Journal of Chromatography A, 1218 (2011) 4045–4051

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Coupling of acetonitrile deproteinization and salting-out extraction with acetonitrile stacking in chiral capillary electrophoresis for the determination of warfarin enantiomers

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ARTICLE INFO

*Article history:*
Received 27 October 2010
Received in revised form 19 April 2011
Accepted 22 April 2011
Available online 6 May 2011

*Keywords:*
Acetonitrile deproteinization
Salting-out extraction
Acetonitrile stacking
Chiral capillary electrophoresis

ABSTRACT

Concurrent sample clean-up and enhancement in detection sensitivity for chiral capillary electrophoresis was demonstrated based on the coupling of salting-out extraction with acetonitrile stacking and the use of dimethyl-beta-cyclodextrin as the chiral selector for the sensitive and enantioselective separation of warfarin enantiomers in urine samples. By optimizing the pH of salting-out extraction, warfarin enantiomers can be efficiently extracted from the aqueous sample solution into a smaller volume organic solvent (acetonitrile) phase. The pressure injection of the enriched acetonitrile phase (containing ca. 1% NaCl) into the CE capillary at 10% capillary volume resulted in additional concentration of the warfarin enantiomers. The limit of detection for both warfarin enantiomers was as low as 1.5 ng/mL in urine sample. Our results show that the novel strategy offers improved sensitivity compared to conventional CE analysis, reaching a combined enrichment factor higher than 1000. Calibration curves of warfarin enantiomers in urine samples were found to be linear between 10 and 1000 ng/mL, and intra- and inter-day precision ($N = 9$) for both warfarin enantiomers in terms of migration time and peak area were found to be within the range of 0.1–0.8% and 1.0–6.7%, respectively. The recovery of warfarin enantiomers from urine was ca. 90%.



## 1. Introduction

Warfarin is widely used as oral anticoagulant in the prevention and treatment of venous and arterial thromboembolism [1]. A major complication associated with the prescription of warfarin is that there is a very large variation in optimal dosage between different patients, which can be attributed to differences in liver metabolism of individual patients, drug–drug interactions and variable ingestion of Vitamin K with food [2]. Moreover, it is important to note that warfarin exist in two different enantiomeric forms (Fig. 1) and are administered orally as racemic mixtures in equal proportion [3]. The enantiomers significantly differ in their interaction with serum albumin, tissue distribution, pharmacokinetic and pharmacodynamic properties. It is found that two enantiomers of warfarin undergo different metabolic pathways in a complex manner involving stereoselective and regioselective cytochrome P450 (CYP)-dependent oxidation to produce hydroxylated metabolites [1]. Stereoselective metabolism of warfarin in human results that (S)-warfarin, which has 3–5 times greater anticoagulant potency than its (R)-congener [4], is metabolized more rapidly than the R-isomer [5]. Warfarin is metabolized by the liver, trace amount (∼ng/mL) of warfarin and many of their metabolites can be found in plasma and urine after oral administration. Therefore, it is useful to develop a simple analytical method that could determine the warfarin enantiomers and/or their related metabolites in biological fluids, such as plasma and urine.

Enantiomeric separation and measurement of warfarin and their metabolites in human plasma and urine have been mainly achieved using high performance liquid chromatography (HPLC) with various chiral columns and detected by UV [6] or UV/fluorescence system coupled with on-line circular dichroism detector [7]. To improve the sensitivity, the chiral liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods were developed for the enantioseparation of warfarin [8], and their metabolites [9] in human plasma.

Capillary electrophoresis (CE) has been demonstrated to be a powerful alternative to HPLC for the chiral separation of various enantiomers (especially chiral drugs) with advantages such as high peak efficiency, reduced sample and chiral reagents consumptions,

*Abbreviations:* CE, capillary electrophoresis; DM-β-CD, dimethyl-beta-cyclodextrin; HPLC, high-performance liquid chromatography; MS, mass spectrometry; LLE, liquid–liquid extraction.

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doi:10.1016/j.chroma.2011.04.067

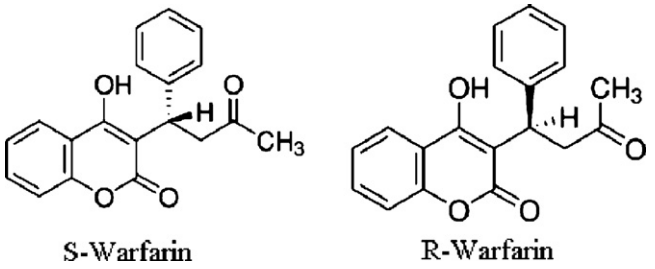


**Fig. 1.** Chemical structures of warfarin enantiomers.

and increased versatility (i.e., in contrast to HPLC with chiral stationary phases, a diverse number and different concentrations of chiral selectors can be conveniently dissolved in the CE run buffer to optimize enantioselective separations) [10].

Enantioseparation of warfarin in CE has been demonstrated by employing a variety of chiral selectors, such as human serum albumin [11], maltodextrins [12], polymeric surfactant [13], ionic liquids [14], and β-cyclodextrin (β-CD) derivatives [15–21]. As reported by Gareil et al. [15], warfarin forms inclusion complexes with β-CD like many other aromatic compounds. Up to now, a variety of β-CD derivatives, including uncharged CDs, like methyl-β-CD (Me-β-CD) [15,16], dimethyl-β-CD (DM-β-CD) [17] and 2-hydroxypropyl-β-CD (2-HP-β-CD) [16], as well as anionic CDs, like sulfobutyl ether β-CD (SBE-β-CD) [18], highly sulfated β-CD (HS-β-CD) [16,19,20], and novel single-isomer 6-monodeoxy-6-mono (hydroxy) alkylamino-β-CDs (mainly used in nonaqueous media) [21] were employed in chiral separation of warfarin.

However, the relatively poor concentration sensitivity of CE when using UV detection due to the limited sample volume and the short optical path length, limits CE application for sensitive detection of trace levels of these anti-coagulant drugs in biological fluids. Mass spectrometry (MS) offers superior selectivity and sensitivity. Capillary electrochromatography (CEC) [22] and micellar electrokinetic chromatography (MEKC) [13] coupling with electrospray ionization mass spectrometry (ESI-MS) have been developed for assay of warfarin enantiomers in human plasma. As an alternative to the use of sensitive detector for improving detection sensitivity in CE, the employment of various on-line sample preconcentration techniques, such as field-amplified sample stacking and sweeping, has been shown to be attractive in terms of costs and enrichment factors [23].

Salting-out extraction, which is a homogeneous liquid–liquid extraction method, has received consideration attention by bioanalysts. The extraction involved a simple one-step solvent extraction of analytes from biological samples (i.e., whole blood, plasma and urine) followed by salting-out the water-miscible organic solvent by adding a huge amount of salt, like magnesium sulfate [24], ammonium sulfate [25], sodium chloride [26], calcium chloride, potassium carbonate, calcium sulfate and magnesium sulfate [27]. The use of organic solvents such as acetonitrile to induce protein precipitation is a simple and effective approach routinely employed in clinical/biomedical laboratories for sample clean-up. Salting-out extraction, which coupling sample clean-up (e.g., acetonitrile deproteinization) with enrichment (via salting-out extraction), is an effective preparation approach for biological sample, and has been widely applied in HPLC [25–27] and LC/MS analysis [24]. However, there are only few reports concerning its use for the clean-up and the subsequent concentration of biological samples prior to CE analysis [28,29], especially CE on-line concentration [30], as the high salt and organic solvent in the matrix may break down the CE stacking of analytes. Shihabi reported a method termed transient "pseudo-ITP" (better known as "acetonitrile stacking") for sample concentration in CE, to address the problem of intolerance to organic solvent and high saline samples [31,32]. Moreover, it has demonstrated that the use of an acetonitrile–salt mixtures in the sample matrix ("acetonitrile stacking") and a chiral surfactant (bile salt) in the run buffer was effective in providing on-line sample enrichment and enantioseparation of enantiomers in CE (in the mode of miceller electrokinetic chromatography) [33].

In the present work, the feasibility and advantages of coupling of acetonitrile deproteinization and salting-out extraction with acetonitrile stacking for biological sample clean-up and enrichment in CE for enantioseparations (using warfarin enantiomers as model analytes) were demonstrated by employing DM-β-CD as the chiral selector and using UV-absorbance for detection. Salting-out extraction efficiencies of warfarin were evaluated as a function of pH, and enrichment factors obtained from the combined enrichment effect of salting-out extraction and acetonitrile stacking were determined and optimized. The usefulness and capabilities of this novel approach, in terms of chiral separation capabilities and the enhanced detection of trace amounts of warfarin enantiomers present in biological fluids, was demonstrated for the CE determination of warfarin enantiomers in urine samples. Analytical figures of merit for urinary warfarin enantiomers in terms of resolution, precision, limits of detection and linearity of the calibration curves were determined.

## 2. Materials and methods

### 2.1. Chemicals

Racemic warfarin and (S)-(+)-6-methoxy-alpha-methyl-2-naphthalene acetic acid were purchased from Aldrich (Milwaukee, WI, USA). Sodium tetraborate, sodium phosphate, sodium chloride, phosphoric acid, and dimethyl-beta-cyclodextrin (DM-β-CD) were obtained from Acros (NJ, USA). Acetonitrile (HPLC grade) were purchased from Lab Scan (Patumwan, Bangkok, Thailand).

### 2.2. Apparatus

All CE separations were carried out using a Beckman P/ACE$^{TM}$ MDQ CE system (Beckman coulter, Fullerton, CA) equipped with a photodiode array detection system. Data were acquired and processed on an IBM model computer using 32 Karat software (Version 4.01, Beckman Coulter). Uncoated fused-silica capillaries (60 cm × 50 μm ID, with an effective length of 50 cm) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The detector wavelength was set at 306 nm and capillary temperature was maintained at 25 °C. All electrophoretic separations were carried out with an applied voltage of 15 kV under normal polarity.

New capillary was conditioned firstly with methanol for 5 min, followed by water for 2 min, and then flushing with 0.1 M NaOH and water for 20 and 10 min, respectively. Prior to start of daily experiments, the capillary was rinsed with 0.1 M NaOH for 15 min, water for 10 min, and separation buffer for 10 min. Between each run, the capillary was rinsed with 0.1 M NaOH for 2 min, water for 2 min, and separation buffer for 2 min.

### 2.3. Preparation of run buffer and sample solutions

The electrophoretic run buffer contained 50 mM sodium tetraborate and 5 mM DM-β-CD, and its pH was adjusted to 8.2 via the addition of 1 M phosphoric acid. The stock solution was prepared by dissolving appropriate amounts of racemic warfarin in acetonitrile to obtain a final concentration of 1000 mg/L. (S)-(+)-6-methoxy-alpha-methyl-2-naphthalene acetic acid was used as the internal standard (with the stock solution prepared at 1000 mg/L in acetonitrile). Sample solutions were prepared by diluting the stock solution of warfarin and internal standard with doubly distilled deionized

water or acetonitrile. All run buffer and sample solutions were filtered through a 0.45 μm membrane filter. The urine sample was obtained by spiking blank urine from a healthy female in our laboratory with the appropriate amount of racemic warfarin and internal standard.

### 2.4. Salting-out solvent extraction procedures

The salting-out solution (5 M NaCl) were prepared by dissolving appropriate amounts of sodium chloride in 100 mM phosphate buffer, and the pH of the salting-out solution was adjusted by mixing with 100 mM phosphoric acid and 100 mM sodium phosphate (both of which prepared in 5 M NaCl) at appropriate volume ratios. To achieve an enrichment factor of ca. 10 based on salting-out extraction, 10 mL of the standard sample solution was mixed with 10 mL of acetonitrile in a 50 mL burette. Afterward, 30 mL of the salting-out solution was added into the burette and the solution mixture was shaken for 5 min to achieve phase separation. To achieve an enrichment factor 100 or higher, the above salting-out extraction procedures were modified by increasing the initial volume of both the standard solution and the acetonitrile from 10 to 25 mL. After the addition of the salting-out solution (65 mL) to induce phase separation (in a funnel), the upper acetonitrile phase was transferred into the burette mixed with the salting-out solution for the second time (at a volume ratio of 1:3 to 1:5).

For the acetonitrile deproteinization and salting-out extraction of real biological samples (urine), appropriate amounts of warfarin enantiomers and internal standard were spiked into 25 mL of urine, and then vortex mixed with 25 mL acetonitrile for 5 min. The mixture was centrifuged for 10 min at 6000 rpm. About 50 mL of the supernatant sample solution was mixed with 65 mL salting-out solution (pH 4.0) to induce phase separation. The next procedure was the same as that for standard solution.

After salting-out extraction, the upper acetonitrile phase was mixed with 1% NaCl solution in a ratio of 2:1 (v/v) prior to injection into CE system.

### 2.5. Extraction efficiency

10 mL of warfarin standard solution (1 mg/L) was mixed with 10 mL of acetonitrile in a 50 mL burette. Then, the salting-out solution at different pH was added to achieve phase separation. The volume of upper acetonitrile phase and the lower aqueous phase were obtained from the burette. The concentrations of warfarin in each phase were determined using UV–Visible spectrophotometry (UV 3010, Hitachi, Japan) with detection wavelength set at 306 nm. The extraction efficiency was calculated by the percentage of warfarin amount in upper acetonitrile phase.

### 2.6. Assay validation

#### 2.6.1. Calibration

Calibration standards were prepared by spiking blank urine with the appropriate amount of racemic warfarin and internal standard solution to yield the final concentrations of 10, 20, 50, 100, 250, 500 and 1000 ng/ml for warfarin, and 200 ng/ml for internal standard. All calibration standards were prepared in triplicate and followed the same extraction procedure as stated above. Calibration curves were plotted by the peak-area ratios of each warfarin enantiomers/internal standard versus concentrations of enantiomers in urine.

#### 2.6.2. Recovery

The recovery was determined in triplicate at four different concentrations (20, 50, 100 and 500 ng/ml for warfarin, and 200 ng/ml for internal standard) by comparing the spiked urine samples to the standard samples containing the equivalent amount of racemic warfarin. All of the solutions were purified and preconcentrated according to the same salting-out procedure.

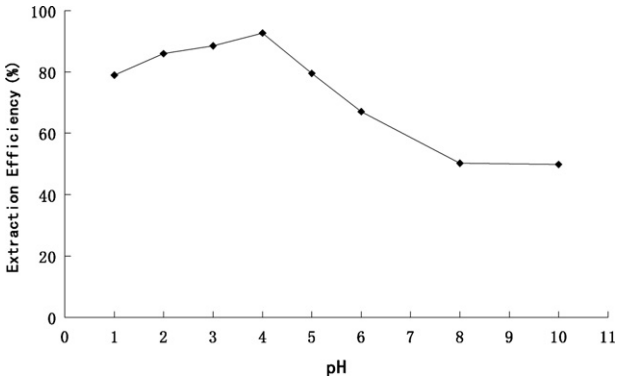


**Fig. 2.** Plot of salting-out extraction efficiency (*E*%) of racemic warfarin as a function of pH. *E*% was calculated by determining the concentrations of warfarin in upper acetonitrile phase relative to the lower aqueous phase upon salting-out extraction using UV–Visible spectrophotometry (detection wavelength set at 306 nm). The original concentration of warfarin in the sample solution was 1 mg/L. Each data point was determined based on an average of three salting-out extractions.

#### 2.6.3. Intra- and inter-day precision

Intra- and inter-day precision were defined as the relative standard deviation (RSD), in terms of peak area and migration time. Intra- and inter-day precision was calculated from spiked urine samples at concentrations of 20, 50, 100 and 500 ng/ml for warfarin, and 200 ng/ml for internal standard. For each of the four spiked concentrations, three separate salting-out extractions and total of 9 consecutive injections were performed. Inter-day precision was determined in two separated days, and the samples employed for day 2 were the same as those for day 1 (stored in capped sample vials over 24 h).

## 3. Results and discussion

### 3.1. Enrichment by salting-out extraction

Salting-out extraction is a classic homogeneous liquid–liquid extraction (LLE) method in which the addition of an appropriate salting-out agent (e.g., NaCl) into the sample solution – composed of a mixture of a water-miscible organic solvent (such as acetotnitrile or acetone) and water – induces the phase separation of the organic solvent from the bulk aqueous phase [34]. Since the organic solvent is initially water-soluble until the ionic strength of the sample solution is increased, there is initially no interface separating the extraction solvent from the sample molecules in salting-out extraction. And thus, when compared to conventional LLE, major advantages of salting-out extraction are that large volumes of non-polar organic solvents and vigorous mechanical shaking are not required to obtain good extraction efficiencies, and evaporation/reconstitution of the sample solvent are not needed to achieve high enrichment factors.

Fig. 2 shows the variation of salting-out extraction efficiency (*E*%) of racemic warfarin as a function of pH. The data indicated that *E*% increased gradually as the pH of the sample solution was increased from 1 to 4, with the *E*% reaching a maximum value of ca. 92.7% at pH 4.0. The *E*%, however, dropped off rapidly as the pH exceeded 4.0, e.g., *E*% falling below 60% between pH 7 and 10. These observations are consistent with the p$K_a$ values reported for warfarin, which has a value of ca. 5.0 in aqueous solutions [35], i.e., warfarin is unionized (neutral) at pH ≤4.0, partly ionized (anionic) at pH 5.0, and completely ionized at pH >7.0. Thus, for the efficient

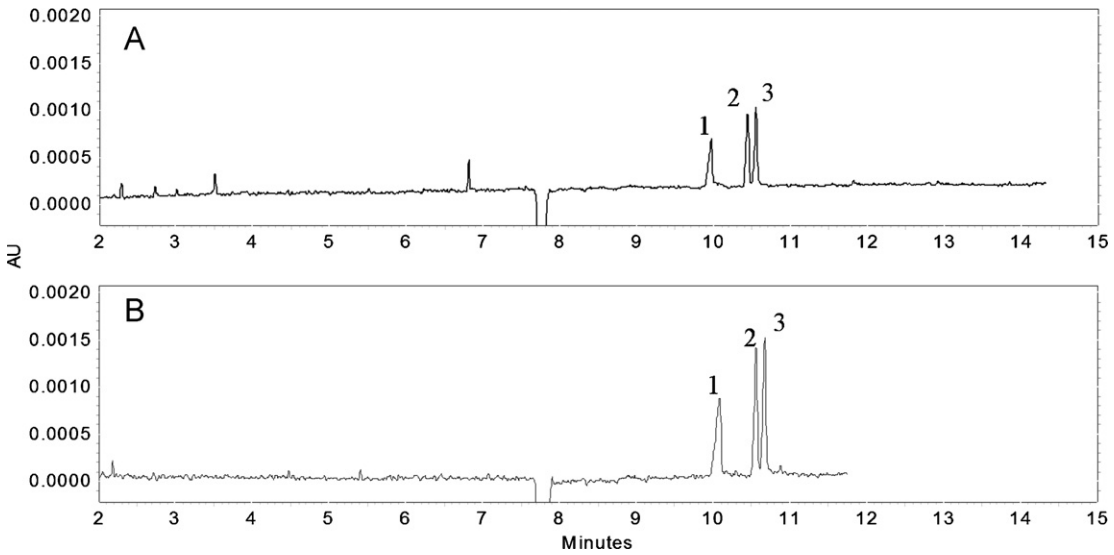


**Fig. 3.** Enantioseparation of racemic warfarin at a concentration of: (A) 100 mg/L without sample enrichment and (B) 10 mg/L with sample enrichment due to salting-out extraction at pH 4.0 (10 mL standard sample solution → 0.8 mL acetonitrile). The run buffer contained 50 mM sodium tetraborate and 5 mM DM-β-CD adjusted to pH 8.2. Pressure injection = 0.5 psi for 5 s and separation voltage = 15 kV. Peak assignments: 1 = internal standard: (A) 200 mg/L and (B) 20 mg/L, 2 = (S)-warfarin, and 3 = (R)-warfarin.

extraction of warfarin enantiomers into the smaller volume organic solvent (acetonitrile) phase in subsequent experiments, the sample solution was adjusted to a pH value of 4.0 to achieve optimal *E*% for the salting-out extraction of the warfarin enantiomers in their neutral, unionized forms.

Fig. 3A shows the enantioseparation of a racemic mixture of warfarin enantiomers (100 mg/L) using DM-β-CD as the chiral selector in the run buffer. The sample solution, with the solvent comprised acetonitrile, and was loaded directly into the capillary by pressure injection (with an injection volume of ca. 0.5% capillary length), i.e., without the effect of sample enrichment by salting-out extraction. Note that it has been previously demonstrated that warfarin (S)-enantiomer migrated ahead of the (R)-enantiomer in uncoated fused silica capillaries, with baseline resolution achievable under alkaline conditions using DM-β-CD as the chiral selector [17]. Thus, under the present experimental conditions, the warfarin enantiomers (existed as anions in the CE run buffer at pH 8.2) were able to form inclusion complexes with the chiral selector (DM-β-CD) and exhibited sufficient differences in formation constants to allow for good separation resolution to be obtained for the (S)- and (R)-warfarin enantiomers, which appeared at ca. 10.3 and 10.5 min, respectively, as shown in Fig. 3A.

Fig. 3B shows the electropherogram of the warfarin enantiomers (10 mg/L) at a concentration 10 times less than that used in Fig. 3A (100 mg/L). The sample was enriched due to the salting-out extraction of the warfarin enantiomers from the bulk aqueous

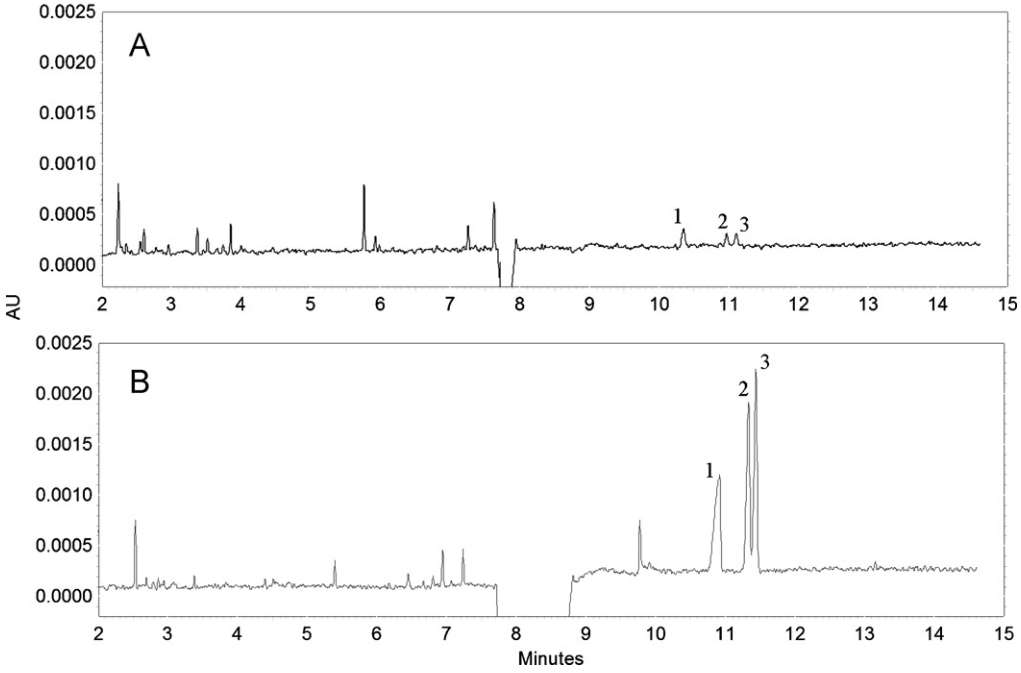


**Fig. 4.** Enantioseparation of racemic warfarin (10 mg/L) with sample enrichment due to acetonitrile stacking: (A) pressure injection: 0.5 psi for 9 s (injected amount = 1% capillary volume) and (B) pressure injection: 0.5 psi for 88 s (injected amount = 10% capillary volume). The sample solution was mixed with 1% NaCl at a ratio of 2:1 (v/v) prior to injection. The concentration of the internal standard was 20 mg/L. Other conditions were the same as those described in Fig. 3.

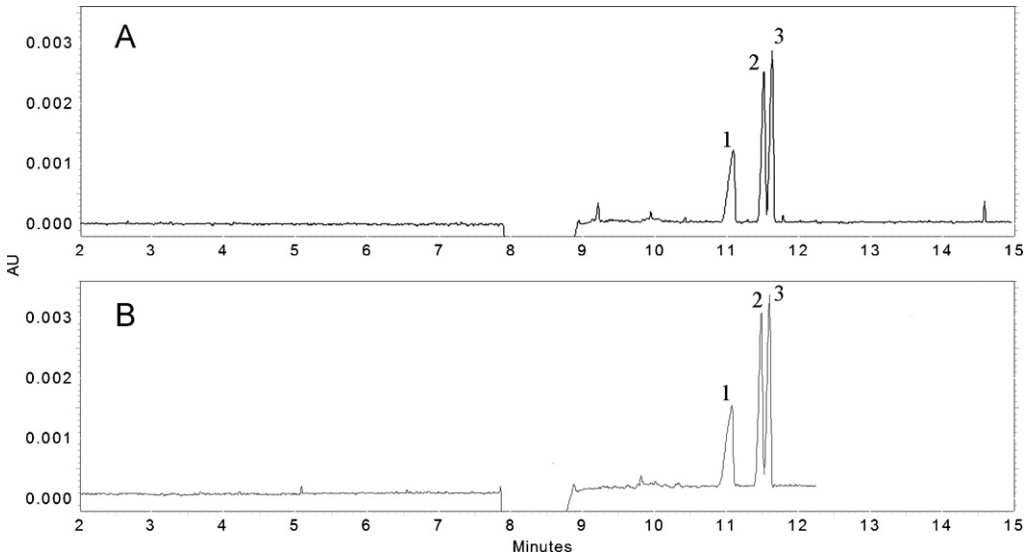


**Fig. 5.** Enantioseparation with sample enrichment due to the combination of salting-out extraction and acetonitrile stacking of racemic warfarin at a concentration of: (A) 1 mg/L. Salting-out extraction: 10 mL sample solution → 0.8 mL acetonitrile and (B) 0.1 mg/L. Salting-out extraction: 25 mL sample solution → 0.15 mL acetonitrile. For (A) and (B) pressure injection at 0.5 psi for 88 s was used to provide an injected amount of 10% capillary volume. The enriched acetonitrile phase was mixed with 1% NaCl at a ratio of 2:1 (v/v) prior to injection. The concentration of the internal standard in (A) and (B) was 2 mg/L and 0.2 mg/L, respectively. Other conditions were the same as those described in Fig. 3.

sample solution (10 mL) into the smaller volume acetonitrile phase (0.8 mL), i.e., allowing for an enrichment factor of ca. 12 to be obtained. By comparing the peak heights of the internal standard, and the (S)- and (R)-warfarin enantiomers between Fig. 3A and B, it can be determined that an experimental enrichment factor of slightly more than 10 can indeed be achieved for the warfarin enantiomers due to salting-out extraction, i.e., similar peak heights and near baseline resolution can be observed for the warfarin enantiomers at a concentration of 100 mg/L (no salting-out extraction) and 10 mg/L (with salting-out extraction). Enrichment factors larger than 10 can be obtained by adjusting the relative amounts of the water-miscible organic solvent (acetonitrile) in the aqueous sample solutions. In fact, an enrichment factor of 100 due to salting-out extraction alone can be readily achieved for the war-

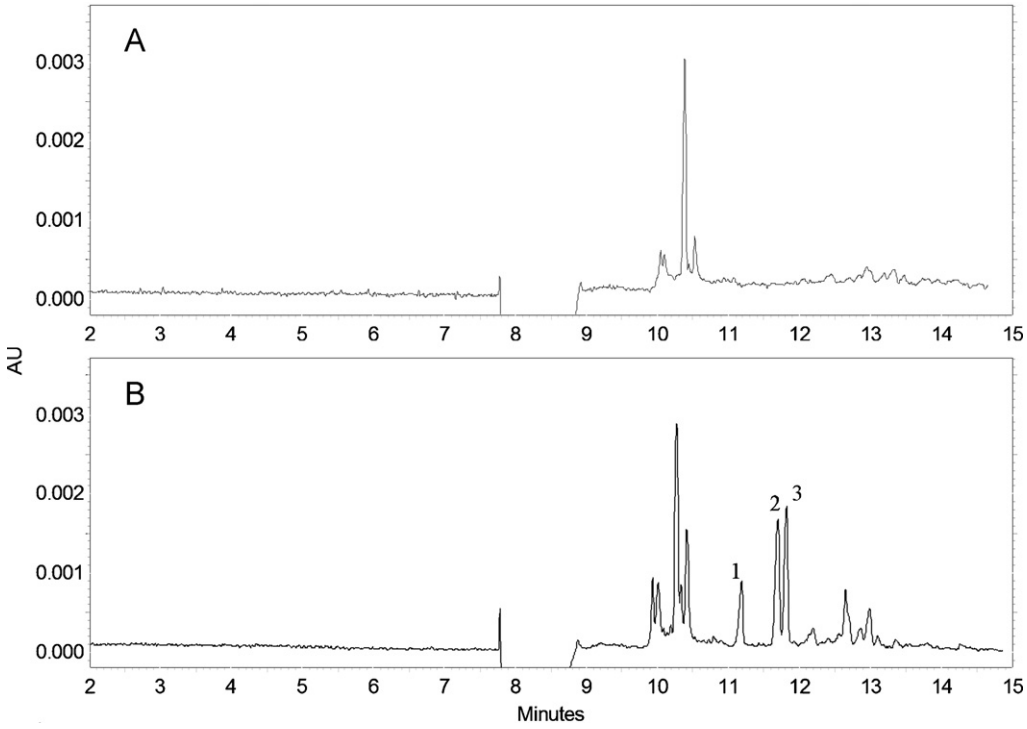


**Fig. 6.** Enantioseparation of racemic warfarin in urine with sample enrichment due to the combination of salting-out extraction and acetonitrile stacking. Urine sample: (A) without spiking and (B) spiked with 0.1 mg/L racemic warfarin and 0.2 mg/L internal standard. Salting-out extraction (pH 4.0): 25 mL urine sample → 0.3 mL acetonitrile at an extraction. The enriched acetonitrile phase was mixed with 1% NaCl at a ratio of 2:1 (v/v) prior to pressure injection (0.5 psi for 88 s yielding an injected amount of 10% capillary volume). Other conditions same as those described in Fig. 3.

**Table 1**
Intra-day and inter-day precision of peak area and migration time for the determination of (S)- and (R)-warfarin spiked into urine.[a]

| Concentration (ng/mL) | RSD (%)[b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day 1 ($N$ = 9) | | | | Day 2 ($N$ = 9) | | | | Inter-day ($N$ = 9) | | | |
| | Area | | Time | | Area | | Time | | Area | | Time | |
| | (S)- | (R)- | (S)- | (R)- | (S)- | (R)- | (S)- | (R)- | (S)- | (R)- | (S)- | (R)- |
| 20 | 1.0 | 1.3 | 0.1 | 0.1 | 2.8 | 2.0 | 0.4 | 0.5 | 6.3 | 1.8 | 0.6 | 0.8 |
| 50 | 1.1 | 1.8 | 0.3 | 0.3 | 2.4 | 5.0 | 0.3 | 0.4 | 2.1 | 3.7 | 0.4 | 0.5 |
| 100 | 2.9 | 3.8 | 0.1 | 0.1 | 2.4 | 4.9 | 0.1 | 0.1 | 2.8 | 4.7 | 0.1 | 0.1 |
| 500 | 4.0 | 5.7 | 0.1 | 0.2 | 4.4 | 6.7 | 0.1 | 0.3 | 4.9 | 6.2 | 0.1 | 0.3 |

[a] Salting-out extraction and acetonitrile stacking conditions were the same as those described in Fig. 6. 0.2 mg/L (S)-(+)-6-methoxy-alpha-methyl-2-naphthalene acetic acid was spiked into the urine samples as an internal standard.
[b] Intra-day and inter-day precision was calculated from samples obtained from three separate salting-out extractions and based on a total of 9 consecutive injections for each of the four spiked concentrations. The samples employed for day 2 were the same as those for day 1 (stored in capped sample vials over 24 h).

farin enantiomers, and a combined enrichment factor of 1000 or higher can be obtained by coupling with acetonitrile stacking (*vide infra*).

### 3.2. Enrichment by acetonitrile stacking

Compared to other on-line sample enrichment techniques, acetonitrile stacking is unique in that the presence of a relatively large amounts of salts in the sample matrix (which normally causes electrodispersion and band broadening in conventional CE stacking methods) actually aids in analyte narrowing/sharpening within the sample zone, and the co-presence of acetonitrile in the sample matrix can be conveniently used for the precipitation of endogenous proteins in biological samples, prior to injection of the samples into CE system [31]. The mechanism of transient pseudo-isotachophoresis has been proposed for acetonitrile stacking, in which fast moving anions (e.g., chloride) in the salts serve as leading ions, whereas water-miscible organic solvents (e.g., acetonitrile) act as the slower moving "terminating ions" [32].

Fig. 4A shows the enantioseparation of (S)- and (R)-warfarin enantiomers (ca. 10 mg/L each) due to the injection of a sample solution consisting of a mixture of acetonitrile and 1% NaCl (2:1, v/v) and the injection amount of ca. 1% capillary volume. When the injection amount was increased from ca. 1% to 10% capillary volume (without changing the chemical composition of the sample solution), Fig. 4B shows that the peak heights of the warfarin enantiomers were increased by ca. 10–15 times, indicating that the use of acetonitrile stacking was able to provide an enrichment factor for the warfarin enantiomers of 10 folds or higher (while maintaining near baseline resolution). Higher enrichment factors can be achieved by increasing the injection volume (e.g. 20–30% capillary volumes); however, this could be accompanied by a marked decrease in enantiomeric resolution.

### 3.3. Enrichment by coupling salting-out extraction with acetonitrile stacking

Fig. 5 shows the enantioseparation of racemic warfarin with sample enrichment due to the combined effect of salting-out extraction and acetonitrile stacking. With an enrichment factor of ca. 12 due to salting-out extraction of 1 mg/L of racemic warfarin (10 mL sample solution → 0.8 mL acetonitrile), Fig. 5A shows that the injection of the enriched warfarin (contained in a sample matrix of 2:1 mixture of acetonitrile and 1% NaCl) at 10% capillary volume resulted in peak heights for both warfarin enantiomers that were slightly higher than those as shown in Fig. 3A, i.e., warfarin enantiomers at a concentration of 100 mg/L (without the effect of salting-out extraction and acetonitrile stacking). These data show that a combined enrichment factor of more than 100 folds can be obtained for the enantiomeric separation of warfarin (with near baseline resolution) by coupling salting-out extraction with acetonitrile stacking. Similarly, Fig. 5B shows that with an enrichment factor of ca. 165 due to salting-out extraction (25 mL sample solution → 0.15 mL acetonitrile), the peak heights obtained for 0.1 mg/L of warfarin enantiomers at 10% injection volume were somewhat higher than those at 100 mg/L (Fig. 3A), indicating that a combined enrichment factor higher than 1000 can be obtained for the enantioselective separation of warfarin enantiomers (with near baseline resolution) by coupling salting-out extraction with acetonitrile stacking.

### 3.4. Determination of warfarin enantiomers in urine

Although direct sample injection analysis of biological fluids has been demonstrated to be feasible in CE [36], some types of sample clean-up procedures (e.g., to reduce protein adsorption onto capillary surfaces) are necessary to ensure predictable and reproducible results, especially under conditions when relatively large volumes of biological fluids (including urine) are injected into the CE capillary to allow for on-line sample concentration.

Fig. 6A shows an electropherogram of a urine sample (blank) from a healthy female, based on the addition of acetonitrile into the sample to effect deproteinization and the combined use of salting-out extraction and acetonitrile stacking to effect analyte concentration. The combined enrichment factor due to salting-out extraction (25 mL urine → 0.3 mL acetonitrile) and acetonitrile stacking (at 10% injection volume) was expected to be ca. 1000 folds (as demonstrated for standard sample solutions). The results indicated that no detectable peaks were observable between 11.5 and 11.8 min, where the warfarin enantiomers were found to appear in the electropherogram for standard sample solutions under the same separation and detection conditions (Fig. 5B). On the other hand, using the same experimental conditions as in Fig. 6A except that the urine sample was spiked with the warfarin enantiomers (0.1 mg/L) and an internal standard, Fig. 6B shows that two near-baseline resolved peaks, corresponding to (S)- and (R)-warfarin, appeared at 11.6 and 11.8 min, respectively, and their peak heights were somewhat higher than those at 100 mg/L (without sample enrichment as shown in Fig. 3A).

To demonstrate how the sensitivity can be enhanced using salting-out solvent extraction or/and acetonitrile stacking technique in urine sample, limit of detections (LODs) of three methods, including conventional CE, salting-out extraction in conventional CE, and method based on the coupling of salting-out extraction with acetonitrile stacking, were estimated based on a signal to noise ratio of 3:1. For conventional CE analysis, spiked urine sample was mixed with acetonitrile in a ratio of 1:1 (v/v) for deproteinization, then the supernatant sample solution was directly injected into CE (injected amount = 1% capillary volume). The LOD of warfarin showed as ca. 3.2 μg/mL ($n$ = 3). With salting-out extraction (25 mL

**Table 2**
Mean recovery of (S)- and (R)-warfarin spiked into human urine.

| | Concentration (ng/mL) | | | |
|---|---|---|---|---|
| | 20 | 50 | 100 | 500 |
| S-warfarin (%) | 96 | 92 | 86 | 82 |
| R-warfarin (%) | 97 | 94 | 88 | 85 |

Salting-out extraction and acetonitrile stacking conditions were the same as those described in Fig. 6. 0.2 mg/L (S)-(+)-6-methoxy-alpha-methyl-2-naphthalene acetic acid was spiked into the urine samples as an internal standard. Three separate salting-out extractions and based on a total of 9 consecutive injections were preformed for each sample.

urine → 0.3 mL acetonitrile), the LOD, shown as 21 ng/mL, was more than 100 times better than that without extraction. After coupling with acetonitrile stacking, the LOD was lowered to 1.5 ng/mL. These results demonstrated that a combined enrichment factor of more than 1000 folds can be obtained for the real sample (urine) analysis of warfarin enantiomers with good separation resolution by coupling salting-out extraction with acetonitrile stacking.

As shown in Table 1, the intra- and inter-day precision in terms of peak area obtained for two separate days was found to be quite good for both warfarin enantiomers within the concentrations of 20–500 ng/mL, with RSD values falling in the range between 1.0% and 6.7% (based on 9 consecutive injections). On the other hand, the intra- and inter-day precision in terms of migration time all fell in the range between of 0.1% and 0.8% RSD within the same concentration range. Since a relatively large sample volume (10% capillary volume) was injected, the good precision in migration time obtained for both warfarin enantiomers in a urine matrix during different days indicated that the use of acetonitrile to induce deproteinization was able to minimize irreproducible results associated with the precipitation and/or adsorption of various proteins within the CE capillary.

Calibration curves obtained from the spiking of warfarin enantiomers into urine within the concentration range of 10–1000 ng/mL yielded the following two linear equations: (S)-warfarin, $y = 0.0234x + 0.260$, with $R^2 = 0.9981$; (R)-warfarin, $y = 0.0183x + 0.231$, with $R^2 = 0.9963$. The recovery of warfarin enantiomers from urine was ca. 90% (Table 2).

## 4. Conclusion

Acetonitrile deproteinization is a common and effective sample pretreatment approach in many research and routine laboratories. Thus, the ability to enhance the concentration detection sensitivity via the addition of an appropriate salt to induce salting-out extraction after acetonitrile deproteinization should be advantageous for sensitive chiral separations of various enantiomers using CE, especially when additional enhancement in concentration detection sensitivity can be achieved by coupling salting-out extraction with acetonitrile stacking. In future work, various enantiomers enriched within the acetonitrile phase (as a result of salting-out extraction) could be injected directly into the capillary for further analyte concentration via the use of on-line enrichment techniques other than acetonitrile stacking, such as large volume sample stacking (LVSS) or sweeping. Also, the present approach should also be applicable for the CE analysis of other biological fluids such as serum and plasma, which contain much higher concentrations of proteins when compared to urine. To this end, the salting-out extraction procedures have to be further improved, especially in terms of reducing the sample volume requirement (e.g., from 25 mL for urine to 1 mL for serum or plasma samples) while maintaining relatively high overall enrichment factors and separation resolution by combining salting-out extraction with acetonitrile stacking or other on-line sample concentration techniques.

## Acknowledgement

The authors wish to thank the financial support of Natural Science Foundation for the Youth, South China University of Technology (x2hgE5090520).

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