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Original Article

Development and validation of an HPLC-FLD method for the determination of ripretinib in rat plasma and application to a pharmacokinetic study

[Desarrollo y validación de un método HPLC-FLD para la determinación de ripretinib en plasma de rata y aplicación a un estudio farmacocinético]

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Abstract

Context: Ripretinib, an FDA-approved kinase inhibitor, has shown promise as a treatment for advanced gastrointestinal stromal tumors.

Aims: To evaluate the pharmacokinetic profile of ripretinib, particularly in pre-clinical studies.

Methods: In this study, we present the first application of high-performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD) for the quantification of ripretinib levels in rat plasma. The developed method was designed to be rapid and simple, employing protein precipitation as the sample preparation step. For accurate quantification, warfarin was utilized as an internal standard. The chromatographic separation was performed on a reversed-phase C_8 column (150 mm × 4.6 mm, 5 µm) with a mobile phase consisting of ammonium acetate buffer (10 mmol/L) and methanol in a ratio of 25:75 (v/v), acidified to pH of 3.8 with acetic acid. The mobile phase was delivered at a flow rate of 1 mL/min. Fluorescence detection was employed to enhance the sensitivity and selectivity of the method. The excitation wavelength was set at 310 nm, while the emission wavelength was set at 390 nm.

Results: Validation of the method followed FDA guidelines for bioanalysis, including linearity (0.1-2.5 µg/mL, R^2 = 0.9984), inaccuracy (4.9-9.1%), RSD% (96.6%), and lower limit of quantification (LLOQ) of 0.1 µg/mL.

Conclusions: The method's reliability and performance were demonstrated in the pharmacokinetic study, making it a valuable tool for *in vivo* and *in vitro* ripretinib studies.

Keywords: ripretinib; HPLC-FLD; pharmacokinetics.

Resumen

Contexto: El ripretinib, un inhibidor de quinasas aprobado por la FDA, ha demostrado ser prometedor como tratamiento para tumores avanzados del estroma gastrointestinal.

Objetivos: Evaluar el perfil farmacocinético del ripretinib, particularmente en estudios preclínicos.

Métodos: En este estudio, presentamos la primera aplicación de cromatografía líquida de alto rendimiento acoplada a un detector de fluorescencia (HPLC-FLD) para la cuantificación de los niveles de ripretinib en plasma de rata. El método desarrollado fue diseñado para ser rápido y simple, empleando la precipitación de proteínas como paso de preparación de la muestra. Para una cuantificación precisa, se utilizó warfarina como estándar interno. La separación cromatográfica se realizó en una columna C8 de fase reversa (150 mm × 4,6 mm, 5 µm) con una fase móvil que consiste en un tampón de acetato de amonio (10 mmol/L) y metanol en una proporción de 25:75 (v/v), acidificado a pH de 3,8 con ácido acético. La fase móvil se administró a una velocidad de flujo de 1 mL/min. Se empleó la detección de fluorescencia para mejorar la sensibilidad y selectividad del método. La longitud de onda de excitación se estableció en 310 nm, mientras que la longitud de onda de emisión se estableció en 390 nm.

Resultados: La validación del método siguió las pautas de la FDA para el bioanálisis, incluida la linealidad (0,1-2,5 µg/mL, R^2 = 0,9984), la inexactitud (4,9-9,1%), el porcentaje de desviación estándar relativa (RSD) (96,6%) y el límite inferior de cuantificación (LLOQ) de 0,1 µg/mL.

Conclusiones: La confiabilidad y el rendimiento del método se demostraron en el estudio farmacocinético, lo que lo convierte en una herramienta valiosa para los estudios *in vivo* e *in vitro* con ripretinib.

Palabras Clave: farmacocinética; HPLC-FLD; ripretinib.

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Abbreviations: ANOVA: Analysis of variance; AUC: Area under the plasma concentration-time curve; AUC_0^{24}: Total area under the concentration-time curve from zero to 24 hours; CL/F: Apparent total clearance; C_{max}: Peak plasma concentration; CMC: Carboxymethylcellulose; CYP: Cytochrome P450; D: Percentage of deviation; FDA: Food and Drug Administration; GIST: Gastrointestinal stromal tumors; HPLC-FLD: High-performance liquid chromatography-fluorescence detector; LLOQ: Lower limit of quantification; QC: Quality control; QCH: High quality control; QCL: Low quality control; QCM: Medium quality control; R^2: coefficient of determination; Rip: Ripretinib; RSD: Relative standard deviation; SD: Standard deviation; SEM: Standard error of mean; SW: Sprague Dawley rats; $t_{1/2}$: Terminal half-life; t_{max}: Time to reach peak plasma concentration; ULOQ: Upper limit of quantitation; UPLC-MS/MS: Quadrupole tandem mass spectrometry; V_d/F: Volume of distribution of the terminal phase; War: Warfarin.

INTRODUCTION

There are growing concerns associated with increased cancer risk. Gastrointestinal stromal tumors (GIST) are the most prevalent gastrointestinal sarcomas. About 5,000 people in the United States were newly diagnosed with GIST in 2019 (Townsend, 2023). Since the initial FDA approval of imatinib, the first kinase inhibitor, in 2001, more than 40 kinase inhibitors have been approved in the United States, showing the growing importance of kinase inhibitors as a crucial class of therapeutic treatments. Food and Drug Administration (FDA) authorized ripretinib (Rip) in 2020 as a kinase inhibitor used to treat advanced gastrointestinal stromal tumors that have not been well treated with previous kinase inhibitors like sunitinib and imatinib. Rip demonstrated a good safety profile and showed favorable efficiency in patients with advanced GIST compared with the previous imatinib treatment (Janku et al., 2020).

Rip, with the structure shown in Fig. 1, has the IUPAC name N {4-bromo-5-[1-ethyl-7-(methylamino)-2-oxo-1,2-dihydro-1,6-naphthyridin-3-yl]2-fluorophenyl} N′-phenyl urea (Dhillon, 2020). From the structure of Rip, we can notice that it possesses several moieties that could have absorption in the UV-visible range; the maximum absorption of Rip was found at a wavelength of 310 nm. This indicates that it can be evaluated using the HPLC coupled with the UV-visible detector. Moreover, the structure of Rip contains the 1,6-naphthyridin moiety, which could cause the molecule to fluoresce due to the presence of a fused aromatic ring and its rigid nature.

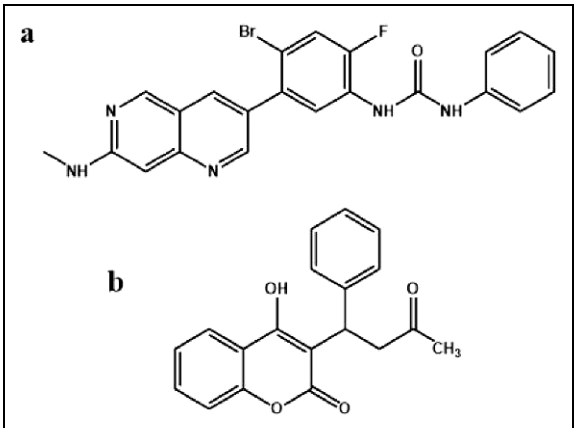

Figure 1. Structures of **(a)** ripretinib and **(b)** warfarin.

The analytical methods currently available for Rip quantification in bio-samples are very limited. In their study, Wang et al. (2021) examined the pharmacokinetic impact of itraconazole and voriconazole on Rip in beagle dogs. They employed ultra-performance liquid chromatography coupled with quadrupole tandem mass spectrometry (UPLC-MS/MS) for the analysis. However, the significant cost associated with the MS detector poses a notable limitation. Consequently, exploring alternative approaches utilizing conventional HPLC would offer substantial cost savings.

Based on the literature survey, neither high-performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD) nor high-performance liquid chromatography coupled with an ultraviolet-visible detector (HPLC-UV-visible) has been utilized thus far for the quantification of Rip. In this study, an HPLC-FLD method has been developed for the extraction and determination of Rip in rat plasma. In addition, the method was successfully applied to a pharmacokinetic study of Rip in Sprague Dawley (SW) rats.

MATERIAL AND METHODS

Chemicals

Rip standard was purchased from Resonance Research lab (Jordan). Warfarin (Fig. 1B), which was used as an internal standard (I.S), was purchased from Sigma-Aldrich (USA). All solvents used in this study were of HPLC grade. Methanol (99.9%), and acetonitrile (99.9%) were from Honeywell (Germany), ethyl acetate (≥99.5%), formic acid (≥98%), acetic acid (≥99.7%), ammonium acetate (≥98%) and sodium hydroxide (99.9%) from Fisher Chemical (USA).

Animals

In this study, Sprague Dawley (SW) rat plasma was used for method development and validation. The plasma was purchased from the Animal House at Petra University in Jordan. Six lots were collected from different SW rat plasma. The pharmacokinetic study was conducted in vivo on ten SW rats obtained from the same animal facility at Petra University. All experimental procedures were carried out under the approval of the Ethical Committee of the Higher Re-

search Council at the Faculty of Pharmacy and Medical Sciences (University of Petra, Amman, Jordan).

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Instrumentation and HPLC-FLD conditions

HPLC-FLD, Shimadzu-20A, with fluorescence detector RF-20A (Shimadzu (Japan)) was used. The chromatographic conditions were selected based on an optimization process to choose the parameters that provide the highest signal and the best resolution. The optimized chromatographic conditions were stationary phase Shim-Pack GIST C8 4.6 mm × 250 mm, 5 µm, and the column temperature was adjusted to 25°C. The mobile phase consisted of 10 mmol/L ammonium acetate acidified with acetic acid (pH 3.8) and methanol (25:75, v/v) under isocratic conditions. The mobile phase was filtered using 0.45-µm filter paper using a vacuum pump, followed by ultrasonic degassing. The detection wavelengths were set at an excitation wavelength of 310 nm and an emission wavelength of 390 nm. The applied flow rate was 1 mL/min, the run time was 8.3 min, and the injection volume was 10 µL.

Standard solutions

Stock standard solutions (100 µg/mL) of Rip and warfarin (I.S.) were prepared in brown volumetric flasks and diluted with methanol. All stock solutions were stored at -20°C for a maximum of one month. Working standard solutions of Rip for calibration were prepared at different concentration levels: 1, 4, 7, 10, 16, 19, 22, and 25 µg/mL. Additionally, quality control (QC) solutions were prepared at three concentration levels: 3 µg/mL for the low QC (QCL), 13 µg/mL for the medium QC (QCM), and 18 µg/ mL for the high QC (QCH). The working standard solution of warfarin was at 11 µg/mL. Calibration standard concentrations are selected based on the concentration range expected in the intended study (FDA, 2018).

Spiked plasma solution for calibration and quality control

The solution was prepared in accordance with FDA guidelines for Bioanalytical Method Validation (FDA, 2018). These guidelines recommend the utilization of six non-zero calibrators, each of which should fall within ±15% of nominal concentrations, with the exception of the lower limit of quantification (LLOQ), where the calibrator range should be within ±20% of the nominal concentrations for each validation run. Given that this method is intended for use in pharmacokinetic studies, the highest expected concentration is 2.02 µg/mL. Consequently, the standard range should span from 0.303 to 2.323 µg/mL. To achieve this, the working standards prepared in the preceding steps were diluted with plasma, thereby covering a range from 0.1 to 2.5 µg/mL, with the LLOQ set at 0.3 µg/mL.

To prepare the calibrator, rat blank plasma was spiked with the pre-prepared Rip working solutions. The calibration curve consisted of eight points with the following concentrations: 0.1, 0.4, 0.7, 1.0, 1.6, 1.9, 2.2, and 2.5 µg/mL.

The selection of QC concentrations during the validation of a pharmaceutical analytical method is critical to ensuring accuracy and reliability. The criteria for selecting QC concentrations are in accordance with guidelines from the FDA. These concentrations are selected as follows: QCL at the lower end of the analytical range, usually about 1-3 times the LLOQ. QCM is generally in the middle of the calibration range. QCH is usually near the upper limit of quantitation (ULOQ) (FDA, 2018). In this study, QC samples were prepared at four different concentration levels: 0.1 µg/mL for the LLOQ, 0.3 µg/mL for the QCL, 1.3 µg/mL for the QCM, and 1.8 µg/mL for the QCH.

Sample preparation

A developed and optimized method was followed for sample preparation. Rat plasma (90 µL) was spiked with 10 µL of Rip 1 µg/mL standard solution; the spiked plasma was stored at -80°C. On the day of analysis, the samples were thawed at room temperature. In a 1.5 mL Eppendorf tube, 10 µL of warfarin solution (11 µg/mL) was added and vortexed for 30 s. Then, 5 µL of 0.1 mol/L sodium hydroxide solution was added and vortexed. After that, 300 µL of cold acetonitrile was added and vortexed gently for 2 min and centrifuged for 10 minutes at 14500 rpm at -5°C. After centrifugation, the supernatant was carefully transferred to a clean glass tube. It was then subjected to evaporation under a stream of nitrogen using a nitrogen evaporator (Biobasel, China) at approximately 40°C. To reconstitute the residue, 100 µL of methanol was added, and the mixture was thoroughly mixed. From this reconstituted solution, 10 µL was taken and injected into an HPLC-FLD system for analysis.

Method validation

In adherence to FDA guidelines (FDA, 2018), method validation was conducted, including lineari-

ty, accuracy, precision, recovery, selectivity, robustness, and assessment of carryover effects.

Linearity

Three calibration curves with eight non-zero levels of Rip (0.1–2.5 µg/mL) were examined for linearity. The peak area ratios of Rip to warfarin were plotted against Rip concentrations. A coefficient of determination (R^2) of 0.995 or higher was considered adequate as evidence of linearity.

Accuracy, recovery, and precision

To assess the accuracy and precision within the same day, plasma samples were spiked with Rip at four different concentration levels, including the LLOQ, QCL, QCM, and QCH concentrations. A total of 24 spiked samples were prepared, with six replicates for each concentration. Additionally, standard calibration samples were also prepared on the same day. All the solutions were injected into the HPLC-FLD instrument. To analyze the data, the peak area ratio obtained from each sample was calculated and compared to the corresponding calibration curve prepared for each batch. The intra-day and interday precision of the method was evaluated by calculating the relative standard deviation % (RSD%) of the different repetitions on the same day and three consecutive days. Intra-day accuracy was evaluated by determining the variation, also known as bias, from the nominal value for each concentration level of the QC samples. Similarly, inter-day accuracy was assessed by comparing the results obtained from the spiked Rip plasma samples on three consecutive days to their respective nominal values.

The efficiency of extraction in the recovery of Rip and warfarin from rat plasma was evaluated at four concentration levels LLOQ, QCL, QCM, and QCH; each was run in six replicates. Recovery was evaluated by comparing the response of the spiked samples with that of the unspiked samples, which represented 100% recovery (FDA, 2018).

Sensitivity

In order to assess the sensitivity of the method, six replicates were analyzed at the LLOQ at 0.1 µg/mL. The LLOQ is assumed to have the lowest non-zero standard analyte concentration on the calibration curve, indicating the lowest concentration at which reliable quantification of Rip could be achieved. The LLOQ response of Rip was required to be at least five times higher than that of the zero calibrators (blank sample). Furthermore, it was necessary to ensure that the bias and RSD for the LLOQ were both below 20%.

Selectivity

To assess selectivity, the potential contribution of plasma sources to the chromatographic response of Rip and warfarin was evaluated. Plasma samples were collected from six different animal lots and subjected to analysis. These plasma samples were spiked with the LLOQ level of Rip (0.1 µg/mL) and warfarin at a concentration of 1 µg/mL. The purpose of this assessment was to ensure that the plasma components did not interfere with the chromatographic response of Rip or warfarin, thereby confirming the selectivity of the method.

Carryover

To evaluate carryover, the presence of an analyte from a previous sample in subsequent samples, a carryover assessment was performed. This involved injecting blank samples, which do not contain the analyte of interest, followed by a ULOQ solution. By analyzing the blank samples after the ULOQ solution, any potential carryover effects could be detected. Carryover is considered significant if there is a noticeable signal or presence of the analyte in the subsequent blank samples, indicating contamination or residue from the previous sample.

In vivo pharmacokinetics study

A total of ten male SW rats, with weights ranging from 150 to 250 g, were obtained from the animal facility located at the University of Petra Pharmaceutical Center (Amman, Jordan). These rats were housed in a controlled environment with a temperature of 25 ± 2°C, a 12-hour light-dark cycle, and a relative humidity of 50%. Measures were taken to ensure their well-being and to maintain a clean and suitable living space for the animals.

The experiment took place during the light cycle, and prior to conducting the experiments, the animals underwent a one-week acclimatization period in the laboratory conditions. During this time, they had unrestricted access to filtered tap water and a standard rat chow diet. The experimental protocol underwent a review by the Ethical Committee of the Higher Research Council at the Faculty of Pharmacy and Medical Sciences (University of Petra, Amman, Jordan) and was approved (Document number UPPC/AH2022/04).

The SW rats were randomly assigned to two groups (10 +10): The Rip-administered group and the vehicle-administered group. Prior to the start of the experiment, the rats were weighed, and their tails were marked for identification purposes. A fasting period of 12 hours was imposed on the rats before the commencement of the experiments. However, they

had continuous access to filtered tap water during the fasting period. This ensured that the rats were in a consistent physiological state and provided a standardized condition for the administration of Rip and the vehicle. Rip was freshly dissolved in 0.5% carboxymethylcellulose (CMC) solution prepared in water, then a single dose of Rip of 25 mg/kg was administered to each rat from the group, and an equivalent volume of 0.5% CMC was administered to each rat from the vehicle group. The dose was chosen according to the FDA reports (FDA, 2018). A volume of 200 µL of the blood samples obtained from the jugular vein was collected in the EDTA tubes. An initial blood sample, a zero-time sample, was collected from each animal.

Blood samples were collected from the rats at specific time intervals following drug administration. The time points for sample collection were 5, 15, and 30 min, and 1, 2, 4, 8, 24, and 48 h. After collection, the blood tubes were subjected to centrifugation at 6000 rpm for 10 min. The clear plasma samples were carefully collected and subsequently stored at -80°C until analysis. The pharmacokinetic parameters, including peak plasma concentration (C_{max}), time to reach peak plasma concentration (t_{max}), terminal half-life ($t_{1/2}$), apparent total clearance (CL/F), apparent volume of distribution of the terminal phase (V_d/F), and total area under the concentration-time curve from zero to 24 hours (AUC_0^{24}), were determined using PK Solver (Zhang et al., 2010).

Statistical analysis

All experimental outcomes were conducted in triplicate, and the results were expressed as mean ± standard deviation (SD). A single-factor analysis of variance (ANOVA) test was performed to estimate significant differences among the results. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Optimization of the method

The fluorescence spectrum of Rip was recorded using Shimadzu HPLC-FLD; the optimal excitation and emission wavelengths were determined to be 310 nm excitation and 390 nm emission wavelengths. These values were adopted throughout this study. Warfarin was chosen as the internal standard due to its matching excitation and emission wavelengths with the analyte, as well as its UV-visible fluorescence property, which enables fluorescence detection. Additionally, it exhibits a reasonable elution time relative to the analyte, ensuring well-defined peak separation.

Mobile phase

The strategy for selecting an appropriate mobile phase in chromatography involves the selection of the organic solvent, pH, buffer solution, and the optimal ratio between the organic solvent and buffer (Bhardwaj et al., 2015). To make accurate choices, several key factors were taken into account. One important factor was the pKa values, as the pKa of Rip is 4.48, which is a weak base, and that of warfarin is 5.0, which is a weak acid. Therefore, ammonium acetate was chosen as the buffer solution, which showed a pH range of 3.8-5.8. Polarity, reflected in the log P values 3.7 and 2.7, was also considered as a key factor. Rip is clearly less polar than warfarin. Thus, increasing the organic ratio in the mobile phase can achieve the required separation and reduce the retention time of Rip.

The third aspect to be considered in optimizing the mobile phase is to determine the optimum pH of the mobile phase. To choose the appropriate pH, the ionization ratios for each compound at different pH values were calculated according to Rosés (2004). At pH 5.8, Rip was 4.8% ionized, and at pH 3.8, Rip was 82.72% ionized.

In the initial stage of the optimization process for the mobile phase, a mixture of ammonium acetate buffer and methanol in a 30:70 (v:v) was employed, and the pH was adjusted to 5.8. The C8 column was selected as the initial stationary phase.

First, a blank plasma sample was introduced into the HPLC-FLD system to confirm the absence of matrix interferences. Then, a standard mixture containing 1 µg/mL of Rip and 1 µg/mL of warfarin was injected, adhering to the selected chromatographic conditions. Subsequently, plasma samples spiked with Rip and warfarin were introduced, as depicted in Fig. 2a. Fig. 2a demonstrates that the elution of warfarin occurs in close proximity to the retention of matrix components at pH 5.8. Consequently, the separation from plasma was enhanced by modifying the organic solvent while maintaining a pH of 5.8. In this pursuit, methanol and acetonitrile were assessed as potential organic solvents. It was observed that acetonitrile provided superior resolution and reduced the overall runtime, as illustrated in Fig. 2b. Nevertheless, methanol was chosen due to its lower cost and its advantage in reducing buffer solution precipitation. Alternatively, an attempt was made to move the warfarin peak away from the plasma peaks by changing the pH values (3.8 and 6.8) of the mobile phase. As observed in Fig. 2c, the best separation was at pH = 3.8. However, warfarin fluorescence intensity decreased significantly at this pH, with a significant shift in retention time from 3.5 to 7.1 min for a warfa-

rin peak, while there was no significant change in the retention time of Rip.

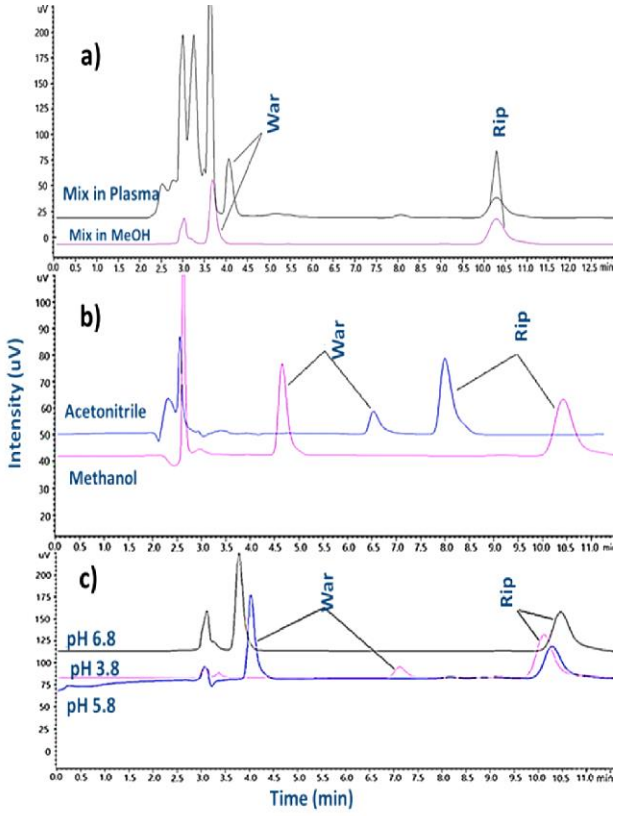

Figure 2. Optimization of chromatographic conditions.
(a) Standard mixture of warfarin (War) and ripretinib (Rip) in methanol and in plasma, **(b)** effects of two solvents in the mobile phase, **(c)** effects of different pH values (pH 3.8, 5.8, 6.8) of the aqueous solution in the mobile phase.

Also, the effect of the percentage of methanol in the mobile phase on elution was investigated. Fig. 3a shows two chromatograms of the analysis mixture using (70 and 75%) methanol at pH 3.8. It was observed that 75% methanol gave satisfactory resolution and a shorter run time. This composition provides the advantages of being isocratic, satisfactory resolution of the peak from each other, and from plasma peak, the shortest run time, and lowest cost.

Stationary phase

The stationary phases tested during this optimization were C18, C8, and biphenyl. It was noted from Fig. 3b that the use of biphenyl as a stationary phase gave a poor resolution of Rip and warfarin. Therefore, it was excluded. On the other hand, Fig. 3b indicates that both columns C8 and C18 gave acceptable resolution. However, the C8 column was selected because it elutes both compounds in a shorter run time.

As a summary, the optimal chromatographic conditions are the C8 stationary phase as a stationary phase and the ammonium acetate in the mobile phase at pH 3.8: methanol (25:75), the wavelength of the detector 310-390 nm. Fig. 3c shows a representative chromatogram of Rip and warfarin obtained after all optimization steps in which the retention time of warfarin and Rip were 5.46 and 6.93 min, respectively.

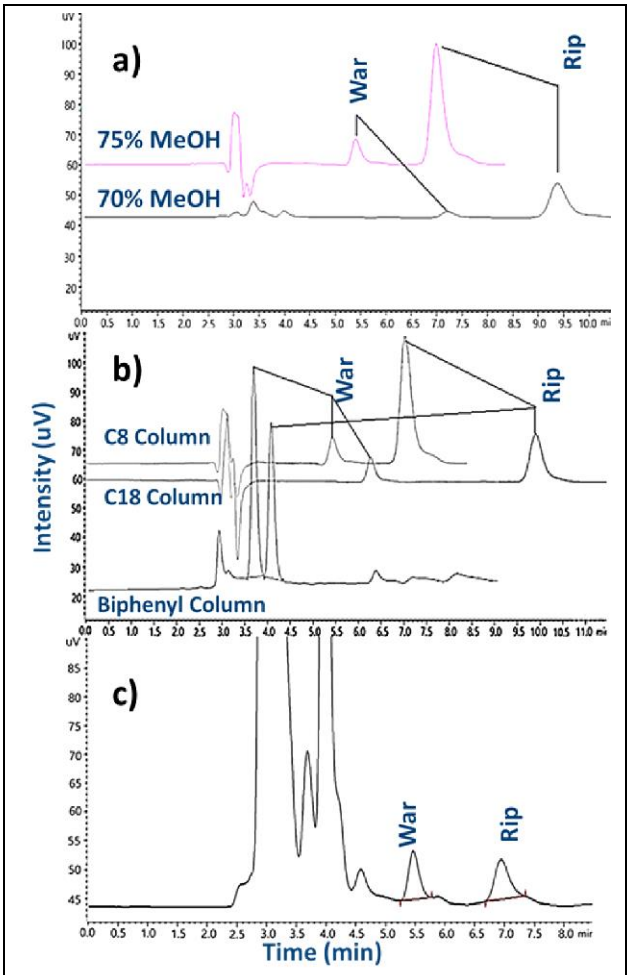

Figure 3. Optimization of chromatographic conditions.
(a) effect of different ratios of methanol in the mobile phase, **(b)** effects of different stationary phases, **(c)** representative chromatogram of warfarin (War) and ripretinib (Rip) after optimization of chromatographic conditions.

System suitability

System suitability parameters were evaluated for the newly developed HPLC-FLD method according to FDA guidelines (FDA, 2018). Table 1 presents the system suitability results in this study and its acceptance criteria according to FDA guidelines, revealing satisfactory system performance.

Optimization of sample preparation

Selection of organic solvent

In this study, three organic solvents (acetonitrile, methanol, and ethyl acetate) were tested as crashing solvents. Attempts to use methanol and ethyl acetate did not yield good results due to the low recovery (75,

60, and 58% for acetonitrile, methanol, and ethyl acetate, respectively). This is consistent with previous studies that demonstrated that acetonitrile is better than methanol in precipitating protein (Yang et al., 2013). Acetonitrile was chosen as the organic solvent because it had the highest recovery percentage, as shown in Fig. 4a. Also, better results were obtained when cold acetonitrile was used for precipitation.

Plasma to organic solvent ratio

To determine the best ratio of plasma to organic solvent, three rations of plasma to acetonitrile (1:3; 1:5; and 1:10) were tested, typically applied in the literature (Henion and Grant, 2017; Polson et al., 2003). By applying a 1:3 ratio, effective protein removal was achieved, as reflected by the high recovery percentage at this ratio, as can be observed from the area of the peaks in Fig. 4b. On the other hand, a larger ratio (5:1 or 10:1) led to more sample dilution and lower recovery.

Table 1. Chromatographic parameters.

Analyte	Retention time t_R (min) ($\bar{x} \pm SD$) (n=6)	Number of theoretical plates (N)	Tailing factor	Capacity factor (k´)	Selectivity factor	Resolution
Criteria		>2000	<2	>1	>1	>1.5
Ripretinib	6.93 ± 0.05	6317	1.29	1.89	1.48	3.75
Warfarin	5.46 ± 0.05	7725	1.21	1.28		
	$p = 8.38 \times 10^{-14}$ ($p<0.05$)					

$\bar{x}$: mean; RSD: Relative standard deviation.

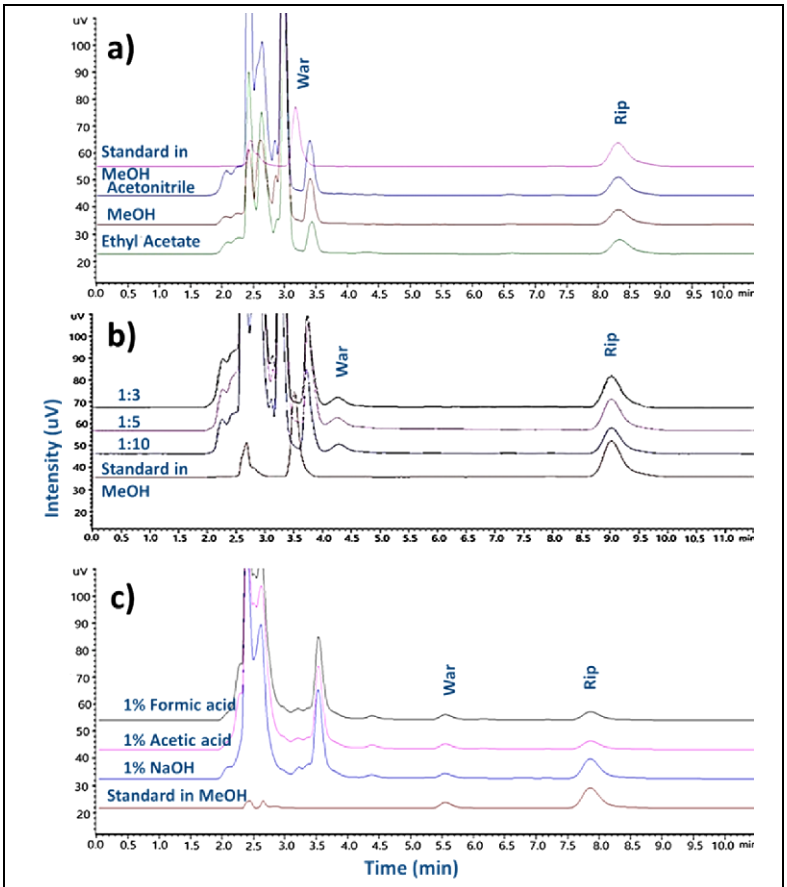

Figure 4. Optimization of sample preparation procedure.

(a) effect of different crashing solvents, **(b)** effect of different plasma: solvent ratios, **(c)** effect of different additives.

Table 2. Summary of three model calibration curves of ripretinib.

Model	Replicate							
	1	2	3	4	5	6	7	8
Ripretinib (µg/mL)	0.10	0.40	0.70	1.00	1.60	1.90	2.20	2.50
($\bar{x}$, n = 3) (µg/mL)	0.089	0.38	0.69	1.05	1.63	1.90	2.22	2.47
RSD (%)	9.2	7.5	0.8	3.6	1.3	2.7	1.3	0.4
D (%)	11.0	5.8	1.0	4.5	2.0	1.9	0.9	1.3
R^2	0.9984							
Slope ± SD (SEM)	0.2825 ± 0.147 (0.0847)							
Intercept ± SD (SEM)	6.5953 ± 0.0314 (0.0181)							

$\bar{x}$: mean; RSD: Relative standard deviation; D: Percentage of deviation; R^2: coefficient of determination; SD: Standard deviation; SEM: Standard error of mean.

Table 3. Model inter-day and intra-day precision and accuracy of the method.

Level	Conc. added (µg/mL)	Intra-day (n = 6)			Inter-day (n = 18)			FDA criteria (%)
		Conc. found (µg/mL)	Bias (%)	RSD (%)	Conc. found (µg/mL)	Bias (%)	RSD (%)	
LLOQ	0.1	0.093	7.3	8.7	0.090	9.2	4.7	±20
QCL	0.3	0.303	0.8	6.1	0.307	4.9	4.2	±15
QCM	1.3	1.400	7.7	3.8	1.384	7.5	4.5	±15
QCH	1.8	1.952	8.4	3.5	1.902	6.2	4.0	±15

Conc.: Concentration; RSD: Relative standard deviation; FDA: Food and Drug Administration; LLOQ: Lower limit of quantification; QCL: Low-quality control; QCM: Medium quality control; QCH: High-quality control.

Additives

Formic acid, acetic acid, and sodium hydroxide have been tested as additives to reduce protein binding (Zhao and Juck, 2018). These compounds can reduce protein binding by disrupting the hydrogen bonds that hold protein molecules together, causing denaturing proteins, which means that they change the shape and structure of the protein molecules. This can make it easier to precipitate proteins and remove them from the samples (Polson et al., 2003). Sodium hydroxide gave the highest recovery rate of Rip from plasma, as shown in Fig. 4c. Therefore, 0.1 M NaOH was chosen as the additive. Alkaline medium increases Rip extraction efficiency by converting it to a non-ionized form, increasing its transfer to the organic layer.

From the previous remarks, the optimal sample preparation includes the use of acetonitrile as the crashing organic solvent, the ratio to plasma (3:1), and 0.1 M NaOH as an additive. These conditions were applied along with the optimized chromatographic conditions in the validation process of the method.

Method validation

Linearity

The linearity of the method was determined by the construction of three calibration curves on three different days with eight non-zero concentrations ranging from 0.1 to 2.5 µg/mL. The mean correlation of determination R^2>0.998 indicated good linearity.

Table 2 shows that the percentage of deviation (D) from the nominal values meets the criteria of all eight non-zero calibrators. The mean linearity results for the three days are summarized in Table 2, including slope, intercept, and standard error.

Accuracy, recovery, and precision

Accuracy and precision were determined according to FDA guidelines (FDA, 2018). Table 3 shows a summary of the intra-day and inter-day precision and accuracy results of three independent days. Intra-day RSD ranged from 3.5 to 8.7 %, while the inaccuracy ranged from 0.8 to 8.4%. The inter-day precision ranged from 4.0 to 4.7%. This indicates the consistency and reproducibility of the measurements across different days.

Table 4. Extraction recovery and matrix effect of ripretinib and warfarin.

Level	Mean peak area of spiked	Mean peak area of unspiked	p-value	Recovery (%) ($\bar{x}$ ± SD)	Matrix effect (%) ($\bar{x}$ ± SD)
LLOQ	138132	141804	4.97×10^{-10}	97.4 ± 3.6	111.3 ± 5.2
QCL	408091	414225	4.52×10^{-12}	98.5 ± 2.8	112.8 ± 4.9
QCM	1392007	1673502	5.40×10^{-19}	83.2 ± 4.3	95.0 ± 3.7
QCH	1946121	2131929	1.16×10^{-18}	91.3 ± 4.5	104.3 ± 2.6
Warfarin	157160	179559	5.85×10^{-14}	87.5 ± 2.7	-

$\bar{x}$: mean; SD: Standard deviation; LLOQ: Lower limit of quantification; QCL: Low quality control; QCM: Medium quality control; QCH: High quality control.

Additionally, the bias ranged from 4.9 to 9.2. This comparison allows for the accurate quantification of the Rip levels in the samples. Importantly,the accuracy and precision of the results obtained in this study meet the acceptance criteria set by the FDA.

Table 4 presents the chromatographic areas of the spiked and unspiked samples, which reflect a significant difference between the two ($p<0.05$). The calculated recovery and matrix effect are also displayed in Table 4. Rip exhibited an average extraction recovery of 92.6% at various levels, whereas warfarin showed a recovery rate of 87.5%. Additionally, the average matrix effect ranged from 95% to 112.8%. This implies the presence of a negative error. Several factors, such as the matrix effect from other sample components, incomplete extraction, or losses during sample handling, could contribute to this error.

Accuracy and recovery are two important performance characteristics that are typically evaluated during chemical analysis method validation.

The FDA provides guidance on the acceptable ranges for accuracy and recovery for different types of analytical methods. For example, the FDA recommends that the accuracy of a method be within 15% of the true value. However, The FDA does not specify a single universally accepted recovery value for bioanalysis across all contexts. The FDA recommends that the recovery of an analyte be at least 70% for most applications.

However, the low accuracy of the method suggests that there is some systematic error. This error could be due to a number of factors, such as calibration errors, matrix effects, or incomplete extraction. It is important to note that a high recovery does not guarantee accurate results. The FDA requires that both accuracy and recovery be evaluated during method validation. This is because it is important to ensure that both the extraction process and the overall method produce accurate results.

Selectivity

Upon examination of the chromatograms depicted in Fig. 5, it was observed that there is no endogenous interference from rat plasma at the retention times corresponding to warfarin (5.46 min) and Rip (6.93 min). The retention times for both compounds were significantly different ($p<0.05$). This indicates that the developed method possesses the necessary selectivity to quantify Rip in rat plasma accurately.

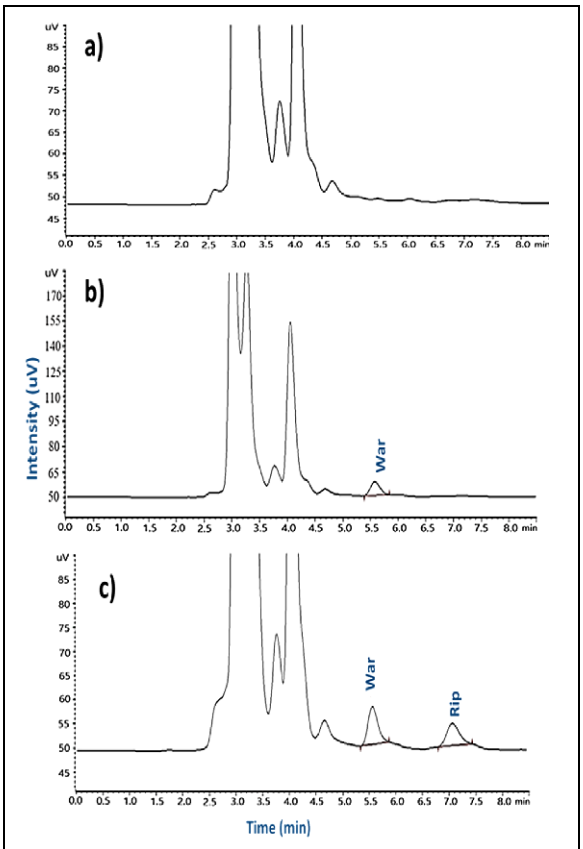

Figure 5. Selectivity test of ripretinib (Rip) and warfarin (War) in rat plasma.
(a) blank, **(b)** warfarin, **(c)** ripretinib and warfarin at LLOQ (0.1 µg/mL).

Carryover test

To assess the presence of carryover, blank samples were injected immediately after the highest concentration calibration standard sample. It was observed that no analyte or internal standard peaks were detected in the blank samples. This indicates that there was no significant carryover of the analyte or internal standard from the previous sample complying with FDA criteria. Meeting these criteria indicates that the method provides reliable and accurate results, aligning with the established standards for bioanalytical analysis.

Comparison with previous studies

Table 5 displays a comparison between the method developed in this study and the previously reported methods. In terms of cost, the developed method employed a considerably more affordable FLD detector compared to the mass spectrometry detector used in previous studies. Furthermore, methanol was opted for as the mobile phase solvent instead of the more expensive acetonitrile.

Table 5. Ripretinib pharmacokinetics parameters after single oral administration.

Parameter	Value (mean ± SD)
$t_{1/2}$ (h)	4.53 ± 0.97
t_{max} (h)	4.00 ± 0.00
C_{max} (ng/mL)	1878.83 ± 262.20
AUC_0^t (ng/mL/h)	16722.33 ± 2489.325
V_d/F (L/kg)	9.74 ± 2.01
Cl/F (L/kg/h)	1.52 ± 0.20

[a]Dose: 25 mg/kg ripretinib in SD rat (n = 10, ripretinib group). $t_{1/2}$: Terminal half-life; t_{max}: Time to reach peak plasma concentration; C_{max}: Peak plasma concentration; AUC_0^{24}: Total area under the concentration-time curve from zero to 24 hours; CL/F: Apparent total clearance; V_d/F: Volume of distribution of the terminal phase; CL/F: Apparent total clearance.

Regarding validation results, the method developed in this study exhibited similar results to previous methods in terms of accuracy, accuracy, and recovery. Despite the higher sensitivity of previously reported UPLD/MS/MS and HPLC/MS/MS methods compared to this method, the achieved sensitivity is adequate, considering that this method will subsequently be applied in pharmacokinetic studies of ripitinib. Since the Cmax of ripitinib in the plasma of male SW rats after a single oral dose was 2.02 µg/mL, according to the European Medicines Agency (2011), the LLOQ should not be more than 5% of Cmax. Accordingly, the detection sensitivity obtained using FLD (LLOQ = 0.1 µg/mL) is considered suitable and satisfactory for quantifying Rip levels in rat plasma (FDA, 2020).

In vivo pharmacokinetic study

The developed and validated HPLC-FLD method was employed for the quantification of Rip in plasma samples obtained from rats. Fig. 6 illustrates representative chromatograms of plasma samples collected prior to the administration of Rip and 4 hours after administration. The average peak plasma concentration of Rip was determined to be 1878.83 ± 262.20 ng/mL. This concentration was reached approximately 4 hours after its administration.

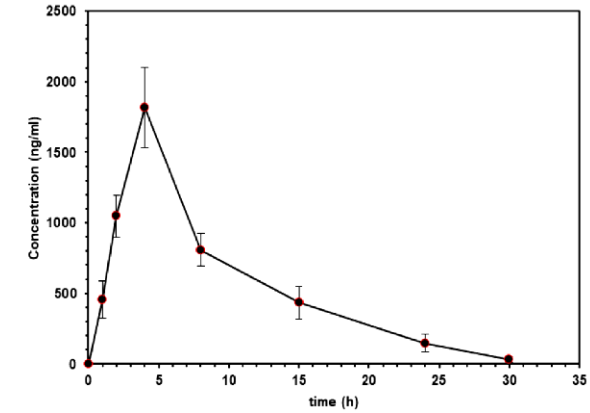

Figure 6. Plasma concentration vs. time profiles of ripretinib following administration of a single oral dose (25 mg/kg) of ripretinib.

Data are shown as mean ± SD (n = 10).

Table 6 presents the pharmacokinetic parameters determined in the study, including the C_{max}, the area under the plasma concentration-time curve (AUC), and the $t_{1/2}$ of Rip. These parameters were calculated based on the plasma concentration vs. time profile shown in Fig. 6.

The pharmacokinetic parameters C_{max}, AUC, and $t_{1/2}$ were found to be consistent with the results reported in a previous study (DCC-2618-03-0037) published in the drug application approved by the FDA (2020). As such, the method was found suitable and valuable for conducting various pre-clinical *in vivo* studies involving the treatment with Rip.

Rip is metabolized by CYP3A to the active metabolite, so from a pharmacokinetic point of view, determination of the active metabolite levels is useful, particularly in applications looking at potential drug-drug interactions of Rip.

Limitations of the study

The amount of Rip metabolites was not quantified using the method presented in this work. However, this may be done as part of future method optimization. Additionally, the study focuses only on single-dose pharmacokinetics, and the method may not take

Table 6. Summary of analytical methods for quantification of ripretinib in biological samples.

Technique	Sample preparation	Mobile phase	Column	Matrix	LLOQ (µg/mL)	Bias (%)	RSD (%)	Recovery (%)	Ref.
UPLC-MS/MS	Protein precipitation	0.1% formic acid and acetonitrile	C18	Beagle dogs' plasma	0.001	2.0-3.3	3.2-4.3	86.36	Wang et al., 2021
HPLC-MS/MS	Protein precipitation	Not mentioned	C8	Human plasma	0.002	0.47-1.88	1.06-7.28	98.8	FDA, 2020
HPLC-FLD	Protein precipitation	Ammonium acetate and methanol	C8	SD rats' plasma	0.1	4.9-9.1	4.0-4.9	92.6	This study

LLOQ: Lower limit of quantification; RSD: Relative standard deviation; Ref.: Reference; UPLC-MS/MS: Ultra-performance liquid chromatography-tandem mass spectrometry; HPLC-MS/MS: High-performance liquid chromatography-tandem mass spectrometry; HPLC-FLD: High-performance liquid chromatography-fluorescence detector.

into account the accumulation of metabolites or other dynamics observed with repeated dosing.

CONCLUSION

In this study, a novel, cost-efficient HPLC-FLD method for the precise determination of ripretinib levels in rat plasma was developed. This method stands out for its speed, precision, accuracy, and selectivity. The chromatographic conditions and extraction procedures were meticulously fine-tuned to attain optimal separation, heightened sensitivity, and maximum recovery, all while minimizing analysis time.

This method was subjected to rigorous validation following FDA guidelines, ensuring its linearity, precision, accuracy, selectivity, and robustness. The method achieved satisfactory ripretinib recovery from plasma samples, averaging 92.6%. It also demonstrated noteworthy sensitivity, detecting ripretinib at concentrations as low as 0.1 µg/mL.

This level of sensitivity enables quantification *in vivo* studies, enabling pharmacokinetic assessments following the oral administration of a single dose (25 mg/kg) to SW rats (n = 10). The method is a valuable and economical analytical tool, especially for research facilities lacking HPLC-MS/MS capabilities. It opens doors for further investigations involving ripretinib, facilitating research in various settings and enhancing the understanding of its pharmacokinetic profile. The pharmacokinetic parameters, including Cmax, AUC, and $t_{1/2}$, obtained in this study were consistent with the findings documented in prior research published in the FDA-approved drug application.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

Bhardwaj SK, Dwivedia K, Agarwala DD (2015) A review: HPLC method development and validation. Int J Anal Bioanal Chem 5(4): 76–81.

Dhillon S (2020) Ripretinib: First approval. Drugs 80: 1133–1138. https://doi.org/10.1007/s40265-020-01348-2

European Medicines Agency (2011) Committee for Medicinal Products for Human Use (CHMP). Guideline on bioanalytical method validation EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2** http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf [Consulted 28 January 2011].

FDA (2018) Center for Drug Evaluation and Research. Bioanalytical Method Validation Guidance for Industry Biopharmaceutics Bioanalytical Method Validation Guidance for Industry Biopharmaceutics Contains Nonbinding Recommendations. https://www.fda.gov/media/70858/download [Consulted 24 May 2018].

FDA (2020) Center for Drug Evaluation and Research. Application number: 213973 Orig1s000 multi-discipline review. Summary Review. Office Director. Cross Discipline Team Leader Review. Clinical Review. Non-Clinical Review. Statistical Review. Clinical Pharmacology Review. Clinical Microbiology/Virology. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2020/213973Orig1s000MultidisciplineR.pdf [Consulted 13 August 2020].

Henion J, Grant R (2017) 2016 ASMS Fall Workshop: Sample preparation for the real-world analysis of samples by mass spectrometry. J Am Soc Mass Spectrom 28(3): 395–398. https://doi.org/10.1007/s13361-016-1568-9

Janku F, Razak ARA, Chi P, Heinrich MC, Mehren MV, Jones RL, Ganjoo K, Trent J, Gelderblom H, Somaiah N, Hu S, Rosen O, Su Y, Ruiz-Soto R, Gordon M, George S (2020) Switch control inhibition of KIT and PDGFRA in patients with advanced gastrointestinal stromal tumor: A phase I study of ripretinib. J Clin Oncol 38(28): 3294–3303. https://doi.org/10.1200/JCO.20.00522

Polson C, Sarkar P, Incledon B, Raguvaran V, Grant R (2003) Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. J Chromatogr B 785(2): 263–275. https://doi.org/10.1016/S1570-0232(02)00914-5

Rosés M (2004) Determination of the pH of binary mobile phases for reversed-phase liquid chromatography. J Chromatogr A 1037(1-2): 283–298. https://doi.org/10.1016/j.chroma.2003.12.063

Townsend H (2023) Surgical management of gastrointestinal stromal tumors. J Adv Pract Oncol 14(6): 541–547. https://doi.org/10.6004/jadpro.2023.14.6.7

Wang HJ, Zhou CY, Su YD, Gou KF, Geng XN, Qiu XJ (2021) The pharmacokinetic effect of itraconazole and voriconazole on ripretinib in beagle dogs by UPLC-MS/MS technique. Drug Des Dev Ther 2021: 4865–4873. https://doi.org/10.2147/DDDT.S337864

Yang Y, Cruickshank CH, Armstrong M, Mahaffey S, Reisdorph R, Reisdorph N (2013) New sample preparation approach for mass spectrometry-based profiling of plasma results in improved coverage of metabolome. J Chromatogr A 1300: 217–226. https://doi.org/10.1016/j.chroma.2013.04.030

Zhang Y, Huo M, Zhou J, Xie S (2010) PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. Comput Methods Programs Biomed 99(3): 306–314. https://doi.org/10.1016/j.cmpb.2010.01.007

Zhao L, Juck M (2018) Protein Precipitation for Biological Fluid Samples Using Agilent Captiva EMR—Lipid 96-Well Plates. USA: Agilent Technologies Inc. https://www.agilent.com/cs/library/applications/5991-9222EN_ppt_biological_fluid_samples_application.pdf [Consulted 23 June 2020].

AUTHOR CONTRIBUTION:

Contribution	Tarawneh IN	Abu Shmeis RM	Al-Foqha'a FM	Alshishani A	Al Hroot J	Zayed A
Concepts or ideas	x	x	x			x
Design	x	x	x	x	x	x
Definition of intellectual content						
Literature search	x		x		x	
Experimental studies	x	x	x	x	x	x
Data acquisition	x	x	x	x	x	x
Data analysis	x	x	x		x	
Statistical analysis	x	x	x	x	x	x
Manuscript preparation	x	x	x		x	x
Manuscript editing	x	x	x			x
Manuscript review	x	x	x	x	x	x

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