three specimens were 6-OH-WAR, 7-OH-WAR, and their corresponding GLUC conjugates (Figure 3). Lower concentrations of 4′-OH-WAR, 8-OH-WAR, and all of the corresponding GLUC metabolites were also detected in these specimens (Figure 3). 10-OH-WAR was not detected in any specimen (Figure 3). Mass spectra for the glucuronidated metabolites presented in this report have previously been reported (10).

The total amount of OH-WAR excreted (free and conjugated) in these urine specimens ranged from approximately 1 to 4 μM (Table 2). These high concentrations demonstrate that both phase I and II metabolic pathways are highly active in the presence of WAR and OH-WARs. The predominance of (*S*)-6- and (*S*)-7-OH-WAR in all three specimens suggests significant CYP2C9 activity (4), while the combined presence of other OH-WARs suggests the involvement of other P450s. WAR, 4′-OH-WAR, 8-OH-WAR, and corresponding GLUC metabolites were found at lower individual concentrations (10–200 nM); however, the combined contribution of all of these metabolites represents multiple detoxification pathways, which when evaluated together demonstrate a large human capacity for detoxifying and excreting warfarin. It is also interesting to note the seemingly enantioselective reactions observed for both phase I and II reactions (Figure 3 and Table 2). For example, it appears that (*S*)-7-OH-WAR is selectively glucuronidated (Figure 3A, B), although some patients appear to have the ability to glucuronidate both (*S*)- and (*R*)-7-OH-WAR (Figure 3A, C).

Data presented also show the importance of considering both phase I and II metabolic pathways while assessing specific P450 contributions. For example, the total amount of glucuronides varied significantly among the three specimens (14–58%), which can cause miscalculation of P450 product ratios if UGT metabolism is ignored. The predicted product ratio of (*S*)-6-OH-WAR:(*S*)-7-OH-WAR is approximately 0.2 (4), which is the case for F85 and F87. The product ratio for F84 is approximately 0.5 when GLUC metabolites are ignored but 0.2 when included. By assessing both P450 and UGT metabolites, all three patients are normalized, and resulting data suggest that (*S*)-6-OH-WAR and (*S*)-7-OH-WAR formation are primarily mediated through CYP2C9 catalysis.

Discussion

The LC-MS/MS approach developed in this study has the unique ability to identify and measure 24 WAR metabolites in human urine. This is the first in vivo report to demonstrate that humans have a high capacity to directly glucuronidate warfarin and its P450-generated metabolites. High concentrations of glucuronides demonstrate that UGT phase II metabolism is very active in humans and represents an important pathway for efficient warfarin detoxification and excretion. The predominance of (*S*)-7-OH-WAR is consistent with the widely acknowledged significance of CYP2C9 in WAR metabolism (15), but other “minor” P450 pathways collectively contribute as much or more than CYP2C9 to the generation of excretable metabolites.

(*S*)-7-OH-WAR, a marker metabolite for CYP2C9 (14), is the most abundant (*S*)-WAR P450 metabolite identified in these studies (61–71%). CYP2C9 also generates (*S*)-6-OH-WAR but with 4-fold less efficiency (4,16). Product ratio analyses show that CYP2C9 alone accounts

for approximately 78–95% of all (*S*)-WAR metabolites. The identities and levels of remaining (*S*)-WAR metabolites are highly variable. (*S*)-8-OH-WAR accounts for 4–20% of the metabolites measured. CYP3A4 is thought to produce this metabolite (4), but the absence of 10-OH-WAR argues against this metabolic pathway. (*S*)-4′-OH-WAR is then probably derived from CYP2C8, CYP2C18, and/or CYP2C19 activity (17).

Limited kinetic investigations with (*R*)-warfarin makes assigning P450 contribution for this isomer difficult. The production of (*R*)-8-OH-WAR in F85 and F87 shows the significance of CYP2C19 in warfarin metabolism (18), although CYP1A2 also produces this metabolite (7). CYP1A2 specifically generates (*R*)-6- and (*R*)-8-OH-WAR at a ratio of 5:1. Increased production of (*R*)-6-OH-WAR in F85 is suggestive of CYP1A2 induction, possibly through tobacco smoke exposures. The major (*R*)-metabolite for F84 and F87 is (*R*)-7-OH-WAR, which is possibly a marker product for CYP2C8 activity (17). As observed for the (*S*)-isomer, there are no measurable levels of (*R*)-10-OH-WAR, and low levels of (*R*)-4′-OH-WAR may indicate CYP2C8 and/or CYP2C18 activity (17).

Glucuronidation presumably promotes the elimination of metabolites through selective transporters and abolishes any VKER binding due to the introduction of a bulky acidic sugar. The 4-hydroxyl group of WAR, the only site available for conjugation, is required for VKER binding (19,20). The preferential glucuronidation of the (*R*)-isomer among the patients may partly explain the lower biological activity of this form of the drug (3), although it is not known whether (*R*)- or (*S*)-WAR bind VKER with differing affinities. WAR oxidation by P450s introduces a second hydroxyl group for conjugation, resulting in more efficient glucuronidation (10). The diversity and levels of glucuronides in urine attest to the significance of UGT activity toward these compounds. Assigning specific UGT contributions at this time is difficult because only racemic mixtures have been used in vitro to assess specific activities and because the isozyme responsible for direct warfarin glucuronidation has not been identified (10). However, some conclusions can be made from human data presented in this report. Production of 6-*O*-GLUC is indicative of UGT1A1 and/or UGT1A10 activity (10), and the contribution of two enzymes is further evidenced in this report by the preferential excretion of the (*S*)-6-*O*-GLUC isomer in F84 and the (*R*)-6-*O*-GLUC isomer in F85 and F87. The glucuronidation of 7- and 8-OH-WARs is more complex due to the potential involvement of UGT1A1, UGT1A3, UGT1A8, UGT1A9, and/or UGT1A10 (10). The presence of 4′-*O*-GLUC is indicative of UGT1A10 activity (10).

Human data presented in this report begin demonstrating the complexity of warfarin detoxification and begin characterizing the significance of both phase I and II enzymes. The significance of phase I metabolism has been the focus of much research for many years, but phase II glucuronidation is just now being recognized as another indispensible step for warfarin detoxification. Little focus has been given to hydroxywarfarin glucuronidation because hydroxywarfarins are generally thought to be biologically inactive. However, early rodent experiments conducted in Karl Link's laboratory show that hydroxywarfarins are fatal to rats receiving 6 mg/kg (21). The significance of this study is limited because only a single dose is used and there is little to no repetition. Nevertheless, recent VKER studies demonstrate that 6-OH-WAR retains similar binding affinity toward the drug target as shown for biologically active warfarin alcohols (20), and it seems that 4′-modifications of warfarin increase affinity toward VKER (20) and anticoagulant efficacy (22). We have also shown that hydroxywarfarins can inhibit P450s in vitro at toxicologically relevant concentrations through a negative feedback mechanism (10). Continued characterization of these metabolic pathways will improve our ability to fully assess the physiological significance of phase I and II warfarin metabolites.

The widespread usage and efficacy of WAR is driving many efforts aimed at developing approaches that improve drug treatment strategies and consequently pharmacological outcomes. This report validates a comprehensive LC-MS/MS method capable of assessing P450 and UGT contribution to warfarin metabolism. Human data confirm that OH-WARs reach high enough concentrations in vivo to serve as substrates for UGTs. Furthermore, it appears that glucuronidation is a major pathway of WAR detoxification and excretion by either direct warfarin conjugation or through subsequent metabolism of OH-WARs. Taken together, both P450 and UGT metabolic pathways are important for facilitating the flux of warfarin through complex metabolic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Vi-Huyen Le and Cheryl Lichti with the Arkansas Department of Health, Public Health Laboratory, and Bob Kobelski with the Centers for Disease Control for their valuable contributions while preparing this manuscript.

References

(1). Lesko L. The critical path of warfarin dosing: finding an optimal dosing strategy using pharmacogenetics. Clin. Pharmacol. Ther 2008;84:301–303. [PubMed: 18714317]

(2). Bristol-Myers. Coumadin tablets. 2005.

(3). Eble JN, West BD, Link KP. A comparison of the isomers of warfarin. Biochem. Pharmacol 1966;15:1003–1006.

(4). Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ, Trager WF. Hydroxylation of warfarin by human cDNA-expressed cyto-chrome P-450: A role for P-4502C9 in the etiology of (*S*)-warfarin-drug interactions. Chem. Res. Toxicol 1992;5:54–59. [PubMed: 1581537]

(5). Kaminsky LS, Zhang ZY. Human P450 metabolism of warfarin. Pharmacol. Ther 1997;73:67–74. [PubMed: 9014207]

(6). Wadelius M, Chen LY, Eriksson N, Bumpstead S, Ghori J, Wadelius C, Bentley D, McGinnis R, Deloukas P. Association of warfarin dose with genes involved in its action and metabolism. Hum. Genet 2007;121:23–34. [PubMed: 17048007]

(7). Zhang Z, Fasco MJ, Huang Z, Guengerich FP, Kaminsky LS. Human cytochromes P450 1A1 and 1A2: *R*-warfarin as a probe. Drug Metab. Dispos 1995;23:1339–1345. [PubMed: 8689941]

(8). Ritter JK. Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chem.-Biol. Interact 2000;129:171–193. [PubMed: 11154740]

(9). Jansing R, Chao E, Kaminsky L. Phase II metabolism of warfarin in primary culture of adult rat hepatocytes. Mol. Pharmacol 1992;41:209–215. [PubMed: 1732719]

(10). Zielinska A, Lichti CF, Bratton S, Mitchell NC, Gallus-Zawada A, Le V-H, Finel M, Miller GP, Radominska-Pandya A, Moran JH. Glucuronidation of monohydroxylated warfarin metabolites by human liver microsomes and human recombinant UDP-glucuronosyltransferases. J. Pharmacol. Exp. Ther 2008;324:139–148. [PubMed: 17921187]

(11). Miller GP, Lichti CF, Zielinska AK, Mazur A, Bratton SM, Gallus-Zawada A, Finel M, Moran JH, Radominska-Pandya A. Identification of hydroxywarfarin binding site in human UDP glucuronosyltransferase 1A10: Phenylalanine90 is crucial for the glucuronidation of 6- and 7-hydroxywarfarin but not 8-hydroxywarfarin. Drug Metab. Dispos 2008;36:2211–2218. [PubMed: 18725508]

(12). Au N, Rettie AE. Pharmacogenomics of 4-hydroxycoumarin anticoagulants. Drug Metab. Rev 2008;40:355–375. [PubMed: 18464049]

(13). Cooper GM, Johnson JA, Langaee TY, Feng H, Stanaway IB, Schwarz UI, Ritchie MD, Stein CM, Roden DM, Smith JD, Veenstra DL, Rettie AE, Rieder MJ. A genome-wide scan for common

genetic variants with a large influence on warfarin maintenance dose. Blood 2008;112:1022–1027. [PubMed: 18535201]

(14). Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. Mass Spectrom. Rev 2007;26:51–78. [PubMed: 16921475]

(15). Rettie A, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ, Trager WF. Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: A role for P-4502C9 in the etiology of (*S*)-warfarin-drug interactions. Chem. Res. Toxicol 1992;5:54–59. [PubMed: 1581537]

(16). Haining RL, Hunter AP, Veronese ME, Trager WF, Rettie AE. Allelic variants of human cytochrome P450 2C9: Baculovirus-mediated expression, purification, structural characterization, substrate stereoselectivity, and prochiral selectivity of the wild-type and I359L mutant forms. Arch. Biochem. Biophys 1996;333:447–458. [PubMed: 8809086]

(17). Kaminsky L, de Morais S, Faletto M, Dunbar D, Goldstein J. Correlation of human cytochrome P4502C substrate specificities with primary structure: Warfarin as a probe. Mol. Pharmacol 1993;43:234–239. [PubMed: 8429826]

(18). Wienkers LC, Wurden CJ, Storch E, Kunze KL, Rettie AE, Trager WF. Formation of (*R*)-8-hydroxywarfarin in human liver microsomes: A new metabolic marker for the (*S*)-mephenytoin hydroxylase, P4502C19. Drug Metab. Dispos 1996;24:610–614. [PubMed: 8723744]

(19). Lee JJ, Fasco MJ. Metabolism of vitamin K and vitamin K 2,3-epoxide via interaction with common disulfide. Biochemistry 1984;23:2246–2252. [PubMed: 6733086]

(20). Gebauer M. Synthesis and structure-activity relationships of novel warfarin derivatives. Bioorg. Med. Chem 2007;15:2414–2420. [PubMed: 17275317]

(21). Eble, JN. Studies on the 4-hydroxycoumarin anticoagulants warfarin and cyclocumarol. University of Wisconsin Library; 1953. p. 22-30.Part II

(22). Lasseur R, Grandemange A, Longin-Sauvageon C, Berny P, Benoit E. Comparison of the inhibition effect of different anticoagulants on vitamin K epoxide reductase activity from warfarin-susceptible and resistant rat. Pestic. Biochem. Physiol 2007;88:203–208.

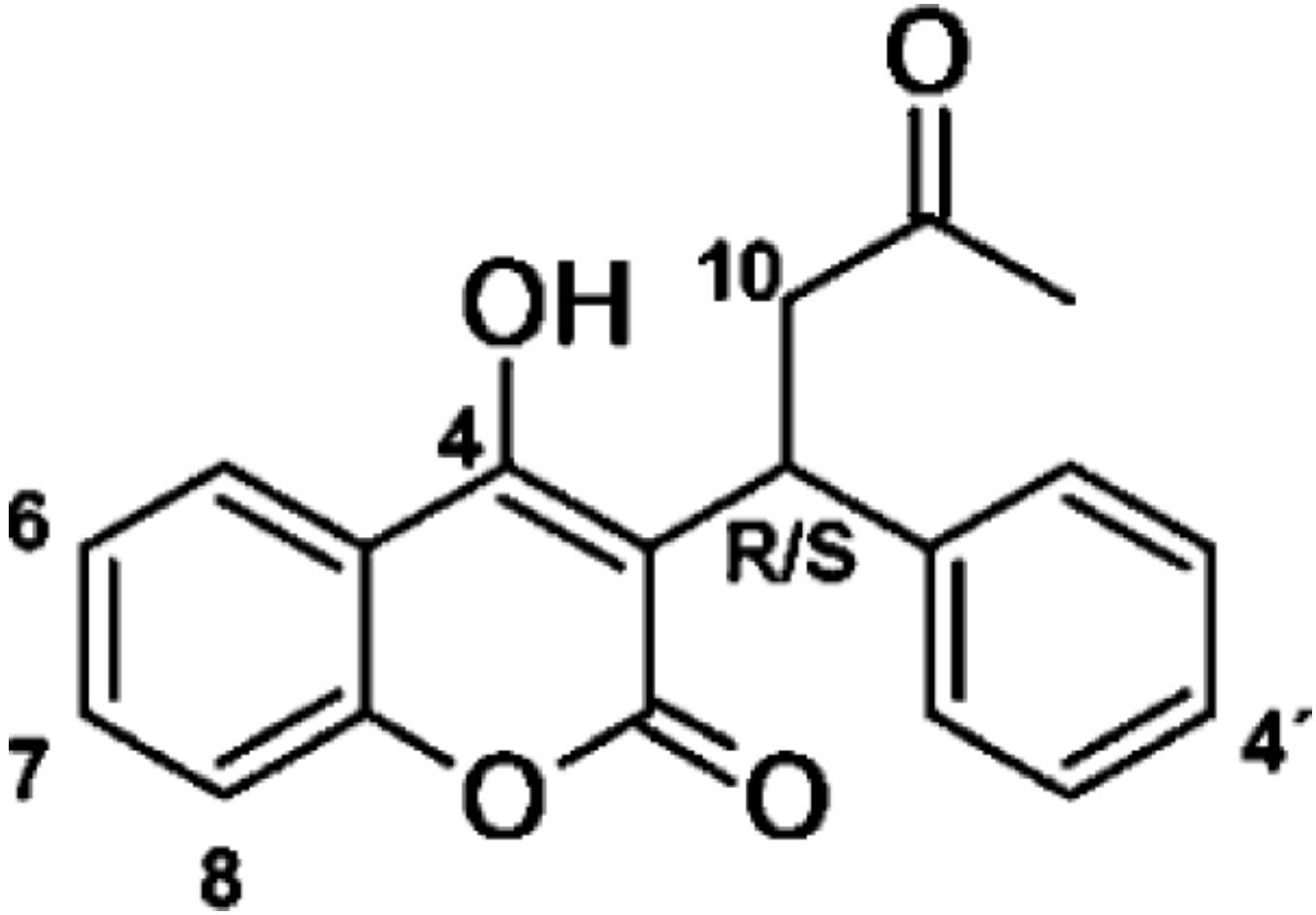

Figure 1.
Schematic representation of warfarin. Numbers indicate active sites for cytochrome P450 hydroxylations and subsequent UGT-catalyzed conjugation.

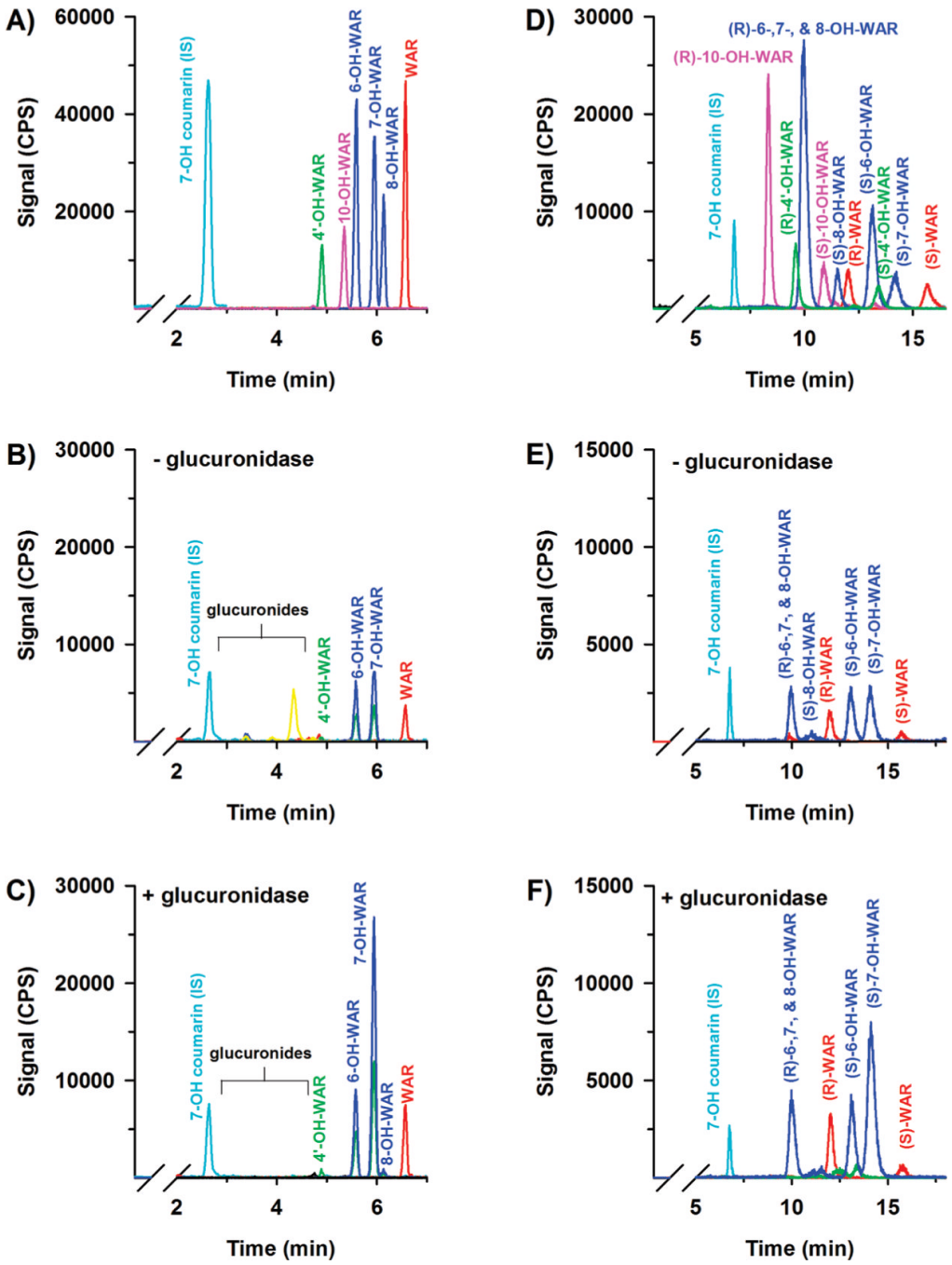

Figure 2.

Representative chromatograms produced during MRM experiments. (A–C) Represent the first analytical phase followed by (D–F) the second chiral analytical phase. Panels A and D represent 100 nm and 50 nM authentic standards, respectively. Panels B and E represent pre β-glucuronidase treatment, while panels C and F represent post β-glucuronidase treatment. All analytical conditions are described in the Materials and Methods. Different color tracings represent unique MRM transitions for each metabolite (see the Supporting Information, Tables 1 and 2).

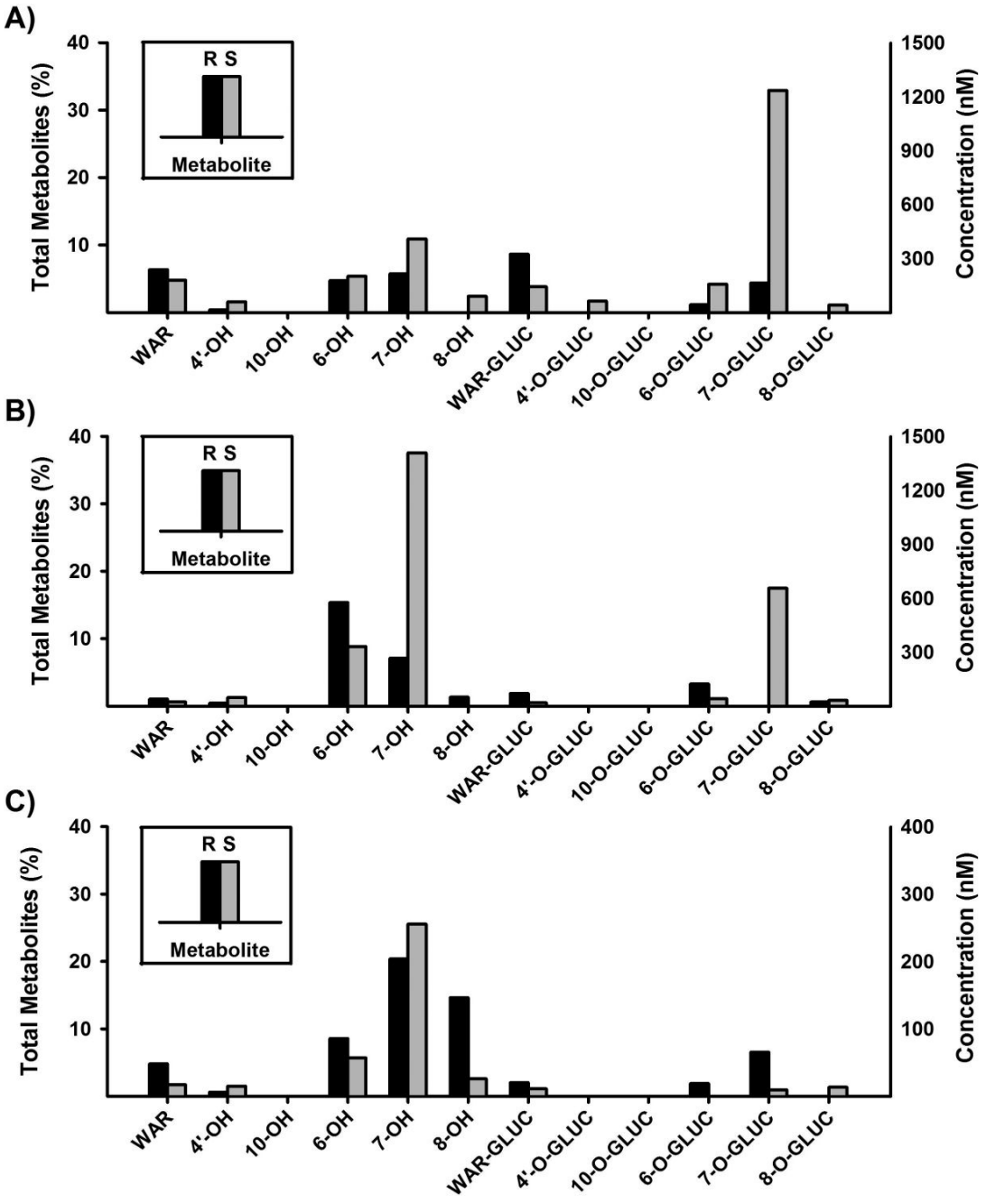

Figure 3.

Metabolic profiles for (A) F84, (B) F85, and (C) F87. Values represent the concentration of each metabolite in urine (nM). Each metabolite is composed of (black) (*R*)- and (gray) (*S*)-enantiomers. All analytical conditions and calculations are described in the Materials and Methods.

Table 1

Summary of Correlation Coefficients, Quality Control Measurements, and Minimum Detection and Reporting Limits[a]

		mean ± % RSD[b]		nM		
analytical phase I	**correlation coefficient (r^2)**	**quality control low (10 nM)**	**quality control high (100 nM)**	**MDL[c]**	**LLQ[e]**	**MRL[d]**
4′-OH-WAR	0.999	10.6 ± 23 (33)	104 ± 17 (24)	<0.5	7	59
10-OH-WAR	0.999	9.4 ± 31 (44)	102 ± 19 (27)	<0.5	9	70
6-OH-WAR	0.999	10.3 ± 21 (30)	101 ± 22 (31)	<0.5	7	53
7-OH-WAR	0.999	10.7 ± 25 (35)	102 − 19 (27)	<0.5	8	65
8-OH-WAR	0.999	11.1 ± 16 (23)	105 ± 15 (21)	<0.5	5	43
WAR	0.999	10.4 ± 23 (33)	105 ± 14 (20)	<0.5	7	58
		mean ± % RSD[b]		**nM**		
analytical phase II	**correlation coefficient (r^2)**	**quality control low (5 nM)**	**quality control high (50 nM)**	**MDL**	**LLQ**	**MRL**
(*R*)-WAR	0.999	5.6 ± 16 (23)	46 ± 22 (31)	<0.25	3	21
(*S*)-WAR	0.998	5.6 ± 12 (17)	55 ± 11 (16)	<0.25	2	17
(*S*)-4′-OH-WAR	0.994	5.4 ± 24 (34)	40 ± 36 (51)	<0.25	4	31
(*S*)-10-OH-WAR	0.994	5.1 ± 51 (72)	45 ± 14 (20)	<0.25	8	64
(*S*)-6-OH-WAR	0.994	5.3 ± 20 (28)	46 ± 17 (24)	<0.25	3	26
(*S*)-7-OH-WAR	0.998	5.3 ± 22 (31)	45 ± 24 (34)	<0.25	4	28
(*S*)-8-OH-WAR	0.999	5.5 ± 15 (21)	51 ± 27 (38)	<0.25	3	20
(*R*)-4′-OH-WAR	0.991	5.4 ± 17 (24)	48 ± 17 (24)	<0.25	3	21
(*R*)-10-OH-WAR	0.991	5.3 ± 19 (27)	53 ± 18 (25)	<0.25	3	25

[a] Values in parentheses represent the errors associated with calculations used to determine glucuronide concentrations. The errors associated with calculations used to determine (*R*)-6-, (*R*)-7-, and (*R*)-8-OHWAR concentrations ranges were 29, 33, and 22% RSD, respectively.

[b] % RSD = 100(CV/mean).

[c] MDL = minimum detection limit.

[d] MRL = minimum reporting limit.

[e] LLQ = lower limit of quantification.

Table 2

Total P450 Metabolism/Contribution Summary[a]

		6-OH-WAR	7-OH-WAR	8-OH-WAR	10-OH-WAR	4′-OH-WAR	total
F84	urinary concentration (nM)	560	1964	111	ND	140	2775
	% conjugated	35	69	31	ND	47	58
	% *S*	35	20	0	ND	56	77
	% *R*	65	80	100	ND	64	33
F85	urinary concentration (nM)	1124	2404	109	ND	94	3731
	% conjugated	15	28	53	ND	ND	27
	% *S*	31	62	2	ND	75	70
	% *R*	69	38	98	ND	25	30
F87	urinary concentration (nM)	204	724	231	ND	20	1179
	% conjugated	12	14	7	ND	ND	14
	% *S*	38	48	31	ND	70	47
	% *R*	62	52	69	ND	30	53

[a]The % S represents the proportion of each metabolite that is in the (*S*)-enantiomeric form. The % conjugated represents the proportion of each phase I metabolite that is glucuronidated. ND, not detected. The error associated with these values is described in Table 1.