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LC-MS/MS method development and validation of an antihistaminic, calcium channel blocker, di-phenyl-methyl-piperazine group containing cinnarizine in human plasma with an application to BA/BE studies in Indian volunteer

Abstract

Antihistamine is drug which antagonises the action of histamine at the H1 receptors. Qualitatively all H1 antihistaminic have similar actions, but there are quantitative differences, especially in the sedative property. These drugs effectively block the histamine induced bronchoconstriction, contraction of intestinal and smooth muscle and triple response. It also suppressed the immediate or type-I hypersensitivity reaction but certain drugs are effective in preventing motion sickness. Cinnarizine is one of the drugs of antihistaminic medication. It is used to treat problems associated with the inner ear and the brain. The medicine is used to treat dizziness and sickness associated with motion sickness. This drug used in the treatment of vertigo and vestibular disorder and heart and blood vessel disorder. Cinnarizine inhibits the contractions of vascular smooth muscle cells by blocking L-type and T-type voltage gated calcium channel and it also binding to dopamine D2 receptor, histamine H1 receptor and muscarinic acetylcholine receptor. Cinnarizine contain diphenylmethylpiperazine group which protonated precursor ion was 369.3 and product ion 167.2 by using 0.1% formic acid in acetonitrile with 0.1% formic acid containing milli Q water added with 10.mM ammonium acetate as a binary flow. Internal standard precursor ion was 268.2 and product ion was 116.1 as a positive mode. The run time was very short 3.5 min and in between this run time 90% aqueous phase flow upto 0.9 min and 10% upto 2.50 min then again 90% flow upto 3.5 min. This method showed very low matrix effect which calculated matrix factor was 0.87 to 0.94 and recovery was very high 86.88% to 99.57%. So this method was very specific, selective, sensitive and reproducible which used for quantification of cinnarizine concentration of unknown Indian healthy human volunteers from their plasma.

Keywords: antihistaminic drug, Cinnarizine, motion sickness, Miniere's disease, Pharmacokinetics

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Introduction

Bioanalytical method development and validation for the quantitation of drugs and metabolites in human plasma has immense importance towards establishing the efficacy of the generic drugs now days. The population specific pharmacokinetic data of a particular drug is essential for the regulatory approvals. Cinnarizine(CAS Number: 298-57-7), an antihistamine and a calcium channel blocker used in the treatment of cerebral apoplexy, post-trauma cerebral symptoms and more commonly for nausea and vomiting due to motion sickness or other sources.[1,2,3] Its ATC classification (Anatomical Therapeutic Chemical) according to WHO is N07CA02.[4] Cinnarizine contain diphenyl methyl piperazine group as a result it promote cerebral blood flow and also used in cerebral arteriosclerosis and Miniere's disease.[5] Vestibular hair cells of the vestibular apparatus of the inner ear send signals to the vomiting centre of the hypothalamus and cinnarizine interfering the signal transmission. Scopolamine has similar type of action but it is more effective than cinnarizine.[6] Due to calcium channel blocking activity cinnarizine acts as a nootropic drug because of its vasorelaxating activity and used as a labyrinthine sedative.[7] A derivative of cinnarizine is the flunarizine which is 2.5 times stronger for treatment of cerebral ischemia.[8] It is used in second line treatment of urticarial vasculitis.[9]

Literature survey reveals that the determination of cinnarizine has been carried out in human plasma by various analytical methods using high-performance liquid chromatography(HPLC) with UV and fluorometric detection and gas chromatography with nitrogen-selective thermionic specific detection etc.[10,11] There are many analytical methods available for determination of the cinnarizine in pharmaceutical dosage forms.[12–15] But no LC-MS/MS method is available for the determination of the same in human plasma with improved sample preparation technique *i.e.* protein precipitation, till date. Therefore, attempts were made in the present study to develop and validate a bioanalytical method for the determination and quantitation of the cinnarizine in human plasma by liquid chromatography-mass spectrometric method. The developed method was also applied to analyse the plasma samples of healthy human volunteers obtained from a comparative pharmacokinetic study of cinnarizine 25mg tablet dosage forms.

Material and methods

Chemical and reagents

HPLC grade acetonitrile (ACN) and ammonium acetate were purchased from Merck (MERCK India Ltd., Mumbai). Other

chemicals and reagents of analytical grade were used throughout the study. Water used in the entire analysis was obtained from Milli-Q water purification system procured from Millipore (Elix, Milli-Q A10 Academic, Bedford, MA, USA) until a resistively of 18.2MΩ was achieved. The blank human plasma with EDTA-K3 anticoagulant was collected from Clinical Pharmacological Unit (CPU) of TAAB Biostudy Services, Kolkata and was stored at −20°C until analysis.

LC-MS/MS instrumentation

Chromatographic analysis with gradation technique was performed on a Shimadzu HPLC system equipped with LC-20AD binary pump, SIL-20A auto-sampler, CTO-10ASvp oven and CBM-20A lite system control compartment. Mass spectrometric detection was performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems/ MDS SCIEX, Toronto, ON, Canada) equipped with an turbo electrospray ionization (ESI) interface. The chromatographic elution of the analytes was performed on a Phenomenex Kinetex 5µ C18 100A 50*3mm column.

Bioanalytical method development by gradation technique

Cinnarizine (CAS No.- 298-57-7) which chemical formula $C_{26}H_{28}N_2$ is chemically (E)-1-(Diphenylmethyl)-4-(3-phenylprop-2-enyl)piperazine which contain two phenyl and one piperazine ring. It is an antihistamine and a calcium channel blocker and selective antagonist of T-type voltage operated calcium ion channel. The exact mass of cinnarizine is 368.2252 (molecular wt. 368.5224), H-bond donar count 0 and H-bond acceptor count 2 and rotable bond count 4. The P^{Ka} value of cinnarizine 7.0 (slightly basic) but neutral in character due to three aromatic ring in which two phenolic ring and one piperazine ring. Due to significant difference with P^{Ka} value and molecular character it was imperative to set optimum condition for plasma extraction, chromatography and mass detection for their simultaneous determination.

Metoprolol used as internal standard (IS). For quantitation used positive polarity to achieve adequate response for their simultaneous analysis. Moreover positive ionization mode is selective and highly sensitive for compounds with low electron affinity. Thus positive ionization mode was selected to fragment the analyte and IS to obtain intense and consistent product ions. The protonated precursor ions $[M+H]^+$ at m/z 369.3 (highest peak), 240.9 (2nd peak), 225.2 (3rd peak), were observed in Q1 MS in which selected parent ion369.3 for cinnarizine and characteristic product ions or fragment ions found in Q3 MS were at m/z 167.2,201.3,152.1. However the most stable and consistent fragment ion selected was m/z 167.2 for piperazine ring (Figure 1).

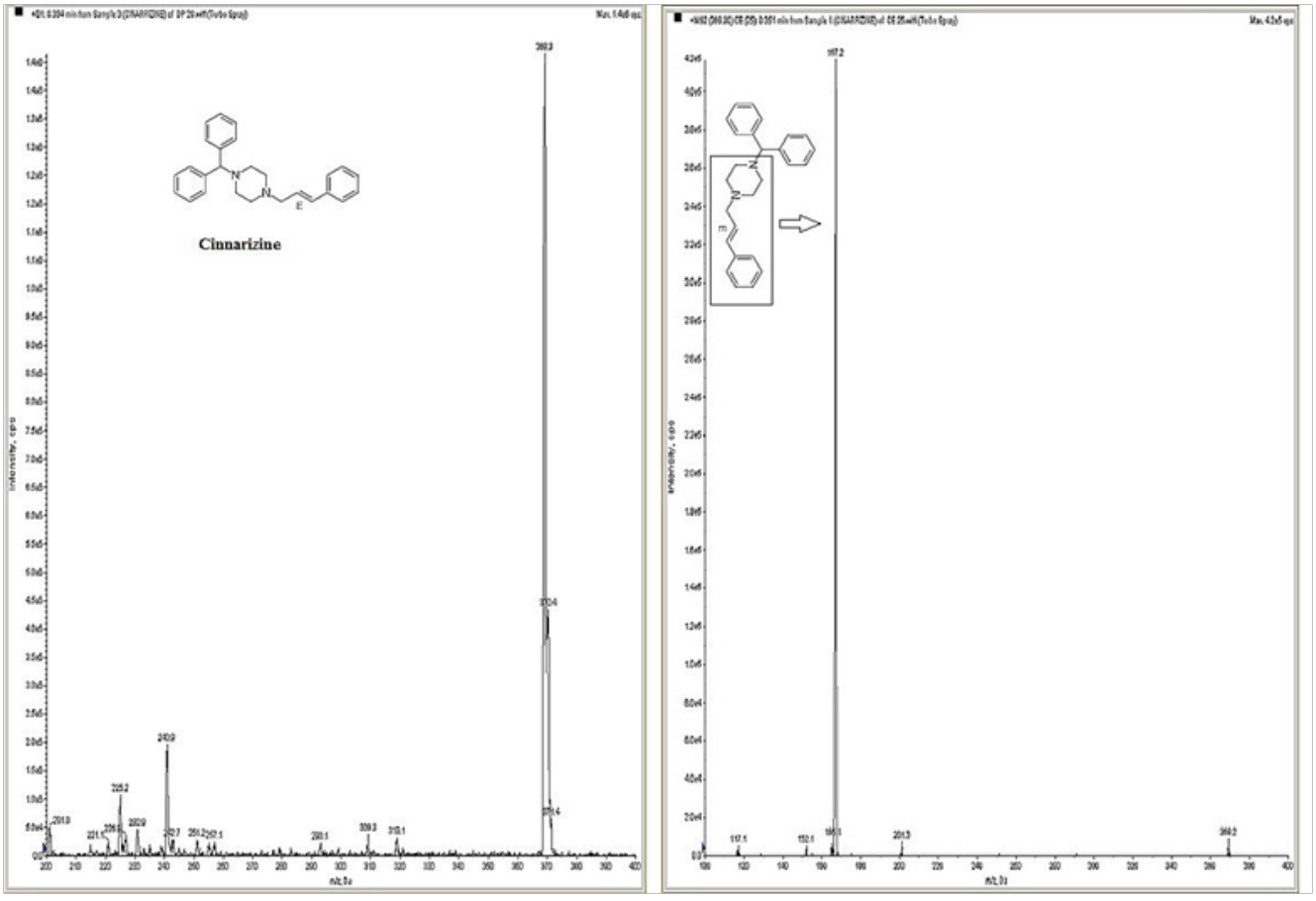

Figure 1 Q-1 and Q-3 Scans of analyte (cinnarizine).

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For the internal standard The protonated precursor ions $[M+H]^+$ at m/z 268.2 (highest peak) 269.3 (2nd peak), 241.2 (3rd peak), 22.9 (4th peak) were observed in Q1 MS for metoprolol and characteristic product ions or fragment ions found in Q3 MS were m/z 116.1, 159.3, 133.1, 74.1, 98.0, 121.2. However the most stable and consistent fragment ion selected was m/z 116.1 for five consecutive rings (Figure 2). The chromatographic elution of the analytes on a Phenomenex Kinetex 5µ C18 100A 50*3mm column was initiated as a rapid, sensitive and rugged analytical method covering the dynamic linear range. The selection of mobile phase was crucial for synchronized determination of the drug having pKa values. Thus, the pH of the mobile phase, buffer concentration, and choice and proportion of diluents were varied, which was important for chromatographic resolution with adequate response to achieve the desired sensitivity. The optimal mass parameters for both the analytes and IS were elaborated in the Table 1.

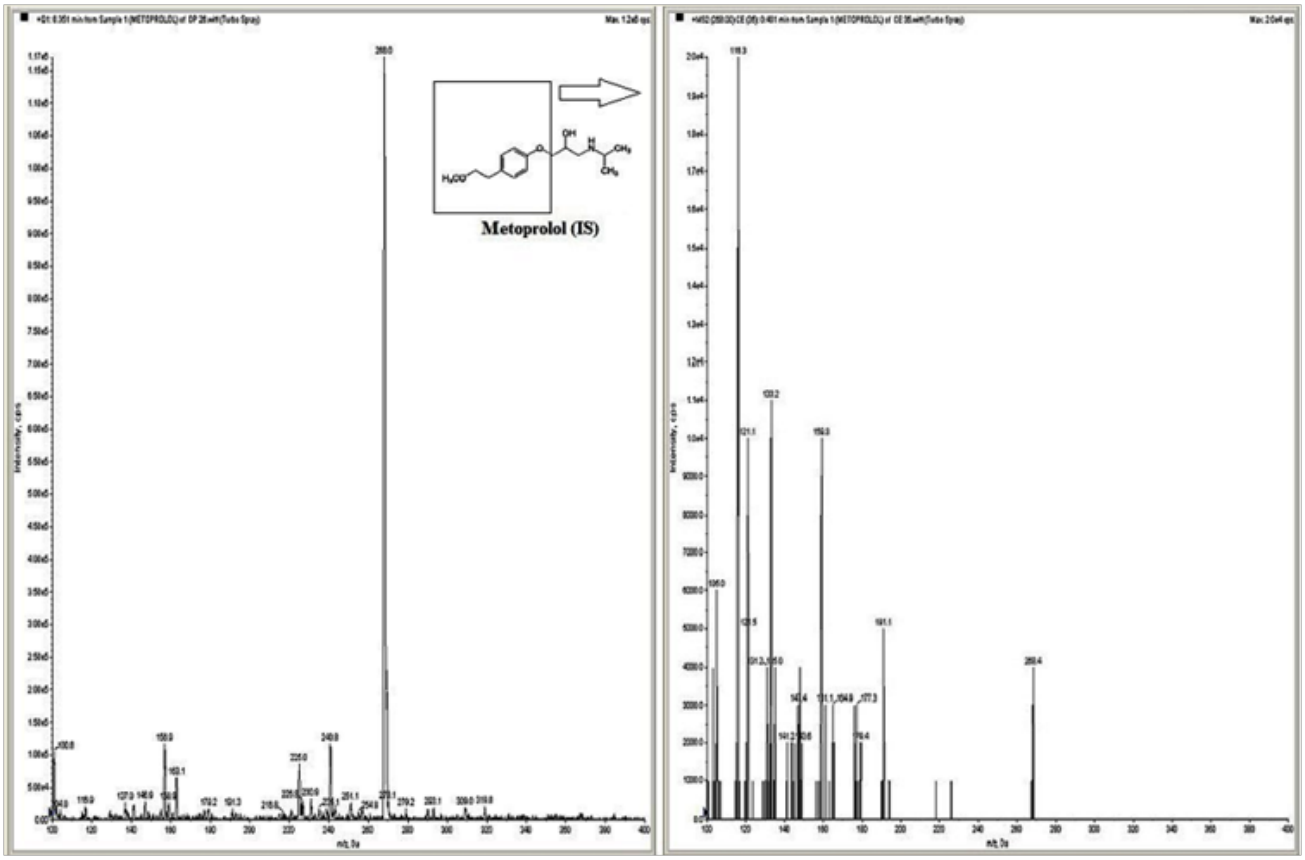

Figure 2 Q-1 and Q-3 Scans of IS (metoprolol).

Table 1 Optimized instrumental (mass) parameters for analyte (cinnarizine) and IS (metoprolol)

Compound dependent parameters				
	Collision energy (CE)	**Declustering potential (DP)**	**Collision cell exit Potential (CXP)**	**Entrance potential (EP)**
Cinnarizine	30	30	4	11
Source dependent parameters				
		Nebulizer gas (gas 1)	55	
		Heater gas (gas 2)	45	
		Ion spray voltage (ISV)	5000	
		Collision activated dissociation (CAD) gas	6	
		Curtain (CUR) gas	10	
		Source temperature (TEM)	400	
		Focusing potential (FP)	400	

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Initially, acetonitrile/methanol with 1 mM ammonium acetate buffer (pH 6.5) gave response for cinnarizine. However, the response was not reproducible. The signal was severely compromised at lower limit of quantitation (LLOQ) levels even after altering the concentration of buffer from 1mM to 10mM. Further, the chromatographic elution was found better with a higher response using an acetonitrile-buffer as compared to a methanol-buffer combination. Moreover, lowering the acetonitrile content in the mobile phase resulted in an increase in the retention of cinnarizine and thereby the analysis time. Subsequent efforts were directed to optimize the pH of the mobile phase and the concentration of the buffer solution as they had significant impact on analyte retention, peak shape, and resolution. At pH above 5.0 the resolution of cinnarizine was affected, which further deteriorated with increase in pH. Thus, to achieve greater reproducibility and better chromatography, low pH buffers were tried.

Better reproducibility and peak shape were observed in case of 0.1% formic acid in acetonitrile, but the signal to noise ratio was not adequate at LLOQ level. A superior signal to noise ratio (≥22) and baseline resolution was obtained for the analytes by 10Mm ammonium acetate buffer with 0.1% (v/v) formic acid together with Milli Q water having apparent pH 3.50 at a flow rate of 0.5000mL/min. In the present study, the chromatographic part was performed by gradation method in which 10% and 90% organic solvent was used for 0.01min to 0.90min and 0.90min to 2.50min of total run time whereas 90% aqueous solvent was used from 2.50min to rest of the total run time (3.50min) for washing purpose The chromatographic elution time for cinnarizine and IS (metoprolol) was found 2.32, and 2.05min respectively, in a total run time of 3.50min.

Plasma extraction and sample preparation: Plasma extraction was performed by Protein precipitation technique, 100µl of plasma was taken and precipitated with 400µl of MeCN containing 5000ng/ml metoprolol (IS) and vortexed for 10min, followed by Centrifugation for 10mins at 12,000rpm at 4°C. 300µl supernatant was taken and transferred to Autosampler vials for injection. Stock solutions of cinnarizine and IS (metoprolol) were prepared by dissolving accurately weighed samples to obtain the concentrations of 1mg/mL. The stock solutions were then gradually diluted with methanol: water: 50: 50 (v/v) to obtain calibration samples of 0.94, 1.87, 3.75, 7.50, 15.00, 30.00, 60.00 and 120.00ng/ml. the concentration of the IS was 5µg/ml throughout the study.

Method validation

The method validation was conducted in accordance with the US-FDA guidelines for selectivity, sensitivity, linearity, precision, accuracy, recovery and stability.[16–18]

Specificity, selectivity and linearity: The specificity and selectivity of the assay was illustrated by the chromatograms of mobile phase run and extract of blank plasma recorded for samples near the C_{max} for 2.00 to 3.00hr for cinnarizine. The linearity of the calibration curve was determined by an unweighted least square regression analysis. Representative calibration curves of cinnarizine from human plasma were depicted in the linearity graph.

Precision and accuracy: Between–run precision and accuracy were determined from the low, medium and high QC samples (LQC, MQC and HQC). A total no of 5 replicates of each QC concentration were assayed on day 1 and a total of 5 replicates each QC concentration were assayed on day 2 and 3. The QC samples concentrations were determined from three different calibration curves that were assayed with QC samples. Within-run precision and accuracy were determined from a total of 5 replicates of each QC concentration. The low, medium and high QC samples (LQC, MQC and HQC) were assayed on day 2. The QC samples concentrations were determined from calibration curves LIN3. Precision was expressed as percent variation (%CV), while accuracy was measured as the percent nominal.

Stability: As per the regulatory guidelines (US-FDA), the freeze thaw, short term (ST), long term (LT) and auto sampler (AS) stability had been performed. Freeze thaw stability percentage should be within 80–120%. As per guidelines the both the ST and LT stability percentage should be within 90–110% and the AS stability percentage should be within 85–115%.

Matrix effect and recovery: In the present study the matrix effect for the internal standard (metoprolol) and analyte (cinnarizine) were also carried out. The matrix effect percentage should be within 85–115% as per the guidelines. The percentage recovery was determined by measuring the peak areas of the analyte and IS from the prepared plasma low, medium and high QC samples. The peak areas of the plasma low, medium and high QC samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the analyte and IS.

Application to a BA/BE studies

The developed and validated LC-MS/MS method was applied for the analysis of the plasma samples obtained from the comparative pharmacokinetic study. Two formulations containing cinnarizine 25mg [test product manufactured by XL Laboratories Pvt. Ltd., India and reference product manufactured by Johnson and Johnson (Philippines) Inc.] was compared in 24 healthy human Indian volunteers in the present study. The study protocol and related other documents were approved by the HURIP Independent Bio-ethics committee, Kolkata, India [Central Drugs Standard Control Organization (CDSCO), New Delhi, India registration: ECR/103/Indt/WB/2013] before initiation of the study.[19,20] The present study was conducted in a randomized and cross-over manner on 24 healthy human volunteer. Written informed consent was obtained from the volunteer before single dosing of the cinnarizine 25mg tablet with 240ml of drinking water on an empty stomach with at least 8-10 hrs fasting condition. It was ensured that the volunteers were exposed in both the products (test or reference) during two clinical phases.[21–23] 15 days wash-out period was maintained between two clinical phases. Total no of 14 blood samples were collected in 5mL K_2EDTA vacationers via an indwelling catheter placed in one of the forearm vein for both the study period. The pre-dose blood sample was collected within a period of 1hr prior to the drug administration. The post-dose blood samples were 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36 and 48hrs. All the blood samples for a specific sampling time point were centrifuged under refrigeration at 3500 rpm and 4°C for 10min. The resulting plasma was separated and stored in suitably labelled polypropylene tubes at –20°C for analysis.[15]

Results and discussion

Method validation

Specificity, selectivity and linearity: Following plasma calibration standards (ng/ml) were prepared -0.94, 1.87, 3.75, 7.50, 15.00, 30.00, 60.00 and 120.00ng/ml for cinnarizine. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were found 0.21ng/

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ml and 0.94ng/ml respectively for cinnarizine. The proposed assay was found linear.

Precision and accuracy: Between–run precision values (% CV) ranged from 7.138% to 11.120% for cinnarizine. Between–run accuracy values (% nominal) were 102.199% for (LLOQ), 96.733% for low QC (LQC), 100.594% for medium QC (MQC) and 103.339% for high QC (HQC) samples. Within-run precision values (% CV) ranged from 5.906% to 11.309% for Cinnarizine. Within-run accuracy values (% nominal) were 98.936% for (LLOQ), 93.737% for low QC (LQC), 94.049% for medium QC (MQC) and 98.871% high QC (HQC) samples. The between run and within run precision results were represented in Table 2. It is evident form the obtained precision and accuracy data of cinnarizine that the values are within the acceptable limit.

Stability: The stability study data were elaborated in Table 3.

Table 2 Precision and accuracy data

Between-run precision and accuracy				
	Concentrations (ng/ml)			
	LLOQ (0.94)	**LQC (2.81)**	**MQC (45)**	**HQC (90)**
Mean±SD	0.961±0.091	2.719±0.302	45.267±4.196	93.005±6.639
C.V.%	9.43	11.12	9.27	7.138
Absolute percent bias (%)	**102.199**	**96.773**	**100.594**	**103.339**
Within-run precision and accuracy				
Mean±SD	0.930±0.064	2.634±0.298	42.322±3.771	88.984±5.255
CV	6.927	11.309	8.911	5.906
Absolute percent bias (%)	**98.936**	**93.737**	**94.049**	**98.871**

Table 3 Stability study data

Concentration (ng/ml)	**LQC (2.81)**	**MQC (45)**	**HQC (90)**
Fresh Samples	2.87	47.53	96.89
After Three Freeze Thaw Cycle	2.45	44.89	92.57
Freeze Thaw Stability (%)	**85.38**	**94.45**	**95.54**
After 24 Hours	3.09	50.76	95.07
Bench Top Stability (%)	**107.52**	**106.8**	**98.13**
After 24 hr in Auto-sampler	2.89	47.44	92.81
Auto-sampler Stability (%)	**100.77**	**99.82**	**95.8**
after 24 hours	2.81	44.4	89.41
Short Term Stability (%)	**97.7**	**93.42**	**92.28**
after 15 days in freezer	2.82	43.92	87.82
Long Term Stability (%)	**98.33**	**92.41**	**90.65**

Bench top: QC samples were kept for 24hrs at room temperature and then processed and analyzed. Percentage stability was found within 98.13% to 106.80% for Cinnarizine.

Auto-sampler stability: The auto sampler stability of cinnarizine ranged between 95.80% to 100.77%.

Freeze thaw stability: The stability of low, medium and high quality control samples were determined after three cycles comparing against freshly thawed samples of the same concentration. The stability found for cinnarizine ranged between 85.38% to 95.54%.

Short term stability: The percentage stability was found within 92.28% to 97.70% for cinnarizine.

Long term stability: The percentage stability range was found within 90.65% to 98.33% for cinnarizine.

Obtained stability data (bench top, auto sampler, freeze thaw, short term and long term) found within the specification of the regulatory guidelines and hence acceptable.

Matrix effect and recovery: The matrix effect of internal standard ranged between 89.38%-94.63%, for Cinnarizine it ranged between 87.01%-94.17%. The percentage recoveries were determined by measuring the peak areas of the drug from the prepared low, medium and high quality control plasma samples. The peak areas of the low, medium and high quality control plasma samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the cinnarizine. Recovery after extraction was found 86.88%--99.57% for cinnarizine. Matrix effect and recovery data for Cinnarizine was elaborated in the Table 4 and Table 5. The values were within the limit and hence accepted. The validation parameters found within the specified regulatory limit, hence acceptable.

Table 4 Matrix effect of analyte and IS

		Matrix effect (Analyte)			
	Sample	**Extracted blank plasma (Area)**	**Aqueous (Area)**	**% of ME**	**Matrix factor**
Mean±SD	LQC	6330.36±880.26	7270.23±957.17	87.01±1.53	0.87±0.01
C.V.%		13.91	13.17	1.75	1.69
Mean±SD	MQC	133963.93±23291.58	149096.59±29811.73	90.31±3.32	0.90±0.03
C.V.%		17.39	19.99	3.68	3.72
Mean±SD	HQC	193900.86±27772.26	205820.37±27520.26	94.17±3.79	0.94±0.04
C.V.%		14.32	13.37	4.02	3.93
		Matrix effect (IS)			
Mean±SD	LQC	387685.40±23649.46	434171.16±26878.23	89.38±4.50	0.89±0.05
C.V.%		6.1	6.19	5.03	5.1
Mean±SD	MQC	546893.58±53016.83	578286.33±53910.29	94.63±4.57	0.95±0.05
C.V.%		9.69	9.32	4.83	5.1
Mean±SD	HQC	400440.39±56338.36	443910.04±70872.45	90.44±3.78	0.90±0.04
C.V.%		14.07	15.97	4.18	4.03

Table 5 Recovery data

	Area		
	LQC	**MQC**	**HQC**
Diluent Sample	452104.7	505610	399916.9
Plasma sample	369293.5	495730.4	363700.9
% Recovery of IS	**81.68**	**98.05**	**90.94**
Diluent Sample	6788.6	137932.4	184481
Plasma sample	6207.95	119836.6	183680.8
% Recovery of Analyte	**91.45**	**86.88**	**99.57**

Comparative pharmacokinetic study in human volunteers

The developed and validated LC-ESI-MS/MS assay method was applied to compare the oral bioavailability of two formulations (test and reference) by conducting the single oral dose, open label, randomized, two period, two sequence, crossover study of 24 healthy Indian volunteers (male) with an average age of 28.08±4.92 years and average BMI of 21.88±1.54Kg/m^2 under fasting condition. The pharmacokinetic parameters like C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$, K_{el}, $T_{1/2}$ are determined for cinnarizine to calculate the relative bioavailability of test preparation Nominal 25mg tablet over the reference preparation of same dosage after oral administration to healthy human volunteers. Single dose, crossover, randomized clinical study was performed healthy human volunteers under fasting condition. 24 volunteers with an average age of 26.79±5.27years and average BMI of 21.24±1.74Kg/m^2 were enrolled in the study. The mean comparative pharmacokinetic parameters for both test and reference product, was elaborated in Table 6. On the basis of comparison of the AUC_{0-t} after single dose administration, relative bioavailability of the test preparation of tablet containing cinnarizine 25mg was found 101.83% of that of the reference preparation, Stugeron tablet containing cinnarizine 25mg. Representative chromatogram for the volunteer plasma analysis is depicted in the Figure 3. The mean plasma concentration against time for cinnarizine (both test and reference product) is represented in Figure 4.

Table 6 Mean pharmacokinetic parameters in 24 volunteers

Mean Pharmacokinetic Parameters	**Reference Preparation (A1)**	**Test Preparation (A2)**
	Mean±SD	
C_{max} (ng/ml.)	38.835±14.858	38.909±11.478
t_{max} (hr.)	2.333±0.381	2.688±0.528
AUC 0-t (ng. hr./ml.)	484.234±206.692	493.078±146.935
AUC 0-¥ (ng. hr./ml.)	557.291±244.375	544.354±181.396
k_{el} ($hr.^{-1}$)	0.053±0.010	0.059±0.012
$t_{1/2}$ (hr.)	13.709±3.105	12.548±4.638
Relative Bioavailability (%)	**100%**	**101.83%**

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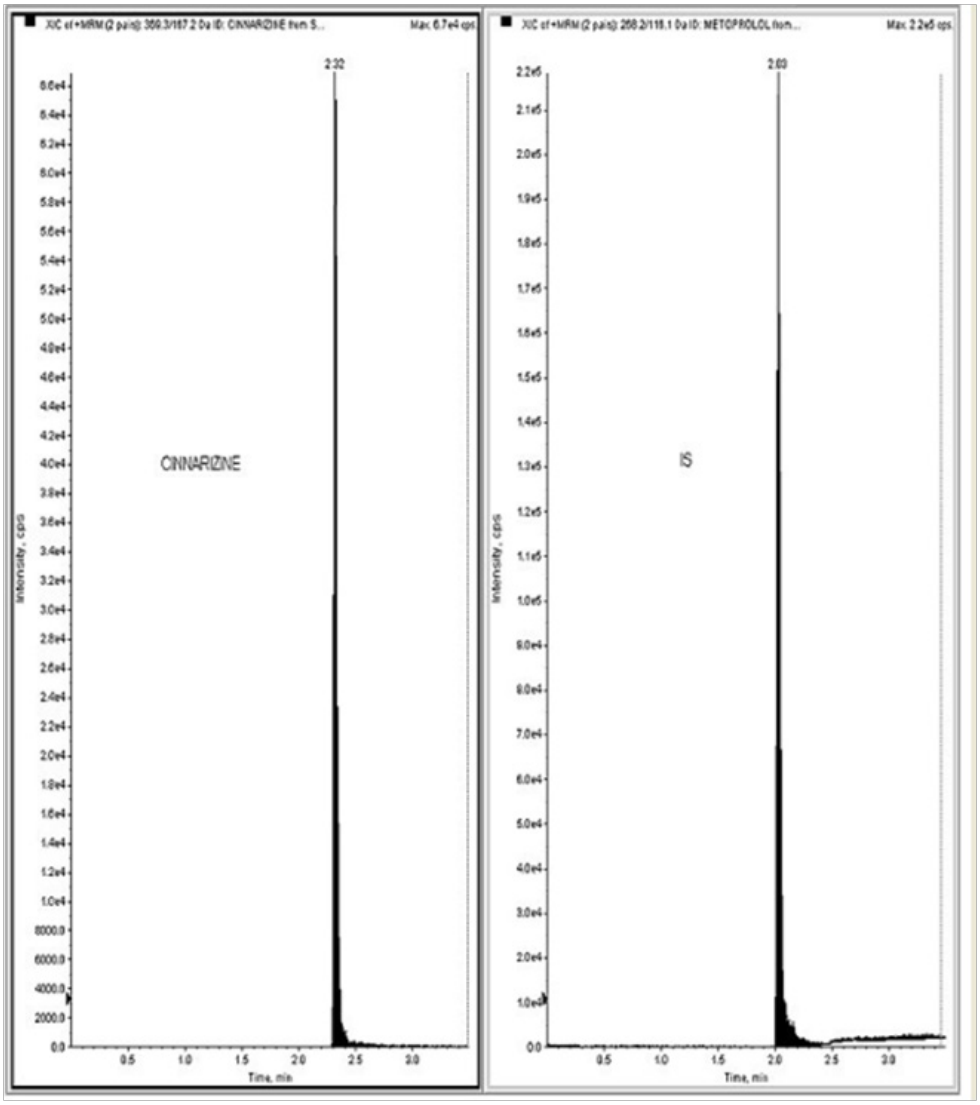

Figure 3 Representative volunteer plasma chromatograms.

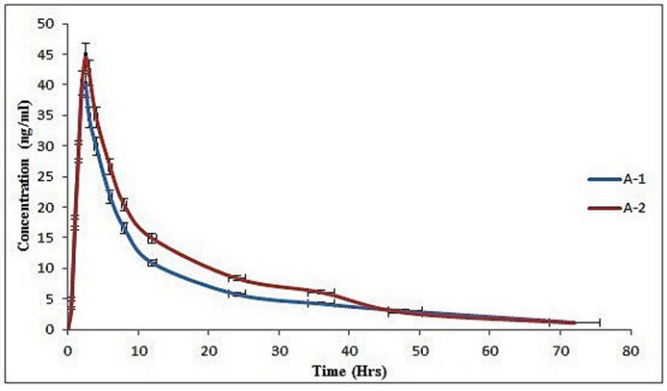

Figure 4 Mean pharmacokinetic profile of cinnarizine.

Conclusion

Efforts were given to develop and validate a bioanalytical method for estimation of Cinnarizine in human plasma by LC-ESI-MS/MS was given by earlier researchers with liquid extraction technique as plasma extraction process. But in the present study, protein precipitation technique was applied which was a cost effective, easy and reproducible extraction process, hence superior to the liquid-liquid extraction technique. The developed method with a short run time (3.5min) for determination and quantification of cinnarizine in human plasma was also validated as per the US-FDA guidelines. The validation parameters found within the specified regulatory limit, hence acceptable. It can be concluded that the developed method was found to be simple, specific, highly selective, sensitive and reproducible. This was applied for the analysis of the volunteer plasma samples obtained from the comparative pharmacokinetic study. On the basis of the pharmacokinetic parameters studied, it can be concluded that the test preparation, tablet containing cinnarizine 25mg was found bioequivalent with the reference preparation. Furthermore, there was no occurrence of adverse event. Dropout from the study was also nil. So it can be concluded that the test and reference preparation was well tolerated by the volunteers.

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Conflicts of interest

Authors declare that there is no conflicts of interest.

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