Toxicology International
DOI: 10.18311/ti/2026/v33i3/55317
INFORMATICS JOURNALS
eISSN: 0976-5131 | Vol 33(3), 2026 | pp. 594-603

Assessment of Unbound Rebastinib in Human Plasma and Serum Albumin by Equilibrium Dialysis Coupled with High-resolution Orbitrap Mass Spectrometry

Suryakant Kumar[1], Bidya Bhushan[1], Bhumika Sharma[2], Balabhau Warkad[1], Rajiv Bharadwaj[3] and Anand Prakash[2]*

[1]*Department of Pharmacy, Banasthali Vidyapith, Jaipur – 304022, Rajasthan, India*

[2]*Department of Bioscience and Biotechnology, Banasthali Vidyapith, Jaipur – 304022, Rajasthan, India; anandprakash@banasthali.in*

[3]*Biopharma Division, Veeda Clinical Research Limited, Bengaluru – 560058, Karnataka, India*

Abstract

Plasma protein binding (PPB) is a critical determinant of drug pharmacokinetics, as only the unbound fraction (f_u) of a drug is available for tissue distribution, target engagement, and clearance. However, the interaction of rebastinib with plasma proteins has not been fully elucidated to date. In this study, the free fraction of rebastinib in human plasma and isolated human serum albumin (HSA) was determined quantitatively using rapid equilibrium dialysis (RED) combined with ultra-high-performance liquid chromatography (UHPLC) - Orbitrap high resolution mass spectrometry operated in parallel reaction monitoring (PRM) mode. The binding study was carried out at 37°C using the RED apparatus under optimised equilibrium conditions. Warfarin and verapamil were used as standards for the method validation. It was found that rebastinib has very high plasma protein binding (99.90 ± 0.01%; f_u = 0.001), but the interaction with albumin was much comaratively less pronounced (87.81 ± 2.32%; f_u = 0.122). Thus, albumin plays an essential role in rebastinib plasma binding, but other plasma proteins may also be involved. Overall, the developed methodology had acceptable stability, repeatability, and extraction recovery for both matrices. The extremely low unbound fraction of rebastinib implies strong protein affinity and can strongly impact its pharmacokinetic profile. As much as is feasible, the present effort endeavours to provide the first comprehensive comparative study of rebastinib interactions with plasma and HSA. It reveals valuable input data for further PBPK studies and pharmacokinetic translation.

Key Findings: Rebastinib exhibited extremely high protein binding in human plasma (99.90%), but relatively less binding was seen in isolated HSA (87.81%). Rapid equilibrium dialysis, along with UHPLC–Orbitrap HRMS, facilitated the accurate determination of rebastinib's ultra-low unbound fraction. The current work is the first comparative investigation on protein binding of rebastinib in human plasma and isolated HSA.

Keywords: Human Serum Albumin , PBPK Modelling, Plasma Protein Binding, Rapid Equilibrium Dialysis , Rebastinib, TIE2 Kinase Inhibitor, UHPLC-Orbitrap HRMS, Unbound Fraction (f_u)

1. Introduction

The extent to which a molecule binds to plasma proteins is an important determinant of drug pharmacokinetics because only the unbound fraction (f_u) is available for tissue distribution, target engagement, and clearance. Drugs with high plasma protein binding (>95%) generally show a limited apparent volume of distribution (V_d), as most of the drug remains confined to the vascular compartment. Extensive binding is also linked to a

Article Received on: 10.03.2026 **Revised on:** 20.05.2026 **Accepted on:** 03.06.2026

*Author for correspondence

prolonged elimination half-life. This occurs because only the unbound fraction is available for metabolism and excretion. Consequently, systemic clearance (CL) is often reduced or altered[1-3].

Variability in plasma protein composition is frequently observed in oncology patients and may contribute to altered exposure of highly protein-bound kinase inhibitors. Drugs with ultralow unbound fractions may be particularly sensitive to disease-associated alterations in plasma protein composition. Altered plasma protein levels associated with inflammation, hepatic dysfunction, renal impairment, or hypoalbuminemia may influence the unbound fraction of highly bound drugs[4-6]. Plasma protein binding is particularly relevant for anticancer agents because many kinase inhibitors exhibit high lipophilicity and narrow therapeutic windows. For highly bound drugs, even modest changes in the unbound fraction may substantially alter tissue exposure and pharmacological response[4]. In clinical environments, alterations in protein binding can also affect the apparent clearance rate, half-life, and possible interactions with other drugs. This becomes more significant for targeted treatments that need prolonged systemic exposure in order to inhibit kinase activity. Consequently, accurate characterisation of the unbound fraction is increasingly recognised as an important parameter during translational pharmacokinetic evaluation and dose optimization[5-7].

Rebastinib is a first-in-class switch-control TIE2 kinase inhibitor currently under clinical investigation for solid tumours and haematological malignancies[8,9]. Signalling from TIE2 contributes significantly to angiogenesis, vascular remodelling and macrophage recruitment in the tumour environment[10,11]. This compound shows effective kinase inhibition properties as well as high lipophilicity (cLogP = 5.2), implying that it could easily bind to the plasma protein[12]. Clinical studies being done on rebastinib have involved its use in conjunction with chemotherapeutics for treating metastatic breast cancers and other advanced solid tumours[13-15]. Due to its lipophilic nature as well as being aromatic, rebastinib (Figure 1) will certainly exhibit extensive binding to plasma proteins; nevertheless, the plasma protein binding properties of rebastinib have not yet been fully explored.

Despite the progression of rebastinib towards clinical evaluation, no quantified data on the plasma protein binding properties of rebastinib have yet appeared in the scientific literature. Specifically, there have been no comparisons of rebastinib binding in whole plasma and in purified serum albumin reported previously. Such information would be useful for gaining insights into the contribution of albumin compared to other plasma proteins, such as α1-acid glycoprotein and lipoproteins that might affect the extent of drug exposure in a diseased state. The rapid equilibrium dialysis (RED) technique is commonly employed in the estimation of plasma protein binding due to its ability to achieve rapid equilibration, minimise non-specific binding, and increase efficiency compared to traditional dialysis approaches. Because of its reproducibility and high accuracy, RED is currently recognised as the gold standard for PPB determination *in vitro*[16-21]. Analysis of very low unbound fractions is challenging because the amount of free drug in the buffer compartment after equilibrium dialysis[22] is low. Variability in analysis could occur due to non-specific binding of the drug to the membrane and plastics, lack of equilibrium, or matrix effect ion suppression in LC–MS analysis[23]. The above difficulties are amplified for highly hydrophobic kinase inhibitors that have high protein binding[1-3]. The use of high-resolution Orbitrap mass spectrometry in PRM mode ensures better selectivity and mass accuracy for the detection of low-abundance samples while minimising endogenous matrix interference[22-23,24].

The present study aimed to quantitatively evaluate the protein binding of rebastinib in human plasma and isolated HSA using RED coupled with UHPLC–Orbitrap HRMS. Warfarin and verapamil were included as reference compounds to assess assay performance. In addition, stability and recovery studies were performed to confirm analytical reliability. These findings provide the first comparative assessment of rebastinib binding behaviour and may facilitate future PBPK modelling and translational pharmacokinetic investigations[25].

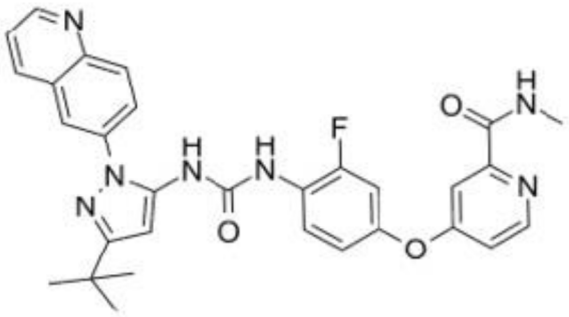


Figure 1. Structure of rebastinib.

2. Materials and Methods

2.1 Chemicals and Reagents

Rebastinib was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Verapamil hydrochloride, warfarin, and carbamazepine (Internal Standard, IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC–MS grade formic acid, water, and acetonitrile were also obtained from Sigma-Aldrich. Phosphate-buffered saline (PBS, pH 7.4) was sourced from Invitrogen (Carlsbad, CA, USA).

Pooled human plasma containing K3EDTA as anticoagulant was obtained from a certified blood bank in Bangalore, India, and stored at −80°C until use. Fatty acid-free human serum albumin (HSA, ≥96% purity) was procured from Sigma-Aldrich. A fresh 4% (w/v) HSA solution was prepared in PBS before analysis. A 10mM stock solution of rebastinib was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C until further use.

2.2 Protein Binding by Rapid Equilibrium Dialysis (RED)

Protein binding experiments were performed using Thermo Scientific Pierce™ Rapid Equilibrium Dialysis (RED) devices with an 8 kDa molecular weight cut-off membrane, previously validated for low nonspecific binding and rapid equilibration. The rapid equilibrium dialysis (RED) device used for protein binding assessment is shown schematically in Figure 2. Dialysis was carried out in a Thermo Scientific Forma Steri-Cycle CO_2 incubator with orbital shaking at 37 °C. Equilibrium time was selected based on literature reports indicating that small molecules typically reach equilibrium within 5 h under RED conditions[18,19]. Samples were analysed by UHPLC- Orbitrap HRMS (Thermo Fisher Scientific) coupled to a Shimadzu Nexera X2 system. Data were acquired and processed using Xcalibur 4.5 SP1[22].

2.2.1 Equilibrium Dialysis

RED plates were preconditioned with aqueous ethanol, followed by Milli-Q water, before use. Rebastinib and reference compounds were spiked into plasma and HSA to achieve a final concentration of 5 µM (n = 3). The plasma chamber (200 µL) contained the spiked matrix, whereas the buffer chamber (350 µL) contained phosphate-buffered saline (PBS, pH 7.4). The assembly was sealed and incubated at 37 °C with orbital shaking at 100 rpm for 5 h[19,20].

Following incubation, samples from both chambers were matrix-matched before protein precipitation to minimise matrix-related ionisation effects during LC–MS analysis[26]. Proteins were precipitated by adding 300 µL of ice-cold acetonitrile containing carbamazepine (0.17 µg/mL) as the internal standard. Samples were centrifuged at 13,000 × g for 10 minutes at 4 °C after being vortexed for two minutes. Supernatants were then moved to HPLC vials for UHPLC–HRMS analysis[22,26]. Stability and recovery experiments were performed under the same incubation conditions used for RED analysis to assess analytical reliability during the 5 h incubation period. Stability was evaluated by comparing analyte responses at 0 h and 5 h, whereas recovery was determined by comparing post-dialysis analyte responses with those of the initial plasma samples[20].

2.3 UHPLC–Orbitrap HRMS Analysis

The unbound fraction (f_u) of rebastinib was determined using rapid equilibrium dialysis (RED) coupled with UHPLC–Orbitrap high-resolution mass spectrometry based on a relative quantification approach. Quantification was performed using analyte-to-internal standard peak area ratios obtained from plasma and buffer compartments processed under identical analytical conditions[21,22]. Before analysis, each analyte was optimised on the Orbitrap HRMS by syringe infusion (2 µM) in Full MS mode. Protonated precursor ions ($[M+H]^+$) were identified and subjected to MS/MS fragmentation using optimised normalised collision energies (NCE).

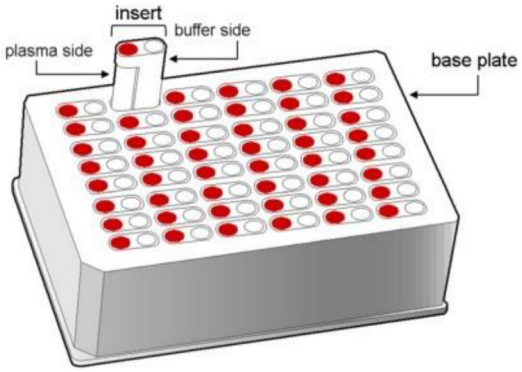


Figure 2. Schematic representation of the rapid equilibrium dialysis (RED) device used for plasma protein binding assessment. The RED insert consists of a plasma chamber and a buffer chamber separated by a semipermeable dialysis membrane, allowing equilibration of unbound drug between compartments during incubation.

The optimised precursor–product ion transitions and acquisition parameters were incorporated into the final PRM method[22]. Quantitative analysis was performed using parallel reaction monitoring (PRM) on an Orbitrap HRMS equipped with a heated electrospray ionisation source (HESI-II) operated in positive ion mode. Target precursor ions defined in the inclusion list were subjected to higher-energy collisional dissociation (HCD), and fragment ions were acquired at a resolution of 17,500 (m/z 200)[23,24]. The selected transitions were as follows: rebastinib m/z 554.2300 → 293.1392; carbamazepine m/z 237.1022 → 194.0962; verapamil m/z 455.2904 → 165.0907; and warfarin m/z 309.1122 → 251.0698. The optimised mass spectrometric parameters of rebastinib, verapamil, warfarin and carbamazepine are summarised in Table 1.

Table 1. Optimised mass spectrometric parameters of rebastinib, verapamil, warfarin and carbamazepine

Optimised Mass Spectrometric Parameters			
Parameter	**Condition**		
Mass Spectrometer	Q Exactive		
Acquisition mode	Full MS SIM		
Scan type	PRM		
Polarity	Positive		
Ion source	HESI		
Aux Gas Flow Rate	18		
Sheath Gas Flow Rate	60		
Capillary temperature	288°C		
Aux gas heater temperature	475°C		
S-lens RF level	55		
Spray voltage	3.50 kV (Positive)		
Scan range	50 to 800 m/z		
Microscans	11		
Resolution	17,500		
AGC Target	2.00E+05		
Maximum IT	100 ms		
Tune File Parameters			
Compound Name	**Q1 (Dalton)**	**Q3 (Dalton)**	**NCE (V)**
Rebastinib	554.23	293.1392	40
Warfarin	309.1122	251.0698	35
Verapamil	455.2904	165.0907	35
Carbamazepine	237.1022	194.0962	40

Chromatographic separation was achieved using a C18 column maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The optimized mobile phase gradient program was employed for analysis. The run time was 5 min, the injection volume was 4 µL, and the flow rate was 0.7 mL/min.

Chromatographic conditions are summarised in Table 2.

Table 2. Chromatographic parameters of rebastinib, verapamil, warfarin and carbamazepine

Analytical column	:	Synergi 4 µm Fusion-RP 80 Å,2 mm x 50 mm
Program type	:	Gradient
Mobile phase A	:	0.1% formic acid in LCMS grade water
Mobile phase B	:	0.1% formic acid in acetonitrile
Flow rate	:	700 µL/min
Sample Extraction technique	:	Precipitation with acetonitrile
Retention time	:	Rebastinib, ~ 3.24 min
		Warfarin ~ 3.10min
		Verapamil: ~ 2.71 min
		Carbamazepine: ~2.70min
Total Run time	:	4 min
Volume of injection	:	4 µL
Column Temperature	:	40 °C
Auto sampler Temperature	:	10 °C

Gradient Program for Rebastinib and Positive Controls/Reference Compounds			
Total Time (min)	**Pump A %**	**Pump B %**	**Curve**
0.1	75	25	6
0.5	75	25	6
1	55	45	6
1.5	25	75	6
3.3	25	75	6
3.51	75	25	6
5	Stop		

Data acquisition and analysis were done using Xcalibur 4.5 SP1 software.

2.4 Calculations for Protein Binding, Unbound Fraction, Stability, and Recovery

Protein binding and related parameters were calculated using peak area ratios of analytes to the internal standard obtained from buffer and plasma samples.

Percentage Protein Binding (% Bound)

$$\%\ Bound\ \mathrm{Pr}otien = 100 - \left(\frac{Peak\ area\ ratio\ of\ buffer\ chamber}{Peak\ area\ ratio\ of\ plasma\ chamber} * 100 \right)$$

Percentage Unbound Drug (% Free)

% Free unbound = 100 – % Bound

Unbound Fraction (fu)

$$Unbound\ Fraction(f_u) = \frac{\%\ Free\ unbound}{100}$$

Plasma Stability (%)

Plasma stability was assessed by comparing peak area ratios of plasma incubated for 5 h and 0 h

$$\%Plasma\ Stability = \frac{Peak\ area\ ratio\ in\ plasma\ at\ 5h}{Average\ peak\ area\ ratio\ in\ plasma\ at\ 0h} * 100$$

Recovery (%)

Recovery (%) was determined by comparing the combined analyte response in donor and receiver compartments after equilibrium dialysis with the initial analyte response before dialysis.

$$\%\mathrm{Recov}ery = \frac{Peak\ area\ ratio\ in\ buffer + Peak\ area\ ratio\ in\ plasma}{Average\ peak\ area\ ratio\ in\ plasma} * 100$$

2.5 Statistical Analysis

Data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). Student's t-test, an unpaired, two-tailed test, was conducted to determine statistical significance for plasma versus HSA binding. Statistical significance was set at $p < 0.001$. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was utilised for all statistical analyses.

3. Results

The UHPLC–Orbitrap HRMS method enabled reliable detection and quantification of rebastinib and the reference compounds under optimised PRM conditions. Representative extracted ion chromatograms (EICs) of rebastinib, verapamil, and warfarin are shown in Figure 3.

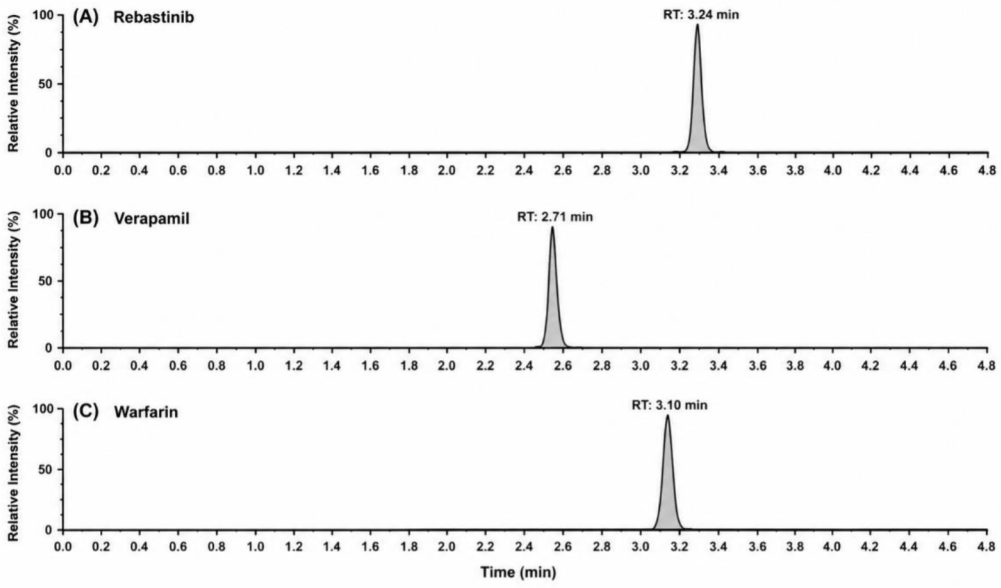


Figure 3. Representative extracted ion chromatograms (EICs) of **(A)** rebastinib, **(B)** verapamil, and **(C)** warfarin obtained using UHPLC–Orbitrap HRMS under optimised chromatographic conditions.

These ions were selected to generate specific fragments that can be used to quantify these compounds, including rebastinib (m/z 293.1392), verapamil (m/z 165.0907), and warfarin (m/z 251.0698). Corresponding product ion spectra are presented in Figure 4.

The EICs and MS spectra of the internal standard carbamazepine has not been presented because it was only used for normalisation purposes. Rebastinib showed a high level of binding to plasma proteins in human plasma, with an average binding of 99.90 ± 0.01%, which corresponded to an unbound fraction (f_u) of 0.001 (Figure 5, Table 3).

In isolated human serum albumin (HSA), rebastinib showed comparatively lower binding (87.81 ± 2.32%; f_u = 0.122), indicating that albumin is a major contributor to binding, while additional plasma proteins may also contribute to the overall plasma binding profile.

A statistically significant difference was obtained for the binding of rebastinib to plasma *vs.* HSA by unpaired Student's t-test ($p < 0.001$). The small standard deviation and percentage coefficient of variation (% CV) values in this experiment signify reliable assay performance. The reference drugs warfarin and verapamil had binding constants of 99.44 ± 0.14% (f_u = 0.0056) and 94.61 ± 2.04% (f_u = 0.0539), respectively, which correlated with those published in the literature, thereby confirming the robustness of our method. The stability and recovery analyses carried out under the RED incubation protocol proved that rebastinib and the other standard drugs gave satisfactory results in terms of analysis (Figure 6, Table 3). It was seen that rebastinib was stable for the five-hour incubation time period at a temperature of 37 °C, and its stability results were 89.32 ± 3.35% in plasma samples and 97.45 ± 7.05% in HSA solutions. The results of recovery for rebastinib were found to be 94.09 ± 7.52% and 99.17 ± 6.53% in plasma and HSA solutions, respectively.

Collectively, these findings demonstrate that the RED–Orbitrap HRMS workflow provided a reliable assessment of rebastinib plasma protein binding and enabled quantification of ultralow unbound drug fractions.

4. Discussion

This paper constitutes, as far as we know, an original contribution. Indeed, the first systematic investigation of rebastinib's plasma protein binding by rapid equilibrium dialysis in conjunction with ultra-high-performance liquid chromatography-Orbitrap high-resolution mass

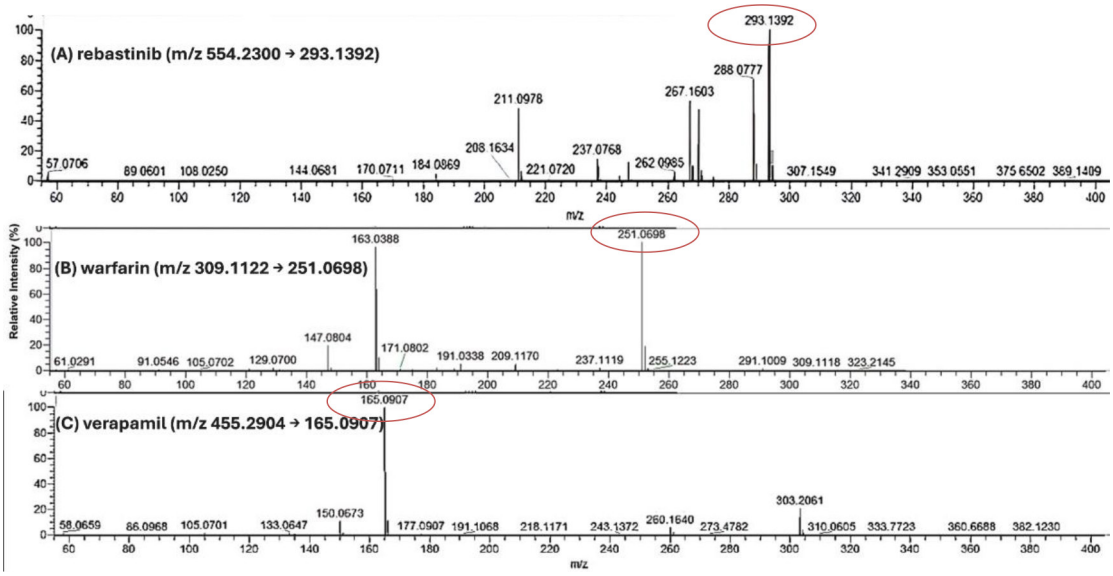


Figure 4. Representative product ion mass spectra of **(A)** rebastinib, **(B)** warfarin, and **(C)** verapamil obtained using Orbitrap HRMS in PRM mode. Characteristic fragment ions used for quantification included rebastinib (m/z 293.1392), verapamil (m/z 165.0907), and warfarin (m/z 251.0698).

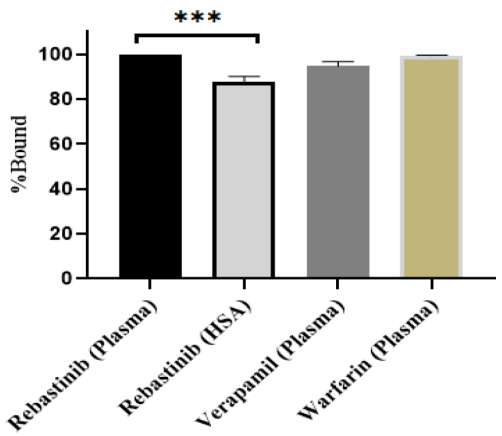


Figure 5. Comparative plasma protein binding (% bound) of rebastinib, verapamil, and warfarin determined using rapid equilibrium dialysis coupled with UHPLC–Orbitrap HRMS. Rebastinib binding was evaluated in both human plasma and isolated human serum albumin (HSA). Data are presented as mean ± SD (n = 3). Rebastinib exhibited significantly lower binding in isolated HSA compared with whole plasma (***p<0.001; unpaired Student's t-test).

spectrometry. Rebastinib demonstrated extensive plasma protein binding with an extremely low unbound fraction, suggesting that a very small amount can remain freely circulating within the bloodstream. Such plasma protein binding behaviour may be attributed to the physicochemical nature of rebastinib, characterised by its highly lipophilic quinazoline structure, aromatic substitution, and amphiphilic nature, thus facilitating its interaction with the hydrophobic plasma protein binding regions. The human serum albumin (HSA) has well-defined sites for drug interaction, such as the Sudlow site I and Sudlow site II, having affinity for lipophilic and heterocyclic drugs[27]. In the case of the isolated HSA system, rebastinib showed comparatively better affinity (87.81%). Thus, albumin can be said to play an important role in the binding of rebastinib to plasma proteins. Nevertheless, due to the presence of significantly high affinity in whole plasma as compared to that in the isolated albumin system, there must be involvement of other plasma components apart from albumin, such

Table 3. Summary of plasma protein binding, unbound fraction (f_u), recovery, and stability parameters of rebastinib and reference compounds (mean ± SD, n = 3)

Comparative Plasma Protein Binding, Recovery, and Stability of Rebastinib and Reference Compounds (mean ± SD, n = 3)							
Compound / Matrix	**% Bound (Mean ± SD)**	**%CV**	**f_u**	**% Recovery (Mean ± SD)**	**%CV**	**% Stability (Mean ± SD)**	**%CV**
Rebastinib – Human Plasma	99.90 ± 0.01	0.01	0.001	94.09 ± 7.52	8.94	89.32 ± 3.35	3.75
Rebastinib – Human Serum Albumin	87.81 ± 2.32	2.64	0.1219	99.17 ± 6.53	6.58	97.45 ± 7.05	7.23
Verapamil – Human Plasma	94.61 ± 2.04	2.15	0.0539	98.04 ± 6.69	6.82	102.43 ± 2.55	2.49
Warfarin – Human Plasma	99.44 ± 0.14	0.14	0.0056	95.31 ± 4.46	4.68	93.58 ± 9.28	9.94

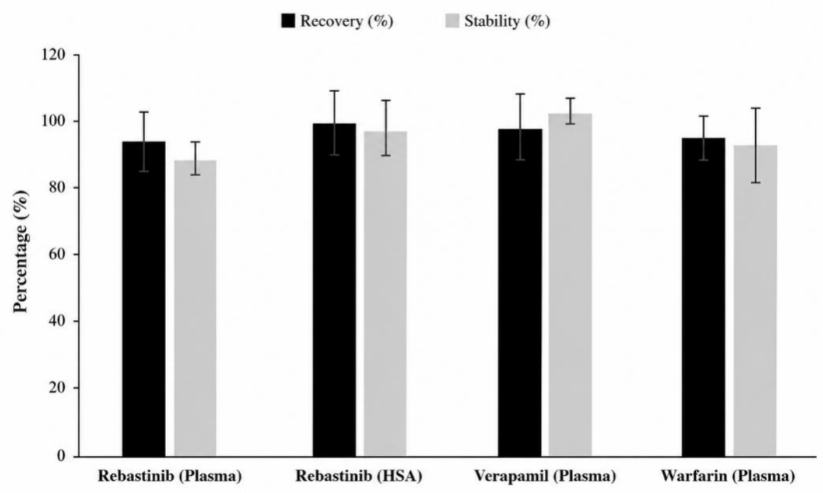


Figure 6. Recovery and stability assessment of rebastinib and reference compounds following 5 h incubation under RED experimental conditions. Data are presented as mean ± SD (n = 3).

as the alpha-1 acid glycoprotein, and lipoproteins, that have affinity for highly lipophilic drugs and weakly basic kinase inhibitors[28].

A high degree of binding has also been observed for other tyrosine kinase inhibitors such as pazopanib (>99 % bound), dasatinib (91-95 % bound), and erlotinib (92-95 % bound) that share high lipophilicity and substantial binding to plasma proteins[29]. High plasma protein binding is usually linked with extended exposure times and low free concentrations of drugs. High plasma protein binding can have a notable effect on the pharmacokinetics of rebastinib through decreasing the amount of freely available compound, which can be distributed throughout the body and metabolised. When talking about highly bound drugs, slight modifications in plasma protein binding can result in large changes in the bioavailability of a freely available fraction. Variability in the composition of plasma proteins occurs rather often in oncology patients due to inflammation, hepatic failure, nutritional status, or critical illness, and can lead to differences in the bioavailability of drugs and their effects in different patients[30-31]. In terms of clinical translation, the very low fraction of free drug in rebastinib can have significant consequences for drug dose and pharmacodynamics prediction.

The combination of the RED technique and PRM analysis using an Orbitrap mass spectrometer ensured the effective and selective determination of ultralow levels of unbound rebastinib. In contrast to low-resolution MRM, high-resolution PRM provides improved selectivity, enhanced mass accuracy, and reduced matrix effects in the case of compounds characterised by very low unbound fractions[32-34]. The obtained recovery and stability data further corroborated the analytical performance of the applied methodology under the specified experimental conditions.

Several limitations should be mentioned. The contribution of separate plasma proteins was not evaluated, and *in vitro-in vivo* correlations were not performed within the framework of the current study. While human serum albumin is chosen as a major plasma binding protein due to its high concentration in blood, further studies concerning the interaction of rebastinib with α1-acid glycoprotein and lipoproteins are needed. In summary, the current study offers a complete understanding of the rebastinib plasma protein binding profile and serves as an essential source of data for further pharmacokinetic studies and PBPK modelling.

5. Conclusion

In this research, for the first time, comparative quantification of rebastinib protein binding to human plasma and HSA is performed via rapid equilibrium dialysis combined with ultra-high performance liquid chromatography with Orbitrap high-resolution mass spectrometry. Extensive binding and ultralow free fraction of the investigated drug were established in human plasma, while less pronounced binding occurred in isolated human serum albumin, suggesting that serum albumin is an important, though not exclusive, binding site in human plasma.

It has been shown that the RED–Orbitrap HRMS method is capable of determining ultralow concentrations of unbound drugs with acceptable analytical parameters in terms of stability, recovery and reproducibility. Based on the results obtained, significant interaction of rebastinib with human plasma proteins was evidenced; therefore, further investigation of rebastinib PK and its mechanism of action is warranted.

Since a very low fraction of drug-free fraction was determined, physiological and pathological changes of plasma proteins may have an impact on the free drug fraction and contribute to pharmacokinetic variability. Further experiments are needed *in vivo*; however, the current findings can be considered valuable information for future PBPK modelling and pharmacokinetics studies of rebastinib.

6. Acknowledgements

The Authors are highly obliged for the kind and needful support of the DBT - funded Bioinformatics centre and the "Workshop in Bioinformatics" project, along with the DST-FIST program under Banasthali Vidyapith, for providing the necessary facility to conduct this study. The authors also express their deepest gratitude to Prof. Ina Shastri, Vice Chancellor, Banasthali Vidyapith, for her support.

7. References

1. Schmidt S, Gonzalez D, Derendorf H. Significance of protein binding in pharmacokinetics and pharmacodynamics. J Pharm Sci. 2010; 99(3):1107-1122. https://doi.org/10.1002/jps.21916 PMid: 19852037.
2. Trainor GL. The importance of plasma protein binding in drug discovery. Expert Opin Drug Discov. 2007; 2(1):51-64. https://doi.org/10.1517/17460441.2.1.51 PMid: 23496037.

3. Ye M, Nagar S, Korzekwa K. A physiologically based pharmacokinetic model to predict the pharmacokinetics of highly protein-bound drugs and the impact of errors in plasma protein binding. Biopharm Drug Dispos. 2016; 37(3):123-141. https://doi.org/10.1002/bdd.1996 PMid: 26531057 PMCid: PMC4844798.
4. Celestin MN, Musteata FM. Impact of changes in free concentrations and drug-protein binding on drug dosing regimens in special populations and disease states. J Pharm Sci. 2021; 110(10):3331-3344. https://doi.org/10.1016/j.xphs.2021.05.018 PMid: 34089711 PMCid: PMC8458247.
5. Al-Qassabi J, Tan SPF, Phonboon P, Galetin A, Rostami-Hodjegan A, Scotcher D. Facing the facts of altered plasma protein binding: do current models correctly predict changes in fraction unbound in special populations? J Pharm Sci. 2024; 113(6):1664-1673. https://doi.org/10.1016/j.xphs.2024.02.024 PMid: 38417790.
6. Buur JL, Baynes RE, Smith GW, Riviere JE. A physiologically based pharmacokinetic model linking plasma protein binding interactions with drug disposition. Res Vet Sci. 2009; 86(2):293-301. https://doi.org/10.1016/j.rvsc.2008.07.003 PMid: 18721993.
7. Jones HM, Dickins M, Youdim K, Gosset JR, Attkins NJ, Hay TL, Gurrell IK, Logan YR, Bungay PJ, Jones BC, Gardner IB. Application of PBPK modelling in drug discovery and development at Pfizer. Xenobiotica. 2012; 42(1):94-106. https://doi.org/10.3109/00498254.2011.627477 PMid: 22035569.
8. Roberts JA, Pea F, Lipman J. The clinical relevance of plasma protein binding changes. Clin Pharmacokinet. 2013; 52(1):1-8. https://doi.org/10.1007/s40262-012-0018-5 PMid: 23150213.
9. Zini R, Riant P, Barré J, Tillement JP. Disease-induced variations in plasma protein levels implications for drug dosage regimens (Part I). Clin Pharmacokinet. 1990; 19(2):147-159. https://doi.org/10.2165/00003088-199019020-00004 PMid: 2199129.
10. Imbs DC, Paludetto MN, Négrier S, Powell H, Lafont T, White-Koning M, Chatelut E, Thomas F. Determination of unbound fraction of pazopanib *in vitro* and in cancer patients reveals albumin as the main binding site. Invest New Drugs. 2016; 34(1):41-48. https://doi.org/10.1007/s10637-015-0304-9 PMid: 26572909.
11. Harney AS, Karagiannis GS, Pignatelli Al-Ani J, Smith BD, Kadioglu E, Wise SC, Hood MM, Kaufman MD, Leary CB, Lu WP, G. The selective Tie2 inhibitor rebastinib blocks recruitment and function of Tie2Hi macrophages in breast cancer and pancreatic neuroendocrine tumors. Mol Cancer Ther. 2017; 16(11):2486-2501. https://doi.org/10.1158/1535-7163.MCT-17-0241 PMid: 28838996 PMCid: PMC5669998.
12. Cortes J, Talpaz M, Smith HP, Snyder DS, Khoury J, Bhalla KN, Pinilla-Ibarz J, Larson R, Mitchell D, Wise SC, Rutkoski TJ. Phase 1 dose-finding study of rebastinib (DCC-2036) in patients with relapsed chronic myeloid leukemia and acute myeloid leukemia. Haematologica. 2017; 102(3):519. https://doi.org/10.3324/haematol.2016.152710 PMid: 27927766 PMCid: PMC5394958.
13. Flynn DL, Kaufman MD, Leary CB, Hood MM, Lu WP, Turner BA, Wise SC, Rudoltz MS, Smith BD. Rebastinib, a selective TIE2 kinase inhibitor, decreases TIE2-expressing macrophages, reduces metastasis, and increases survival in murine cancer models. Cancer Res. 2015; 75(1 Suppl):615. https://doi.org/10.1158/1538-7445.CHTME14-PR01
14. DrugBank Online. Rebastinib (DB13005). https://go.drugbank.com/drugs/DB13005
15. Barre J, Chamouard JM, Houin G, Tillement JP. Equilibrium dialysis, ultrafiltration, and ultracentrifugation compared for determining the plasma-protein-binding characteristics of valproic acid. Clin Chem. 1985; 31(1):60-64. https://doi.org/10.1093/clinchem/31.1.60 PMid: 3917383.
16. Toma CM, Imre S, Vari CE, Muntean DL, Tero-Vescan A. Ultrafiltration method for plasma protein binding studies and its limitations. Processes. 2021; 9(2):382. https://doi.org/10.3390/pr9020382
17. Tsai TH. Assaying protein unbound drugs using microdialysis techniques. J Chromatogr B Analyt Technol Biomed Life Sci. 2003; 797(1-2):161-73. https://doi.org/10.1016/j.jchromb.2003.08.036 PMid: 14630148.
18. Waters NJ, Jones R, Williams G, Sohal B. Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding. J Pharm Sci. 2008; 97(10):4586-4595. https://doi.org/10.1002/jps.21317 PMid: 18300299 PMCid: PMC10031514.
19. Choi B, Han C, LaRochelle JR, Pitsawong W, Houde D. Improved rapid equilibrium dialysis-mass spectrometry (RED-MS) method for measuring small molecule-protein complex binding affinities in solution. J Am Soc Mass Spectrom. 2024; 35(12):2785-2789. https://doi.org/10.1021/jasms.4c00334 PMid: 39495757.
20. Thermo Fisher Scientific. Pierce™ Rapid Equilibrium Dialysis (RED) Device Inserts User Guide. Pub. No. MAN0011571. Waltham, MA: Thermo Fisher Scientific. https://www.thermofisher.com
21. Anampa JD, Flynn DL, Leary C, Oh S, Xue X, Oktay MH, Condeelis JS, Sparano JA. Phase Ib clinical and pharmacodynamic study of the TIE2 kinase inhibitor rebastinib with paclitaxel or eribulin in HER2-negative metastatic breast cancer. Clin Cancer Res. 2025; 31(2):266-277. https://doi.org/10.1158/1078-0432.CCR-24-2464 PMid: 39531537 PMCid: PMC11818423.

22. Jiang WT, Tang LX, Pan J, Su CJ, Shen Z, Hu ZH, Zhan ZB, Shi AM. Development and validation of an UHPLC-Orbitrap-HRMS method for rapid determination of endogenous L-carnitine in patients on hemodialysis/peritoneal dialysis and its application to promote rational drug use. Ann Transl Med. 2022; 10(2):103. https://doi.org/10.21037/atm-21-6784 PMid: 35282068 PMCid: PMC8848361.
23. Salis S, Dore S, Rubattu N, D'Ascenzo V, Cherchi FT, Lollai SA. The development and validation of an LC-Orbitrap-HRMS method for the analysis of four tetracyclines in milk and its application to determine oxytetracycline concentrations after intramuscular administration in healthy Sarda ewes and those naturally infected with ***Streptococcus uberis***. Separations. 2024; 11(8):242. https://doi.org/10.3390/separations11080242
24. Wang P, Wang J, Xu Q, Zou H, Gao X, Ren H, *Cao Y*. Development and validation of a novel UPLC-Q-Orbitrap HRMS method for determining p-nitroaniline and its metabolites in blood. Front Chem. 2025; 13:1631203. https://doi.org/10.1016/S0731-7085(00)00476-3 PMid: 11275431.
25. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. Parallel reaction monitoring for high resolution and high mass accuracy quantitative targeted proteomics. Mol Cell Proteomics. 2012; 11(11):1475-1488. https://doi.org/10.1074/mcp.O112.020131 PMid: 22865924 PMCid: PMC3494192.
26. Smith DA, Di L, Kerns EH. The effect of plasma protein binding on *in vivo* efficacy: misconceptions in drug discovery. Nat Rev Drug Discov. 2010; 9(12):929-939. https://doi.org/10.1038/nrd3287 PMid: 21119731.
27. Sudlow GDJB, Birkett DJ, Wade DN. The characterization of two specific drug binding sites on human serum albumin. Mol Pharmacol. 1975; 11(6):824-832. https://doi.org/10.1016/S0026-895X(25)10691-3 PMid: 1207674.
28. Israili ZH, Dayton PG. Human alpha1-acid glycoprotein and its interactions with drugs. Drug Metab Rev. 2001; 33(2):161-235. https://doi.org/10.1081/DMR-100104402 PMid: 11495502.
29. Widmer N, Bardin C, Chatelut E, Paci A, Beijnen J, Levêque D, Veal G, Astier A. Review of therapeutic drug monitoring of anticancer drugs. Part two: targeted therapies. Eur J Cancer. 2014; 50(12):2020-2036. https://doi.org/10.1016/j.ejca.2014.04.015 PMid: 24928190.
30. Di L, Kerns EH. Profiling drug-like properties in discovery research. Curr Opin Chem Biol. 2003; 7(3):402-408. https://doi.org/10.1016/S1367-5931(03)00055-3 PMid: 12826129.
31. Naidong W, Ring PR, Midtlien C, Jiang X. Development and validation of a sensitive and robust LC-tandem MS method for the analysis of warfarin enantiomers in human plasma. J Pharm Biomed Anal. 2001; 25(2):219-226. https://doi.org/10.1016/j.xphs.2020.09.012 PMid: 32941852 PMCid: PMC12070811.
32. Jones RS, Chang JH, Flores M, Brecht E. Evaluation of a competitive equilibrium dialysis approach for assessing the impact of protein binding on clearance predictions. J Pharm Sci. 2021; 110(1):536-542. https://doi.org/10.1007/s10637-015-0304-9 PMid: 26572909.
33. Zsila F, Iwao Y. The drug binding site of human α1-acid glycoprotein: insight from induced circular dichroism and electronic absorption spectra. Biochim Biophys Acta - Gen Subj. 2007; 1770(5):797-809. https://doi.org/10.1016/j.bbagen.2007.01.009 PMid: 17321687.
34. Yuan ZQY, Qiao C, Yang ZC, Yu L, Sun LN, Qian Y, Zhang XH, Meng L, Zhang XY, Wang YQ. The impact of plasma protein binding characteristics and unbound concentration of voriconazole on its adverse drug reactions. Front Pharmacol. 2020; 11:505 https://doi.org/10.3389/fphar.2020.00505 PMid: 32390847 PMCid: PMC7194128.

Author Contributions

Conceptualisation: S.K. and A.P.; Methodology: S.K., B.B., and R.B.; Investigation: S.K., B.B., and B.S.; Data Curation: S.K. and B.B.; Writing – Original Draft: S.K., B.B., B.S., and BW; Writing – Review and Editing: R.B. and A.P.; Supervision: A.P. All authors have read and approved the manuscript.

Access this journal online	
Website: https://informaticsjournals.com/journals/toxi/home	**Quick Response Code**