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Development of a chiral micellar electrokinetic chromatography-tandem mass spectrometry assay for simultaneous analysis of warfarin and hydroxywarfarin metabolites: Application to the analysis of patients serum samples

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Abstract

The enantioseparation of warfarin (WAR) along with the five positional and optical isomers is challenging because of the difficulty to simultaneously separate and quantitate these chiral compounds. Currently, no effective chiral CE-MS methods exist for the simultaneous enantioseparation of WAR and all its hydroxylated metabolites in a single run. Polymeric surfactants (aka. molecular micelles) are particularly compatible with micellar electrokinetic chromatography-mass spectrometry (MEKC-MS) because they have a wider elution window for enantioseparation and do not interfere with the MS detection of chiral drugs. Using polysodium *N*-undecenoyl-L, L-leucylvalinate (poly-L,L-SULV) as a chiral pseudophase in MEKC-MS baseline separation of WAR, its five metabolites along with the internal standard was obtained in 45 min. This is in comparison to 100 min required for separation of the same mixture with packed column CEC-MS using a vancomycin chiral stationary phase. Serum samples were extracted with mixed-mode anion-exchange (MAX) cartridge with recoveries of greater than 85.2 % for all WAR and hydroxywarfarin (OH-WAR) metabolites. Utilizing the tandem MS and multiple reaction monitoring mode, the MEKC-MS/MS method was used to simultaneously generate calibration curves over a concentration range from 2–5000 ng/mL for *R*- and *S*-warfarin, 5–1000 ng/mL for *R*- and *S*-6-, 7-, 8- and 10-OH-WAR and 10–1000 ng/mL for *R* and *S*-4'-OH-WAR. For the first time, the limits of detection and quantitation for most WAR metabolites by MEKC-MS/MS were found to be at levels of 2 and 5 ng/mL, respectively. The method was successfully applied for the first time to analyze WAR and its metabolites in plasma samples of 55 patients undergoing WAR therapy, demonstrating the potential of chiral MEKC-MS/MS method to accurately quantitate with high sensitivity.

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Keywords

Warfarin/hydroxylated-warfarin metabolites; Polymeric surfactant; optical / positional isomers; MEKC-MS versus.CEC MS; SIM versus MRM; CYP mediated warfarin metabolism

1. Introduction

Warfarin (WAR), the most commonly prescribed anticoagulant, is clinically administered as a racemic mixture of *R*- and *S*-WAR to treat and prevent thromboembolism [1–3]. Despite its extensive use, WAR therapy is complicated by a narrow therapeutic range and wide inter-individual response to treatment [4]. The *S*-WAR is 2–5 times more potent anticoagulant, and is metabolized much quicker (~ 1.5 times faster) than *R*-WAR [5]. Therefore, different *R*- and *S*-form of WAR are differentially metabolized by stereoselective metabolism. The time-consuming and laborious procedure in collecting urine samples can be eliminated by directly determining WAR enantiomers in the human plasma. In addition, to understand the mechanisms for relationships between WAR therapy and patient response, the investigation of the metabolic processes in serum samples is necessary and critical.

WAR metabolism is further characterized by a process termed as regioselective metabolism catalyzed by several important cytochrome (CYP) P450 members (Figure 1). For example, the more potent *S*-WAR exclusively catalyzed by CYP2C9 to form 7-OH-WAR although 6-OH-WAR and 4-OH-WAR are also formed [6]. In contrast, *R*-WAR is metabolized to all five OH-WAR metabolites but 10-OH-WAR and 6-OH-WAR are the most abundant metabolites [7–8]. Reported drug interaction [9] and genetic studies [10–12] indicate that many of these *P450s* enzymes may play a clinical role in patient response to WAR therapy. Therefore, better understanding of the contributions of the CYP enzymes toward total WAR metabolism is warranted.

There has been significant interest in developing sensitive methods for monitoring WAR and its metabolites in serum samples. Currently, the most common enantioselective analytical technique used for the determination of WAR and its metabolites in biological samples is high performance liquid chromatography (HPLC) based on either achiral or chiral methods and detection by UV [13–16], combination of UV/fluorescence and circular dichroism [17, 18] as well as mass spectrometry (MS) [3, 19–25]. Generally, multiple research groups focus on resolving *R*- and *S*-WAR [3, 13–15, 23], but not simultaneous enantiomeric separation of all OH-WAR enantiomers. A more recent study reported an elegant method for the analysis of WAR and OH-WAR enantiomers in plasma samples by ultra performance liquid chromatography (UPLC)-MS/MS using a combination of two columns (phenyl achiral column and chiral Astec Chirobiotic V column) connected in series with identical mobile phase [25]. However, HPLC method requires large volumes of organic solvent. In addition, relatively large injection volume requirement in HPLC is also unfavorable when the patient samples are volume-limited. For example, when extensive research is needed to understand the correlation of metabolite formation with genetic data and other separation techniques, capillary electro-phoresis (CE) has a clear advantage.

The technique of CE has been extensively developed as a complementary enantioselective analytical technique to conventional HPLC in the past decade [26–31]. Electrokinetic chromatography (EKC) using low molecular mass chiral selectors (e.g., charged cyclodextrins, macrocyclic antibiotics, crown ethers, unpolymerized micelles) are capable of separating both charged and uncharged molecules simultaneously with high efficiency, selectivity and flexibility. A weak point, which still remains using the aforementioned chiral selectors for CE is UV-detection sensitivity. Coupling EKC or MEKC with ESI-MS is very

attractive and advantageous. However, direct coupling of MEKC to MS is problematic using conventional surfactants forming micelles. This is mainly due to production of background ions from surfactant monomers resulting in cluttering of mass spectrum and signal suppression [32, 33]. Several methods have been introduced to overcome these limitations of MEKC-MS including: (i) use of high-molecular-mass polymeric surfactants [34–36], (ii) use of the partial filling technique [37], (iii) counter-migration [38], and the use of atmospheric pressure photoionization [39, 40]. Among the various approaches for chiral CE-ESI-MS, the use of high molecular mass polymeric chiral surfactants (also called molecular micelles or micelle polymers) is considered to be a simple and convenient for a variety of chiral compounds. This is because molecular micelles are covalently stabilized micellar aggregates, which remain intact in solution phase inside the capillary. In addition, they are not fragmented in the gas phase of the mass spectrometer, showing notable advantages. These includes zero critical micelle concentration, very stable micellar structure, low surface activity, low volatility as well relatively narrow polydispersity and significantly less MS signal suppression. All of the aforementioned characteristics make polymeric surfactants very compatible chiral selector for on-line MEKCESI-MS [34–36, 41–43].

In our previous study [35], MEKC-ESI-MS method for the analysis of WAR enantiomers was developed, using a polymeric surfactant, polysodium *N*-undecenoyl-L, L-leucyl-valinate (poly-L, L-SULV), as a pseudostationary phase. The method was applied to the determination of only *R*- and *S*-WAR in patient samples. Thus, the simultaneous enantio-separation of WAR and its hydroxylated metabolites by MEKC-MS remains unexplored. This article begins with the development of MEKC-MS method for simultaneous separation of WAR and all OH-WAR metabolites. Comparison of the MEKC-MS versus packed column CEC-MS as well comparison of single quadrupole and triple quadrupole mass spectrometer are provided in details for such analysis. Optimization of solid phase extraction (SPE) procedure for generation of calibration curve, improving limit of quantitation and limitation of detection (LOD) of WAR and metabolites in serum samples using MEKC-ESI-MS/MS with excellent sensitivity and specificity is illustrated. Finally, the MEKC-ESI-MS/MS study was expanded to apply the method to analysis of 55 patient samples receiving WAR therapy.

2 Materials and methods

2.1 Chemicals and reagents

Racemic WAR, racemic 4', 6-, 7-, 8- and 10-OH-WAR, non-racemic diclofenac sodium (used as internal standard (I.S.)), HPLC-grade acetonitrile (ACN), methanol (MeOH), ethanol (EtOH) and isopropanol (IPA), analytical-grade ammonium acetate (as 7.5 M NH₄OAc solution) and dipeptide (L-leucylvalinate, L,L-SULV) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium hydroxide and acetic acid were obtained from Fisher Scientific (Springfield, NJ, USA). The 3 μm vancomycin chiral stationary phase (CSP) was donated by Advanced Separation Technologies, Inc. (Whippany, NJ, USA). Water used in all experiments was triply deionized and obtained from Barnstead Nanopure II water system (Barnstead International, Dubuque, IA, USA). Oasis mixed-mode anion-exchange (MAX) cartridges (3cc, 100 mg) were supplied by Waters (Milford, USA). The dipeptide surfactant monomer of sodium (L, L-SULV) was synthesized following the procedure described in reference [44]. The surfactant monomer was polymerized using a total dose of 20 M rad of ⁶⁰Co γ-radiation by Phoenix Memorial Laboratory (University of Michigan, Ann Arbor, MI). Blank and patient's human plasma were obtained from Mercer University Southern School of Pharmacy (Atlanta, GA, USA) and stored under –80 °C until the assay was performed.

2.2 Preparation of Background electrolytes and sheath liquids

The background electrolyte (BGE) was prepared by diluting the 7.5 M NH_4OAc stock solution and then adjusting to the desired pH values with acetic acid or ammonium hydroxide. The chiral MEKC running buffers were prepared by adding various amounts of poly-L, L-SULV and organic solvent to the BGE solution. The molar concentration of the poly-L,L-SULV was calculated using the molecular mass of its respective monomer (referred to as equivalent monomer concentration, EMC). The CEC mobile phases were prepared by adding various amounts of organic solvent to the BGE solution. The sheath liquids for both MEKC and CEC were prepared by mixing aqueous NH_4OAc buffer (adjusted to the desired pH value prior to mixing) with appropriate volume ratio of MeOH. The running MEKC buffer, CEC mobile phase and sheath liquid were filtered with 0.45 μm PTFE membranes and degassed for 20 min before use.

2.3 Preparation of standard solutions, plasma samples and calibration curves

Individual stock solutions of racemic warfarin, racemic 4', 6, 7, 8, 10-OH-WAR, and internal standard (I.S) were prepared by dissolving the appropriate amount of each authentic standard in ACN/ H_2O (40/60, v/v) at 1.0 mg/mL. A mixture containing all analytes was prepared by taking certain aliquot of each stock solution and diluting to the desired concentration with ACN/ H_2O (40/60, v/v). The elution order of each analyte was established by spiking the standard mixture or the plasma sample with a certain concentration of individual authentic standard.

Calibration standards were prepared by spiking a 250- μL of blank human plasma with 5 μL of standard stock solutions (0.1, 0.25, 1.25, 2.5, 5, 12.5, 25, 125 and 250 $\mu\text{g/mL}$) of racemic mixtures of WAR and all its five hydroxylated metabolites, 2.5 μL of I.S. solution at a concentration of 1 mg/mL to yield the final concentrations of 2, 5, 25, 50, 100, 250, 500, 2500 and 5000 ng/mL of *R*- and *S*-WAR, *R*- and *S*-OH WARF metabolites, respectively. All spiked calibration standards were prepared in triplicate and followed the same plasma solid phase extraction (SPE) procedure as discussed below.

MAX cartridges used for the plasma sample extraction were preconditioned with 2 mL of MeOH, followed by 2 mL of water. Aliquots (250 μL) of patient plasma sample, 2.5 μL of I.S. solution and 250 μL of 10 % (v/v) perchloric acid or 4 % (v/v) phosphoric acid were transferred to a 1.5 mL microcentrifuge tube. After vortex mixing for 30 s, followed by centrifugation [10 min at 1000 rpm, (9279g)], the supernatant (~ 450 μL) was loaded on the preconditioned MAX cartridge. The loaded sample on the cartridge was washed with 2 mL of 2 % (w/w) NH_4OH and then 2 mL of H_2O . Finally, the analytes were eluted with 2 mL of ACN/MeOH/formic acid (50/50/5, v/v/v). The eluate was collected in a 5 mL screw cap vial and the solvent was evaporated to dryness in a vacuum chamber under a stream of air. The residue was reconstituted with 50 μL of ACN / H_2O (40/60, v/v) for analysis by MEKC-MS/MS.

Calibration curves were obtained by plotting the peak-area ratio of each respective enantiomer to the I.S. versus concentrations of enantiomers in plasma. Recovery of WAR and its metabolites were determined by comparing the peak area of the blank plasma sample at various concentrations to those of the original aqueous standard solutions.

2.4 Instrument

The MEKC-ESI-MS and CEC-ESI-MS experiments were carried out on an Agilent CE system interfaced to an Agilent 1100 series MSD single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA) equipped with an Agilent CE-MS adapter kit (G1603A), and an Agilent CE-ESI-MS sprayer kit (G1607). The sheath liquid was delivered

using an Agilent 1100 series isocratic HPLC pump with a 1:100 splitter. Nitrogen was used as both nebulizing gas and drying gas. The Agilent ChemStation and CE-MSD add-on software were employed for instrument control and data analysis. Fused-silica capillary 375 μm O.D., with 50 μm I.D. (118 cm total length) and 75 μm I.D. (65 cm total length) obtained from Polymicro Technologies, Phoenix, AZ, USA) was used for MEKC-MS and CEC-MS, respectively. The CEC-MS capillary column was prepared in two steps. The first step involved the formation of an internal taper at the outlet end of the capillary that is 10 μm wide at its narrowest, which has been described elsewhere [45]. In the second step, the internal taper column was slurry packed with vancomycin CSP following the procedure reported earlier [46, 47] using a Knauer pneumatic pump (Wissenschaftliche Gerätebau, Dr. Ing. Herbert Knauer GmbH, Berlin, Germany). After the column was packed, an inlet frit was burned using a home-made frit burner. A typical CEC-MS column consists of 40 cm packed and 25 cm unpacked segment.

The MEKC-ESI-MS/MS experiments were performed on an Agilent CE system interfaced to an Agilent 6410 series triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA), in which the same sheath liquid delivery setting was used as described for MEKC-ESI-MS. Nitrogen was not only used as a nebulizing gas and drying gas but also as a collision gas. Agilent MassHunter Workstation (version B.02.01) was the software used for data acquisition, qualitative and quantitative analysis. A 120 cm long fused-silica capillary (375 μm O.D., 50 μm I.D.) was used for MEKC-ESI-MS/MS experiments.

2.5 CEC-MS operating conditions

Before the CEC-ESI-MS run, the column was preconditioned following the procedure reported earlier [46, 47]. All injection was performed electrokinetically at 6 kV for 3 s. The separation voltage was set at 25 kV, employing a voltage ramp of 3 kV/s. During the separation, a 12 bar external pressure was applied to the inlet buffer vial in order to suppress bubble formation and maintain a stable current. As for the MEKC experiments, before the capillary was installed into the CE-MS nebulizer, it was flushed with 1 M NH_4OH for 40 min and water for 10 min at 45 $^\circ\text{C}$. After installation of the capillary into the sprayer, it was flushed with 1M NH_4OH and water for 3 min each before injection. As a preconditioning step, the capillary was rinsed with actual running buffer containing poly-L-SULV for 4 min. After each run, the capillary was flushed with water (3 min), NH_4OH (4 min) and water (3 min). The separation voltage was set at 30 kV, employing a voltage ramping of 3 kV/s. To ensure a good run-to-run repeatability, a fresh buffer vial was always used for each MEKC/MS and MEKC-MS/MS run. Analytes were kept at 25 $^\circ\text{C}$ in the autosampler and injected hydrodynamically at the pressure of 5 mbar for variable times.

The noise level and S/N ratio were calculated by the Agilent ChemStation software (Version 10.02) or by Agilent Mass Hunter Workstation (version B.02.01). The S/N ratio was calculated by taking the ratio of the peak height and noise, where the noise is measured as six times the standard deviation of peak to-peak noise of the linear regression of the baseline by the software. The value for noise used in this work is determined from the baseline selected in the time range closest to the eluting peak. Resolution (Rs) was calculated using the following equation:

$$Rs = \frac{2 \times (t_{R2} - t_{R1})}{W_1 + W_2} \quad (1)$$

where t_{R1} and t_{R2} are the retention times of the first and second eluting peaks. W_1 and W_2 are the peak widths for the first and second eluting peaks measured at the baseline. The retention times and the peak widths were calculated by Agilent MassHunter Workstation (version B.02.01).

For the single quadrupole MS, unless otherwise stated, the following ESI-MS conditions were used: sheath liquid, MeOH/H₂O (80/20, v/v) containing 5 mM NH₄OAc at pH 6.8; sheath liquid flow rate, 5 μ L/min; capillary voltage, - 3000 V; fragmentor voltage, 91 V; drying gas flow rate, 6.0 L/min; drying gas temperature, 200°C; nebulizer pressure, 4 psi. The ESI-MS detection was performed in the selective ion monitoring mode. The negative [M-H]⁻ ions were monitored at 307 m/z for WAR and 323 m/z for 4'-, 6-, 7-, 8- and 10-OH-WAR

For the triple quadrupole MS, the sheath liquid composition, sheath liquid flow rate and spray chamber conditions were the same unless otherwise stated as follows: drying gas flow rate, 8.0 L/min; fragmentor voltage set at 125 V for WAR and OH-WAR and 75 V for I.S.; collision cell gas supply pressure, 30 psi; collision energy was set at 20 eV for WAR and OH-WAR and 5 eV for I.S. The optimization of the fragmentation pathways of WAR and OH-WAR metabolites by changing the collision energy from 10 to 30 eV in a 5 units increments was studied by CE-ESI-MS/MS analysis in the product ion scan mode (Agilent Mass Hunter Workstation (version B.02.01)) (Figure S1). The optimal multiple reaction monitoring (MRM) in the negative ion mode was used to monitor WAR (m/z 307→m/z 161), 4'-OH-WAR (m/z 323→m/z 161), 6-, 7- and 8-OH-WAR, (m/z 323→m/z 177), 10-OH-WAR (m/z 323→m/z 250) and I.S. (m/z 294→m/z 250), which were the same as previously reported in the references [21, 24].

3. Results and discussion

3.1 Optimization of chiral MEKC-ESI-MS separation conditions

As mentioned earlier, the MEKC-MS using high molecular mass polymeric surfactants provide significantly less signal suppression, improving the separation and detection of chiral compounds [34–36]. The use of dipeptide amide polymeric surfactants such as poly-L-SULV in MEKC was reported to have broad enantioselectivity [48]. Furthermore, recently we reported that MEKC-MS of *R/S* WAR could be achieved with high enantioselectivity using poly-L, L-SULV compared to other single amino acid and dipeptide surfactants of various head groups [35]. Because the monohydroxylated WAR metabolites such as 4'-, 6-, 7-, 8- and 10-OH-WAR are positional isomers with similar chromatographic and electrophoretic properties, they generally elutes in close proximity in both HPLC, and EKC [16, 21, 22]. Accordingly, poly-L,L-SULV was chosen as a pseudostationary phase for sequential optimization of MEKC parameters to achieve simultaneous enantioseparation of WAR and OH-WAR metabolites.

3.1.1 Effect of pH on simultaneous enantioseparation of warfarin and its metabolites—The pH of the volatile BGE not only affects the charge on the analyte and chiral polymeric surfactant (possessing ionizable head group) but also the magnitude of the electroosmotic flow (EOF) as well as MS detection sensitivity. In our previous study [35], the migration time and chiral resolution for WAR decrease with an increase in pH from 5.5 to 8.0 and best enantioseparation was obtained at the lowest pH of 5.5. Therefore, to achieve simultaneous enantioseparation of WAR, as well as 4'-, 6-, 7-, 8- and 10-OH-WAR, relatively narrow range of pH was investigated by decreasing the pH value very slowly by 0.10 pH units from pH 5.5–5.0. Figure 2 A–B shows overlaid chromatograms at two representative pH values of 5.5 and 5.0. Clearly, a decrease in pH from 5.5 to 5.0 results in increase in migration time (due to decrease in EOF) but also widens the migration window for simultaneous enantioseparation. Note that not only selectivity changes are observed for some of the positional isomers, but overall chiral *Rs* of closely spaced stereoisomers increases at pH 5.0 (Fig. 2D) compared to pH 5.5 (Fig. 2C). The enantiomers of WAR as well as 4'-, 6-, 7- and 10-OH-WAR (except 8-OH-WAR) were all baseline separated at pH 5.0, though some overlap was observed between few positional isomers.

3.1.2 Effect of type of organic solvent—Addition of organic solvent to MEKC buffer could influence several viscosity of the BGE, the mobilities of analytes and chiral selector as well as the solubility of enantiomers and the EOF. More importantly, the addition of organic solvent seems to change the partitioning behavior between the chiral analytes and chiral surfactant. Consequently, different types of organic solvent were investigated for the simultaneous enantioseparation of WAR and OH-WAR. Three polar protic solvents (MeOH, EtOH and IPA) and one polar aprotic solvent (ACN) were each added from 0–20% (v/v) to the BGE to find the most volume fraction. It could be observed from Figure 3 that among the four tested organic solvent, MeOH was the best organic solvent with improve peak shapes compared to EtOH or IPA. Several differences are noted when comparing ACN versus MeOH solvents. First, ACN provided similar peak shapes and efficiency but the run times were much faster compared to MeOH due to lower viscosity/dielectric constant ratio of the former solvent resulting in faster EOF. Second, the positional isomers of 6-OH-WAR were only partially resolved from 8-OH-WAR, and the optical resolution of optical isomer of 7-OH-WAR was much lower using ACN compared to MeOH. When the ACN or MeOH content exceeded 15% (v/v), the resolution of both optical and positional isomers of OH-WAR decreases (data not shown). Nevertheless, 15% (v/v) MeOH is the solvent of choice as it allows a better migration time difference between positional isomers of OH-WAR.

3.2 Comparison of MEKC-ESI-MS versus CEC-ESI-MS

Although simultaneous enantioseparation of WAR from the OH-WAR metabolites is required in MEKC-UV, it is not essential in MEKC-MS because different m/z of WAR and OH-WAR allows easy identification. For example, under optimized pH and organic solvent composition, one of the later eluting enantiomer of 6-OH-WAR the early enantiomer of WAR in total ion chromatogram (TIC) (Figure 4A) but both WAR and 6-OH-WAR can easily distinguishable in EIC mode (Figure 4B vs. 4C). Nevertheless, simultaneous separation of all positional isomers of OH-WAR metabolites (Figure 1) in MEKC-MS is of utmost importance due to same m/z .

Encouraged by our earlier results on simultaneous enantioseparation of β -blockers [47], we attempted to explore the possibility of utilizing vancomycin as a packing material in combination with single-frit column to perform chiral CEC-MS of WAR and OH-WAR metabolites. Several mobile phase combinations of ACN and MeOH were tried Although the chiral selectivity for each hydroxylated metabolite (except 10-OH WAR) was very high, simultaneous separation of positional isomers by CEC-MS seems very challenging under both mobile phase conditions noted in Figure 5A and B. Clearly, the optical isomers of 10-OH-WAR was better separated using the mobile phase of ACN / H₂O (45/55, v/v) containing 10 mM NH₄OAc at pH 4.0 (Figure 5C) compared to the mobile composition of ACN / MeOH/ H₂O (30/50/20, v/v/v) (Figure 5 D). This is because both ACN and MeOH compete for hydrogen bonding sites on vancomycin CSP with the analytes. One possible explanation is that ACN is a relatively poor bonding donor than MeOH [49], the competition with the analyte for the hydrogen bonding sites on the CSP is less without MeOH in the mobile phase. Therefore, stereoisomers of 10-OH-WAR are able to bond more strongly to the CSP resulting in improved separation. Further decreasing the % ACN in the mobile phase, the R_s of 10-OH-WAR was not significantly improved, but the total run time of the CEC-ESI-MS was obviously increased due to the decrease of electroosmotic flow (data not shown). Optimization of other mobile phase parameters such as type and concentration of other volatile buffers, pH and temperature provided no improvement in simultaneous enantioseparation. Attempts were also made to improve the resolution of six enantiomeric pairs using other organic solvent (i.e., isopropanol, butanol) (data not shown). Hence, the CEC-MS chromatogram shown in Figure 5 is the optimized one.

Comparing the two CE-MS modes, clearly shows that chiral resolution of ($\pm$)-WAR and its -OH metabolites was possible via CEC-MS in ~100 min (Figure 5A). On the other hand, MEKC-ESI-MS (Figure 4A) using poly-L,L-SULV, provided simultaneous resolution of all positional and optical isomers (with the exception of 8-OH WAR) with much shorter analysis time of less than 40 min but suffers from relatively lower sensitivity due to some suppression effect of the poly-L,L-SULV surfactant. As a result, with the aim of increasing the detection sensitivity to enable the application of WAR and its metabolites in biological samples, coupling of MEKC with a triple quadrupole mass spectrometer was the next obvious approach investigated.

3.3 Comparison of MEKC-MS and MEKC-MS/MS

Both single quadrupole MS and triple quadrupole MS were used to compare the S/N of the blank plasma spiked with WAR and its five -OH metabolites at a concentration of 1 $\mu\text{g/mL}$. As shown in Fig.6A, the MEKC-MS provided a S/N of < 15 for a standard mixture of WAR and OH-WAR. However, the use of multiple reaction monitoring mode (i.e., MEKC-MS/MS, Fig.6B) provided much higher S/N at the same injection size of 5 mbar for 2 s. For example, the $S/N_{avg} \sim 7$ fold higher, i.e., 103 for WAR and ranged from 35 to 110 for OH-WAR. One of the advantage of triple quadrupole MS is that the S/N ratio can be further improved by using more diluted sample and increasing the injection size to 5 mbar 300 s without compromising chiral resolution. For example, the $S/N_{avg} \sim 300$ fold higher, i.e., 5016 for 0.25 $\mu\text{g/mL}$ for WAR and ranged from 350–1178 for 0.05 $\mu\text{g/mL}$ of OH-WAR (data not shown). This is because single quadrupole systems contain only one mass filtering quadrupole, whereas triple quadrupole systems consist of three quadrupoles, which drastically reduces noise resulting in higher detection sensitivity. In addition to higher sensitivity, better selectivity was obtained for positional isomers in MRM mode in MEKC-MS/MS (Fig.6D) compared to the SIM mode in MEKC-MS (Fig.6C).

3.4 Optimization of chiral MEKC-ESI-MS/MS assay

3.4.1 Optimized enantioseparation of warfarin, its hydroxylated metabolites and internal standard by chiral MEKC-ESI-MS/MS—Although both coumachlor (COU) and diclofenac have been previously reported as I.S. for the analysis of WAR [17, 24, 35], diclofenac was chosen as I.S. for quantitation due to its shorter retention time compared to COU. Other reasons includes its easy accessibility, good SPE-MAX clean-up recovery ($> 80\%$), better separation from WAR and OH-WAR metabolites as well as excellent ESI-MS response. Due to the observed suction effect of the nebulizer, injection times were evaluated by keeping the injection pressure constant at 5 mbar to achieve highest S/N without sacrificing the chiral resolution. When the injection time increases from 80 to 300 s, the S/N_{avg} increases ~ 8 folds (Fig.7A), without influencing the chiral R_s values of closely eluting stereoisomers (e.g., WAR, 6-, 7-OH-WARs, Fig 7B). Because only a maximum of 0.78 % of total capillary length of the sample was injected at 300 s in this work, the increase of the S/N_{avg} of analyte is mostly attributed to the increasing sample loaded volume with increasing injection time. Therefore, 300 s of injection time was chosen as optimum because it establishes good compromise between chiral R_s and MS/MS sensitivity. The enantiomers of a standard mixture of WAR, its five OH-WARs and the I.S. were all best resolved within 45 min with high sensitivity (Figure 8). For each OH-WAR, the R and S -enantiomers were identified for the elution order of parent drug, WAR, which indicates R -enantiomer eluted first followed by the S -enantiomer. Traditional HPLC, which requires chiral and achiral columns for the separation of WAR and its OH-WAR metabolites is difficult due to too much back pressure generated at the required flow rate. Furthermore, the peak widths from the first column should be narrow to allow sufficient time to load on to the second column. To overcome these drawbacks, recently, simultaneous enantioseparation of WAR and its five chiral metabolites was obtained by UPLC-MS/MS method [25] in nearly 16 min using a

combination of phenyl and Chriobiotic V column. However, the UPLC method required two columns in series, which restricts the choice of mobile phase composition to a narrow range, which can be used for separation.

3.4.2 Recovery and method validation—Liquid-liquid extraction (LLE) and solid phase extraction (SPE) were previously compared for extractions of WAR and OH-WAR for human plasma samples [24]. The extraction recoveries of WAR and 7-OH-WAR were less than 50% by LLE and relatively high (>80%) using MCX and MAX. We investigated the recoveries of WAR and its metabolites in blank plasma. Two type of SPE (MAX and C18) cartridges as well two acids (HClO₄ and H₃PO₄) used as deproteinizing reagents were compared. For example, recoveries of WAR, 7-OH-WAR and I.S. were > 85 % using a combination of MAX cartridge and 4% (v/v) H₃PO₄ (Table 1, middle column), which were significantly higher compared to the use of 10% (v/v) HClO₄ (Table 1, left column). The combination of C18 cartridge and 4% (v/v) H₃PO₄ were also compared but the recoveries with C18 cartridge were < 30% (Table 1, right column). Therefore, the MAX cartridge with 4% (v/v) H₃PO₄ as the deproteinized reagent was chosen as optimum combination to study recoveries of WAR, IS, and OH-WAR at three different concentration levels (i.e. 25, 100, and 500 ng/mL) in human plasma. The recoveries were between 90.1 – 110.7 % for WAR, and 85.2 – 112.9 % for OH-WARs with the RSDs between 5.3 and 8.7 % (Table 1).

To determine the concentration of WAR and OH-WAR metabolites in patients' plasma samples, chiral MEKC-ESI-MS/MS was applied to develop a chiral assay. Under the optimum conditions, the simultaneous determination of WAR and OH-WAR was examined by generating calibration plots of all enantiomers in blank plasma. The calibration curves displayed good linearity in the range of 2–5000 ng/mL for *R*- and *S*-warfarin ($r^2 = 0.9993$ – 0.996), 5–1000 ng/mL for *R*- and *S*-6-, 7-, 8- and 10-OH-WAR and 10–1000 ng/mL for *R* and *S*-4'-OH-WAR with r^2 values > 0.99 (Figure S2 and S3). In this work, the LOD is defined as the lowest concentration of the analyte resulting in a *S*/*N* of 10:1. The LOQ is defined as 3 times the LOD. The LOD and LOQ were 0.5 ng/mL and 2 ng/mL for WAR, 1.5 and 5 ng/mL for 6-, 7-, 8- and 10-OH-WAR, and 3 and 10 ng/mL for 4'-OH-WAR, respectively (Table 2). The developed chiral MEKC-ESI-MS/MS provided much higher sensitivity of WAR and OH-WAR enantiomers than those using UV analysis (75–180 ng/ml) [14–16] and comparable to those LC-MS/MS detection [24, 25]. The intra-day and inter-day reproducibilities at low, medium and high concentration levels were 10.1 % RSD (Table 2).

3.4.3. Profiling enantiomeric warfarin and hydroxywarfarins in human plasma

—The developed chiral MEKC-ESI-MS/MS assay was next applied to the simultaneous determination of concentrations of WAR and OH-WAR in plasma samples obtained for a total of 55 patients. Table S1 showed the metabolite profiles for all 55 subjects plasma samples. The *R*- and *S*-WAR were quantifiable in all of the patients. Mean plasma concentrations with of *R* and *S*-WAR were 1187.5 ± 717.8 and 636.9 ± 432.1 ng/mL, respectively, which is in accordance with the literature data [16, 50]. Concentration ratio of *R*- to *S*-WAR ranged from 0.37 to 5.14 with mean value of 2.10 ± 0.95 was observed, which is consistent with the literature data [16]. By analyzing the data for *R*/*S* ratio of WAR enantiomers (Table S1, column 1) using chiral MEKC-ESI-MS/MS method, it is convenient to screen whether the patients are normal or patients have a mutant gene (CYP2C9*2 or *3) [35]. The normal patients possess an *R*/*S* ratio of WAR enantiomers above 1 where the *R*-enantiomer is more concentrated than *S*-enantiomer. In contrast to the normal patient, the mutant patients have an *R*/*S* ratio of WAR enantiomers below 1. Among the 55 patients screened in Table S1, there are 51 patients with (*R*/*S*)-WAR >1 and 4 patients with (*R*/*S*)-WAR <1. The representative electrochromatograms of the normal versus mutant patient's samples are shown in Figure 9. Clearly, the peak height of *R*- WAR > *S*-WAR in normal

subject (patient 11, left electrochromatogram). As expected, for the same subject, there is much lower peak height of *R*-7-OH-WAR compared to *S*-7-OH WAR (i.e. $R < S$). Although the peak height ratio of *R/S*-WAR is reversed in mutant subject (patient 5, right electrochromatogram), the trend in *R/S* ratio for OH-WAR metabolites remains is not reversed.

For all 55 patients' plasma samples, 8-OH-WAR was observed in 15% of these patients, but was below the LOQ. Plasma concentrations of *R*-4'-OH-WAR, *R*-7-OH-WAR, *S*-6-OH-WAR and *S*-10-OH-WAR were above the LOQ in 5%, 11%, 24% and 4% of these 55 subjects, respectively. Nevertheless, *R*-6-OH-WAR, *R*-10-OH-WAR, *S*-4'-OH-WAR and *S*-7-OH-WAR were able to be quantified in 85%, 95%, 95% and 98% of these 55 subjects, respectively. In 95% and 4% of these patients, the major observed metabolites were *S*-7-OH-WAR and *R*-10-OH-WAR confirming that the formation of these two major isomers are catalyzed by human CYP2C9 and CYP3A4, respectively. The formation of metabolites of *S*-WAR decreases in the order $7\text{-OH} \gg 4'\text{-OH} \sim 6\text{-OH} > 10\text{-OH} \sim 8\text{-OH}$ (Table S1) suggesting that *S*-WAR is metabolized primarily by CYP2C9 to 7-OH-WAR. The mean plasma concentrations of *R*-OH-WAR are ranked in the following order: $10\text{-OH} > 6\text{-OH} \sim 7\text{-OH} \sim 4'\text{-OH} \sim 8\text{-OH}$ (Table S1) confirming that *R*-WAR is metabolized primarily by CYP3A4 to 10-OH-WAR. Even though the 51 normal and 4 mutant subjects have different *R/S*-WAR ratios, the order of concentrations for the enantiomers of each metabolite are the same. For example, the concentration of 4-OH-WAR and 7-OH-WAR have the order of $R < S$, whereas 6-OH-WAR and 10-OH-WAR have the order of $R > S$ (Table S1, and Figure 9). Furthermore, in four subjects (5, 15, 54, 55, mutant gene) not only the enantiomeric ratio of *R/S* WAR is < 1 , but also the concentration of major WAR metabolites, (e.g., *S*-7-OH WAR) was significantly lower with a mean concentration of 17 ± 14.2 ng/mL compared to majority of the normal subjects with a mean concentration of 135.6 ± 105.7 ng/mL.

A comparison between our results with others revealed striking similarities and differences. There is a common agreement on the major metabolites but not the minor ones. In a previous study, only the racemic forms of OH-WAR enantiomers were quantified among 204 patients undergoing warfarin therapy. In addition, racemic 7-OH WAR and 10-OH WAR were major plasma metabolites, while 4'-OH-WAR was undetectable in all patients [16]. This novel chiral MEKC-ESI-MS/MS method allowed quantitation of *R*-4'-OH-WAR and *S*-4'-OH-WAR in 5% and 95% of the plasma samples, respectively, which was not observed in the study by Locatelli [16] and Jones [25]. This is most likely due to the lower LOQ of this novel chiral MEKC-ESI-MS/MS method compared to the previous method. Therefore, our results clearly demonstrate that this novel chiral MEKC-ESI-MS/MS method is suitable for enantiomeric profiling of WAR and its metabolites in human plasma.

4. Conclusions

For the first time, a novel and sensitive chiral MEKC-ESI-MS/MS method is developed and validated for profiling of regioselective and stereoselective OH-WAR isomers along with the parent drug, WAR using a polymeric surfactant, poly-L,L-SULV, as a pseudostationary phase. The method provides excellent simultaneous separation of WAR and all OH-WAR metabolites in 45 min and was superior to the packed column CEC-MS using vancomycin chiral stationary phase. The successful analysis of 55 patient samples demonstrated the potential of the method to simultaneously and accurately quantitates WAR and its metabolites present in human plasma with high sensitivity. A diagnostic chiral test was also developed to distinguish between *R/S* WAR ratios in normal and mutant subjects. In general, subjects with mutant gene not only have the lower enantiomeric ratio of *R/S* warfarin ($R/S < 1$), but also the concentration of major WAR metabolite, e.g., *S*-7-OH-WAR, was significantly lower compared to majority of the normal subjects. In addition, this

novel chiral MEKC-ESI-MS/MS method allows the quantitation of *R*-4'-OH-WAR and *S*-4'-OH-WAR in 5% and 95% of the plasma samples, respectively, due to the lower LOQ compared to the previous reported method [16, 25]. Thus, the developed methodology marks a significant advancement in the profiling of WAR and its hydroxylated metabolites in patients undergoing WAR therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Develop a novel CMEKC-MS/MS for simultaneous profiling of warfarin and metabolites.
- Comparison of MEKC-MS versus CEC-MS revealed that MEKC-MS-MS provide higher peak capacity and shorter run times
- MEKC-MS/MS provide better *S/N* and LOD than MEKC-MS
- LOD of warfarin and OH-warfarin metabolites were as low as 0.5 ng/mL and 1.5 ng/mL, respectively.
- 55 patient samples were successfully analyzed.

Figure 1.

Structures of (A) WAR and its sites of hydroxylation (indicated by arrow) to generate monohydroxylated WAR metabolites by cytochrome P450s (B) diclofenac (DFC) sodium (used as internal standard). The asterisk indicates stereogenic center.

Figure 2.

Effect of pH on simultaneous enantioseparation of WAR and its metabolites. pH 5.0 (A, C) and pH 5.5 (B, D). Conditions: 118 cm long (375 μm O.D., 50 μm I.D.) fused silica capillary. Buffer: 25 mM NH_4OAc /25 mM poly-L,L-SULV. Applied voltage, +30 kV, injection, 5 mbar, 2 s. Spray chamber parameters: nebulizer pressure: 4 psi, drying gas temp.: 200 $^{\circ}\text{C}$, drying gas flow: 6 L/min; capillary voltage: -3000 V; fragmentor voltage, 91 V; SIM mode; sheath liquid: MeOH/ H_2O (80/20, v/v), 5 mM NH_4OAc , pH 6.8 with flow rate of 0.5 mL/min; Sample concentration: 10 $\mu\text{g/mL}$ in ACN/ H_2O (40/60, v/v).

Figure 3.

Effect of type of organic solvent on simultaneous enantioseparation of WAR and its hydroxylated metabolites. MeOH (A), ACN (B), EtOH (C) and IPA (D). Conditions: buffer 25 mM NH_4OAc pH 5.0 / 25 mM poly-L,L-SULV containing organic solvent. Other MEKC-ESIMS conditions are the same as in Fig. 2.

Figure 4.

Optimized enantioseparation of WAR and its hydroxylated metabolites by MEKC-ESIMS (A–C). Conditions: buffer 25 mM NH₄OAc/25 mM poly-L,L-SULV containing 15% (v/v) MeOH at pH 5.0. Other MEKC-ESI-MS conditions are the same as in Fig. 2.

Figure 5.

Optimized enantioseparation of WAR and its hydroxylated metabolites by CEC-ESIMS (A–D). Conditions: 40 cm long vancomycin packed column (total long 65 cm, 375 μm O.D. and 75 μm I.D.). Running buffer, ACN / H₂O (45/55, v/v), 10 mM NH₄OAc at pH 4.0 (A) and ACN / MeOH/ H₂O (30/50/20, v/v/v), 10 mM NH₄OAc at pH 4.0 (B). Applied voltage, + 25 kV, injection, 5 kV, 3 s. Spray chamber and sheath liquid conditions are the same as in Fig. 2.

Figure 6.

Comparison of the detection of WAR and its hydroxylated metabolites by MEKC-MS (A, C) and MEKC-MS/MS (B, D). For MEKC-MS, all the conditions are the same as in Fig. 4A. MEKC-MS/MS conditions: 120 cm long (375 µm O.D., 50 µm I.D.) fused silica capillary. Buffer: 25 mM NH₄OAc pH 5.0, 25 mM poly-L,L-SULV with 15 % (v/v) MeOH. Injection: 5 mbar for 2 s, Voltage: 30 kV; Spray chamber parameters: drying gas temperature: 200 °C, drying gas flow rate: 8 L/min, nebulizer pressure: 4 psi, capillary voltage: -3000 V, collision energy: 20 eV for all except 5 eV for I.S., fragmentor voltage: 125 V for all except 75 V for I.S.. Sheath liquid: MeOH/H₂O (80/20, v/v) with 5mM NH₄OAc, sheath liquid flow rate: 5 µL/min.

Figure 7.

The effect of injection time on S/N ratio (A) and chiral resolution (B) of WAR and OHWAR metabolites. All other conditions are the same as Fig. 6B. Analyte: 0.1 $\mu\text{g/mL}$ of WAR, 0.2 $\mu\text{g/mL}$ of each OH-WAR, and 10.0 $\mu\text{g/mL}$ of I.S. in ACN/ H_2O (40/60, v/v).

Figure 8.

Extracted ion electrochromatograms of a standard mixture of WAR and OH-WAR under optimized injection conditions of 5 mbar for 300 s. All other conditions are the same as Fig. 6B. Analyte: 0.1 $\mu\text{g/mL}$ of WAR, 0.2 $\mu\text{g/mL}$ of each OH-WAR, and 10.0 $\mu\text{g/mL}$ of I.S. in ACN/H₂O (40/60, v/v). For WAR and each OH-WAR, the *R*-enantiomer eluted first followed by the *S*-enantiomer.

Figure 9.

Extracted ion electrochromatograms of subject 11 and subject 5 (with mutant gene CYP2C9*2 or *3). The MEKC-MS/MS conditions are the same as in Fig. 8.

Table 1

Comparison of recovery using three different solid phase extraction (SPE) methods

Test compound ^{a)}	MAX with 10 % (v/v) perchloric acid as deproteinized reagent	MAX with 4 % (v/v) phosphoric acid as deproteinized reagent	C18 with 4 % (v/v) phosphoric acid as deproteinized reagent
R-warfarin	24.6% ^{b)}	91.9%	30.4%
S-warfarin	25.1%	100.9%	29.8%
R-7-OH-war	28.6%	85.7%	23.8%
S-7-OH-war	30.5%	97.3%	25.9%
I.S.	9.2%	86.7%	15.8%

^{a)} The concentrations of WAR and 7-OH-WAR were 500 and 100 ng/mL, respectively. The concentration of I.S. was 10 µg/mL.

^{b)} Mean values (n = 3) with %RSD < 9.0%.

Table 2

Method validation parameters of calibration curves for the determination of warfarin, 4'-, 6-, 7-, 8- and 10-OH-warfarins in human blood plasma.

Validation parameter	WAR		4'-OH-WAR		6'-OH-WAR		7'-OH-WAR		8'-OH-WAR		10'-OH-WAR	
	R	S	R	S	R	S	R	S	R	S	R	S
Interday repeatability (% RSD) (n=3)	medium	8.1	7.7	8.8	9.2	10.1	9.3	7.6	6.9	9.7	8.0	7.5
	low	5.3	5.6	6.8	6.2	5.8	6.1	4.9	4.5	6.3	4.2	4.5
	medium	4.5	4.7	6.1	6.3	5.1	6.6	4.3	4.8	6.1	4.9	5.3
Interday repeatability (%RSD) (n=4)	high ^{a)}	5.0	4.1	5.9	5.3	5.7	5.2	3.9	4.5	6.7	4.6	3.9
Limit of detection (ng/mL) ^{b)}		0.5	0.5	3	3	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Limit of quantitation (ng/mL) ^{c)}		2	2	10	10	5	5	5	5	5	5	5
Linear range (ng/mL)		2 – 5000	10 – 1000	5 – 1000	5 – 1000	5 – 1000	5 – 1000	5 – 1000	5 – 1000	5 – 1000	5 – 1000	5 – 1000
Correlation coefficient, r ²		0.9966	0.9993	0.9999	0.9997	0.9999	0.9980	0.9990	0.9993	0.9974	0.9929	0.9947

^{a)} Low, medium and high concentrations were 50, 250, 2500 ng/mL for S- or R-WAR, respectively, and 25, 100, 500 ng/mL for S- or R-OH-WAR, respectively.

^{b)} The limit of detection (LOD) was defined as the lowest concentration of the analyte resulting in a signal-to-noise ratio (S/N) of 10:1.

^{c)} The limit of quantitation (LOQ) was defined as 3 times the LOD.