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A Validated Stability Indicating LC-MS/MS Bio-analytical Method for Analysis of Non-Small Cell Lung Cancer Drug (Mobocertinib) in Human Plasma

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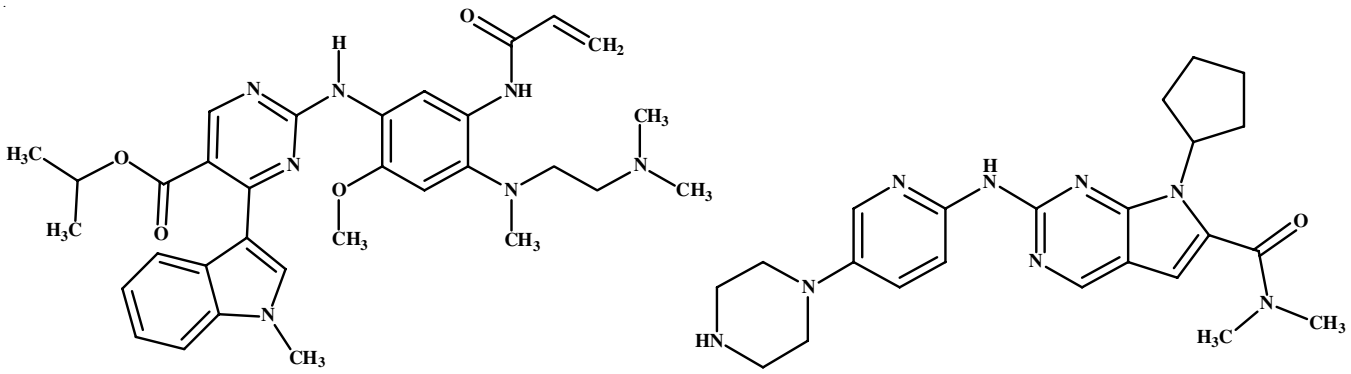
An anticancer drug mobocertinib was prescribed for the patients suffering with non-small cell lung cancer. The literature reveals that no analytical method reported for evaluation of mobocertinib in biological samples and hence this study planned to propose a simple and sensitive LCMS/MS method for quantification of mobocertinib in spiked plasma using similar action drug ribociclib as internal standard. The extraction of mobocertinib along with ribociclib was performed using a simple protein precipitation and liquid–liquid extraction with dichloromethane. The Synergi, RP 80A (100 × 4.6 mm; 4 µ) column with 0.05 M ammonium acetate at pH 4.1 with 1 % formic acid, methanol and acetonitrile at 45:25:30 (v/v) at 0.5 mL/min isocratic flow was optimized and finalized for resolution of mobocertinib and internal standard. The resolved analytes were monitored with mass analyzer operated in multiple reaction monitoring positive ion mode. The characteristic mass transition at *m/z* 586 → 202 and *m/z* 435 → 199 was observed for mobocertinib and ribociclib, respectively. The method produces calibration curve linear in the level of 0.25 ng/mL to 300 ng/mL with a sensitive detection limit of 0.075 ng/mL. The % RSD in intraday precision was calculated to be 0.64, 0.36, 0.55 and 0.68 whereas in inter-day precision was obtained as 0.95, 1.07, 0.56 and 0.28 in HQC, MQC, LQC and LLOQ, respectively. The stability during handling, extraction and as well as analyzing the mobocertinib solution was evaluated by performing various stability studies. The analytes were confirmed to be acceptable in every studied stability studies. The validation study produces acceptable results in all parameters and can successfully utilized for evaluation of mobocertinib in the biological samples.

Keywords: Mobocertinib, Ribociclib, LC-MS/MS, Plasma extraction, Stability evaluation.

INTRODUCTION

World Health Organization projects 2.08-folds increase in the deaths due to cancer and places top position by over-training ischemic heart disease [1]. Among the various types of cancers, the mortality due to lung cancer was reported to be leading one [2]. The non-small cell lung cancer treatment drug mobocertinib belongs to tyrosine kinase inhibitor class [3]. It works by targeting epidermal growth factor receptor having mutations in exon 20 region. It was approved for the treatment of metastatic non-small cell lung cancer in adult patients [4] and is recognized as orphan drug by FDA. The commonly reported side effects while using mobocertinib (Fig. 1a) are decreased appetite, diarrhea, rash, musculoskeletal pain, fatigue, nausea, vomiting, dry skin, stomatitis and paronychia [5].

Bioanalysis refers to the examination and quantification of trace level analytes, including biomarkers, medicines and their metabolites, within biological samples such as plasma, tissue, urine, *etc*. The bioanalysis requires a suitable bioanalytical method that has the capabilities for analyzing trace level drugs in biological samples [6]. The industry guidance for validation of bioanalytical method given by USFDA was utilized for developing and validating the bioanalytical method for analysis of analytes in the biological samples [7]. The bioanalytical laboratories considered LC-MS/MS is the best choice for resolution, detection and quantification of trace level compounds in biological samples. This is because LC-MS/MS possesses high selectivity and sensitivity [8]. Prior to carry bioanalysis, it was significant to know the structure along with chemical properties of analytes.



Mobocertinib **Ribociclib (internal standard)**

Fig. 1. Structure of analytes [mobocertinib and ribociclib (internal standard)]

Present study proposed to develop a sensitive bioanalytical method for quantification of mobocertinib in human plasma. To our best of knowledge, no method reported in the literature for the evaluation of mobocertinib in the biological samples. Hence, this work planned to optimize an effective bioanalytical method for quantification of mobocertinib in biological samples like human plasma. In the process of method development and validation, similar class drug ribociclib (Fig. 1b) was utilized as internal standard. Moreover, this method doesn't utilize isotopic labelled internal standard, which was considered as general practice during bioanalytical method development. This reduces the cost of analysis as the isotopic labelled products were significantly high cost with less availability. This study utilizes easily available and similar class drug ribociclib as internal standard that facilitates high extraction efficiency with high recoveries.

EXPERIMENTAL

Pure drug mobocertinib with 98.25% purity was procured from Zydus Takeda Healthcare Pvt. Ltd., Navi Mumbai, India whereas ribociclib internal standard with 98.75% purity was procured from Novartis Pharmaceuticals, Hyderabad, India. The liquid chromatography grade acetonitrile, methanol and Milli-Q water along with reagent grade dimethyl sulfoxide, formic acid and ammonium acetate were brought from Merck, India. Heparinized healthy human plasma were procured from local diagnostic laboratory, Guntur, India.

Instruments: An LCMS/MS equipment consists of alliance 2695 (Waters corporation, Japan) system with ZQ mass analyzer (model LAA 1369, Waters corporation, Japan). An auto-injector with 0.1-1500 µL sample injection capacity equipped with a system that was maintained at 25 ºC and the spectral integration was conducted with Masslynx 4.2 (Waters Corp., Japan) software. Mobocertinib and ribociclib were resolved with Synergi, RP 80A (100 × 4.6 mm; 4 µ) column. The mass analyzer was functioned in multiple reaction monitoring (MRM) electrospray ionization positive (ESI^+) mode.

Sample extraction: The QuEChERS technique was followed and liquid-liquid extraction procedure was utilized for extracting mobocertinib and ribociclib from plasma. Various solvents were tested for effective extraction and based on the extraction efficiency, diethyl ether was finalized as extracting solvent for extraction of mobocertinib and internal standard from plasma. The extraction procedure comprises, an exactly pipetted 20 µL of human plasma spiked to 80 µL of acetonitrile solution having various concentrations of mobocertinib and 50 ng/mL of internal standard to induce plasma protein precipitation. Then, it was vortex for 5 min and centrifuged at 5000 rpm at 4 ºC for 5 min. The resultant supernatant solution was taken in a 96-well plate and used during the analysis.

Quality control samples and standard preparation: The mobocertinib standard stock at 1000 µg/mL and internal standard at 1000 ng/mL stock solution was prepared using dimethyl sulfoxide as diluent. The serial dilution mobocertinib working dilutions were carried from stock solution by utilizing methanol diluent. The ribociclib internal standard at strength of 50 ng/mL was prepared with methanol diluent. The spiked linear dilutions were arranged by adding 5 µL of solution having selected concentration of mobocertinib and 50 ng/mL of internal standard into 15 mL human plasma to obtain mobocertinib final concentration of 0.25, 5, 25, 50, 100, 150, 200 and 300 ng/mL.

Method development: The method development was initiated by following the guidelines issued by FDA [7,9]. The extracted analyte solution was utilized in the method development process. The method optimization was performed by analyzing the analyte solution in various method conditions. The method parameters were changed systematically and based on achieved results, optimized conditions were concluded and were proceeded for validation.

Method validation: The FDA [7,9] bioanalytical method validation guidelines were adopted for validating the method developed for evaluation of mobocertinib in human plasma. The method selectivity was considered as the method can analyze the analytes with any interference from endogenous compounds possible in plasma. It was assessed by analyzing blank plasma brought from six independent sources. The simultaneous experiment was carried for analysing the zero sample (plasma spiked with no analytes), zero blank (spiked with internal standard only) and a standard solution prepared by spiking both standard and internal standard. The chromatographic results were observed thoroughly for identifying the interference of endogenous compounds.

The method sensitivity was considered as minimal analyte concentration that can produce precision and accuracy within

acceptable level and was expressed as LLOQ (lower limit of quantification). This concentration can produce response ≥ 5 times response than zero blank analysis response. The calibration curve range in linearity was evaluated with standard solution in wide rage *i.e.* from LLOQ to 2000 ng/mL and each linear level contains 50 ng/mL of ribociclib. The area response of resultant chromatograms and peak area ratio of mobocertinib to ribociclib was tabulated. The peak area ratio *versus* analyte strength was utilized for calibration construction. Based on correlation coefficient, the calibration range and regression equation of mobocertinib in the proposed method was evaluated.

In the calibration range, four different levels such as high (HQC), medium (MQC), low (LQC) and LLOQ were utilized for evaluating method accuracy and precision. The selected level solutions were independently prepared in the same day (n = 6) for intraday, in three different days (two in each day) for interday precision and accuracy study. Based on the results, deviation of experimental results with theoretical concentrations were evaluated to determine the method, which was expressed as relative error (RE) in percentage. The % RE was calculated using the following formula:

$$\text{Relative error (\%)} = \frac{\text{Absolute error}}{\text{True value}} \times 100$$

The % accuracy of less than 15% in all levels studied except LLOQ, where in it should be within 20% was treated as acceptable and accurate.

The percent relative standard deviation (% RSD) was obtained for the peak area response at each level studied to assess the precision of the method as:

$$\text{RSD (\%)} = \frac{\text{Standard deviation}}{\text{Average}} \times 100$$

The % RSD of less than 15% in all levels studied except LLOQ, where in it should be within 20 % was treated as acceptable and precise. The matrix effect, recovery and process efficiency of the proposed method was evaluated in triplicates in three levels in calibration range *i.e.* HQC, LQC and MQC. The QC samples in the selected levels were prepared independently and analyzed in this proposed procedure. The area was used for evaluating the % recovery. The human plasma components interference for evaluating mobocertinib was assessed in matrix effect. The plasma samples collected from six independent laboratories was analyzed in the proposed procedure and the peak area ratio response was evaluated in the presence and absence of matrix. The % variation (%CV) was calculated and the result less than 15% was designated to be acceptable as per guidelines. The comparison of area response achieved for samples analysing before and after precipitation was evaluated in the recovery study. This experiment was performed in three levels in linearity range and the % recovery was calculated.

The mobocertinib stability in plasma evaluated in three calibration levels under different experimental as well as handling conditions like bench-top, freeze-thaw, long-term, autosampler and reinjection stability. Freeze-thaw stability of mobocertinib was evaluated by analyzing the sample prepared by freezing the sample at –20 ºC and thawing to room temperature at least for three cycles in 12 h. The sample preserved one month at –20 ºC and the preserved sample was bring to room temperature and was analyzed in long term stability. The QC dilutions were preserved in autosampler for 24 h at 10 ºC and then analyzed in the proposed method for evaluating the autosampler of the method.

RESULTS AND DISCUSSION

Various extraction protocols were utilized to achieve high recoveries of mobocertinib from human plasma. The precipitation of protein with organic solvent was studied as first extraction choice and produces significantly poor recoveries. Then liquid-liquid extraction protocol using various organic solvents in single or various combinations were tried. Among the solvents tested, acceptable recoveries were yielded with diethyl ether and hence were utilized as solvent for extracting mobocertinib from plasma. Fig. 2 illustrates the % extractions observed in various concentrations of mobocertinib using different solvents.

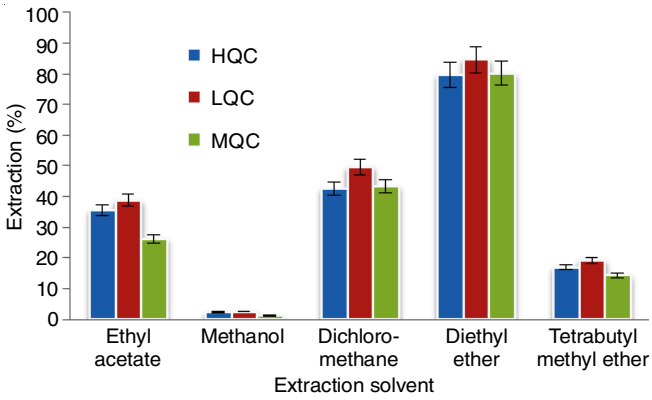


Fig. 2. Extraction recovery results

The optimization of LC-MS/MS parameters was concluded by producing symmetric shaped peaks for analytes with no interference of impurities/plasma matrix. The positive ion ESI mode was finalized since it produces high intense signals with very nominal or negligible noise. The optimized mass operating parameters are tabulated in Table-1. The precursor ions $[M + H]^+$ was observed at *m/z* 586 for mobocertinib and 435 for ribociclib. The most abundant product ion that produces great senstivitity was noticed at *m/z* 202 for mobocertinib and 199 for ribociclib internal standard. Based on the signal intensity of product ions, the MRM transition was finalized as 586 > 202 for mobocertinib (Fig. 3a) and 435 > 199 for ribociclib internal standard (Fig. 3b).

TABLE-1
OPTIMIZED MASS PARAMETER DATA FOR ANALYSIS OF MOBOCERTINIB AND RIBOCICLIB

Parameter	Mobocertinib	Ribociclib
Precursor ion (*m/z*)	586	435
Product ion (*m/z*)	202	199
Collision energy (v)	21	30
Cell exit potential (v)	12	18
Entrance potential (v)	15	11
Declustering potential (v)	42	38

The optimal resolution of mobocertinib and ribociclib internal standard with short elution time with significantly less noise was finalized by studying various chromatographic parameters. The chromatographic requirements was fulfilled with the use of 0.05 M ammonium acetate at pH 4.1 (adjusted with 1% formic acid), methanol and acetonitrile at 45:25:30 (v/v) at 0.5 mL/min flow. The sample at 5 µL quantity was injected in to 100 mm length Synergi, RP 80A (4 µ internal diameter) column. The procedure elutes at retention time of 1.89 min and 3.88 min for mobocertinib and ribociclib, respectively.

The chromatographic response of spiked and unspiked sample analysis suggest that no interfering peaks noticed in the unspiked chromatogram whereas the spiked chromatogram shows peaks representing analytes such as mobocertinib and

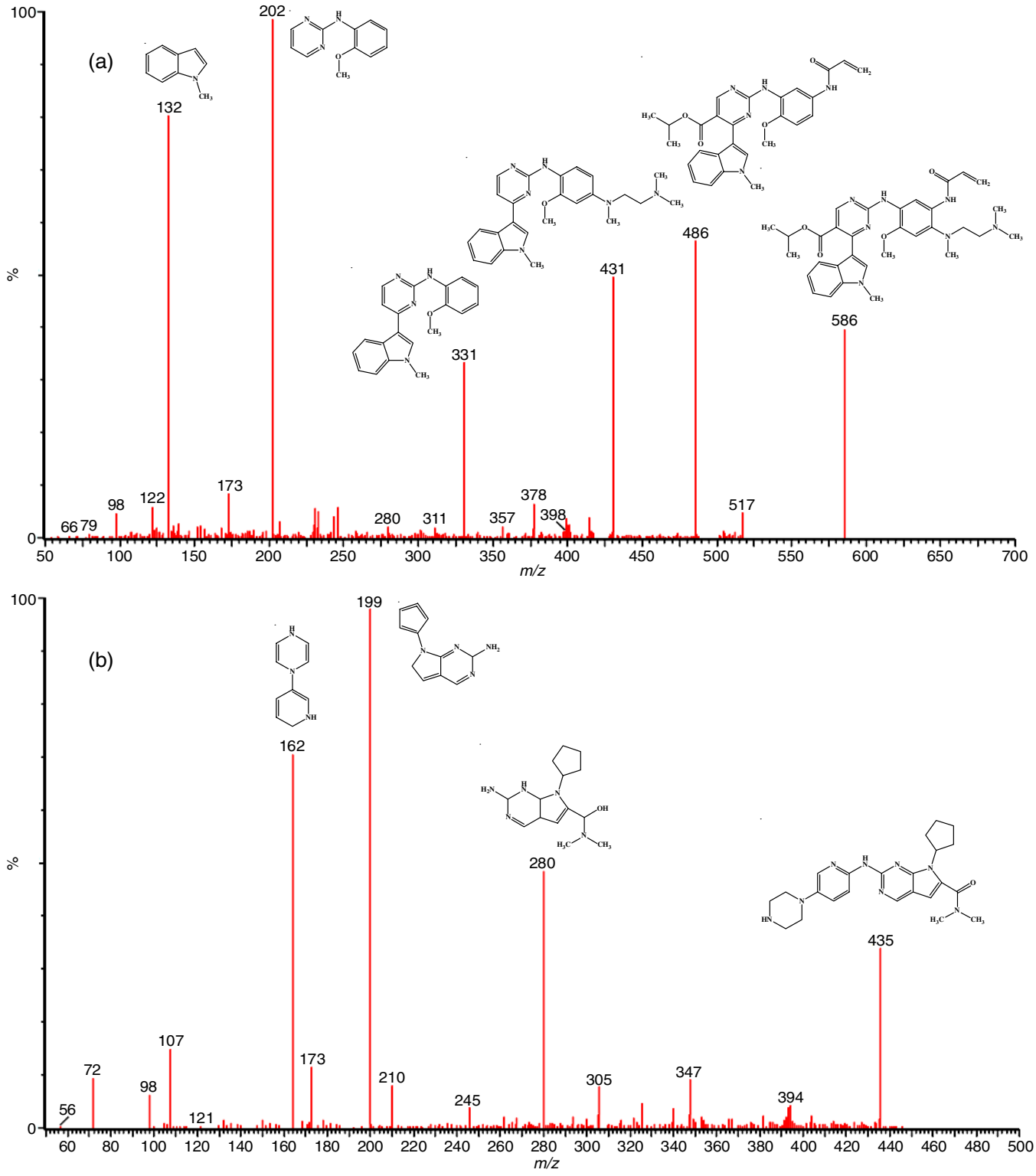


Fig. 3. Mass spectra obtained in the study method (a) mobocertinib; (b) ribociclib internal standard

ribociclib internal standard. This suggests that this method was specific for analysis of mobocertinib. The appearance of previous analysis sample in the present analysis chromatogram was evaluated by performing carryover test. It was confirmed from the obtained results, there is no carryover effect was observed in the proposed method.

The LLOQ of mobocertinib in this proposed method was assessed based on peak intensity at lower concentration that produces ≥ 5 times than blank response. The LLOQ was derived as 0.25 ng/mL advised that this procedure sufficiently selective and sensitive for evaluation of mobocertinib in plasma. The calibration curve proved to be linear in 0.25-300 ng/mL concentration level with $y = 0.0143x + 0.052$ as regression equation with 0.9996 correlation. The results are presented in Table-2.

TABLE-2
LINEARITY RESULTS

Conc. (ng/mL)	Peak response		Ratio of mobocertinib and ribociclib
	Mobocertinib	Ribociclib	
0.25	16352.4	294156.3	0.056
5	46262.8	295151.2	0.157
25	122927.2	294847.6	0.417
50	231168.9	294116.2	0.786
100	419425.6	295007.8	1.422
150	645257.1	294693.4	2.190
200	852162.8	294161.7	2.897
300	1285659.3	293263.4	4.384

The precision and accuracy experiment was conducted in four QC levels *i.e*. HQC, MQC, LQC and LLOQ and results observed in this study are illustrated in Table-3. The % RSD in intraday precision was calculated to be 0.64, 0.36, 0.55 and 0.68, whereas in interday precision was obtained as 0.95, 1.07, 0.56 and 0.28 in HQC, MQC, LQC and LLOQ, respectively. The % recovery was observed in level of 99.51-100.24 in intraday precision and 98.89-100.37 in interday precision. The data gotten in the proposed method assures that this method was appropriate in terms of precision and accuracy.

The % recovery of mobocertinib was calculated to be 97.24 ± 0.586, 96.45 ± 1.089 and 93.35 ± 0.707, respectively in HQC, LQC and MQC levels with % RSD of less than 2. The obtained recovery data indicating that the sample processing procedure proposed in this study provide adequate recovery for mobocertinib along with internal standard. The process efficiency of the method was confirmed to be high with significantly no matrix effect. The results observed in precision study are tabulated in Table-4.

The mobocertinib stability under different experimental and handling conditions was evaluated at three QC levels *i.e*.

TABLE-4
RECOVERY RESULTS

QC level	Concentration obtained (ng/mL)	Recovery (%)	RSD (%)
HQC (300 ng/mL)	291.72 ± 1.758	97.24 ± 0.586	0.60
MQC (100 ng/mL)	96.45 ± 1.089	96.45 ± 1.089	1.13
LQC (5 ng/mL)	4.67 ± 0.035	93.35 ± 0.707	0.76

Mean ± SD, n = 3

HQC (300 ng/mL), MQC (100 ng/mL) and LQC (5 ng/mL) level in calibration range. The results are tabulated in Table-5. Insignificant change of less than 2 % was observed when samples were analyzed after 6 h of incubation in bench top stability whereas the variation in concentration was observed to be very nominal. The sample store for 1 month at –20 ºC and then analyzed in this optimized method for evaluating the long term stability of mobocertinib. The % stability was observed as 98.39 in HQC, 96.65 in MQC and 92.17 in LQC with very nominal % RSD of less than 2 suggest that adequate results was produced in the proposed method after long term storage of analytes.

The spiked mobocertinib solution was analyzed after three freeze–thaw cycles and the % stability was analyzed. An acceptable % RSD in every studied level and acceptable % recovery in each analysis was observed in the proposed method. The recoveries of mobocertinib in auto-sampler and reinjection stability was observed to be under the permissible level suggest that the method was stable. Stability study results of mobocertinib performed under various experimental conditions are tabulated in Table-5.

The human plasma pre-treatment involves precipitation of protein using acetonitrile and then extracted with dichloromethane solvent in liquid-liquid extraction procedure. This extraction procedure recovers analytes with very high extraction efficiency. The extracted analytes were resolved by utilizing 100 mm Synergi C_{18} column that produces effective resolution of mobocertinib and internal standard from biological matrix using 1% formic acid, methanol and acetonitrile at 45:25:30 (v/v) at 0.5 mL/min isocratic flow. The proposed method elutes 1.89 min and 3.88 min for mobocertinib and internal standard. The analysis ends within shortest 5 min run time that facilitates large sample analysis with minimal time consumption.

When operated in positive ion mode, it was observed that the mass spectra of mobocertinib and the internal standard exhibited high intensity signals with minimal noise during the retention time. This facilitates sensitive detection of analytes at very minimal analyte concentration. The LLOQ was observed at 0.25 ng/mL with calibration curve linear in LLOQ to 300 ng/mL range. The data noted in accuracy and precision study

TABLE-3
PRECISION AND ACCURACY RESULTS

QC level	Intra-day precision (n = 6)			Inter-day precision (n = 6)		
	Conc. obtained (ng/mL)	Accuracy (%)	RSD (%)	Conc. obtained (ng/mL)	Accuracy (%)	RSD (%)
HQC (300 ng/mL)	300.09	100.03	0.64	300.38	100.12	0.95
MQC (100 ng/mL)	99.03	99.03	0.36	99.63	99.63	1.07
LQC (5 ng/mL)	5.012	100.24	0.55	5.01	100.37	0.56
LLOQ (0.25 ng/mL)	0.249	99.51	0.68	0.25	98.89	0.28

TABLE-5
STABILITY RESULTS OF MOBOCERTINIB

Test	QC level	Conc. obtained (ng/mL)	Stability (%)	RSD (%)
Bench top stability	HQC (300 ng/mL)	298.76	99.59	1.00
	MQC (100 ng/mL)	99.66	99.66	0.96
	LQC (5 ng/mL)	5.01	100.29	1.33
Freeze–thaw stability	HQC (300 ng/mL)	280.74	93.58	0.36
	MQC (100 ng/mL)	94.94	94.94	0.58
	LQC (5 ng/mL)	4.47	89.36	0.68
Long term stability	HQC (300 ng/mL)	286.17	95.39	0.63
	MQC (100 ng/mL)	96.65	96.65	0.57
	LQC (5 ng/mL)	0.430	92.17	0.43
Auto-sampler stability	HQC (300 ng/mL)	299.63	99.87	0.97
	MQC (100 ng/mL)	99.33	99.33	1.32
	LQC (5 ng/mL)	4.84	96.70	0.65
Reinjection stability	HQC (300 ng/mL)	299.89	99.96	1.11
	MQC (100 ng/mL)	99.26	99.26	0.96
	LQC (5 ng/mL)	5.01	100.26	0.63

meets the standards prescribed by internal guidelines suggest that the method was precise and accurate.

Conclusion

This study presents a sensitive and convenient LC-MS/MS method for evaluation of mobocertinib in the biological samples such as human plasma. The effective strategy for extracting mobocertinib and ribociclib as internal standards involved the utilization of a straightforward and uncomplicated protein precipitation method in conjunction with liquid-liquid extraction using diethyl ether. The method produces calibration curve liner in LLOQ (0.25 ng/mL) to 300 ng/mL range with acceptable validation results as per USFDA bioanalytical method validation guidelines. Hence, this method was adequately suitable for trace level identification and quantification of mobocertinib in human plasma.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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