

Re-Validation of New Develop Highly Sensitive, Simple LCMS/MS Method for the Estimation of Rohitukine and its Application in ADME/Pre-Clinical Pharmacokinetics

Amarinder Singh^{1,2*}, Priya Wazir¹, Pankaj Chibber¹, Nitika Kapoor³, Amit Kumar¹, Utpal Nandi¹, Sumit G Gandhi^{2,3}, Surjeet Singh^{1,2}, Manoj Kumar Tikoo¹, Ram Vishwakarma⁴ and Gurdarshan Singh^{1,2*}

¹PK-PD-Toxicology Division, CSIR-Indian Institute of Integrative Medicine, Jammu, Jammu and Kashmir, India

²Academy Council of Scientific and Industrial Research (AcSIR), New Delhi, India

³Plant Biotechnology Division, CSIR-Indian Institute of Integrative Medicine, Jammu, Jammu and Kashmir, India

⁴CSIR-Indian Institute of Integrative Medicine, Jammu, Jammu and Kashmir, India

Abstract

The purpose of the research was to develop a simple, rapid, accurate, reproducible and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for determination of Rohitukine, a chromone alkaloid in plasma. The chromatographic separation was achieved with high resolution RP18e Chromolith column (100 × 4.6 mm, 2 μm) employing a isocratic composition of organic solvent acetonitrile with 0.1% (v/v) formic acid (80:20, %v/v) at a flow rate of 0.5 mL/min. Triple quadrupole mass spectrometry with positive electrospray ionization (ESI) technique operating in multiple reaction monitoring used to estimate MS/MS ion transitions like 306.05>245.10 and 306.05>231.05 for Rohitukine and 330.30>97.0 for IS. Simple single step protein precipitate method was used for sample preparation. The method was validated for specificity, linearity, accuracy, precision, recovery, matrix effect and stability as per FDA guidelines. Linearity of the analyte was acquired throughout the concentration range from 0.1 ng/mL to 1000 ng/mL in mice plasma. Pharmacokinetic study was performed on female BALB/c mice through oral (20 mg/kg) and intravenous (2 mg/kg) route where the oral bioavailability of Rohitukine obtained was 84%. The bioanalytical method was successfully used for determination of plasma protein binding study, permeability and microsomal stability in mouse, rat and human liver microsomes.

Keywords: LC-MS/MS; Validation; Microsomes; Permeability; Plasma protein binding; Rohitukine

Introduction

Rohitukine, a chromone alkaloid has growing significant interest in the natural product drug discovery. Its occurrence naturally limited to only four plant variety such as *Amoora rohituka* and *Dysoxylum binectariferum* (Meliaceae) whereas *Schumannioophyton magnificum* and *Schumannioophyton problematicum* (Rubiaceae) [1,2]. Among these plant sources, trunk bark of *Dysoxylum binectariferum* is the extensively used source for isolation of Rohitukine due to extremely high yields [2].

The parent plant is well known for anti-inflammatory, immunomodulatory, anticancer activities [3,4]. Whereas, Rohitukine is found to arrest mitotic clonal expansion during adipogenesis and improves dyslipidemia [5]. It is also been reported as anti-cancer, gastroprotective, anti-inflammatory [6], immune-modulator, anti-fertility, antileishmanial, CNS depressant and anti-implantation [7,8]. Moreover, Rohitukine synthetically modified chemical analogue named flavopiridol (a anti-cancer molecule) is under phase II clinical trial [9-11]. However, detailed pharmacological investigation on Rohitukine is still under way.

Chemically, Rohitukine is 5,7-Dihydroxy-8-[(3R,4S)-3-hydroxy-1-methyl-4-piperidinyl]-2-methyl-4H-chromen-4-one having molecular formula of C₁₆H₁₉NO₃ with molecular weight of 305.32 g/mol. Chemical structure is shown in Figure 1.

The primordial reason for drug failure during drug development phase was inadequate pharmacokinetic and intolerable toxicity properties rather than inefficacy. Therefore, it becomes foremost important to determine biopharmaceutical (physicochemical properties like Log D, Log P, solubility, microsomal stability etc.) along with optimization of efficacy at early lead identification process. This strategically process refinement improves the overall quality of drug candidates, success probability, shorten the duration of drug development and ultimately reduction in expenses. As of now inadequate pharmacokinetics data of

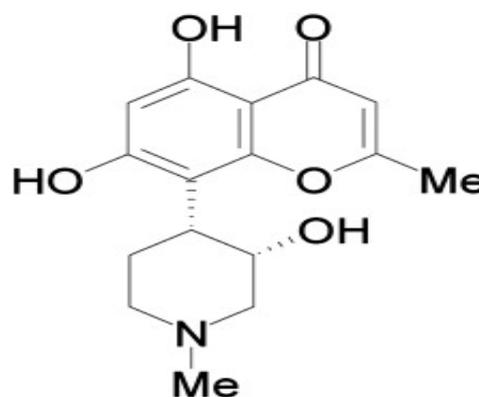


Figure 1: Chemical structure of Rohitukine.

Rohitukine is available in the existing literature. So, there is a critical need to explore absorption, distribution, metabolism, and excretion (ADME) properties of Rohitukine.

***Corresponding authors:** Dr. Gurdarshan Singh, Principal Scientist and Head, Department of PK-PD-Toxicology Division, CSIR-IIIM, Jammu-180 001, Jammu and Kashmir, India, Tel: +919419795920; E-mail: singh_gd@iiim.ac.in

Amarinder Singh, Senior Research Associate, Syngene International, Biocon Park, Plot No. 2 and 3, Bommasandra, IV Phase, Bangalore-560 099, Karnataka, India, Tel: +919086366928; E-mail: amarinder_pharma@yahoo.co.in

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A very simple, rapid, highly sensitive, accurate, reproducible method has been developed for quantification of Rohitukine in BALB/c mice plasma using LCMS/MS, where simple mobile phase composition is used; total run time is short for high throughput analysis; easier one step matrix extraction with improved sensitivity. All the parameters are vital for analysis of any analyte in biological matrix. The method is validated for specificity, recovery, linearity, accuracy, precision and stability as per FDA guidelines [12]. This method is successfully applied for estimation of pharmacokinetics, plasma protein binding, microsomal stability and permeability.

Materials and Methods

Chemicals

Rohitukine (purity~95%) was isolated from leaves of *Dysoxylum binectariferum* plant by Plant Biotechnology Division, CSIR – Indian Institute of Integrative Medicine, Jammu, India. Acetonitrile and water (LC/MS grade) were purchased from Fisher Chemical Pvt. Ltd. Testosterone (internal standard; IS), reduced nicotinamide adenine dinucleotide phosphate (NADPH), magnesium chloride, phosphate buffer were procured from Sigma Aldrich Pvt. Ltd and sodium EDTA (analytical grade) was purchased from Himedia laboratories Pvt. Ltd. Liver microsomes of rat, mouse and human were procured from Invitrogen Life Sciences. Reagents used in the study were of analytical grade.

Instrumentation and chromatographic conditions

Liquid chromatographic analysis was performed employing Shimadzu LCMS 8030 system (Shimadzu, Kyoto, Japan). The system consisted of LC-30AD quaternary pumps, a SIL-30AD autosampler, a DGU-20AR degasser and CTO-20 AC column oven. The chromatographic separation was achieved using high resolution RP18e Chromolith column (100 × 4.6 mm, Merck, Darmstadt, Germany). The mobile phase used is a mixture of acetonitrile and 0.1% (v/v) formic acid in water (80:20 v/v). The flow rate was kept at 0.5 mL/min and column oven temperature was maintained at 26 ± 3°C. Triple-quadrupole tandem mass spectrometer (TQD-MS) coupled with an ESI ion source executing in both positive and negative ion mode. ESI source was operated in positive ion mode and MRM mode was used for quantification. The precursor/product ion pairs were monitored at m/z 306.05>245.10, 306.05>231.05 for Rohitukine and m/z 330.30>97.0 for the IS. All the data was acquired and processed using Lab Solution Analyst Software, version 5.72, from Shimadzu Corporation.

Preparation of Stock, standard and quality control samples

Stock solutions of 1 mg/mL concentration each for Rohitukine and IS were prepared in DMSO. For Rohitukine, working stock solutions were prepared by diluting it with acetonitrile to prepare quality control (QC) and calibration standards (CS). Testosterone (IS) stock solution was further diluted with acetonitrile and spiked into standards and quality control samples at a concentration of 200 ng/mL (Stock solution concentration: 20 µg/mL).

The CS was prepared by spiking 10 µL of respective working stock solutions of Rohitukine into 100 µL of blank plasma matrix and then 10 µL of IS (Stock solution concentration: 20 µg/mL), followed by extraction with 880 µL cold acetonitrile. The final concentrations for CS were 0.1, 0.25, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng/mL in the matrix. Different stock solutions of Rohitukine were used for preparation of lower limit of quantification (LLOQ; 0.25 ng/mL), low quality control (LQC; 20 ng/mL), middle quality control (MQC; 400 ng/mL) and high

quality control (HQC; 800 ng/mL). All the stock solutions were kept in the refrigerator for further use.

Sample processing

Simple one step plasma protein precipitation method was used for the extraction of Rohitukine from the plasma. All the plasma samples were processed through this method. For this 100 µL of plasma (containing Rohitukine) was taken, 10 µL of IS (Stock solution concentration: 20 µg/mL), was added, followed by vortex mix for 30 sec. Then, 880 µL of cold acetonitrile (0.1% formic acid) was added and vortexed for 2 minutes, to facilitate dissemination of drug into extraction solvent. The samples were then subjected to centrifugation at 14000 rpm for 10 min at 4°C to settle down the precipitated matrix. After that, 800 µL of supernatant was transferred into the HPLC vials and finally injected into the LC-MS/MS for the analysis [13].

Method Validation

Method validation is the foremost important for the biopharmaceutical investigation of any compound. The developed LC-MS/MS method for Rohitukine was fully validated (based on accuracy, precision, selectivity, specificity, linearity, recovery and stability studies) according to the US FDA Guideline for Bioanalytical method development [12].

Selectivity and specificity

Selectivity and specificity is the ability of the method to differentiate and quantify the analyte in the presence of potential interfering substances in the sample. The interfering substance can be xenobiotics, analyte decomposed product or endogenous proportion. It was determined by analyzing the blank plasma samples from six different healthy Balb/c mice with that of Rohitukine and IS-spiked plasma sample (n=6). At least four samples out of six ought to have five times less response compare to response at LLOQ level in the same matrix.

Determination of Linearity and LLOQ

The standard calibration curve was attained via plotting of peak area ratio of Rohitukine to IS versus the corresponding Rohitukine concentrations (0.1, 0.25, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng/mL) and linear regression analysis was done for assessment of linearity that is, correlation coefficient (r^2) should be 0.995 or better. The calibration curve consist of a blank sample (plasma sample without IS and drug), a zero sample (plasma sample with IS) and ten samples covering the all concentration of analyte. The lowest calibration curve standard which could be persuadable with accuracy and precision was considered as the lower limit of quantification (LLOQ). According to FDA guideline, response at LLOQ concentration of analyte should be at least ten times that of blank plasma response with ± 20% deviation and each standard concentration was the acceptable within deviation of ± 15% from the nominal concentration [14].

Accuracy and precision

Intra- and inter-day accuracy and precision were determined by analyzing six replicates of QC samples at LLOQ (0.25 ng/mL), LQC (20 ng/mL), MQC (400 ng/mL) and HQC (800 ng/mL) levels, in BALB/c mice plasma for three successive days. The approved acceptance limit of deviation for all the QCs were ± 15% standard deviation (SD) from the nominal values with a precision of ± 15% relative standard deviation whereas for LLOQ, the limit was ± 20% of SD according to FDA guideline.

Recovery

The extraction efficacy was determined by comparing peak area of Rohitukine in extracted plasma samples with neat concentration at LQC, MQC and HQC levels. IS recovery was evaluated at 500 ng/mL.

Matrix effect

Matrix effect is a most critical factor responsible for sensitivity and specificity of any Bioanalytical method. Post column infusion method was adopted to determine wheater the unwanted plasma constituents interfere during the ionization of analyte and IS. This is done by comparing the responses of plasma at different QC standard (n=6) with the analyte response of neat standard at equivalent concentration [15]. A deviation of $\pm 15\%$ from the nominal concentration was acceptable for each concentration except at LLOQ, where acceptable range is 20%.

Determination of analyte stability

Stability studies were performed throughout different phases of bioanalytical method development. The process sample stability was estimated by determining any response/concentration deviation beyond the acceptable criteria, comparing plasma samples that were injected immediately and after 24 hours after loading into autosampler at 4°C. Stability of the analyte in plasma stored in freezer at -80°C for 30 days was assessed using QC samples. Also, three repeated freeze-thaw cycles stability of analyte in plasma at -20°C was accessed using another set of QC samples [16].

Microsomal Stability

Determination of metabolic stability of lead compound is a fundamental requirement during early stage of drug development. It helps in evaluation of chances of drug failure during clinical investigation [17,18]. Therefore, we have evaluated the microsomal stability of Rohitukine in three different species liver microsomes namely rat, mouse and human and the quantification was accomplished using the above mentioned LC-MS/MS.

For this, 10 μM of Rohitukine was prepared in phosphate buffer saline (100 mM) (final DMSO concentration below 0.1%). The reaction mixture was performed in triplicates, containing 0.25 mg/ml microsomal protein, 100 mM phosphate buffer (pH 7.4), and 2.5 mM NADPH in a final volume of 500 μL . Verapamil (3mM) was used as positive control. Sample without NADPH serves as negative control. Incubation was done using water bath maintained at 37°C. 100 μL of reaction mixture was quenched at different intervals 0, 0.25, 0.5, 1, 2 hours using 900 μL cold acetonitrile and Supernatant collected after centrifuged at 14000 rpm for 10 min. analysis was done using LC-MS/MS [19].

Protein Binding Study

An earlier published method with some minor modification [20] was adopted during this study, in order to determine the plasma protein binding of Rohitukine in mice plasma. Rapid equilibrium dialyses (RED) device consist of insert, which is separated into two chambers via dialysis membrane was used. Chamber 1 was filled with 300 μL phosphate buffer (10 mM, pH 7.40, while chamber 2 was filled with mice plasma (200 μL). Three replicates (n=3) were prepared and Rohitukine at a concentration of 10 μM was spiked to plasma containing chamber 2. Equilibrium was achieved by incubating samples at 37 °C for 5 hour. After equilibrium, 100 μL of the resulting plasma and buffer sample from different chambers were promptly extracted separately with cold acetonitrile to recover the analyte and analyzed by LC-MS/MS. The extent of binding of Rohitukine in the equilibrium dialysis experiments

is determined using following eq (1):

$$fb = \frac{\text{Chamber 1} - \text{Chamber 2}}{\text{Chamber 1}} \times 100 \quad 1$$

where fb is the bounded fraction, Chamber 1 is the concentration of compound in the plasma and Chamber 2 is the concentration of the compound in the phosphate buffer. Standard drug (testosterone) was also study to authenticate the assay procedure via comparing percentage plasma protein binding value of the standard compound with the literature reported value.

Parallel Artificial Membrane Permeability Assay (PAMPA)

Passive diffusion is one of the important parameter for the appropriate movement of compound across the cell membranes. The PAMPA method allows estimation of the compound permeability, on the basis of physical and chemical properties and thus gives important information about extent of absorption via passive diffusion.

Permeation experiment was carried out using a hydrophobic PVDF 96-well filter plate (Millipore, Molsheim, France). The donor plate well were coated with 15 μL mixture of 5% (v/v) hexadecane in n-hexane (liquid membrane) and dried overnight at room temperature. In each well of acceptor plate, 300 μL phosphate buffer (100mM, pH 7.4) containg 5% DMSO was added. Solutions of the Rohitukine (100 μM , 200 μL) was added thereafter to donor plate,. The donor-acceptor plate sandwich was raped with wet cloth in order to avoid evaporation during incubation for 8 hours in rotating water bath shaker maintained at 37°C and 120 rpm. Thereafter, concentration of Rohitukine in the donor and acceptor wells were determined using LCMS/MS [21]. Standard drug (testosterone) was used as quality control sample to verify the assay results variation with reported value.

Permeability coefficient calculation

The value of permeability coefficients (logP) of Rohitukine and standard drug testosterone was calculated using Eq's (2), (3) and (4):

$$P_{app} = CX - \ln I - \frac{\text{Drug acceptor}}{\text{Drug equilibrium}} \quad 2$$

$$C = \frac{V_D X V_A}{(V_D + V_A) \times A \times T \text{ (sec.)}} \quad 3$$

$$\text{Drug equilibrium} = \left(\frac{C_D X V_D + C_A X V_A}{V_D + V_A} \right) \quad 4$$

where V_A and V_D are the volumes in the acceptor and the donor wells, respectively, T is the incubation time in seconds(s), A is the filter (0.48 cm^2), C_D and C_A are the concentration of test substance in donor and acceptor well.

Pharmacokinetics Study

Animals husbandry and maintenance

Male BALB/c mice having weight 25-28 g were procured from in-house animal facility of CSIR-Indian Institute of Integrative Medicine, Jammu, India after Institutional Animal Ethics Committee approval (66/84/2/16) for pharmacokinetic study. Polypropylene cages were used to house animals, maintaining standard laboratory conditions of 12 h/12 h light/dark cycle at $25 \pm 2^\circ\text{C}$ temperature, $45 \pm 15\%$ relative humidity. The animals were acclimatized with the laboratory conditions

for one week prior to the experiments with standard pellet diet along with water *ad libitum*.

Dose preparation

The formulation base used for intravenous delivery of Rohitukine contained 5% DMSO, 5% Solutol: absolute alcohol (1:1, v/v) and 90% sterile normal saline (% v/v/v). Whereas for oral dosing 3% DMSO, 2% absolute alcohol, 75% PEG 200 and 20% normal saline was used as vehicle.

Experimental design

Rohitukine was administered at a dose of 20 mg/kg and 2 mg/kg of body weight oral and intravenous route, respectively. Each study done by using total twenty five animals, divided into five groups for sparse sampling. Animals were fasted for 4 h prior to oral dosing with water *ad libitum*.

Blood sampling and processing during pharmacokinetic study

Blood samples were obtained at different time intervals (0, 0.083, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 and 24.0 h) during pharmacokinetic study after i.v and oral dosing. Collection was done in sterile micro centrifuge tubes containing 5% sodium-EDTA in distilled water (% v/v) as an anti-

coagulant. Plasma was obtained by centrifuge blood samples at 5000 rpm for 10 min and stored at -20°C until analysis. During extraction, 100 µl of the plasma was taken to which 10 µl of the IS (Stock solution concentration: 20 µg/mL) was added, followed by processing and analysis of samples as mentioned in previous section. Data obtained after quantification was multiplied with factor of ten, to make the representation of plasma volume to 1 ml. Plasma concentration-time data of Rohitukine were calculated by non-compartmental method using PK solutions software (Version 2.0) for Pharmacokinetics Data Analysis (Summit Research Services, Colorado, USA).

Results and Discussion

Optimization of LC and MS conditions

To optimize MS conditions for quantification of Rohitukine and IS, ionization was carried out in positive and negative polarity mode and the optimal MS conditions for detection of both the analyte and IS were achieved in positive ion mode with ESI interface. The MS/MS parameters such as DL temperature, curtain gas, collision gas were optimized to get intense parent and product ions. The substantially yielded $[M+H]^+$ ions were m/z 306.05 for Rohitukine and m/z 330.30 for IS. Whereas during MRM scan mode, the transitions at m/z 306.05>245.10, 306.05>231.05 for Rohitukine and m/z 330.30>97.0 for IS were preferred (due to higher abundance and greater stability), for

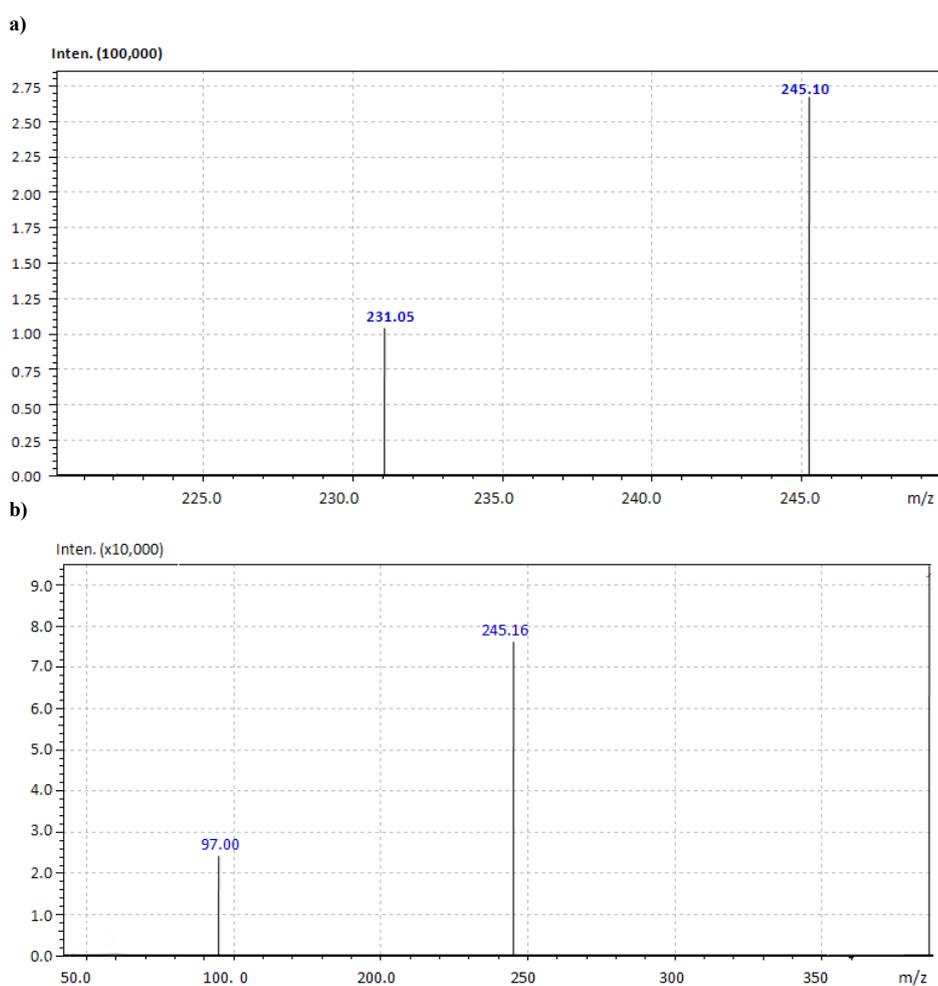


Figure 2: Typical MRM spectrum of rohitukine and IS.

quantitative analysis (Figure 2).

After careful consideration of many columns and mobile phase, chromolith RP 18e column (100 × 4.6 mm) with simple isocratic composition of acetonitrile and 0.1% formic acid in water (80:20, %v/v) at a flow rate of 0.5 mL/min gave symmetrical peak shapes and improved response at low level of detection. This yielded retention time of 3.3 min for Rohitukine that allowed high throughput analysis. Testosterone was preferred as IS because extraction behavior, similarity in chromatographic retention and ionization with that of Rohitukine.

Method validation

Specificity: There was no significant interfering peak was observed because endogenous compounds at the retention time of analyte and IS which was found to be 3.3 and 3.7 min, respectively. Figure 3a and 3b represents the chromatograms of the blank plasma and samples spiked with Rohitukine /IS.

Linearity and LLOQ: Calibration curve of Rohitukine was plotted over ten concentrations from 0.25 to 1000 ng/mL. The calibration range was found to be linear and reproducible over the multiple tested concentrations. The regression equation of Rohitukine concentration

over its peak area ratio was found to be $y=223.733x+0.0001$, where X is the concentration of Rohitukine and Y is the respective peak area ratio of analyte to IS. The regression coefficient (r^2) is 0.995. The lower limit of quantification (LLOQ) was established at 0.25 ng/mL with the coefficient of variation of <20%, indicates the sensitivity of the method and limit of detection (LOD) was 0.1 ng/ml.

Accuracy and precision:

Intra-day (Within-run) accuracy and precision: Analyzing replicate at different concentrations of Rohitukine in mice plasma performed for within-run accuracy and precision evaluations. Six replicates of QC samples (LLOQ, LQC, MQC, and HQC) samples were analyzed twice at a interval of 4 hours (within a day). The intra-day precision was found to be 0.61 to 4.32% at different QC samples whereas accuracy value was range from 94.02 to 105.98% (Table 1).

Inter-day (Between-run) accuracy and precision: The between-run accuracy and precision were assessed by the repetitive analysis of mice plasma samples containing different concentrations of Rohitukine. Six replicates of QC samples (LLOQ, LQC, MQC and HQC levels) were analyzed for three consecutive days and mean results were expressed

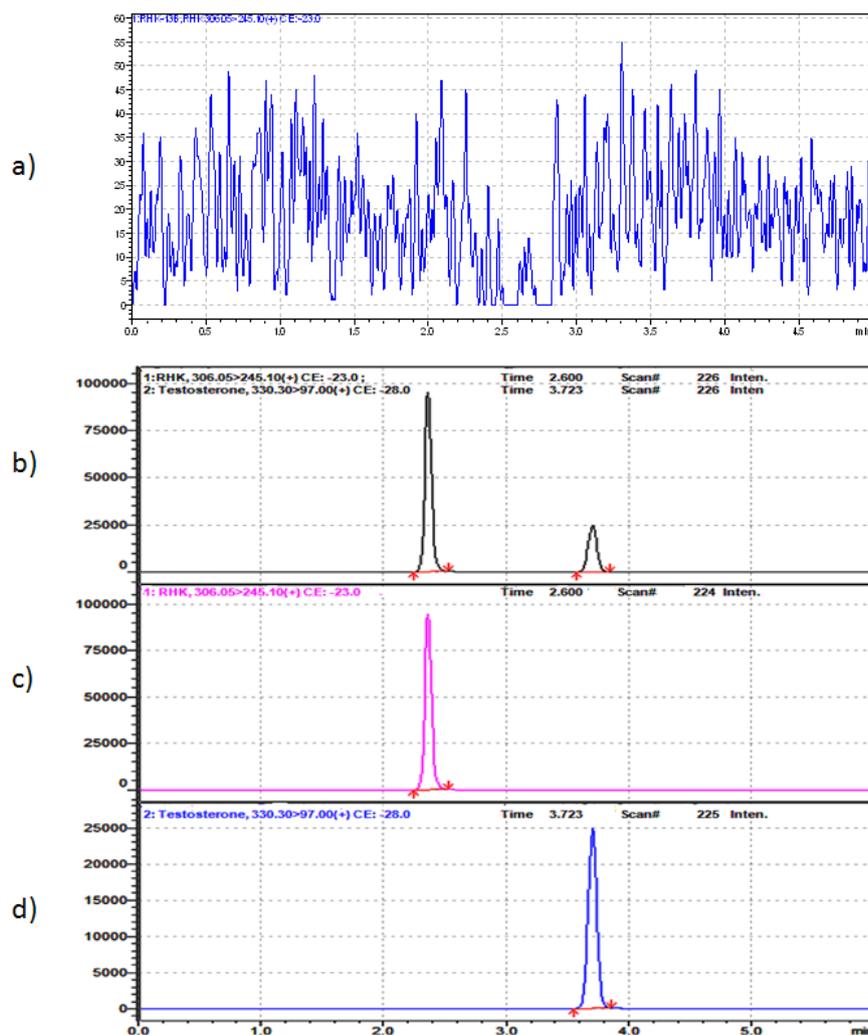


Figure 3: Typical MRM chromatogram/representative chromatogram of a) blank Plasma b) plasma sample obtained after 0.083 hrs of oral administration c) Rohitukine spiked in blank plasma d) IS spiked in blank plasma.

Quality Control (Spiked concentration of Rohitukine; ng/mL)	Inter-day (n=24)			Intra-day (n=6)	
	Measured Concentration (Mean \pm SD; n=6)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)
LLOQ (1.9 ng/ml)	1.76 \pm 0.09	4.87	92.4	2.9	94
LQC (20 ng/ml)	18.81 \pm 1.40	7.43	94.1	4.3	99
MQC (400 ng/ml)	392.86 \pm 5.27	1.34	98.2	0.7	97
HQC (800 ng/ml)	796.05 \pm 4.81	0.6	99.5	0.6	##

SD: Standard Deviation; %RSD: percentage relative standard deviation; %RSD=(SD/Mean) \times 100

Accuracy (%)=100-(Mean measured concentration-Nominal Concentration)/Nominal Concentration

Table 1: Precision and Accuracy data for rohitukine.

Spiked concentration of Rohitukine (ng/mL)	Peak area (Mean \pm SD)		% Recovery
	Extracted Rohitukine response	Un-extracted Rohitukine response	
LQC (20 ng/ml)	4277.0	6022.5	71.0
MQC (400 ng/ml)	89545.5	102072.7	87.7
HQC (800 ng/ml)	177722.7	210495.3	84.4

Table 2: Recovery data of Rohitukine in Plasma (n=6).

Storage conditions	Precision (%RSD)	Accuracy (%)
At 0 hr		
LQC (20 ng/ml)	6.7	99.15
MQC (400 ng/ml)	1.5	100.33
HQC (800 ng/ml)	1.05	98.72
6 hr at room temperature		
LQC (20 ng/ml)	6.08	99.45
MQC (400 ng/ml)	1.47	100.15
HQC (800 ng/ml)	0.92	100.2
48 hr in Autosampler Stability at 4°C		
LQC (20 ng/ml)	3.28	91.69
MQC (400 ng/ml)	0.97	96.09
HQC (800 ng/ml)	0.99	99.60
Freeze and Thaw stability after 24 hours (at -20 \pm 5°C)		
LQC (20 ng/ml)	3.07	94.20
MQC (400 ng/ml)	2.57	89.67
HQC (800 ng/ml)	2.98	94.15
Long term stability (1 month at -80°C)		
LQC (20 ng/ml)	4.01	88.75
MQC (400 ng/ml)	5.35	90.02
HQC (800 ng/ml)	2.67	97.15

Table 3: Stability study data for Rohitukine (n=6).

as accuracy and precision in %RSD of the analytical method (Table 1). The between-run precision (% RSD) was found to be 0.60 to 7.43% at different QC samples whereas accuracy value was ranged from 92.43 to 107.57%.

Recovery: The mean % recovery for LQC, MQC, and HQC are 71.0, 87.7 and 84.4, respectively. The recovery of IS was 98.84%. The extent of analyte recovery was found to be convincing in terms of consistent, precise and reproducible. Protein precipitation utilizing single step extraction via cold acetonitrile was proved to be effective enough to extract both the analytes (Table 2).

Matrix effect: The matrix effect determined at three different concentrations (LQC, MQC and HQC) for Rohitukine. Matrix effect on the estimation of Rohitukine and IS were found to be negligible. During multiple single runs, it has been observed that plasma matrix

did not interfere with analyte and IS peak, and assured that method developed was specific, selective and devoid of any matrix effect.

Stability: The autosampler stability samples were found to be stable for short term and 24 hours at 4°C. Precision varies from 2.57 to 3.07% whereas 89.67 to 94.20% in accuracy, during the freeze and thaw stability study samples at each QC levels, which depicts that analyte was stable in the specified experimental conditions. Same nature of the compound was followed during its long term stability evaluation, which means plasma sample can be stored at least 4 weeks in the mentioned conditions (Table 3).

Microsomal stability

The metabolic stability results for Rohitukine demonstrated that it exhibits little metabolism by mouse microsomal (9%) compared to

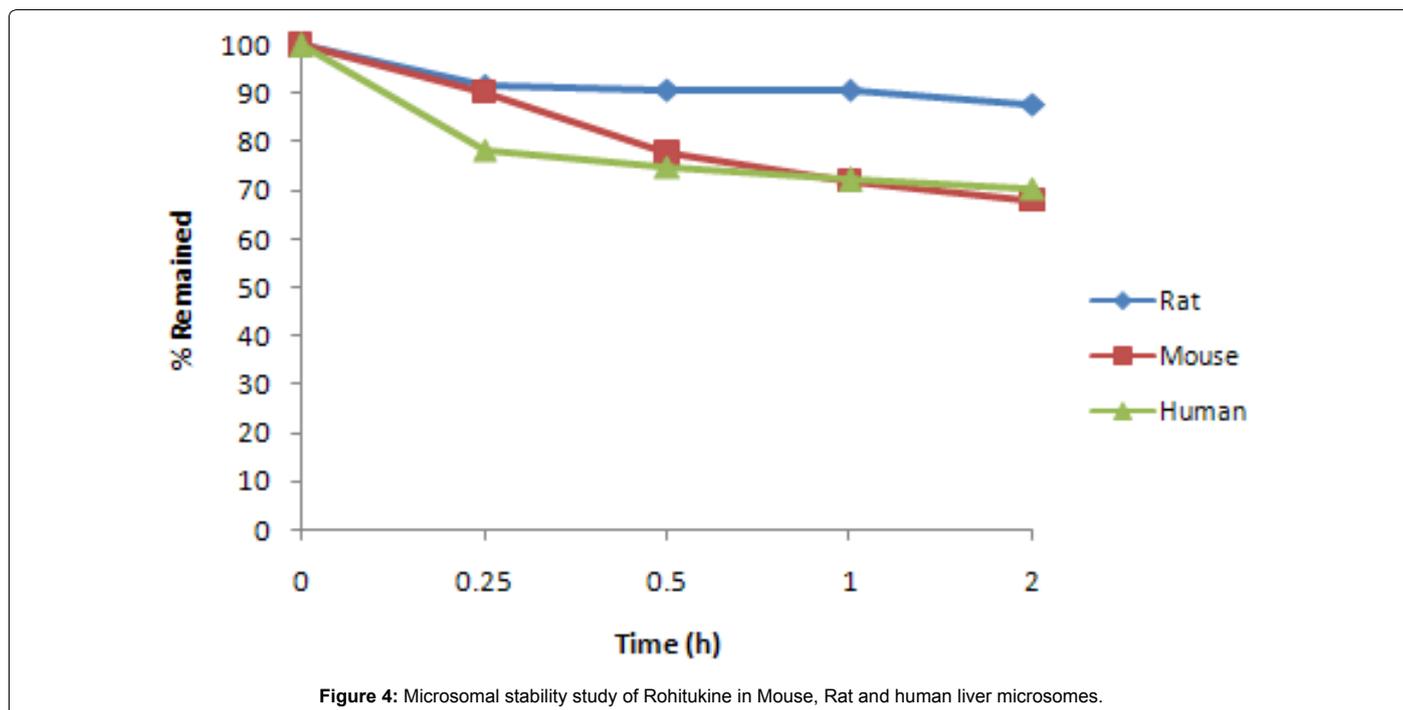


Figure 4: Microsomal stability study of Rohitukine in Mouse, Rat and human liver microsomes.

S. No.	Compound name	Concentration Used	Liver Microsomes concentration: 0.25 mg/ml		
			% Metabolized		
			Mouse	Rat	Human
1	Verapamil (Standard)	3 μ M	69.53	34.18	81.18
2	Rohitukine	5 μ M	9.19	22.26	25.05

Table 4: Data for Microsomal stability study of rohitukine.

Dose	Mean Plasma Concentration (ng/ml) \pm SD							
	0.083 hr	0.25 hr	0.5 hr	1 hr	2 hr	4 hr	8 hr	12 hr
Rohitukine 20 mg/kg p.o.	-	6564.79 \pm 327.81	3699.85 \pm 151.80	1404.71 \pm 186.11	786.74 \pm 195.06	543.95 \pm 169.67	84.38 \pm 15.03	42.58 \pm 4.48
Rohitukine 2mg/kg i.v.	1653.77 \pm 136.04	1124.27 \pm 120.45	368.50 \pm 46.19	247.30 \pm 22.04	45.62 \pm 15.29	ND	ND	ND

PO: Oral; IV: Intravenous; ND: Not Detected; Values are Mean \pm SD; N=3; ND=Not Detected

Table 5: Mean Plasma concentration of rohitukine after oral (20 mg/kg p.o.) and i.v (2 mg/kg i.v.) administration in BALB/c mice.

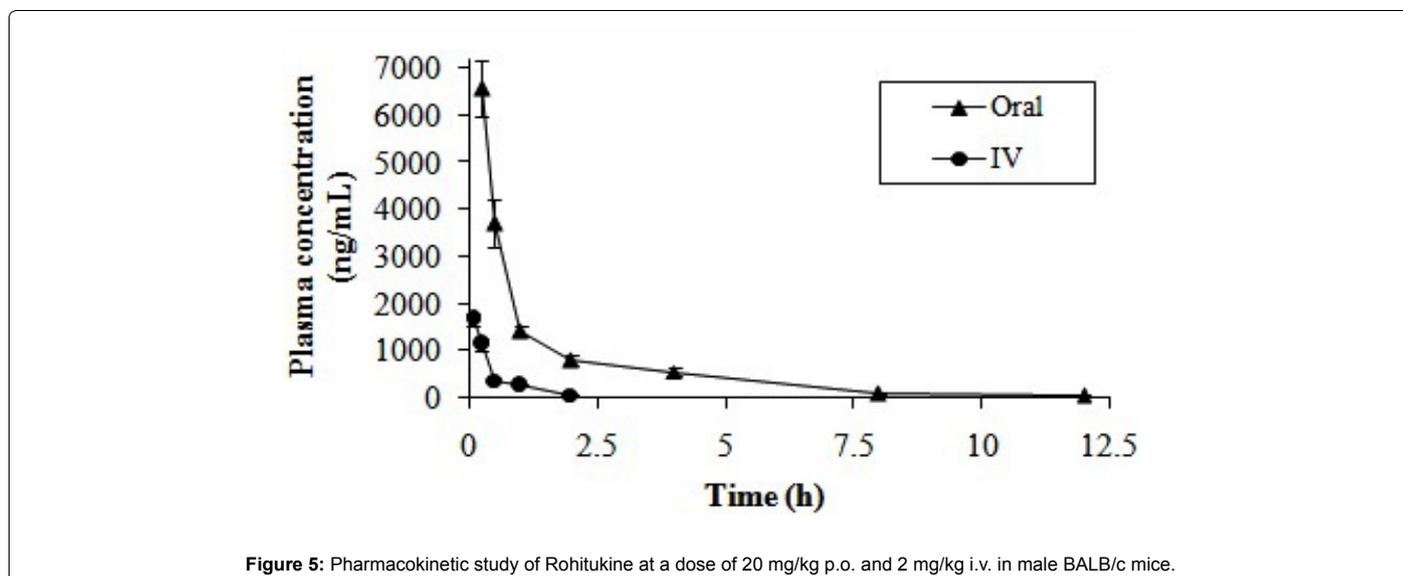


Figure 5: Pharmacokinetic study of Rohitukine at a dose of 20 mg/kg p.o. and 2 mg/kg i.v. in male BALB/c mice.

Parameters	Oral (20 mg/kg p.o.)	Intravenous (2 mg/kg i.v.)
C_{max} (ng/mL)	6564.7	-
T_{max} (hr)	0.3	-
$T_{1/2}$ (hr)	2.17	0.48
$AUC_{(0-t)}$ (ng.hr/mL)	7316.8	871.4
$AUC_{(0-\infty)}$ (ng.hr/mL)	7450.5	903.2
V_d (L/kg)	8.43	1.54
K_{el} (L/hr/kg)	2.68	2.2
MRT (hr)	2.3	0.5
Relative Bioavailability (F %)	84	-

where C_{max} : Maximum concentration; T_{max} : Time at which drug concentration reaches maximum in plasma; $T_{1/2}$: Half life in hours; AUC: Area under the curve; V_d : Volume of distribution; K_{el} : Elimination constant; MRT: Meant resident time

Table 6: Pharmacokinetic parameters of rohitukine after oral (20 mg/kg) and intravenous (2 mg/kg) administrations in male BALB/c mice.

moderately metabolism by rat and human Microsomes (22.26 and 25% respectively) (Figure 4 and Table 4). Results are validated using verapamile as a standard drug. Result indicates that Rohitukine bear acceptable metabolic stability.

Protein binding assay

Equilibrium dialysis is one of the best method for enumerate the plasma protein-drug binding, as nonspecific binding did not have any role in evaluation of free drug fraction [22]. Plasma Protein binding for Rohitukine was found to be 40.257% whereas testosterone was 97.82%, which is approximately equivalent to literature reported value of 98%. Value represents concentration of drug in plasma (unbound drug) that can reach the receptor site and elucidate pharmacological effect.

PAMPA assay

The permeability coefficient of Rohitukine was determined using PAMPA assay. The assay was performed utilizing an artificial membrane, originally proposed by Ottaviani et al. [23,24]. Log p value of Rohitukine was found to be -3.86 and assay was validated by simultaneously determining log p value of testosterone which was found to be -3.11 (found close to the reported literature).

Pharmacokinetic study

The developed and validated LC-MS/MS method was applied for the estimation of Rohitukine pharmacokinetics in male BALB/c mice after a single oral (p.o.) and intravenous (i.v) dose at 20 and 2 mg/kg body weight, respectively (Figure 5). Pharmacokinetic parameters of Rohitukine after an oral and i.v administration are mentioned in the Tables 5 and 6. During the study, we have observed rapid increase in plasma concentration within 0.25 h after dosing, thereafter decline in plasma concentration to LLOQ level by 12 h. The maximum plasma concentration (C_{max}) achieved after oral administration was 6564.7 ng/mL at T_{max} 0.3 h. The mean terminal half life ($t_{1/2}$) was found to be 2.3 h upon oral administration. Area under the plasma curve ($AUC_{0-\infty}$) was obtained to be 7316.8 ng h/mL. The oral bioavailability of the compound was 84%. Results of study indicated fast oral absorption within 15 minutes. This property could be the advantageous in pre-clinical or clinical situations like inflammation, analgesic or immune modulation.

Conclusion

The method we developed and validated is highly sensitive, specific, reproducible and accurate to quantify Rohitukine in mice plasma. It has several advantages with respect to single step sample preparation, simple

mobile phase composition, short run time that is, high throughput analysis and high sensitivity. This LC-MS/MS method was effectively applied for quantitative estimation of Rohitukine in the plasma and evaluation of pharmacokinetics, plasma protein binding, permeability and microsomal stability studies. Rohitukine is fast absorbing plant based molecule with promising high oral bioavailability. This method will help in fast routine estimation of Rohitukine for preclinical and clinical study.

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