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LC/MS/MS warfarin assay – An emerging tool for the early detection of cytochrome P450-associated drug–drug interactions in drug discovery [1]

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Abstract. The LC/MS/MS warfarin assay, combining stereo- and regioselective cytochrome P450 (CYP) form-specific warfarin hydroxylation with sensitive and specific LC/MS/MS detection technique, is emerging to be a promising tool for the study of CYP-associated drug–drug interactions for new chemical entities (NCEs) during drug discovery process.

1. Introduction

Cytochromes P450 (CYPs), a superfamily of enzymes, are responsible for the metabolism of the majority of drugs and xenobiotics. In contrast to more than fifty human CYP genes discovered to date (http://drnelson.utmem.edu/CytochromeP450.html), important drug-metabolizing CYP enzymes are rather few, and most of them in the subfamilies of CYP1A, 2C, 2D and 3A [1,2]. Though capable of diverse reactions, P450s most commonly catalyze monohydroxylation (and its derivatives such as dealkylation). One such example is CYP-mediated metabolism of anticoagulant warfarin. The C9 asymmetric carbon of warfarin gives rise to R- and S-enantiomeric forms, and several important human CYP members stereo- and regioselectively convert warfarin to a series of monohydroxylated metabolites, which has been extensively studied, and well delineated over the last decade (Fig. 1) [3,4].

In addition to the functional analyses of individual metabolic CYP forms including the allelic variants and active site modeling, one of the utilities of stereo- and regioselective warfarin hydroxylation recently emerging in drug discovery is the early detection of potential drug–drug interactions for new chemical entities (NCEs) [5,6]. Drug–drug interactions, mainly due to the inhibition or induction of metabolic CYPs, are significant factors of high attrition rates of NCEs in pre- and clinical development. Inhibition of the metabolic enzymes of concurrent drugs would potentially lead to undesirable accumulations of concurrent drugs and elevate the possible toxic side effects. *Vice versa*, enzyme induction may accelerate the rate of drug metabolism, thus potentially attenuate, even abolish the therapeutic effects of the drugs. Additionally, CYP1A1, an extrahepatic CYP member, which plays an insignificant role in

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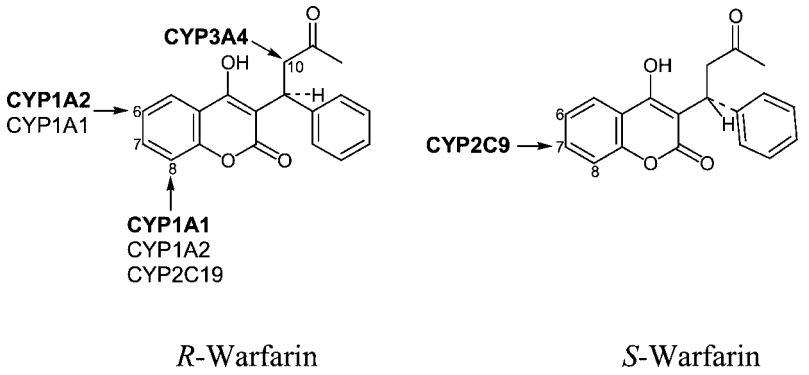

Fig. 1. Human CYP-mediated stereo- and regioselective warfarin metabolism. Major responsible CYP forms are in bold font, and minor forms are in plain font.

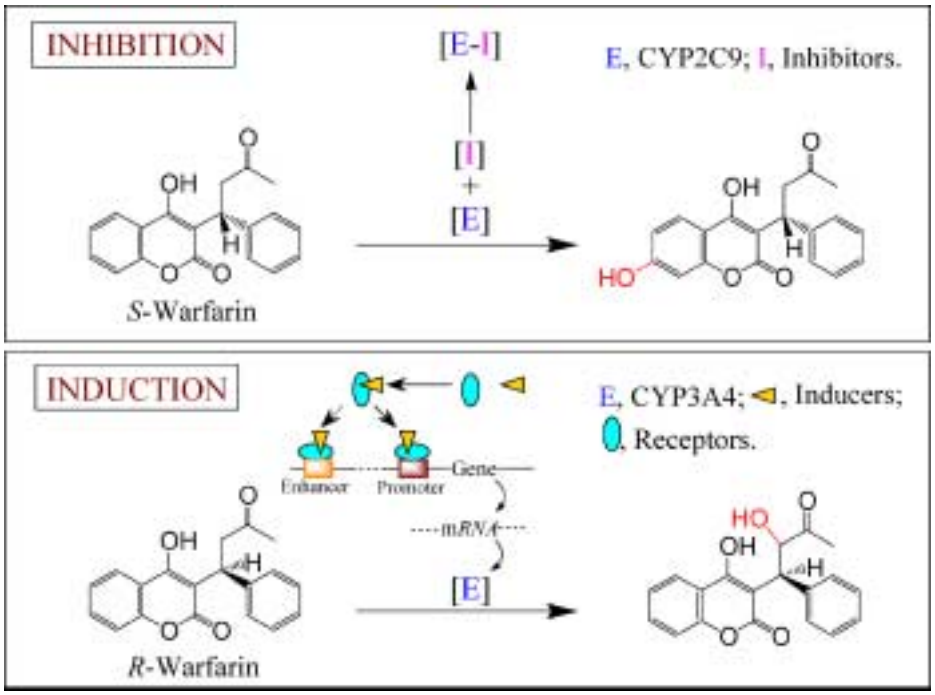

Fig. 2. Schematic diagram of CYP associated drug–drug interactions using warfarin as probe substrate. In the case of enzyme inhibition, drugs or xenobiotics may directly bind to metabolic CYPs reversibly or irreversibly, thus reducing the amount of free enzyme and catalytic activities. In the case of induction, drugs or xenobiotics may serve as ligands for the nuclear receptors. The ligand-bound receptors (*trans*-regulators) then interact with the DNA regulatory elements at the upstream region of CYP genes (*cis*-regulators), and consequently activate the CYP gene transcription.

drug metabolism, is responsible for bioactivating several classes of environmental or food procarcinogens, most known for polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene [7]. Obviously, the inducibility of CYP1A1 has to be avoided for NCEs by evaluating at the early phases of drug development. Therefore, as illustrated in Fig. 2, warfarin enantiomers are well suited for the assessment of potential CYP-NCE interactions, especially for the inhibition and induction of CYP1A1, CYP1A2, CYP2C9, and CYP3A4 [3–5].

The quantitative analyses of warfarin and its hydroxylated metabolites *in vitro* and *in vivo* started at least twenty-five years ago. A variety of detection, especially chromatography-based, methods have been developed during that long period, including the most recent LC/MS/MS method developed in our laboratory [8]. In this article, I will review the qualitative and quantitative characteristics of that newly developed LC/MS/MS technique, in comparison with other existing analytical methods for warfarin metabolism. Moreover, to explicitly demonstrate the advances of the LC/MS/MS method, especially the great potential for drug–drug interaction studies using human cells, the materials presented here combine what originally published and some of recent results [8].

2. Experimental techniques

2.1. Brief summary of conventional detection methods for warfarin metabolism

In the past, considerably efforts have been made to develop analytical methods to quantify oxidative warfarin metabolites in various biological matrices. Most, if not all, of the quantitative methods were chromatography-based. These methods include thin-layer chromatography (TLC), gas chromatography (GC) with mass spectrometric detection (MS), high performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescent (FLD), and mass spectrometric detection, etc. [9–14]. The LC/UV and GC/MS method have been more commonly exploited among these established methods, thus the instrumental conditions of these methods are briefly described.

The HPLC/UV detection technique was reported by Fasco *et al.* [11], which is probably one of the earliest but reliable quantitative methods. Combining the reverse phase C18 column, the gradient mobile phase at the pH close to the pK_a of warfarin, and the detection of UV absorbance at or near the UV_{max} of warfarin and metabolites (approx. 310 nm), that method was capable of resolving and detecting all hydroxylated warfarin metabolites known to form in humans with reasonable sensitivity.

Meanwhile, GC/MS methods with similar principal techniques, probably developed by the efforts from more than one research group, were exemplified by what reported by Bush et al. [10]. The condition of GC/MS operation was considered typical, such as the application of helium carrier gas, and oven temperature program (temperature increased to 250°C at 30°C/min after 2 min isothermal at 160°C, and held for 8–12 min). MS source temperature was set at 200°C, electron impact ionization potential (EI) at 70 eV, and detection at selected-ion monitoring (SIM) mode.

Besides the LC/UV and GC/MS, other techniques including LC/FLD and LC/MS are also useful, at least to some extent, for the metabolic studies of warfarin. However, the applicability of these techniques is rather restricted due to the intrinsic technical limitations. For instance, LC/FLD (12), similar to the LC/UV method with the exception of fluorescent detection ($Ex_{320\ nm}/Em_{380\ nm}$), is sensitive and quite specific for the detection of 7-hydroxywarfarin, and possibly 6-hydroxywarfarin, however it is incapable of detecting other warfarin metabolites due to the lack of fluorescence. On the other hand, LC/MS methods with single quadrupole mass analyzers [13,14], sharing the similar LC techniques as HPLC/UV or HPLC/FLD, were developed at early phase of LC/MS interfacing technologies. Therefore, these LC/MS methods were for qualitative rather than quantitative applications. Even for qualitative analyses, such as the structural elucidation and metabolite identification, the LC/MS methods were also limited, largely due to the incapability of generating characteristic product ions. Therefore, contrary to the early establishment of LC/MS methods, successful applications of these LC/MS technologies in warfarin metabolic studies were rarely reported either qualitatively or quantitatively.

It is worthwhile to mention that the modified methods using HPLC/UV and HPLC/FLD were used in our laboratory in the past [6]. We thus have the direct experience in comparison between these techniques and the newly developed LC/MS/MS method, which is described in detail in the following sections.

2.2. LC/MS/MS assay for the quantitative analyses of warfarin hydroxylation

2.2.1. Instrumental conditions

The LC/MS/MS instrument was composed of a HP1100 HPLC system and a SCIEX API-2000NT triple quadrupole (MS/MS) mass spectrometer. The HPLC system consisted of an autosampler, a column compartment, and a binary pump. A computer software Analyst was applied to control the instrument operation and the data acquisition. Warfarin metabolites were separated on a C18 column (Waters Symmetry Shield RP_{18} 100 $\times$ 2.1 mm column) at a flow rate of 250 μl/min. The gradient of mobile phases (10 mM ammonium acetate, pH 4.6, and acetonitrile), demonstrated by the composition of acetonitrile, was 30% (0–1 min), 50% (6 min), 95% (6.5–8.5 min), and 30% (9 min and after). The total run time, including equilibrium period, was 15 min. Under negative ionization, the mass spectrometer (MS) was operated at the multiple reaction monitoring (MRM) mode for quantitative analysis, and synchronized with the HPLC. The curtain, nebulizer, turbo gas, and collision-associated dissociation (CAD) gas were typical, with 350°C source temperature and -4500 V ion spray potential, and optimized MRM parameters [8]. Quantification was based on the peak area ratio of the metabolite over the internal standard against the respective concentration of the metabolite.

2.2.2. Enzyme assays

Cytochrome P450-mediated warfarin metabolism and *in vitro* drug–drug interaction studies were performed in reconstituted enzyme systems containing human subcellular liver fractions such as microsomal preparations, or intact cells (primary or transformed).

Briefly, the CYP-mediated warfarin hydroxylation was taken place either in Tris buffer (50 mM, pH 7.4) containing NADPH (2.3 mM) and microsomal proteins (2 mg/ml) or recombinant enzymes (25 pmol) in a test tube, or in the phosphate-based saline (PBS) with or without the supply of NADPH in a cell culture well. The concentration of substrate R-warfarin was between 200 μM to 4 mM, and that of S-warfarin between 40 to 200 μM. If CYP inhibitory potency of a compound, such as CYP3A4 inhibitor ketoconazole, was evaluated, the testing compound was added to the reaction buffer prior to initiation of the reaction. The reaction was carried out at 37°C under atmosphere pressure and terminated by chilling on ice followed by the immediate addition of equal volume of methanol solution, which contained internal standard mebendazole. If CYP inducibility of a compound, such as CYP1A inducer 2,3,7,8-tertrachlorodibenzo-p-dioxin (TCDD), was evaluated, human primary hepatocytes or transformed cells, e.g., colorectal tumor cell line LS174T, were be exposed to the testing compound for 72 hr. The warfarin hydroxylation activities of the treated cells were determined at the end of exposure.

The samples were prepared by protein precipitation method applying the centrifugation in a benchtop centrifuge at 14,000 rpm for 5 min. The supernatant was filtered into a HPLC injection vial through spin tube filter (0.45 μ), and analyzed by LC/MS/MS.

2.2.3. Protein detection

The CYP expression in the culture cells was analyzed by Western immunochemical detection. At the end of the treatment, the cells were harvested and cell lysates were prepared. Proteins were resolved in a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked by blotting buffer (PBS containing 0.05% Tween 20 and 5% non-fat dried milk) and

probed by anti-human CYP antibodies diluted in blotting buffer. The membrane was later exposed to secondary antibodies labeled with horseradish peroxidase (HRP), and the substrate of the HRP (enhanced chemiluminescence reagent). CYP proteins were detected by luminescence using an X-ray developer.

3. Results and discussion

3.1. HPLC condition and optimization

To facilitate the LC/MS application, modifications from the conventional HPLC condition were made including the use of a narrow-bore reverse phase LC/MS column with corresponding slower flow rate but more rapid gradient, and also the reduced salt concentration in the aqueous mobile phase [11]. Most importantly, the mobile phase pH, the crucial factor for the separation of the hydroxylated metabolites, was determined as shown in Fig. 3. The resolution of 6-, 7-, and 8-hydroxywarfarin strictly depends on pH of the mobile phase, and was at the best at pH 4.6, which is near the pK_a of warfarin. Interestingly, the metabolites tend to be eluted out of the column earlier at higher pH, indicating that the polarity of the hydroxylated metabolites increases along with the raise of buffer pH due to the enhancement of ionization potential of the hydroxyl hydrogen, as in the case of warfarin.

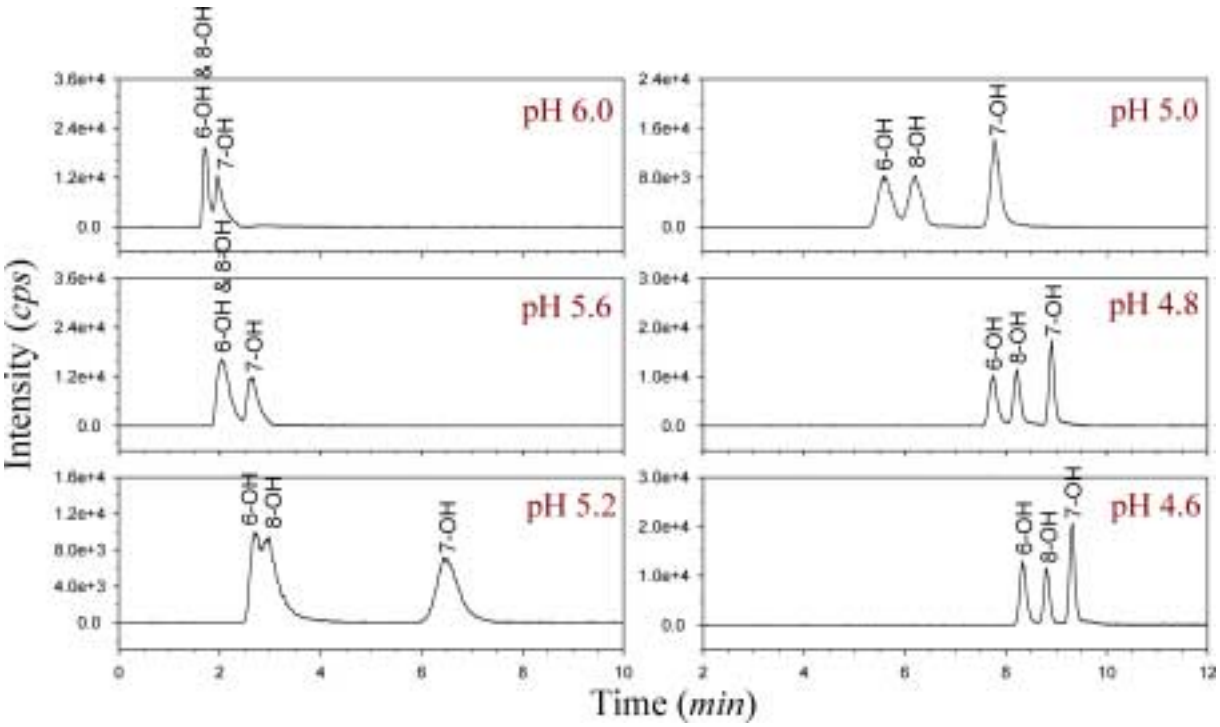

Fig. 3. Effect of mobile phase pH on the separation of 6-, 7-, and 8-hydroxywarfarin. The mobile phases were all 10 mM ammonium acetate and acetonitrile. The mobile phase pH was adjusted by the acidification of the aqueous mobile phase using acetic acid.

3.2. MS/MS fragmentation and interpretation

MS/MS spectra were generated by the conversion of MRM Q3 selected ion monitoring as described to ion scanning. Interestingly, the fragmentations of quasi-molecular ions of warfarin and the metabolites in the gas phase, though mostly derived from the dissociations of the common C–C bonds, were distinguishable.

As shown in Fig. 4, the majority of the product ions of warfarin, the metabolites, and 4′-chlorowarfarin were easily assigned, due to the cleavages at two of the three C–C bonds connected to the C9 asymmetric carbon. For instance, the product ions of warfarin at m/z 161 and 250, the counterparts of the product ions at m/z 177 and 266 of 6-, 7-, and 8-hydroxywarfarin, were the hydroxycoumarin and benzylhydroxycoumarin ion, which were indeed an even-electron ion (EE^-) and a radical ion ($OE^{-\cdot}$), as proposed in Fig. 5. Therefore, the fragmentations were quite similar among all metabolites, with the exception of the ion at m/z 305 for 10-hydroxywarfarin, which is likely produced by the feasible H_2O elimination of aliphatic hydroxyl group. However, corresponding to the single product ion at m/z 250 from warfarin, all of the hydroxywarfarins, including 6-, 7-, 8-, and 10-hydroxywarfarin, generated product ion pairs, instead of product ions, which differed by 1 atomic mass unit (*amu*). Moreover, 4′-chlorowarfarin, similar to warfarin, produced single MS/MS daughter ion at m/z 284, apparently the counterpart of the radical ion at m/z 250 for 10-hydroxywarfarin or warfarin. It is worthwhile to mention that the abundant quasi-molecular ion of 4′-chlorowarfarin was selected at Q1, in order to avoid the potential formation of the MS/MS product ion cluster due to the isotopic effect of chloride. Evidently, the formation of the

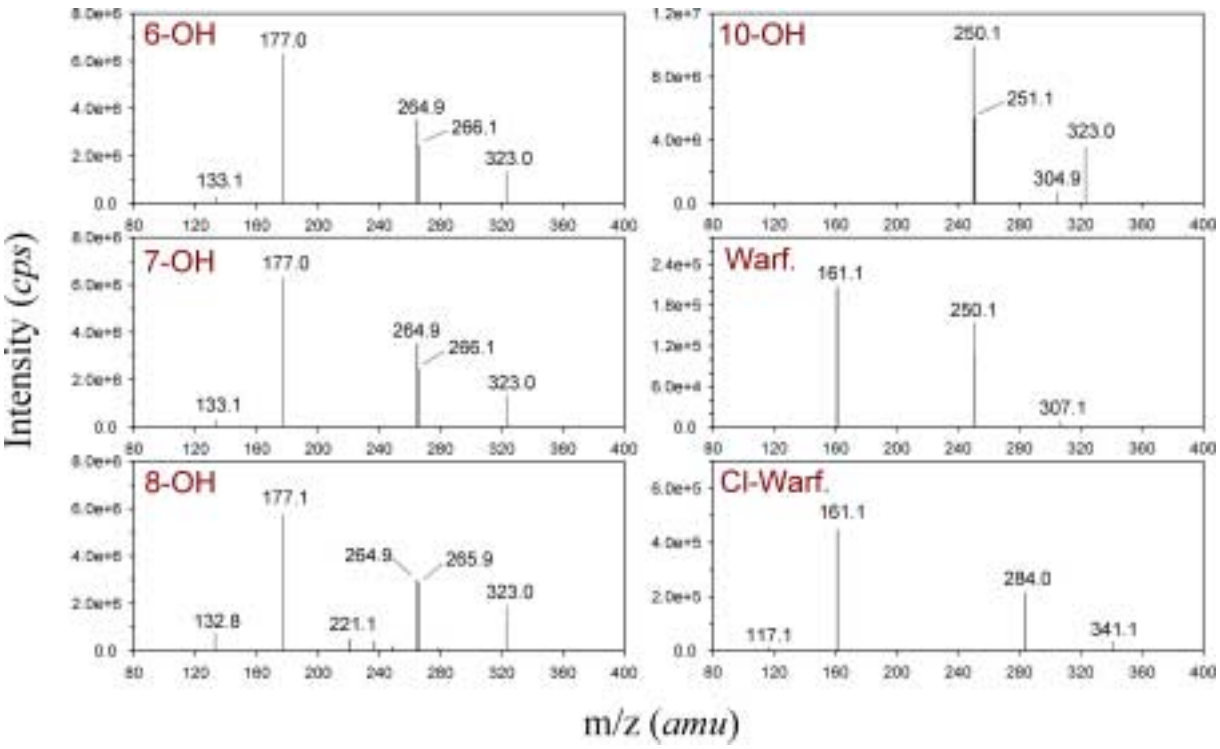

Fig. 4. MS/MS spectra of 6-, 7-, 8-, and 10-hydroxywarfarin, warfarin, and 4′-chlorowarfarin. The spectra were generated online after the HPLC injection and separation of the standard mixture. The injection amount was 1 nmol for each compound. HPLC and MS operation condition were described in Experimental techniques.

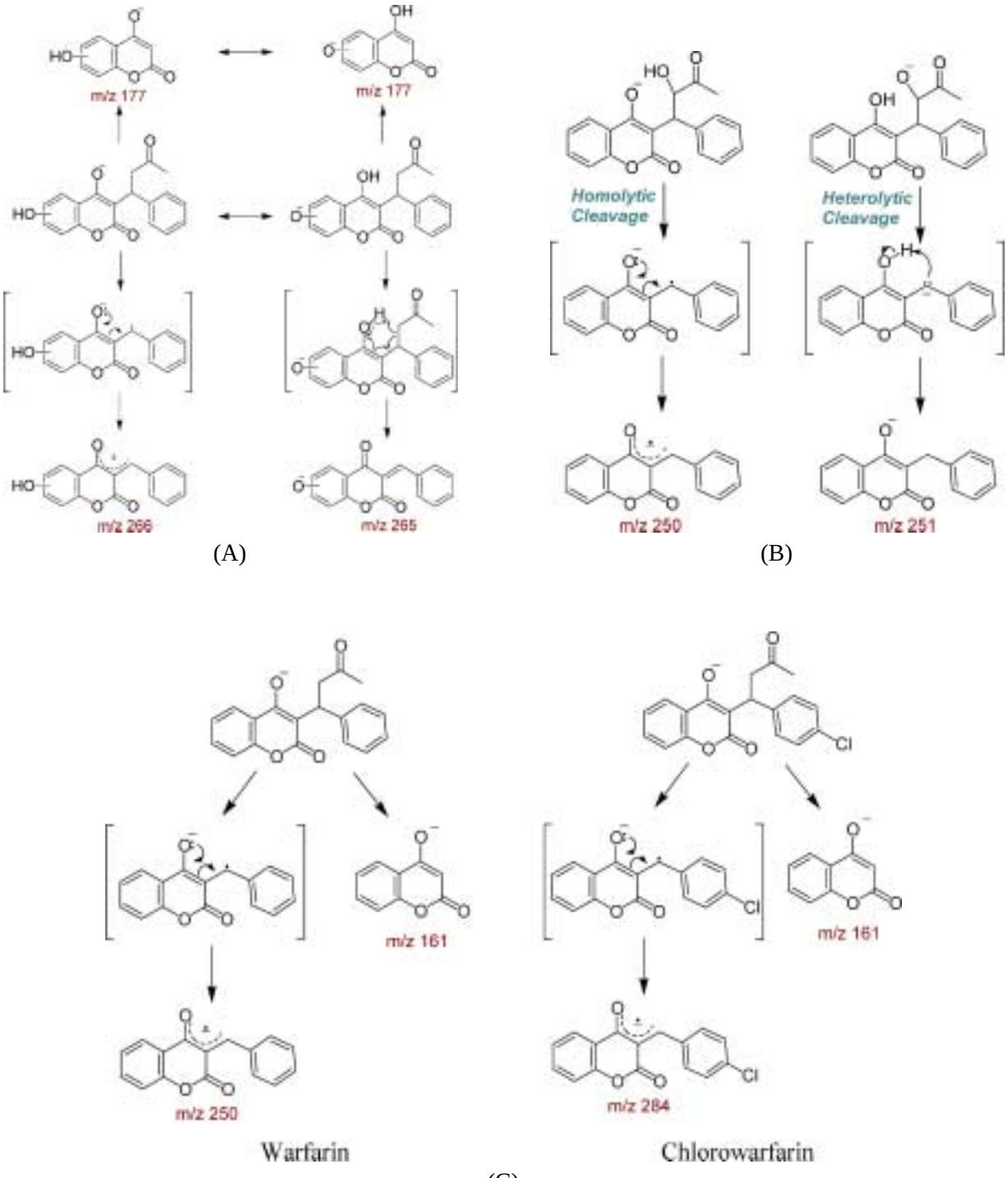

Fig. 5. Proposed MS/MS fragmentation pathways of 6-, 7-, 8-hydroxywarfarin (A), 10-hydroxywarfarin (B), and warfarin and 4′-chlorowarfarin (C).

product ion pairs from the metabolites, is likely associated with the existence of two hydroxyl groups, and less restrained by the position of the second hydroxyl group besides the warfarin hydroxyl group at C4 position of the coumarin moiety. Therefore, the deprotonation at two hydroxyl groups on the molecules would likely result in two distinctive fragmentation pathways for 6-, 7-, and 8-hydroxywarfarin to form an EE^- and a radical ion $OE^{-\cdot}$ of benzyl-dihydroxycoumarin, or for 10-hydroxywarfarin to form an EE^- and an $OE^{-\cdot}$ of benzyl-hydroxycoumarin, as shown in Fig. 5, thus generating the ion pairs differed by 1 *amu*. In contrast, the deprotonation at the single hydroxyl group of warfarin or 4′-chlorowarfarin at the C4 position of the coumarin moiety would only result in single fragmentation pathway to generate an $OE^{-\cdot}$, but not an EE^-, of benzyl-hydroxycoumarin after the cleavage at the C9-C10 bond, thus not producing the ion pair differed by 1 *amu*.

3.3. LC/MS/MS quantitative performance

The assay performance was described in detail in the original publication [8]. It is herein briefly stated. Calibration curves were generated by nonlinear regression analyses of the peak area ratio (Y) against the concentration of the metabolite standard (X) spiked in the reaction mixture. Quadratic ($Y = a + bX + cX^2$) and power equation ($Y = a \cdot X^b$), fitting the data best, were applied for the quantification. The assay accuracy was defined as the percent difference, the difference between the mean concentration detected and the respective concentration divided by the respective concentration. The assay precision was expressed as the coefficient of variation, the standard deviation of the concentration detected divided by the mean concentration detected. The assay was validated, and the accuracy and the precision were all within 15% (20% for the lowest concentration level or limit of quantification) deviation from the respective concentrations (Fig. 6). Moreover, the sensitivity and specificity of the as-

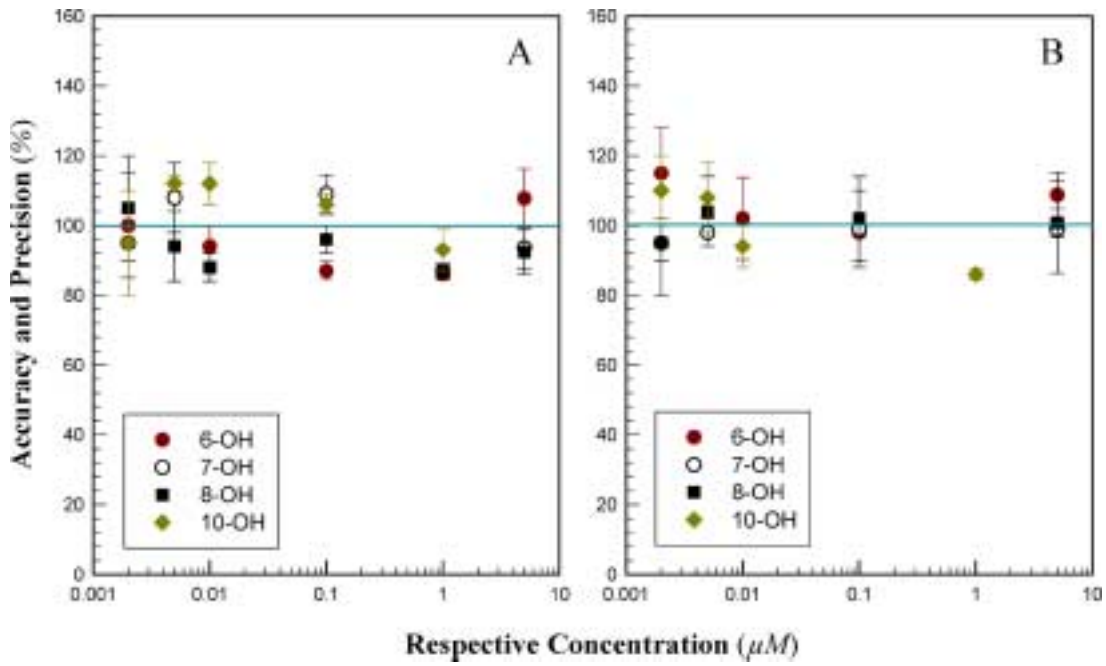

Fig. 6. Intra- (A) and inter-run validation of 6-, 7-, 8-, and 10-hydroxywarfarin in reaction mixture containing microsomal preparations (B).

Table 1
Comparison of existing methods for the quantification of warfarin hydroxylated metabolites

Techniques	Operational and performance characteristics						
	Accuracy	Sensitivity	Specificity	Broadness[1]	Speed	Simplicity	Applicability
GC/MS	Good	Excellent	Fair	Good	Good	Poor	Good
LC/UV	Good	Fair	Good	Excellent	Fair	Good	Good
LC/FLD	Good	Good	Excellent	Poor	Good	Good	Poor
LC/MS	Fair	Fair	Fair	Good	Fair	Good	Fair
LC/MS/MS	Good	Excellent	Excellent	Good	Good	Good	Excellent

[1]Number of the metabolites could be detected.

say were also superior due to the complete separation of the coumarin moiety-hydroxylated metabolites and MRM MS detection mode. Typically, the metabolites of 50 fmol quantity, or at 2 nM concentration with 25 μl sample injection, were detected with approximately a signal-to-noise ratio of 5. Therefore, this assay is roughly two-magnitude more sensitive than the conventional HPLC/UV method and avoids tedious and potentially problematic sample preparation procedures as required for GC/MS method [10,11]. The head-to-head comparison of the assay with other existing methods for *in vitro* warfarin metabolic studies was summarized in Table 1. Undoubtedly, the quantitative performance of the LC/MS/MS assay excels that of any other existing methods.

3.4. Assay application

As mentioned earlier, the characteristic human CYP-mediated stereo- and regioselective warfarin hydroxylation has been utilized for a variety of CYP studies, from fundamental mechanistic investigations of CYP catalytic reactions to phenotypic profiling in population. Moreover, as a pair of warfarin enantiomers could be used to probe the activities of most major drug-metabolizing CYP members with the exception of CYP2D6, CYP-mediated warfarin hydroxylation is especially useful to determine CYP profile and to study CYP metabolism-associated drug–drug interactions, the important aspects for NCE selection, optimization, and preclinical safety evaluation in drug discovery [5,6]. Therefore, I would like to provide a couple of examples to illustrate the applicability and advances of the LC/MS/MS assay related to such aspects, which would be of interest for both academia and industries.

3.4.1. CYP inhibition

The majority of prescribed drugs were metabolized, and usually inactivated by CYP members. Among these drug-metabolizing CYP forms, CYP3A4 is exceptionally crucial since it is the most abundant hepatic CYP member and responsible for approximately 50% or more of total CYP-mediated drug metabolism. Other important drug-metabolizing CYP members include CYP1A2, CYP2C9, and CYP2D6. Therefore, the probability of inhibiting the metabolism of concurrent drugs, which are metabolized by these CYP members, would be anticipated high in the clinic. In this regard, LC/MS/MS warfarin assay is ideal not only to assess the potential CYP inhibition for the development of NCEs, but also to predict the likelihood and thus to prevent the potential occurrence of clinical drug–drug interactions for the existing and newly developed therapeutic agents. Therefore, as part of preclinical safety evaluation, we routinely determine CYP inhibition profiles for our lead compounds and drug candidates using this LC/MS/MS warfarin assay, with the addition of a CYP2D6-specific LC/MS/MS bufuralol assay [6]. As shown in Fig. 7, 10 μM CYP3A4 inhibitor ketoconazole inhibited at least 95% of R-warfarin 10-hydroxylation activity in the reconstituted system containing pooled human liver microsomal preparations, whereas, 50 μM CYP2C9 inhibitor sulphaphenazole suppressed approximately 75%

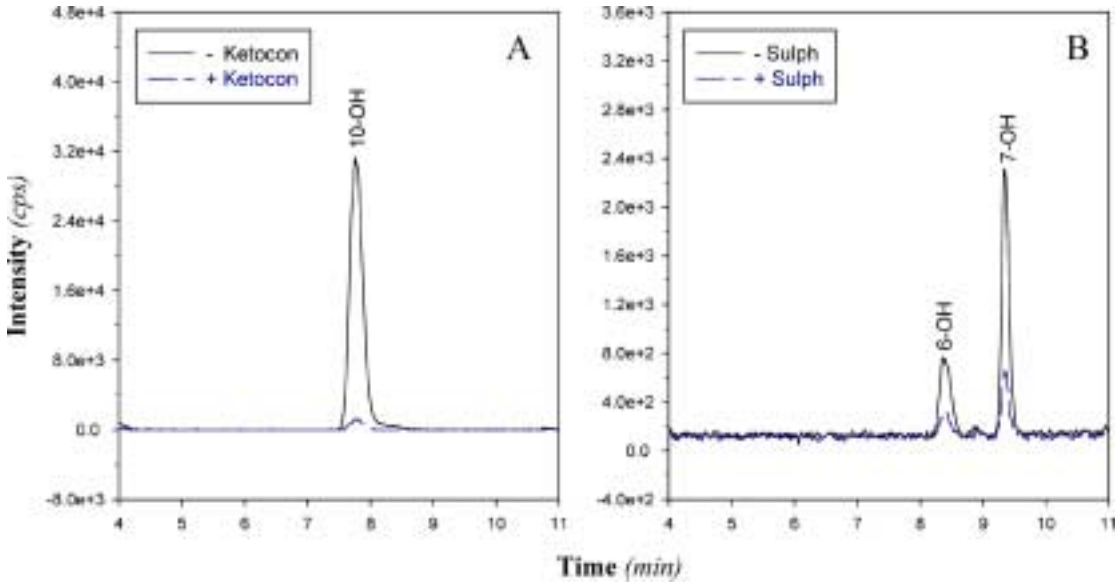

Fig. 7. CYP form-specific inhibition determined by warfarin hydroxylation activities. Inhibition of CYP3A4-mediated *R*-warfarin 10-hydroxylation by ketoconazole in the reconstituted system containing pooled human liver microsomal preparations (A); inhibition of CYP2C9-mediated *S*-warfarin 7-hydroxylation by sulphaphenazole in the reconstituted system containing recombinant human CYP2C9 (B). The procedures of *in vitro* inhibition studies, and the condition of LC/MS/MS operation were described in Experimental techniques. 2 mM R-warfarin, 100 μM S-warfarin, 10 μM ketoconazole, and 50 μM sulphaphenazole were applied for the studies.

of S-warfarin 7-hydroxylation activity in the reaction mixture containing recombinant human CYP2C9, as determined by this LC/MS/MS assay. Therefore, our assay was capable of detecting the inhibitory effects on CYP3A4 and CYP2C9 activity by the prototypic CYP form-specific inhibitors. These prototypic inhibitors, being regularly applied, have served as positive controls of the assay, and their inhibitory potencies were used as references for those of the testing compounds.

3.4.2. CYP induction

CYP induction, the other aspect of CYP-associated drug–drug interactions, has also been recognized as a potential toxicological issue recently. In addition to the possible attenuation of drug efficacy, elevated bioactivation of drugs or xenobiotics could be one of the most serious consequences of enzyme induction. One of such examples is the induction of CYP1A1, an extrahepatic enzyme that bioactivates several well-known procarcinogens including benzo[a]pyrene. However, contrary to relative simple enzyme inhibition, the underlying mechanism for enzyme induction is usually complicated, and difficult to delineate because of the complex regulation of protein expression, which is indeed largely unknown in humans. Moreover, thorough investigations of CYP induction have been further hindered historically due to either the lack of form-specific substrates for the differentiation of the activities of CYP members in the same subfamilies such as CYP1A1 and CYP1A2, or the lack of sensitive assays for the detection of CYP activities in cell culture. Again, LC/MS/MS warfarin assay with the CYP1A1 and CYP1A2-differentiating hydroxylation regioselectivity and the fmol range detection capability is well suitable for such studies. As demonstrated in Fig. 8, 2,3,7,8-tertrachlorodibenzo-p-dioxin (TCDD) induced CYP1A expression and activities in the culture of colorectal cell line LS174T. Interestingly, contrary to the lack of identification of the induced enzyme by the polyclonal anti-CYP1A antibodies due to the cross-reactivity, the similar R-warfarin 6- and 8-hydroxylation activity in the cells after TCDD exposure suggested that

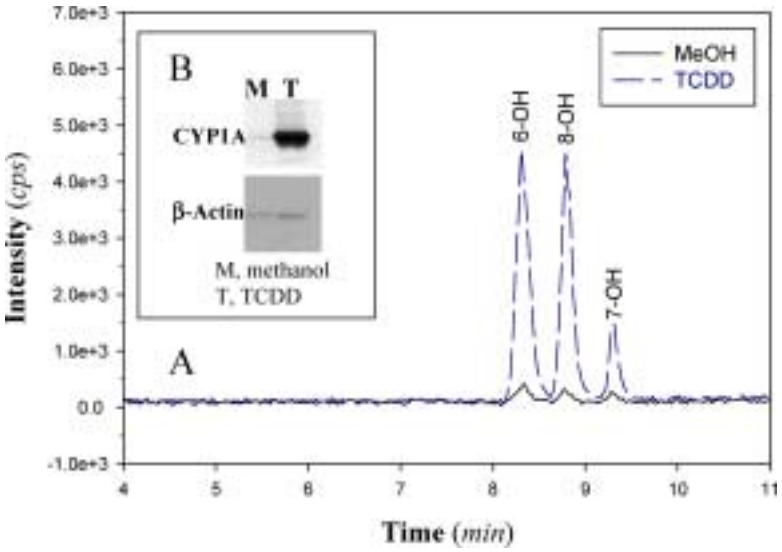

Fig. 8. Effect of TCDD on *R*-warfarin hydroxylation (A) and CYP1A protein expression in LS174T cells (B). The procedures of cell culture and treatment, protein detection, and the condition of LC/MS/MS operation were described in Experimental techniques. The final TCDD concentration in the culture media was 20 nM.

CYP1A1 was mainly, if not exclusively, induced by TCDD in LS174T cells [3]. This was further proven by the lack of CYP1A2 detection in these TCDD-treated cells using monoclonal anti-CYP1A2 antibodies. The monoclonal antibodies only recognized human CYP1A2, thus did not cross-react with CYP1A1 (data not shown). In addition to transformed cell lines, the assay has been successfully and frequently applied to study CYP induction in primary culture of human hepatocytes, which is the major advance of the assay and described extensively in the original publication [8].

4. Summary and outlook

LC/MS/MS warfarin assay, combining CYP form-specific stereo- and regioselective warfarin hydroxylation with sensitive and specific LC/MS/MS detection technique, is promising for the characterization of the functionality of CYP1A1, CYP1A2, CYP2C9, and CYP3A4 in humans. In the process of drug discovery, this assay would become one of powerful tools to early detect potential CYP inhibition and induction for lead compound selection and optimization, and to estimate the likelihood of CYP-associated drug–drug interactions in clinical trials of drug candidates. Compared to other existing analytical methods for warfarin metabolism, this assay offered unmatched wide range of applicability, especially for the metabolic studies in cell culture. Therefore, I would expect that the LC/MS/MS warfarin assay is going to be one of the standard methods for CYP inhibition and induction studies in both industries and academia since the LC/MS/MS instruments become more and more accessible to biomedical scientists besides mass spectrometry specialists.

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