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Short communication

Simultaneous measurement of *S*-warfarin, *R*-warfarin, *S*-7-hydroxywarfarin and *R*-7-hydroxywarfarin in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Clinically used anticoagulant warfarin is usually available as a racemic mixture of *S*- and *R*-warfarin that are both metabolized mainly by cytochrome P450 isoenzymes. Determination of warfarin enantiomers and their enantiomeric 7-hydroxywarfarin (7-OH-warfarin) metabolites in the human plasma sample allows probing of cytochrome P450 isoenzyme activities and detection of ingestion of warfarin-containing products for the investigation of unexplained bleeding. The present study aims to develop a sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for simultaneous detection of *S*-warfarin, *R*-warfarin, *S*-7-OH-warfarin and *R*-7-OH-warfarin in human plasma. Plasma samples were extracted with mixed-mode cation-exchange (MCX) cartridge with recoveries of greater than 87.0% for all four analytes. The selectivity of 7-OH-warfarin from other monohydroxylated warfarin metabolites such as 4′-, 6-, 8- and 10-hydroxywarfarins using a Chirobiotic V chiral column and multiple reaction monitoring (MRM) was addressed. The developed LC–MS/MS method is simple, specific and has been fully validated with satisfactory accuracy and adequate reproducibility with limit of quantification (LOQ) of 5 ng/ml for all four analytes. The method was successfully applied to analyze the steady state plasma concentrations of the four analytes in 30 patients.

1. Introduction

Warfarin, the most commonly prescribed anticoagulant, is clinically available as a racemic mixture of *S*-warfarin and *R*-warfarin. *S*-Warfarin, which is 3–5 times more potent than the *R*-warfarin, is primarily metabolized by cytochrome P450 (CYP) 2C9 forming the inactive *S*-7-hydroxywarfarin (*S*-7-OH-warfarin) [1,2].Genetic polymorphisms of CYP2C9 have a significant effect on the clearance of the *S*-warfarin [3] and are found to be associated with lower warfarin dosage requirement and increased risk of major bleeding events during the induction phase of warfarin therapy [4]. *S*-Warfarin has been used for probing the CYP2C9 activities of variant polymorphisms by assessing unbound oral clearance of *S*-warfarin, *S*-7-OH-warfarin formation clearance and the ratio of *S*-warfarin to *R*-warfarin [5,6]. Determination of warfarin enantiomers and their enantiomeric 7-OH-warfarin metabolites in the human plasma sample can eliminate the time-consuming and laborious procedure of urine sample collection. In addition, confirmation of the presence of warfarin or its major metabolite is required for the investigation of accidental or intentional ingestion of warfarin-containing products. Therefore, it would be ideal to have a simple analytical method that could determine the warfarin enantiomers and their related enantiomeric 7-OH-warfarin in a same plasma sample.

There are quite a few analytical methods developed for the determination of warfarin enantiomers and/or their hydroxylated metabolites in biological matrices/human plasma. Enantiomeric separation and measurement of *S*-warfarin and *R*-warfarin in human plasma samples [7–10] or 7-OH-warfarin in human urine [11] have been mainly achieved by using high performance liquid chromatography (HPLC) with various chiral columns and detected by UV [7–10] or UV/fluorescence system coupled with on-line circular dichroism detector [11]. Furthermore, simultaneous enantioseparation of warfarin and 4′-, 6-, and 7-OH-warfarin standard mixtures was demonstrated by capillary zone electrophoresis using highly sulfated β-cyclodextrin as chiral selector [12]. Generally, these reported methods mainly focus on the enantiomeric separation of warfarin but not their hydroxylated metabolites.

Although a more recent study has reported the determination of *S*-warfarin, *R*-warfarin, *S*-7-OH-warfarin and *R*-7-OH-warfarin in human plasma by column switching (BSA-C8 precolumn and Chiralcel OD-RH chiral column) HPLC-UV approach [13], mass

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spectrometry (MS) has been receiving more attention due to its superior selectivity and sensitivity. LC–MS/MS has been employed for the achiral separation and determination of racemic warfarin and/or their monohydroxylated metabolites in human plasma [14,15], urine [15] or *in vitro* metabolism study [16]. The chiral LC–MS/MS method developed by Naidong et al. was for the enantiomeric separation of only *S*- and *R*-warfarin in human plasma [17]. Thus, simultaneous determination of *S*-warfarin, *R*-warfarin and their major enantiomeric 7-OH metabolites in human fluid by mass spectrometric technique remains to be explored. The present study was proposed aiming to develop a sensitive and highly specific LC-MS/MS method for simultaneous quantitative analysis of *S*-warfarin, *R*-warfarin, *S*-7-OH-warfarin and *R*-7-OH-warfarin in human plasma. In the mean time, the selectivity of 7-OH-warfarin from other monohydroxylated metabolites such as 4′-, 6-, 8- and 10-OH-warfarin were demonstrated.

2. Materials and methods

2.1. Materials

S-warfarin, *R*-warfarin, and racemic standards including 4′-, 6-, 7-, 8- and 10-OH-warfarin, were purchased from Ultrafine Chemicals (Manchester, UK). Diclofenac sodium (used as internal standard (IS)) was purchased from Sigma (St. Louis, MO, USA). Ammonium acetate and acetic acid were obtained from BDH Laboratory (Poole, England). Phosphoric acid was purchased from APS Fine Chemicals (Seven Hills, Australia). Acetonitrile and methanol (both of HPLC grade) were obtained from Tedia Company Inc. (Fairfield, USA) and Fisher Scientific UK Limited (Leicestershire, UK), respectively. All other reagents were at least analytical grade and used without further purification. Distilled and deionized water was used for the preparation of all solutions. Oasis mixed-mode cation-exchange (MCX) and mixed-mode anion-exchange (MAX) cartridges (1cc, 30 mg) were supplied by Waters (Milford, USA).

2.2. Preparation of standard solutions and calibration curves

Individual stock solutions of *S*-warfarin, *R*-warfarin, racemic monohydroxylated warfarin (1 mg/ml) were prepared by dissolving the appropriate amount of each authentic in acetonitrile. For quantitative analysis, working standard mixture was prepared by mixing and diluting the stock solutions of *S*-warfarin, *R*-warfarin and racemic 7-OH-warfarin with water to yield concentrations of 2000 ng/ml for *S*- and *R*-warfarin, and 1000 ng/ml for *S*- and *R*-7-OH-warfarin. This working standard mixture was further diluted with water to yield concentrations of 250 ng/ml for *S*- and *R*-warfarin, and 125 ng/ml for *S*- and *R*-7-OH-warfarin. Standard solution of IS was prepared by dissolving the appropriate amount of diclofenac sodium in water to yield a concentration of 2 μg/ml.

Calibration standards were prepared by spiking blank human plasma (200 μl) with the appropriate amount of working standard mixture and 500 μl of internal standard solution to yield the final concentrations of 5, 10, 25, 50, 100, 250, 500, 1000 and 1500 ng/ml for *S*- or *R*-warfarin and 2.5, 5, 12.5, 25, 50, 125, 250, 500 and 750 ng/ml for *S*- or *R*-7-OH-warfarin, respectively. All calibration standards were prepared in triplicate and followed the same sample clean-up procedure as stated below. Calibration curves were plotted by the peak-area ratios of each analyte/internal standard versus concentrations of analytes in plasma.

2.3. Plasma sample preparations

MCX used for the plasma sample extraction was preconditioned with 1 ml of methanol, followed by 1 ml of water. To 200 μl plasma sample, 500 μl of internal standard solution (2 μg/ml) and 200 μl of 10% phosphoric acid (v/v) were added. After vortex mixing for 15 s, the mixture was loaded on the preconditioned MCX cartridge. The cartridge was flushed with 1 ml of 0.1 M HCl before the analytes were eluted with 1 ml methanol. The eluent was then evaporated to dryness in a Centrivap concentrator (Jouan, Virginia, USA), and the residue was reconstituted with 200 μl of 60% acetonitrile. An aliquot of 50 μl was injected into the HPLC–MS/MS system.

2.4. Instrumentation

The LC–MS/MS system, consisted of an ABI 2000 Q-Trap triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI), two Perkin-Elmer PE-200 series micro-pumps and auto-sampler (Perkin-Elmer, Norwalk, CT, USA), was used to perform the analysis. The chromatographic separation was achieved by using a chiral Astec Chirobiotic V column (250 mm × 4.6 mm i.d., 5 μm particle size, Supelco) which was protected by a Astec Cyclobond I guard column (20 mm × 4.0 mm i.d., 5 μm particle size, Supelco).

2.5. LC–MS/MS condition

The elution gradient for HPLC analysis consisted of two solvent compositions: acetonitrile (A) and 10 mM ammonium acetate (pH 4.4 adjusted by glacial acetic acid) (B). The gradient began with 10% eluent A for 7 min, changed linearly to 40% A in 5 min and remained for further 3 min before changing back to 10% A and equilibrating for 3 min prior to next injection. Throughout the LC process the flow rate was set at 1 ml/min. The temperatures of auto-sampler and the analytical column were set at 4 °C and ambient, respectively. The sample injection volume was 50 μl. Prior to the ESI source, 60% of the LC eluent was split off and only 40% of the effluent was introduced.

The mass spectrometer was set at negative ionization mode. Typical instrumental conditions were: ion spray voltage at −4500 V; nitrogen as nebulizer gas, auxiliary gas, curtain gas and collision gas at 30, 80, 30 and 3 psi, respectively; auxiliary gas temperature at 450 °C and interface heater temper at 100 °C. Other instrumental parameters were analyte specific and were optimized prior to analysis. Data acquisition was conducted at multiple reaction monitoring (MRM), with m/z 307 → m/z 161 for warfarin, m/z 323 → m/z 161 for 4-OH-warfarin, m/z 323 → m/z 177 for 6-, 7- and 8-OH-warfarin, m/z 323 → m/z 250 for 10-OH-warfarin and m/z 294 → m/z 250 for IS.

2.6. Method validation

The specificity of the method was investigated by comparing the chromatogram of blank plasma spiked with standard solutions to the samples collected from subjects after administration of warfarin. The intra-day precision and accuracy were determined within one day by analyzing five replicates of the quality control samples at concentrations of 15, 150 and 800 ng/ml for *S*- or *R*-warfarin, and 7.5, 75 and 400 ng/ml for *S*- or *R*-7-OH-warfarin. The inter-day precision and accuracy were determined on three separate days. The intra- and inter-day precision were defined as the relative standard deviation (RSD) and the accuracy was determined as the percentage bias from nominal concentration (%).

Extraction efficiency was determined for the quality control samples at concentrations of 10, 50, 250, 1000 ng/ml for *S*- or *R*-warfarin and 5, 25, 125, 500 ng/ml for *S*- or *R*-7-OH-warfarin. The absolute extraction recoveries were calculated by comparing the peak areas of the samples to that of the unextracted standard solutions containing the equivalent amount of analytes ($n = 3$). The limit

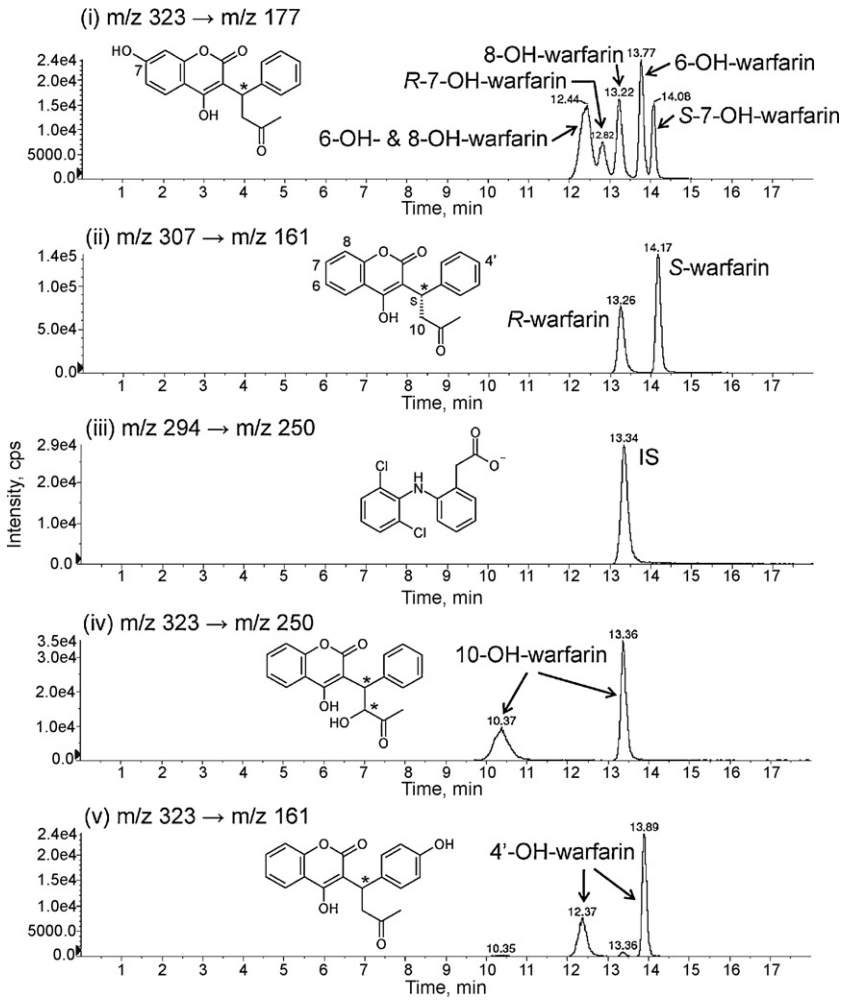

Fig. 1. Extracted ion chromatograms of standard mixture containing 1000 ng/ml of *R*-warfarin, *S*-warfarin, racemic 4′-, 6-, 7-, 8-, 10-hydroxywarfarin and diclofenac (IS).

of detection (LOD) was defined as the lowest concentration of the analyte resulting in a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration of spiked plasma samples that can be determined with sufficient precision and accuracy, i.e. RSD less than 20% and relative error of ±20%.

Freeze–thaw stability of the plasma samples was evaluated by exposing quality control samples to three freeze (−80 °C)–thaw (room temperature) cycles before sample preparation. The stability of the samples in auto-sampler was evaluated by analyzing extracted quality control samples after being placed in the auto-sampler at ambient for 24 h.

2.7. Clinical applications on patient blood samples analyses

Ten milliliters of peripheral blood were obtained from eleven patients who were stabilized on warfarin therapy with INR ranged 2–3 after prior approvals from both the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee and patients.

3. Results and discussion

3.1. HPLC–MS/MS

Although the current study focus mainly on the determination of *R*-, *S*-warfarin and their *R*-, *S*-7-OH-warfarin enantiomers, separations from other monohydroxylated warfarin metabolites such as 4′-, 6-, 8- and 10-OH-warfarin cannot be neglected during method development since these metabolites are positional isomers of 7-OH-warfarin with similar UV absorption profile and generally eluted in close proximity under achiral LC condition [10,15,16]. The extracted ion chromatogram of standard mixture containing *R*- and *S*-warfarin, and racemic 4′-, 6-, 7-, 8- and 10-hydroxywarfarin at 1000 ng/ml level is shown in Fig. 1. The identification of each analyte was made by comparing the retention time of individual authentic standard. The *R*- and *S*-7-OH-warfarin enantiomers were identified with reference to the elution order of these analytes reported in literature [11,13], though the assignment of *R*-/*S*- of other monohydroxylated warfarin metabolites have not been made due to unavailability of enantiomeric standard or relevant literature. As shown in Fig. 1, *R*- and *S*-warfarin are baseline resolved. Among the six enantiomers of 6-, 7-, 8-OH-warfarin, the major metabolite of warfarin, i.e. *S*-7-OH-warfarin (elute at 12.8 min), was separated from 6-, 8-OH-warfarin and *R*-7-OH-warfarin (elute at 14.1 min). Similarly, enantiomeric separation of 6- and/or 8-OH-warfarin was observed, though one of the enantiomers of 6-OH was co-eluted with an enantiomer of 8-OH-warfarin (at 12.4 min). The enantiomers of 4′- and 10-OH-warfarin are also baseline resolved, respectively. Two peaks at 10.4 and 13.4 min correspond to those from 10-OH-warfarin enantiomers. Previous investigation found that *R*-warfarin was metabolized mainly to (9*R*;10*S*)-10-OH-warfarin and *S*-warfarin was metabolized mainly to (9*S*;10*R*)-10-OH-warfarin enantiomer with only trace of the (9*R*;10*R*)- and (9*S*;10*S*)- diastereomers [18]. We there-

fore, postulated that the two stereoisomers of 10-OH-warfarin standard belong to the (9*R*;10*S*)-10-OH-warfarin and (9*S*;10*R*)-10-OH-warfarin. To date, this is the first report on the simultaneous enantioseparation of warfarin and 4′-, 6-, 7-, 8- and 10-OH-warfarin metabolites with superior specificity offered by LC/MS/MS.

Although both naproxen and diclofenac have been previously reported as IS for the analysis of warfarin [10,11,14], diclofenac was chosen in the current study due to its easy accessibility, good MCX recovery (>80%), reasonable retention time and no interference with the other analytes. Several mobile phase composition and organic modifier (e.g. acetic acid/diethylamine/ammonium acetate/formic acid) have been evaluated, only the reported mobile phase and LC profile achieves optimal separation. Gradient elution of the analytes from Astec Chirobiotic V column with a relative high content (90%) of buffer for the first 7 min was found to be necessary for the separation of all OH-warfarin analytes. Although it may lead to peak broadening for one of the 10- and 4′-OH-warfarin stereoisomers, the peaks sharpened when the organic phase increases to 40%. With a comprise of sensitivity for early eluting compounds, the separation of *R*-, *S*-warfarin and *R*-, *S*-7-OH-warfarin was achieved with sufficient selectivity and sensitivity.

The linearity was 5–1500 ng/ml for *R*- and *S*-warfarin and 5–750 ng/ml for *R*- and *S*-7-OH-warfarin. The LOD and LOQ were 1.5 and 5 ng/ml, respectively, for all analytes. The LOQ obtained by this method are much lower than those using UV analysis (75–180 ng/ml) [8,10] and comparable to those from column switching LC–DAD detection [13]. The intra-day ($n = 5$) accuracy and precision at low, medium and high concentration levels (Table 1) were 85.5–105.8% (<10.2% RSD), and those for inter-day were 94.7–108.9% (<8.5% RSD), which meets the criteria of 15% bias and within 15% RSD as stated in the guidance on Bioanalytical Method Validation from FDA (May 2001).

Table 1
Inter- and intra-day accuracy and precision for *S*-, *R*-warfarin and *S*-, *R*-7-OH-warfarin.

Tested compound	Nominal value (μg/ml)	Intra-day (n = 5)		Inter-day (n = 3)	
		% Accuracy	%RSD	% Accuracy	%RSD
S-Warfarin	15	104.7	9.8	108.9	4.9
	150	99.2	7.4	109.2	6.2
	800	85.5	3.8	90.9	6.1
R-Warfarin	15	96.6	12.7	105.2	7.8
	150	105.8	4.8	106.2	8.5
	800	96.3	7.5	97.1	7.4
S-7-OH-warfarin	7.5	98.8	3.9	103.9	4.3
	75	97.9	4.3	102.9	4.7
	400	90.1	9.3	94.7	4.2
R-7-OH-warfarin	7.5	104.7	10.2	102.8	1.7
	75	98.7	4.7	101.1	4.7
	400	97.4	9.2	99.4	6.5

3.2. Sample extraction and stability test

Liquid–liquid extraction (LLE) using diethyl ether:chloroform [13], ethyl acetate [8] or ether [9–11,17], and solid phase extrac-

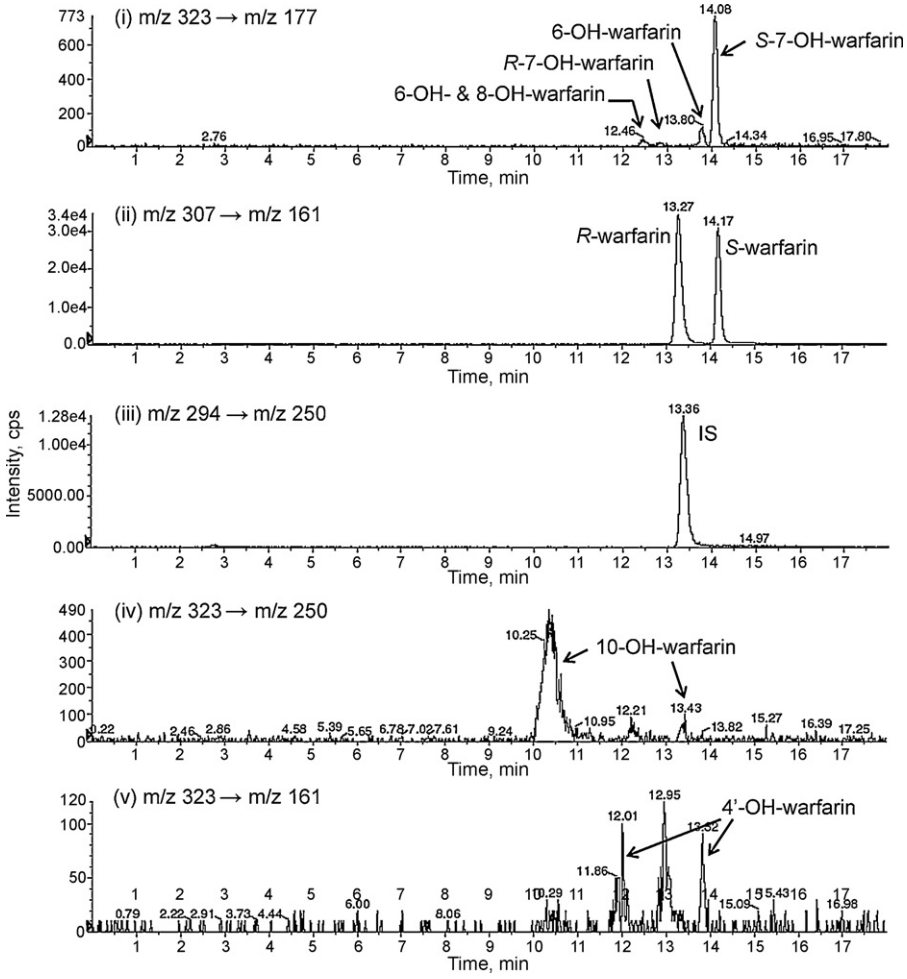

Fig. 2. Extracted ion chromatograms of subject S034.

Table 2
Steady state plasma concentrations of *S*-warfarin, *R*-warfarin, *S*-7-OH-warfarin and *R*-7-OH-warfarin in 30 patients.

Patient ID	Dosage level (mg)	Plasma concentration (ng/ml)			
		R-Warfarin	*S*-Warfarin	*R*-7-OH-warfarin	*S*-7-OH-warfarin
S001	2.50	449	237	<5	51
S002	2.75	794	343	6	54
S003	2.50	774	390	12	168
S004	6.25	497	421	11	69
S005	3.75	813	382	22	346
S006	2.75	786	298	11	123
S008	3.25	658	207	5	75
S009	3.50	603	329	<5	46
S010	8.50	1161	310	17	195
S011	5.25	768	359	8	72
S012	3.50	519	169	13	120
S013	3.00	495	187	7	69
S014	3.00	747	349	9	134
S015	2.25	809	430	8	53
S016	6.00	1254	884	<5	130
S018	4.00	649	308	11	185
S019	2.25	544	237	5	56
S020	3.50	769	310	8	153
S021	3.00	834	244	<5	64
S022	2.00	412	103	<5	90
S027	2.25	948	440	7	91
S029	4.00	655	471	<5	83
S030	3.50	838	351	10	80
S031	3.00	1005	438	5	86
S033	3.00	767	524	8	73
S034	3.00	808	274	<5	58
S035	4.00	911	353	10	146
S039	7.25	1356	753	10	196
S040	1.75	754	345	5	93
S041	4.50	579	352	6	85

tion using either C_{18} type [7,15] or MCX [14] have been reported for extractions of warfarin and/or OH-warfarin from human plasma samples. In our preliminary studies, we have tried LLE using ether:hexane and solid phase extraction using both MCX and MAX cartridges and found that the extraction recoveries of warfarin and 7-OH-warfarin were rather low (<50%) by LLE and relatively high (>80%) using MCX and MAX, which is even higher than that obtained with Bakerbond C_{18} cartridges (~80%) [7], MCX cartridge was chosen for the current study due to its less sample preparation time. The extraction recoveries of the four analytes spiked at four different concentration levels (i.e. 15, 50, 250 and 1000 ng/ml for *S*-/*R*-warfarin, 5, 25, 125 and 500 ng/ml for *S*-/*R*-7-OH-warfarin) in human plasma are found to be 98.1–108.7% for *S*-warfarin, 88.6–101.9% for *R*-warfarin, 91.5–101.0% for *R*-7-OH-warfarin and 87.0–98.0% for *S*-7-OH-warfarin. The stability experiments in autosampler indicated that the percentage of all four analytes remained after 24 h at ambient temperature was 83.5–99.6% for *S*- and *R*-warfarin (at 15, 150 and 750 ng/ml) and 91.9–97.7% for *S*- and *R*-OH-warfarin (at 7.5, 75 and 375 ng/ml). In addition, 3-cycle freeze–thaw stability tests showed that 99.4–112.4% (<10% RSD) of all four analytes remained after at least three freeze–thaw cycles.

3.3. Clinical application to the in vivo patient plasma sample analyses

The validated LC–MS/MS method has been applied to simultaneous determination of the steady state concentrations of warfarin enantiomers and their enantiomeric seven hydroxylated metabolites in plasma obtained from subjects with INR between 2 and 3. The representative chromatograms of a plasma sample obtained from a subject is shown in Fig. 2. Under the current assay condition, no interference from the endogenous plasma was observed at the retention time of both the analytes and the internal standard. The steady states plasma concentrations of *S*-warfarin, *R*-warfarin, *S*-7-OH-warfarin and *R*-7-OH-warfarin obtained from selected subjects were presented in Table 2. Although 4′-, 6-, 8- and 10-OH warfarin have also been found in the patient samples, 7-OH warfarin followed by 10-OH-warfarin are the predominant metabolites of warfarin in human plasma [12,15]. Comparing the steady state concentrations of *S*-warfarin, *R*-warfarin versus that reported previously for Asian and Caucasian populations [6,7,11,14,19], it was found that the *S*-warfarin/*R*-warfarin ratio (mean of 0.47 ± 0.14), the steady state plasma concentrations of *S*-warfarin (360 ± 157 ng/ml) and *R*-warfarin (765 ± 224 ng/ml), obtained from the current study were all within the range of that have been reported. Thus, it further proves the validity of the current developed method.

4. Conclusions

A simple and specific HPLC–MS/MS analytical method for simultaneous determination of enantiomers of warfarin and their 7-OH-warfarin metabolites in human plasma has been developed and fully validated with satisfactory accuracy and adequate reproducibility. The developed assay method demonstrated for the first time on the simultaneous determination of plasma concentrations of not only *S*-warfarin and *R*-warfarin, but also their major hydroxyl metabolites, *S*-7-OH-warfarin and *R*-7-OH-warfarin, which has provided a valuable tool for monitoring the *in vivo* concentrations of warfarin enantiomers and their enantiomeric metabolites and the effect of genetic polymorphisms and co-administered drugs/herbs on warfarin metabolism.

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