

Enantiomeric separation and quantitation of warfarin and its metabolites in human plasma by LC-MS/MS

Ahmed Abd-Alazim Mostafa^{1,2,*}

¹ Pharmaceutical Chemistry Department, Faculty of Pharmacy, Helwan University, Egypt, Ein Helwan, 11795, Cairo, Egypt

² Therapeutics Research Centre, School of Medicine, The University of Queensland, Princess Alexandra Hospital, Woolloongabba, QLD, 4102, Australia

* Corresponding author at: Pharmaceutical Chemistry Department, Faculty of Pharmacy, Helwan University, Egypt, Ein Helwan, 11795, Cairo, Egypt. Tel.: +202.3.7836552. Fax: +202.2.5541601. E-mail address: ahmed34@yahoo.com (A.A. Mostafa).

ARTICLE INFORMATION



DOI: 10.5155/eurjchem.7.3.357-362.1470

Received: 18 June 2016

Received in revised form: 12 July 2016

Accepted: 14 July 2016

Published online: 30 September 2016

Printed: 30 September 2016

KEYWORDS

Warfarin
 LC-MS/MS
 Metabolites
 Enantiomers
 Chiral chromatography
 Enantiomeric separation

ABSTRACT

The enantiomeric separation of warfarin (WAR) enantiomers and its hydroxy metabolites positional isomers is described in this work. The utilization of chiral chromatography coupled to Tandem mass spectrometry helps to achieve that. The developed method was able to separate *R,S*-warfarin and enantiomers of 4,7,10-hydroxy warfarin in human plasma. Plasma samples were processed with simple protein precipitation with acetonitrile. The chromatographic separation was done on chiral Astec Chirobiotic V column with gradient flow of 0.1% aqueous formic acid and 0.1% formic in acetonitrile:water (95:5, v:v). The mass spectroscopic detection was done via negative mode multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer coupled with positive electrospray ionization (ESI). Linearity of the method was valid over a concentration range of 125.00-0.13 µg/mL for racemic WAR, 47.000-0.088 µg/mL for racemic 4'-OH-WAR, 56.00-0.18 µg/mL for racemic 7-OH-WAR and 56.00-0.02 µg/mL for racemic 10-OH-WAR. The LLOQs were found to be 0.13 µg/mL for racemic WAR, 0.088 µg/mL for racemic 4'-OH-WAR, 0.18 µg/mL for racemic 7-OH-WAR and 0.02 µg/mL for racemic 10-OH-WAR. The method was validated for matrix effect, intra- and inter-day precision, freeze/thaw and storage stability. Accuracy and precision were within acceptable range (<15%). Enantiomeric forms of WAR and its metabolites were separated by the chiral column. Standards for *R*- and *S*-WAR identified peak 2 as *S*-WAR while metabolite peaks could not be definitively identified. Peak 2 for 7-OH-WAR gave higher blood levels, while the opposite applied to 10-OH-WAR.

Cite this: *Eur. J. Chem.* 2016, 7(3), 357-362

1. Introduction

Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)chromen-2-one) is one of the most frequently prescribed anticoagulant (Figure 1). It was administrated orally in the form of racemic mixture *R*- and *S*-WAR as treatment and prophylaxis of Venous Thromboembolism [1-3]. *S*-WAR is 3-5 times potent than *R*-enantiomer, metabolised by P450 (CYP) 2C9 to form inactive *S*-7-hydroxy metabolite (7-OH-WAR) [4,5]. Genetic variation in CYP2C9 impact the clearance of *S*-WAR with increasing the risk of bleeding during induction period of treatment [6-8]. The metabolism of WAR show a regioselectivity for the products, for example *S*-WAR is metabolised to 7-OH-WAR as major product, however 4,6-OH-WAR were produced as minor product. On the other hand, *R*-WAR is metabolised to 4,6,7,8, 10-OH-WAR, however 6,10-OH-WAR are the major products [9,10]. WAR is widely used for treatment and prevention of thrombosis however, its uses always accompanied by bleeding.

Due to its narrow therapeutic index and genetic metabolic variations it is hard to reach a safe therapeutic action [11], it was strongly recommended to monitor WAR level in blood to insure that warfarin is working safely and effectively [12]. Many drugs have a chiral centre in their structures, producing

enantiomers, having different potencies and activities [13]. Because of enantiomers have the same physical and chemical properties; it cannot separate through achiral stationary phase. Chiral stationary phase plays an important role in enantiomers separation by chromatography [14]. The difference in potency between *R,S*-WAR has results stress to develop a sensitive, robust and selective method for separation and analysis of enantiomers of WAR and its metabolites in plasma. This obviously will facilitate drug monitoring and saving time and simplify the producers of urine sample collection [15]. There are many HPLC methods coupled with UV, fluorescence and mass detectors used for analysis of WAR and its metabolites. Although some of them used for chiral separation of warfarin and hydroxylated metabolites, they used non-specific detectors as UV and fluorescence [16-18]. Reported LC/MS methods for WAR were use either achiral separation or chiral separation with only limited consideration of metabolites [3,15,19]. The novelty of my method comes from it used for separation of enantiomers of WAR and three of its metabolites.

The main aim in this study is to develop and validate a simple and rapid method for the simultaneous quantitation of enantiomers of WAR and its metabolites in clinical samples.

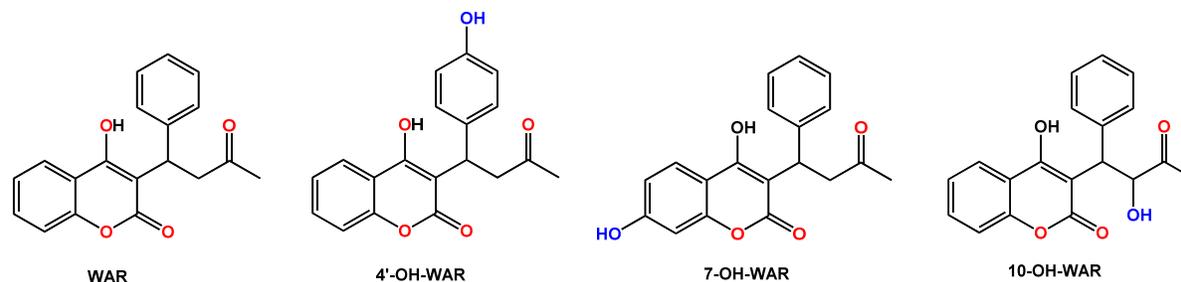


Figure 1. Chemical structures of the analytes: Warfarin (WAR), 4-hydroxy warfarin (4'-OH-WAR), 7-hydroxy warfarin (7-OH-WAR), 10-hydroxy warfarin (10-OH-WAR).

2. Experimental

2.1. Materials and standards

Warfarin, 4-hydroxy warfarin (4'-OH-WAR), 7-hydroxy warfarin (7-OH-WAR), 10-hydroxy warfarin (10-OH-WAR) and 4-hydroxy nitro phenol (IS) were purchased from Sigma (Germany). All solvents were HPLC grade and were obtained from Merck (Kilsyth, Australia). Blank human plasma was kindly donated by the Australian Red Cross.

2.2. Equipment

The analysis was performed using an API 2000 (Applied Biosystems/MDS Analytical Technologies Inc., Foster City, CA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and a Valco diverter valve. The HPLC system consisted of a Shimadzu SLC-10A VP system controller with three LC-10AD pumps and a SIL-20AC-HT autosampler operated at 4 °C.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was performed on a chiral Astec ChirobioticV column (100 × 4.6 mm, 5 μm particle size) with an Astec Cyclobond I guard column (20 × 4.0 mm, 5 μm). Gradient flow of a 5:95 mixture of 0.1% aqueous formic acid and acetonitrile elutes the analytes with good chromatographic separation. The negative-ion mass spectrometric detection method utilised electrospray ionization and the multiple reaction monitoring (MRM) mode. Optimisations of mass spectrometric conditions were done for each compound by continuously infusing a standard solution (1 μg/mL in mobile phase) at 10 μL/min. The optimized parameters were as follows: turbo ion spray temperature, 400 °C; ion spray voltage, 5000 V; declustering potential (DP), -60 V (WAR), -65 V (4'-OH-WAR), -40 V (7-OH-WAR), -50 V (10-OH-WAR) and -46 V 4-nitro phenol (IS); entrance potential (EP), -9 V (WAR), -9V (4-OH-WAR), -8 V (7-OH-WAR), -5 V (10-OH-WAR) and -12 V (IS); collision energy (CE), -25 V (WAR), -30 V (4'-OH-WAR), -30 V (7-OH-WAR), -30 V (10-OH-WAR) and -25 V (IS); collision cell entrance potential (CEP), -16.2 V (WAR), -16 V (4-OH-WAR), -16.6 V (7-OH-WAR), -16.6 V (10-OH-WAR) and -20.41 V (IS); collision cell exit potential (CXP), -14 V (WAR), -10 V (4-OH-WAR), -10 V (7-OH-WAR), -10 V (10-OH-WAR) and -10 V (IS). The MRM ion transition were 307 → 161.1 for WAR, 323 → 161.1 for 4'-OH-WAR, 323 → 177 for 7-OH-WAR, 323 → 250 for 10-OH-WAR and 137.9 → 107 for (IS). Applied Biosystems Analyst version 1.4.2 software was used to control the LC-MS/MS system, collect and analyse the data.

2.4. Preparation of stock and working solutions

Four 250 μg/mL stock solutions of each analyte WAR, 4'-OH-WAR, 7-OH-WAR and 10-OH-WAR were prepared by

dissolving each compound in phosphate buffer (100 mM, pH = 7.4). These were mixed with buffer to produce a combined stock at 125 μg/mL for WAR, 47 for 4'-OH-WAR, and 56 μg/mL for 7-OH-WAR and 10-OH-WAR. Quality control stock solutions at the same concentrations were prepared independently.

A 125 μg/mL stock solution of IS was prepared in methanol. The working solution of IS then was prepared by diluting an aliquot of stock solution with phosphate buffer (100 mM, pH = 7.4) to achieve the concentration of 62.5 ng/mL. All stock solutions were kept at -20 °C until use, whilst the working solutions were kept at 4 °C and discarded within 30 days.

2.5. Preparation of calibration standards and quality control (QC) samples

An upper limit of quantification (ULOQ) calibration standard was prepared by spiking the combined standard stock into pre-screen human plasma to 125 μg/mL for WAR, 47 μg/mL for 4'-OH-WAR, and 56 μg/mL for 7-OH-WAR and 10-OH-WAR. Eight further calibration standards were prepared by serial dilution with plasma to obtain concentration ranges of 125-0.13 μg/mL for WAR, 47.000-0.089 μg/mL for 4'-OH-WAR, 56.00-0.18 μg/mL for 7-OH-WAR and 56.00-0.02 μg/mL for 10-OH-WAR. Since the supplied standard are racemic the above standards concentration are divided by two to obtain the concentrations of each enantiomer.

Quality control samples were prepared by spiking blank plasma with the quality control stock solution to concentration of 100, 50 and 1 μg/mL for WAR and 40, 20 and 0.3 μg/mL for the other analytes. The calibration standards and quality control samples were stored at -70 °C before use.

2.6. Sample preparation

An aliquot (50 μL) of plasma to be tested was mixed with 50 μL IS working solution and 200 μL of acetonitrile were added to promote protein precipitation. After vortex-mixing for 10 s and centrifuging at 1000 × g for 10 min, approximately 200 μL of the supernatant was transferred into a HPLC vial and 5 μL was analysed by LCMS.

2.7. Method validation

2.7.1. Selectivity and matrix effect

To determine whether variation in the composition of human plasma would be likely to cause ion suppression or other matrix effect on the measured analyte levels, five different lots of blank human plasma were spiked with all analytes at both high and low concentration and assayed against a calibration set prepared in a sixth lot of plasma. The accuracy (% of normal concentration) and precision (%R.S.D.) are determined.

Table 1. Matrix effect results of WAR and its metabolites (n = 5).

Analyte	Nominal concentration (ng/mL)	Matrix effect (%)	%R.S.D.
Racemic WAR	0.26	96.2	8.10
	62.50	102.8	3.66
Racemic 4'-OH-WAR	0.17	110.5	6.70
	23.00	97.3	5.20
Racemic 7-OH-WAR	0.36	103.2	10.00
	28.00	103.8	5.60
Racemic 10-OH-WAR	0.04	104.2	9.20
	28.00	98.5	3.47

2.7.2. Linearity and lower limit of quantification (LLOQ)

The stock solutions are composed of racemic mixture from *R,S*-WAR and metabolites. Enantiomers are equimolar in racemic mixture. The linearity of the method was determined by analysing eight calibration standard samples at concentrations ranges of 62.500-0.065 µg/mL for enantiomers of WAR, 23.500-0.044 µg/mL for enantiomers of 4'-OH-WAR, 28.00-0.09 µg/mL for enantiomers of 7-OH-WAR and 28.00-0.01 µg/mL for enantiomers of 10-OH-WAR. The acceptable tolerance for accuracy and precision was 20% for LLOQ and 15% for other standard points. The calibration curve was constructed by least squares quadratic regression of the peak area ratios of each analyte to IS obtained against the corresponding concentrations using a weighting factor of 1/[Concentration]². The LLOQ was defined as the lowest concentration in the calibration curve with acceptable precision and accuracy.

2.7.3. Accuracy and precision

The intra-day accuracy and precision were evaluated by analysing five replicates of quality controls (QCs) at three concentration levels in a single batch using a freshly prepared calibration curve. Additional QC samples were also analysed on five different days in order to assess inter-day accuracy and precision. Precision was represented by percent relative standard deviations (%R.S.D.) while the accuracy was percentage of the calculated concentration.

2.7.4. Stability test

The post-preparative stability was determined by comparing the level found in freshly prepared samples to processed samples after 24 h in the autosampler at 4 °C. Short-term stability in plasma for 3 h (bench storage) was determined at ambient temperature (24±3 °C) at concentrations of QC samples. The stability was also tested after three freeze/thaw cycles using QC samples of three different concentration levels. The samples were stored at -80 °C between freeze/thaw cycles, and then they were thawed by allowing them to stand at room temperature for approximately 30 min. The samples were then returned to the -80 °C freezer for 24 h.

3. Results and discussion

3.1. LC-MS/MS optimization and sample preparation

During the development of the method, using of different achiral columns was not able to separate enantiomers, because it has the same physical and chemical properties especially same retention time. Using of chiral column, in which achiral silica gel (SiO₂) is converted into a chiral stationary phase by a reaction with a chiral molecule to form active chiral centre. Each enantiomer will bind to active chiral centre unequally and separation will be occurred. Astec Chirobiotic V column was used; it has active site with Vancomycin which contains 18 chiral centres surrounding three pockets or cavities. These allow good separation and resolution of enantiomers. In

comparison to other published method, the retention times of WAR and metabolites were short [19].

Different chromatographic conditions were investigated to optimize sensitivity, peak shape and separation. The use of water acetonitrile and formic acid as mobile phase was found to be preferable for analyte separation and ionization than the use of ammonium acetate.

Based on the chemical structures of the analytes, electrospray ionization operated at negative ion mode was used for LC-MS/MS analysis to provide optimum sensitivity and selectivity. Deprotonation of phenolic OH of each analyte ([M-H]⁻). This form was found to be dominant ions in the Q1 scan, and were used as the precursor ions to obtain Q3 product ion spectra. Multiple reaction monitoring (MRM) was used to decrease interference of matrix components [20,21]. The MRM ion transition were 307 → 161.1 for WAR, 323 → 161.1 for 4'-OH-WAR, 323 → 177 for 7-OH-WAR, 323 → 250 for 10-OH-WAR and 137.9 → 107 for IS in Figure 2.

3.2. Method validations

3.2.1. Selectivity and matrix effect

Six different lots of blank human plasma were checked for any false positive MS responses. No interferences from endogenous plasma substances were observed and a good separation of the analytes was achieved using the described LC-MS/MS conditions. As shown in Table 1, no obvious matrix effects were found for all the analytes as the results ranged from 96.2 to 110.5% which is within the acceptable limit.

3.2.2. Linearity and LLOQ

Calibration curves in spiked human plasma were linear over the range of 125.00-0.13 µg/mL for racemic WAR, 47.000-0.088 µg/mL for racemic 4'-OH-WAR, 56.00-0.18 µg/mL for racemic 7-OH-WAR and 56.00-0.02 µg/mL for racemic 10-OH-WAR. The linearity of standard curves (*r*²) for all analytes were greater than 0.99 using 1/*c*² weighting. For each point of calibration standards, the back calculated concentrations from the equation of calibration curves were within ±15% deviation. The calibration curve had a reliable reproducibility across the calibration range. The LLOQs were found to be 0.13 µg/mL for racemic WAR, 0.088 µg/mL for racemic 4'-OH-WAR, 0.18 µg/mL for racemic 7-OH-WAR and 0.02 µg/mL for racemic 10-OH-WAR, with acceptable accuracy 98.1% for racemic WAR, 96.7% for racemic 4'-OH-WAR, 98.9% for racemic 7-OH-WAR and 97.6% for racemic 10-OH-WAR and precision 1.70-6.78%.

3.2.3. Accuracy and precision

QC samples at three concentration levels (low, medium and high) with five replicates at each level were processed and analysed on the same day and also on five separate days to determine intra- and inter-day accuracy and precision for each analyte. As summarized in Table 2, the accuracy ranged from 90.1-106.5 for racemic WAR, 87.6-98.5 for racemic 4'-OH-WAR, 93.2-107.5 for racemic 7-OH-WAR and 91.1-107.4 for racemic 10-OH-WAR and the precision was within 11% for all analytes.

Table 2. Intra- and inter-day accuracy (% of nominal concentration) and precision (% RSD) of WAR and its metabolites in human plasma.

Analyte	Nominal concentration (µg/mL)	Intra-day (n=5)		Inter-day (n=15)	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Racemic WAR	0.5	99.2	10.04	100.8	6.94
	50	103.1	4.74	105.9	1.84
	100	106.5	3.64	90.1	3.74
Racemic 4'-OH-WAR	0.1	91.2	6.24	93	2.64
	20	87.6	9.84	106	3.44
	40	98.5	1.34	92.3	6.54
Racemic 7-OH-WAR	0.2	105.2	4.14	106.2	3.14
	22	96.6	7.94	110.4	1.34
	50	107.5	1.74	93.2	2.94
Racemic 10-OH-WAR	0.2	97.7	2.64	98.2	3.24
	22	91.1	8.64	107.4	6.34
	50	99.2	6.64	95.2	6.64

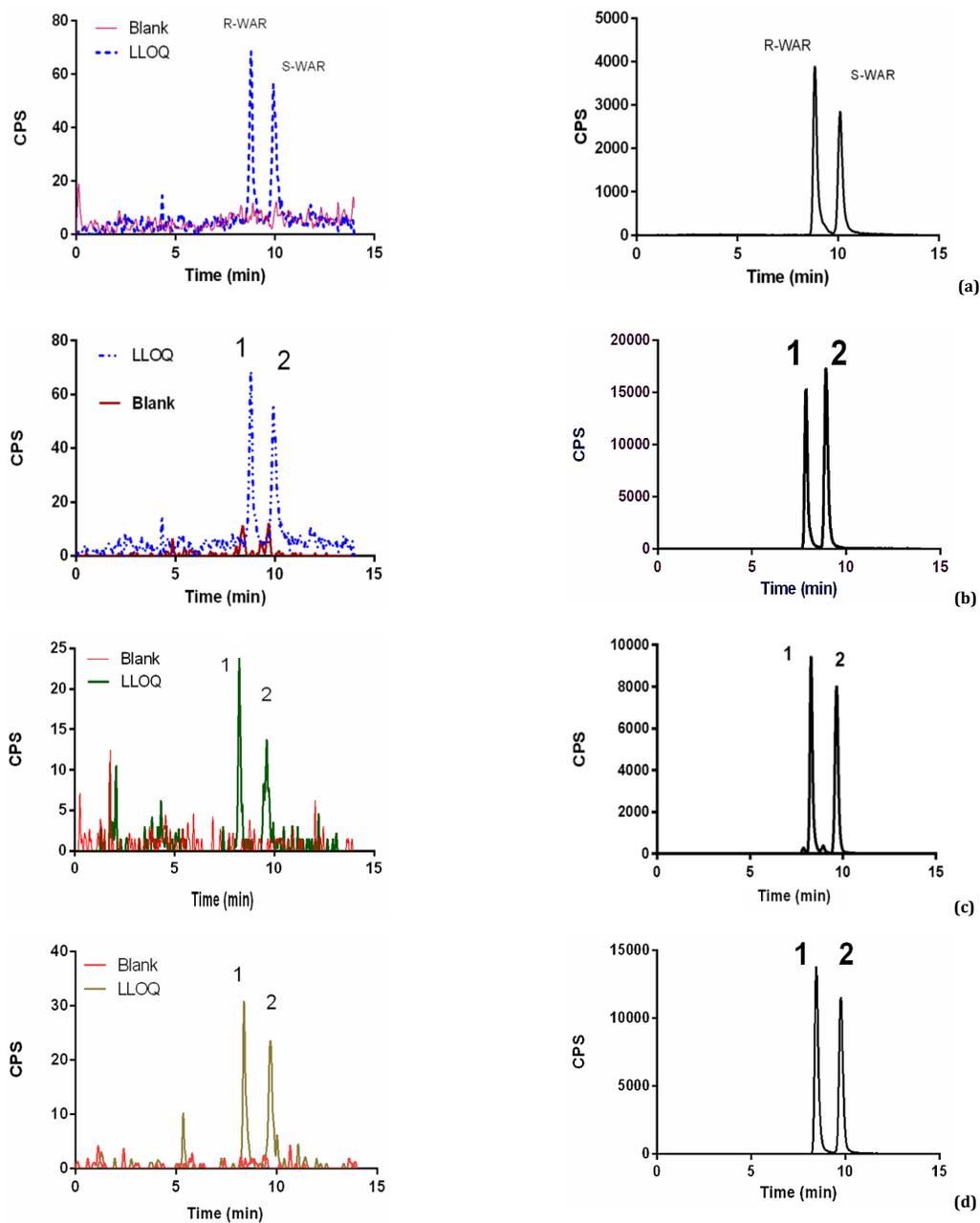
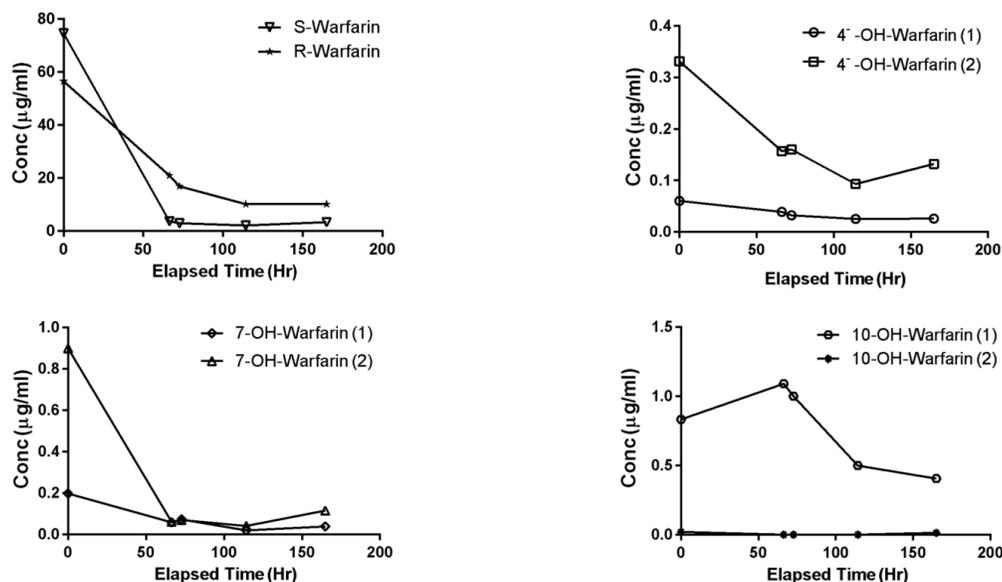
**Figure 2.** Representative extracted ion chromatogram (XIC) of blank plasma and calibration standards of WAR and metabolites at lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ); (a) R-WAR and S-WAR (b) 10-OH-WAR (c) 4'-OH-WAR (d) 7-OH-WAR.

Table 3. Stabilities of WAR and its metabolites in human plasma QC samples (n=3).

Analyte	Nominal concentration (µg / mL)	% Loss/gain in stability study		
		Post-preparative	Freeze-thaw	Short term
Racemic WAR	50	+3.44 %	+5.82 %	-2.5 %
Racemic 4'-OH-WAR	20	+1.33%	+8.53%	-2.33%
Racemic 7-OH-WAR	22	-1.56 %	+2.60 %	+1.45 %
Racemic 10-OH-WAR	22	+4.15%	+3.16 %	+3.10%

**Figure 3.** Plasma concentration versus time profiles of enantiomer of warfarin and 3 of its metabolites.

3.2.4. Stability

As shown in Table 3, buffered plasma samples containing racemic WAR, racemic 4'-OH-WAR, racemic 7-OH-WAR and racemic 10-OH-WAR were stable for up to 3 h at room temperature and for at least three freeze/thaw cycles. The prepared samples were stable for 24 h in the cooled auto-sampler. The relative deviations were within $\pm 15\%$ for all analytes at the different conditions studied.

3.3. Application to clinical study

This method was applied to morning the metabolites of WAR in plasma of patient under treatment enantiomeric forms of WAR and its metabolites were separated by the chiral column. Standards for *R*- and *S*-WAR identified peak 2 as *S*-WAR while metabolite peaks could not be definitively identified. Peak 2 for 7-OH-WAR gave higher blood levels, while the opposite applied to 10-OH-WAR, Figure 3.

The 1st and 2nd eluted peaks of WAR and metabolites were identified as the *R*- and *S*- enantiomers, respectively, based on known human metabolic pathways for WAR [22]. *R*-10-OH-warfarin is the major metabolite in clinical samples from patients undergoing warfarin therapy, via the CYP3A4 pathway (Peak 1 was the dominant peak). Hydroxylation at the 7- position is catalysed by CYP2C19 and CYP2C9, but CYP2C9 predominates with an almost 1000-fold higher affinity for *S*-WAR than *R*-WAR (Peak 2 was the dominant peak). Identification of the two 4'-OH-WAR peaks is less clear-cut, as multiple enzymes (e.g. CYP2C8 and CYP3A4) are likely to contribute to this enzymatic pathway. In comparison to previous methods our method was able to separate WAR and metabolites enantiomers from each other [15,19].

4. Conclusions

In summary, we have developed a rapid, accurate and robust LC-MS/MS method for simultaneous quantification of

WAR and three of its main metabolites in human plasma. We used chiral chromatography to separate enantiomers of each analyte and have successfully applied this method for analysis of patient plasma under WAR treatment.

References

- Bern, M. M.; Lokich, J. J.; Wallach, S. R.; Bothe, J. A.; Benotti, P. N.; Arkin, C. F.; Greco, F. A.; Huberman, M.; Moore, C. *Ann. Intern. Med.* **1990**, *112*, 423-428.
- Ridker, P. M.; Goldhaber, S. Z.; Danielson, E.; Rosenberg, Y.; Eby, C. S.; Deitcher, S. R.; Cushman, M.; Moll, S.; Kessler, C. M.; Elliott, C. G.; Paulson, R.; Wong, T.; Bauer, K. A.; Schwartz, B. A.; Miletich, J. P.; Bounameaux, H.; Glynn, R. J. *N. Engl. J. Med.* **2003**, *348*, 1425-1434.
- Radwan, M. A.; Bawazeer, G. A.; Aloudah, N. M.; AlQuadeib, B. T.; Aboul-Enein, H. Y. *J. Chromatogr. B Biomed. App.* **2012**, *26*, 6-11.
- Kaminsky, L. S.; Zhang, Z. Y. *Pharmacol. Ther.* **1997**, *73*, 67-74.
- Rettie, A. E.; Korzekwa, K. R.; Kunze, K. L.; Lawrence, R. F.; Eddy, A. C.; Aoyama, T.; Gelboin, H. V.; Gonzalez, F. J.; Trager, W. F. *Chem. Res. Toxicol.* **1992**, *5*, 54-59.
- Scordo, M. G.; Pengo, V.; Spina, E.; Dahl, M. L.; Gusella, M.; Padriani, R. *Clin. Pharmacol. Ther.* **2002**, *72*, 702-710.
- Loebstein, R.; Yonath, H.; Peleg, D.; Almog, S.; Rotenberg, M.; Lubetsky, A.; Roitman, J.; Harats, D.; Halkin, H.; Ezra, D. *Clin. Pharmacol. Ther.* **2001**, *70*, 159-164.
- Giuseppe, L.; Elena, R.; Antonio Maria, L.; Valerio De, S. *Curr. Med. Chem. Cardiovasc. Hematol. Agents.* **2004**, *2*, 311-326.
- Miller, G. P.; Jones, D. R.; Sullivan, S. Z.; Mazur, A.; Owen, S. N.; Mitchell, N. C.; Radomska-Pandya, A.; Moran, J. H. *Chem. Res. Toxicol.* **2009**, *22*, 1239-1245.
- Redman, A. R.; Zheng, J.; Shamsi, S. A.; Huo, J.; Kelly, E. J.; Ho, R. J. Y.; Ritchie, D. M.; Yuen, Y. H. *Clin. Appl. Thromb. Hemost.* **2008**, *14*, 29-37.
- Piatkov, I.; Rochester, C.; Jones, T.; Boyages, S. *Toxins (Basel)* **2010**, *2*, 2584-2592.
- Hirsh, J. *Am. J. Cardiol.* **1995**, *75*, 39B-42B.
- Gübitz, G. *Chromatographia* **1990**, *30*, 555-564.
- Sekhon, B. S. *Int. J. Pharmtech. Res.* **2010**, *2*, 1584-1159.
- Zuo, Z.; Wo, S. K.; Lo, C. M. Y.; Zhou, L.; Cheng, G.; You, J. H. S. *J. Pharm. Biomed. Anal.* **2010**, *52*, 305-310.
- Locatelli, I.; Kmetec, V.; Mrhar, A.; Grabnar, I. *J. Chromatogr. B Biomed. Sci. Appl.* **2005**, *818*, 191-198.
- Ring, P. R.; Bostick, J. M. *J. Pharm. Biomed. Anal.* **2000**, *22*, 573-581.
- Malakova, J.; Pavek, P.; Svecova, L.; Jokesova, I.; Zivny, P.; Palicka, V. *J. Chromatogr. B Biomed. Sci. Appl.* **2009**, *877*, 3226-3230.

- [19]. Wang, X.; Hou, J.; Jann, M.; Hon, Y. Y.; Shamsi, S. A. *J. Chromatogr. A* **2013**, *1271*, 207-216.
- [20]. Heneedak, H. M.; Salama, I.; Mostafa, S.; El-Kady, E.; El-Sadek, M. *Biomed. Chrom.* **2015**, *29*, 998-1007.
- [21]. Mostafa, A.; Medley, G.; Roberts, D. M.; Mohamed, M. S.; Elshanawani, A. A.; Roberts, M. S.; Liu, X. *J. Chromatogr. B Biomed. App.* **2011**, *879*, 2234-2238.
- [22]. Kim, S. Y.; Kang, J. Y.; Hartman, J. H.; Park, S. H.; Jones, D. R.; Yun, C. H.; Boysen, G.; Miller, G. P. *Drug Metab. Lett.* **2012**, *6*, 157-164.