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Original Article

AN LC-MS/MS BASED BIOANALYTICAL APPROACH TO RESOLVE PHARMACOKINETIC INVESTIGATION OF ACOTIAMIDE HYDROCHLORIDE AND ITS APPLICATION TO BIOEQUIVALENCE STUDY

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ABSTRACT

Objective: Acotiamide, a prokinetic drug used to treat Functional Dyspepsia, which acts by modulating gastric motility. However, in this present study, a simple and accurate bioanalytical method was developed for the estimation of Acotiamide in human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and validated according to US-FDA guideline.

Methods: The method was developed in blank human blood plasma; propranolol was used as internal standard (IS). Protein Precipitation technique was followed for the extraction of the drug from the plasma sample. In liquid chromatography, the C18 analytical column (50 x 3 mm, particle size-5 µm) was used; as a mobile phase, 0.1% formic acid in Mili Q water, and ACN with methanol (1:1) used, at 0.50 ml/min flow rate. Detection was done by positive electrospray ionization (ESI) with a run time of 7 min in multiple reaction monitoring (MRM) mode. Eight calibration concentrations were taken, ranging from 1.5625-200 ng/ml for Acotiamide. Different stability studies were performed and obtained results found within the acceptable range. Moreover, a comparative pharmacokinetic analysis was done in 24 healthy human volunteers in a single dose, randomized, crossover study.

Results: The precursor to production reaction was; m/z 451.200 → 271.200 for Acotiamide and m/z 260.300 → 116.100 m/z for IS. The obtained calibration curve was linear, with a mean r^2value 0.9953. Among the pharmacokinetic parameters, C_{max} and T_{max} were 25.71±2.31,23.61±2.32 ng/ml; 2.54±0.12, 2.43±0.21 h for reference and test samples, respectively.

Conclusion: No major adverse events were noted in the clinical phase, the developed method was accurate and linear; obtained pharmacokinetic parameters hence represented.

Keywords: Acotiamide, LC-MS/MS, Pharmacokinetics, Bioequivalence, Mass spectrometry, US-FDA guideline

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INTRODUCTION

Functional Dyspepsia (FD) is a disorder associated with gastric discomfort of the gastroduodenal region. It can affect any age or any socioeconomic class of society. The actual cause of FD is still not clear, it is believed that an irregular lifestyle and unhealthy food habit may be an important factor for such discomforts [1]. This also reduces the quality of life in individuals. The symptoms of FD include bothersome postprandial fullness, early satiety, epigastralgia, and epigastric burning, etc. FD is further divided into two branches i) postprandial distress syndrome (PDS, characterized by postprandial fullness and early satiation) and ii) epigastric pain syndrome (EPS, characterized by epigastric pain and burning) [2]. Treatment of EPS includes involved with antacids or acid neutralizers, but the treatment of PDS includes prokinetic agents. Previously, other prokinetic agents such as Domperidone, a dopamine receptor antagonist was used for the management of gastroparesis or nausea. But it exhibited a serious elevation of plasma prolactin level. Afterward, Cisapride a non-selective 5HT4 blocker, was withdrawn for its cardiovascular safety issue. Acotiamide is a classic example of a prokinetic agent, which shows its prokinetic activity by inhibiting acetylcholine-esterase release in peripheral nerve endings and suppressing degradation of acetylcholine, resulting in gastric motility [3, 4]. Moreover, during the postprandial state, Acotiamide also inhibits duodenal and colonic motility in conscious dogs [5].

Acotiamide hydrochloride trihydrate is used in treatment for functional dyspepsia diagnosed by Rome III criteria recently approved in Japan [6]. Acotiamide is an amide containing a basic drug with a molecular weight of 450.56. Acotiamide exerts its therapeutic activity by inhibiting the muscarinic receptor, which results in enhanced acetylcholine release and via inhibition of acetylcholinesterase (AChE) activity in the stomach. However, the gastroprokinetic activity of Acotiamide not responsible for the prolongation of the QT interval, thus, it is safe in terms of cardiac safety issues [7, 8].

LC-MS/MS can be used for the bioanalysis of drugs in the body [9]. So in this present study, a simple and sensitive method using LC-MS/MS for the quantification of Acotiamide from human blood plasma was developed to evaluate the pharmacokinetic parameters. Protein precipitation, a simple and cost-effective and less time-consuming technique, was followed for the extraction purpose of analyte and IS from human blood plasma.

MATERIALS AND METHODS

Study design and volunteer enrolment

A randomized, open-label, two treatment, two-period, two sequences, single-dose, crossover, comparative, pharmacokinetic study of two different formulations of Acotiamide i.e.; film-coated tablets containing Acotiamide Hydrochloride Hydride 100 mg in 24 healthy human volunteers to evaluate the comparative pharmacokinetic parameters and comparative oral bioavailability. The study was conducted following the guidelines of the Central Drug Standard Control Organization (CDSCO), New Delhi, India set for BA/BE Studies [10]. Before the initiation of the study, healthy volunteers were enrolled and confirmed that volunteers are healthy and were not taking medications that influence esophageal motility.

Furthermore, an informed consent form was obtained from each volunteer, as per the Declaration of Helsinki. In each study period, after overnight fasting, drug administration was done with 240 ml of water. From each volunteer 182 ml blood samples were taken in 5 ml EDTA containing vials at different times i.e., 0.5, 1.0, 1.5, 2.0, 2.25, 2.5, 2.75, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0 and 72.0 h. The blood samples were centrifuged in the cold centrifuge at 3500±10 rpm and 4±1 °C for 10 min for separation of plasma. The separated plasma was properly labeled with volunteer code and sampling time in polypropylene tubes and stored at–20±5 °C for analysis purposes. The following pharmacokinetic parameters C_{max}, T_{max}, Kel, $t_{1/2}$, $AUC_{0-t,}$ and AUC_{0-inf} were evaluated with the plasma samples, and the relative bioavailability of test and reference was estimated [11]. The calculated 90% Confidence Interval should fall within 80 to 125% for the Test/Reference ratios for AUC_{0-t}, $AUC_{0-inf,}$ and C_{max} to conclude bioequivalence.

Method development and validation

Chemicals and reagents

Active Pharmaceutical Ingredient (API) of Acotiamide was procured from Akums Drugs and Pharmaceuticals Limited, Delhi, India, whereas Propranolol (Internal Standard) was obtained from SubhamBiopharma, Mumbai, India. Test and reference samples were also obtained from Akums Drugs and Pharmaceuticals Limited, Delhi-110034, India and Lupin Limited, Sikkim-737132, India, respectively. Formic Acid of AR Grade, water was prepared from the milli-Q water purification system until a conductivity of 18.2 mΩ was reached that supplied by millipore (elix, milli-Q A10 Academic, Bedford, mA, USA), methanol of HPLC grade. Acetonitrile, Dimethyl Sulfoxide of HPLC Grade.

Stock solution and working standards

The stock solution of analyte and IS (Propanolol) of 1 mg/ml were prepared in DMSO and kept in-20° C freezer. The stocks were then further diluted with a given mobile phase organic solvent to obtain the working solution of 1 µg/ml for method developments and further proceedings. After tuning the method with 1 µg/ml stock of both analyte and IS following replicates of working standards were spiked in plasma for obtaining the calibration curve.

LC condition

The LC used Shimadzu Controller CBM 20Alite with binary pumps of Shimadzu LC20AD, which equipped with a Shimadzu SIL20A auto-sampler used in the following study. Separation of Acotiamide and Propanolol (IS) was carried out during different stages of analysis on a C18 column [PhenomenexKinetex, 50 mm × 3 mm (length ×inner diameter) particle size of 5 µm], to fulfill the study objectives. The mobile phase was used under gradient conditions and the composition was 0.1% formic acid in Milli Q water with an observed pH of 3.2 (aqueous solvent) at pump A and as organic solventACN and methanol (1:1) at pump B at the flow rate 0.50 ml/min. The total LC run time was set at 7 min and the injection volume was 10 µL for each sample. However, the organic flow in gradient method was set at 10% flow from pump B (0.1 min to 1.0 min of total run time) and 90 % flow was given of organic solvent from 1.0 min to 3.0 min and again back to 10% of the organic flow from 3.0 to 7.0 min to obtain an accurate chromatographic condition. The autosampler temperature was set at 15 °C and the pressure of the LC system was not more than 6000 psi.

Mass equipment and optimization of mass parameters

Acotiamide and Propranolol (IS) after separation by LC technique during method development, bioanalytical method validation and in biological samples were analyzed using an LC-MS/MS system (API 2000, Applied Biosystems/MDS SCIEX, Toronto, Canada) by a triple quadrupole mass spectrometer coupled with turbo electrospray ionization (ESI) interface. For tuning parameters of the system 1µg/ml of analyte and IS was scanned at a flow rate of 10 µl/min. Q1 and Q3 scan of both Analyte and IS was done individually, and then it was processed for MRM scanning of Analyte and IS mixture for development of the bioanalytical method is optimized chromatographic condition summarized in table 1. The entire bioanalytical method was carried out in positive ionization mode.

Table 1: Different mass parameters of instrument and component (analyte and IS) dependent parameters

Parameters	Values	
Instrumental parameters		
Source temperature (°C)	400	
Curtain Gas	30	
CAD gas	5	
Ion spray voltage	5500	
Gas 1	45	
Gas 2	45	
Component dependent parameters		
	Drug	**IS**
Declustering potential (DP)	83	33
Entrance potential (EP)	9.5	8.5
Focusing potential (FP)	398	393
Collision energy (CE)	29	28
Cell exit potential (CXP)	3.5	3

Plasma extraction and sample preparation

A simple, less time consuming and accurate method of protein precipitation was followed for preparation validation and volunteer samples. 100 µl of blank plasma thawed at room temperature and taken in 2 ml Eppendorf tube, 50 µl of the stock solution along with 50 µl of IS was mixed in Eppendorf tube and mixed by a vortex mixer for 1 min. Then, 300 µl of cold Acetronile was added and mixed and followed by vortex mixing again for 5 min. The resulted mixture was then was centrifuged at 12000 rpm for 10 min at 4±1 °C using a cold centrifuge. The upper supernatant layer was separated and put into an autosampler vial for injection purposes in LC-MS/MS. However, for the preparation of volunteer plasma samples, 150 µl of plasma sample is mixed with 50 µl IS and the remaining procedure was the same followed described previously.

Method Validation

Validation of the developed bioanalytical method was carried out according to 'Guidance for Industry: bioanalytical method validation' by the USFDA and EMA standard guideline. Different validation parameters including selectivity and specificity, accuracy and precision, linearity and lower limit of quantitation (LLOQ), recovery and matrix effect, and stability studies (Short term, Benchtop, Freeze-thaw, Autosampler stability, long term stability) was evaluated accordingly [11, 12, 16].

Selectivity and specificity

Selectivity is the ability of a bioanalytical method to differentiate and quantify the analyte in the presence of other components in the sample. The specificity of the method was assessed by six different

sources of blank plasma. This plasma was spiked with standard concentrations of the analyte along with IS at low, medium, and high-quality control samples, i.e., LQC, MQC, and HQC.

Accuracy and precision

Different QC concentrations of Drug and IS were prepared as QC batch in the Plasma sample and were analyzed with the developed method. The result was obtained and was expressed using standard calibration curves for 3 d (PA batch Day 1, Day 2, Day 3), to obtain inter-day precision and accuracy. Whereas, intraday precision and accuracy were obtained analysis of 5 sets of QC batch, extracted by blank plasma with different concentrations along with LLOQ of Acotiamide on three cumulative days, respectively. The precision was calculated in terms of

coefficient of variation (CV (%) = standard deviation/mean x 100),

and the accuracy was calculated as the

Absolute Percent Bias (APB (%) = measured concentration/ targeted concentration x 100).

The acceptance criteria of intra and inter-day batch for Precision and Accuracy batch were 20% or better for LLOQ and 15% or better for the rest of QC concentrations and the accuracy was within±20% or better for LLOQ and within±15% or better for the rest of QC concentrations.

Linearity and lower limit of quantitation (LLOQ)

Eight calibration standards i.e., 1.5625, 3.125, 6.25, 12.50, 25, 50, 100, 200 ng/ml of acotiamide with a known and equal concentration of IS were added in blank plasma and analyzed to establish the Linearity of the method. The linearity range must be within±20% of the LLOQ and±15% other than LLOQ. The analyte peak in the LLOQ sample should be identifiable and reproducible with a precision of±20% and an accuracy of 80% to 120% according to standard guidelines [11, 12].

Recovery and matrix effect

The matrix effect is defined as the amount of erratic value produced during analysis due to the presence of endogenous substances present in a biological matrix. Sometimes, the alteration may cause a serious deviation of actual value, which is not permitted according to the guideline. Therefore, the matrix effect was calculated from QC standards with spiked plasma, at different concentrations. And the Recovery is calculated as QC samples spiked with the analyte or are with analyte spiked in blank plasma to ensure the reproducibility of the method [13].

Stability studies

Different QC concentration samples spiked into blank plasma and kept in the deep freezer and were frozen at-20 °C, then it was thawed at room temperature and analyzed according to the protocol to check the Freeze-thaw Stability in three cumulative periods. The Autosampler stability of prepared samples established in Autosampler vials at a defined temperature at 15±2 °C for 24 h compared with freshly prepared control concentrations. In addition to that, the Benchtop stability was evaluated with the QC samples at room temperature which was kept for 8 h and was compared with fresh QC samples. The spiked analytes and IS in blank plasma kept in room temperature for 6 h and the short term stability was established against fresh control concentrations. However, Long term stability was necessary to evaluate with prepared QC samples prepared on day 1 of validation till 15thday and compared with freshly prepared QC samples according to USFDA and EMA guidelines. The precision and accuracy for the stability samples must be within ≤15% and±15% respectively of fresh QC concentrations [11-13].

RESULTS AND DISCUSSION

Liquid chromatography

The presence of an amide group in the parent structure makes it basic; therefore, the acidic mobile phase was used for separation. Though, different mobile phases for organic pumps ranging from ACN or Methanol with different buffers of different pH used to obtain a good resolution with acceptable peak shape. However, after a few trials and errors, 0.1% formic acid as a modifier in Mili Q water with an observed pH of 3.2 (aqueous solvent) at pump A and as organic solvent ACN and methanol (1:1) at pump B was used using gradient technique. In LC parameters, the gradient flow of aqueous flow of 90% flow was set to obtain maximum retention of the matrix interferences by LC. Whereas Phenomenex Kinetex C18; 50 x 3 mm column was used as a stationary phase which showed better retention of the matrix components that provide good separation and peak shapes [13].

Mass spectrometry

For the optimization of mass parameters each analyte and IS was introduced into the mass quadrupole initially during the method development phase Haward syringe pump method was used where 10 µl of flow was maintained. As Acotiamide contains a basic amide group, which can readily accept proton for ionization, positive ionization mode was used, which showed a maximum response. After system tuning the method was checked in MRM mode using electrospray as ionization (ESI) source. The mass parameters, i.e., Curtain Gas, CAD gas, Ion spray voltage, Gas 1, Gas 2 was set and optimized, and simultaneously compound dependent parameters (listed in table 1) also set to obtain good resolution and symmetrically shaped peaks in chromatography. The most abundant protonated ions $[m+H]^+$ obtained by collision-induced dissociation of Acotiamide and IS (Propanolol) in the Q1 spectrum, was used as production in Q3. The precursor to production reactions was m/z 451.200→ 271.200for Acotiamide and m/z 260.300→ 116.100 for IS. The ionization pattern of Acotiamide and IS (Propanolol) from Q1 to Q3 shown in fig. 1 and fig. 2, respectively. M/z and Retention time of analyte and IS were set as acceptance criteria for the identification parameter of compounds [14, 15]. However, identification and quantification of the analytes were evaluated and produced in chromatograms according to the ratios of signal-to-noise (S/N), S/N ≥ 3, and S/N ≥ 10, respectively. Final MRM scan of Analyte and IS shown in fig. 3.

Method validation

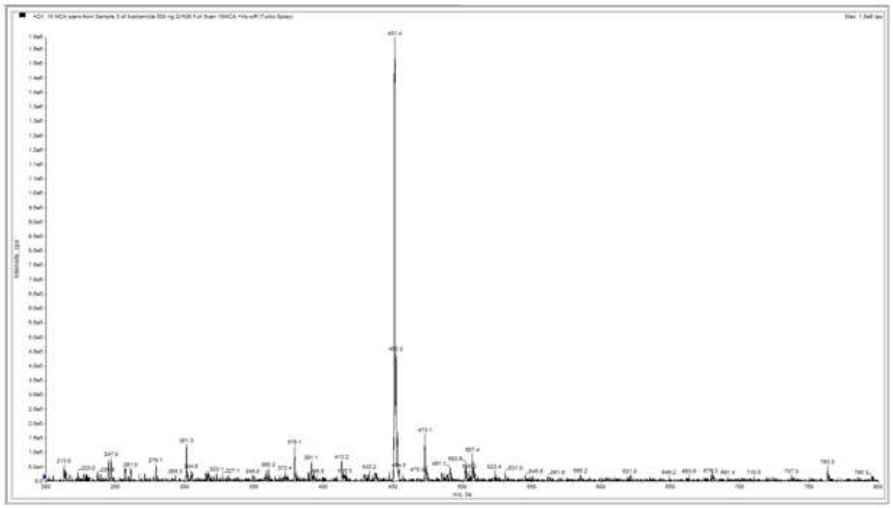
Method validation was carried to establish the acceptance of a prepared method according to the US FDA and EMA guidelines along with other published acceptance criteria [11, 12]. Standard validation parameters i.e., linearity, accuracy, precision, recovery, and sample stability, were studied and obtained results with chromatograms shown below. As the study and method mainly govern with plasma samples, it was highly recommended to check the matrix effect to find any endogenous interactions if any.

Selectivity and specificity

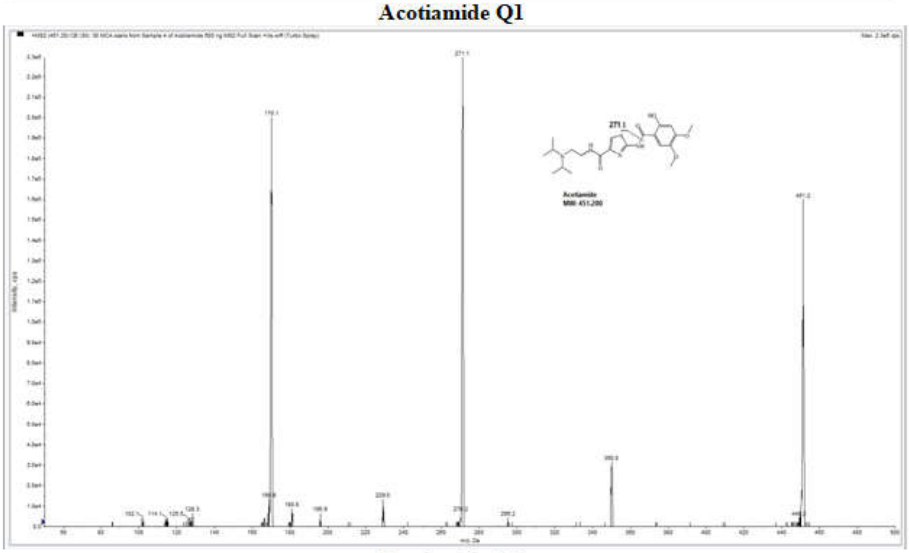
No interfering peaks were observed at the retention times of Acotiamide and are in human blank plasma. It showed that the developed analytical method was specific for the analysis.

Accuracy and precision

The intraday and inter-day accuracy and precisions were checked using high medium and low QC samples prepared in human plasma for Acotiamide and IS. The precision of both intra and inter-day was calculated from the relative standard deviation. Interday precision obtained by analyzing the same QC batch and by the same extraction procedure in previously tuned LC-MS/MS methods (n=3 d). Inter day precision values (%CV) ranged from 4.514 to 8.917 %. Inter day accuracy values (% nominal) were 103.25% for (LLOQ), 102.02% for low QC (LQC), 94.88% for medium QC (MQC), and 96.52% for high QC (HQC) samples. Intraday precision values (%CV) ranged from 5.658% to 8.389%. Intraday accuracy values (% nominal) were 91.26% for (LLOQ), 103.51% for low QC (LQC), 102.45% for medium QC (MQC) and 96.58% for high QC (HQC) samples. Inter day and Intraday Accuracy and Precision for Acotiamide summarized in table 2. These obtained results indicated that the developed method was accurate and reproducible.



Acotiamide Q1



Acotiamide Q3

Fig. 1: Ionization pattern of acotiamide from Q1 to Q3

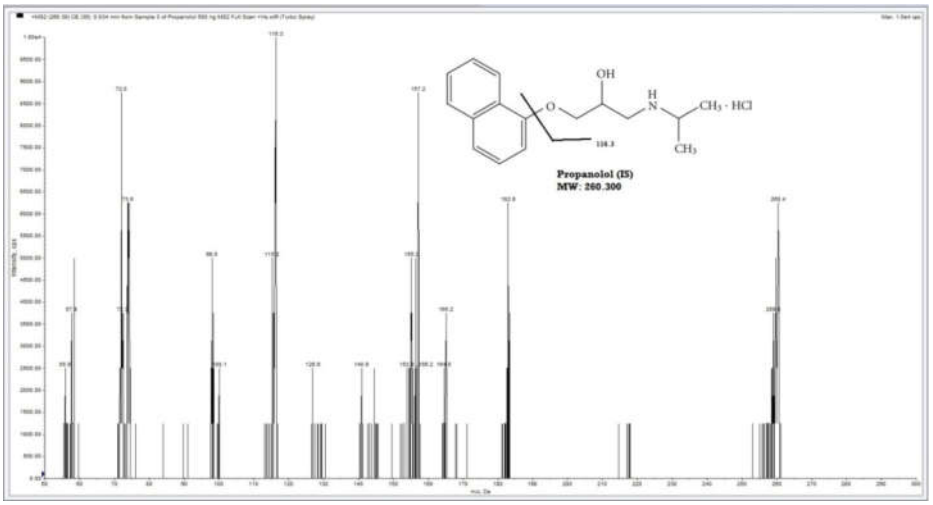


Fig. 2: Ionization pattern of IS (Propanolol) Q3

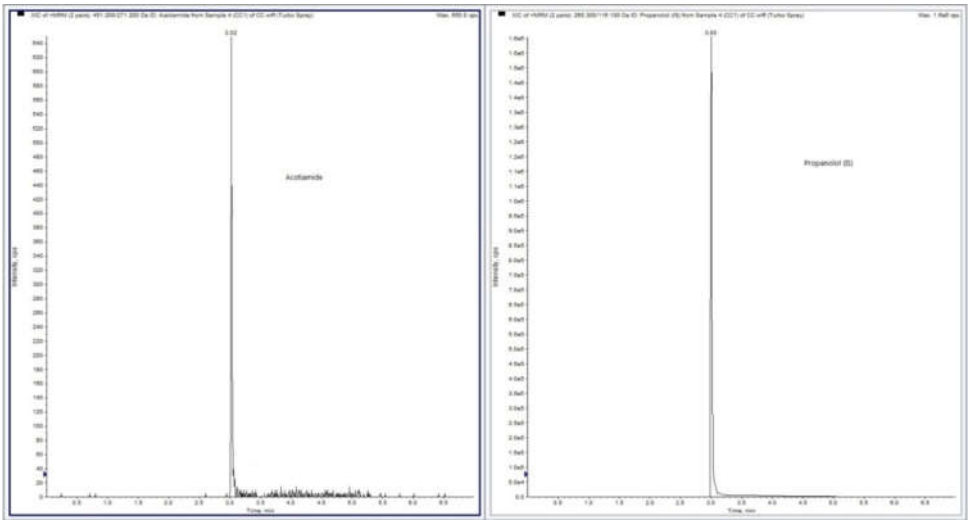


Fig. 3: Typical MRM chromatograms of Acotiamide and Propanolol (IS) in blank plasma spiked of Acotiamide at LLOQ (1.5625 ng/ml) with IS

Table 2: Inter day and intraday accuracy and precision for acotiamide (n=5)

	(Between run) inter-day precision and accuracy				(Within run) intraday precision and accuracy			
	Mean	**SD**	**C. V.%**	**Accuracy**	**Mean**	**SD**	**C. V.%**	**Accuracy**
LLOQ 1.5625 ng/ml	1.613	0.144	8.917	103.25	1.426	0.113	7.897	91.26
LQC 4.6875ng/ml	4.782	0.276	5.776	102.02	4.852	0.275	5.658	103.51
MQC 75 ng/ml	71.161	3.212	4.514	94.88	76.834	6.446	8.389	102.45
HQC 150 ng/ml	144.776	9.268	6.402	96.52	144.868	8.453	5.835	96.58

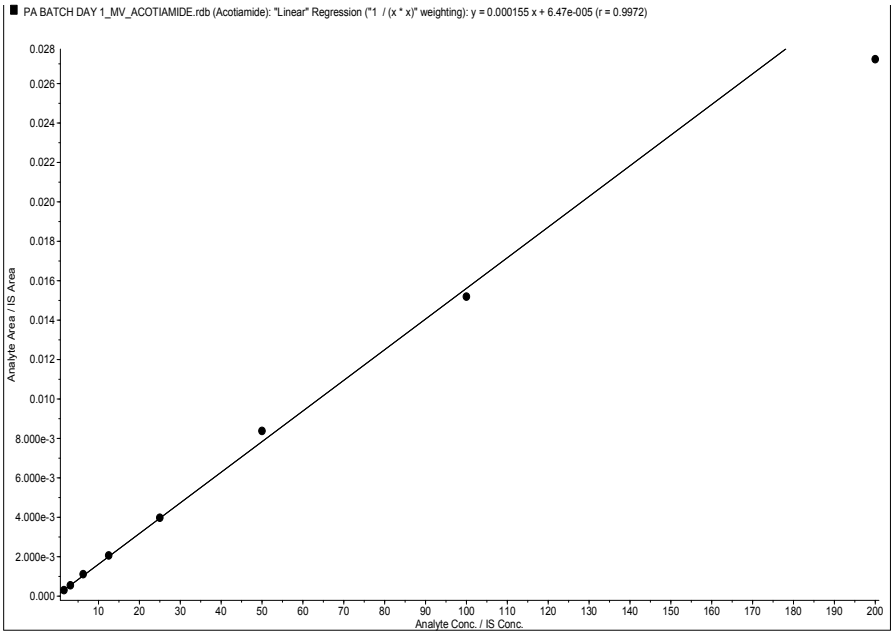


Fig. 4: The representative calibration curve of PA batch Day 1 for acotiamide

Linearity, the lower limit of detection (LOD) and lower limit of quantitation (LLOQ)

LOD was found 0.78125 ng/ml and LLOQ was 1.5625 ng/ml in this validated method and the intra and inter-day CV% was determined between 1.28 to 14.15, which proved that the method was sensitive enough and validated precisely. Hence, a representative chromatogram showing LLOQ is shown in fig. 3.

The eight-point standard curve was linear over the wide range of calibration 1.5625 to 200 ng/ml, which gives acceptable results during the quantification of unknown concentration of the drug in human plasma by plotting AUC of analyte AUC of IS on the curve. Calibration curve of PA batch day 1 for Acotiamide shown in fig. 4. The mean correlation coefficient for the linearity equation was determined to be 0.9953 using the regression equation y=0.0001x+642333.33 (y = mx + c) where y and x denote analyte

area IS area and analyte concentration/IS concentration, respectively.

Recovery and matrix effect

Recovery results have shown that the maximum recovery was carried out with both Acotiamide and IS above 90%. The extraction recovery was achieved to be satisfactory as it was consistent, precise, and reproducible. Hence single-step protein precipitation technique used in this method proved to be efficient and simple enough to extract drugs with IS from human plasma.

No significant matrix effect, i.e., ion suppression or enhancement was noticed by comparing the area under the curve (AUC) ratios of the extracted QCs and IS with the AUC of unextracted QCs and IS acquired from the injecting aqueous solution prepared at same concentrations. No interferences from endogenous compounds were found in the six different sources of human plasma. Therefore, it shows that the extracts were ''clean'' with no co-eluting ''unseen'' components which interfere with the ionization of both the analyte and IS. Recovery and matrix effect of Acotiamide summarized in tables 3 and 4, respectively.

Table 3: Recovery of acotiamide and IS from plasma

	Acotiamide		IS	
	Mean area	**(%) recovery**	**Mean area**	**(%) recovery**
LQC (4.6875 ng/ml)	2737.85	93.68	3382127.94	95.70
MQC (75 ng/ml)	39645.69	91.49	3362235.21	95.41
HQC (150 ng/ml)	76306.48	92.00	3444570.48	102.60

Table 4: Matrix effect of acotiamide from a different source of human plasma (n=5)

	Matrix effect (% ME)					Mean	SD	%CV
	1ST SET	**2ND SET**	**3RDSET**	**4TH SET**	**5TH SET**			
LQC (4.6875 ng/ml)	90.13	83.81	85.22	90.61	106.32	91.22	8.95	9.81
MQC (75 ng/ml)	90.62	84.49	91.71	78.30	88.13	86.65	5.43	6.27
HQC (150 ng/ml)	87.24	77.18	82.92	94.43	94.97	87.35	7.60	8.70

Stability studies

The different stability studies all carried out with freshly prepared QC concentrations of different concentrations. Freeze-thaw stability was checked with QC concentrations spiked in blank plasma which was kept in the deep freezer and thawed in room temperature, compared with freshly prepared QC samples. The stability of Acotiamide ranges between 96.34% to 106.43%. It was also found in the Short term (24 h) stability study, low, medium and high QC samples were compared against freshly prepared QC samples of the same concentration. The short term stability of Acotiamide ranges between 100.80%-108.55% after three cycles. Another stability parameter, Autosampler stability, was checked with QC samples stored in Autosampler vials for 24 h with freshly prepared samples. The obtained results of Autosampler stability ranged from 85.94% and 97.08%. Long term stability of 30 d was checked for low and high-quality control samples against samples. The obtained result ranges from 88.91% and 98.37%. All the stability study results of all QC samples summarized in table 5.

Table 5: Stability of different QC standards in the different storing condition of acotiamide (mean±SD, n=5)

Stability studies	QC samples	mean±SD	%CV
Short term Stability	LQC (4.6875ng/ml)	5.02±0.28	100.80
	MQC (75 ng/ml)	78.61±4.33	108.55
	HQC (150 ng/ml)	144.62±4.62	106.47
Auto Sampler Stability	LQC (4.6875ng/ml)	4.28±0.26	85.94
	MQC (75 ng/ml)	65.08±1.76	89.87
	HQC (150 ng/ml)	131.87±2.67	97.08
Bench Top Stability Study	LQC (4.6875ng/ml)	4.88±0.44	98.11
	MQC (75 ng/ml)	71.47±2.33	98.70
	HQC (150 ng/ml)	143.70±10.89	105.79
Long Term Stability	LQC (4.6875ng/ml)	4.43±0.23	88.91
	MQC (75 ng/ml)	69.32±4.38	95.72
	HQC (150 ng/ml)	133.62±5.27	98.37
Freeze-Thaw Stability	LQC (4.6875ng/ml)	4.80±0.13	96.34
	MQC (75 ng/ml)	76.27±6.28	105.33
	HQC (150 ng/ml)	144.57±9.43	106.43

Pharmacokinetics evaluation and application of the method

A comparative clinical study was conducted in 24 healthy human volunteers consisting of two different phases of the study, in each phase volunteers received alternatively test and reference drug formulations according to the prescribed manner [17-20]. The utility of the study was also emphasized on establishing the measure of the safety of Acotiamide in healthy volunteers. Generally, it was found in earlier literature that, Acotiamide undergoes a rapid absorption, therefore, the maximum plasma level reaches within the first hour, and it was found that within the study period, no adverse reactions occurred to any volunteer in both phases. So, it can be concluded that the finished product is safe in the Indian population.

In this present study, it was found that both the treatments were well tolerated and no SAE (serious adverse event) was reported from the clinical aspect. The pharmacokinetic comparison of both test and reference was shown in table 6. The representative chromatogram for the volunteer plasma sample is shown in fig. 5. The mean terminal half-life of orally administered Acotiamide reference and test medications were 23.49±5.09 and 24.54±4.86 h, respectively. In addition to that, the mean AUC_{0-t} (421.52±101.96 and 417.72±128.93ng. hr./ml for test and reference respectively) and C_{max} (25.71±2.31 and 23.61±2.32 ng./ml for test and reference respectively) were not significantly different, and the 90% confidence interval obtained in the statistical evaluation and shown in table 7 (using the SAS software and ANOVA) [21]. It was found that the obtained 90% confidence interval satisfactory following US-FDA and the EMA guideline. Mean drug plasma concentration-time profile curve of Acotiamide is shown in fig. 6.

Table 6: Pharmacokinetic parameters in 24 volunteers with the reference and test preparation (mean±SD)

Pharmacokinetic parameters	Reference preparation (A1)	Test preparation (A2)
C_{max} (ng/ml)	25.71±2.31	23.61±2.32
t_{max} (h)	2.54±0.12	2.43±0.21
AUC 0-t (ng h/ml)	421.52±101.96	417.72±128.93
AUC 0-∞ (ng h/ml)	511.75±163.30	512.78±198.55
k_{el} (h)	0.031±0.005	0.029±0.005
$t_{1/2}$ (h)	23.49±5.09	24.54±4.86
Relative bioavailability (%)	100 %	99.10%

Table 7: 90% Confidence interval calculation for untransformed and Ln transformed pk parameters

Acotiamide 100 mg tablet		
	Untransformed	**Ln transformed**
C_{max}	M =0.92, 90% CI [0.8797, 0.9603].	M =0.98, 90% CI [0.9733, 0.9867].
AUC_{o-t}	M =0.98, 90% CI [0.8625, 1.0975]	M =1, 90% CI [0.98, 1.02].
AUC_{0-inf}	M =0.99, 90% CI [0.8523, 1.1277]	M =1, 90% CI [0.98, 1.02].
Relative bioavailability	99.10%	

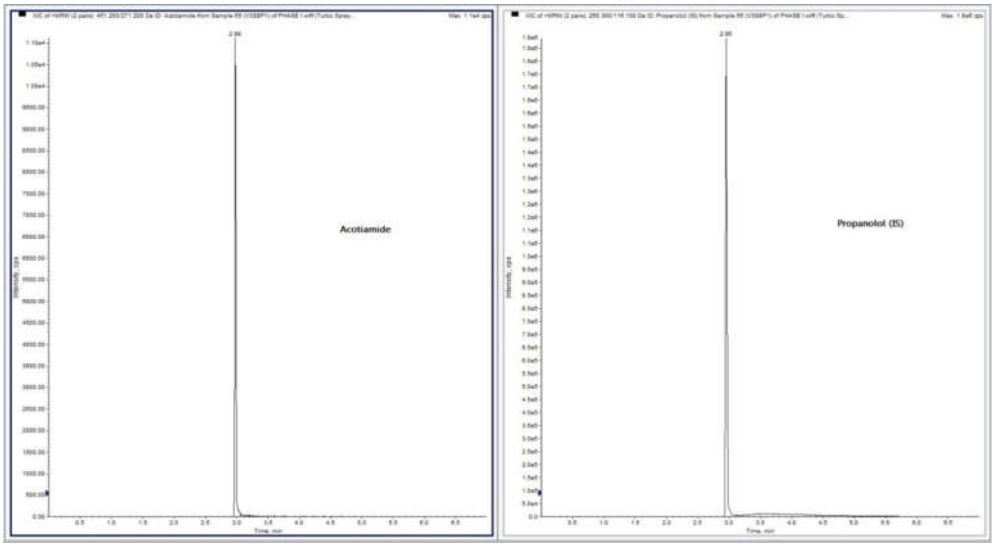


Fig. 5: Representative MRM chromatograms of volunteer plasma samples of (A) Acotiamide and (B) Propanolol (IS) in human volunteer plasma

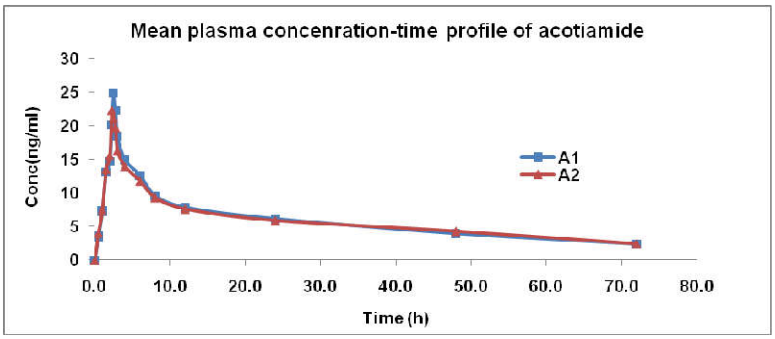


Fig. 6: Graphical representation of mean plasma concentration versus time of Acotiamide 100 mg single oral dose of test and reference preparation in 24 healthy human volunteers, N. B.: A1: Reference drug product; A2: Test drug product

DISCUSSION

The method developed for quantification of Acotiamide was done in human plasma and pharmacokinetic parameters were calculated after oral administration in healthy human volunteers. As we are aware by the prokinetic nature of Acotiamide, so the determination of the pharmacokinetic parameters was so essential to establish the tangible efficacy of Acotuamide. In this study, comparative bioavailability was studied of two different brands. It was founded that the half-life of Acotiamide is about 24.54±4.86 h, so from the

pharmacodynamic point of view, the therapeutic concentration persists for a longer period. For the development of the bioanalytical method to determine pharmacokinetic parameters using LC-MS/MS, a simple method was developed, which involved an easy, time-efficient, and cost-effective protein precipitation technique for the extraction of drugs in plasma samples.

After analyzing the clinical data of Acotiamide, it was found that the drug is safe upon administration in healthy human subjects. In each study period, no adverse event was found; therefore, the drug is well tolerated in individuals. The comparative pharmacokinetic outcome showed that the test and reference product having an almost similar pattern of pharmacokinetics. Although it was found that the test is bioequivalent comparing to the reference product. The drug here, Acotiamide was first introduced by Japan as a prokinetic agent, which enhances acetylcholine release from enteric neurons by antagonizing muscarinic receptors (M1)and acetylcholinesterase inhibition, which results enhancing gastric emptying [14]. In the formulation, Acotiamide hydrochloride hydrate was used, which is highly water-soluble and offers a good absorption rate in the systemic circulation.

On the other hand, so far to our knowledge, none of the reported bioanalytical procedures describe a method for the determination of Acotiamide in human plasma by LC-MS/MS. Previously, the only pharmacokinetic study was carried out using LC-MS/MS done in Rat plasma, so it was essential to evaluate the pharmacokinetic study in human plasma to establish a firm clinical significance in the human population [22]. In the present study, attempts were made to develop and validate a sensitive, selective, precise, accurate, rapid, and economical method for the determination of Acotiamide in human plasma by LC-MS/MS.

CONCLUSION

Based on a comparison of the AUC0-t for Acotiamide after single-dose administration, the relative bioavailability of the test was found to be 99.10% compared to reference preparation. No occurrence of adverse events was reported by the subjects throughout the study duration. Therefore it may be concluded that the test preparation was found to be bioequivalent with the reference preparation. Finally, a simple, sensitive, selective, and rapid LC-MS/MS bioanalytical method has developed for the determination of Acotiamide in human plasma for the very first time. The developed method is at par with the standard industry guidelines for validation. The developed bioanalytical method is demonstrated highly specific due to the inherent selectivity of tandem mass spectrometry. The validation results have been proved that the developed LC-MS/MS method is satisfied with all the parameters within range, i.e., specificity, sensitivity, linearity, precision, accuracy, and stability. A simple and convenient extraction procedure, i.e. protein precipitation technique (PPT) with used only 150 µL of human plasma makes this method more advantageous for the bioanalysis of Acotiamide. The resulted pharmacokinetic parameters would be helpful to provide some guidance to clinical application and investigation.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors hereby declare that they have no conflict of interest either to disclose.

REFERENCES

1. Ramadevi K, Kalyan VR, Abeer RM. A study on the effect of dietary factors on functional gastrointestinal disorders in women of HA'IL region in Saudi Arabia. Asian J Pharm Clin Res 2018;11:202-7.
2. Hironobu M, Norihisa I, Mayumi O, Daisuke I, Eiko O, Shunji I, *et al.* Acotiamide has no effects on esophageal motor activity or esophagogastric junction compliance. J Neurogastroenterol Motil 2018;24:241-7.
3. Tack J, Janssen P. Acotiamide (Z-338, YM443), a new drug for the treatment of functional dyspepsia. Expert Opin Investig Drugs 2011;20:701–12.
4. Kusunoki H, Haruma K, Manabe N. Therapeutic efficacy of acotiamide in patients with functional dyspepsia based on enhanced postprandial gastric accommodation and emptying: randomized controlled study evaluation by real-time ultrasonography. J Neurogastroenterol Motil 2012;24:540–5.
5. Nagahama K, Matsunaga Y, Kawachi M, Ito K, Tanaka T, Hori Y, *et al.* Acotiamide, a new orally active acetylcholinesterase inhibitor, stimulates gastrointestinal motor activity in conscious dogs. J Neurogastroenterol Motil 2012;24:566–74.
6. Attia KA, Nassar MW, El-Olemy A, Ramzy S. Spectrofluorimetric determination of acotiamide hydrochloride trihydrate in the presence of its oxidative degradation product. Luminescence 2018;1–6. https://doi.org/10.1002/bio.3480.
7. Lacy BE, Talley NJ, Locke GR. Current treatment options and management of functional dyspepsia. Aliment Pharmacol Ther 2012;36:3–15.
8. Karamanolis GP, Tack J. Current management of functional dyspepsia: impact of Rome III subdivision. Ann Gastroenterol 2012;25:96–9.
9. Tijare LK, Rangari NT, Mahajan UN. A review on bioanalytical method development and validation. Asian J Pharm Clin Res 2016;9:6-10.
10. Dhiman H, Shubhasis D, Murari MP, Easha B, Nilendra C, Pradipta S, *et al.* LC–MS/MS assay for quantitation of enalapril and enalaprilat in plasma for bioequivalence study in Indian subjects. Future Science OA 2017;3:1-19.
11. United States Food and Drug Administration. Guidance for Industry: Bioavailability and Bioequivalence Studies for orally Administered Drug Products; 2000.
12. The European Agency for the Evaluation of MedicinalProducts EMA Committee for Proprietary Medicinal Products (CPMP) Note for guidance on the investigation of Bioavailability and Bioequivalence, London; 2001.
13. Dhiman H, Shubhasis D, Pradipta S, Dibya D, Umesh Chandra H, Tapan KP. LC-MS/MS determination of 4-hydroxy nimesulide, an active metabolite of nimesulide and application to bioequivalence study in Indian subjects. Eur J Mass Spectrom 2019;25:399-411.
14. Altan E, Masaoka T, Farre R, Tack J. Acotiamide, a novel gastroprokinetic for the treatment of patients with functional dyspepsia: postprandial distress syndrome. Expert Rev Gastroenterol Hepatol 2012;6:533–44
15. Dhiman H, Shubhasis D, Easha B, Pradipta S, Umesh CH, Tapan KP. A rapid LC-ESI-MS/MS method for the quantitation of salicylic acid, an active metabolite of acetylsalicylic acid: application to *in vivo* pharmacokinetic and bioequivalence study in Indian healthy male volunteers. Appl Clin Res Clin Trials Regul Aff 2015;2:90-102.
16. Sourav D, Dhiman H, Shubhasis D, Easha B, Chiranjit S, Tapan KP. Method development, validation and plasma analysis of etoricoxib using LC-MS/MS in Indian healthy human volunteers. Indian Drugs 2019;56:34-41.
17. Shubhasis D, Dhiman H, Anwesha B, Easha B, Pragnya C, Pradipta S, *et al.* Comparative bioavailability study of two antiretroviral FDC containing abacavir 600 mg and lamivudine 300 mg in healthy human Indian volunteers. J Appl Biopharm Pharmacokinet 2015;3:18-26.
18. Shubhasis D, Dhiman H, Anwesha B, Easha B, Murari MP, Balaram G, *et al.* Bio-analytical method development and validation of tadalafil with a special emphasis on pharmacokinetic study in healthy indian subjects for the ods formulation. Curr Anal Chem 2015;11:175-83.
19. Bapi G, Hira C, Dhiman H, Amlan KS, Pradipta S, Easha B, *et al.* A comparative pharmacokinetic study of a fixed dose combination for essential hypertensive patients: a randomized

crossover study in healthy human volunteers. Drug Res 2013;63:177-84.

20. Amlan KS, Debotri G, Dhiman H, Pradipta S, Sujata GD, Tapan KP, *et al.* A rapid LC-ESI-MS/MS method for the quantitation of choline, an active metabolite of citicholine: application to *in vivo* pharmacokinetic and bioequivalence study in Indian healthy male volunteers. J Pharma Biomed Anal 2012;71:144-7.
21. Amlan KS, Debotri G, Dhiman H, Pradipta S, Hira C, Sujata GD, *et al.* Biopharmaceutical evaluation of active metabolite of fenofibrate and atorvastatin in human plasma by LC-ESI-MS/MS method. J Pharm Res 2012;5:2347-51.
22. Jing Li, Rui Huang, Zhi Wang, Haijun Qu, Meijuan Sun, Zhenhuan Zhao, *et al.* Development and validation of a sensitive and specific lc–ms-ms method for the determination of acotiamide in rat plasma. J Chromatogr Sci 2016;54:1004–9.