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SIMULTANEOUS LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRIC DETERMINATION OF WARFARIN ENANTIOMERS IN HUMAN PLASMA AND ITS APPLICATION TO A BIO-EQUIVALENCE STUDY IN INDIAN POPULATION

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Abstract: Warfarin (WAR), one of the most commonly used oral anticoagulant in the US and across the globe, is a drug of choice for millions. It's a unique drug of its kind due to the multiple pharmacological and pharmacokinetic properties. Although highly efficacious, positive clinical outcomes during WAR therapy depend on maintaining a narrow therapeutic range for the drug. This goal is challenging due to large inter-individual variability in patient response, which has been attributed to diversity in drug metabolism. WAR is given as a racemic mixture and evidence suggest differences of *R* and *S*-WAR in their therapeutic activities and metabolism. Previous investigation of WAR metabolism has been hampered by the inability to quantify the individual enantiomers with reliable accuracy and within short time period. To overcome these limitations a single, simple, selective and rapid LC–MS/MS method is reported. The reported method separated individual *R* and *S* enantiomers of WAR with a short run time of just 4 min. per sample using *R* WAR d5 and *S* WAR d5 as internal standards. WAR enantiomers were resolved to reveal unique insight into the stereo-specific metabolism of WAR. The mean recovery was 98.9 % and 84.6 % for *R* WAR and *S* WAR respectively. The coefficient of variation of the assay was less than 5.5 % and 7.2 % for *R* WAR and *S* WAR with an accuracy of 93.1 % to 105.8 % and 94.5 % to 102.6 % for *R* WAR and *S* WAR respectively. Stability was evaluated under different conditions including bench top, freeze and thaw, auto sampler and long term. The validated method was applied for the determination of individual WAR enantiomers in human plasma samples from a bioequivalence study of 5mg fixed dose formulation in 10 healthy Indian subjects. Assay reproducibility was demonstrated by reanalysis of 10% incurred samples.

Keywords: Warfarin; Chiral; LC-MS/MS; Human Plasma; Enantiomers; Bioequivalence

INTRODUCTION

Warfarin (WAR) (4-hydroxy-3-(3-oxo-1-phenylbutyl) coumarin) is one of the most commonly used oral anticoagulants [1]. Given the presence of a centre of chirality at C9, the compound exists in two enantiomeric forms, (*R*) - and (*S*)-WAR. The drug, however, is administered as a racemic mixture. (*S*)-WAR has been reported to have a faster metabolism and an anticoagulant potency that is 2–5 times higher than that of (*R*)- WAR. The anticoagulant effect is mediated by the inhibition of vitamin K epoxide reductase complex 1 [2]. Phase I metabolism of WAR is stereoselective [3]. It's a unique drug of its kind due to the multiple pharmacological and pharmacokinetic properties. WAR has a high interpatient variability and narrow therapeutic index which requires continuous monitoring of its plasma concentration, the prothrombin time and the international normalized ratio followed by a dosage adjustment. WAR in pure form exists as a racemic mixture consisting of equal amounts of *R* and *S* enantiomers. WAR is highly metabolized in the body in a stereo specific pathway catalyzed by cytochrome P450 [4]. The properties of WAR, such as narrow therapeutic index, high protein binding, CYP dependent metabolism and a very high elimination half-life render to be prone to many drug interactions [5]. Elimination half-life of WAR is relatively long (10-16 hours in animals and 40-46 hours in humans), causing a dramatic increase in the anticoagulant effect upon concomitant administration of WAR with other drugs causing drug-drug interactions [6].

There are various published HPLC assays for WAR enantiomers using UV or fluorescence detections ([7-11]. Recently, LC-MS/MS methods were also published for the determination of racemic WAR and WAR enantiomers. [4, 12-14]. All the published methods had a long chromatographic run time and generally required complex extraction procedures to remove interferences, which were obviously impractical for high throughput analysis. Therefore, it was necessary to develop a simple, precise and sensitive method for the determination of WAR enantiomers in human plasma which will give high throughput analysis in short interval of time. The present work comes up with a rapid, simple, sensitive and precise isocratic reversed-phase HPLC-MS/MS method for the determination of WAR enantiomers in human plasma with a

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quantification limit sufficiently low to support stereoselective pharmacokinetic studies.

MATERIALS AND METHODS

Materials and Chemicals:

Reference standards of WAR enantiomers and d5 WAR enantiomers with 99% purity were purchased from Syncom (Groningen, Netherlands). The HPLC grade solvents viz. methanol and acetonitrile were purchased from J.T. Baker INC. (Phillipsburg, NJ, USA). LC-MS grade formic acid was procured from Sigma-Aldrich Co. (Bangalore, India). Strata-X 33µm polymeric reversed phase (30mg/1mL) solid phase extraction cartridges and HPLC grade water were procured from E. Merck Ltd. (Mumbai, India). Blank human blood was collected with Na Heparin as anticoagulant from healthy and drug free volunteers. Plasma was separated by centrifugation at 3000 RPM at 10°C, and stored at –20°C.

Instrumentation and Analytical Conditions:

The chromatographic separation and quantification was achieved by a liquid chromatography system, LC-20AD (Shimadzu, Kyoto, Japan) coupled with mass spectrometer, API-3000 (ABS Sciex, Canada). The chiral column, Chiralpak AS-3R (150 x 4.6 mm, 3µ) from Daicel Chemicals Ind. Ltd. (Hyderabad, India) was used for separation of WAR enantiomers and internal standards. Mobile phase of 0.1% formic acid with acetonitrile in the ratio of 5:95 (*v/v*) was pumped isocratically at flow rate of 1.0 mL/min. Auto sampler temperature was set at 10°C and the injection volume was 2µL. The column oven temperature was maintained at 40°C and the total LC run time was 4.0 min.

The MS/MS system was operated in the multiple reaction monitoring (MRM) mode for monitoring the transition of the deprotonated molecular ion *m/z* 307.2 to the product ion *m/z* 161.1 for WAR enantiomers and the transition of the deprotonated molecular ion *m/z* 312.2 to the product ion *m/z* 161.1 for the d5 WAR enantiomers. The instrument response was optimized for WAR enantiomers and internal standards by infusing a constant flow of a solution of the drug dissolved in mobile phase.

Electrospray ionization (ESI) was performed in the negative ion mode. The source temperature was set to 500°C with ion spray voltage of -3500. Nitrogen gas was used as the CAD gas. The curtain gas was kept at 15. Compound dependant parameters set for R-WAR and its IS were Decluster Potential: -70 and -45 V; Focusing potential: -240 and -200 V; Entrance Potential: -10 V for both; collision energy: -30 eV for both; Cell Exit Potential: -10 V for both. The compound dependant parameters set for S- WAR and its IS were Decluster Potential: -70 and -45 V; Focusing potential: -240 and -200 V; Entrance Potential: -10 V for both; collision energy: -30 eV for both; Cell Exit Potential: -10 V for both respectively. Q1 and Q3 were maintained at unit resolution and the dwell time was kept at 200ms. The instrument was interfaced with computer running analyst version 1.4.2 software.

Preparation of standards and quality control samples:

WAR is a light sensitive drug; hence, calibration standard and quality control samples preparation, sample processing and bioanalysis were carried out under sodium vapor light. Stock solutions of WAR enantiomers and IS were prepared by dissolving the test compounds in methanol to obtain 1000 µg/mL concentration for each enantiomer. Stock solutions of WAR enantiomers prepared were serially diluted to prepare working solutions in required concentration range with diluent methanol: water (60:40, v/v). Two separate stock solutions of *R*-WAR and *S*-WAR were prepared for bulk spiking of calibration curve and quality control samples for the method validation experiment and subject analysis. The calibration standards and quality control (QC) samples were prepared by spiking 5% of the total plasma volume with working solutions. Calibration standards were prepared at concentration of 10.0, 20.0, 50.0, 100.0, 200.0, 400.0, 650.0 and 800.0 ng/mL for R- WAR and S-WAR. Similarly, quality control samples (QC's) were prepared at four different concentrations namely, 10.0 (LLOQ), 30.0 (LQC), 350.0 (MQC) and 600.0 (HQC) ng /mL for *R*-WAR and *S*-WAR. Sufficient calibration standards and quality control samples were prepared to validate the method. Aliquots of the standards and quality controls were stored at -20°C until used for validation runs and subject analysis.

Sample preparation:

200 µL plasma sample was taken in polypropylene tube (Tarsons, India) and 25 µL of IS dilution (working solution of 5.000 µg/mL each of *R*-d5-WAR and *S*-d5-WAR) was added to it. The contents were vortexed to mix. 200µL of 0.2% formic acid in water was added to each sample. The contents were vortexed for 30 seconds and centrifuged for 5 minutes at 14000 RPM. After centrifugation the samples were loaded on Strata-X 30mg/1mL polymeric reversed phase SPE cartridges preconditioned with 1mL of methanol and equilibrated with 1mL of HPLC water. The plasma matrix was drained out from the extraction cartridges by applying positive nitrogen pressure. The sorbent bed was washed with 1 mL of HPLC water. The analytes and internal standards were eluted with 1 mL of mobile phase and transferred into autosampler vials for injection. 2µL of the sample was injected into the LC-MS/MS system through the autosampler.

Method Validation:

Validation experiments of the method were carried out according to USFDA guidelines [15].

Selectivity and Cross selectivity: Selectivity was performed using 10 different sources of blank plasma comprising of 6 normal, two hemolysed and two lipemic. These blank plasma samples were processed as per the extraction method and their response was assessed at the retention time of the analytes and the internal standards with six LLOQ samples for *R*-WAR and S-WAR (prepared from the screened blank plasma, which had the least interference). Cross Selectivity was performed to check the possibility of cross contribution of one enantiomer at the retention time and MRM of the other enantiomer. To assess the cross selectivity blank matrix was spiked with concentration of *R*-WAR at LQC level in duplicate; similarly blank matrix was spiked with concentration of *S*-WAR at LQC level in duplicate. These spiked LQC samples were processed along with LLOQ samples which were spiked separately for both the analytes. The mean response of the interfering peak at the retention time of one enantiomer in the replicate LQC samples was compared against the processed LLOQ samples of the other enantiomer and vice versa, which should be ≤20% of the mean response of the LLOQ samples.

Carry Over: Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs. The design of the experiment comprised blank plasma, LLOQ, upper limit of quantitation (ULOQ) followed by blank plasma to check for any possible interference due to carryover.

Linearity and lower limit of quantification: The linearity of the method was determined by analyzing three standard plots associated with a eight-point standard calibration curve. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/X^2$) linear regression. The calculation was based on the peak area ratio of analyte versus the area of internal standard. The concentration of the analyte were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/X^2$). Several regression types were tested and the linear regression (weighted with $1/\text{concentration}^2$) was found to be the simplest regression. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0% and for LLOQ it should not be more than ±20.0%.

Precision and Accuracy: The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analysed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of % accuracy and coefficient of variation (% CV) respectively.

Matrix Effect and Recovery: The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in replicate (n=4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found should fall within the acceptable limit set i.e. the RSD of area ratio to be within ± 15% at each level tested.

Absolute recoveries of the analytes were determined at the three different quality control levels viz. LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the unextracted standard mixtures (prepared in the elution solution at the same concentrations as the extracted samples) representing 100% recovery.

Dilution intergrity and Stabilities: The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of ½ and ¼th concentration were prepared and the concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

Stability experiments were conducted to evaluate different conditions that plasma samples may encounter during sample shipment as well as pre- and post-processing such as several freeze-thaw cycles and short term storage of plasma samples at room temperature. All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison samples with identical concentration. Stock solutions and working solutions of analyte and IS were checked for short term stability at room temperature and long term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. For extracted sample conditions such as Autosampler stability, processed sample stability (at room

temperature), bench top stability (at room temperature), and freeze–thaw stability at 3 and 5 freezing (at -20°C) and thawing at room temperature cycles were performed at LQC and HQC using six replicates at each level. Long term stability of spiked plasma samples stored at –20° C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.

Bioequivalence study design and incurred sample reanalysis: The bioequivalence study was conducted with a single fixed dose of a test (5mg tablets from a Generic Company) and a reference Coumadin 5 mg tablets of Bristol Mayers Squibb GmbH & Co. KGaA, Munchen, Germany, in 10 normal, healthy, adult, male and female human subjects under fasting conditions. Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization, E6 Good Clinical Practice [16]. The subjects were orally administered a single dose of test and reference formulations after recommended wash out period of 35 days. Blood samples were collected at 0.00 (pre-dose), 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 12.00, 16.00, 24.00, 48.00, 72.00, 96.00 and 120.00 hours after oral administration of test and reference formulation. Samples at 48.00, 72.00, 96.00 and 120.00 hours were collected on ambulatory basis. Plasma was separated by centrifugation and kept frozen at –20°C until analysis. WAR is a light sensitive drug; hence, dispensing, dosing, blood sample collection, sample processing and bioanalysis were carried out under sodium vapor light. Standard meals were provided to the subjects at 4.00, 9.00 and 13.00 hrs. after dosing in each study period. Water intake was unmonitored except restriction for 1 hr. pre dose and post dose. The pharmacokinetic and statistical parameters of *R*-WAR and *S*-WAR were estimated by SAS® version 9.2 (SAS institute Inc. USA). ANOVA was performed on log transformed pharmacokinetic parameters C_{max} and $AUC_{0\text{-}120}$. To assess bioequivalence, two one sided 90% confidence intervals were calculated for test by reference ratios of geometric least square means of C_{max} and $AUC_{0\text{-}120}$ for both enantiomers. T_{max} was calculated by nonparametric Wilkoxon test. An incurred sample reanalysis (assay reproducibility test) of 10% samples was also conducted by random selection of subject samples. The selection criteria included samples which were near the Cmax and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the values should not be more than ±20% [17].

RESULTS AND DISCUSSIONS

Chromatographic conditions:

The objective of this study was to develop a simple, selective and rapid LC-MS/MS method for quantification of WAR enantiomers in human plasma. To resolve WAR enantiomers several types of chiral columns were tried including macrocyclic glycopeptide based, protein based and polysaccharide based. But, incompatibility with Electrospray ionization, long run time and high back pressure on glycopeptide based and protein based columns insisted us to go for polysaccharide based reverse phased column, Chiralpak AS-3R. This column brought down the run time of enantioseparation to just 4.0 minutes, most appropriate for the rapid and high throughput analysis. To find the best eluting solvent system, various combinations of methanol/acetonitrile along with buffers (ammonium trifluoroacetate/acetic acid, ammonium formate/formic acid, ammonium acetate/acetic acid, ammonium bicarbonate/ammonium hydroxide) having different ionic strengths (1–10mM) in the pH range of 3.0–10.0 and volume ratios were tested. For better peak shape and higher response, the buffer selected for this study was 0.1% formic acid because of its maximum response, volatilization and compatibility to MS. Different column temperatures were also tested from 25°C to 50°C, and concluded that the resolution improved with 40°C column temperature. Based upon these results, the mobile phase composition was set at 0.1% formic acid buffer-acetonitrile, (5:95, v/v).

Method Validation:

Selectivity and Cross Selectivity: In the negative ESI mode, deprotonated molecules at *m/z* 307.2 and 312.2 were observed as the most abundant ions for WAR enantiomers and d5 WAR enantiomers, respectively. The transitions of *m/z* 307.2 $\longrightarrow$ 161.1 for WAR enantiomers and 312.2 $\longrightarrow$ 161.1 for d5 WAR enantiomers were chosen in MRM mode. The product ion spectra of $[M\text{-}H]^-$ ions of WAR enantiomers and d5 WAR enantiomers are shown in Fig. 01.

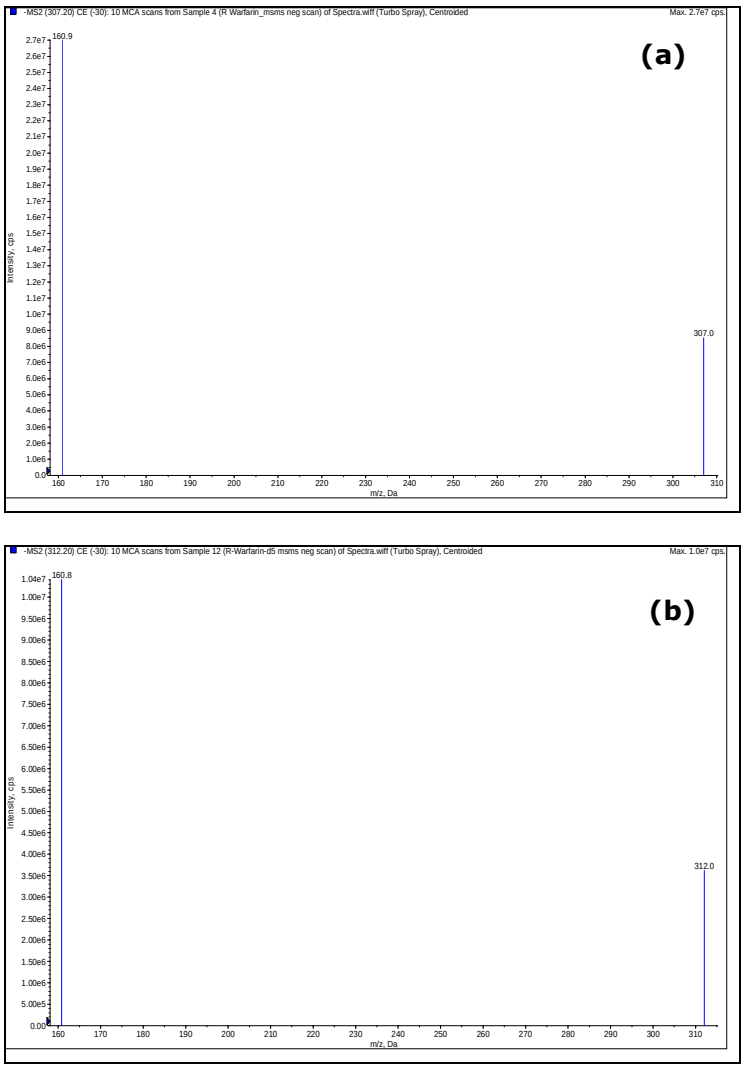

Figure 1: Representative Product ion spectra of $[M-H]^-$ (a) WAR enantiomers and (b) d5-WAR enantiomers.

Selectivity of the method was assessed by comparing the chromatograms of blank plasma samples from 10 different sources with the corresponding LLOQ samples. Typical chromatograms of a blank plasma sample, a blank plasma sample spiked with *R*-WAR and *S*-WAR at LLOQ and respective IS are shown in Fig. 02 and Fig. 03. Percent interference observed was less than 2.00% and 0.03% at RT and MRM of WAR enantiomers and d5 WAR enantiomers, respectively. Whereas, cross selectivity exercise showed 2.03% contribution of *R*-WAR at the retention time and MRM of *S*-WAR and 1.11% for the *vice versa.*

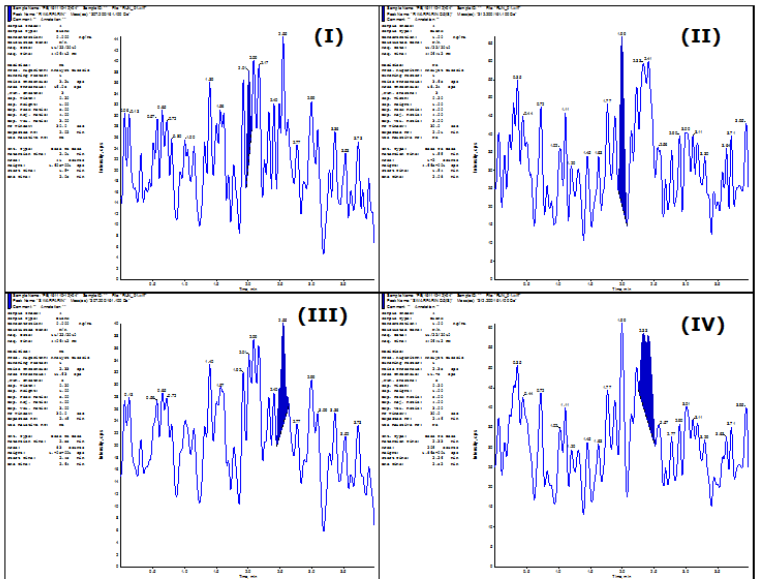

Figure.2: Representative MRM chromatograms of Blank plasma samples of (I) *R*-WAR, (II) *R*-d5-WAR (IS), (III) *S*-WAR and (IV) *S*-d5-WAR (IS) in human plasma.

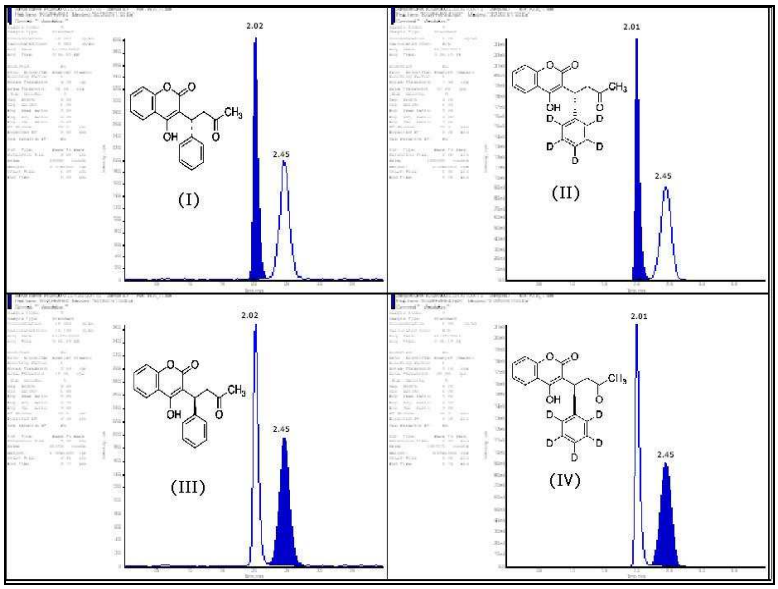

Figure.3: Representative MRM chromatograms of blank human plasma sample spiked with (I) *R*-WAR (10.0ng/mL), (II) *R*-d5-WAR (5.0 µg/mL) (IS), (III) *S*-WAR (10.0ng/mL) and (IV) *S*-d5-WAR (5.0 µg/mL) (IS)

Carry Over: Carry over was evaluated at less than 1.52% and 0.04% at RT and MRM of WAR enantiomers and d5 WAR enantiomers, respectively, with 2µL injection volume, which shows that the rinsing solution of Acetonitrile-Water (50:50 v/v) is good enough to clean the injection needle and port.

Linearity and lower limit of quantification: The linearity was evaluated based on the average of eight calibrators analyzed on three separate days. Acceptable linearity was achieved in the range of 10.0–800.0 ng/ml for *R*-WAR and *S*-WAR. For *R*-WAR, the slope was 0.0012 with an intercept of 0.00374. A slope

of 0.0053 with an intercept of 0.0041 was determined for *S*-WAR. The correlation coefficients (R^2) for both enantiomers were greater than 0.995 in all validation batches.

LLOQ samples (*N*=6) were analyzed in each validation run to evaluate sensitivity in a robust manner. The validated assay utilized an LLOQ of 10.0ng/ml for *R*-WAR and *S*-WAR and resulted in a signal-to-noise ratio (S/N) of approximately 135 and 95 for both, respectively. A typical LC–MS/MS chromatogram of the LLOQ sample is shown in Fig. 3. Reliable precision (RSD% ≤5.5) and accuracy (%≤105.8) for *R*-WAR was obtained. Similarly, reliable precision (RSD% ≤7.2) and accuracy (%≤102.6) were obtained for *S*-WAR (Table 1).

Table No. 01: Precision and accuracy of quality control samples of WAR enantiomers

	LLOQ		LQC		MQC		HQC	
	***R*-WAR**	***S*-WAR**	***R*-WAR**	***S*-WAR**	***R*-WAR**	***S*-WAR**	***R*-WAR**	***S*-WAR**
Day 1								
N	6	6	6	6	6	6	6	6
Mean	10.654	10.506	28.437	29.205	361.446	362.038	572.546	574.403
RSD%	3.2	1.4	2.5	2.4	1.8	1.3	1.3	2.6
Accuracy %	106.5	105.1	94.8	97.4	103.3	103.4	95.4	95.7
Day 2								
N	6	6	6	6	6	6	6	6
Mean	11.110	10.822	29.281	29.796	355.434	357.699	562.775	585.304
RSD%	2.4	6.4	3.3	1.4	1.3	1.1	1.2	1.3
Accuracy %	111.1	108.2	97.6	99.3	101.6	102.2	93.8	97.6
Day 3								
N	6	6	6	6	6	6	6	6
Mean	9.990	9.446	26.799	27.439	343.705	339.377	540.640	540.742
RSD%	4.3	3.7	1.3	1.3	1.3	0.7	2.0	1.5
Accuracy %	99.9	94.5	89.3	91.5	98.2	97.0	90.1	90.1
Interday								
N	18	18	18	18	18	18	18	18
Mean	10.585	10.258	28.172	28.813	353.528	353.038	558.653	566.816
RSD%	5.5	7.2	4.5	4.0	2.6	3.0	2.8	3.9
Accuracy %	105.8	102.6	93.9	96.0	101.0	100.9	93.1	94.5

Precision and Accuracy: The back-calculation results for all calibration standards showed ≤7.1% RSD and 91% to 112% accuracy for *R*-WAR and ≤9.0% RSD and 92% to 112% accuracy for *S*-WAR for all three validation curves as summarized in Table 2. The precision and accuracy of the method was determined by analyzing six replicates of QC samples at low (30.0 ng/ml, LQC), medium (350.00 ng/ml, MQC), and high levels (600.0 ng/ml, HQC) for *R*-WAR and *S*-WAR in three separate batches, Table 1. For *R*-WAR, the precision was in the range of 2.6–4.5% RSD and the accuracy was in the range of 93–101% over the three concentration levels evaluated in all the three batches. The precision and accuracy of the LQC, MQC, and HQC for *S*-WAR was in the range of 2.8–4.0% RSD and 94–101%, respectively, over these batches. These results demonstrate that the method provides excellent precision and accuracy.

Table No. 02: Precision and accuracy of calibration standards of WAR enantiomers

ng/mL	**10.00**	**20.00**	**50.00**	**100.00**	**200.00**	**400.00**	**650.00**	**800.00**
***R*-WAR**								
N	3	3	3	3	3	3	3	3
Mean	9.688	20.673	52.993	111.658	207.707	402.945	612.063	730.562
RSD%	0.7	0.1	1.7	7.1	0.8	2.8	2.6	4.2
Accuracy %	96.9	103.4	106.0	111.7	103.9	100.7	94.2	91.3
ng/mL	**10.00**	**20.00**	**50.00**	**100.00**	**200.00**	**400.00**	**650.00**	**800.00**
***S*-WAR**								
N	3	3	3	3	3	3	3	3
Mean	9.731	20.520	53.074	111.567	206.356	401.355	616.720	740.575
RSD%	1.7	2.9	1.0	9.0	0.9	2.8	1.1	1.3
Accuracy %	97.3	102.6	106.1	111.6	103.2	100.3	94.9	92.6

Recovery and Matrix Effect: The mean absolute recoveries of *R*-WAR determined at 30.00, 350.00 and 600.00 ng/mL were 87.4%, 113.5% and 95.7%, respectively. The mean absolute recoveries of *S*-WAR determined at 30.00, 350.00 and 600.00 ng/mL were 74.8%, 96.0% and 82.9%, respectively. The mean absolute recovery of *R*-d5-WAR and *S*-d5-WAR were 100.8% and 85.0%, respectively (Table 3).

Table No. 03: Results for Recovery

Analyte	N	Mean (Peak Area)		SD		RSD		Recovery
		Samples[A]	Samples[B]	Samples[A]	Samples[B]	Samples[A]	Samples[B]	
LQC	6							
R-WAR		41002	35760	1524.5	955.6	3.7	2.7	87.4
S-WAR		51357	38437	560.6	834.0	1.1	2.2	74.8
R-d5-WAR		1050335	1006290	28922.3	21731.9	2.8	2.2	95.9
S-d5-WAR		1182400	941987	19946.4	23857.5	1.7	2.5	79.7
MQC	6							
R-WAR		335982	381033	5191.0	6767.3	1.5	1.8	113.5
S-WAR		439372	421909	4462.7	7905.5	1.0	1.9	96.0
R-d5-WAR		940929	932434	8836.7	16475.2	0.9	1.8	99.1
S-d5-WAR		1101968	926897	16885.6	16745.4	1.5	1.8	84.1
HQC	6							
R-WAR		618219	591501	5633.3	6533.5	0.9	1.1	95.7
S-WAR		824686	683250	11754.7	13938.8	1.4	2.0	82.9
R-d5-WAR		860799	923687	12590.0	18586.7	1.5	2.0	107.3
S-d5-WAR		1035826	945413	9387.4	11500.5	0.9	1.2	91.3

Note: [A]Samples spiked with pure standards solutions of both enantiomeric WAR and d5 WAR pairs in mobile phase (neat samples). [B]Extracted human plasma samples spiked with pure standards solutions of both enantiomeric WAR and d5 WAR pairs.

Minimal matrix effect for *R*-WAR and *S*-WAR was observed from the six different plasma lots tested. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 2.17% for *R*-WAR and 2.04% for *S*-WAR. For the internal standard the RSD of the area ratios over both LQC and HQC levels was less than 4.28% and 3.83% for *R*-d5-WAR and *S*-d5-WAR respectively. This indicated that the extracts were "clean" with no co-eluting compounds influencing the ionization of the analyte and the internal standard.

Dilution integrity: The dilution integrity of the method was determined by analyzing six replicates of DIQC samples (1350.00ng/ml) for *R*-WAR and *S*-WAR after diluting for ½ and ¼ times with blank plasma. For *R*-WAR, the precision was 3.39% and 1.97% RSD and the accuracy was 92.9% and 97.2% over the two dilution levels, respectively. For *S*-WAR, the precision was 0.86% and 1.82% RSD and the accuracy was 93.3% and 96.8% over the two dilution levels, respectively. Results show that samples with concentration greater than the upper limit of the standard curve could be quantified with reliable accuracy after being diluted with blank matrix.

Stabilities: Plasma stability data is shown in Table 4. Stock solutions of WAR enantiomers were stable for 22 h at room temperature and 7 days at refrigerated temperature. Bench top and autosampler stability for *R*-WAR and *S*-WAR revealed that WAR enantiomers were stable in plasma for at least 23 h at room temperature and processed samples were stable for at least 53 h in auto sampler at 10°C. It was confirmed that repeated freezing and thawing (five cycles) of spiked plasma samples at LQC and HQC level did not affect the stability of WAR enantiomers and were found stable for minimum five freeze and thaw cycles. The long term stability results also indicated that WAR enantiomers were stable in human plasma for up to 74 days at a storage temperature of -20°C.

Table No. 04: Stability of WAR enantiomers under various conditions

	LQC		HQC	
	***R*-WAR**	***S*-WAR**	***R*-WAR**	***S*-WAR**
	(30.0 ng/mL)	**(30.0 ng/mL)**	**(600.0 ng/mL)**	**(600.0 ng/mL)**
Bench top stabiliy (room temperature, 23 h), *N*=6				
Mean	27.6	28.9	570.3	577.0
RSD%	2.7	4.4	3.0	2.0
Accuracy%	91.9	96.3	95.0	96.2
Freeze-Thaw stability (5 Cycles, -20°C), *N*=6				
Mean	28.3	28.9	558.7	563.4
RSD%	2.8	3.2	2.7	2.0
Accuracy%	94.2	96.3	93.1	93.9
Autosampler stability (10°C, 53 H), *N*=6				
Mean	27.1	28.3	546.6	539.6
RSD%	4.8	5.5	1.4	1.4
Accuracy%	90.2	94.3	91.1	89.9
Long term stability (-20°C, 74 D), *N*=6				
Mean	28.4	28.3	569.4	570.3
RSD%	2.2	3.1	1.6	2.2
Accuracy%	94.5	94.2	94.9	95.0

Application of the method in healthy human subjects and Incurred sample results:

The validated method was successfully applied for the assay of WAR enantiomers in 10 healthy, Indian male and female subjects. Fig. 4 shows the plasma concentration vs. time profile for WAR enantiomers under fasting condition. Table 5 summarizes the mean pharmacokinetic parameters after oral administration of 5mg tablets of WAR test and reference formulation. About 660 samples including the calibration and QC samples along with subject samples were analyzed during a period of 3 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The C_{max}, T_{max} and AUC_{0-120} for both the enantiomers obtained in the present work were comparable with the available literature. No statistically significant differences were found between the two formulations in any parameter. The ratios of mean log-transformed parameters (C_{max} and AUC_{0-t}) and their 90% CIs were all within the defined bioequivalence range of 80–125%. These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. The % change for assay reproducibility in 10% incurred samples was within ±10.0% for both the enantiomers. This authenticates the reproducibility of the proposed method.

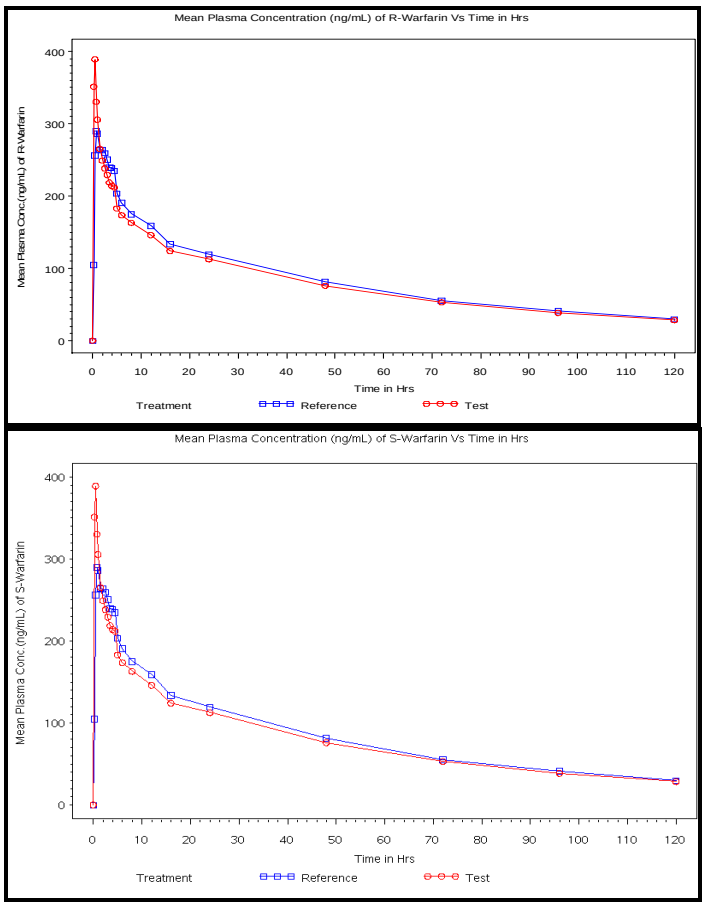

Figure.4: Mean plasma concentration-time profile of WAR enantiomers after oral administration of test (5mg of Warfarin tablet from a Generic company) and a reference (Coumadin 5 mg tablets of Bristol Mayers Squibb GmbH & Co. KGaA, Munchen, Germany) formulation to 10 healthy volunteers.

Table No. 05: Mean pharmacokinetic parameters following 5mg oral dose of Warfarin test and reference formulation to 10 healthy Indian subjects under fasting condition.

	R-WAR		*S*-WAR	
	Test	**Reference**	**Test**	**Reference**
	Mean±SD	**Mean±SD**	**Mean±SD**	**Mean±SD**
C_{max} (ng/mL)	408.34±58.39	396.22±61.27	406.29±59.56	384.52±68.42
T_{max} (h)	0.43±0.17	1.12±1.17	0.46±0.15	1.23±1.02
AUC_{0-120} (h*ng/mL)	13245.45±2224.57	13902.43+2302.06	7593.34±2342.76	8012.85±2096.88
AUC_{0-inf} (h*ng/mL)	24482.56±5764.12	24962.77±4893.93	12256.65±7801.42	12486.32±5446.87
K_{el} (h^{-1})	0.01±0.002	0.01±0.002	0.02±0.004	0.02±0.004
$t_{1/2}$	60.53±10.53	61.25±10.53	49.28±15.26	49.93±16.29
AUC_{0-t}/AUC_{0-inf} Ratio	73.02±5.73	73.61±5.62	81.72±7.13	81.95±6.84

Where, Cmax: maximum plasma concentration; Tmax: time point of maximum plasma concentration; AUC_{0-120}: area under the plasma concentration–time curve from 0 h to 120 h; SD: standard deviation; K_{el}: Elimination rate constant; $t_{1/2}$: Elimination half life

CONCLUSION

A simple, selective and rapid method for the simultaneous estimation of WAR enantiomers in human plasma was developed and validated using high-performance liquid chromatographic separation and electrospray ionization tandem mass spectrometric detection in negative mode. The validated method can be applied to pharmacokinetic studies for simultaneous estimation of WAR enantiomers. This method is an excellent analytical option for rapid and simultaneous quantification of WAR enantiomers in human plasma.

The baseline separation of the enantiomers was achieved within 4.0 min using a Chiralpak AS-3R column in the reversed-phase mode at simple isocratic LC

conditions. The sample preparation using SPE was straightforward, simple, and easy for automation, thereby enabling a high throughput capability for analyzing WAR enantiomers while providing very clean samples for bioanalytical assays. The bioanalytical assay yields highly reproducible chromatographic and statistical results when quantifying enantiomeric WAR and provides an accurate and precise format for analyzing subject samples obtained from clinical studies.

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