ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 219–226

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Development and validation of a sensitive and robust LC–tandem MS method for the analysis of warfarin enantiomers in human plasma

Weng Naidong *, Paula R. Ring, Camilla Midtlien, Xiangyu Jiang

Department of Bioanalytical Chemistry, Covance Laboratories, Inc., 3301 Kinsman Boulevard, Madison, WI 53704, USA

Received 1 August 2000; received in revised form 19 September 2000; accepted 27 September 2000

Abstract

A liquid chromatography–tandem mass spectrometry method (LC-MS-MS) was developed and validated for measuring warfarin (WAR) enantiomers (*R*-WAR and *S*-WAR) in human EDTA plasma. Liquid–liquid extraction using ethyl ether was used to extract the analytes from the plasma. Baseline resolution of *S*- and *R*-WAR as well as the internal standard enantiomers (*S*- and *R*-*p*-ClWAR, *S*-IS and *R*-IS) was achieved on a β-cyclodextrin column with a mobile phase of acetonitrile–acetic acid–triethylamine (1000:3:2.5, v/v/v). The retention times are 6.9, 8.0, 7.0, and 7.9 min for *S*-WAR, *R*-WAR, *S*-IS and *R*-IS, respectively. The detection was by monitoring *S*- and *R*-WAR at m/z 307 → 161 and *S*- and *R*-IS at m/z 341 → 161 using (−) ESI. The standard curve range was 1–100 ng ml^{-1} for both *S*- and *R*-WAR. The inter-day precision and accuracy of the quality control (QC) samples were < 7.3% relative standard deviation (RSD) and < 7.3% bias for *S*-WAR, and < 6.5% RSD and < 5.8% bias for *R*-WAR, respectively. Analyte stability during sample processing and storage were established. Method ruggedness was demonstrated by the reproducible performance from analysis of clinical samples.

Keywords: Warfarin enantiomers; LC-MS-MS

1. Introduction

Warfarin (WAR), which acts by inhibiting vitamin K-dependent coagulation factors, is a common anticoagulant agent used in humans. Fig. 1 shows the structure of WAR. WAR exists in two enantiomers, *S*- and *R*-WAR, with the *S*-form having several times the pharmacological activity of the *R*-form in human [1]. Both enantiomers are believed to bind at the same site on human serum albumin [2] but appear to have different equilibrium constants for their interactions at this site [3–6]. *S*-WAR was reported to be more quickly metabolized than *R*-WAR [7]. Potential drug–drug interactions with WAR are enormous. In order to support drug–drug interaction studies, a

* Corresponding author. Tel.: +1-608-2422652; fax: +1-608-2422735.
E-mail address: naidong.weng@covance.com (W. Naidong).

0731-7085/01/$ - see front matter
PII: S0731-7085(00)00476-3

sensitive, reliable and quick analytical method is required. Previously developed HPLC methods with UV or fluorescence detection are adequately sensitive for most of drug-drug interaction studies [8–10] but for some studies, a LLOQ of 1.0 ng ml^{-1} in human plasma is required. A LC-MS-MS method for quantitative analysis *S*- and *R*-WAR in biological fluids has not been reported.

We present in this paper the development and validation of a sensitive and robust LC-MS-MS method for analysis of *S*- and *R*-WAR in human plasma. The internal standard is *p*-chlorowarfarin (*p*-ClWAR).

2. Experimental

2.1. Chemicals and reagents

Racemic WAR was from USP (Rockville, MD), and the internal standard (IS) *p*-ClWAR was from Sigma (St. Louise, MO). All organic solvents used were of analytical grade and purchased from Sigma or Fisher (Fair Lawn, NJ). HPLC grade water was also purchased from Fisher. Control sodium EDTA or sodium heparin human plasma was purchased from Biochemed (Winchester, VA).

2.2. Calibration standards and quality control samples

Two primary stock solutions of WAR were prepared from separate weights for standards and quality control samples (QC samples). Methanolic solutions of WAR primary stocks and substocks were prepared under yellow light and stored in dark. Working standards were prepared fresh daily by spiking 50 μl of 20-fold concentrated solutions into1.0 ml of blank control EDTA plasma. The final concentrations of *S*- or *R*-WAR in plasma standards were 1.0, 2.0, 5.0, 10.0, 25.0, 50.0, 75.0, and 100.0 ng ml^{-1}. Three levels of QC samples, 3.5, 15, and 80 ng ml^{-1}, were prepared, aliquoted and stored frozen at −20°C with the clinical samples to be analyzed.

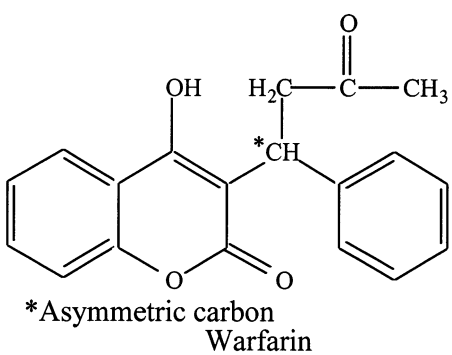

Fig. 1. Chemical structures of WAR.

2.3. LC – tandem mass spectrometry method

The LC-MS-MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and a PE Sciex API 3000 tandem mass spectrometer (Torondo, Canada) with (−) ESI. The analytical column, β-cyclodextrin column of 5 μm, 250 × 4.6 mm ID and a β-cyclodextrin guard column of 5 μm, 50 × 4.6 mm ID were from Astec (Whippany, NJ). The injection volume was 10 μl, run time was 10 min and flow rate was 1.0 ml min^{-1}. The column was maintained at room temperature. After about 20–30 min equilibration with a mobile phase consisting of acetonitrile–acetic acid–triethylamine (1000:3:2.5, v/v/v), the new columns showed consistent retention times of the analytes. Without any treatment, one column could be used for at least five hundreds injections of the extracted samples.

Sensitivity of multiple reaction mode (MRM) was optimized by testing with an infusion of 1 μg ml^{-1} WAR in a mixture of methanol and water (1:1, v/v). The drying gas (nitrogen) flow rate was 8 l min^{-1}. The electrospray source was operated with a capillary voltage of 4.5 kV, an orifice voltage of −56 V, and a source temperature of 400°C. The ring voltage was −150 V, and Q1 energy was 1 eV. The preselected precusor ion masses, which passed the first MS, went into the collision cell. The fragmentation occurred at collision energy of 26 eV. Nitrogen was used as collision gas. The product ions with the preselected masses produced from the fragmentation in the collision cell passed the second MS and were detected. The Q3 energy was 3 eV. The dwell time

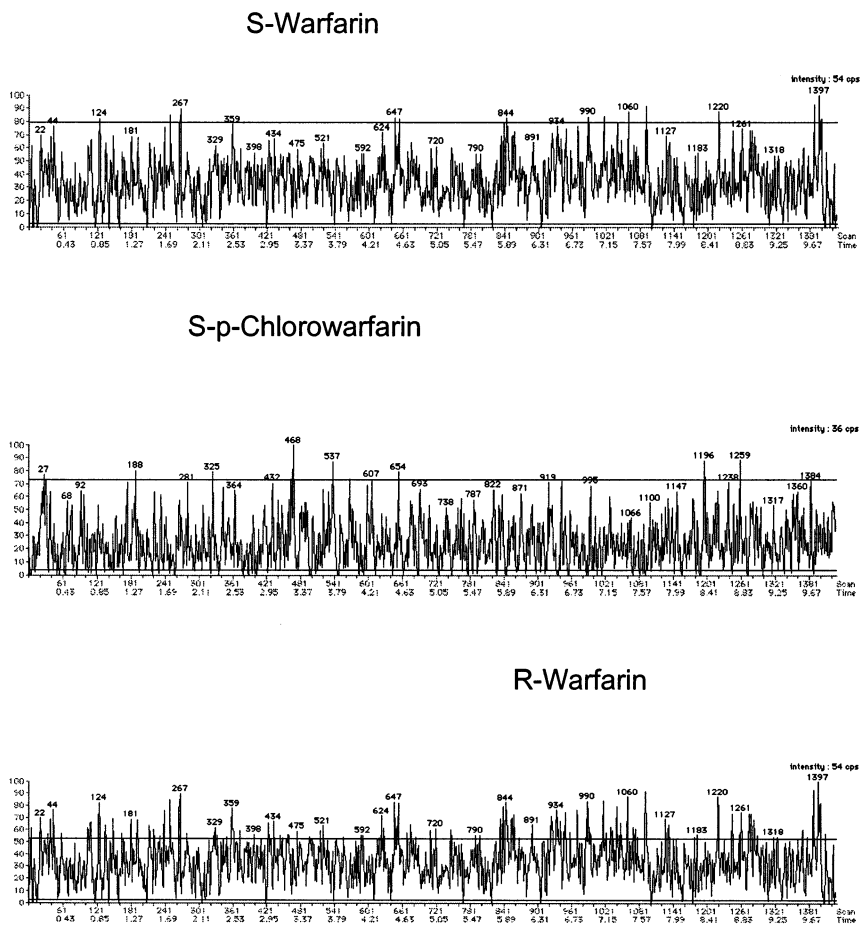

Fig. 2. Chromatogram of a blank plasma sample.

was 200 ms. The following ions (precursor to product) were monitored: *S*- and *R*-WAR, 307 → 161; *S*- and *R*-IS: 341 → 161.

Chromatograms were integrated using MacQuan. A weighed $1/x^2$ linear regression was used to determine slopes, intercepts and correlation coefficients (r^2), where x is the concentration of the analyte.

2.4. Sample preparation

To 1.0 ml plasma sample, 50 μl of IS solution (1.0 μg ml^{-1} in methanol) and 0.7 ml of 1 N sulphuric acid were added. After mixing, 5 ml of ethyl ether was added to extract analytes and IS. After shaking for 15 min at 60–100 cpm, the samples were centrifuged at 2000 rpm for 5 min. The aqueous layer was frozen in a dry-ice bath and the ether layer was decanted into a clean tube, evaporated to dryness under a stream of nitrogen, and reconstituted in 0.20 ml of acetonitrile. An aliquot of 10 μl was injected onto the LC-MS-MS.

2.5. Validation of the liquid chromatography–tandem mass spectrometry method

The method was validated by three consecutive analytical curves on three separate days. Each calibration curve contained a single set of calibration standards and six replicates of QC samples at

each concentration level. Each curve also contained other test samples such as stability samples of processing and storage. Calibration standards, QC samples and other test samples were randomized through the curves. An extracted blank sample was always placed after the ULOQ (upper limit of quantitation) to determine carry-over of the LC-MS-MS system.

Analyte stability was tested by subjecting QC samples through multiple freeze-thaw cycles (F/T cycles), on the bench at room temperature (bench-top), or at −20°C in the freezer for a long period

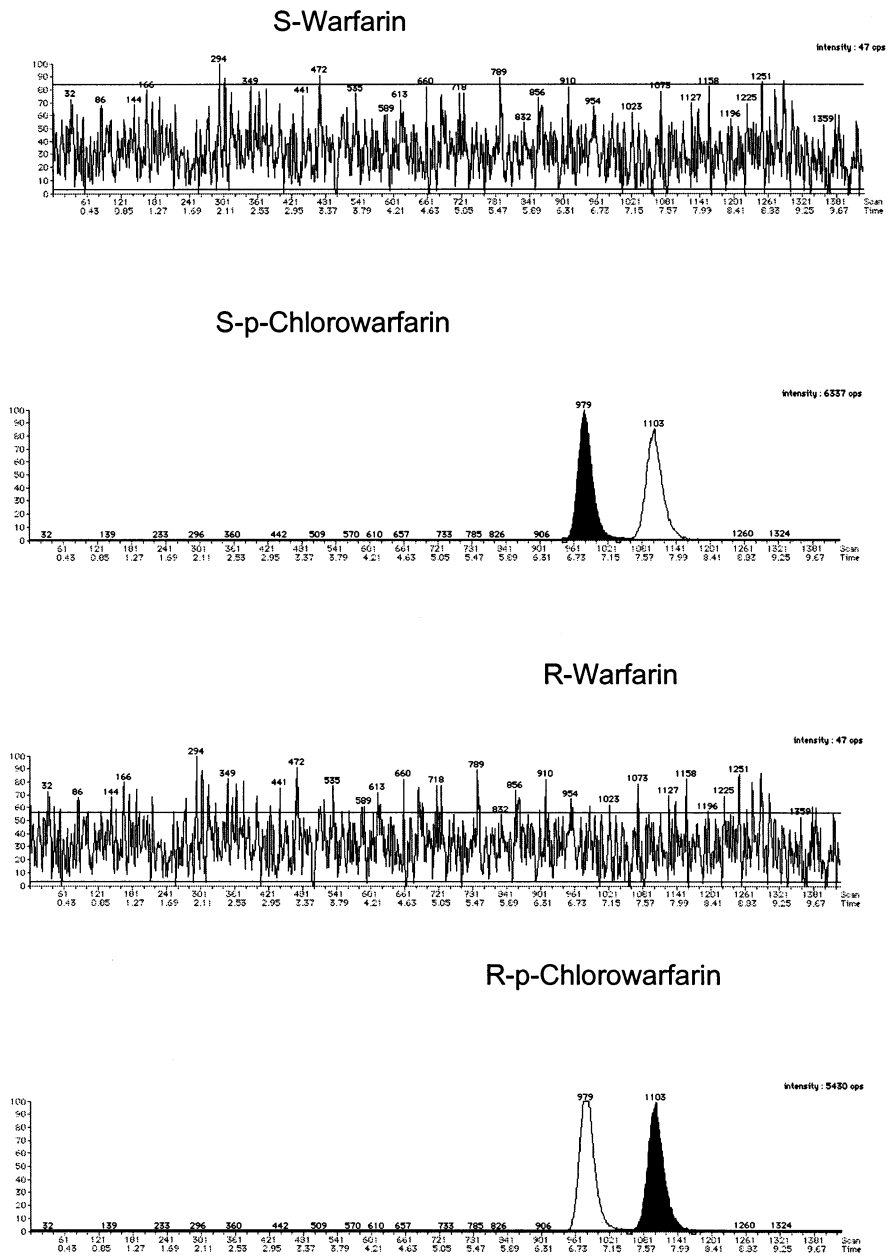

Fig. 3. Chromatogram of a blank plasma sample spiked with only *p*-chlorowarfarin (IS).

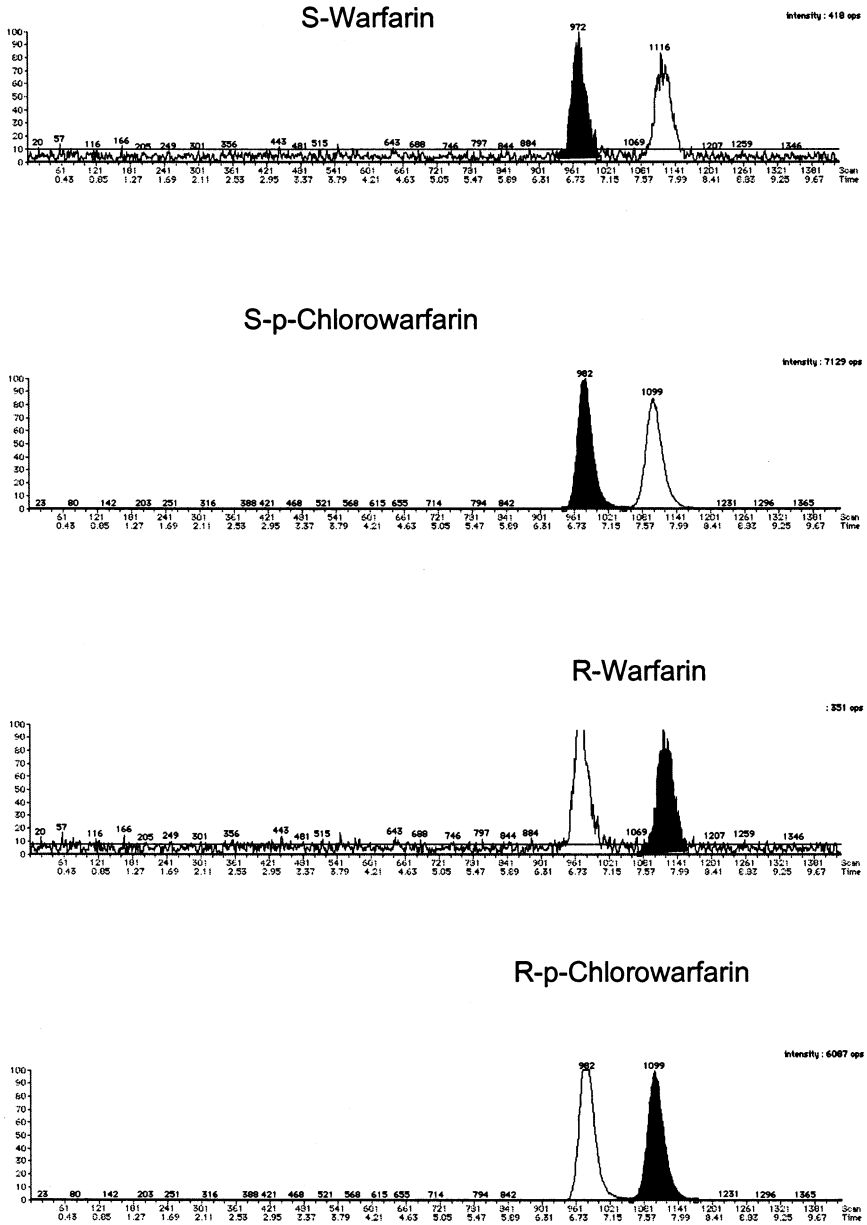

Fig. 4. Chromatogram of a blank plasma sample spiked with *p*-chlorowarfarin (IS) and 1.0 ng ml^{-1} *S*- and *R*-warfarin.

(storage). Post-extraction analyte stability was also determined.

Recoveries of the analytes were determined by comparing the peak area of the analytes extracted from plasma with those prepared in acetonitrile.

The method selectivity was evaluated by screening eight lots of blank sodium EDTA plasma and six lots of blank sodium heparin plasma.

Table 1
Precision and accuracy of calibration standards ($n = 3$)

	1.0	2.0	5.0	10.0	25.0	50.0	75.0	100.0 (ng ml^{-1})
S-WAR								
Mean	0.990	2.0	5.17	10.4	25.3	50.8	71.7	97.1
RSD	1.3%	3.8%	1.9%	3.5%	3.8%	4.5%	9.3%	7.6%
Bias	−1.0%	0%	+3.5%	+3.5%	+1.1%	+1.6%	−4.4%	−2.9%
R-WAR								
Mean	0.995	1.98	5.17	10.4	25.2	50.6	72.5	96.5
RSD	0.8%	1.1%	4.5%	4.0%	2.6%	4.3%	8.4%	7.2%
Bias	−0.5%	−1.2%	+3.4%	+3.0%	+0.8%	+1.3%	−3.3%	−3.5%

Table 2
Precision and accuracy of quality control samples

	S-WAR (ng ml^{-1})				*R*-WAR (ng ml^{-1})			
	3.50	15.0	80.0	80.0[a]	3.5	15.0	80.0	80.0[a]
Intraday (n = 6)								
Mean	3.59	14.6	84.1	87.7	3.55	14.6	84.9	87.6
RSD	5.9%	3.5%	4.6%	3.1%	5.4%	2.7%	4.6%	2.6%
Bias	+2.5%	−2.7%	+5.2%	+9.6%	+1.5%	−2.8%	+6.1%	+9.5%
Interday (n = 18)								
Mean	3.62	14.5	85.8		3.60	14.4	85.5	
RSD	5.2%	7.3%	5.1%		5.1%	6.5%	5.1%	
Bias	+3.3%	−3.1%	+7.3%		+4.1%	−3.4%	+5.8%	

[a] Samples were diluted ten folds with blank plasma prior to analysis.

3. Results and discussion

3.1. Liquid chromatography–tandem mass spectrometry method

The selection of β-cyclodextrin column for resolving *S*- and *R*-WAR enantiomers is based on our previous experiences [8,10]. In order to minimize quantitation bias caused by matrix effect, an IS with retention time identical or similar to the analyte but resolved on the tandem mass spectrometry is usually preferred. This bias, caused by inconsistent matrix effects from individual samples, has been identified as the major failure for some of the earlier LC-MS-MS methods [11–14]. Previously used IS in HPLC-UV methods, such as naproxen [8] or oxybenzone [10], might not be suitable for LC-MS-MS of WAR because of drastic retention difference from WAR. Oxybenzone eluted too close to the solvent front where the matrix suppression might be more severe. The retention for naproxen was quite long, which decreased sample through put. *p*-ClWAR enantiomers have almost identical retention times as *S*- and *R*-WAR and were chosen as the IS.

Table 3
Stability of the sample

Three Freeze/thaw stability	
S-WAR	100.8–104.0%
R-WAR	102.6–105.4%
Bench-top stability (24 h)	
S-WAR	100.9–107.3%
R-WAR	102.3–108.6%
Extracts stability (24 h at ambient temperature)	
S-WAR	99.9–102.7%
R-WAR	99.7–102.2%

Table 4
Cross-validation between sodium heparin and sodium EDTA plasma ($n = 6$)

	S-WAR (ng ml^{-1})			*R*-WAR (ng ml^{-1})		
	3.50	15.0	80.0	3.50	15.0	80.0
QC samples in sodium heparin plasma and a calibration curve in EDTA plasma						
Mean	3.40	14.9	79.9	3.64	15.1	80.8
RSD	5.2%	7.1%	8.8%	3.6%	4.7%	5.6%
Bias	−2.8%	−0.6%	−0.1%	+3.9%	+0.7%	+1.0%
QC samples in EDAT plasma and a calibration curve in sodium heparin plasma						
Mean	3.23	13.8	76.8	3.36	13.8	78.4
RSD	6.2%	4.8%	4.1%	3.9%	2.8%	2.6%
Bias	−7.7%	−8.2%	−4.0%	−4.0%	−7.8%	−2.0%

ESI with negative (−) mode provided better sensitivity than ESI with positive (+) mode. This is in agreement with the theory of pre-formed ion [15,16] since the pH value of water–TEA–acetic acid (100:0.25:0.3, v/v/v) is 7.0 and replacement of water with acetonitrile should not change the pH significantly. The pK_a for WAR is 5.0 and in a mobile phase at pH 7.0, WAR and IS carry negative charges.

3.2. Selectivity, sensitivity and linearity

Eight lots of human sodium ETDA plasma and six lots of human sodium heparin plasma were tested for interference. All were clean in the WAR and IS regions. There were no interference from IS contributing to the WAR m/z channel. No interference peak was observed in the IS channel when an extracted WAR ULOQ sample (100 ng ml^{-1} *S*- and *R*-WAR without IS) was injected onto the LC-MS-MS system. The signal to noise from an extracted LLOQ sample (1.0 ng ml^{-1}) was at least 15 to 1 for both *S*- and *R*-WAR. Six lots of EDTA plasma spiked with WAR at LLOQ showed RSD and bias of 8.9% and −6.6% for *S*-WAR and 5.0% and −10.6% for *R*-WAR. Carry-over from ULOQ to blank sample was not noted, indicated by no peaks corresponding to WAR or IS enantiomers were observed in the blank sample that was injected right after the ULOQ sample. Calibration curves were linear using $1/x^2$ linear regression in the concentration range from 1.0 to 100.0 ng ml^{-1}, with correlation coefficients greater than or equal to 0.9974 for all curves. Chromatograms of blank plasma, blank plasma spiked with IS only, and blank plasma spiked with WAR at LLOQ and IS are shown in Figs. 2–4.

3.3. Precision and accuracy

Table 1 shows the validation data on accuracy and precision of each standard concentration. Table 2 presents the inter-day and intra-day accuracy and precision of QC samples. For partial volume analysis, the samples were diluted ten-folds with blank plasma prior to analysis. The data show that this method is consistent and reliable with low values of RSDs and bias.

3.4. Absolute recovery

Absolute recoveries were determined by comparing the peak areas of extracted QC samples with the peak area of recovery standards (unextracted equivalents of extracted QC samples). Here, we did not attempt to determine whether the lower signal in the extracted samples than the unextracted solutions is due to extraction or due to matrix effects. If existing, the matrix effects did not cause quantitation bias as evidenced by the fact of low RSDs and bias values obtained with LLOQ spiked into six individual lots of plasma (Section 3.2). Calculated absolute recovery is 95.7% for *S*-WAR, 92.5% for *R*-WAR, 75.5% for *S*-IS and 72.4% for *R*-IS. The excellent absolute

recoveries for *S*- and *R*-WAR indicated good extraction efficiency and little matrix effects. Somewhat lower absolute recoveries for IS enantiomers were probably due to lower extraction efficiency rather than matrix effects. Since IS enantiomers chromatographically coeluted with WAR enantiomers and their structures are very similar, one would expect identical matrix effects for both compounds. Since the recoveries for IS enantiomers were very consistent and tracked WAR enantiomers in the process of extraction and chromatography, attempt to further improve IS recoveries was not pursued.

3.5. Stability of the analytes

The protocol of the stability tests was designed to cover the anticipated conditions that the clinical samples may experiences. Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis, appeared to have little effect on the quantitation of *S*- and *R*-WAR in human plasma. QC samples stored in a freezer at −20°C remained stable through the course of the validation (21 days). Extracted calibration standards and QC samples were allowed to stand at ambient temperature for 24 h prior to injection. No effect on quantitation of the calibration standards or QC samples was observed. Stability results are summarized in Table 3.

3.6. Cross-validation to sodium heparin plasma and method robustness

One set of QC samples prepared in sodium heparin plasma was run against the calibration standards in EDTA plasma. Another set of QC samples prepared in EDTA plasma was run against the calibration standards in sodium heparin plasma. The results shown in Table 4 indicate this method is accurate and precise for measuring *S*- and *R*-WAR in human plasma. The method robustness has been indicated by two analysts who used two analytical columns and two LC-MS-MS plate-forms during the analysis of clinical trial samples.

4. Conclusion

A sensitive and reliable chiral LC-MS-MS method for the measurement of *S*- and *R*-WAR enantiomers in human plasma has been successfully developed and validated. A structure-similar compound, *p*-chlorowarfarin, is used as IS. Enantiomers of the IS coeluted with *S*- and *R*-WAR, which minimized the potential bias caused by inconsistent matrix effects and improved the method ruggedness. Electrospray with negative ion detection mode, (−) ESI, was used to improve sensitivity. The LLOQ is 1.0 ng ml^{-1}.

References

[1] I.A. Choonara, B.P. Haynes, S. Cholerton, A. Breckenridge, B.K. Park, Br. J. Clin. Pharmacol. 22 (1986) 729–732.
[2] G. Sudlow, D.J. Birkett, D.N. Wade, Mol. Pharmacol. 12 (1976) 1052–1061.
[3] R.A. O'Reilly, Mol. Pharmacol. 7 (1971) 209–218.
[4] C. Lagercrantz, T. Larsson, I. Denfors, Comp. Biochem. Physiol. 69C (1981) 375–378.
[5] J.H.M. Miller, G.A. Smail, J. Pharm. Pharmacol. 29 (1977) 33.
[6] B. Loun, D.S. Hage, Anal. Chem. 66 (1994) 3814–3822.
[7] S. Toon, L.K. Low, M. Gibaldi, W.F. Trager, R.A. O'Reilly, C.H. Motley, D.A. Goulart, Clin. Pharmacol. Ther. 39 (1986) 15–24.
[8] W. Naidong, J.W. Lee, J. Pharm. Biomed. Anal. 11 (9) (1993) 785–792.
[9] A.S. Prangle, T.A.G. Noctor, W.J. Lough, J. Pharm. Biomed. Anal. 16 (1986) 1205–1212.
[10] P.R. Ring, J.M. Bostick, J. Pharm. Biomed. Anal. 22 (2000) 573–581.
[11] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.
[12] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Mass Spectrom. 7 (1996) 882–889.
[13] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972A–986A.
[14] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347–357.
[15] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64–71.
[16] J.B. Fenn, J. Am. Soc. Mass Spectrom. 4 (1993) 524–529.