IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736.Volume 9, Issue 2 Ver. II (Feb. 2016), PP 06-14
www.iosrjournals.org

Development and Validation of Clavulanic Acid by Negative Ion Mode in Human Plasma Using Lcms/Ms: Application to Pharmacokinetic and Bio-Equvivalance Study.

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Abstract: *A high performance liquid chromatography tandem mass spectrometric simple protein precipitation method for the estimation of Clavulanic acid (CLV), in human plasma in Negative ion mode was developed and validated using Sulbactam as internal standard (IS). Sample preparation was accomplished by using 250 µL of human plasma. The reconstituted samples were chromatographed on Zorbax Eclipse XDB C18, 150*4.6 mm, 5µm column using a mobile phase consisting of HPLC grade acetonitrile: 5mM ammonium acetate (70:30, v/v). The method was validated over a concentration range of 25.589 to 3004.454 ng/mL. The validation provides the results of selectivity, sensitivity, matrix effect, calibration standards and quality control samples data, precision and accuracy data, recovery, various stabilities, run size evaluation and dilution integrity. The study Quantified by the transition of 198.00→136.00, 232.00→139.90 for Clavulanate and Sulbactam respectively. Mean extracted recovery and precision were 58.61 and ≤10.16. The drug was stable in whole blood up to 3 hrs 30 mins. Concomitant drug effect investigated results are within their limits, not compromised with potentially interfering concomitant medication. Application of rapid validated method was applied successfully for the healthy volunteers cross over subject sample analysis in bio-analytical department.*

Keywords: *Clavulanic acid, LC-MS/MS, Protein Precipitation, Human Plasma, Validation.*

I. Introduction

Clavulanic acid (Fig-1) is chemically (2R, 3Z, 5R)-3-(2-HydroxyEthylidene)-7-Oxo-1-azabicyclico [3.2.0] heptane-2-carboxylic acid and is highly sensitive to temperature and P^H. It is a potent inhibitor of beta-Lactamase, enzymes produced by bacteria resistant's to penicillin and cephalosporin. Industrial production of this molecule by strains of streptomyces clavuligerus in complex media and resources for this molecule synthesis is inexpensive [(1)]. Clavulanic acid is co-administered with amoxicillin there is no substantial variation of the pharmacokinetics of either drug compared with their separate administration [(2)]. It is widely metabolized in the liver with half life around 1 hour and protein binding approximately 20-30% [(3)]. It is a suicide inhibitor and covalently bonding to a serine residue in the active site of the beta-Lactamase and restructures this to more reactive species, attacked by another amino acid active site, inactivating the enzyme permanently.

Clavulanic acid used with penicillin's has been associated with an increased incidence of cholestatic jaundice, during therapy it is sensitive to hepatitis and associated jaundice usually self-limiting with rarely fatal [(4-5)]. Clavulanic acid is chemically unstable in its crude form like other beta-lactam compounds due to susceptibly of the carbonyl group associated to the beta-lactam ring to suffer an acidic (H+) or alkaline (OH−) catalyzed attack by water molecules [(6, 7)]. Several authors investigated the stability of Clavulanic acid in buffered solutions at different pHs and observed that degradation follows pseudo-first-order kinetics, highly influenced by catalysis caused by the buffering salts used to maintain constant pH and maximal stability was estimated to be around pH-6.39 [(8-10)]. Several Chromatographic methods for the determination of Clavulanic acid by HPLC-UV [(11-12)] and LC-MS/MS [(13-14)] in various biological fluids, simultaneous analysis of Clavulanic acid with amoxicillin in human plasma by LC-MS/MS [(15-19)] and UPLC-MS/MS [(20-21)] have been reported, among all these methods few are sensitive and some are less sensitive, but in all these methods Clavulanic acid analyzed along with amoxicillin. However as per the literature investigation there is no LC-MS/MS method for single Clavulanic acid estimation in human plasma. HPLC-UV methods for Clavulanic acid are low sensitive, pH adjustable mobile phase and longer run time with more human plasma volumes for processing. However among these the current developed method is highly sensitive and more productive.

Fig.1: Chemical Structures of Analyte (Clavulanic acid) & IS (Sulbactam)

II. Experimental

2.1 Chemicals and reagents:

Clavulanate Lithium Lot no: K0J130 with purity 96.4% as is basis by HPLC was purchased from USP (Bangalore, India). Internal Standard Sulbactam Sodium Batch no: VL/S-SB-046 with purity 99.95% by HPLC was purchased from Vivan Life Sciences Pvt. Limited, India. Analytical grade chemicals, solvents and reagents were used for this study. Strata X Polymeric sorbent cartridges from Phenomenex (Batch-no: S300-189) and highly purified Milli-Q water from Millipore (Bangalore, India) were also used in this test method.

Concomitant Drug Details:

Drug Name	Lot/Batch no	Purity(%)	Supplier
Pantoprazole magnesium	VL/S-PT-055/b	98.87	Vivan Life Sciences
Diphenhydramine hydrochloride	VL/S-DH-024/d	99.38	Vivan Life Sciences
Ibuprofen	VL/S-IP-004/c	99.95	Vivan Life Sciences
Dicyclomine hydrochloride	VL/S-DC-021/b	99.87	Vivan Life Sciences
Nicotine bitartrate dihydrate	G2H304	99.90	USP
Caffeine	J1D241	99.80	USP
Paracetamol	4	99.9	EU Reference Standard

*All the stock solutions of concomitant drugs were prepared in methanol.

2.2 Standard & sample solution preparations:

Standard solutions of Clavulanic acid and Sulbactam (IS, internal standard) 1 mg/mL prepared by 100% HPLC grade water and 100% methanol respectively. Further, stock solutions were diluted to suitable concentrations using a mixture of acetonitrile and HPLC grade water (Diluent) in the ratio of (60:40 v/v) for spiking into plasma to obtain calibration curve (CC) standards, quality control (QC) and dilution integrity quality control (DIQC) samples. For the preparation of calibration curve standards and quality control samples two separate stock solutions were prepared and used. All other final dilutions (system suitability dilutions, aqueous mixture, etc.) were prepared in mobile phase. Calibration curve standard consisting of a set of ten non-zero concentrations ranging from 25.589 to 3004.454 ng/mL and five quality control samples concentrations of 25.969 ng/mL (LLOQ QC, lower limit of quantification quality control), 75.274 ng/mL (LQC, lower quality control), 310.408 ng/mL (MQC1, medium quality control-1), 1443.757 ng/mL (MQC2, medium quality control-2), and 2555.322 ng/mL (HQC high quality control). Quality control samples for dilution integrity were prepared by spiking about 1.60 times highest standard concentration of Clavulanate (4855.111 ng/mL). From these, two times dilution and four-times dilution was performed. Standard stock and working solutions were stored at 2-8^{0}C, while plasma samples spiked with Calibration curve Standards (CSs) and Quality Controls were stored at -70^{0}C until use.

2.3 Protocol of Sample extraction:

The samples were thawed at room temperature and vortexes to ensure complete mixing of the contents. 250µL of the plasma sample was pipette out into 4 mL polypropylene RIA vial tubes, 25 µL of internal standard dilution (4983.383 ng/mL of Sulbactam) was added to it and vortexed, except in blank plasma samples where 25 µL diluent was added and vortexed. Then 50µL of 2% ortho-phosphoric acid buffer was added and vortex. To this 3.0 mL of mobile phase was added and vortexed. Then the samples were centrifuged at 4000 rpm for 20 minutes at 4°C and the supernatant was transferred into auto sampler loading vials and 20 µL was used for injection in to the chromatographic system.

2.4 Instrumentation:

The chromatographic experiment was carried out using Shimadzu (BE-LC-08) system interfaced with high sensitive API 4000 (Applied Bio-System SCIEX) tandem mass spectrometry and results are exposed by using the Analyst software Version 1.4.2.

2.5 Chromatographic & Spectrometry conditions:

The analysis was completed with Zorbax Eclipse XDB C18, 150x4.6 mm, 5µm (Agilent Technologies) using the isocratic mobile phase [Acetonitrile: 5mM ammonium acetate (70:30, v/v)], sonicated for 10 minutes and filtered through 0.45µm membrane filter. Flow rate of 1.0 mL/minute (with splitter-50:50-split ratio) was used. Before running the instrument, the column was equilibrated for 15 minutes with an initial flow and aliquot samples of 20µL were then injected into the column. Chromatographic run time was 2.50 min with 1.10 retention times of CLV and Sulbactam. Mass spectrometry detection parameters for both Analyte (CLV) and IS (Sulbactam) were shown in below table.

Parameter	Clavulanic acid	Sulbactam
Ionization mode	Negative	Negative
Detection (m/z)	198.00 (parent) and 136.00 (product)	232.00 (parent) and139.90 (product)
Ion Spray Voltage (ISV)	-4500 V	-4500 V
Temperature (TEM^{0}C)	450 ^{0}C	450 ^{0}C
Curtain Gas (CUR)	20 psi	20 psi
Collision Gas (CAD)	5 psi	5 psi
GS1	25 psi	25 psi
GS2	35 psi	35 psi
Declustering Potential (DP)	-40 V	-55 V
Collision Energy (CE)	-10 V	-20 V
Collision Cell Exit Potential (CXP)	-5 V	-5 V
Entrance Potential (EP)	10 V	10 V
Dwell time	200 ms	200 ms

III. Results And Discussion

Today pharmaceutical industry faced major challenges are finding different techniques and new ways to enhance productivity, reduce costs whilst still ultimately developing new therapies that improve human health. Generally bioanalytical methods are applied for quantitative estimation of drugs and their metabolites in physiological matrices, and could be applied to studies in the area of human clinical pharmacology and nonhuman pharmacology/toxicology that involves evaluation and interpretation of bioequivalence, pharmacokinetic and toxicokinetic studies. The most important bioanalytical services include method development, method validation and subject sample analysis (method application).

3.1 Method Development:

Earlier investigations were involved in the switching polarity mode, but for optimum results the present study was deals with single polarity mode (-ve) for main drug and internal standard (Sulbactam), and their MS parameters were discussed above the table. The most stable and abundant product ions for CLV and IS in Q3 MS spectra were observed at m/z 136.0 and 139.9.The daughter ion at m/z 136.0 for CLV was due to the elimination of water and carboxylic acid group from the precursor ion. The developed test method was run robust isocratic condition by Zorbax Eclipse XDB C18 column. Several presented methods have used acetonitrile mutually with either formic acid or ammonium acetate as an additive for mobile phase selection. Acetonitrile has been found to be more compatible than methanol with ESI and gives better ionization efficiencies [15]. At initial development study several combinations of acidic buffers (ammonium acetate/acetic acid, ammonium formate/formic acid) with acetonitrile having different ionic strengths (1-10 mM) in the pH range of 3.0-6.5 were tested. Finally high quality chromatographic results were observed using Zorbax Eclipse XDB C18, 150*4.6 mm, 5µm (Make: Agilent Technologies) column using a mobile phase consisting of HPLC grade acetonitrile: 5mM ammonium acetate (70:30, v/v).

In all three general conservative extraction techniques [solid phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PPT)] our test method was employed for simple and cost effective protein precipitation technique for the analysis of CLV in human plasma. Initial trials conducted using LLE with common organic solvents of methyl tert-butyl ether, dichloromethane and n-hexane provided somewhat improved recovery compared to PPT method but matrix interference was also observed. Consequently SPE trials were also carried out on Phenomenex Strata-X (30 mg, 1 cc) cartridge, provided good results for both the analyte and IS but it was highly expensive technique and slightly time consuming when compared to PPT technique. In PPT method also different protein precipitants like acetonitrile, methanol and trichloroacetic acid were tried, in that highly qualified results were observed by using 50µL of 2% ortho-phosphoric acid buffer combined with 3 mL of mobile phase [Acetonitrile:5mM Ammonium acetate (70:30 v/v)]. The results of this assay method were discussed below by stepwise.

IV. Validation

4.1 System Suitability:

System suitability performed by running the mixture of analyte with internal standard and the concentration of analyte corresponds to middle concentration of calibration range. System performance is checked by acquiring the results of signal to noise ratio, retention time and relative standard deviation which should be in their limits.

4.2 Selectivity & Sensitivity, Carry over test (COT):

Selectivity & sensitivity parameter evaluated by running the eight different blank plasma lots (including one Haemolyzed and one Lipemic) were processed without Analyte and internal standard. The same eight lots were processed at LLOQ level to estimate the presence of any interference at the retention time of Analyte and Internal standard. If the experiment does not meet the acceptance criteria the method must be altered and documented.

Carry-over assessment was performed in each analytical run so as to ensure that it does not affect the precision and accuracy of the planned method. The results demonstrate that insignificant carry-over (≤ 20 % of the average drug response in COT LLOQ samples and for the internal standard should be ≤ 5 %) observed in extracted blank plasma (without analyte and IS) after consequent injection of highest concentration of calibration standard (aqueous and extracted) at the retention time of analyte and IS. The calculations were done by using below formula.

$$\% \text{ of drug carry over} = \frac{\text{Drug response in COT BLANK after High Conc. run}}{\text{Average drug response in COT LLOQ}} \times 100$$

4.3 Matrix effect:

In bioanalysis, special focus on matrix effect for calculation of endogenous or exogenous components in biological fluids while using mass spectrometric methods, as it may be revealed in diverse and unexpected forms impacting accuracy of the data generated because of ion suppression or enhancement in the analyte signal. The matrix effect expressed as matrix factor (MF) was determined at low & high concentration levels. The MF measured from the peak area response of the analyte and IS separately and the ratio was used to find the IS-normalized MF. The precision for IS normalized matrix factor at LQC and HQC level was found to be 7.75% and 5.40% respectively, IS normalized factor was 0.908 for LQC and 0.943 for HQC as shown in the table 1.

Table: 1 Matrix effect of Clavulanic acid.

	LQC				**HQC**			
Matrix Lot. No.	**Area of Analyte**	**Area of Internal Standard**	**Area Ratio**	**IS Normalized Matrix Factor**	**Area of Analyte**	**Area of Internal Standard**	**Area Ratio**	**IS Normalized Matrix Factor**
PLASMA-LOT-1	12270	459355	0.0267	0.84	399821	432148	0.9252	0.89
PLASMA-LOT-2	12376	430409	0.0288	0.90	393708	401489	0.9806	0.94
PLASMA-LOT-3	12125	457068	0.0265	0.83	414281	440290	0.9409	0.90
PLASMA-LOT-4	12365	451775	0.0274	0.86	402164	414548	0.9701	0.93
PLASMA-LOT-5	13002	452433	0.0287	0.90	408666	428256	0.9543	0.91
PLASMA-LOT-6	12336	432568	0.0285	0.90	400581	412748	0.9705	0.93
Lipemic-Plasma	15993	493158	0.0324	1.02	436947	408446	1.0698	1.02
Hemolyzed-Plasma	15714	490832	0.032	1.01	442574	417447	1.0602	1.02
	Mean			0.908	Mean			0.943
	SD			0.0704	SD			0.0509
	% CV			7.75	% CV			5.4

*CV: coefficient of variation; LQC: low quality control; HQC: high quality control

*Avg area ratio of LQC in Absence of Matrix Ions: 0.03182

* Avg area ratio of HQC in Absence of Matrix Ions: 1.04373

Calculations of IS normalized factor by the following formula:

$$\text{IS normalized Matrix Factor} = \frac{\text{Peak response area ratio in presence of matrix ions}}{\text{Mean peak response area ratio in absence of matrix ions}}$$

4.4 Extraction Recovery & Linearity:

To evaluate the recovery study by checking the extraction efficiency of developed method at three concentration levels (low, medium and high) compared with the non-extracted samples of analyte and of internal standard. There is no criterion applied for this experiment, but it should be consistent, reproducible and precise. The mean overall recovery of Clavulanic acid was 58.61% with a precision range of 0.95 to 2.11% and of internal standard Sulbactam was 65.08% recovery with a precision ranging from 0.93 to 1.65 %. (Table: 2)

Table: 2 Results of Recovery

	LQC Response		MQC Response		HQC Response	
	Extracted	**Non Extracted**	**Extracted**	**Non Extracted**	**Extracted**	**Non Extracted**
	18013	32716	355896	595768	620793	1020951
	17843	33261	356097	583004	635219	1046432
	18104	33332	353058	573919	633698	1069560
	18148	32390	351813	573599	630846	1068845
	17517	31790	352339	580699	635192	1082735
	18669	32527	346963	584659	649731	1056286
Mean	18049	32669.3	352694.3	581941.3	634246.5	1057468.2
S.D.	380.52	576.7	3338.03	8187.64	9321.7	21775.01
C.V (%)	2.11	1.77	0.95	1.41	1.47	2.06
N	6	6	6	6	6	6
% Recovery	55.25		60.61		59.98	
Overall mean recovery			58.61			
S.D.			2.929			
%Difference			5.36			

Linearity measures the relationship between known concentrations of calibration curve to instrument response. Current developed method covers the dynamic concentration range of 25.5 to 3004 ng/mL, built with nine point's coverage. A regression equation with a weighting factor of 1/ (concentration ratio) ^{2} of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Clavulanic acid in human plasma. Details of five calibration curve plots were shown in the table 3&4.

Table: 3&4 Linearity Data of Clavulanic acid:

Nominal concentration ng/mL									
CC points, range	**STD-A 25.589**	**STD-B 51.178**	**STD-C 102.356**	**STD-D 204.711**	**STD-E 602.093**	**STD-F 1204.185**	**STD-G 1802.672**	**STD-H 2403.563**	**STD-I 3004.454**
1	25.641	51.972	97.636	207.591	592.604	1236.660	1812.392	2437.950	2955.184
2	25.511	51.576	102.942	200.117	607.550	1223.018	1778.009	2398.639	3013.499
3	26.547	46.667	105.889	200.589	595.014	1265.451	1796.745	2367.103	3051.459
4	26.064	50.456	98.661	199.191	602.516	1217.653	1867.889	2422.433	3012.554
5	26.067	50.571	100.654	188.350	602.184	1240.411	1867.319	2464.168	2999.745
Mean	25.966	50.248	101.156	199.168	599.974	1236.639	1824.471	2418.059	3006.488
S.D.	0.3661	1.8819	2.9819	6.1789	5.4343	16.6755	36.8631	33.2099	30.9384
C.V.%	1.41	3.75	2.95	3.1	0.91	1.35	2.02	1.37	1.03
% Nominal	101.47	98.18	98.83	97.29	99.65	102.70	101.21	100.60	100.07

CC no.	Slope	Intercept	r	r^2
1	0.0003	-0.0005	0.9997	0.9994
2	0.0003	-0.0007	0.9999	0.9998
3	0.0003	0.0002	0.9989	0.9978
4	0.0003	0.0008	0.9997	0.9994
5	0.0005	0.0005	0.9992	0.9984

4.5 Precision & Accuracy:

The precision of the assay was measured by the percent coefficient of variation and accuracy should be absolute value of the ratio of the calculated mean value to the respective nominal value, expressed in percentage over the five concentrations of LLOQ QC, LQC, MQC1, MQC2 and HQC samples during the course of validation. Table 5 examine the results of intra-day & inter-day assay precision and accuracy for Clavulanic acid, established method was highly precise and accurate because of all QC levels including LLOQ QC were met the acceptance criteria.

Table: 5 Intra-day & Inter-day Precision and Accuracy Data:

Nominal Conc. (ng/mL)	Intra-batch (n = 12; single batch)			Inter-batch (n = 30; single batch)		
	Mean conc. found (ng/mL)	**% CV**	**Accuracy %**	**Mean conc. found (ng/mL)**	**% CV**	**Accuracy %**
25.969	27.086	10.16	104.30	27.481	10.06	105.82
75.274	78.664	4.97	104.50	78.075	6.93	103.72
310.408	316.033	3.87	101.81	309.893	6.13	99.83
1443.757	1321.830	3.13	91.55	1323.775	2.93	91.69
2555.322	2312.160	5.37	90.48	2372.555	5.10	92.85

*CV: coefficient of variation, n: Number of replicates

4.6 Dilution Integrity and Ruggedness:

To evaluate the dilution integrity should be any samples fall on above the ULOQ in subject sample analysis will be repeated as Dilution integrity samples, but diluting the sample with screened blank matrix appropriately (to bring the concentration within the validation range) and back calculate by applying dilution factor (at least five determinations per dilution factor). Dilution integrity samples (12 sets) were prepared by spiking 1.60 time's of highest standard concentration (4855.111 ng/mL). In that 6 sets are twice dilution and another 6 sets were fourth dilution, analyzed along with calibration curve standards (undiluted) of concentration range and results were tabulated in table 6.

Table: 6 Results of Dilution Integrity:

	Nominal Concentration (ng/mL)			
	Two times dilution (1-6)		Four times dilution (1-6)	
S. No	**4855.111**	**% Accuracy**	**4855.111**	**% Accuracy**
1	4696.917	96.74	4822.709	99.33
2	4807.967	99.03	4994.818	102.88
3	4743.628	97.7	4904.061	101.01
4	4912.438	101.18	5019.846	103.39
5	4737.751	97.58	4922.728	101.39
6	4809.355	99.06	5092.847	104.9
Mean	4784.676		4959.502	
S.D	76.24066		95.7161	
C.V %	1.59		1.93	
%Nominal	98.55		102.15	

Method ruggedness experiment performed by different analyst, different column of same make and different set of solvents & solutions on the same instrument with ≤ 7.71% precision and ≤ 109.37% accuracy.

4.7 Concomitant Drug effect:

Concomitant drug effect was investigated to ensure the precision and accuracy is not compromised with potentially interfering concomitant medication. For this study Pantoprazole, Paracetamol, Ibuprofen, Diphenhydramine, Caffeine, Dicyclomine and Nicotine drugs were spiked in screened plasma at the concentration equivalent to their individual C_{max} concentration level. Precision and accuracy for this analysis were ≤ 4.26 and 103.40% respectively.

4.8 Run size/Production batch evaluation:

Run size evaluation was carried out to assess the integrity of the samples analyzed in a long run during study sample analysis. For this 40 sets of each of LQC, MQC1, MQC2 and HQC samples were processed and analyzed for run size evaluation along with freshly spiked calibration curve standards and quality control samples (Low, Middle and High QC samples). 150 QC's out of 160 QC's of run size evaluation, 24 QC's out of 24 QC's of freshly prepared QCs were within 15% of their respective nominal values. Based on this outcome the test method should be highly precise and accurate.

4.9 Stability:

For Stability evaluation of Chemical entity in a given matrix under specific conditions has been carried out at different time intervals. The aim of this stability study is to detect any degradation of the analyte of interest during the entire period of sample collection, processing, storing, preparing and analysis [22]. Generally, stability should be determined at two (low & high QC's) concentration levels, compared with freshly prepared samples of same concentrations using screened blank biological matrix and analysis was carried out along with fresh calibration curve. Different stability study details were discussed on table 7&8.

Table 7: Stability Results@ Aqueous medium

Storage conditions	Fresh stock Conc.(ng/mL), Mean response (n=6)	Stability stock Conc.(ng/mL), Mean response (n=6)	%Stability
Std. stock. Soln. stab. @RT, 8 Hrs	1018458.9, 609332.5	1022519.9, 601756.2	98.36
Spiking Soln. stab. @RT, 8 Hrs	29535.308, 609332.5	29653.077, 603963.0	98.68
Std. stock Soln. stab.@2-8^{0}c, 6 days	1021458.074, 702478.0	1022519.9, 698047.3	99.27

* n: Number of replicates, RT: Room temperature, Hrs: Hours, m:minutes

Table 7: Stability Results@ Biological matrix (Human plasma)

Storage conditions	Mean Fresh Conc.(ng/mL)	Mean Stability Conc.(ng/mL)	%Change
Bench top Stability @RT, 8 Hrs	75.141	77.853	3.61
	2450.787	2470.554	0.81
Freeze-thaw Stability @-70^{0}C	75.141	73.901	-1.65
	2450.787	2416.151	-1.41
Short-term Stability @-20^{0}C, 5days	75.141	75.470	0.44
	2450.787	2452.965	0.09
Wet Extract Stability @2-8^{0}C, 48 Hrs	75.141	77.629	3.31
	2450.787	2456.465	0.23
Auto sampler Stability @2-8^{0}C, 53 Hrs	75.141	74.3838	-1.01
	2450.787	2458.1702	0.30
Re-injection Stability @ 26 Hrs	80.5443	78.0987	-3.04
	2285.7825	2328.0437	1.85
Whole blood Stability @ 3 Hrs 30 m	0.03732	0.03628	-2.79
	1.2739	1.26762	-0.49

For calculations of

$$\% \text{ Stability} = \frac{\text{Mean Response of stability samples x Fresh stock Concentration}}{\text{Mean response of Fresh stock samples x Stability stock concentration}} \times 100$$

$$\% \text{ Change} = \frac{\text{Mean stability Concentration - Fresh stock Concentration}}{\text{Fresh stock concentration}} \times 100$$

V. Conclusion

A rapid and simple LC-MS/MS method has been described for the estimation of Clavulanic acid in human plasma is highly sensitive, accurate and precise when compared to previously reported methods for single molecule judgment. The simple protein precipitation and a chromatographic run time of 2.50 min per sample make it an attractive procedure in high-throughput bioanalysis of this method. The linear dynamic range established was adequate to determine the plasma concentration of Clavulanic acid in a clinical study involving healthy subjects. In addition, matrix effect and stability of analyte in plasma was extensively studied. Further, run size evaluation, concomitant drug effect results proved the reproducibility of the projected method.

Acknowledgements

The authors wish to thank of my guide Dr.G.Venkata reddy, Dr.T.Shobha Rani, colleagues, friends and the management of GVK Bio Science Private Ltd. Hyderabad for providing facilities and also for their constant support and help.

Respective chromatograms:

Fig: 2 Representative Chromatogram of Blank Plasma Sample of Clavulanic acid

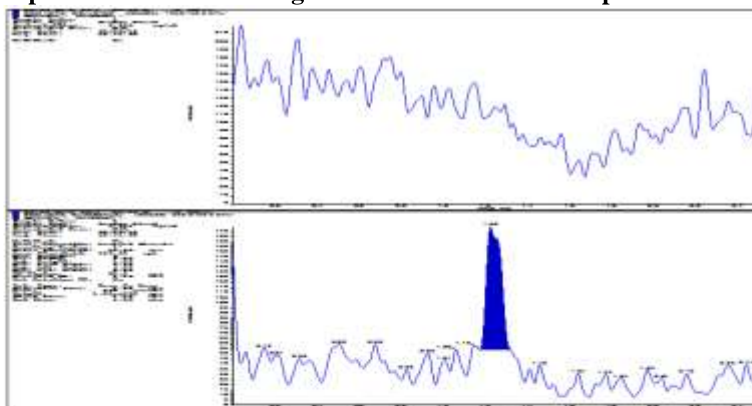


Fig: 3 Representative Chromatogram of LLOQ QC Sample of Clavulanic acid

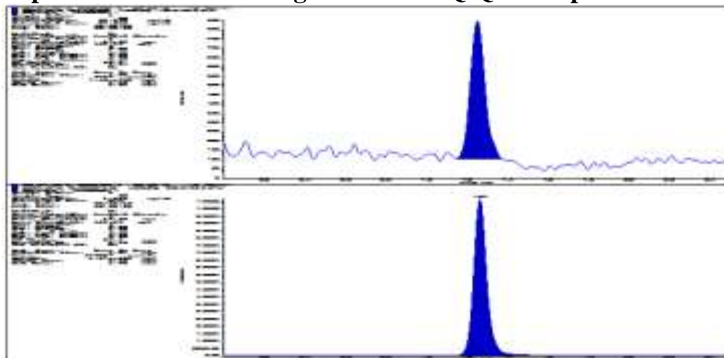


Fig: 4 Representative Chromatogram of LQC Sample of Clavulanic acid

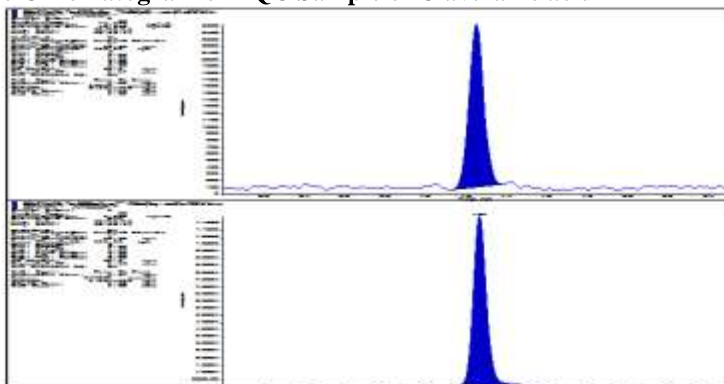


Fig: 5 Representative Chromatogram of MQC2 Sample of Clavulanic acid

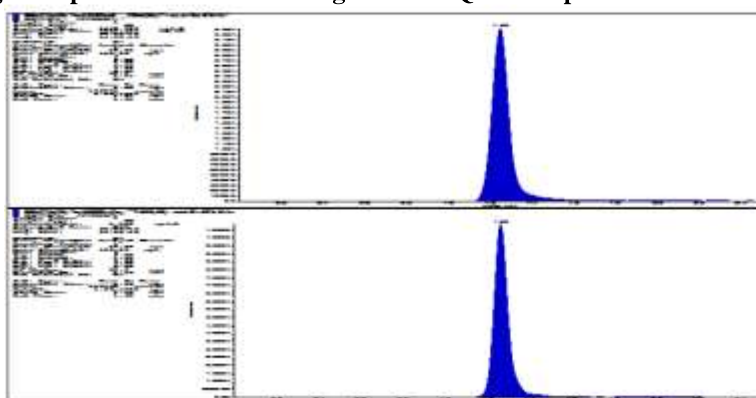
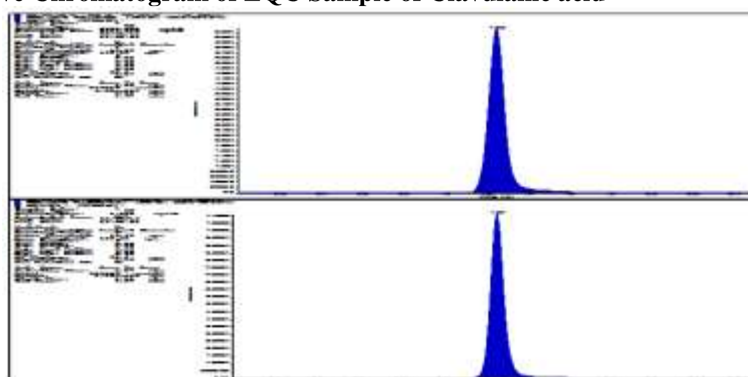


Fig: 6 Representative Chromatogram of LQC Sample of Clavulanic acid



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