IJPSR (2022), Volume 13, Issue 10 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 16 February 2022; received in revised form, 28 March 2022; accepted, 03 May 2022; published 01 October 2022

NOVEL LIQUID CHROMATOGRAPHY / TANDEM MASS SPECTROMETRY METHOD OF WARFARIN IN BUFFERED RAT PLASMA AND ANALYSIS PLASMA PROTEIN BINDING ASSAY SAMPLES AS ITS APPLICATION

Ravi Akki Reddy *, Pratima Srivastava, Sudhir Tiwari, Sachin Arjun Atole and Prabhakara Reddy Kolli

Aragen Life Sciences Pvt. Ltd, Bommasandra Jigani Link Road, Jigani, Bangalore - 562106, Karnataka, India.

Keywords:

Plasma protein binding, LC-MS/MS, Rat plasma, Method validation

Correspondence to Author:
Ravi Akki Reddy

Aragen Life Sciences Pvt. Ltd, Bommasandra Jigani Link Road, Jigani, Bangalore - 562106, Karnataka, India.

E-mail: ravi.akkireddy@aragen.com

ABSTRACT: Sensitive and selective liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was developed and validated for warfarin (WAR) in buffered SD (Sprague Dawley) rat plasma (PBS and Plasma: 50:50, v/v) using propranolol (PRP) as internal standards (IS) with the Plasma protein binding (PPB) assay samples to a determination of free and total warfarin. Plasma protein precipitation extraction technique with acetonitrile containing internal standard was used for extraction of the analyte. A generic gradient method with a short run time of 2.0 min was developed for the analysis of warfarin with a C18 analytical column (Synrgi 4 µm Fusion-RP, 50X2 mm), mobile phase composed of aqueous phase: 0.1% formic acid containing Milli-Q-water and Organic Phase 0.1% formic acid containing acetonitrile. Detection was performed on a triple quadrupole mass spectrometer that employed the Positive electrospray ionization (ESI) technique, operating in multiple reaction monitoring (MRM), with the transitions of m/z 309.2-163.1. The analysis was carried out over a linear concentration range of 1–1200 nM, where the 0.1% of the free fraction of the compound was able to quantify by this method, which resulted in successfully quantifying the tightly bound compounds; the method was validated following the FDA guidelines[5] for bioanalytical method. All obtained recoveries were higher than 95.0%, while the accuracy was 88.14–113.05%, and the relative standard deviation was below 10.0%. Successfully developed LC-MS/MS method used to analyze the plasma protein binding assay samples. Where WAR is the standard idle compound for plasma protein binding, it shows species-specific % free fraction.

INTRODUCTION: Warfarin is an anticoagulant medication. It's often used to prevent blood clots, including deep vein thrombosis and pulmonary embolism and strokes in persons with atrial fibrillation, valvular heart disease, or prosthetic heart valves [1, 2, 3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.13(10).4205-13

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.13(10).4205-13

There are two types of drugs in blood: bound and unbound. A fraction of a drug may get bound to plasma proteins while the rest remains unbound, depending on the drug's affinity for plasma proteins. If protein binding is reversible, a chemical equilibrium between bound and unbound states will occur [4, 5].

WAR is highly binding (> 98%) to rat plasma proteins [6], having the species-specific binding nature; in addition, it has a high degree of reproducibility, making it to opt as a control compound for PPB assay. Protein binding nature

varies with species as follows Human>Rat>dog> mice [7].

Majority of the drugs are bound to proteins in the plasma. The extent to which the drugs bind to the proteins varies significantly. The degree of drug-protein binding may have an impact on the efficacy of a medication. Both plasma proteins and tissue proteins can bind to drugs. The pharmacokinetics (PK) of a drug can be affected by plasma protein binding in a variety of ways [8]. A significant factor of drug disposal is drug binding to plasma proteins. Major plasma proteins, glycoproteins, albumins, and globulins responsible for bound. The most prevalent protein in blood plasma is albumin. Many medications do not reach the site of action in time to interact with the target tissues. Furthermore, the bound drug is retained in the bloodstream, whilst the unbound (free) portion is processed or eliminated. The binding has a significant impact on drug pharmacodynamics as well (PD). Because only the free drug interacts with receptors, it can only have a therapeutic impact. Human serum albumins are common blood proteins that bind medicines, and lipoproteins bind to albumins in large amounts [9, 10, 11].

Chemical Structures:

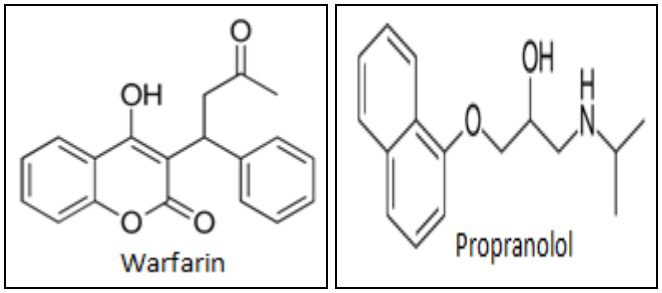


FIG. 1: CHEMICAL STRUCTURES OF WARFARIN AND PROPRANOLOL

The overall objective of the research was to establish the warfarin validated method in the PPB assay [11, 12, 13, 14] conditions; warfarin is exhibited highly binding (> 98%) [6] to rat plasma proteins [6], having the species-specific binding nature [2] in addition to these it has a high degree of reproducibility made it to opt it as idle control compound for PPB assay control.

MATERIALS AND METHODS:

Chemicals and reagents: Warfarin, Propranolol HCL, Formic acid and PBS tablets were procured from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methanol were procured from JT baker (USA) all chemicals were LC-MS grade or of the highest purity available; rat Plasma was an in-house animal facility (Aragen life sciences).

Equipment: Chromatographic analysis was carried out using an ExionLC HPLC system (ABSCIEX, Canada). Mass spectrometry was carried out using a triple quadrupole SCIEX QTRAP 6500 + (ABSciex, Canada), thermo mixer make of Thermofisher Scientific (Waltham, Massachusetts, United States), Mix mate and Centrifuge made of Eppendorf (Hamburg, Germany).

Stock and Working Solutions: 10 mM master stock solutions were prepared in the 2 mL vials by weighing 3.5 mg of the warfarin in DMSO solvent, then diluted with methanol to obtain a final concentration of 1mM secondary stock from secondary working stock, a series of WAR calibration and quality control (QC) spiking solutions were prepared in glass vials by diluting the diluting solution (methanol: water; 50:50 (v/v)). Neat calibration standards were prepared at 25, 50, 312.5, 2100, 5250, 15000, 24000, and 30000nM (nanomolar) by adding 20 µL of each neat calibration solution to 480 µL of rat pooled plasma. QC samples were prepared at 25.92 Lower limits of quantification (LLOQ), 115.2 Low-quality control (LQC), 14400 Middle-quality control (MQC), and 24000 High-quality control (HQC) nM by adding 100 µL of QC neat solution to 2400 µL of rat plasma and stored at -80 ºC. Preparations were scaled up or down as required. Same were aliquoted 35 µL in individual vials and stored at -80 ºC until analysis.

Internal Standard (IS) Preparation: 1.0 mg/mL of master stock solutions were prepared in the 2 mL vials by weighing 1.2 mg of the propranolol in DMSO solvent from master stock to 50 ng/mL in the acetonitrile.

LC conditions: A generic gradient methodology used for chromatographic separation. The stationary phase was Fusion C18 with 4 µm particle diameter (Phenomenex, USA). The stationary phase column dimensions were 50 × 2.0 mm. The mobile phase flow rate was 0.8 mL/min with a split ratio of 1:1 to the ionization source.

The mobile phase consisted of 0.1% formic acid in water as aqueous component and 0.1% formic acid acetonitrile as an organic modifier.

A generic gradient LC method (time (min) /% B = 0.00/5, 0.3/5, 1.0/90, 1.4/90, 1.5/5, 2.0/5) with a short run time of 2.0 min was developed for the analysis of WAR in plasma samples. The column and autosampler were maintained at 40 ºC and 8 ºC, respectively.

MS/MS Conditions: QTRAP 6500+ mass spectrometer was used for the analysis, the Turbo Ion spray source was operated with typical settings as follows: Ionisation mode-Positive; Curtain gas-40 psi; Nebuliser gas (GS1)- 55 psi; Heater gas (GS2)- 65 psi; Ion spray voltage- 5500V; Temperature- 550ºC. The mass spectrometer was set up to perform in MS/MS mode and to run in MRM scan mode. The molecular ions of WAR and PPL were formed using the declustering potentials (DP) 80V and 120V, respectively.

In MRM mode, the most abundant molecular ions were selected at m/z 309.2 and was fragmented to m/z, 163.10 at a collision energy of 21V with 12 CAD gas setting. Molecular ion m/z, 260.10 of PPL was fragmented to m/z, 183.10 at a collision energy of 26V with 12 CAD gas settings. Peak areas for all components were automatically integrated using Analyst software version 1.7.2.

Sample Preparation: The plasma protein precipitation extraction technique used for the sample preparation procedure as follows, 25 µL of plasma samples/Standards/Quality controls samples were aliquoted into a 96-well plate and 25 µL of Phosphate buffer saline (equal quantity for matrix matching) mixed for 10 seconds at 1000 RPM on mix mate, followed by 200 µL of acetonitrile containing internal standard added. The samples were vortex for 5 min at 1000 RPM, then centrifuged at 4000 RPM for 15 min at 4 ºC; from centrifuged samples, 100 µL of the supernatant was transferred into a new 96 well plate (LC-MS/MS-loading plate) and added equal quantity of 100 µL of acetonitrile: water; 50:50(v/v), mixed well and loaded on LC-MS/MS for analysis.

RESULTS AND DISCUSSION:

Bioanalytical Method Validation: The Food and Drug Administration (FDA) guidelines [6] for the bioanalytical method 5 were followed for complete validation of the developed method by calculating all the validation parameters as follows:

Selectivity: The selectivity of the developed method was tested by screening six different batches of blank rat plasma.

The peak area of blank plasma samples was measured and compared to samples of blank plasma spiked with the WAR at their LLOQ levels, observed the results within the range. Blank and zero calibrators should be free of interference at the retention times of the analyte should be ± 20% LLOQ, and the IS should not exceed 5% of the average IS responses of the calibrators and QCs.

Carryover: The carryover after the ULOQ has assessed the effect of carryover on unknown samples concentrations by injecting the Bank, LLOQ, ULOQ, and Blank. Carryover was observed within the acceptable range. Carryover should not exceed 20% of LLOQ.

Sensitivity: The sensitivity checked by injecting the LLOQ of the calibration curve defines the sensitivity (LLOQ). The signal-to-noise ratio was observed to be > 8.

The analyte response at the LLOQ should be ≥ five times the analyte response of the zero calibrators, accuracy should be ± 20% of nominal concentration, and precision should be ± 20% CV.

Linearity and Range: The calibration curves were linear in the range of 1–1200 nM for WAR. The blank and zero samples were used to verify the absence of interference but were excluded from the regression analysis. The regression equations were as follows:

Linearity Calculations: Y = mx + c WAR, Y = 0.00112X − 0.000463, r = 0.9972 and SD = 6.272, Y = Peak area ratio of the analyte to the internal standard, X=Concentration of the analyte in nM. The r values, slopes, and intercepts were calculated utilizing the linear regression 1/X2 analysis. 75% and a minimum of six non-zero calibrator levels and ± 15% of nominal concentrations, except at LLOQ where the calibrator should be ± 20% of the nominal concentrations should meet the above criteria in each batch validation run.

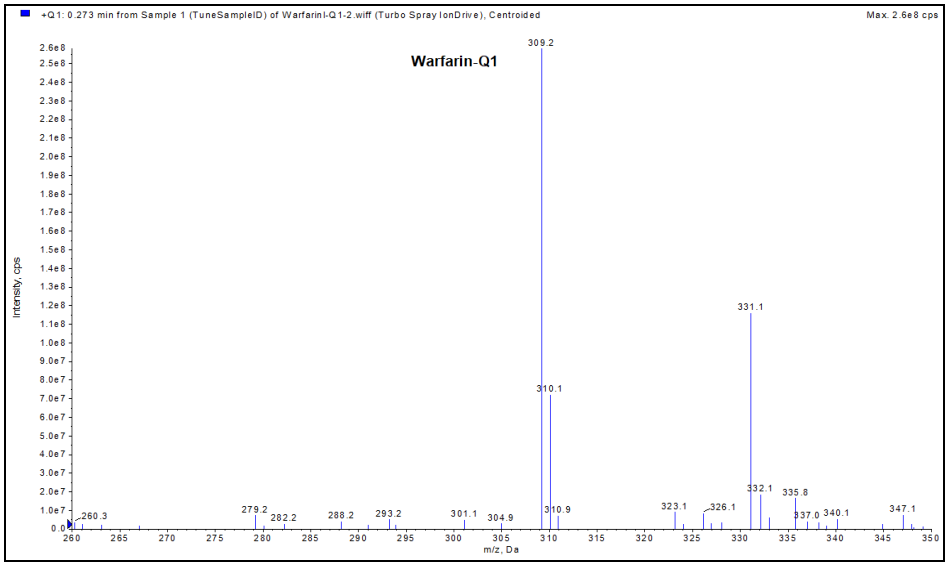


FIG. 2: REPRESENTATIVE WARFARIN PARENT (Q1: M+H) + ION MASS SPECTRA

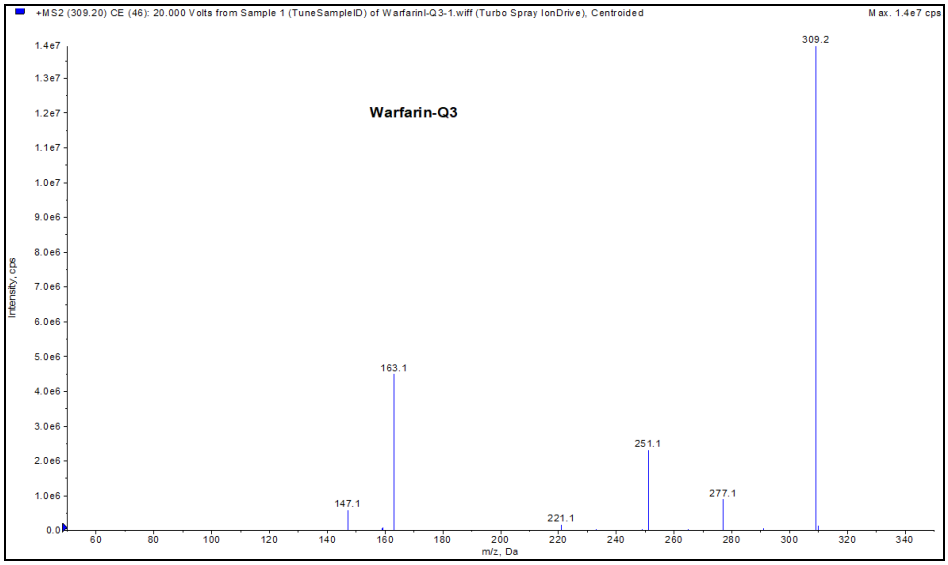


FIG. 3 REPRESENTATIVE WARFARIN PRODUCT (Q3) ION MASS SPECTRA

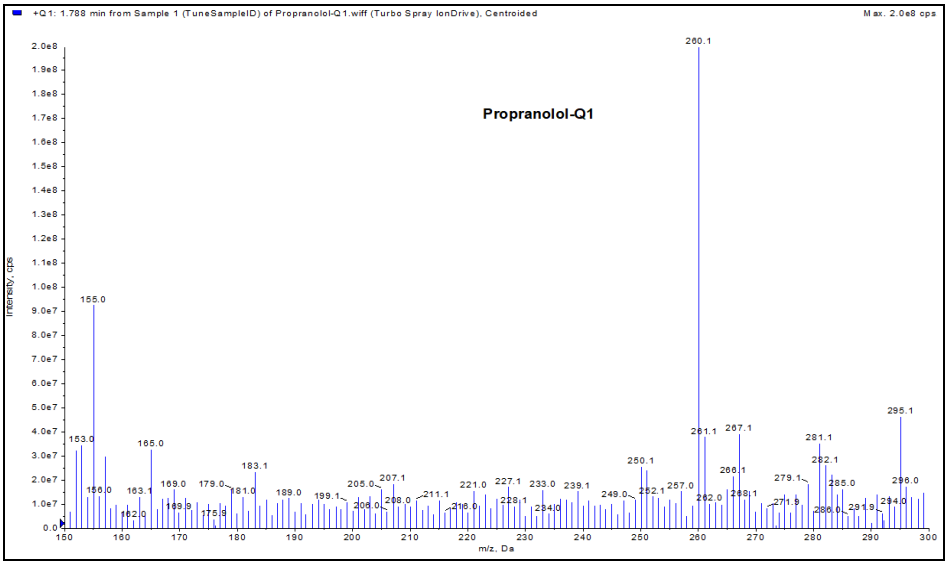


FIG. 4: REPRESENTATIVE PROPRANOLOL PARENT (Q1: M+H)+ ION MASS SPECTRA

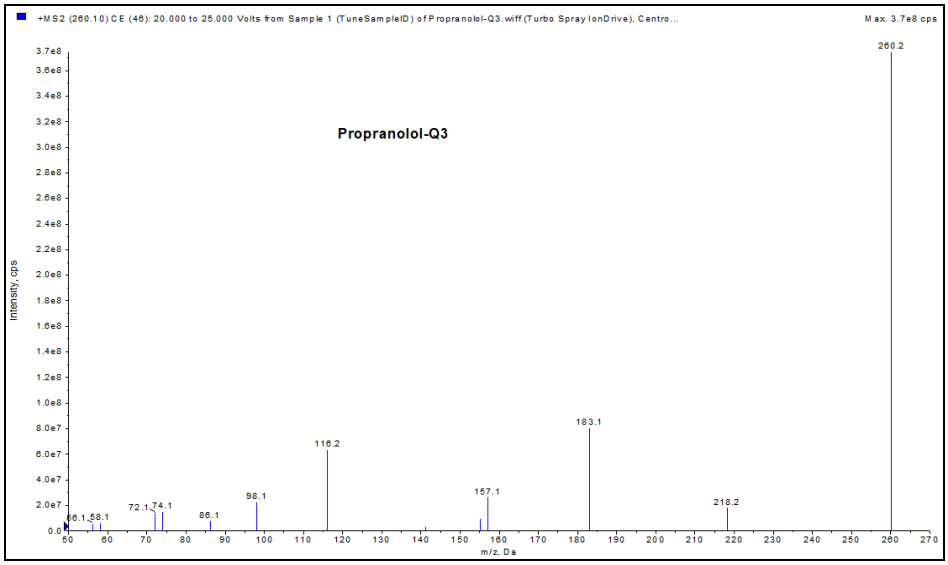


FIG. 5 REPRESENTATIVE PROPRANOLOL PRODUCT (Q3) ION MASS SPECTRA

TABLE 1: LC-MS/MS PARAMETERS SELECTED FOR THE QUANTIFICATION OF WARFARIN USING PROPRANOLOL AS AN INTERNAL STANDARD

Analyte	Q1	Q3	DP(V)	EP(V)	CE(V)	CXP(V)
Warfarin	309.2	163.1	80	10	21	10
Propranolol	260.1	183.1	120	10	26	10

Q1: Parent ion; Q3: Product ion; DP: Declustering potential; EP: Entrance potential; CE: Collision energy; CXP: Cell exit potential.

Accuracy and Precision: Inter-day and intraday accuracy and precision were evaluated at four different concentrations levels (LLOQ, LQC, MQC, and HQC) using six replicates for both analytes analyzed over 3 days. The accuracy and precision were determined and expressed in percentage accuracy and coefficient of variation (%CV), respectively. Acceptance criteria are ± 15% of nominal concentrations, except ± 20% at LLOQ**.** The examination of the spiked plasma samples revealed that the assay's intraday accuracy has varied between 90.78% and 111.88%, with a precision (%CV) in the range of 2.60–11.48% for WAR, **Table 2.**

TABLE 2: INTRADAY AND INTER DAY WARFARIN ACCURACY AND PRECISION OF QUALITY CONTROL SAMPLES IN RAT PLASMA

Batch ID	Parameter	Concentrations in nM			
		LLOQC	LQC	MQC	HQC
Intraday-Batch-1	Nominal Concentration (nM)	1.037	4.608	576.000	960.000
	Mean Calc. Concentration (nM)	1.160	4.291	522.881	928.016
	% Accuracy	111.88	93.13	90.78	96.67
	% CV	9.08	6.32	3.45	2.60
Intraday-Batch-2	Mean Calc. Concentration (nM)	1.010	4.231	567.007	958.403
	% Accuracy	97.40	91.81	98.44	99.83
	% CV	5.38	4.00	4.05	3.81
Intraday-Batch-3	Mean Calc. Concentration (nM)	1.098	4.281	544.121	976.835
	% Accuracy	105.88	92.90	94.47	101.75
	% CV	9.63	7.77	5.54	8.07
Inter day	Mean Calc. Concentration (nM)	1.052	4.381	562.005	971.646
	% Accuracy	101.48	95.07	97.57	101.21
	% CV	11.48	8.30	4.79	7.34

Recovery: The recoveries of WAR and IS were calculated at the three QC levels (six replicates). As the recovery describes the efficiency of the extraction of analytes from the matrix samples, thus, the results are shown in **Table 3** prove the efficiency of the extraction protocol introduced by the proposed method, where the recoveries of the analyte were satisfactory and consistent.

The mean of the recovery values was 98.48% with precision (RSD%) 2.71 for WAR, while is recovery values were 99.84% with precision (RSD%) 2.23.

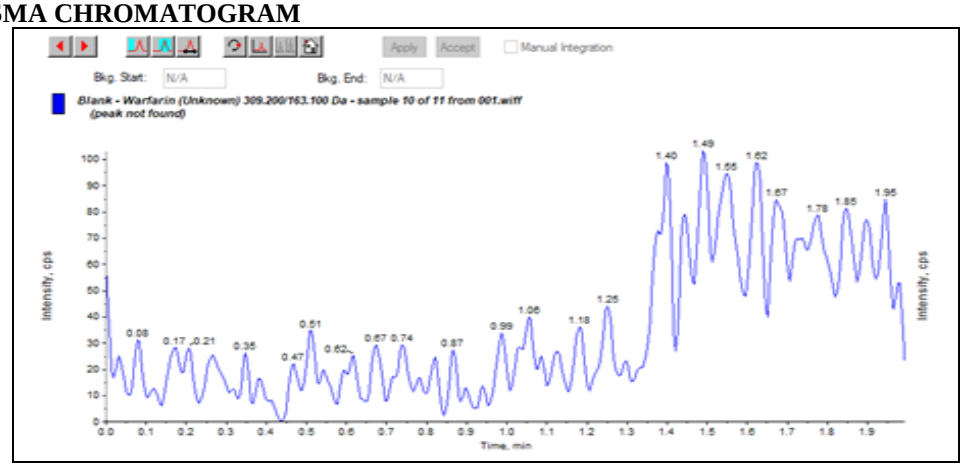
Plasma recovery samples were spiked with WAR at LQC, MQC, and HQC levels, extracted, and spiked with is post-extraction. Reference samples were prepared by spiking WAR and are into blank plasma post-extraction. Is recovery was performed in an analogous way. Recovery was calculated as follows:

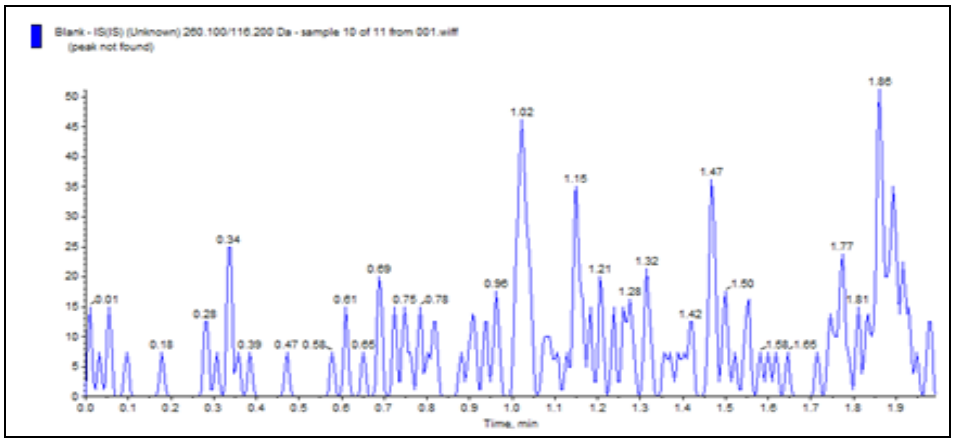
Recovery (%) = Mean peak area ratio of extracted sample / Mean peak area ratio of post extracted sample × 100 %

TABLE 3: RECOVERY DATA OF WARFARIN IN RAT PLASMA

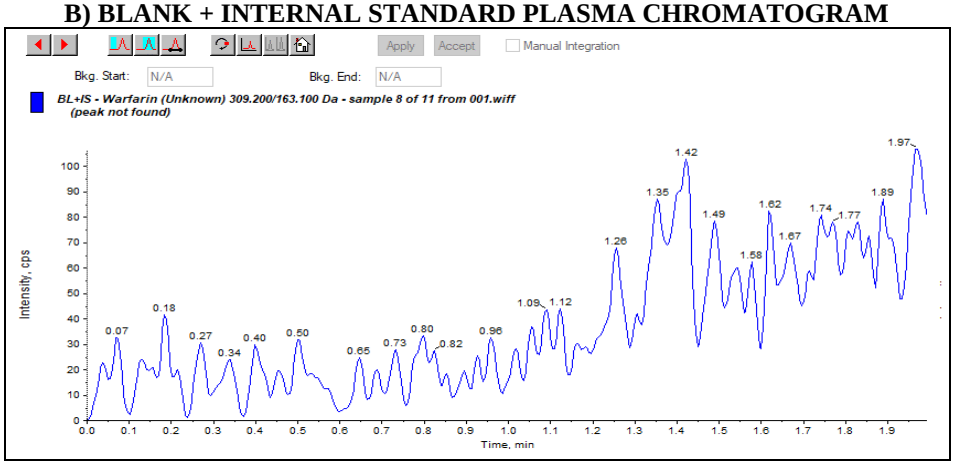
Batch ID	Parameter	Warfarin recovery at each level			
		LQC	MQC	HQC	LQC
Recovery Batch	Nominal Concentration (nM)	4.608	576.000	960.000	4.608
	Recovery (%)	98.91	97.61	98.93	98.91
	%CV	2.96	3.26	1.90	2.96

A) BLANK PLASMA CHROMATOGRAM





B) BLANK + INTERNAL STANDARD PLASMA CHROMATOGRAM



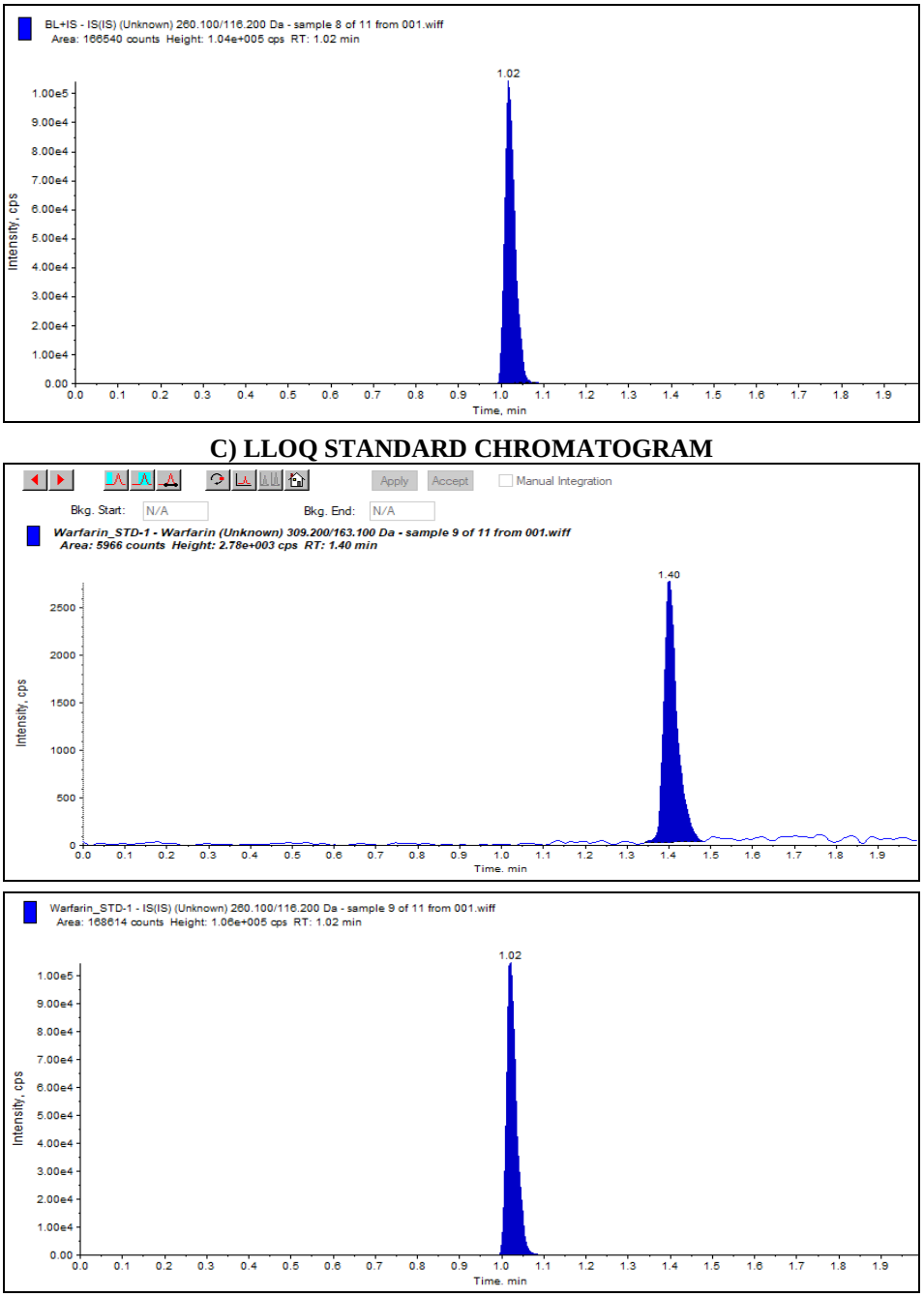


FIG. 6: REPRESENTATIVE LC–MS/MS CHROMATOGRAMS OF WARFARIN. (A) BLANK PLASMA (B) BLANK + INTERNAL STANDARD (C) 1 NM_-LLOQ CALIBRATION STANDARD

Dilution Integrity: Dilution integrity is performed to check if samples dilution would interfere with the accuracy and precision of results [6]. Quality control samples (Five and Ten times the HQC concentration) were prepared and diluted by factor of 5 and 10 with blank matrix to conduct the dilution integrity. Accuracy values for dilution integrity were found to be 92.40 and 92.68% for WAR.

Acceptance Criteria Accuracy: ± 15% of nominal concentrations, Precision: ± 15% CV.

Stability: The stability of WAR in rat plasma was assessed at LQC and HQC levels, different stability experiments processed sample stability, benchtop stability, and repeated freeze-thaw cycles;

Processed sample stability was evaluated by leaving processed QC samples in the auto-sampler at 8 °C for 1 day (24 h) followed by analysis. This study is conducted to determine the consequences of an infrequent delay in the injection of extracted samples on the analyte stability.

Freeze-thaw stability was examined by investigating the QC samples' stability through four freeze-thaw cycles after being kept to freeze for 24 h. Samples were then thawed unassisted at room temperature for 2 h or even more, then kept to freeze again at −80 °C overnight for every freeze-thaw cycle.

Benchtop stability was examined by investigating the QC samples' stability through kept on the bench for 6 h then processed along with the freshly prepared samples.

The stability of the QC samples was investigated by comparing their recoveries under the different stability conditions with those of freshly prepared samples. Samples were considered stable if the mean concentration at each QC level was within acceptable limits (±15%) with RSD% not exceeding 15%, as demonstrated in **Table 4.**

TABLE 4: STABILITY PARAMETERS SUMMARY DATA FOR THE WARFARIN

Batch ID	Parameter	Warfarin Stability	
		LQC	HQC
Auto sampler stability	Nominal Concentration (nM)	4.608	576.000
	Mean Calc. Concentration(nM)	4.428	952.350
	% CV	7.47	3.52
	% Stability	96.10	99.20
Bench top stability	Mean Calc. Concentration (nM)	4.136	949.626
	% Accuracy	4.26	4.71
	% CV	89.75	98.92
Freeze thaw stability	Mean Calc. Concentration (nM)	4.320	964.808
	% CV	9.01	4.78
	% Stability	93.74	100.50

Plasma Protein Binding Study Application: Rapid equilibrium dialysis (RED) technique was used the perform the PPB experiment; the RED insert contains donor (plasma side) and receiver (buffer side) chambers separated by semi-permeable cellulose membrane (molecular weight cut-off of 6-8 kDa), same were placed in the base plate than in donor chamber 200 µL of 1 µM spiked plasma and 350 µL of PBS was spiked into the receiver chamber of RED inserts in triplicate and incubated at 37 ºC for 5 h using thermomixer with constant shaking (450 rpm), the experiment as carried out as per the PPB protocol and at the end of incubation, 25 µL of donor and receiver samples were matrix equilibrated with opposite matrix (25 µL of plasma/buffer sample was matched with 25 µL of blank buffer/plasma).

The samples were extracted as mentioned above, and similarly calibration curve and quality control samples were prepared. Same subjected for analysis on LC-MS/MS. The following equations calculated % bound/unbound fraction:

Fu: R*100/D % Bound = 100- Fu % Recovery = 100 * (R*X + D*Y) / T0*Z % Stability = 100 * T5/ T0 fu: Unbound fraction; R: Receiver concentration; D: Donor Concentration T0: 0 min concentration; T5: 5h sample concentration X: Donor volume (200 µL), Y: Receiver volume (350 µL), Z: T0 volume (200 µL).

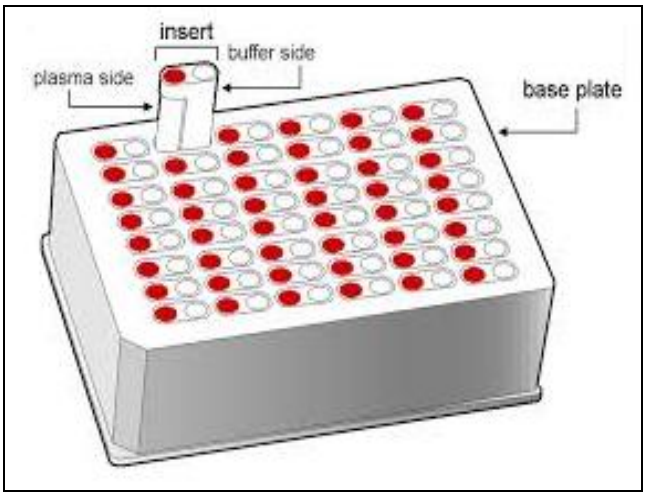


FIG. 7: REPRESENTS THE EXPERIMENT BASE PLATE AND RED INSERT DESIGN USED

TABLE 5: WARFARIN PLASMA PROTEIN BINDING DATA OF THREE DIFFERENT EXPERIMENTS

Experiment	% Fu	% Bound	% Recovery	%Stability
1	1.26	98.74	98.08	92.14
2	1.29	98.71	88.68	87.89
3	0.92	99.08	109.02	105.24

From the three experiments %Fu: 0.82 to 1,29, reported free fraction18,19 values 1.1 to 1.8.

CONCLUSION: A novel HPLC–MS/MS method was established and validated to estimate warfarin in SD rat plasma. The developed method's results were satisfactory and confirmed its selectivity, accuracy, and precision. Reliable and reproducible recoveries were obtained for the analytes and are from rat plasma, with minor interference from the matrix.

Owing to the LLOQ, the proposed method was effectively utilized to estimate plasma concentrations of warfarin in the PPB study. Where the 0.1% of free fraction of compound is able to quantify by the current developed method, which is unique to currently available quantification methods.

ACKNOWLEDGEMENT: I would like to thank Aragen life sciences Pvt Ltd, Bangalore, for conducting this study.

CONFLICTS OF INTEREST: Authors declare no conflicts of interest to declare.

REFERENCES:

1. Ageno W and Alexander S: Gallus: Ann Wittkowsky, Mark Crowther, Elaine M. Hylek. Oral Anticoagulant Therapy 10.1378/chest.11-2292 (2012).2017.5512.
2. Aiidit S, Soh YC, Yap CS, Khan TM and Neoh CF: Effect of standardized warfarin treatment protocol on anticoagulant effect. Comparison of a Warfarin Medication Therapy Adherence Clinic with Usual Medical Care: doi.org/10.3389/fphar.2017.00637
3. Haaland GS, Ragnhild S, Falk and Oddbjørn Straume: James B. Lorens: association of warfarin use with lower overall cancer incidence among patients older than 50 years. JAMA Intern Med 2017; 177(12): 1774-1780. doi:10.1001/jamainternmed.2017.55.
4. Zi-Qing-Yun Yuan, Chun Qiao, Zhi-Cheng Yang, Lei Yu, Lu-Ning Sun, Yi Quan and Xue Hui Zhang: The impact of plasma protein binding characteristics and unbound concentration of voriconazole on its. Adverse Drug Reactions: doi.org/10.3389/fphar.2020.00505.
5. Gardiner P, Cox RJ and Grime K: Plasma protein binding as an optimizable parameter for acidic drugs. Drug Metabolism and Disposition August 2019; 47(8) 865-873.
6. Grbac RT, Yerino P and Barbara JE: High content automated plasma protein binding screening and definitive assays using rapid equilibrium dialysis for drug development. Xeno Tech LLC 16825 W 116th St, Lenexa, KS 66219 USA).
7. MacFaul, Philip, Colclough, Nicola, Ruston, Linette, Wood and Matt: Species differences in drug plasma protein binding. MD-CAR-03-2014-000148.R1.
8. Karolina Wana: Biological barriers and the influence of protein binding on the passage of drugs across them 10.1007/s11033-020-05361-2 (2020).
9. Philip Gardiner, Rhona J. Cox and Ken Grime: Plasma protein binding as an optimizable parameter for acidic drugs. Drug Metabolism and Dispos 2019; 47(8): 865-873.
10. Bruno Charlier, Albino coglianese, Federica De Rosa and Ugo De Grazia: The Effect of Plasma Protein Binding on the Therapeutic Monitoring of Antiseizure.
11. Li Di: Review An update on the importance of plasma protein binding in drug discovery and development: doi/abs/10.1080/17460441.2021.1961741
12. Li Di: Review, An update on the importance of plasma protein binding in drug discovery and development: doi/abs/10.1080/17460441.2021.1961741.
13. Zayed A, Babaresh WM, Darweesh RS and El-Elimat T: Simultaneous determination of warfarin and 7-hydroxywarfarin in rat plasma by HPLC-FLD: 10.2478/acph-2020-0025
14. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CV). FDA Guidance for industry: Bioanalytical Method Validation 2013.

How to cite this article:
Akkireddy R, Srivastava P, Tiwari S, Atole SA and Kolli PR: Novel liquid chromatography / tandem mass spectrometry method of warfarin in buffered rat plasma and analyse plasma protein binding assay samples as its application. Int J Pharm Sci & Res 2022; 13(10): 4205-13. doi: 10.13040/IJPSR.0975-8232.13(10).4205-13.