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Development and Validation of High Performance LCMS Methods for Estimation of Ebastine and Carebastine in Human Plasma

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ABSTRACT

A simple and specific method for simultaneous determination of Ebastine and Carebastine by liquid chromatography-tandem mass spectrometry operated in positive ionization mode was developed and validated. The column used was a BDS Hypersil C_{18}, 50 mm × 4.6 mm, 5µm with a flow rate of 0.6 mL/min, without splitter for the chromatographic analysis with flow rate of 0.6 mL/min. Solid phase extraction method was applied. Mass parameters, 496.2/261.0 and 672.2/479.3/261.2 were chosen for analysis. Linearity was established in human plasma covering the concentration range of 0.051 ng/mL to 31.099 ng/mL for Ebastine and 1.013 ng/mL to 1005.451 ng/mL for Carebastine using (Ebastine D6 & Carebastine D6) as internal standards. Different parameters such as linearity, range, precision, accuracy, ruggedness and robustness, limit of detection (LOD) and limit of quantification (LOQ) were used for full validation of the method. The results were found to be acceptable as per the guidelines of International Conference on Harmonization (ICH). The method found to be novel, rapid, linear, precise, accurate, robust and rugged and can be successfully applied for the routine analysis of Ebastine and Carebastine with more sensitivity and covers wider range of quantitation. The method also found to be useful and economical.

Keywords: Ebastine and Carebastine, LCMS, Validation, Solid phase extraction, ICH.

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INTRODUCTION

This research paper details the development and validation of LCMS method for estimation of Ebastine and Carebastine in API. The IUPAC name of Ebastine is 4-(4-benzhydryloxy-1-piperidyl)-1-(4-tert-butylphenyl) butan-1-one and IUPAC name of Carebastine is 2-[4-[4-[4-[di(phenyl)methoxy]piperidin-1-yl]butanoyl]phenyl] -2-methylpropanoic acid. [1] Ebastine is a H_1 antihistamine with low potential for causing drowsiness and is a type of non-sedating second generation antihistamine which is used for allergic disorders. Clinical trials showed that it could effectively decrease symptoms of intermittent or seasonal allergic rhinitis and chronic idiopathic urticaria by blocking

histamine receptors. Upon ingestion, Ebastine undergoes extensive first-pass metabolism to its active metabolite Carebastine. Ebastine is generic and available globally under many brands. Ebastine molecular formula is $C_{32}H_{39}NO_2$ with average molecular weight 469.669 g/mol and available in white powder form. Carebastine molecular formula is $C_{32}H_{37}NO_4$ with average molecular weight 499.64 g/mol and available in white powder form. [2-18]

Several methods were reported in literature for the determination of Ebastine and its metabolite and Ebastine with different drugs with varying analytical techniques for its estimation with its active metabolite Carebastine and in combination of drugs like phenylephrine hydrochloride with UV spectrophotometric assay method [3], Ebastine and Montelukast sodium [1], Reverse Phase high performance liquid chromatography (RP-HPLC) method [7], and by high performance liquid chromatography (HPLC) [12, 17] by spectrometric methods [10] electrochemical determination [14] and gas chromatography-mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) [2] and ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). [11] The reported method of quantification in LC-MS/MS was validated over the concentration range of 0.1–10 ng/mL for Ebastine and 0.2–200 ng/ mL for Carebastine in human plasma, respectively. [11]

To meet the requirement of pharmaceutical studies with wider range of dosage forms it was essential to develop more sensitive and a wider quantitation range of method. Linearity for the current method was established in human plasma covering the concentration range 0.051 ng/mL to 31.099 ng/mL for Ebastine and 1.013 ng/mL to 1005.451 ng/mL for Carebastine which was lower than the reported method for Ebastine and covered a wide range for the drug and metabolite upto 31.099 ng/mL for Ebastine and 1005.451 ng/mL for Carebastine. So that it helps to apply the method for a wide range of the dosage with varying strength. The method was validated successfully and applied to bioequivalence study in healthy indicant subjects with required accuracy and precision.

MATERIALS AND METHODS

Materials: Ebastine, Carebastine, Ebastine D6 and Carebastine D6 (IS) was supplied by Clearsynth labs Pvt. ltd., Mumbai. Methanol was obtained from Finar chemicals Ltd., Ahmadabad, India. The LCMS instrument used here was AB Sciex, API 4000.

Selection of solvent: Solubility of drug was checked in different solvents and LCMS spectra recorded.

Preparation of stock and working standard solutions for pure drug

Ebastine: Transferred accurately weighed (about 5 mg) Ebastine into a 5 mL volumetric flask and dissolved in methanol. Made up the volume with methanol and vortexed. Concentration of the resultant solution was about 1000µg/mL.

Carebastine: Transferred accurately weighed (about 5 mg) Carebastine into a 5 mL volumetric flask and dissolved in methanol. Made up the volume using methanol and vortexed. Concentration of the resultant solution was about 1000µg/mL. This solution was further diluted with methanol to get working standard solution of 10µg/mL of drug.

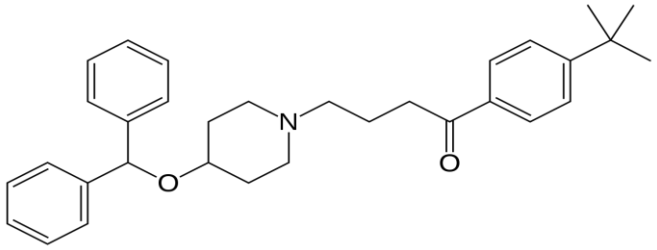

Fig. 1: Structure of Ebastine

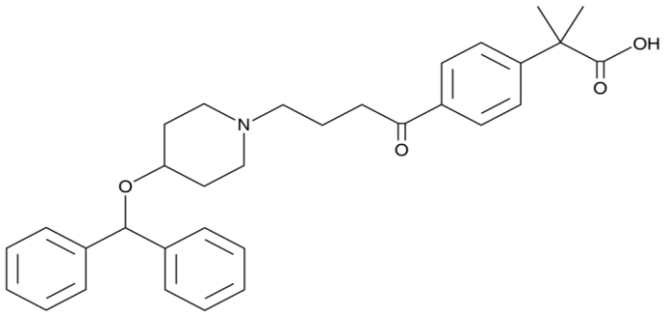

Fig. 2: Structure of Carebastine

Preparation of internal standard stock solution

Ebastine D6: Transferred accurately weighed (about 5 mg) Ebastine D6 into a 5 mL volumetric flask and dissolved in methanol and made up the volume using methanol and vortexed. Concentration of the resultant solution was about 1000µg/mL.

Carebastine D6: Transferred accurately weighed (about 5 mg) Carebastine D6 into a 5 mL volumetric flask and dissolved in methanol and made up the volume using same and vortexed. Concentration of the resultant solution was about 1000µg/mL.

Preparation of internal standard working solution

Diluted 10µL of Internal Standard-1 stock solution to 5 mL using dilution solvent to get IS dilution approx. 2µg/mL and dilute 10µL of Internal Standard-2 stock solution to 5 mL using dilution solvent get IS dilution approximately 2µg/mL. The 0.25 mL IS dilution 1 and 1.25 mL of IS dilution 2 dilute to 25 mL using dilution solvent (Methanol: Water 50:50 v/v) to get IS dilution of about 20 ng/mL (IS1) and 100 ng/mL (IS2) concentration.

Preparation of sample solutions

Sample solutions of different concentration from 0.051 ng/mL to 31.099 ng/mL for Ebastine and 1.013 ng/mL to 1005.451 ng/mL for Carebastine were prepared from above stock solution and diluted with mobile phase.

Preparation of sample solutions

To prepare sample solutions of Methanol: Water: 50:50 v/v, 500 mL of Methanol, 500 mL of Milli-Q water into a 1000 mL of reagent bottle, mixed and sonicated.

Preparation of Buffer-01 (10mM Ammonium Acetate)
Approximately weighed 0.7712g of ammonium acetate dissolved in 1000 mL of milli-q-water and adjusted solution pH to 9.0 with ammonia and filtered.

Mobile phase: (Buffer-01: Acetonitrile: Formic acid: 30:70:0.1 % v/v)
To 800 mL of acetonitrile added 200 mL of buffer into a 1000 mL of volumetric flask, and then added 1 mL of formic acid mix and sonicated.

Preparation of Dilution solvent (Methanol: water 50:50 % v/v)
Approximately 250 mL of Milli-Q-water into a 500 mL reagent bottle and then added 250 mL of Methanol and sonicated well.

Preparation of sample solutions: Sample solutions of different concentrations from 0.051 ng/mL to 31.099 ng/mL for Ebastine and 1.013 ng/mL to 1005.451 ng/mL for Carebastine were prepared from above stock solution and diluted with mobile phase.

Extraction Procedure
To an aliquot of 200µL of spiked plasma/subject samples, 50µL of working solution was added and vortexed for 10s. Samples were then loaded on Strata-X-C 33 micro extraction cartridges which were conditions with 1.0 mL methanol, followed by 1.0 mL of water. Washing of samples was done with 2 x1.0 mL of water, followed by drying of cartridges for 2.0 min by applying nitrogen. The elusion of Ebastine and Carebastine and Internal Standards were done using 500µL of mobile phase solution into pre-labeled vials, and 5µL was used for injection in the chromatographic system.

Chromatographic Conditions
Injection Volume: 5 µL
Column: BDS Hypersil C_{18}, 50 mm × 4.6 mm, 5µm
Flow rate: 0.6 mL/min, without splitter

Instrumentation
Quantitation was achieved with LCMS-MS detection in positive ion mode using an AB Sciex API- 4000 mass spectrometer equipped with a Turboionspray™ interface consisting of a BDS Hypersil C_{18} column (50 mm × 4.6 mm, 5µm), a binary LC-20 AD prominence pump an auto sampler and a solvent degasser of Agilent 1200. Solid phase extraction method was used. Detection of the ions was carried out in the multiple reaction monitoring (MRM) mode by monitoring the transition pairs Quadruples Q1 and Q3 were set on unit resolution. Mass parameters, 496.2/261.0 and 672.2/479.3/261.2 were chosen for analysis. The analysis data obtained were processed by Analyst software™ Analyst 1.6.1.

Method Validation
The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation. [13] The method was validated for the parameters of Linearity, Concentration Range, Accuracy, Precision (Repeatability, Intermediate Precision), Ruggedness, Matrix Effect, Selectivity, Robustness, Limit of Detection and Limit of Quantification. [13]

Specificity and Selectivity
Specificity of the method was checked for any interference in eight different lots of human blank plasma including one lipemic and one hemolyzed with the proposed extraction procedure. Selectivity was assessed, by comparing the chromatograms of blank plasma and plasma spiked with lowest standard from eight different sources including lipemic and hemolyzed (or donors). Cross analyte effect (cross talk) was evaluated by spiking the highest concentration individually and monitored all Multiple Reaction Monitoring (MRM) channels for interferences.

Precision and Accuracy
All validation experiments were carried out at five (LOQQC, LQC, MQC-1, MQC-2 and HQC) QC levels. For the determining of intraday accuracy and precision, a replicate (n=6) analysis of plasma samples were performed on the same day. The inter-day accuracy & precision were assessed by analysis of three batches on different days. The precision was expressed as Relative Standard Deviation (RSD %) and accuracy as Relative Error (RE %).

Calibration Curve
Plotting of the peak area ratio of the transition pair of analytes to that of IS against the nominal concentration of calibration standards was done to obtain calibration curves. Blank sample and zero samples were run with each calibration curve. The acceptance criterion for each back-calculated standard concentration was + 15% deviation from the nominal value except at LLOQ, which was set at + 20%.

Recovery and Matrix effect
The recovery of Ebastine and Carebastine and ISs were determined by comparing the responses of the analytes extracted from replicate QC samples (n = 6) with the response of analytes from post extracted plasma standard sample at equivalent concentration. Recovery was determined at low, mid and high quality control concentrations, whereas the recovery of the ISs was determined at a single concentration. The matrix of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analytes from neat samples at equivalent concentrations. Matrix effect was determined at two levels with six different sources of plasma (out of eight, six were normal K_2EDTA plasma, one was lipemic and one was hemolyzed with the same anticoagulant) (LQC and HQC) for Ebastine and Carebastine and for ISs. Dilution integrity was performed to extend the upper concentration limit with acceptable precision & accuracy.

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Stabilities

Stability of Ebastine and Carebastine and IS (Ebastine D6 and Carebastine D6) stock solutions were evaluated after storage at room temperature for 119 hours 06 & 119 hours 32 minutes. Mean peak area ratios for stability solutions were compared to mean peak area ratios determined from freshly prepared solution Other stabilities were also determined in matrix samples such as Freeze thaw, bench top stability at room temperature & refrigerator, wet extract stabilities and long term stabilities. The areas of stability samples and freshly prepared samples were compared to determine mean % nominal concentration during stability period.

Table 1: Summary of the Experimental Parameters of Ebastine in K_2EDTA Human Plasma

Experimental Parameters	Results
Analyte	Ebastine
Biological Matrix	K_2EDTA Human Plasma
Specificity and Selectivity % CV	7.06%
Analytical range	0.051 to 31.099 ng/mL
Sensitivity: Precision, Accuracy	13.11%, 89.54%
Recovery	
Ebastine % CV , % Recovery	10.72%, 58.96%
Ebastine D6 % CV, % Recovery	3.32%, 73.44%

Table 2: Summary of the Experimental Parameters of Carebastine in K_2EDTA Human Plasma

Experimental Parameters	Results
Analyte	Carebastine
Biological Matrix	K_2EDTA Human Plasma
Specificity and Selectivity % CV	3.68%
Analytical range	1.013 to 1005.451 ng/mL
Sensitivity: Precision , Accuracy	8.65%, 105.22%
Recovery	
Carebastine % CV, % Recovery	8.76%, 77.33%
Carebastine D6 % CV, % Recovery	2.56%, 77.36%

RESULTS AND DISCUSSION

LC-MS/MS Method Development

Though currently there are several methods available to determine Ebastine and Carbastine with various techniques, the aim of the present work was to develop a highly sensitive and rapid method to meet the requirement of wide range of dosage forms in pharmacokinetic studies in healthy humans. Multiple existing chromatographic methods were Ebastine in combination of different drugs and couple of methods with its active metabolites. In this method we developed Ebastine and its active metabolite Carebastine in simultaneous estimation with more sensitive detection than reported for Ebastine and covered wider range for both Ebastine and Carbastine using LC-MS/MS instrumentation and solid phase extraction (SPE) employing a deuterated IS, Which helped in controlling any variability during extraction and analyte ionization.

To meet the requirement of pharmaceutical studies with wider range of dosage forms it is essential to develop more sensitive and a wider quantitation range of method. Linearity for the current method was established in human plasma covering the concentration range 0.051 ng/mL to 31.099 ng/mL for Ebastine and 1.013 ng/mL to 1005.451 ng/mL for Carebastine. Which is lower than the reported method for Ebastine and covered a wide range for the drug and metabolite upto 31.099 ng/mL for Ebastine and 1005.451 ng/mL for Carebastine. So that it helps to apply the method for a wide range of the dosage with varying strength. The method was validated successfully and applied to bioequivalence study in healthy indicant subjects with required accuracy and precision.

Mass spectrometer parameters were optimized for Ebastine and Carebastine in the positive ESI mode. The Q1 MS spectra of the analyte and IS showed abundant protonated molecular ion at Mass parameters, 496.2/261.0 and 672.2/479.3/261.2. In this method we have chosen analysis by solid phase extraction method which is economical and easy to process.

Specificity and Selectivity

The retention times of Ebastine & Carebastine were found to be 3.61 & 1.71 minutes respectively. The overall chromatographic run time is 6.00 minutes. During the validation, blank K_2EDTA plasma samples were evaluated and all plasma lots were found to be satisfactory that there were no interfering peaks in the blank plasma at the retention times of Ebastine & Carebastine or internal standard as seen in the chromatograms. Representative Calibration Curve for Ebastine & Carebastine in K_2EDTA human plasma are shown in Figure 1 and 2.

Linearity

Linearity was established by preparing an eight-point standard calibration curve in K_2EDTA human plasma covering the concentration range 0.051 ng/mL to 31.099 ng/mL for Ebastine and 1.013 ng/mL to 1005.451 ng/mL for Carebastine using (Ebastine D6 & Carebastine D6) as internal standards.

Calibration standards were prepared and six batches of precision and accuracy were analysed (Table 1 and 2). Calibration curves were calculated by least-squares linear regression analysis of the response ratios (analyte/IS) in calibration standards with 1/x2 weighting.

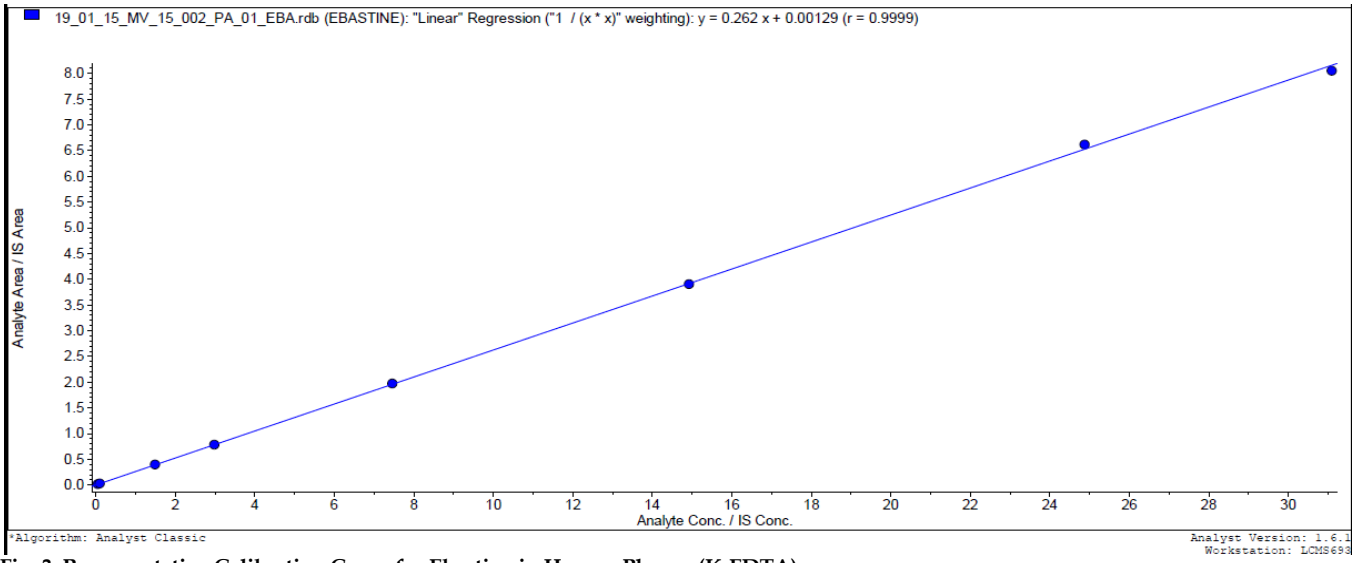

Fig. 3: Representative Calibration Curve for Ebastine in Human Plasma (K_2EDTA)

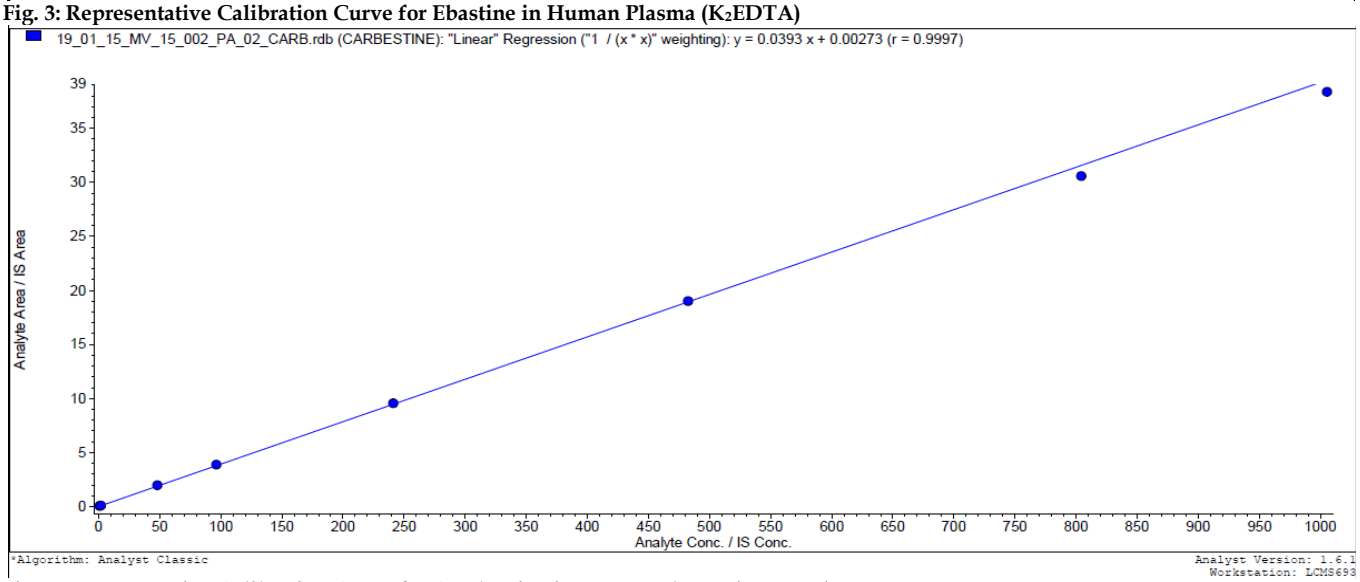

Fig. 4: Representative Calibration Curve for Carebastine in Human Plasma (K_2EDTA)

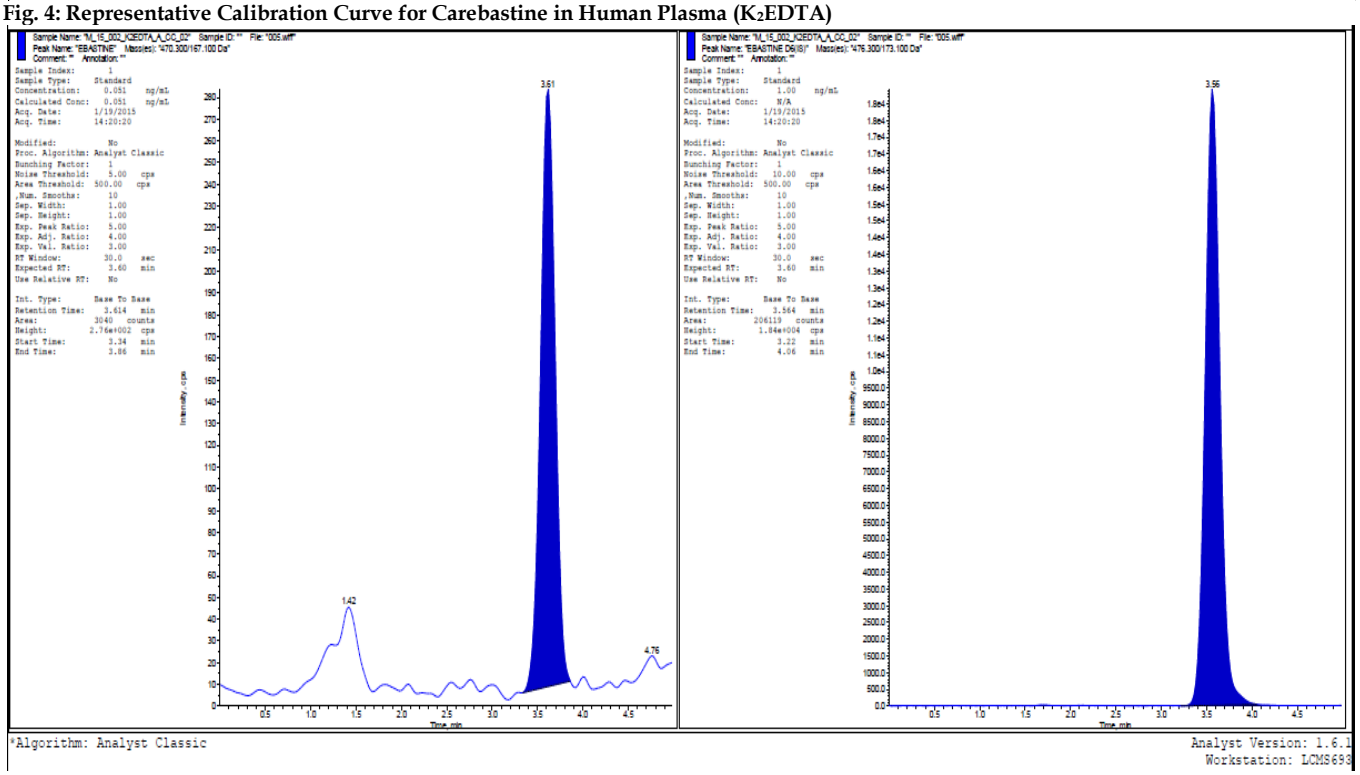

Fig. 5: Chromatogram of the LLOQ Calibration Curve Standard for Ebastine with Internal Standard (Ebastine D6)

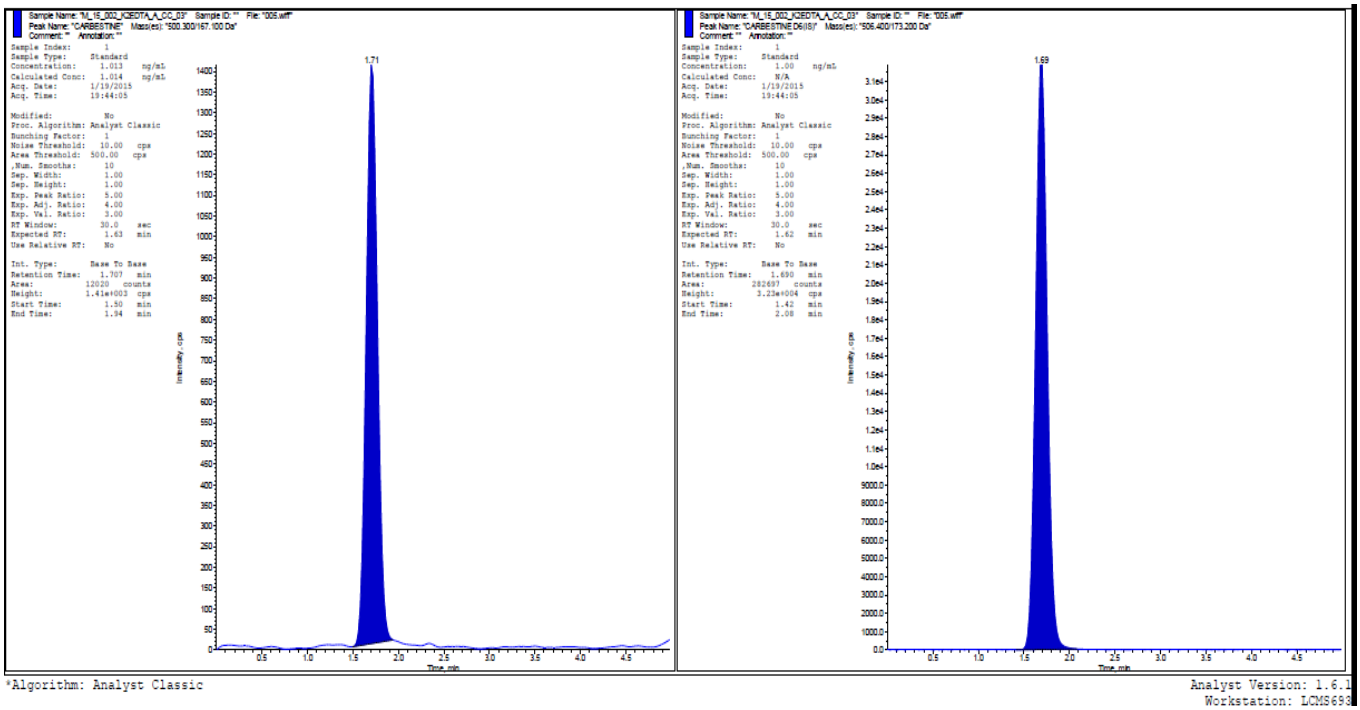

Fig. 6: Chromatogram of the LLOQ Calibration Curve Standard for Carebastine with Internal Standard (Carebastine D6)

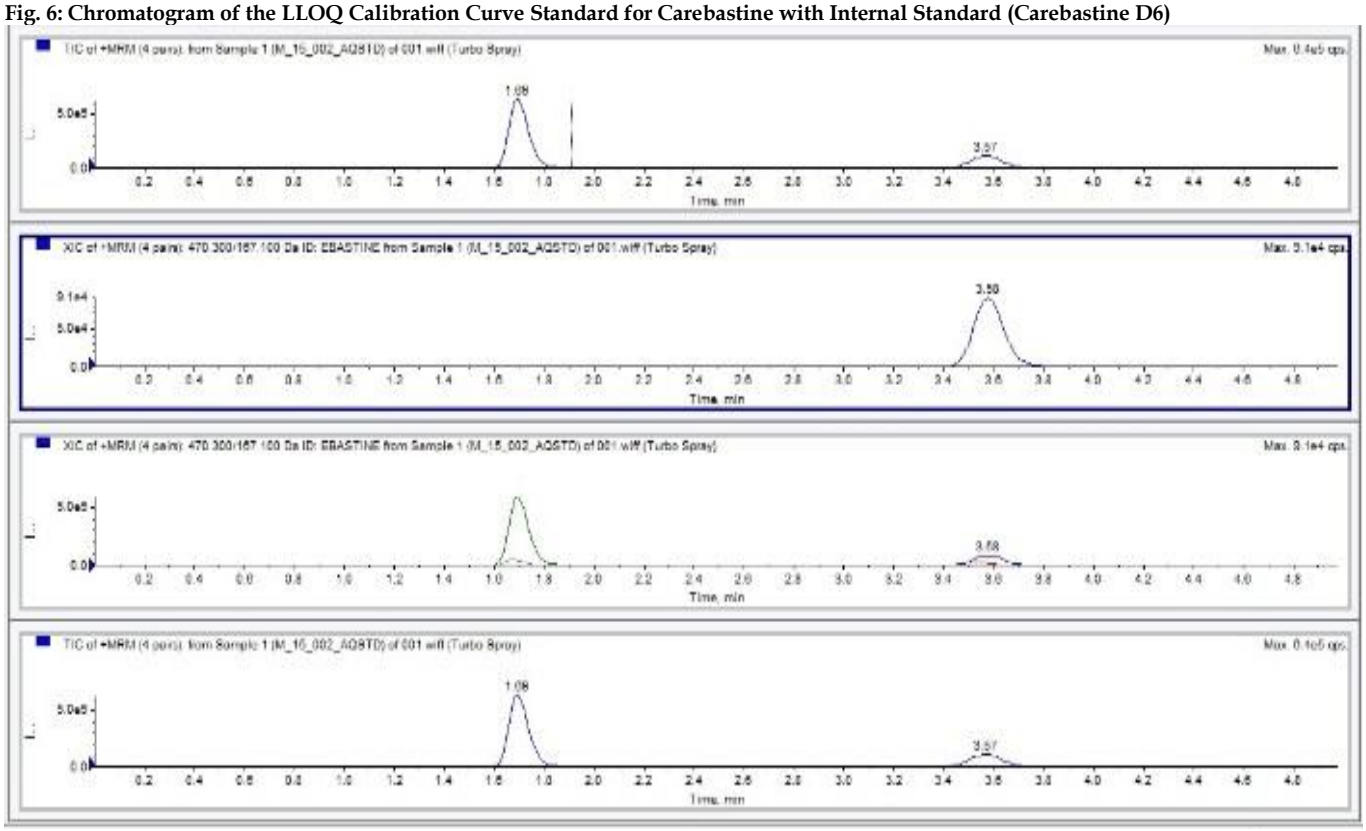

Fig. 7: Chromatographic separation of Ebastine, Carebastine, Ebastine D6 and carebastine D6 (Internal Standards)

Precision and Accuracy

The results for intra-day and inter-day precision and accuracy for Ebastine and Carebastine in plasma quality control samples are summarized in Table 1 and 2. The intra-day precision found to be 13.11% for Ebastine and 8.65% for Carebastine and accuracy was 89.54% for Ebastine and 105.22% for Carebastine.

Recovery

Recovery of Ebastine and Carebastine from K_2EDTA human plasma was determined by comparing peak areas of extracted QCL, QCM and QCH samples with peak areas determined from freshly prepared unextracted (aqueous) samples prepared at similar concentrations in mobile phase. Mean overall % recovery was 58.96% and overall %CV was 10.72% for Ebastine and % recovery was 77.33% and %CV was 8.76% for Carebastine and % recovery was 73.44% and %CV was 3.32% for IS (Ebastine D6) and 77.36% and %CV was 2.56% for IS (Carebastine D6).

Matrix Effect

The matrix effect was evaluated by analyzing QC samples. Average matrix factor values (matrix factor =

response of post-spiked concentrations/ response of neat concentrations) obtained for Ebastine and Carebastine and ISs. There were no significant matrix effects observed for any of the analytes or the ISs.

Stability

The stability of the analytes in human plasma under different temperature and timing conditions was evaluated. QC samples were subjected to long-term storage conditions at room temperature and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels of low QC and high QC values for Ebastine and Carebastine with six determinations for each. For process stability, the results indicated that the difference in the back-calculated concentration from time 0 to 119 h allowed us to conclude that processed samples are stable at least for 119 h at room temperature. Freeze and thaw stability results indicated that the repeated freeze and thawing (five cycles) did not affect the stability of Ebastine and Carebastine.

A sensitive and selective LC-MS/MS method to simultaneously quantitate Ebastine and Carebastine in K_2EDTA in human plasma over the concentration range 0.051 ng/mL to 31.099 ng/mL for Ebastine and 1.013 ng/mL to 1005.451 ng/mL for Carebastine was successfully validated. This method is suitable for subject sample analysis and incurred sample analysis to support bioequivalence/bioavailability and/or pharmacokinetic studies involving formulations of Ebastine and Carebastine.

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