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Original Article

BIOANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF RIBOCICLIB AND LETROZOLE AND ITS APPLICATION TO PHARMACOKINETIC STUDIES USING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objective: An easy, quick, precise, active and reproducible UPLC technique was developed for the bioanalytical method of Ribociclib and Letrozole using Lapatinib as the internal standard.

Methods: This article summarizes the recent progress on bioanalytical UPLC method using phenyl column (100x2.1 mm, 1.7µ) column and an organic mobile phase of 0.1% Tri fluoro acetic acid and Acetonitrile in 50:50 with a flow of 0.5 ml/min. An injection volume of 5 microliters was used. Lapatinib was used as an internal standard.

Results: The drugs were found at Letrozole (m/z 435.46/216.55), Ribociclib (m/z 506.34/167.43) and Lapatinib (internal standard, m/z 582.37/184.29), respectively. Tests were performed in less than five minutes on Ribociclib (r^2 = 0.99953; concentration range: 2 to 40 ng/ml) and Letrozole (r^2 = 0.99915; concentration range: 0.025 to 0.5 ng/ml). The study's precision and recovery results were determined to be accurate. Accuracy, precision, recovery, matrix effect and stability results were found to be within the suitable limits. Simple and efficient method was developed and utilized in pharmacokinetic studies to see the investigated analyte in body fluids.

Conclusion: The application denotes all the parameters of system suitability, specificity, linearity and accuracy are in good agreement with USFDA guidelines and applied effectively for the investigation of pharmacokinetic studies in rabbit.

Keywords: Ribociclib, Letrozole, Development, Validation, Rabbit plasma

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INTRODUCTION

In the treatment of hormonally sensitive breast cancer [1, 2] after surgery, letrozole is an aromatase inhibitor [3] offered under many trade names, including Femara (by Novartis). Letrozole's antiestrogen [4] effect has been demonstrated to be beneficial as a pretreatment for misoprostol-induced abortion [5]. Instead of pricey and unavailable mifepristone, it may be used [6]. Letrozole is occasionally used to treat gynecomastia [7, 8]; however, it is most successful if discovered early on in the condition's progression (such as in users of anabolic steroids). In male patients with nonobstructive azoospermia [9, 10], letrozole has been demonstrated in certain trials to help enhance spermatogenesis [11]. Mice treated with letrozole had their growth plates take longer to fuse, according to a study that examined the drug [12]. Letrozole has been demonstrated to be successful in one short-statured [13] teenage male when combined with growth hormone [14]. Endometriosis [15] patients have benefited from letrozole treatment. It has been shown that letrozole decreases serum oestrogen levels in endometrial stromal sarcomas. A promising alternative for the long-term treatment of this condition is letrozole. The amount of uterine myoma tumours was also effectively decreased with an aromatase inhibitor in one research [16]. Aromatase inhibitors provide a rapid beginning of action and prevent the early gonadotropin flare. Sweating, heat flushes, arthralgia (joint pain) [17], and weariness are the most prevalent adverse effects. Symptoms of hypoestrogenism [18] are often reported as adverse effects. Osteoporosis has been linked to long-term usage of bisphosphonates in particular patient groups, such as post-menopausal women or osteoporotics [19].

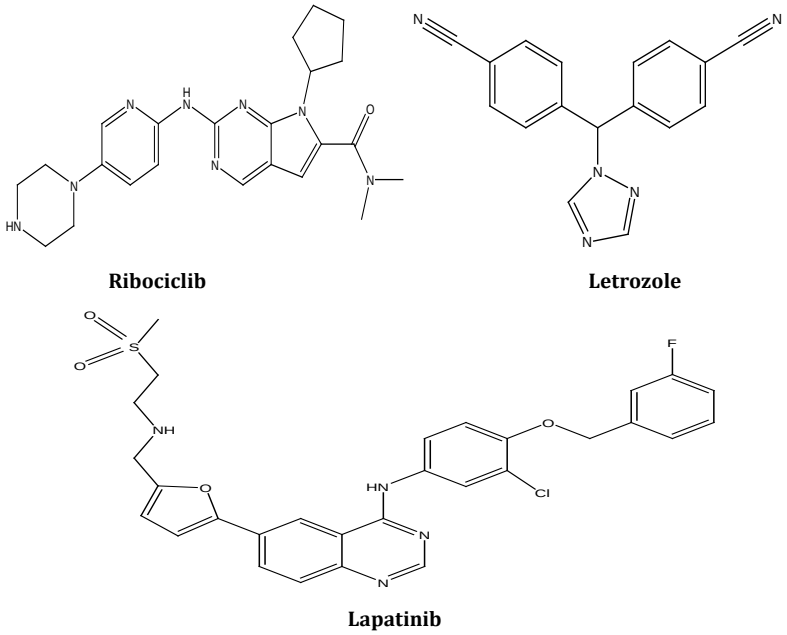
Inhibitors of cyclin D1/CDK4 (Cyclin-dependent kinase 4) [20] and CDK6 (Cyclin-dependent kinase 6) [21] are found in Ribociclib, which is marketed by Novartis under the brand names Kisqali and Kryxana. This drug is used to treat some types of breast cancer. The combination of aromatase inhibitors and fulvestrant [22] for the treatment of pre/perimenopausal or postmenopausal women with HR-positive, HER2-negative (protein) advanced or metastatic breast cancer, as an initial endocrine-based therapy [23], is recommended for the treatment of postmenopausal women with HR-positive, HER2-negative advanced or metastatic breast cancer. Also being tested as a therapy for other drug-resistant tumours [24]. Novartis and Astex Pharmaceuticals collaborated on its creation and are the proud owners. All three MONALEESA clinical studies with diverse endocrine therapy partners, independent of menopausal state or treatment line, have shown that Ribociclib is the only CDK4/6 inhibitor with a demonstrable benefit on overall survival. Anemia [25] and neutropenia [26] (in 75% of patient's vs 5% in placebo groups) were the most prevalent adverse effects in trials, as were reduced blood cell counts, particularly neutropenia (18 percent vs. 5 percent). Gastrointestinal problems [27] were also widespread, such as nausea [28] (52 percent vs. 29 percent) and diarrhea [29] (35 percent vs. 22 percent), as well as alopecia [30] (33 percent vs. 16 percent). Fig. 1 represents the chemical representation of Ribociclib and Letrozole and Lapatinib (Internal standard).

In the present research, UPLC was used for the simultaneous quantification of Ribociclib and Letrozole in rabbit plasma. Until now, there were no quantification methods for Ribociclib and Letrozole. UPLC offers better separation and can thus yield more information in a short period of time than HPLC. Thus, UPLC separation technique was employed for the purpose of separating Ribociclib and Letrozole.

Experimental study

Solutions and reagents

Zydus Cadila, an Ahmadabad provided the APIs of Ribociclib ($C_{23}H_{30}N_8O$), Letrozole ($C_{17}H_{11}N_5$), and Lapatinib (Internal Standard, $C_{29}H_{26}ClFN_4O_4S$). Acetonitrile (99.99 percent purity), water (Milli Q), and trifluoroacetic acid (99.99 percent purity) were acquired from Merck in Worli and Mumbai, India.



Ribociclib **Letrozole**

Lapatinib

Fig. 1: Chemical structures of Ribociclib, Letrozole and Lapatinib

Equipment

The experiment was carried out using Agilent1290 Infinity II LC System with a variable PDA detector [31-33], which was developed by the researchers. The data collection and processing were carried out using the chromatographic programme Empower-2.0.

Preparation of standard and internal control samples

Preparation of stock solution-A

Accurately weighed and transferred 8 mg of Ribociclib pure powder into a 100 ml volumetric flask. Approximately 70 ml of diluent was added, sonicated for 15 min to dissolve, and then diluted to the volume. One mill liter of the above solution was further transferred to 100 ml volumetric flask, made up to the volume with diluents. This is the stock solution-A.

Preparation of stock solution-B

Accurately weighed and transferred 5 mg of Letrozole pure powder into a 100 ml volumetric flask. Approximately 70 ml of diluent was added, sonicated for 15 min to dissolve, and then diluted to the volume. Two mill liters of the above solution was further transferred to 100 ml volumetric flask, made up to the volume with diluents. After that one mill liter of the above solution was further transferred to 100 ml volumetric flask, made up to the volume with diluents. This is the stock solution-B.

Preparation of standard stock solution

One mill liter of stock solution-A and stock solution-B were transferred to 10 ml volumetric flask, made up to the volume with diluents.

Preparation of internal standard stock solution

Accurately weighed and transferred 8 mg of Lapatinib pure powder into a 100 ml volumetric flask. Approximately 70 ml of diluent was added, sonicated for 15 min to dissolve, and then diluted to the volume. One mill liter of the above solution was further transferred to 100 ml volumetric flask, made up to the volume with diluents. After that one mill liter of the above solution was further transferred to 10 ml volumetric flask, made up to the volume with diluents. This is the internal standard stock solution.

Preparation of standard solution

For standard preparation, 200 µl of plasma was taken and 300 µl of ACN into a 2 ml centrifuge tube and 500 µl of standard stock solution and 500 µl of IS and 500 µl of diluents were added and vortexed for 10 min. These samples further subjected for centrifuge at 5000rpm for 30 min. Collect the solution and filter through 0.45µ nylon syringe filter and the clear solution was transferred into vial and injected into a system.

Bioanalytical method validation

The method was validated [34-39] in selective, sensitive, linearity, accuracy and precise, matrix condition, recovery study, re-injection reproducibility and stability.

Animal parameters

In vivo pharmacokinetic studies, 6 healthy white New Zealand female rabbits (body weight in between 2.0-2.5 kg) were obtained from Biological E Limited, Hyderabad, India. The protocol of animal study was approved by the institute of animal ethics committee (Reg. No: 1074/PO/Re/S/05/CPCSEA). The animals were housed in similar laboratory conditions with access to endive, carrots, fresh corn (few amount only); the animal feed should be kept at temperature of 36±3°C and humidity was 51-63%. Before experimentation, all animals were fasted overnight and had water adlibitum.

Selectivity

Selectivity was carried out by investigating the rabbit plasma specimens from 6 various rabbits to test for obstruction from unknown specimens at retention times of Ribociclib, Letrozole and IS.

Matrix effect

The matrix effect for Ribociclib and Letrozole was eavluated by comparing the peak zone proportion in the post extracted plasma samples from 6 different medication-free plasma samples and slick recovery samples. Trails were carried out in triplicate with six separate lots of plasma at MQC levels with acceptable accuracy (percent CV) of ≤15%.

Precision and accuracy

It was evaluated at a lower quantification limit (LLOQ), low-quality control (LQC), medium quality control (MQC), and high-quality control (HQC) levels by replication analysis of quality control specimens (n=6). The level of CV should be less than 15%, except for LLOQ, where it should be less than 20%.

Recovery

By analyzing the six samples reproduce at each internal control concentration, is by extracting the Ribociclib and Letrozole. By comparing the height areas of extracted standards to the height areas of unextracted standards, recovery was evaluated.

Carry over

Carry over [40, 41] was performed with the analyte retained by the chromatographic system during the matrix with an analyte concentration ULOQC and above, diluting this sample with blank matrix.

Dilution integrity

Dilution integrity ought to be exhibited by spiking matrix above the ULOQC with an analyte concentration and diluting this test with a blank matrix.

Stability

Stability of stock solution was achieved by comparing the area response of the analyte in the stability [42] samples with the region response of the specimen prepared from the fresh stock solution. Sample Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is a smaller amount than 15% as per US FDA guidelines. The perfectness of spiked rabbit plasma stored at room temperature was evaluated for twenty-four hrs. The stability of spiked rabbit plasma stored at RT in an autosampler was evaluated for twenty-four hrs. The autosampler stability (LQC, MQC and HQC) was evaluated by comparing the extract plasma samples that were injected immediately with the samples that were re-injected after storing with wet extract stability at room temperature after 12 h and 18 h at 2-8 °C. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately with the samples that were re-injected after storing in the dry extract stability at room temperature after 12 h and 18 h at-20 °±3 °C. Freeze-thaw stability was conducted by comparing the steadiness samples that had been frozen at-31 °C and thawed 3 times with freshly spiked internal control samples. The short-term stability was conducted 7 d at 7 °C. For long-term stability evaluation, the concentrations obtained after 24 h were compared with the initial concentration.

Pharmacokinetic study

Before experimentation, all animals are starved overnight and had water ad-libitum. Topical anaesthetic procedure was used. Pharmacokinetic evaluation was performed for Ribociclib and Letrozole formulation. The samples were administered to each rabbit under fasting conditions. After oral administration of Ribociclib and Letrozole, blood samples were collected from rabbit marginal ear vein using a 25-guage, 5/8 inch needle by clipping the marginal ear vein with a paper clip shown in fig. 1 with volume of 0.5 ml to 1.0 ml at 0.5,1,1.5,2,2.5,3,3.5,4,5,10,20,30,40 and 50 h. The blood was collected in Eppendorf containing 10% EDTA solution. Blood was centrifuged at 5000 rpm for 30 min at 2-8 °C temperature. The clear supernatant plasma were collected and stored at-30 °C till its analysis. After the study, the animals were returned to the animal house for rehabilitation.

The pharmacokinetic parameters for Ribociclib and Letrozole oral administration were determined from plasma concentration data. Pharmacokinetic parameters like AUC, Cmax, T_{max} the time at which C_{max} occurred, K_{el}, $t_{½}$, K_a and MRT were calculated using the data. Data was measured by the trapezoidal rule method from time zero to infinity of the concentration-time curve. C_{max} and T_{max} were obtained from the graph.

RESULTS AND DISCUSSION

To acquire the best chromatographic conditions, we used different buffers with acetonitrile as mobile phase in different ratios for isocratic and gradient mode was tested. The mobile phase composition was modified at each trial to enhance the resolution and also to achieve acceptable retention times. Finally, 0.1% tri fluoro acetic acid and ACN in isocratic mode at 50:50 v/v ratio was selected as mobile phase because it gives a maximum response of the selected drugs. In the optimization method, we used different stationary phases like $C_{18,}$ C_8 and CN-propyl was used. From the different trials we get good peak shapes of Ribociclib and Letrozole by using C_8 column of dimensions 50 mmx1.0 mm, 1.7 µ connected to a PDA detector. Mobile phase flow rates were performed at 0.5 ml/min. By applying the above conditions, we get the retention times of the two drugs Ribociclib and Letrozole as 1.564 min and 3.045 min, respectively. The method under progress has been validated pursuant to USFDA guidelines [43]. Fig. 2, shows the standard chromatogram.

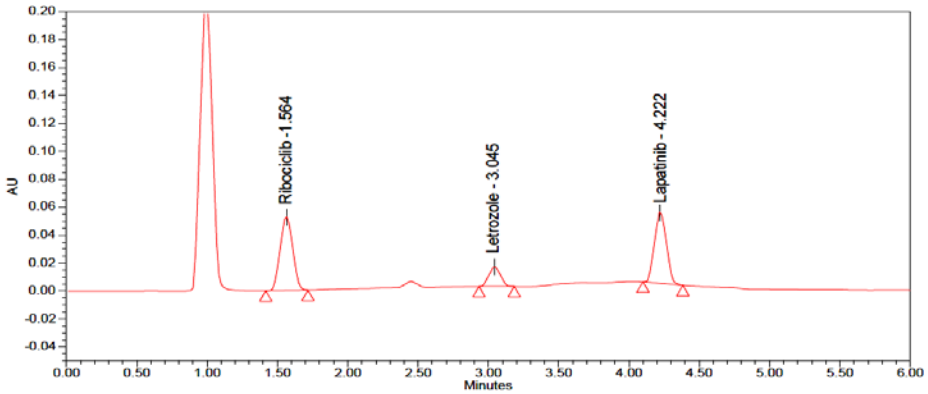


Fig. 2: Chromatogram of standard

Specificity

The specificity of the method to research Ribociclib and Letrozole simultaneously is proved. The chromatograms of blank as shown in fig. 3. The chromatogram of blank rabbit plasma and standard having no interference peaks were observed.

Matrix effect

Matrix effect [44] results of Ribociclib and Letrozole at LQC, HQC levels were 98.35, 99.17% and 98.73, 99.57%. Where % CV of both drugs was found at LQC and HQC levels 1.25, 0.37 and 1.05, 0.76. The results indicate that the matrix influence on the ionization of analytes and the internal requirements have been within the acceptable limit.

Linearity

The region at its height proportions in adjustment norms were relative under focus. The range of linearity for Ribociclib of this method was 20-40 ng/ml and 0.025-0.5 ng/ml for Letrozole (fig. 4). The curves of calibration were appeared over the linear

concentration range [45] and the correlation coefficient was found to be beyond 0.99953 for Ribociclib and 0.99915 for Letrozole at different QC levels. Linearity and correlation results of Ribociclib and Letrozole are shown in table 1.

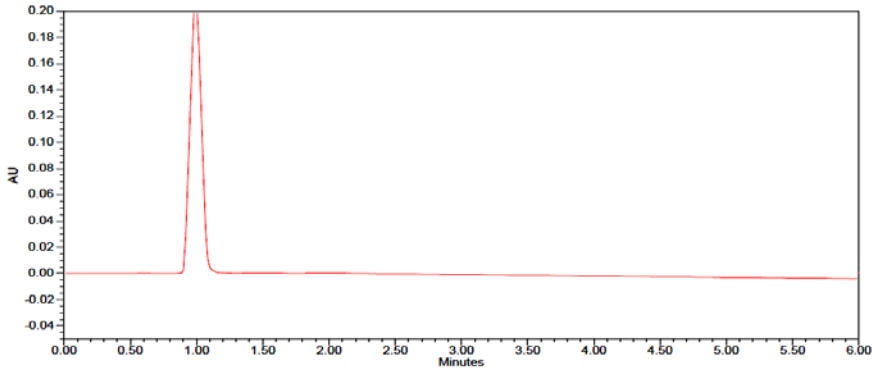
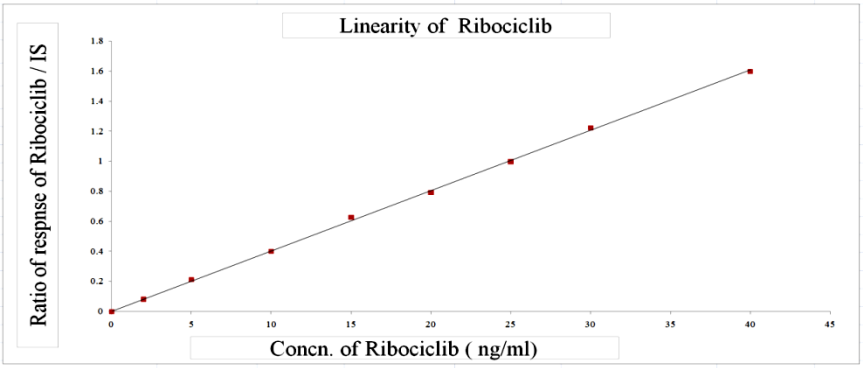


Fig. 3: Chromatogram of blank

Table 1: Results of linearity

Linearity	Ribociclib		Letrozole	
	Conc.(ng/ml)	Area response ratio	Conc.(ng/ml)	Area response ratio
1	2.00	0.082	0.03	0.007
2	5.00	0.213	0.06	0.020
3	10.00	0.400	0.13	0.038
4	15.00	0.628	0.19	0.052
5	20.00	0.793	0.25	0.073
6	25.00	0.997	0.31	0.092
7	30.00	1.223	0.38	0.111
8	40.00	1.597	0.50	0.147
Slope		0.0403	Slope	0.2944
Intercept		0.00266	Intercept	0.00022
CC		0.99953	CC	0.99915



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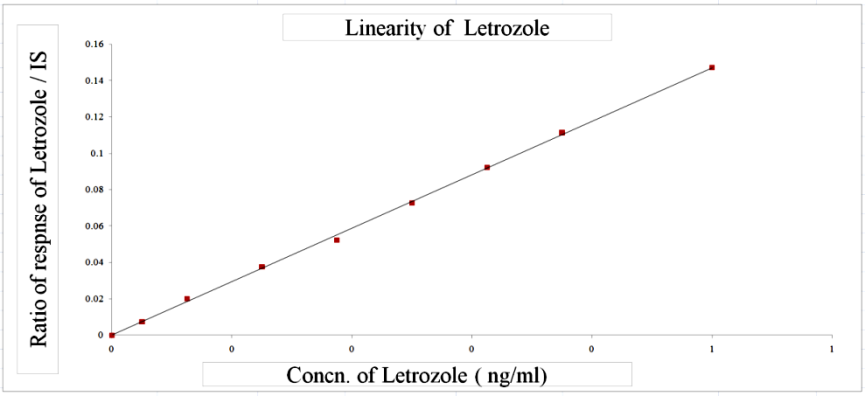

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Fig. 4: Calibration plots of (A) Ribociclib and (B) Letrozole

Table 2: Precision and accuracy of ribociclib

QC name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	2	10	20	30
QC sample-1	2.021	10.154	20.153	30.154
QC sample-2	2.057	10.142	20.127	30.112
QC sample-3	2.032	10.174	20.151	30.209
QC sample-4	2.038	10.126	20.224	30.153
QC sample-5	2.067	10.198	20.163	30.164
QC sample-6	2.017	10.104	20.172	30.118
Mean	2.039	10.150	20.165	30.152
SD	0.020	0.034	0.033	0.035
%CV	0.97	0.33	0.16	0.1
Accuracy	101.07	100.62	99.96	99.64

Mean+SD (n=6)

Table 3: Precision and accuracy of letrozole

Qc name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	0.025	0.125	0.255	0.375
QC sample-1	0.027	0.123	0.257	0.374
QC sample-2	0.023	0.122	0.254	0.378
QC sample-3	0.024	0.129	0.258	0.372
QC sample-4	0.025	0.124	0.253	0.376
QC sample-5	0.022	0.128	0.251	0.375
QC sample-6	0.027	0.127	0.283	0.371
Mean	0.025	0.126	0.259	0.375
SD	0.002	0.003	0.012	0.002
%CV	8.37	2.30	4.58	0.53
Accuracy %	98.81	99.6	102.37	98.81

(n=6)

Precision and accuracy

By pooling all individual assay results of different internal control samples, the accuracy and precision [46] were calculated. It was obvious, based on the data provided, that the strategy was precise and effective. The precision results of Ribociclib and Letrozole was shown in table 2, 3. Ribociclib accuracy results in quality control samples 99.64-101.07 and Letrozole accuracy results in quality control samples 98.81-102.37.

Recovery

The recoveries for Ribociclib and Letrozole at LQC, MQC and HQC levels the results demonstrated that the bio-analytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. The recoveries for Ribociclib (99.43%-100.79%) and Letrozole (98.74%-100.11%) at LQC, MQC and HQC levels and % CV ranged from 0.32-1.56 for Ribociclib and 0.74-1.92 for Letrozole; the results demonstrated that the bio-analytical method had good extraction efficiency.

Ruggedness

The percent recoveries and percent CV of Ribociclib and Letrozole determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples. The results proved method is ruggedness. The percent recoveries ranged from 98.53–101.23% for Ribociclib and 99.73%-100.24% for Letrozole. The %CV values ranged from 0.43-1.29 for Ribociclib and 0.38–1.58 for Letrozole.

Autosampler carryover

Peak area response of Ribociclib and Letrozole wasn't observed within the blank rabbit plasma samples after successive injections of LLQC and ULQC at the retention times of Ribociclib and Letrozole. In autosampler carryover [47] this method doesn't exhibit autosampler carryover.

Stability

The benchtop stability of Ribociclib and Letrozole was investigated by a stock solution was prepared and stored at room temperature for 18 h, in case of autosampler stability the stock solution was stored for 24h in auto sampler at room temperature gives reliable stability behaviour under these conditions. Assessment of freeze thaw stability, the stock arrangement was stored for 24 h at (-28±5) °C, in wet extract stability, the stock solution was stored for 18h at 2-8 °C, in dry extract stability, the stock was stored for 18h at (-20±3) °C. The short term stability shows stability of drugs was stored for 7 d at (5±3) °C, and in long term stability, the stock was stored for 28 d at (-20±3) °C and inject into the UPLC. Compare the stability results of freshly arranged stock solution with stock solution made before 24h. From this we observed a % change of Ribociclib and Letrozole was 1.27% and 0.75%, respectively, it indicates that solutions are stable up to 24 h [48, 49].

At room temperature Ribociclib and Letrozole were stable in plasma for different conditions. It was evaluated that LQC, MQC and HQC levels continued freezing and defrosting of plasma specimens spiked with Ribociclib and Letrozole, didn't influence its stability. It was clear from long-term stability that Ribociclib and Letrozole were stable at a capability temperature of-30 °C up to 24 h. The overall stability results of Ribociclib and Letrozole were shown in table 4 and 5.

***In vivo* pharmacokinetic evaluation**

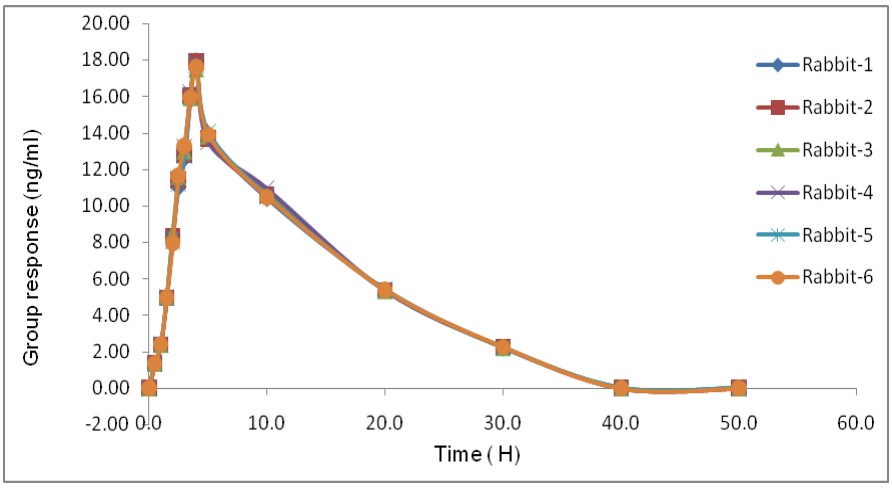
The plasma concentration-time profiles of Ribociclib and Letrozole in rabbit are shown in fig. 5. The graph indicated a bell-shaped curve in both cases of the experimental formulation. Ribociclib and Letrozole could be traced to be present in the blood for 4 h and 2 h after oral administration, which indicates the effectiveness of drug release from the formulation.

The pharmacokinetic parameters C_{max}, T_{max}, $T_{1/2}$, K_{el}, K_a, AUC_{0-t}, $AUC_{0-\infty}$, were calculated and the data is shown in table 6. The C_{max} for Ribociclib and Letrozole were found to be 17.79 ng/ml and 0.2 ng/ml, respectively. The T_{max} for Ribociclib and Letrozole were found to be 4h and 2h, respectively. The $T_{1/2}$ values were 30 h and 40 h, respectively for Ribociclib and Letrozole. The AUC_{0-t} for Ribociclib and Letrozole were found to be 274 and 4 ng-hr/ml respectively.

Table 4: Stability results of ribociclib

Stability experiment spiked plasma		Mean conc. (n=6,ng/ml)	Std dev (n=6,ng/ml)	%CV
Bench top stability	LQC	10.025	0.324	0.74
	MQC	20.147	0.118	1.25
	HQC	29.962	0.012	0.26
Auto sampler stability	LQC	10.154	0.217	0.57
	MQC	20.139	0.165	0.65
	HQC	30.118	0.194	1.18
Long term(Day28) stability	LQC	9.986	0.115	0.83
	MQC	10.254	0.136	1.39
	HQC	30.057	0.158	0.84
Wet extract stability	LQC	10.547	0.214	0.47
	MQC	20.054	0.178	1.13
	HQC	29.869	0.163	0.69
Dry extract stability	LQC	10.152	0.154	2.67
	MQC	20.347	0.058	1.34
	HQC	30.113	0.462	1.58
Freeze thaw stability	LQC	10.068	0.247	0.93
	MQC	19.941	0.192	0.84
	HQC	30.038	0.084	0.99
Short term stability	LQC	10.057	0.147	1.83
	MQC	19.273	0.132	1.64
	HQC	29.175	0.117	0.27

(n=6)



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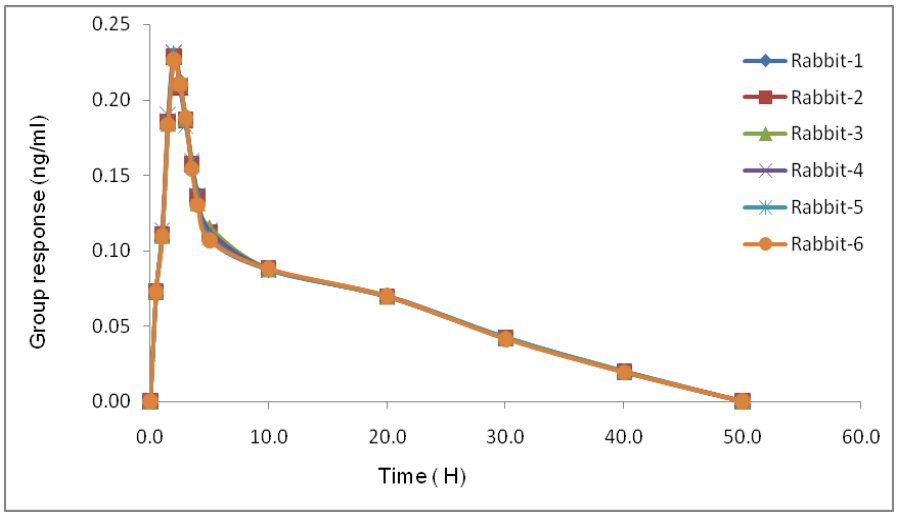

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Fig. 5: Recovery plot of (A) Ribociclib and (B) Letrozole

Table 5: Stability results of letrozole

Stability experiment spiked plasma		Mean conc. (n=6,ng/ml)	Std dev (n=6,ng/ml)	%CV
Bench top stability	LQC	0.122	0.094	1.73
	MQC	0.257	0.214	0.78
	HQC	0.379	0.112	0.33
Auto sampler stability	LQC	0.129	0.069	1.59
	MQC	0.252	0.314	0.74
	HQC	0.374	0.124	0.53
Long term	LQC	0.126	0.185	0.84
(Day 28)stability	MQC	0.259	0.174	1.33
	HQC	0.378	0.124	0.46
Wet extract stability	LQC	0.121	0.165	1.68
	MQC	0.253	0.047	0.65
	HQC	0.376	0.035	1.27
Dry extract stability	LQC	0.118	0.118	1.55
	MQC	0.249	0.162	0.73
	HQC	0.381	0.094	0.68
Freeze thaw stability	LQC	0.132	0.187	2.17
	MQC	0.267	0.134	1.11
	HQC	0.368	0.185	0.76
Short term stability	LQC	0.122	0.169	2.38
	MQC	0.246	0.284	0.65
	HQC	0.389	0.256	1.27

(n=6)

Table 6: Pharmacokinetic parameters of ribociclib and letrozole

Pharmacokinetic parameters	Ribociclib	Letrozole
AUC_{0-t}	274 ng-h/ml	4 ng-h/ml
C_{max}	17.79 ng/ml	0.2 ng/ml
$AUC_{0-\infty}$	274 ng-h/ml	4 ng-h/ml
T_{max}	4 h	2 h
$T_{1/2}$	30 h	40 h

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity, $AUC_{0-□}$: Area under the curve up to the last sampling time, C_{max}: The maximum plasma concentration, T_{max}: The time to reach peak concentration, $T_{1/2}$: Time the drug concentration

CONCLUSION

The first time a new UPLC technique has been successfully developed and validated for the evaluation of Ribociclib and Letrozole in 6 min rabbit plasma. After intravenous administration, Ribociclib and Letrozole were rapidly absorbed from the rabbit body and shows the pharmacokinetic behaviour. Here the described method is fast, rugged, and reproducible and it can be applied for pharmacokinetic studies successfully and to check the investigated analyte concentrations in body fluids with acceptable accurate results and good linear concentration range. These studies are warranted to validate our results in the near future as a reference. This method was validated as per USFDA guidelines.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

Author declares that there have been no conflicts of interest.

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