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A VALIDATED LC-MS/MS METHOD FOR PHARMACOKINETIC STUDY OF AFATINIB IN HEALTHY RABBITS

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Keywords:

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ABSTRACT: A liquid chromatography-tandem mass spectrophotometric (LC–MS/MS) method was developed for quantification of Afatinib in rabit plasma employing Solid Phase Extraction (SPE) technique. Developed method was validated for specificity, precision, accuracy, recovery, and stability characteristics. Chromatographic separation was achieved on Gemini, 5µ C18, 50 × 4.60 mm with 30:70 v/v of 10 mM ammonium acetate in water: Organic Mixture (methanol : acetonitrile, 80:20% v/v) as an isocratic mobile phase with a flow rate of 1.0 ml/min. the developed LC-MS method was applied to assess pharmacokinetics parameters of afatinib (20 mg) tablet in healthy rabbits. Six Male albino rabbits weighing 2.0 - 2.5 kg were randomly selected for the pharmacokinetic study. The tablets were administered to rabbits using a balling gun. Blood samples (0.6 ml) were withdrawn from the marginal ear vein before dosing (zero time) and at time intervals of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 20, 24, 36 and 48 hr after administration. Plasma was separated by centrifugation at 5000 rpm for 10 min and the plasma concentrations of afatinib at different times were determined by LC-MS/MS. Pharmacokinetic parameters was calculated. Afatinib showed T_{max} of 3.833 ± 0.752 and mean C_{max}, $AUC0_{\rightarrow t}$ and $AUC_{0\rightarrow\alpha}$ for Test formulation is 675.83 ± 22.031, 6213.417 ± 257.922 and 6474.61 ± 258.45 respectively.

INTRODUCTION: Afatinib, irreversible tyrosine kinase inhibitor of the ErbB family of receptors and is effective in treating tumors of various types [1 - 7]. The recommended daily oral dose is 40 mg/day, it can be increased to a maximum of 50 mg/day, or decreased to a minimum of 20 mg/day, depending on tolerability[8 - 9]. The preclinical pharmacokinetics and metabolism of afatinib were studied in several animal species (mice, rats, rabbits and Go¨ttingen minipigs). In all species, metabolism was minimal; excretion of unchanged parent compound accounted for >50, >60, >72, and >87 % in the rat, Mouse, minipigs and rabbit, respectively (Boehringer Ingelheim, unpublished data) [10 - 11]. To characterise the clinical pharmacokinetics of afatinib in a large number of patients with advanced solid tumours, a meta-analysis of five phase I trials and one phase II trial in patients with advanced solid tumours (n = 221) who received afatinib (10 - 100 mg doses) was performed using non-compartmental analysis [12 - 17].

In an another study it was found that Mild to moderate hepatic impairment had no clinically relevant effect on the pharmacokinetics of a single 50 mg dose of afatinib[18]. Few LC-MS/MS methods were reported for human pharmacokinetic studies of afatinib alone [19] or in combination [20] with other drugs. In patients with solid tumors, Tmax of afatinib occur approximately 2 - 5 hr after oral dosing and decline afterwards in an at least bi-exponential manner.

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Afatinib metabolism is minimal and the unchanged drug predominantly excreted in the faeces and approximately 5% in urine. (Boehringer Ingelheim, unpublished data) [8, 10]. The affinity of afatinib in binding with haemoglobin covalently results decreased plasma concentration levels [8]. Hence it is essential to develop more sensitive analytical methods to determine the concentration of afatinib from plasma samples. To best of our knowledge, no published LC-MS/MS based methods for pharmacokinetic study of afatinib in healthy rabbits. Therefore a liquid chromatography-tandem mass spectrophotometric (LC–MS/MS) method was developed, validated and applied for quantification of Afatinib in rabit plasma employing Solid Phase Extraction (SPE) technique. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with any marketing formulation of afatinib in human volunteers.

Experiment:

Apparatus and Software: The HPLC system with an auto sampler was a Shimadzu LC- 20A Dvp (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex, Canada) API 4000 Tandem mass spectrometer. The auto sampler was SIL-HTC from Shimadzu, Japan. The solvent delivery module was LC-20AD from Shimadzu, Japan. The chromatographic integration was performed by Analyst software (version: 1.4.2; Applied Biosystems).

Chemicals and Reagents: Afatinib and Afatinib D3 (IS) were procured from Unichem Laboratories Ltd., Mumbai, India, ammonium acetate was procured from Merck Specialities Pvt. Ltd., Mumbai, India. Water used was collected from water purification systems (Milli Q, Milli Pore, USA) installed in laboratory. Methanol and acetonitrile were of HPLC grade and were supplied by J. T. Baker, USA. Hyderabad. The study was approved by Institutional Ethical committee no: VCP/IAEC/2016-44.

Calibration standard Solutions: Stock solutions of Afatinib and Afatinib D3 internal standard (IS) were prepared in methanol. Further dilutions were carried out in 70% methanol. Calibration standards of eight concentration levels were prepared freshly by spiking drug free plasma with Afatinib stock solution to give the concentrations of 2.00, 4.00, 20.00, 40.0, 80.0, 160, 300 and 600 ng/ml.

Quality Control Standards: Lowest quality control standards, Median quality control standards and highest quality control standards were prepared by spiking drug free plasma with Afatinib to give solution containing 6, 240 and 480ng/ml respectively. They were stored at -200c till the time analyzed.

Chromatographic Conditions: Chromatographic separation was performed on a Gemini, 5µ C18, 50 × 4.60 mm with 30: 70 v/v of 10 mM ammonium acetate in water: Organic Mixture (methanol: acetonitrile, 80:20% v/v) as an isocratic mobile phase with a flow rate of 1.0 ml/min. Injection volume was 5µl. Total analysis time of single injection was 2.00 minutes. Column oven temperature and auto sampler temperature was set to 40 °C and 5 °C, respectively.

Mass Spectrometric Conditions: The LC eluent was split (75%), and approximately 0.25 ml/min was introduced and Quantitation was achieved with MS/MS detection in negative ion mode for the analytes and IS using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with Turboion spray™ interface at 400 ºC. The ion spray voltage was set at 5500 V. The source parameters *viz*., the nebulizer gas, curtain gas, CAD gas were set at 40, 40 and 5 psi, respectively. The compound parameters *viz*. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) for MT and MT-D3 were similar and are -55, -25, -10, -6 V. For Afatinib and Afatinib D3 the DP, CE, EP and CXP were -55, -24, -10 and -18 V. A Turbo ion spray interface (TIS) operated in negative ionization mode was used for the detection. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of m transitions of *m/z* 344.10/194.00 for Afatinib and m/z 347.30./197.00 for Afatinib D3. Quadrupoles Q1 and Q3 were set on unit resolution.

Study Design: Six Male albino Rabbits (weighing about 2.5 kg) were selected as the animal model. The age of the rabbits was 8 - 12 weeks. The Rabbits selected for the study had no medication for two weeks prior to the study. Twelve hours

before drug administration, food was withdrawn from the rabbits until 24 hr post-dosing, while, water was available for rabbits throughout the study. The tablets were administered to rabbits using a balling gun. Blood samples (0.6 ml) were withdrawn from the marginal ear vein before dosing (zero time) and at time intervals of 0.5, 1,1.5, 2, 2.5, 3, 4, 5, 6,7 8, 10, 12, 20, 24, 36, 48 hr after administration. For each animal the total number of blood samples drawn during the study was 17. EDTA disodium salt was used as an anticoagulant. Plasma was separated by centrifugation at 5000 rpm for 10 min and the resulting plasma sample from each blood sample was divided into two aliquots and stored in suitably labeled polypropylene tubes at – 20 °C until used. All the plasma samples were analysed under the construction of standard calibration curve of afitanib in rabbit's plasma. The afatinib concentrations in the rabbit plasma samples was calculated using the calibration curve, obtained after linear regression of the peak area ratio (afitanib/afitinib-D3) versus the concentration of afitinib.

Sample Preparation Method: To 200µl of plasma, 50 µl of Afatinib D3 (1µg/ml) was added and vortexed. Add 200 µL of Extraction buffer (0.1N Sodium hydrogen Carbonate in water) to all samples and vortex for about 30 seconds. Centrifuge all samples at 4000rpm for 2 minutes by using refrigerated centrifuge maintained at 10 ± 2°C. Arrange the required number of pre-labeled Orochem DVB LP 30 mg/1 mL extraction cartridges on EZYPRESS® 48 - 48 Position Positive Pressure Processor. Condition the cartridges with 1.0mL of methanol followed by 1.0 mL water. Load about 400µL of the prepared samples on conditioned cartridges carefully. Wash the cartridges with 1mL of Water followed by 1mL of Methanol in water, 10% v/v; Dry the cartridges for 2 minutes by applying positive pressure at maximum flow rate or by applying full vacuum. Elute the contents from the cartridges with 1 mL of Methanol into pre-labeled tubes and vortex to mix. Transfer appropriate volume of samples into pre-labeled Autosampler vials, and inject by using HPLC-ESI-MS/MS.

Pharmacokinetic Analysis: Single dosage pharmacokinetic parameters were calculated using PK Solver tool from plasma drug concentration-time data by non-compartmental methods. The maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}) were obtained directly from the observed concentration-time profiles. Linear trapezoidal rule was used to estimate the area under the plasma concentration versus time curve (AUC) from 0 to the last measurable concentration (AUC 0-_t). The area under the plasma concentration versus time curve from 0 to infinity (AUC 0-_∞) was calculated as AUC 0-t+ Ct/ke, where Ct was the last measurable concentration. Ke was the elimination rate constant. The terminal elimination half-life ($t_{1/2}$) was calculated as 0.693/Ke.

Validation:

Specificity: A solution containing 2.0 ng/ml was injected on to the column under optimized chromatographic conditions to show the separation of Afatinib from impurities and plasma. The specificity of the method was checked for the interference from plasma.

Linearity: Spiked concentrations were plotted against peak area ratios of Afatinib to internal standard and the best fit line was calculated .Wide range calibration was determined by solutions containing 2 ng/ml - 600 ng/ml.

Recovery Studies: The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against unextracted Quality control samples at HQC, MQC and LQC.

Precision and Accuracy: Intraday precision and accuracy was determined by analyzing quality control standards (6, 240 and 480 ng/ml) and LLOQ Quality control standard (2.00 ng/mL) five times a day randomly, interday precision and accuracy was determined from the analysis of each quality control standards (6, 240 and 480 ng/ml) and LLOQ Quality Control standards (2.00 ng/mL) once on each of five different days.

Matrix Effect: The matrix effect for the intended method was assessed by using chromatographically screened human plasma. Concentrations equivalent to LQC and HQC of Afatinib were prepared with six different lots of plasma and are injected.

RESULTS:

Results of Method Validation: The chromatography observed during the course of validation was acceptable and representative chromatograms of standard blank, HQC, MQC and LQC samples are shown in **Fig. 1 - 4**.

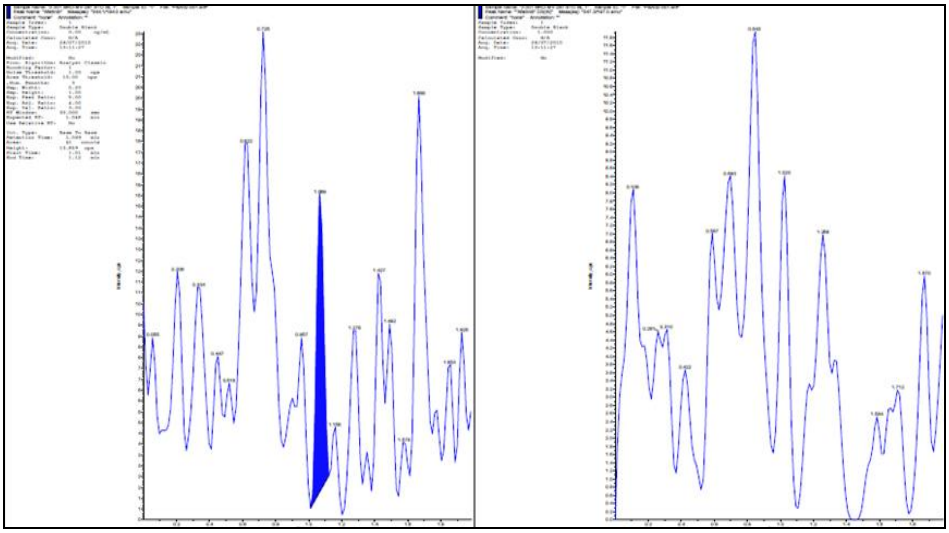

FIG. 1: REPRESENTATIVE BLANK CHROMATOGRAMS OF AFATINIB AND IS IN BLANK PLASMA

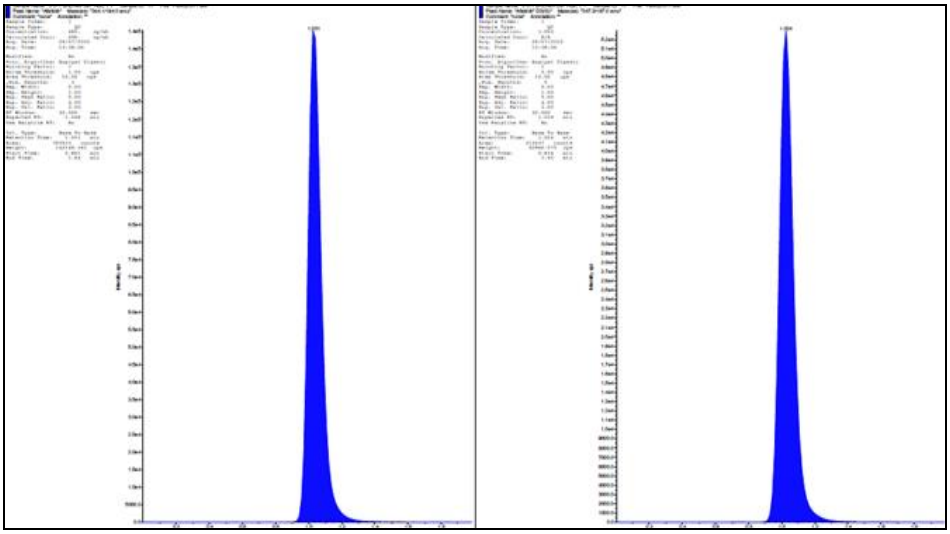

FIG. 2: REPRESENTATIVE HQC-CHROMATOGRAMS OF AFATINIB IN PLASMA WITH INTERNAL STANDARD

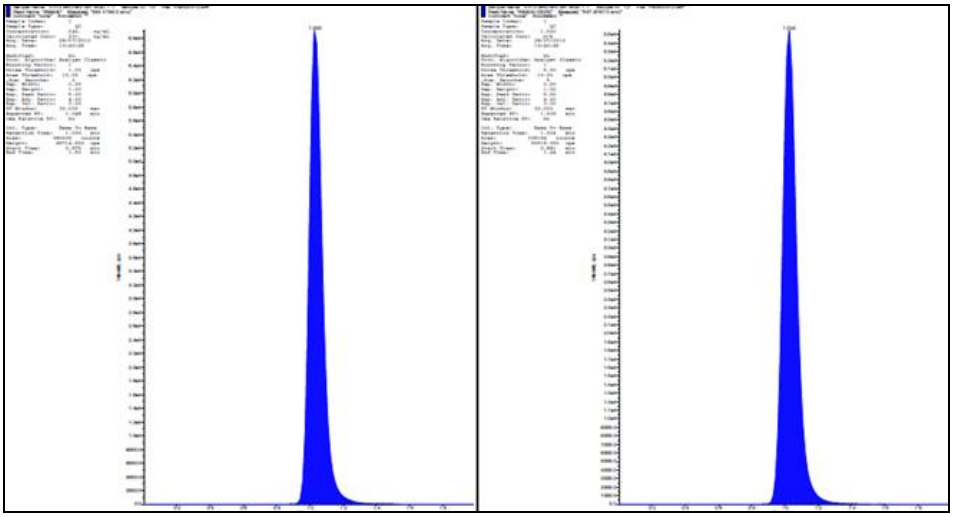

FIG. 3: REPRESENTATIVE MQC - CHROMATOGRAMS OF AFATINIB AND ITS INTERNAL STANDARD

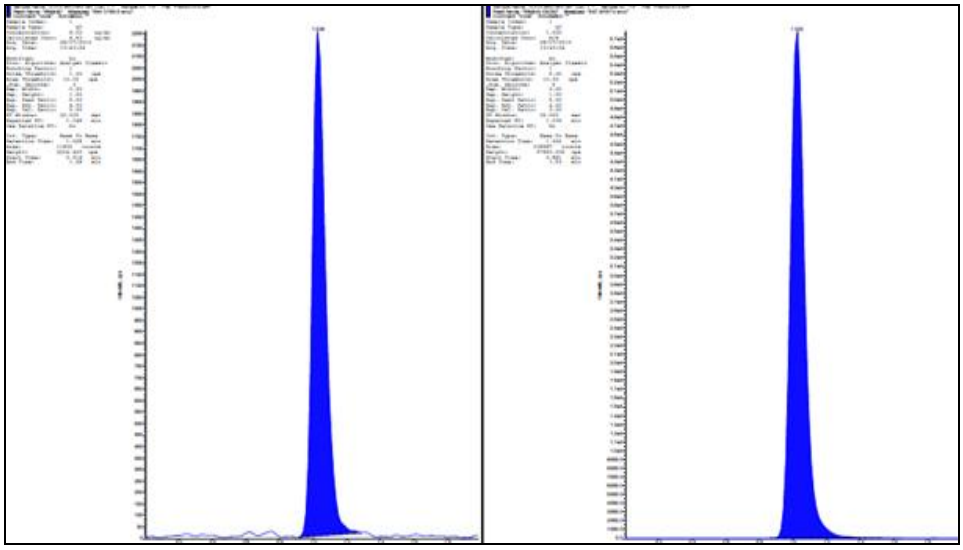

FIG. 4: REPRESENTATIVE CHROMATOGRAMS OF AFATINIB AND ITS INTERNAL STANDARD AT LQC LEVEL

The method developed was validated for linearity, accuracy and precision, and stability as per ICH guidance [21 - 27]. The results of validating parameters are given below.

Linearity: The three calibration curves (peak area ratio *vs*. Concentration) were linear over working range of 2 ng/ml to 600 ng/ml with eight point calibration used for quantification by linear regression **Fig. 5**. The regression equation for the analysis was Y = 0.0053x + 0.0018 with coefficient of correction (r^2) = 0.9956. The % bias and precision (% CV) observed for the calibration curve standards was found to be -8.0 to 9.0 and ≤ 4.93 for Afatinib **Table 1**.

TABLE 1: LINEARITY STANDARDS OF AFATINIB

	Afatinib							
STD ID (in ng/mL)	**STD 8 (2.0)**	**STD 7 (4.0)**	**STD 6 (20)**	**STD 5 (40)**	**STD 4 (80)**	**STD 3 (160)**	**STD 2 (300)**	**STD 1 (600)**
N				28				
Mean	1.96	4.09	21.8	42.8	79.7	156	288	552
SD	0.0851	0.149	0.548	1.86	3.32	7.11	9.86	27.2
% CV	4.34	3.64	2.51	4.35	4.17	4.56	3.42	4.93
% Bias	-2.00	2.25	9.00	7.00	-0.38	-2.50	-4.00	-8.00

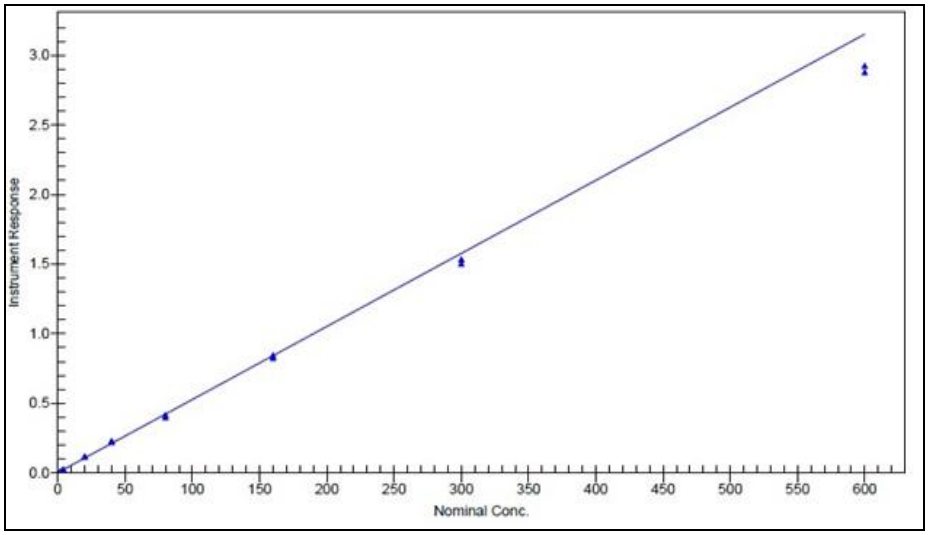

FIG. 5: SPIKED CONCENTRATIONS (2 ng/ml - 600 ng/ml) WERE PLOTTED AGAINST PEAK AREA RATIO *vs* CONCENTRATION WITH NINE POINT CALIBRATION USED FOR QUANTIFICATION BY LINEAR REGRESSION

Recovery: The % mean recovery for Afatinib in LQC (6 ng/ml), MQC (240 ng/ml) and HQC (480 ng/ml) was 112.5%, 113.1% and 112.7% respectively **Table 2**.

TABLE 2: THE % MEAN RECOVERY OF AFATINIB FOR LQC, MQC AND HQC

Replicate no.	HQC		MQC		LQC	
	Extracted Peak Area Ratio	Un-extracted Peak Area Ratio	Extracted Peak Area Ratio	Un-extracted Peak Area Ratio	Extracted Peak Area Ratio	Un-extracted Peak Area Ratio
1	2.176	2.160	1.133	1.118	0.027	0.027
2	2.176	2.135	1.110	1.103	0.029	0.029
3	2.186	2.119	1.111	1.115	0.032	0.033
4	2.118	2.211	1.122	1.113	0.031	0.030
5	2.127	2.140	1.090	1.086	0.027	0.027
Mean	2.1566	2.1530	1.1132	1.1070	0.0292	0.0292
SD	0.03156	0.03557	0.01599	0.01302	0.00228	0.00249
% CV	1.46	1.65	1.44	1.18	7.81	8.53
% Mean Recovery	112.7			113.1		112.5
% Overall Recovery			112.8			
% Overall CV			0.3			

Intraday and Inter-day Precision: The mean intraday and inter-day precision of the method was found to be 1.31 - 5.16% for the quality control samples. This is within the acceptance limits of precision is 15%. The limit of Quantification was found to be 2 ng/ml. at such concentration the mean inter day and intraday precision was found to be 9.11% and 6.53% respectively. Which are within the acceptance limits of precision is 20% **Table 3**.

TABLE 3: INTRA-DAY AND INTER-DAY QUALITY CONTROL SAMPLES FOR AFATINIB

QC	Afatinib (ng/mL)			
Intra-batch	**LLOQ QC (2 ng/mL)**	**LQC (6 ng/mL)**	**MQC (240 ng/mL)**	**HQC (480 ng/mL)**
Mean	1.91	6.25	220.00	500.00
SD	0.0841	0.239	2.88	7.09
%CV	4.40	3.82	1.31	1.42
% Bias	-4.50	4.17	-8.33	4.17
Mean	1.64	6.64	227.00	496.00
SD	0.0851	0.222	4.30	6.80
%CV	5.19	3.34	1.89	1.37
% Bias	-18.00	10.67	-5.42	3.33
Mean	1.82	5.83	215	451
SD	0.182	0.301	4.49	11.5
%CV	10.00	5.16	2.09	2.55
% Bias	-9.00	-2.83	-10.42	-6.04
Inter-batch	**LLOQ QC (2 ng/mL)**	**LQC (6 ng/mL)**	**MQC (240 ng/mL)**	**HQC (480 ng/mL)**
Mean	1.79	6.24	221.00	482.00
SD	0.163	0.418	6.23	24.30
%CV	9.11	6.70	2.82	5.04
% Bias	-10.50	4.00	-7.92	0.42

Matrix effect: The % CV for HQC and LQC samples was observed 1.73% and 5.98% respectively **Table 4**, which are within 15% as per the acceptance criteria.

TABLE 4: MATRIX EFFECT OBTAINED WITH SIX DIFFERENT LOTS OF PLASMA

QC ID	LQC	HQC
Actual conc.	**6 (ng/mL) (area ratio)**	**480 (ng/mL) (area ratio)**
1	0.026	1.809
2	0.028	1.893
3	0.028	1.901

4	0.028	1.863
5	0.027	1.863
6	0.031	1.869
Mean	0.0280	1.8663
± SD	0.00167	0.03234
% CV	5.98	1.73

Results of Pharmacokinetic Studies: The Pharmacokinetic parameter of afatinib was calculated from the plasma concentration-time curves using pk solver software. Also, the area under the plasma concentration-time curve from 0 to 48 hr (AUC0-48) was calculated using trapezoidal rule. Afatinib showed T_{max} of 3.833 ± 0.752 and mean $C_{max,}$ $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow \alpha}$ for Test formulation is 675.83 ± 22.031, 6213.417 ± 257.922 and 6474.61 ± 258.45 respectively The results were presented in **Table 5**, **Table 6** and **Fig. 6**.

TABLE 5: CALCULATED PLASMA CONCENTRATIONS IN RABBITS AT EACH TIME POINT

Time points in hours	Calculated concentrations (ng/ml)							
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5	Rabbit 6	Avg	SD
0		0	0	0	0	0	0	0
0.5	16	18	19	12	15	18	16.33	2.58
1	34	29	32	31	37	34	32.83	2.79
1.5	52	58	60	58	52	56	56	3.35
2	71	81	79	69	65	69	72.33	6.28
2.5	84	96	91	87	93	89	90	4.29
3	77	83	89	82	88	93	85.33	5.75
4	68	75	76	70	76	81	74.33	4.68
5	63	58	73	66	69	78	67.83	7.14
6	58	54	64	62	62	67	61.17	4.58
8	55	61	61	57	54	49	56.17	4.58
12	52	56	57	49	47	41	50.33	5.99
16	49	53	50	42	36	35	44.17	7.63
24	46	49	45	40	33	32	40.83	7.08
36	34	38	38	31	29	33	33.83	3.66
48	0	0	0	0	0	0	0	0

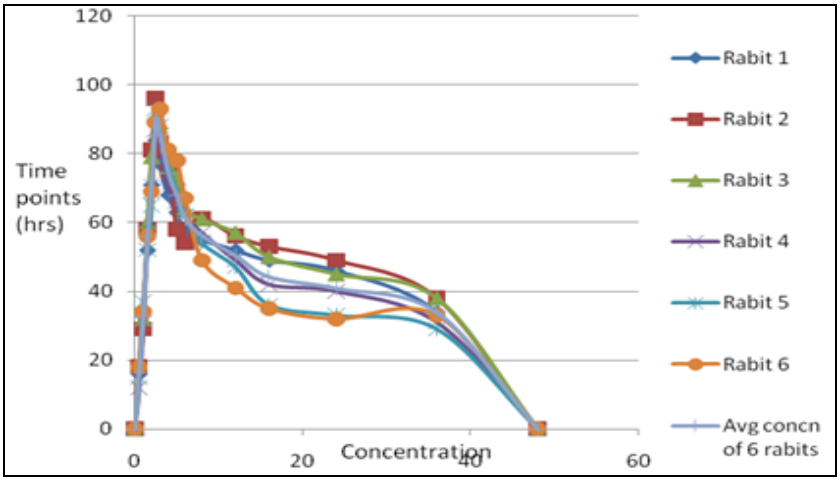

FIG. 6: PLASMA TIME PROFILE CURVES OF TEST ANIMALS

TABLE 6: CALCULATED MEAN VALUES OF PK PARAMETERS FOR TEST ANIMALS

Parameters	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5	Rabbit 6	Mean	SD
C_{max}	643	675	694	694	693	656	675.8	22
log C_{max}	2.808	2.829	2.8414	2.841	2.841	2.8169	2.83	0.01
T_{max}	3	4	5	4	4	3	3.833	0.75
log T_{max}	0.477	0.602	0.699	0.602	0.602	0.4771	0.577	0.09
$t_{1/2}$	8.331	9.563	6.7059	8.113	6.028	9.1414	7.98	1.37
log $t_{1/2}$	0.921	0.981	0.8265	0.909	0.78	0.961	0.896	0.08

Ke	0.083	0.072	0.1033	0.085	0.115	0.0758	0.089	0.02
log Ke	-1.08	-1.14	-0.986	-1.068	-0.94	-1.12	-1.06	0.08
AUC0-$_t$	5874	5940	6218	6455	6306	6488.5	6213	258
log AUC 0-$_t$	3.769	3.774	3.7937	3.81	3.8	3.8121	3.793	0.02
AUC-0-inf_obc	6150	6229	6469.5	6735	6471	6791.8	6475	258
log AUC-0-inf_obc	3.789	3.794	3.8109	3.828	3.811	3.832	3.811	0.02

CONCLUSION: The bio-analytical methodology for determination of Afatinib described in this manuscript is highly specific, rugged and rapid for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for clinical trial samples with desired sensitivity, precision, accuracy and high throughput. The method involved a simple and specific sample preparation by solid phase extraction followed by isocratic chromatographic separation in 2.0 min. The overall analysis time is promising compared to other reported procedures for Afatinib. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with any marketing formulation of Afatinib in human volunteers.

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CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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