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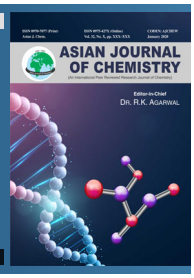

Development and Validation of Novel HPLC Bioanalytical Analysis Method for Acalabrutinib: An Anticancer Drug in Human Plasma

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The aim of the work is to develop and validate the bioanalytical RP-HPLC method for determination of acalabrutinib in plasma with nifedipine drug as internal standard. Liquid-liquid extraction with diethyl ether and methanol in the ratio of 50:50 (v/v) was used for the extraction of drugs from the biological matrix. The optimized chromatography conditions consist of methanol, acetonitrile and 0.1% orthophosphoric acid in the ratio of 45:35:20 (v/v) as a mobile phase with KNAUER Eurospher II C18 Column (250 × 4.6 mm, 5µ) as stationary phase. Isocratic elution with 0.9 mL flow separates acalabrutinib at 4.6 min and nifedipine at 6.8 min. The method was validated as per ICH guidelines and linear calibration curve was obtained for the peak area ratio of acalabrutinib and nifedipine compound across a range of 50-3000 ng/mL. Greater than 90% recoveries were obtained for acalabrutinib. The relative standard deviation (%RSD) was found to be < 5% for precision studies. Hence, the method was found to be suitable for the analysis of acalabrutinib in spiked human plasma and is used for the pharmacokinetic study.

Keywords: RP-HPLC, Acalabrutinib, Nifedipine.

INTRODUCTION

Bioanalytical method demonstrates that a particular method used for quantitative measurement of drugs or analytes in a given biological matrix, such as blood, plasma, serum or urine is reliable and reproducible for the intended use [1,2]. Determination of drugs in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic study data which supports regulatory filings [3,4]. The quality of these studies is directly related to the quality of the underlying bioanalytical data. It is therefore validation of these bioanalytical methods are established and disseminated to the pharmaceutical community [5].

Acalabrutinib drug is a second generation Bruton′s tyrosine kinase inhibitor with potential antineoplastic activity. It is used for treatment of adult patients with mantle cell lymphoma (MCL) a type of non-hodgkin lymphoma [6]. The drug is available as oral admistration and active metabolite half-life was found to be 6.9 h. After administration of a single 100 mg radiolabeled acalabrutinib dose in healthy subjects, 84% of the dose was recovered in the feces and 12% of the dose was recovered in the urine. Less than 1% of the dose was excreted as unchanged acalabrutinib. The drug has common side effects include headaches, feeling tired, low red blood cells, low platelets and low white blood cells [7,8]. Literature survey reveals that there are very few analytical methods have been reported regarding the estimation of acalabrutinib [9-13].

There are only two formulation analysis methods have reported by using HPLC and LC/MS-MS methods [9,10]. Though the LC-MS/MS method has less runtime however, the proposed RP-HPLC method is easily available to analysis of acalabrutinib and has great calibration range and potential validation results. Hence, the proposed RP-HPLC method is more useful method for quantification of acalabrutinib in plasma.

EXPERIMENTAL

HPLC grade solvents like methanol, water and orthophosphoric acid (85%) were purchased from Thermo-Fisher Scientific India Pvt. Ltd., Mumbai, India. HPLC grade acetonitrile was purchased from Merck chemicals.

The chromatographic system used is Agilent 1100 series HPLC with Quaternary G1311 A pump, COLCOM G1316A thermostat column temperature control, Thermostatic auto sampler G 1329A with sample volume of 0.1-1500 µL and variable programmable UV detector (G 1314 A). The instrument was operated and integrated with Agilent chem station LC software. The separation of compounds was achieved by using KNAUER Eurospher II C18 Column (250 × 4.6 mm, 5µ).

Preparation of mobile phase: Methanol, acetonitrile and 0.1% orthophosphoric acid in the ratio of 45:35:20 (v/v) and sonicated the solution for 10 min to ensure the homogeneous mixing using ultrasonicator, and then it was filtered through 0.45 µ nylon membrane filter paper using vacuum filtration set. The solution was stored at room temperature and used within 7 days from the date of preparation. Later the mixture was degassed.

Preparation of diluent: An equal ratio of methanol and acetonitrile was used as diluent in the analysis. For the preparation of diluent, 50 mL of methanol was transferred into a 100 mL reagent bottle and 50 mL of acetonitrile was added, mixed and sonicated for 5 min. The solution was stored at room temperature and used within 7 days from the date of preparation.

Preparation of sample solution: Different organic extraction solvents *viz*. dichloromethane, methanol, acetonitrile, chloroform and diethyl ether were evaluated. Diethyl ether and methanol combination proved to be the most efficient extracting solvent. Blood samples from local diagnostic lab was collected in heparinized tubes and immediately placed on ice and taken to the lab.

The tubes were centrifuged at 5000 rpm for 5 min at room temperature for separation plasma. The plasma samples were stored at -30 ºC. Acalabrutinib drug standard and nifedipine drug as internal standard was premixed with plasma. A liquid-liquid extraction method was employed by using diethyl ether and methanol in the ratio of 50:50 (v/v) for the extraction of drugs from the biological matrix. Diethyl ether (50 mL) was added to 50 mL of methanol and vortex for 30 s, and then centrifuged at 4 ºC at 5000 rpm. The blank plasma sample was also prepared in a similar procedure by adding 1 mL of spiked plasma into extraction solution and vortex for 30 s, and then centrifuged at 4 ºC at 5000 rpm. Supernatant of these solutions were kept in a HPLC vial.

Preparation of solutions: Stock solutions (100 µg/mL) of acalabrutinib and nifedipine were appropriately diluted with diluents solution to get working standard solutions with concentrations of 50-3000 ng/mL. Aliquots of 0.9 mL of blank human plasma were spiked with 0.1 mL of the working standard solutions to get calibration curve standards containing 50, 100, 250, 750, 100, 1500, 2000 and 3000 ng/mL of acalabrutinib. The internal standard nifedipine samples were similarly prepared and maintained at constant concentration of 100 µg/mL. Three concentrations [50 ng/mL low quality control (LQC), 1000 ng/mL middle quality control (MQC) and 3000 ng/mL high quality control (HQC)] were selected for validation.

Method development and optimized conditions: Various chromatography parameters like columns, mobile phase, flow, and column temperatures were tested during development of the analytical method. The initial aim of the development is to develop a sensitive condition in order to detect the drug at very low concentration, where usual concentrations of the drug exist at biological samples. Different columns like C8 and C18 of the same length and diameter were tested and also mobile phase with solvents ratio were tested. UV detector wavelength was selected as iso-absorptive point *i.e*. 242 nm. The mobile phase holdup time, resolution, acalabrutinib drug peak asymmetry of acalabrutinib and nifedipine and quantity of fractions defined by the reading of area integrations from the chromatograms were assessed. The concentration of tested samples was 10 µg/mL throughout development. By keeping the same parameters and conditions other method parameters like mobile phase flow, injection volume, temperature of the column were optimized to get efficient chromatogram.

Method validation: The validation of the developed method was conducted as per the recommendations of US FDA guidelines [14]. System suitability was studied at the middle of quantification (MQC) of 1000 ng/mL by comparing blank responses of plasma. Accuracy was estimated as the mean RE while the precision was measured in terms of RSD. For each of the above validation tests, the analysis was performed at three QC concentrations (low, medium and high), with six determinations for each concentration. Stability of acalabrutinib in human plasma was evaluated under different conditions *viz*. three freeze-thaw cycles, stability of long term for 30 days and stability of short term at room temperature for 6 h. All the validation stability studies were performed at LQC, MQC and HQC concentrations. The obtained results were compared with the nominal concentration of the analytes.

RESULTS AND DISCUSSION

The chromatographic estimation of acalabrutinib using nifedipine as an internal standard was optimized after several trials using the C8 and C18 columns mobile phase with different ratios of orthophosphoric acid buffer and at various pHs. Optimized chromatography conditions include methanol, acetonitrile and 0.1% orthophosphoric acid in the ratio of 45:35:20 (v/v) (pH 5.4) as mobile phase with KNAUER Eurospher II C18 Column (250 × 4.6 mm, 5µ) at 242 nm. The flow rate of the mobile phase 0.9 mL/min with isocratic elution at ambient temperature for 10 min was successfully achieved the separation of acalabrutinib and nifedipine at 4.6 and 6.8 min of retention time with high resolution. The optimized chromatography conditions are presented in Table-1 and the chromatograms of blank and system suitability are shown in Figs. 1 and 2.

All analytes eluted rapidly with good resolution within 10 min without any interfering of plasma matrix components with the analyte peaks (Fig. 2). Acalabrutinib was eluted at 4.6 min wheres as nifedipine eluted at 6.8 min with 11.63 resolution. Hence, no interfering endogenous peaks were identified on the chromatogram that the method possesses high specificity. Peak shape and retention time (R_t) were found to be same as that of pure standards.

Calibration curve is presented to confirm the relationship between the peak area ratios and the concentration of acalabru-

TABLE-1
OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Condition	Results
Mobile phase	Methanol, acetonitrile and 0.1% orthophosphoric acid in the ratio of 45:35:20 (v/v)
Pump mode	Isocratic
pH	5.4
Diluents	Mobile phase
Column	KNAUER Eurospher II C18 Column (250 × 4.6 mm, 5 µ)
Column temp.	Ambient
Wavelength	242 nm
Injection volume	20 µL
Flow rate	0.9 mL/min
Run time	10 min

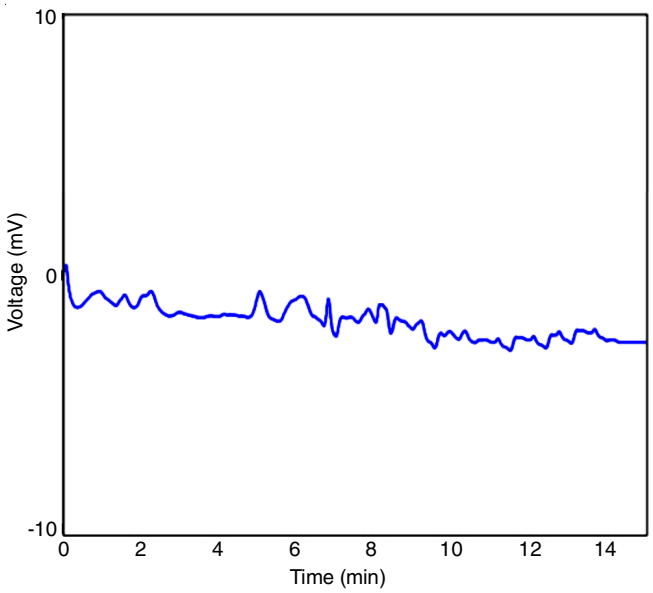

Fig. 1. Blank chromatogram of plasma with diluents and further subjected to liquid-liquid extraction

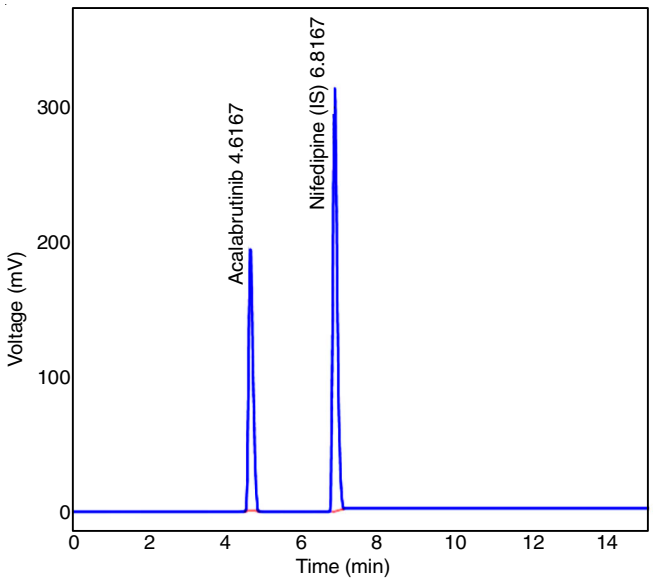

Fig. 2. System suitability chromatogram of plasma spiked drug of acalabrutinib with nifedipine (IS) no interfering endogenous after liquid-liquid extraction

tinib in the standard samples. The linearity of the method was evaluated at eight concentration range including the LQC. The calibration curve was found to be linear in the range 50-3000 ng/ mL, with a correlation coefficient (r^2) of 0.9993. The data of calculated calibration standards are presented in Table-2 and linear calibration graph is shown in Fig. 3.

TABLE-2
LINEARITY TEST RESULTS

Conc. (ng/mL)	Peak area observed for		Ratio of standard/ IS	Sample Id
	Acalabrutinib - Standard	Nifedipine - IS		
50	20861.1	228268.9	0.091	PSCC 1
100	34504.9	227956.0	0.151	PSCC 2
250	68833.4	229172.3	0.300	PSCC 3
500	112306.0	228808.3	0.490	PSCC 4
750	155767.5	227045.4	0.686	PSCC 5
1000	197131.6	229457.6	0.859	PSCC 6
1500	295009.9	229251.6	1.286	PSCC 7
2000	386717.7	228065.1	1.695	PSCC 8
3000	588128.5	229127.0	2.566	PSCC 9

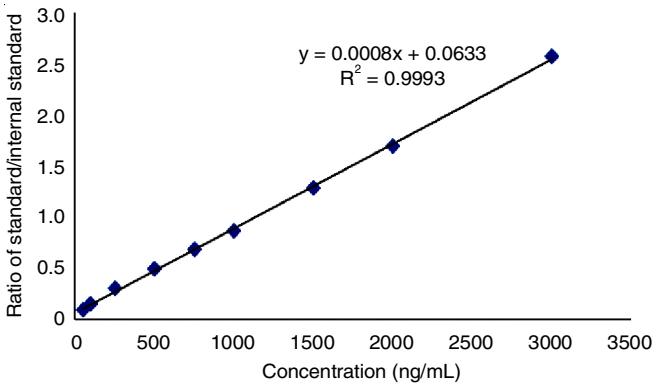

Fig. 3. Calibration curve which ratio of acalabrutinib to internal standard nifedipine

Acalabrutinib concentrations in QC samples, recovery and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve. The regression equations were y = 0.0008x + 0.0633, where y indicates the ratio of analyte to internal standard and x indicates the plasma concentration. The lower limits of quantification (LLOQs) under the optimized conditions were 50 ng/mL acalabrutinib, which was determined from visual method of detection. The precision evaluation was assessed by repeated analysis of plasma samples containing different concentrations of acalabrutinib with nifedipine drug as internal standard on separate occasions. Six replicates of LQC, MQC and HQC samples. Recovery of acalabrutinib drug was evaluated by comparing mean analyte responses of six extracted samples of LQC, MQC and HQC samples. The results of intraday precision found that mean % recovery was 99.80% with a RSD of 0.94 for HQC, 98.39% with a RSD of 0.94 for MQC and 99.59% with a RSD of 0.39 for LQC, respectively. The interday precision results with three concentrations were found that mean % recovery was 100.08% with a RSD of 0.62 for HQC, 97.93% with a RSD of 0.82 for MQC and 99.64% with a RSD of 0.27

TABLE-3
PRECISION AND RECOVERY STUDY WITH VARIOUS QC CONCENTRATIONS

Parameter	Concentration	Drug estimated (%)	Standard deviation	CV (%)	Accuracy (%)
Intra-day precision	HQC	98.37-101.03	0.94	0.94	99.80
	MQC	97.26-99.60	0.94	0.95	98.39
	LQC	99.31-100.0	0.39	0.40	99.59
Inter-day precision	HQC	99.37-101.06	0.62	0.62	100.08
	MQC	97.80-99.00	0.82	0.84	97.93
	LQC	99.15-99.93	0.27	0.27	99.64
Recovery	HQC	88.02-89.58	0.56	0.63	88.95
	MQC	92.53-94.30	0.86	0.92	93.36
	LQC	84.14-85.00	0.33	0.39	84.67

TABLE-4
STABILITY STUDY WITH VARIOUS QC CONCENTRATIONS

Parameter	Concentration	Drug estimated (%)	Standard deviation	CV (%)	Accuracy (%)
Short term stability	HQC	99.33-100.41	0.46	0.46	99.60
	MQC	96.53-97.79	0.44	0.45	97.17
	LQC	90.60-91.31	0.23	0.26	90.89
Long term stability	HQC	95.51-97.59	0.83	0.86	96.47
	MQC	91.81-94.33	1.03	1.11	93.33
	LQC	87.40-87.95	0.19	0.22	87.72
Freeze thaw stability	HQC	98.06-98.96	0.34	0.34	98.47
	MQC	95.80-96.26	0.20	0.21	96.00
	LQC	88.67-89.51	0.35	0.39	89.10

for LQC, respectively (Table-3) and shows that method is precise and accurate within the acceptable limits.

The range of percent coefficient of variation (CV) for accuracy was found to be from 0.27 to 0.95. The range of between run nominal value percentage was found to be from 99.80 to 100.08 at HQC. The range of recovery for MQC was 97.93 to 98.39 with %CV 0.84 to 0.95. The range of recovery for LQC was 99.59 to 99.64 with %CV 0.27 to 0.40. The mean recovery values were 88.95%, 93.36% and 84.67% at HQC, MQC and LQC, respectively. No outcome of quantization for acalabrutinib and nifedipine was observed in the matrix effect. The results for accuracy and precision and stability effect are given in Tables 3 and 4.

Stability studies were performed to evaluate the stability of acalabrutinib in plasma after exposing to various stress conditions like long term, short term and freeze thaw. Short term stability results at HQC were found that standard deviation of 0.46 with mean recovery 99.60%, at MQC standard deviation 0.44 with mean recovery 97.17% and at LQC standard deviation of 0.23 with mean recovery 90.89%. Long term stability results at HQC were found that standard deviation of 0.83 with mean recovery 96.47%, at MQC standard deviation 1.03 with mean recovery 93.33% and at LQC standard deviation of 0.19 with mean recovery 87.72%. Freeze thaw stability results at HQC were found that standard deviation of 0.34 with mean recovery 98.47%, at MQC standard deviation 0.20 with mean recovery 96.00% and at LQC standard deviation of 0.35 with mean recovery 89.10%. The stability studies were presented in Table-4. The outcomes of other parameters like precision, accuracy, reproducibility, effect of potentially interfering drugs, dilution integrity, were found to be within the acceptance criteria as per ICH and USFDA guidelines.

Conclusion

A simple, sensitive, accurate and precise RP-HPLC method was developed and validated for the estimation of acalabrutinib in plasma with nifedipine drug. The present method was employed with liquid-liquid extraction of the plasma spiked drug and successfully validated. The results of all the validation and stability studies were found in acceptable range of recovery. The developed RP-HPLC method is efficient and can be used in pharmacokinetics studies as well as in the monitoring of the acalabrutinib in biological samples.

CONFLICT OF INTEREST

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

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