Development and validation of bio analytical liquid chromatography and mass spectrometry method for quantification of upadacitinib in human plasma

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Abstract
In the present investigation, a simple, rapid and sensitive liquid chromatography and mass spectrometry (LC/MS) method was developed for quantification of Upadacitinib (UCB) in spiked human plasma. The drug UCB was extracted by liquid –liquid extraction using diethyl ether and chloroform in the ratio of 75:25 (v/v). The drug Leflunomide (LFM) was used as an internal standard. The extracted drug mixture was followed by the liquid chromatography mass spectrometry (LC/MS) analysis and electrospray ionization interface. The chromatographic separation of UCB was carried out on Phenomenex Luna C18 column (100mm X 2.0 mm X 5µm) at ambient temperature followed by isocratic elution of 0.05% formic acid in methanol and acetonitrile in the ratio of 65:30(v/v). The method is concluded at a flow rate of 0.8 mL/min, 262 nm of UV detector wavelength. Protonated ions formed by electrospray ionization were recorded in the positive mode and were used to detect the analyte (UCB) with internal standard (LFM). The mass detection was made by monitoring the fragmentation of m/z 381.0 for UCB m/z 271.0 for LCM on mass spectrometer.

The method was validated for accuracy, precision, linearity and recovery. The assay was linear over the entire range of calibration standards i.e 2.5-500ng/mL. The recoveries of the UCB after liquid-liquid extraction at 5, 20, 35 ng/mL were 100.7, 99.4 and 99.8. The lowest limit of the analytical method of UCB was 2 ng/mL in spiked human plasma. The developed method was successfully validated and can be used for determination of the pharmacokinetic parameters of the UCB in biological samples.

Keywords: Upadacitinib, LC/MS, Internal standard, Validation, Bioanalytical method.

Introduction

Upadacitinib (UCB) is a second generation Janus kinase target inhibitor approved by FDA in 2019 for the treatment of patients with rheumatoid arthritis (RA), therapeutic targets and other inflammatory diseases[1,2]. The drug is used for patients with moderately to severely active rheumatoid arthritis who have had an inadequate response or intolerance to methotrexate. Rheumatoid arthritis is a chronic autoimmune inflammatory disease affecting the peripheral joints which are characterized by synovial inflammation, hyperplasia, autoantibody production, cartilage damage and bone destruction, leading to co morbidities.

JAK is STAT (signal transducers and activation of transcription) which has four subtypes and is responsible for homeostatis in humans by initiating many signaling cascades. The inhibitors of the JAK enzyme family have shown efficacy in treating certain inflammatory and autoimmune diseases such as rheumatoid arthritis and Crohn's disease. UCB inhibits the JAK kinase enzyme inhibitor that is selective for JAK1, JAK2, JAK3 and tyrosine kinase 2 subtypes[3,4]. UCB may indicate as monotherapy or in combination with methotrexate. The chemical structure of UCB is presented in figure 1.

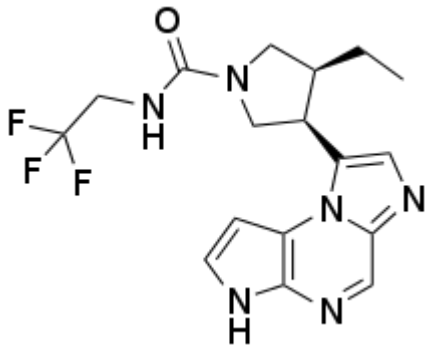

Figure 1: Structure of Upadacitinib

High performance liquid chromatography (HPLC) – mass spectrometry (MS) is a powerful analytical technique that is becoming increasingly used in the clinical setting[5]. It has been demonstrated as a routine technique for application in pharmaceutical analysis that affords high throughput, god sensitivity and selectivity. The present study deals with the development and validation of a method for the quantitative determination of UCB in human plasma with liquid-liquid extraction. Several studies like efficiency study, safety profile study and formulation release characteristics study and pharmacokinetics studies are reported with the UCB drug. Hence the present study is a novel approach for development of HPLC-MS method for analysis of UCB in spiked human plasma[6-8].

Material and Methods

Chemicals and reagents: HPLC- grade organic solvents like methanol, acetonitrile were purchased from Merck, Mumbai. HPLC-grade water was purchased from Thermo Fisher Scientific India Private Limited, Mumbai. Analytical grade chemicals like formic acid, diethyl ether and chloroform were purchased from Thermo Fisher Scientific India private limited, Mumbai.

Instrumentation: Analysis and validation were performed using Waters 2695 Alliance HPLC system (Waters Corporation, Milford, MA, USA) consisting of a quaternary pump, auto-injector, column compartment with temperature control, online degasser using Waters 487 UV detector. The LC is interfaced with a mass spectrometer coupled with an electrospray ionization source operated in the positive mode. Mass spectrometry analysis was carried out with Micromass ZQ mass detector model LAA 1369 (Micromass Ltd., UK). The data interpretation was carried out with masslynx software.

Preparation of solutions

Preparation of mobile phase: Methanol, acetonitrile and 0.05% formic acid in the ratio of 65:30:05 (v/v) respectively were mixed and sonicate the solution for ten minutes to ensure the homogeneous mixing using ultrasonicater and then it was filtered through 0.45 µ nylon membrane filter paper using vacuum filtration set. An equal ratio of methanol and acetonitrile was used as diluent in the analysis. For the preparation of diluent solution, 50mL of methanol was transferred into a 100 mL reagent bottle and 50mL of acetonitrile was added, mixed and sonicated for 5 minutes.

Preparation of calibration standards and quality control samples: The calibration standards were prepared by spiked human plasma with UCB standard drug with corresponding internal standard (LFM) to obtain 2.5, 5, 10, 20, 40, 80, 150, 300, 500 ng/mL respectively. The developed method was validated with the 5, 40, 500 ng/L to respect low-quality control (LQC), middle-quality control (MQC) and high-quality control (HQC) respectively.

Sample extraction procedure: Sample extraction trails were carried by liquid-liquid extraction by using different organic solvents like dichloromethane, diethyl ether, chloroform, methanol and acetonitrile etc. Finally, the combination of organic solvents diethyl ether and chloroform in the ratio of 75:25 (v/v) was successfully used for extraction drugs from the biological matrix. For extraction, 1ml of collected frozen plasma was added with 50mL of diethyl ether and chloroform in the ratio of 75:25 (v/v) in multi-tube vortexer. After centrifugation, the upper organic phase was separated in a conical flask.

Finally, the residue was dissolved in the diluent for the preparation of quality control samples. The blank plasma solution was prepared by following the same procedure without addition of any drugs.

Chromatographic and mass conditions: Chromatographic separation was performed by using C18 Phenomenex® Luna C18 column (100 mm × 2.0 mm, 5 µm particle size) at column temperature of 27^0C. To optimize the mobile phase, analysis of mixture containing 500 ng/mL of UCB and known fixed concentration of LFM IS in acetonitrile, methanol and water was performed consecutively used each of the investigated mobile phase combinations. All chromatographic separations using each of the investigated mobile phase combination at a flow rate of 0.5 ml/min. Methanol, acetonitrile and 0.05% formic acid in the ratio of 65:30:05 (v/v) was the optimum condition in terms of the sensitivity and peak shape of the compound UCB and IS.

The mass spectrometer analysis of UCB was carried out by optimizing the curtains gas, collision gas, ion spray voltage, source temperature parameters. The optimum mass spectrometer conditions for UCB are achieved at capillarity voltage at 3.0V, cone voltage at 40V, extractor voltage at 3V and source temperature at 300 ^{0}C at nitrogen gas flow 300psi. The conformation ion transition for quantification was m/z 281.0 for compound UCB and m/z 271.0 for LFM internal standard respectively.

Results

A simple, rapid, selective and sensitive LC-MS method was developed for the determination of UCB in human plasma. The consideration of a suitable internal standard is an important factor for achieving the accuracy and precision of quantitative analysis. Leflunomide (LFM) was appropriately chosen as an internal standard since it is stable in plasma and reproducible in LC-MS system. The samples were chromatographed to determine to which extent endogenous plasma components may contribute to peak interference at retention times of the UCB.

Furthermore, the equal volume of the spiked plasma of standard UCB and LFM (IS) represents the exact location of peaks and ratio of the extracted UCB for plasma with this methodology. The fragmentation mass spectra of UCB and LFM (IS) are shown in figure 2 and 3.

The precursor ions of UCB and LFM (IS) were found at m/z 381.0 and 271.0 respectively. The product ion spectrum of UCB was similar to that reported. After fragmentation, the stable product ion of the UCB was observed at m/z 256.0. Therefore, the appropriate multi reaction monitoring (MRM) transitions of the m/z 381.0 were selected and were also found specific for detection of UCB. The detailed mass spectrometry parameters are listed in table 1.

The chromatographic conditions were optimized to achieve a good resolution, symmetric peak shape as well as short run time. The proposed method provides fast analysis with a retention time of 1.51 min for UCB and 2.18 for LFM (IS) respectively. The standard chromatogram and mass spectra under proposed conditions are presented in figure 4.

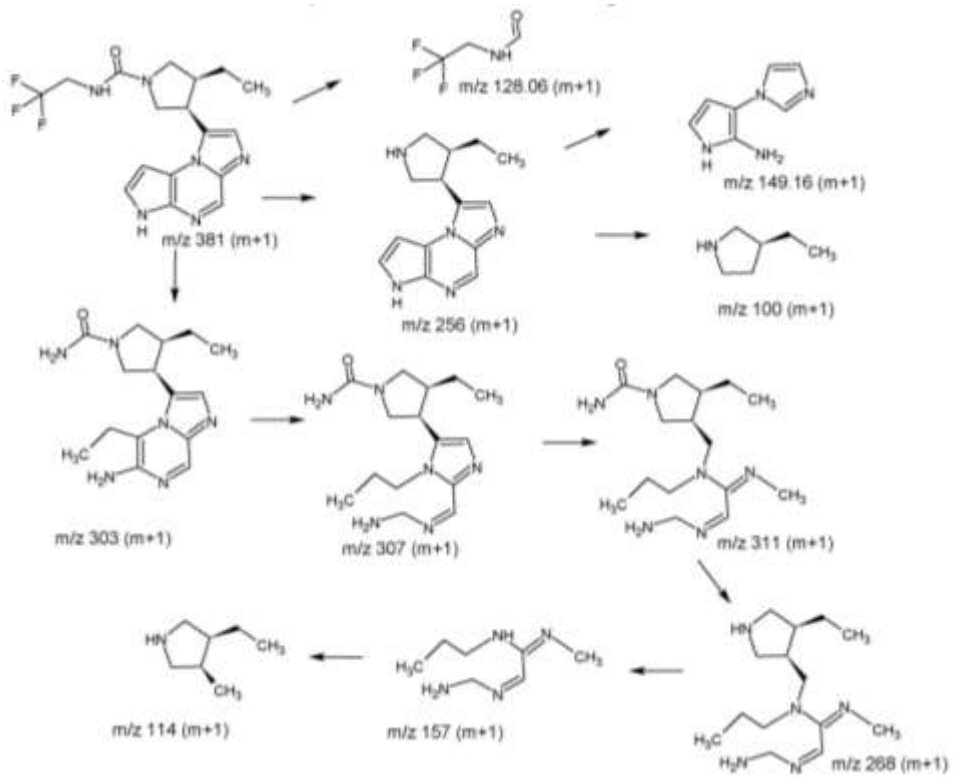

Figure 2: Mass fragmentation pattern of UCB

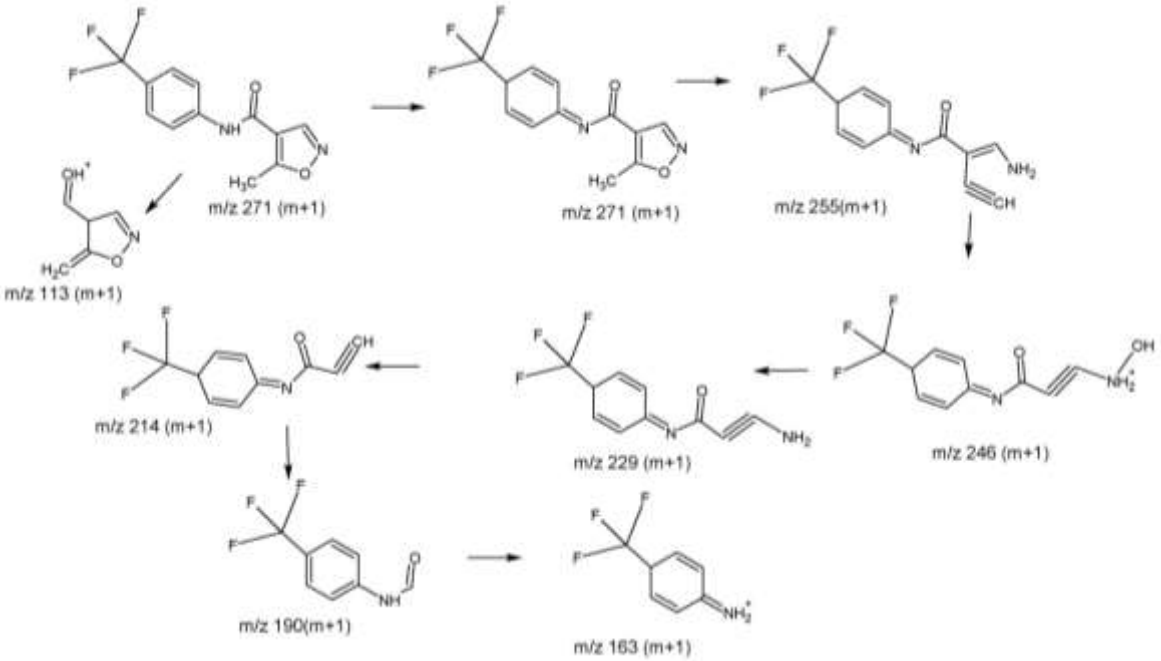

Figure 3: Mass fragmentation pattern of LFM (IS)

Table 1
Optimized mass spectrometry conditions

S.N.	MS Condition	Results
1	Nitrogen Gas Flow	300 Psi
2	Capillary voltage	3.0 kV
3	Cone voltage	40V
4	Extractor voltage	3V
5	Source temperature	300^0C

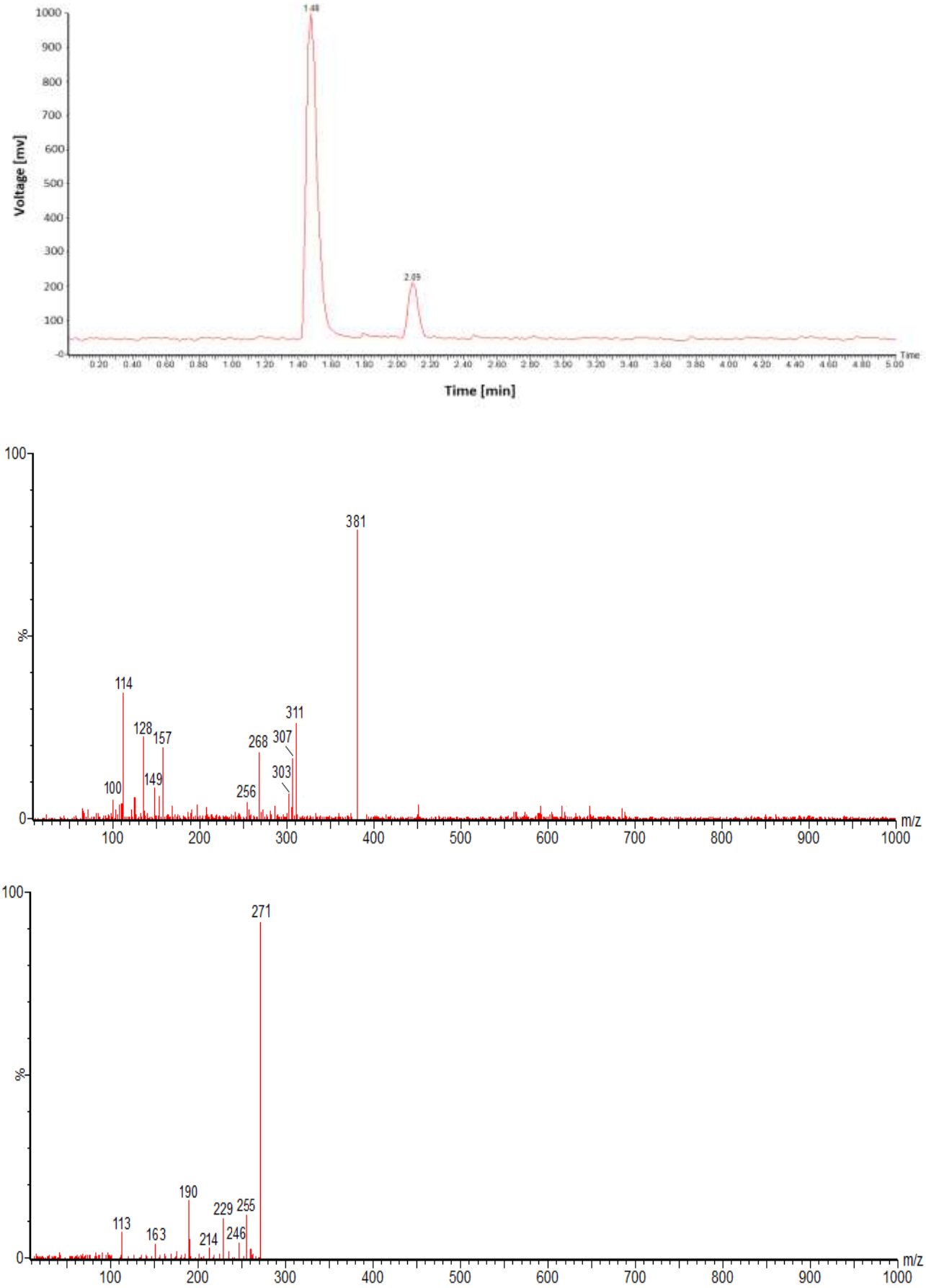

Figure 4: System suitability chromatogram of UCB at 1.48 min and LFM (IS) at 2.09 min. and the mass spectra of UCB (A) and LFM internal standard (B)

Table 2
Optimized chromatographic conditions

S. N.	LC Condition	Results
1	Mobile phase	Methanol, Acetonitrile and 0.05% Formic acid in the ratio of 65:30:05 (v/v)
2	Pump mode	Isocratic
3	pH	4.8
4	Diluents	Mobile phase
5	Column	Phenomenex® Luna C18 column (100 mm × 2.0 mm, 5 µm)
6	Column Temp	Ambient
7	Wavelength	262 nm
8	Injection Volume	10 µL
9	Flow rate	0.8 mL/min
10	Run time	5 min

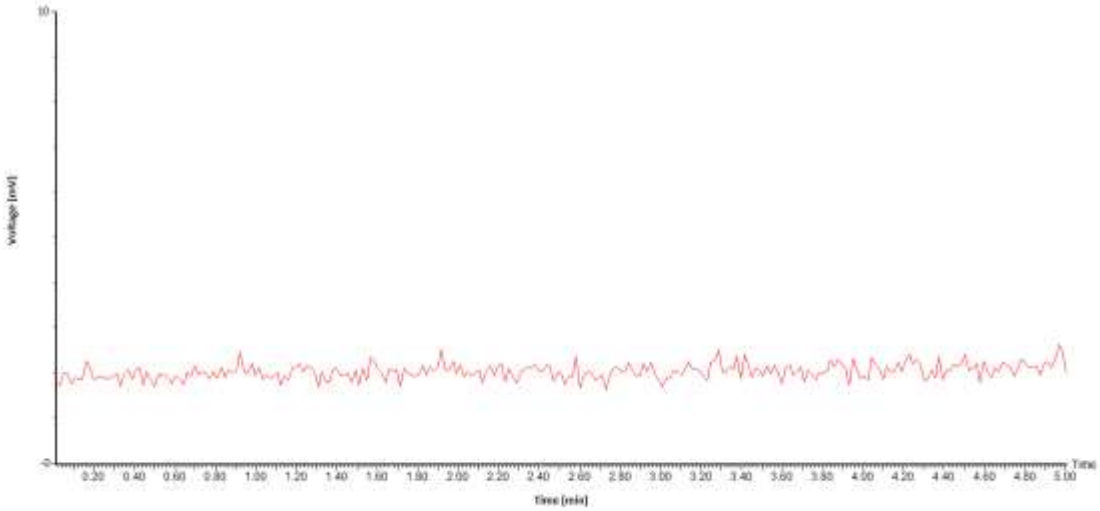

Figure 5: Chromatogram of blank plasma sample

Table 3
Results of calibration curve (Linearity)

S N.	Concentration in µg/ml	Peak Area observed for		Ratio of Standard/IS	Sample Id
		Upadacitinib - Standard	Leflunomide - IS		
1	2.5	10484.3	51241.8	0.205	PSCC 1
2	5	21635.1	51336.7	0.421	PSCC 2
3	10	29857.6	51320.1	0.582	PSCC 3
4	20	42576.9	51336.2	0.829	PSCC 4
5	40	98574.6	51350.4	1.920	PSCC 5
6	80	191535.9	51243.6	3.738	PSCC 6
7	150	344976.5	51224.8	6.735	PSCC 7
8	300	669685.3	51269.7	13.062	PSCC 8
9	500	1111529.8	51357.1	21.643	PSCC 9

The selectivity of the method from the endogenous plasma was investigated by analyzing the three sets of blank human plasma. The absence of the interfering compounds in the chromatogram for UCB and IS demonstrated selectivity in the matrix. Figure 5 represents chromatograms of blank plasma, blank plasma spiked with internal standard and blank plasma spiked with UCB and IS.

No co-eluting peak was eluted with UCB at LLQC level. The linearity was tested by assessing the signal response of UCB in human plasma at a concentration range of 2.5- 500 ng/mL. The calibration points were 2.5, 5, 10, 20, 40, 80, 150, 300, 500 ng/mL. The calibration curve was constructed by plotting the concentrations of UCB against the peak area ratio of UCB and LFM (IS). The proposed analytical method

was linear over the concentration range with coefficient of determination (r^2) of > 0.99. The results of calibration curves are summarized in table 3 and calibration curve is presented in figure 6.

Intra and interday precision studies were done by injecting quality control dilutions (5, 40, 500 ng/mL) and peak areas were calculated for % relative standard seviation (RSD) values. Results concluded the repeatability of the method including both sample processing and chromatographic measurement. The percentage of deviation of the mean from true values expressed as relative error (RE) and the % CV served as the measure of accuracy and precision respectively. Summary of precision and accuracy data are presented in table 4 and 5. All the results of inter and intraday precision are within the acceptable limits of validation. The % RSD of intraday precision results was found 0.33, 0.24 and 0.74 at HQC, MQC and LQC respectively. The % RSD interday precision results were found as 0.60, 0.19 and 1.45 at HQC, MQC and LQC respectively.

Accuracy was calculated after repeated analysis of six different runs. Recovery results were subjected to statistical analysis and % RSD was recorded. The % RSD is a ratio of standard deviation of mean in percent. % RSD values were small indicating good accuracy of results. The recovery analysis was conducted at HQC, MQC and LQC levels of quality control samples. The % recovery was found between 96-61-98.97, 83.58-84.37 and 95.19-7.08 for HQC, MQC and LQC respectively. Recovery results are presented in table 6, 7 and 8.

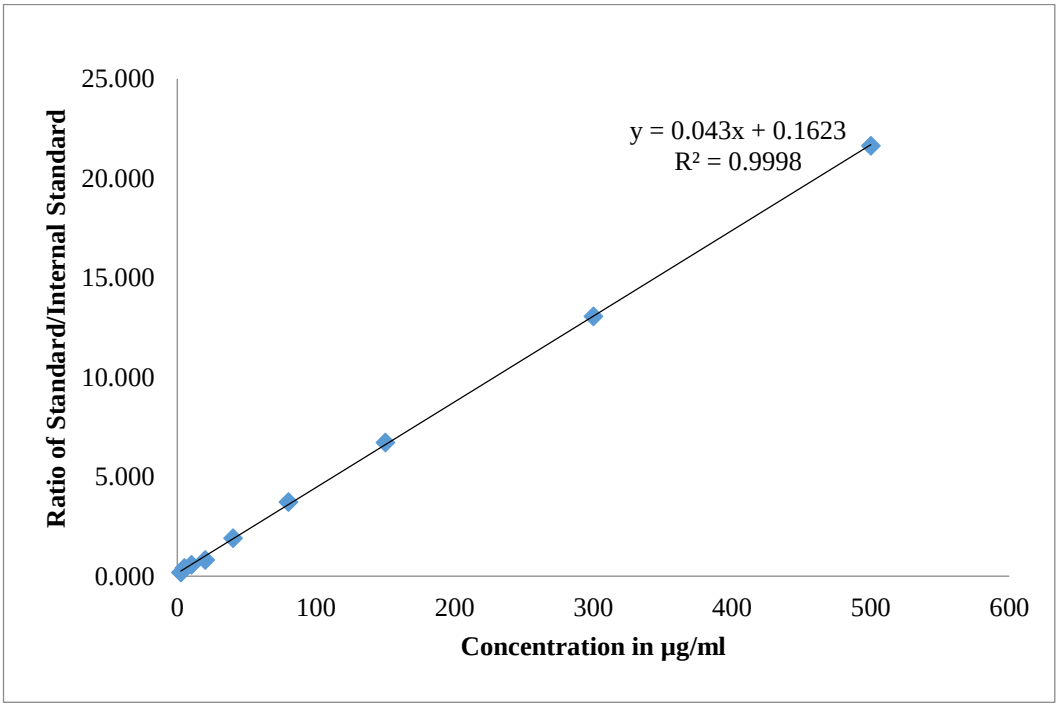

Figure 6: Calibration graph of UCB

Table 4
Results of intraday precision studies

S.N.	Injection	Ratio of Peak area of standard/IS at HQC	Ratio of Peak area of standard/IS at MQC	Ratio of Peak area of standard/IS at LQC
1	1	21.625	1.917	0.413
2	2	21.608	1.913	0.413
3	3	21.596	1.918	0.421
4	4	21.520	1.920	0.415
5	5	21.433	1.919	0.417
6	6	21.580	1.908	0.413
	SD	0.072	0.005	0.003
	Average	21.6	1.9	0.4
	%CV	**0.33**	**0.24**	**0.74**

Table 5
Results of interday precision studies

S.N.	Injection	Ratio of Peak area of standard/IS at HQC	Ratio of Peak area of standard/IS at MQC	Ratio of Peak area of standard/IS at LQC
1	1	21.647	1.913	0.408
2	2	21.695	1.913	0.409
3	3	21.639	1.912	0.422
4	4	21.498	1.920	0.421
5	5	21.390	1.920	0.417
6	6	21.732	1.915	0.412
	SD	0.130	0.004	0.006
	Average	21.6	1.9	0.4
	%CV	**0.60**	**0.19**	**1.45**

Table 6
Results of recovery studies at HQC

S.N.	Test	Sample ID	Peak Area Obtained			Ratio of Peak area of standard/IS	% Drug estimated
			Aqueous	Extracted	IS		
1	Recovery at HQC	R01	1107972.9	1070372.0	49644.2	21.561	96.61
2		R02	1105749.8	1094161.3	49749.2	21.994	98.95
3		R03	1106861.4	1095445.5	49799.5	21.997	98.97
4		R04	1105305.2	1074595.2	49797.7	21.579	97.22
5		R05	1110196.0	1092406.2	50131.5	21.791	98.40
6		R06	1106083.3	1079788.6	49720.4	21.717	97.62
7	**Nominal Conc.**			**500µg/ml**			
8	**N**			**6**			
9	**SD**		1813.872	10913.748	168.980	0.192	0.968
10	**Average**		1107028.1	1084461.5	49807.1	21.8	97.96
11	**% CV**		**0.16**	**1.01**	**0.34**	**0.88**	**0.99**
12	**Accuracy (%)**		97.96				

Table 7
Results of recovery studies at MQC

S.N.	Test	Sample ID	Peak Area Obtained			Ratio of Peak area of standard/IS	% Drug estimated
			Aqueous	Extracted	IS		
1	Recovery at MQC	R08	120193.7	100739.7	50341.5	2.001	83.81
2		R09	120129.7	100400.2	50119.8	2.003	83.58
3		R10	120259.1	101456.7	51028.4	1.988	84.37
4		R11	120335.9	100870.6	51244.6	1.968	83.82
5		R12	120225.7	101351.8	50935.3	1.990	84.30
6		R13	120319.9	101292.0	51235.7	1.977	84.19
7	**Nominal Conc.**			**40µg/ml**			
8	**N**			**6**			
9	**SD**		77.916	414.687	475.203	0.014	0.317
10	**Average**		120244.0	101018.5	50817.5	2.0	84.01
11	**%CV**		**0.06**	**0.41**	**0.94**	**0.68**	**0.38**
12	**Accuracy (%)**		84.01				

Table 8
Results of recovery studies at LQC

S.N.	Test	Sample ID	Peak Area Obtained			Ratio of Peak area of standard/IS	% Drug estimated
			Aqueous	Extracted	IS		
1	Recovery at LQC	R15	21544.2	20589.7	51534.4	0.400	95.57
2		R16	21565.9	20529.4	51760.7	0.397	95.19
3		R17	21442.5	20816.2	52523.9	0.396	97.08
4		R18	21330.0	20453.5	53314.7	0.384	95.89
5		R19	21351.7	20572.2	53012.0	0.388	96.35
6		R20	21561.2	20587.4	56507.4	0.364	95.48
7	**Nominal Conc.**			**5µg/ml**			
8	**N**			**6**			
9	**SD**		107.013	121.481	1802.240	0.013	0.688
10	**Average**		21465.9	20591.4	53108.8	0.4	95.93
11	**%CV**		**0.50**	**0.59**	**3.39**	**3.37**	**0.72**
12	**Accuracy (%)**		95.93				

Table 9
Results of Short term Stability

Injection	HQC		MQC		LQC	
	Ratio of Peak area of standard/IS	% Drug estimated	Ratio of Peak area of standard/IS	% Drug estimated	Ratio of Peak area of standard/IS	% Drug estimated
1	493.651	98.73	39.970	99.92	4.851	97.03
2	492.304	98.46	39.991	99.98	4.843	96.86
3	498.909	99.78	39.994	99.98	4.944	98.89
4	494.757	98.95	39.867	99.67	4.879	97.59
5	495.242	99.05	39.727	99.32	4.893	97.86
6	491.720	98.34	39.915	99.79	4.863	97.26
SD	2.581	0.516	0.103	0.257	0.037	0.737
Average	494.4	98.89	39.9	99.78	4.9	97.58
%CV	**0.52**	**0.52**	**0.26**	**0.26**	**0.76**	**0.76**

Table 10
Results of Long term Stability

Injection	HQC		MQC		LQC	
	Ratio of Peak area of standard/IS	% Drug estimated	Ratio of Peak area of standard/IS	% Drug estimated	Ratio of Peak area of standard/IS	% Drug estimated
1	468.822	93.76	36.155	90.39	4.449	88.99
2	458.990	91.80	36.311	90.78	4.439	88.77
3	463.082	92.62	36.188	90.47	4.559	91.17
4	456.780	91.36	36.262	90.65	4.487	89.73
5	456.046	91.21	36.726	91.81	4.484	89.69
6	456.936	91.39	36.702	91.76	4.469	89.37
SD	4.973	0.995	0.256	0.641	0.042	0.850
Average	460.1	92.02	36.4	90.98	4.5	89.62
%CV	**1.08**	**1.08**	**0.70**	**0.70**	**0.95**	**0.95**

Limit of detection and limit of quantification: The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting the progressively low concentration of the UCB standard solution under the chromatographic conditions. LOD and LOQ were calculated directly from the calibration plot. The LOD and LOQ of the current method were found 0.75 ng/mL and 2.5 ng/mL respectively where σ is the standard deviation of intercept and S is the slope of the calibration plot. The method was confirmed for sensitivity by estimating LOD and LOQ.

Stability: In bench top stability, six replicates of LQC, MQC and HQC (5, 40 and 500 ng/mL) of UCB were analyzed at 0 to 6 hours at room temperature and deviation was calculated. In freeze-thaw-stability, six replicates of LQC, MQC and HQC (5, 40 and 500 ng/mL) of UCB were prepared, frozen at – 20^0C and analyzed two and three freeze thaw cycles. Long term stability was examined for 14 days by taking the six replicates of LQC, MQC and HQC (5, 40 and 500 ng/mL) of UCB. The mean concentration was taken into consideration which was compared with 0-day sample concentration. The % recovery of the UCB at short term stability was found to be 98.46-99.78 at HQC, 99.32-99.98 at MQC and 96.86-98.89 at LQC respectively.

The % recovery of the UCB at long term stability was found to be 91.21-93.76 at HQC, 90.39-91.81at MQC and 88.77-91.17at LQC respectively. The % recovery of the UCB at freeze thaw stability was found to be 92.93-95.69 at HQC, 94.86-96.30at MQC and 91.85-93.87at LQC respectively. The acceptable recovery results of the method concluded that the method is stable. The stability results are presented in table 9, 10 and 11. Hence the developed liquid chromatographic and mass spectrometry methods for quantification of UCB in plasma can be applicable for the drug interaction in study of patients under drug treatment.

Discussion

The complexity of biological matrices can significantly affect accuracy and precision of analytical method. Co-elution of endogenous phospholipids with an analyte can cause ion suppression or enhancement that also dramatically impact on quantitative LC-MS. Hence besides the selection of analytical technique, sample preparation is another key factor for quantitative analysis of UCB in human plasma samples. The sample extraction step must ensure an effective extraction of target analytes, adequate sample clean-up and high sample throughput. Liquid-liquid extraction has been found as simple and effective extraction method among other available methods.

In the present study, different solvents as individual and combination mixtures have been used for extraction of the UCB in blood plasma. Among all studied, diethyl ether and chloroform in the ratio of 75:25 (v/v) has been found effective for extraction of UCB along with its internal standard LFM from blood sample with good recovery and less sample preparation time.

The precursor ions of UCB and LFM (IS) were found at m/z 281.0 and 271.0 respectively. Product ion spectrum of UCB was similar to that reported. After fragmentation, the stable product ion of the UCB was observed at m/z 256.0. Therefore, the appropriate multi reaction monitoring (MRM) transitions of the m/z 281.0 were selected and also found to be specific for detection of UCB. The method was observed to be selective and does not show any matrix effect. The calibration curve was found to be linear over a wide concentration range of 2.5 to 500 ng/mL.

The developed method was compared with the existing literature and confirms that the method was the best choice for the routine analysis of UCB in biological samples and may be applied for the pharmacokinetic studies.

Table 11
Results of Freeze thaw Stability

Injection	HQC		MQC		LQC	
	Ratio of Peak area of standard/IS	% Drug estimated	Ratio of Peak area of standard/IS	% Drug estimated	Ratio of Peak area of standard/IS	% Drug estimated
1	473.922	94.78	38.394	95.98	4.603	92.06
2	464.653	92.93	37.946	94.86	4.592	91.85
3	470.068	94.01	38.192	95.48	4.694	93.87
4	478.461	95.69	38.520	96.30	4.624	92.48
5	476.458	95.29	38.292	95.73	4.677	93.54
6	474.660	94.93	37.821	94.55	4.600	92.01
SD	4.973	0.995	0.267	0.668	0.043	0.864
Average	473.0	94.61	38.2	95.49	4.6	92.63
%CV	**1.05**	**1.05**	**0.70**	**0.70**	**0.93**	**0.93**

Conclusion

The present study was undertaken to develop a sensitive method for quantification of UCB in human plasma. The assay employed with UCB provided advantages in terms of simplicity of liquid – liquid extraction, low cost analysis and appropriate reproducibility. The efficiency of LLE and chromatographic runtime of 5.0 miuntes per sample allowed the method to be useful for routine analysis. The developed LC-MS method was validated in accordance with guidelines and confirmed to be specific, selective, accurate, precise, sensitive and stable.

The validated method can be applied for bioequivalence study and routine measurement in pharmacokinetic studies to quantify the levels of UCB in human volunteers under therapy of rheumatoid arthritis (RA).

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