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A LC-MS/MS METHOD FOR THE QUANTIFICATION OF CLAVULANATE IN HUMAN PLASMA BY NEGATIVE ION MODE AND ITS PHARMACOKINETIC APPLICATION

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ABSTRACT

A high performance liquid chromatography tandem mass spectrometric simple protein precipitation method for the estimation of Clavulanate potassium (CLV), in human plasma in Negative ion mode was developed and validated by using Sulbactam (SLB) as internal standard (IS). Sample preparation was accomplished by using 250 µL of human plasma. The reconstituted samples were chromatographed on Zorbax Eclipse XDB C_{18} 150x4.6 mm, 5µm column using a mobile phase consisting of HPLC grade acetonitrile: 5mM ammonium acetate (70:30, v/v). The flow rate was 1.0 mL/min.The method was validated over a concentration range of 25.220 to 8095.123ng/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. Totally five precision and accuracy batches were performed during the entire validation and intra-day and inter-day precision and accuracy were proved. Concomitant drug effect investigated results are within their limits, not compromised with potentially interfering concomitant medication. The method was validated and was suitable for the quantitation of clavulanate from plasma samples in a pharmacokinetic study.

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INTRODUCTION

Clavulanate potassium [1] is a beta-lactamase inhibitor that helps prevent certain bacteria from becoming resistant to amoxicillin. Amoxicillin and clavulanate potassium is a combination medicine used to treat many different infections caused by bacteria, such as sinusitis, pneumonia, ear infections, bronchitis, urinary tract infections, and infections of the skin. Potassium clavulanate is a Clavulanic acid and its salts and esters[2,3]. The acid is a suicide inhibitor of bacterial beta-lactamase enzymes from Streptomyces clavuligerus[4]. Administered alone, it has only weak antibacterial activity against most organisms, but given in combination with other beta-lactam antibiotics[5] it prevents antibiotic inactivation by microbial lactamase. Clavulanate Potassium is a semi-synthetic beta-lactamase inhibitor[6] isolated from streptomyces. Clavunalate potassium contains a beta-lactam ring and binds strongly to beta-lactamase at or near its active site. This protects other beta-lactam antibiotics from beta-lactamase catalysis[7,8]. This drug is used in conjunction with beta-lactamase susceptible penicillins to treat infections caused by beta-lactamase producing organisms. As per the literature, only two analytical methods had been reported for the determination of Clavulanate. One method reported by Avinash Gaikwad et al [9] has employed Solid Phase Extraction (SPE) in which ampicillin and amoxacillin D_4 were used as internal standards. This method invoved different optimization conditions with run time of 1.5 min. K.A.Chaitanya et al.[10] reported simultaneous estimation along with Solid-Phase extraction which involves run time of 2.5 min. But in all these methods Clavulanic acid analyzed along with amoxicillin. However as per the literature investigation there is less LC-MS/MS methods 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[11] is highly sensitive and more productive. The chemical structures of Clavulanate and Sulbactam were shown in Fig.1. We report a fully validated LC-MS/MS assay for the quantification of clavulanate in a small volume (250 µL) of human plasma with short run time. This assay was successfully applied to a pilot pharmacokinetic study in healthy volunteers.

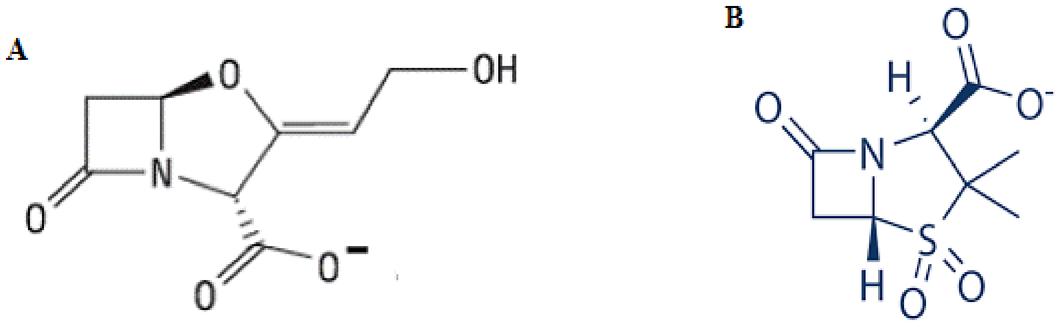


Fig. 1: Chemical structures of Clavulanate (A) and Sulbactam (B).

MATERIALS AND METHODS

Chemicals and materials

The reference samples of Clavulanate (99.11%) was produced from Clearsynth Labs Ltd., (Mumbai, India) and Sulbactam as IS (99.41%) was procured form Vivan Life Sciences Pvt. Ltd., (Mumbai, India). Water used for the LC–MS/MS analysis was prepared from Milli–Q water purification system procured from Millipore (Bangalore, India). Acetonitrile of HPLC grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Analytical grade ammonium acetate was purchased from Merck Ltd., (Mumbai, India). The control human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).

Instrumentation and chromatographic conditions

An HPLC system (Agilent Technologies) consisting of a Zorbax Eclipse XDB C_{18} 150x4.6 mm, 5µm; a binary LC–20 AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–20A_3) were used for the study. Aliquots of the processed samples (20 µL) were injected into the column employing an isocratic mobile phase, a 70:30 (v/v) mixture of acetonitrile and 5mM ammonium acetate was delivered at 1.0 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MS-MS detection in negative ion mode for all the analytes and the internal standard using a MDS Sciex API–4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 450 ºC. The ion spray voltage was set at -4500 V. The source parameters viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 25, 20, 35 and 5 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were -40, -10, -10, -5 V and -55, -20, -10, -5 V for IS. Detection of the ions was carried out in the multiple reaction monitoring mode (MRM) by monitoring the transition pairs of m/z -198.00 (parent ion) to the m/z -136.00 (product ion) for Clavulanate m/z -232.00 (parent ion) ion to the m/z -139.90 (product ion) for the IS. Quadrupoles Q1 and Q3 were set on unit and low resolution. The analysis data obtained were processed by Analyst software™ (version 1.4.2).

Preparation of stock solutions of analytes and IS

Initial stock solutions (1.000 mg/ml) of Clavulanate was prepared in Acetonitrile. Further dilutions were prepared in a mixture of acetonitrile and water (30:70, v/v; diluent) and also used for preparation of calibration standards and quality control (QC) samples. The stocks were stored at 2-8°C and found to be stable for 5 days 14 hrs. All other final dilutions (system suitability dilutions, aqueous mixture, etc.) were prepared in mobile phase. Weighed approximately 2.2 mg of Sulbactam transferred to a 2 mL volumetric flask, dissolved in HPLC grade methanol and made up the volume with the same to produce a solution of 1.000 mg/mL. The stock solution was stored in refrigerator 2-8°C and used for maximum of 5 days 14 hours. The stock solution was diluted to suitable concentration using diluent for internal standard dilution.

Preparation of calibration curve standards and quality control samples

Calibration samples were prepared by spiking 950 µL of human plasma with the suitable working standard solution of the analyte (50 µL dilution of Clavulanate). Calibration curve (CC) standards of Clavulanate in blank plasma were prepared by spiking correct volume of the working solutions, giving final concentrations ranging from 25.220 to 8095.123 ng/mL for Clavulanate. The CC samples were analyzed along with the quality control (QC) samples for each batch of plasma samples. The QC samples were prepared at six different concentration levels of 25.232 (lower limit of quantification, LLOQ), 75.773 (low quality control, LQC), 896.719 (middle quality control, MQC-1), 4113.390 (MQC-2) and 6139.388 (high quality control, HQC) ng/mL for Clavulanate in blank plasma. All the prepared plasma samples were stored at –70±10°C.

Sample preparation

Before beginning of the processing make ensure that the experiment is done under the presence of yellow monochromatic light. The samples were thawed at ice water bath and vortexed to ensure complete mixing of the contents. 250 µL of the plasma sample was pipetted into pre-labelled RIA vial tubes 15 mL glass tubes, 25 µL of internal standard dilution (10.000 µg/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 2.0 mL of mobile phase was added and vortex. And centrifuge the samples at 4000 rpm for 20 minutes at 4°C. Transfer the supernatant to autosampler amber colored loading vials and load in to the auto sampler.

Method validation

The validation of the method was carried out as per EMEA and US FDA guidelines. The parameters determined were selectivity, specificity, matrix effect, method rugeddness, linearity, precision, accuracy, recovery, stability, run size evaluation and dilution integrity.

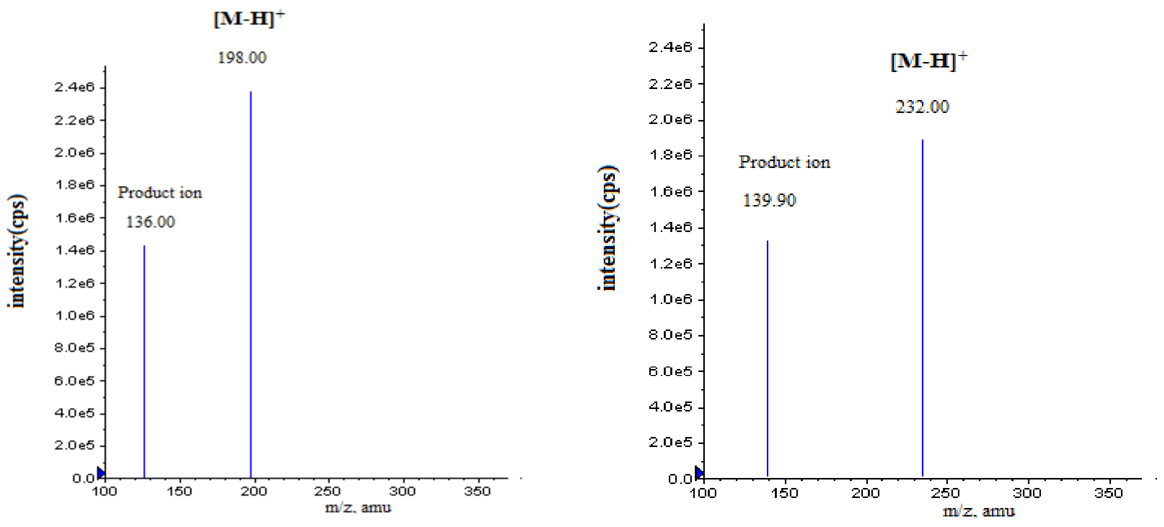
Pharmacokinetic study design

A pharmacokinetic study was performed in healthy male subjects (n = 6). The ethics committee approved the protocol and the volunteers provided with informed written consent. Blood samples were collected following oral administration of clavulanate at pre-dose and 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.17, 1.33, 1.50, 1.67, 1.83, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00 and 24.00 h, in K_2 EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at -70 °C till their use. Along with the clinical samples, the QC samples at low, middle 1, middle 2 and high concentration levels were also assayed. Plasma concentration–time profile of Clavulanate was analyzed by non-compartmental method using WinNonlin Version 5.1. An incurred sample re-analysis (ISR) was also conducted by selecting the 12 subject samples (2 samples from each subject) near C_{max} and the elimination phase. The percent change in the value should not be more than ±20%.

RESULTS AND DISCUSSION

Mass spectrometry

MS parameters were optimized by infusing the standard analyte solution of 100 ng/mL into the mass spectrometer having electrospray as the ionization source and operating in the multiple reaction monitoring (MRM) mode. The signal intensities obtained in negative mode were much higher than those in positive ion mode. Protonated form of each analyte and IS, $[M-H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. Here the signal intensities obtained are in negative mode includes m/z -198.00 (parent ion) to the m/z -136.00 (product ion) for Clavulanate m/z -232.00 (parent ion) ion to the m/z -139.90 (product ion) for the IS. Quadrupoles Q1 and Q3 were set on unit and low resolution was shown in Fig. 2.



A **B**

Fig.2: Product ion mass spectra of $[M\text{-}H]^+$ of Clavulanate (A) and Sulbactam (B).

Method development

To develop a rapid, sensitive and simple assay method for the extraction and quantification of Clavulanate during method development different options were tried to optimize chromatography parameters. The selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. LC-MRM is a very powerful tool for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized at a concentration of 100 ng/mL to maximize the response for the analyte.

Chromatographic conditions

Chromatographic conditions, especially the composition of the mobile phase, column type, flow rate and column oven temperature were optimized through several trials to achieve good resolution and increased intensity of the signals of the analyte and IS, as well as for the short run time. It was found that a mixture of acetonitrile: 5mM ammonium acetate (70:30, v/v) could achieve this purpose and was finally adopted as the mobile phase. Zorbax Eclipse XDB C_{18} 150x4.6 mm, 5µm column gave good peak shape and response even at lowest concentration level for the analyte and IS. The mobile phase was operated at a flow rate of 1.0 mL/min. The retention time of analyte and the IS were low enough allowing a small run time of 2.50 min.

Optimization of extraction procedure

Protein Precipitation (PPT) was employed for sample preparation in this work for extraction purpose. It provides high recoveries of the drugs. The use of stable labeled isotopes of the analyte and IS is recommended for bioanalytical assays to increase assay precision and limit variable recovery between analyte and the IS. For an LC-MS/MS analysis, utilization of stable isotope-labeled drugs as IS proves to be helpful when a significant matrix effect is possible. At the initial stages of this work, several compounds were investigated to find a suitable IS and finally Sulbactam was found to be best for the present purpose rather than labeled isotopes.

System suitability

The system suitability was performed daily prior to start of the analysis. A mixture of analyte at medium level concentration and IS at working concentrations constutes the system suitability solution and it is injected. The precision (%CV) for the system suitability test were found to be less than 1 % for both retention time and area ratio.

Selectivity and chromatography

The degree of interference by endogenous plasma components with the analytes and IS was confirmed by checking chromatograms derived from processed blank plasma sample. As shown in Fig.3 no significant direct interference in the blank plasma traces was observed from endogenous substances in drug free plasma at the retention time of the analytes.

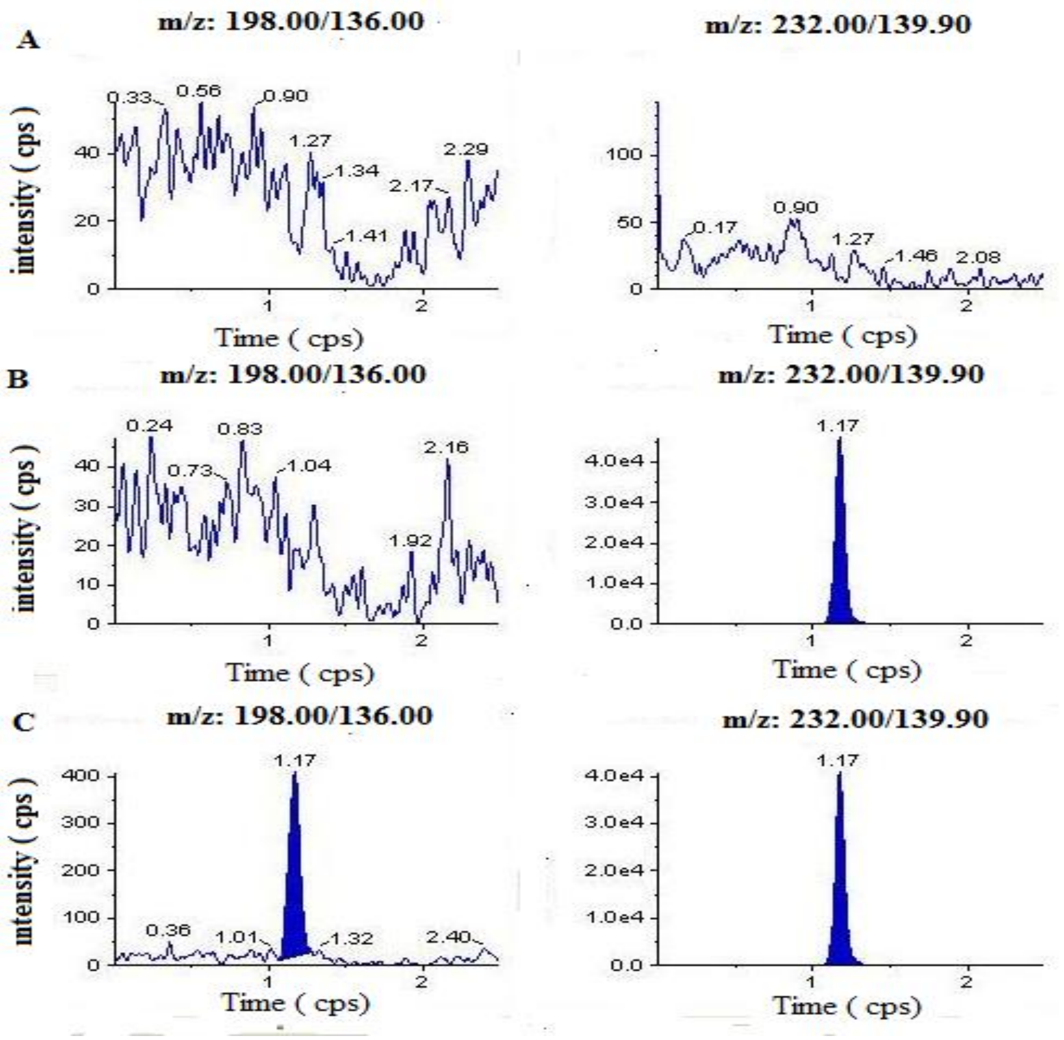


Fig.3: Typical MRM chromatograms of Clavulanate (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS.

Sensitivity

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 5.35% and 102.94% for Clavulanate respectively.

Matrix effect

PPT technique was employed to get the clear and neat sample thereby endogenous components will be eliminated and clear sample will be obtained also matrix effect will be reduced or nullified. The results found were well within the acceptable limits as shown in Tab.1. No significant matrix effect was observed in all the lots of human plasma for the analyte at low and high quality control concentrations.

Tab.1: Matrix effect of Clavulanate in human plasma (*n* = 6).

Plasma lot	LQC (ng/mL)		HQC (ng/mL)	
	Concentration found (mean ± SD; ng/mL)	% Accuracy	Concentration found (mean ± SD; ng/mL)	% Accuracy
Lot 1	82.68 ± 2.51	109	6324.16 ± 85.79	103
Lot 2	79.49± 2.04	105	6219.58 ± 34.02	101
Lot 3	75.28 ± 2.63	99.4	6285.71 ± 91.13	102
Lot 4	76.95± 1.52	102	6174.36 ± 45,36	101
Lot 5	77.31 ± 1.23	102	6190.10 ± 87.11	101
Lot 6	75.23 ± 1.25	99.3	6150.79 ± 60.00	100

Ruggedness

Ruggedness of the method was proved by analyzing one precision and accuracy batch by a analyst who is not involved in the particular study and these samples after processing were run on another instrument of the same make. The precision (%CV) values obtained at LLOQ, LQC, MQC1, MQC2 and HQC were 6.39 %, 6.35%, 1.76%, 2.42% and 1.88% respectively and the accuracy values at these levels were 107.67, 103.27, 104.01, 101.15 and 102.51%.

Linearity

Ten-point calibration curve was found to be linear over the concentration range of 25.220-8095.123 ng/mL for Clavulanate. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration-detector response relationship for both the analytes in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥0.99.

Precision and accuracy

The results for intra-day and inter-day precision and accuracy in plasma quality control samples are summarized in Tab.2. The intra-day and inter day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle 1, middle 2 and high quality control level, whereas within 20% at LLOQ QCs level. The intra-day and inter-day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle 1, middle 2 and high quality control level, whereas within 100± 20% at LLOQ QCs level. The results revealed good precision and accuracy.

Tab.2: **Precision and accuracy data for Clavulanate.**

Quality control	Run	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
Within batch precision (Six replicates at each concentration)				
LLOQ	1	26.24±1.81	6.90	104
	2	26.21±1.07	4.07	104
	3	23.60±2.39	10.1	93.5
	4	22.45±3.56	15.9	89.0
	5	27.17±1.73	6.39	108
LQC	1	77.52±3.77	4.87	102
	2	76.09±2.35	3.09	100
	3	80.94±4.74	5.85	107
	4	72.20±3.10	4.29	95.3
	5	78.25±4.97	6.35	103
MQC1	1	961±17.7	1.84	107
	2	934±21.0	2.25	104
	3	949±13.5	1.43	106
	4	963±15.0	1.56	107
	5	933±16.4	1.76	104
MQC2	1	4312.39±93.5	2.17	105
	2	4177.83±42.4	1.01	102
	3	4227.60±35.6	0.84	103
	4	4345.78±44.3	1.02	106
	5	4160.82±101	2.42	101
HQC	1	6474.25±84.96	1.31	106
	2	6322.61±98.54	1.56	103
	3	6436.50±73.52	1.14	105
	4	6566.28±46.78	0.71	107
	5	6293.21±118	1.88	103
Intra–day variations (Twelve replicates at each concentration)				
LLOQ		24.90±2.23	8.96	98.7
LQC		78.51±4.37	5.57	104
MQC1		941.19±18.6	1.97	105
MQC2		4202.72±45.45	1.08	102
HQC		6379.56±102	1.60	104

Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 25.232, 75.773, 896.719, 4113.390 and 6139.3688 ng/mL, respectively.

Recovery

A simple Protein Precipitation Technique (PPT) proved to be robust and provided cleanest samples. The recoveries of the analytes and the IS were good and reproducible. The mean overall recoveries (with the precision range) of Clavulanate were 28.74% with a precision range of 0.43-5.20% respectively. The recovery of the IS was 26.29% with a precision range of 1.43- 5.05%.

Stability studies

In the different stability experiments carried out viz. bench top stability (8 h), autosampler stability (81 h), stability of samples at -20°C (04 days, 18h) repeated freeze–thaw cycles (four cycles cycles), reinjection stability (45 h), wet extract stability (77 h. 04min at 20±5°C) and long term stability at -70 °C is yet to be proved the mean % nominal values of the analytes were found to be within ±15% of the predicted concentrations for the analytes at their LQC and HQC levels shown in Tab. 3. Thus, the results were found to be within the acceptable limits during the entire validation.

Tab. 3: Stability data for Clavulanate in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Process[a]	75.77	75.81 ± 2.44	3.21	100
	6139.39	5910.71± 52.18	0.88	96.28
Process[b]	75.77	77.22 ± 2.24	2.90	102
	6139.39	5953.64 ± 70.27	1.18	99.4
Bench top[c]	75.77	76.49 ± 2.87	3.75	101
	6139.39	6022.59 ± 104.97	1.74	98.10
FT[d]	75.77	75.47 ± 2.75	3.65	99.59
	6139.39	5971.22 ± 44.16	0.74	97.26
Reinjection[e]	75.77	78.44 ± 7.10	9.05	103
	6139.39	6490.66 ± 116.09	1.79	103
Wet Extract[f]	75.77	77.67 ± 1.61	2.07	103
	6139.39	5956.28 ± 53.03	0.89	97.02

[a] after 81 h in autosampler at 10°C; [b] after 4 days 18 h at -20°C; [c] after 8 h at room temperature; [d] after 4 freeze and thaw cycles; [e] after 45 h of Reinjection;
[f] at room temperature for 77 h

Run size evaluation

The total number of samples that can be analyzed in a single run and integrity of the samples demonstrates run size evaluation. The batch size includes 44 sets of each of LQC, MQC1, MQC2 and HQC samples were processed and analyzed for run size evaluation along with calibration curve standards. 172 QC’s out of 176 QC’s of run size evaluation were within 15% of their respective nominal (theoretical) values.

Dilution integrity

The upper concentration limits can be extended 26604.015 ng/mL for Clavulanate by a 1/2 or 1/4 dilution with screened human blank plasma. The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 1.38% and 95.91% of their nominal. The coefficients of variation (%CV) for 1/2 and 1/4 dilution samples were less than 5% for all the analytes.

Pharmacokinetic study results

The sensitivity and selectivity of this method tested for Clavulanate concentrations in human plasma samples collected from healthy male volunteers (*n* =6). No interference was found in subject blank plasma as shown in Fig.4. The mean plasma concentrations against time profile of Clavulanate are depicted in Fig.5. The pharmacokinetic parameters estimated are shown in Tab.4.

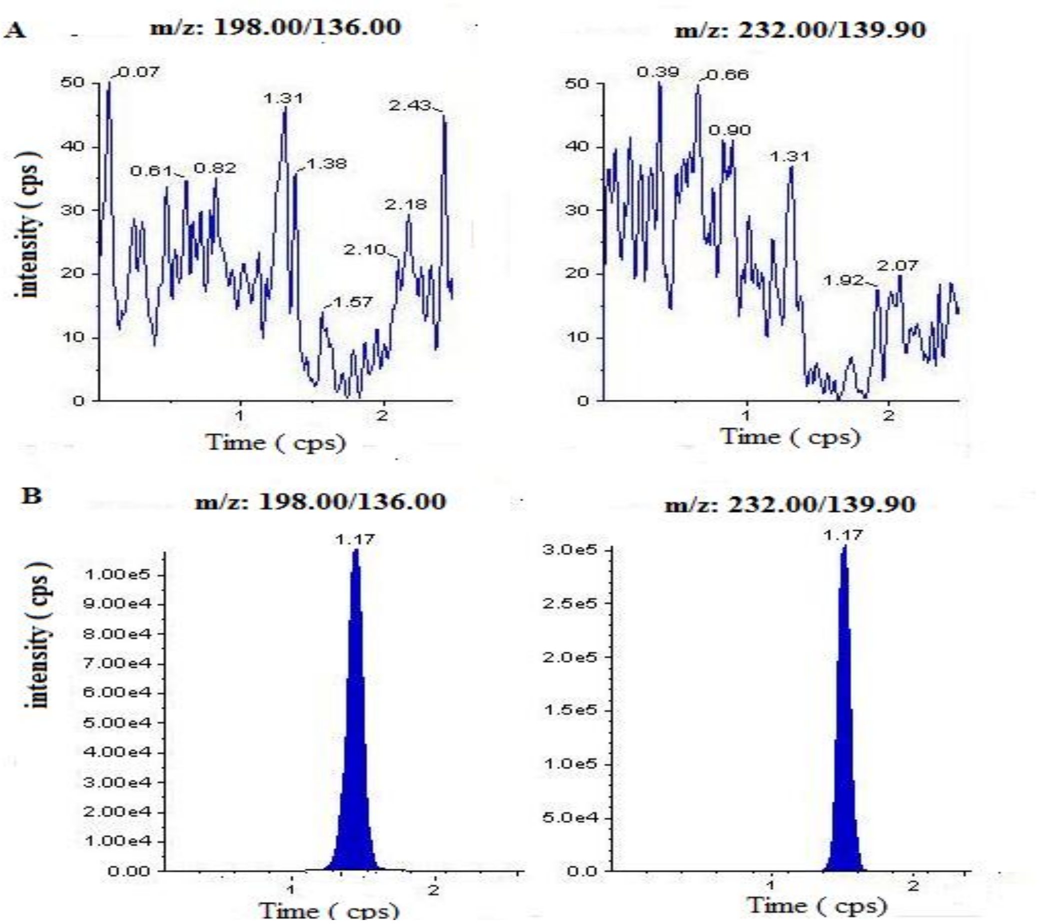


Fig.4: MRM chromatograms resulting from the analysis of subject blank plasma sample (A) and 0.83 h subject plasma sample (B), after the administration of potassium 57 mg/5mL oral suspension. The sample concentration was determined to be 2094 ng/ml.

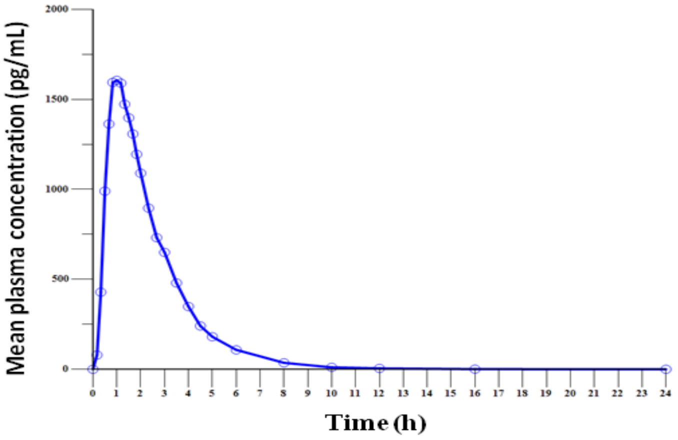


Fig.5: Mean plasma concentration–time profile of Clavulanate in human plasma following oral administration of potassium 57 mg/5ml oral suspension to healthy volunteers (*n*=6).

Tab.4: Pharmacokinetic parameters of Clavulanate (5 mL of oral suspension i.e., 57 mg/mL Clavulanate) formulation in healthy, adult, human male subjects under fed condition (n=6, Mean ± SD).

PK Parameters	Mean ± SD
	57 mg/mL
t_{max} (h)	1.03±0.17
C_{max} (ng/mL)	1662±329.3
$AUC_{0–t}$ (ng h/mL)	4189±1154
$AUC_{0–inf}$ (ng h/mL)	4201±1159
$t_{1/2}$ (h)	1.33±0.53
Kel (h^{-1})	0.58±0.20

Incurred sample reanalysis

In Crystal City III meeting the FDA has demonstrated the necessity of incurred sample reanalysis evaluation and the importance of assay reproducibility by using dosed subject samples. Incurred sample reanalysis (ISR) was performed using two plasma samples from each subject and re–assayed in a separate batch run. The differences in concentrations between the ISR and the initial values for all the tested samples were less than 20% which was shown in Tab.5 indicating good reproducibility of the present method.

Tab.5: Incurred samples re-analysis data of Clavulanate.

Sample	Clavulanate 57 mg/5mL oral suspension		
	Initial conc. (ng/mL)	Re-assay conc. (ng/mL)	Difference [a] (%)
1	1722.668	1701.32	-1.25
2	79.128	81.294	2.70
3	1579.180	1590.28	0.70
4	170.828	165.449	-3.20
5	1831.465	1811.2	-1.11
6	111.356	117.301	5.20
7	2040.724	2044.4	0.18
8	88.181	89.012	0.94
9	1370.301	1401.2	2.23
10	90.902	92.224	1.44
11	1180.360	1197.72	1.46
12	128.600	129.346	0.58

[a] Expressed as [(initial conc.−re–assay conc.)/average]×100%.

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. The future scope of work includes a novel method which was helpful for carrying out pharmacokinetic study.

CONFLICTS OF INTEREST

All authors have none to declare.

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