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Comparison of enzyme kinetics of warfarin analyzed by LC–MS/MS QTrap and differential mobility spectrometry

Abdul Naveed Shaik[a,b,*], Richard Grater[b], Mukesh Lulla[b], David A. Williams[a], Lawrence L. Gan[c], Tonika Bohnert[b], Barbara W. LeDuc[a]

[a] Department of Pharmaceutical Sciences, MCPHS University, 179 Longwood Ave., Boston, MA 02115, USA
[b] Department of Drug Metabolism and Pharmacokinetics, Biogen, 14 Cambridge Center, Cambridge, MA 02140, USA
[c] Development Center for Biotechnology, Taipei 221, Taiwan

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ABSTRACT

Warfarin is an anticoagulant used in the treatment of thrombosis and thromboembolism. It is given as a racemic mixture of *R* and *S* enantiomers. These two enantiomers show differences in metabolism by CYPs: *S*-warfarin undergoes 7 hydroxylation by CYP2C9 and *R*-warfarin by CYP3A4 to form 10 hydroxy warfarin. In addition, warfarin is acted upon by different CYPs to form the minor metabolites 3′-hydroxy, 4′-hydroxy, 6-hydroxy, and 8-hydroxy warfarin. For analysis, separation of these metabolites is necessary since all have the same *m*/*z* ratio and similar fragmentation pattern. Enzyme kinetics for the formation of all of the six hydroxylated metabolites of warfarin from human liver microsomes were determined using an LC–MS/MS QTrap and LC–MS/MS with a differential mobility spectrometry (DMS) (SelexION™) interface to compare the kinetic parameters. These two methods were chosen to compare their selectivity and sensitivity. Substrate curves for 3′-OH, 4′-OH, 6-OH, 7-OH, 8-OH and 10-OH warfarin formation were generated to determine the kinetic parameters (K_m and V_{max}) in human liver microsomal preparations. The limit of quantitation (LOQ) for all the six hydroxylated metabolites of warfarin were in the range of 1–3 nM using an LC–MS/MS QTrap method which had a run time of 22 min. In contrast, the LOQ for all the six hydroxylated metabolites using DMS interface technology was 100 nM with a run time of 2.8 min. We compare these two MS methods and discuss the kinetics of metabolite formation for the metabolites generated from racemic warfarin. In addition, we show inhibition of major metabolic pathways of warfarin by sulfaphenazole and ketoconazole which are known specific inhibitors of CYP2C9 and CYP3A4 respectively.

1. Introduction

Warfarin (Coumadin®, (±) 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-chromen-2-one) (Fig. 1), is an orally active anticoagulant used in the treatment and prevention of venous thromboembolism or atrial fibrillation. Warfarin is administered as a racemic mixture of *R* and *S* warfarin, pharmacologically, *S*-warfarin is approximately 3–5 times more potent than the *R*-enantiomer and is more rapidly cleared from the body [1]. Warfarin is excreted mainly in the urine, terminal half-life of *R*-warfarin ranges from 37 to 89 h, while *S*-warfarin ranges from 21 to 43 h [2].

Warfarin acts by inhibiting the synthesis of vitamin K-dependent coagulation factors II, VII, IX, and X, along with the anticoagulant proteins C and S [3]. Vitamin K is the essential cofactor required in the post-translational modification of these clotting factors, because it promotes the synthesis of γ-carboxyglutamic acid required for biological activity [4]. Warfarin inhibits the C1 subunit of the vitamin K epoxide reductase (VKORC1) enzyme complex, resulting in a decrease in reduced vitamin K1 [5]. The peak anticoagulant effect is not observed until 72–96 h following inhibition, when the pre-existing reduced form of vitamin K is depleted. Therefore, a single dose of warfarin has a duration of action of 2–5 days [6].

Warfarin undergoes metabolism by cytochrome 450 (CYP), with both the pure enantiomers showing CYP mediated metabolism to form various hydroxylated metabolites, 3′-hydroxy, 4′-hydroxy, 6-hydroxy, 7-hydroxy, 8-hydroxy, and 10-hydroxy warfarin metabolites (Fig. 1). The two enantiomers are stereoselectively metabolized by different CYPs: *S*-warfarin predominantly under-

* Corresponding author at: Department of Pharmaceutical Sciences, MCPHS University, 179 Longwood Ave., Boston, MA 02115, USA. Fax: +1 407 313 7030.
E-mail address: naveed.shaik@my.mcphs.edu (A.N. Shaik).

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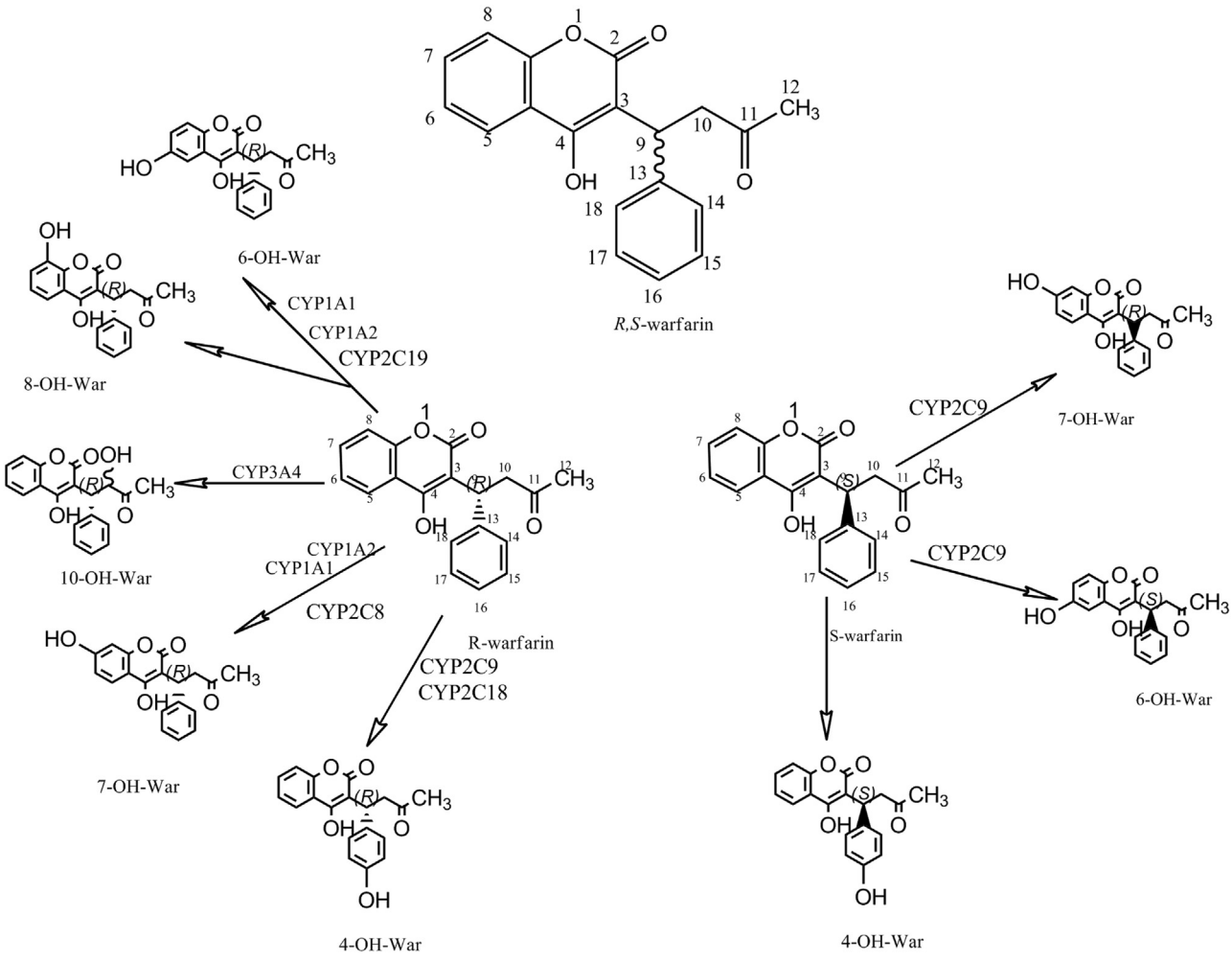

Fig. 1. Metabolic pathway of warfarin enantiomers.

goes 7-hydroxylation by CYP2C9 while *R*-warfarin preferentially forms 10-hydroxy warfarin via CYP3A4. *S*-warfarin is approximately 90% oxidized by CYP2C9 and *R*-warfarin is approximately 60% oxidized by CYP1A2 and CYP3A4 [7]. Apart from these major metabolites, warfarin is also metabolized by CYPs 1A1, 1A2, 2C8, 2C18, and 2C19 to form 4′-OH, 6-OH, and 8-OH warfarin [8,9]. Both 7-OH warfarin and 10-OH warfarin inhibit CYP2C9, thus preventing further formation of the CYP2C9 mediated 7-hydroxy metabolite [10].

Few methods have been reported for quantitation of warfarin and all of its six hydroxylated metabolites using either HPLC or LC–MS/MS [11,12]. The most recently reported method has a LOD of 0.1 nM, but separated only 7-OH and 10-OH warfarin [13]. We are reporting a highly sensitive LC–MS/MS method with QTrap that has been developed and validated for simultaneous determination of warfarin plus its six hydroxylated metabolites from human liver microsomes (HLM). This method has a low limit of quantitation (LLOQ) of 1 nM for all the metabolites. The method reported here uses 8-cyclopentyl-1,3-dipropyl xanthine (CPDPX) as internal standard (IS) at a concentration of 10 ng/mL. This method has been applied to study the enzyme kinetics of the CYPs involved in hydroxylation of warfarin. Furthermore, a chiral method was developed using LC–MS/MS QTrap with a chiral column (Chiracel, Chiral Technologies, PA, USA) for separation of pure warfarin enantiomers.

Separately, another LC method using a differential mobility spectrometry (DMS) interface was developed and used to quantitate all of the six hydroxylated warfarin metabolites. The ions in DMS are separated in trajectory based on difference in migration rates between the high and low electric field portions of the applied asymmetric, high frequency, waveform RF voltage known as separation voltage (SV). These two electric fields are applied alternately, high field is applied between the electrodes for a short period of time followed by low field which is applied in the opposite direction for a longer duration. Differences between the low-field and high-field mobility of an analyte ion causes it to oscillate and ultimately drift in perpendicular direction, losing its trajectory [14,15]. An ion which loses its trajectory is steered back to center by the application of an offset potential, known as the compensation voltage (CoV) which nullifies the drift. The CoV is a compound-specific parameter that can be used to selectively filter out all other ions. Rapid switching of the compensation voltage parameter allows the user to concurrently monitor many different compounds [15] and also separate co-eluting isomeric compounds and metabolites using their different CoV values [16,17]. A commercially available interface (SelexION™, Applied Biosystems, Framingham, MA, USA) exploits DMS principles and has the potential to provide enhanced selectivity for quantitation in complex matrices [18], and separate the ions, was used for this study.

The objective of this study was to separate all the metabolites and the pure enantiomers in a single run, reduce the run time for the separation of metabolites, study and compare the kinetics of warfarin metabolism obtained by using ESI (electrospray ionization) and differential mobility spectrometry using a DMS interface. We report the kinetics of formation for all of the hydroxylated metabo-