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RESEARCH ARTICLE

BIO- ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF BETAHISTINE DIHYDROCHLORIDE IN HUMAN PLASMA BY LC-MS/MS

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ABSTRACT

The objective of this research was to develop and validate a simple, sensitive and specific Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) quantification of Betahistine in human plasma using Betahistine-d4 as Internal Standard (IS). The analytical method consists of liquid-liquid extraction of plasma sample followed by the determination of Betahistine by a LC–MS/MS. The analyte was separated on a Zorbax Eclipse XDB -C18 (150 x 4.6 mm, 5 µ) column with an isocratic mobile phase of Acetontrile: 0.1% formic acid (80: 20 v/v) at a flow rate of 0.8 mL/min. The protonated ions were formed by a turbo ionspray in a positive mode was used to detect analyte and internal standard (IS). The MS/MS detection was made by monitoring the fragmentation of *m/z* 137.1→94.0 for Betahistine and *m/z*140.2→94.10 for internal standard on a mass spectrometer. The method was validated with the correlation coefficients of (r2)≥0.9997 over a linear concentration range of 10.00-501.20 pg/mL. This method demonstrated intra and inter-day precision within 1.1–1.6% and 0.2–0.54% and accuracy within 98.04-101.85% and 98.04–101.14% for BET.

INTRODUCTION

Betahistine Dihydrochloride (BET) is chemically N-Methyl-2-pyridineethanamine well known Anti Vertigo drug[1]. The chemical formula of Betahistine Dihydrochloride is C8H12N2.2HCl and its molecular weight is136.194. It is official in Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), European Pharmacopoeia (EP), and United States Pharmacopoeia (USP). In which, USP[2] and IP[3] describe Liquid chromatographic method for estimation. While BP[4] and EP[5] describe potentiometric method for estimation. Several analytical methods have been reported for the determination of Betahistine Dihydrochloride such as HPLC for estimation Betahistine Dihydrochloride in human serum[6]. It also shows colorimetric method[7], HPLC[8], Voltammetric method[9], LC-MS/MS [10-13] for the estimation of Betahistine in tablet.

Literature survey reveals that only few methods were reported for estimation of BET in human plasma by LC-MS/MS method. How ever there is a need to develop simple, sensitive, rapid, accurate and precise LC-MS/MS method for estimation of Betahistine in human plasma. Hence this research is carried out by LC-MS/MS.

MATERIAL AND METHODS

Instrumentation

The Agilent 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) connected to the API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) with turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Reagents / Materials

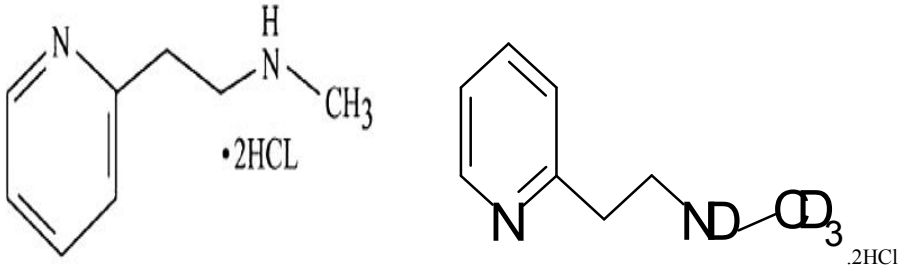
Betahistine Dihydrochloride (Figure1A) was obtained from Cadila Pharmaceuticals, India. Betahistine Dihydrochloride-D4 (BETD4) was procured from Clear Synth, India. Water (HPLC Grade), (Figure1B). Formic Acid (analytical grade) were purchased from Merck, Mumbai, India. Acetonitrile (HPLC Grade), ethyl acetate, and dichloromethane (HPLC grade) were obtained from J.T. Baker, USA. Human plasma was procured from Navjeevan Blood Blank, Hyderabad. Milli Q water was taken from the in-house Milli-Q system.

Detection

Detection was done by turboionspray (API) positive mode with unit resolution. For BET, mass transitions were obtained

from 137.10 m/z (parent ion) to 94.00 m/z (product ion). Similarly, BETD4 mass transitions were obtained from (140.20 m/z) (parention) to 94.10 m/z (product ion).

(Acetontrile: 0.1% formicacid, 80:20, v/v), and the sample was transferred into autosampler vials and injected into the LC-MS for study.



A B

Figure 1 Chemical structure of A)Betahistine Dihydrochloride and B)Betahistine Dihydrochloride-D4

Chromatographic conditions

Chromatographic separation was performed using an Xbridge Zorbax Eclipse XDB -C18 (150 x 4.6 mm, 5 µ) at a temperature of 40ºC. The mobile phase was composed of Acetontrile: 0.1% formic acid (80: 20, v/v) at a flowrate of 0.8mL/min. Deuterated BETD4 (Internal standard-IS) was used as the appropriate IS interms of chromatography and extractability. BET and BETD4 were eluted at 4.52 min, approximately, with a total runtime of 8 min for each sample.

Preparation of standards and quality control samples

Standard stock solutions of BET (10.0mg/mL) and BETD4 (10.0 mg/mL) were prepared in Acetontrile. The IS spiking solution (100.0 pg/mL) was prepared in mobile phase solution (Acetontrile: 0.1% formicacid (80: 20, v/v) from BETD4 stock solution. Standard stock solutions and IS spiking solutions were stored in refrigerator conditions of 2–8ºC until analysis. Standard stock solutions of BET (10.0 mg/mL) were added to drug-free screened human plasma to obtain concentration levels of 10.2, 20.1, 35.1,70.2, 100.3, 200.6, 301.0, 401.3 and 501.6 pg/mL for analytical standards, and 10.2 (LLOQ), 30.1 (LQC), 250.1 (MQC) and 461.6 pg/mL (HQC) for quality control (QC) standards, and stored in the freezer at 30ºC until analysis. The aqueous standards were prepared in a mobile phase solution(Acetontrile: 0.1% formicacid (80:20, v/v) and stored in the refrigerator at 2–8ºC until analysis.

Biological Matrix

Human plasma containing Sodium Heparin as anticoagulant was used as a biological matrix during method validation. Selectivity and sensitivity tests were performed before bulk spiking.

Sample preparation

The LLE method was used to isolate BET and BETD4 from human plasma. For this purpose, 50 µL of BETD4 (10 pg/mL) and 200 µL of plasma sample were added to the labelled polypropylene tubes and vortexed briefly for about 5 min. Thereafter, 50 µL of 0.1M NaOH solution and 3mL of extraction solvent (in the ratio of ethylacetate: dichloromethane 80:20(v/v)) were added and vortexed for about 10 min. Next, the samples were centrifuged at 4000 rpm for approximately 5 min at ambient temperature. From each, a supernatant sample was transferred into labelled polypropylene tubes and evaporated to a dryness of 40ºC briefly, and then reconstituted with a mobile phase solution

Method Validation

The validation was performed as per FDA guidelines to evaluate the method in terms of linearity response, sensitivity, selectivity, precision and accuracy (within-batch and between-batch/inter- day), stabilities (freeze-thaw, bench top, short-term and long-term stock solutions, working solutions and long term stability in matrix), carryover effects, recovery, dilution integrity, matrix effect, matrix factor, autosampler re-injection reproducibility and ruggedness experiment[10].

System suitability

System suitability experiment was performed by injecting six consecutive injections at least once in a day with using aqueous MQC solution. System performance experiment was performed by injecting sequence of injections at the beginning of analytical batch and %CV was calculated.

Selectivity and sensitivity

Selectivity was performed by analyzing human blank plasma samples from six different sources (donors) with an additional hemolyzed group and lipedimic group to test for interference at the retention times of analytes. The sensitivity was compared with the lower limit of quantification (LLOQ) of the analyte with its blank plasma sample. The peak area of blank samples should not be more than 20% of the mean peak area of the limit of quantification (LOQ) of BET and 5% of the mean peak area of BETD4.

Calibration of standard curve (Linearity and range)

The linearity of the method was determined by using standard plots associated with nine point standard curve including LLOQ and ULOQ. Concentration of calibration curve standards was calculated against the calibration curve and the linearity of the method was evaluated by ensuring the acceptance of precision and accuracy of calibration curve standards. Two consecutive calibration curve standards should not be beyond the acceptance criteria. The lower limit of quantification (LLOQ) was the lowest concentration at which the precision expressed by relative standard deviations (RSD, CV %) is better than 20% and the accuracy (bias) expressed by relative difference of the measured and true value was also lower than 20%.

Precision and accuracy

The within-run and between-run percentage mean of precision and accuracy of the BET were measured by the percent coefficient by using six replicate samples of variation over the concentration range of LLOQ (Lowe limit), LQC (Low), MQC

(Middle) and HQC (high) quality control samples for the three precision and accuracy batches to their nominal values. The acceptable %coefficient of precision and accuracy should be less than 15%. The between and within batch % mean precision and accuracy for LQC, MQC and HQC samples were within the range of 85.00-115.00% and for the LLOQ within the range of 80.00-120.00% respectively.

Recovery

The % mean recoveries was determined by comparing the mean peak area of the 6 replicates of extracted plasma quality control samples at high, middle 1&2 and low concentrations against respective mean peak area of the six replicates of un-extracted quality control samples at high, middle and low concentrations. A recovery of more than 50% was considered adequate to obtain required sensitivity. The % mean Internal Standard recovery was determined by comparing the mean peak area of internal standard in the extracted plasma quality control samples at MQC concentration against the mean peak area of internal standard in the un-extracted quality control samples at MQC concentration.

Dilution integrity

The dilution integrity of the method was evaluated by diluting the stock solution prepared as spiked quality control sample at concentration 1.5-2 times above the concentration of the highest standard in the calibration curve in the screened biological matrix. Conduct dilution integrity experiment by using six replicates each of diluted quality control (1/2) and diluted quality control (1/10) samples. Process and analyze the diluted quality control samples along with freshly spiked calibration curve standards and at least two sets of batch qualifying quality controls (at lower and higher).

Ruggedness

Ruggedness of the method was evaluated by using different analyst and different column of the same make and model or different equipment of the same make and model. The ruggedness experiment should meet the acceptance criteria for linearity and intra-batch accuracy & precision.

Matrix effect

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by determining the matrix factor, which was calculated as follows:

$$\text{Matrix Factor} = \frac{\text{Peak response ratio in presence of extracted matrix (post extracted)}}{\text{Peak response ratio in aqueous standards}}$$

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with aqueous standards of same concentration. The overall precision of the matrix factor is expressed as coefficient of variation (CV %) and %CV should be < 15%.

Stability of Betahistine(BET) and Betahistine-D4 IS (BETD4)

Short term stock solution stability

Short term stock solution stability for BET and internal standard were performed at the stock concentration by using six consecutive injections of aqueous standard equivalent to ULOQ concentration and working concentration respectively after storage of at least 6 hours at ambient temperature. Stability was assessed by comparing the stock dilutions of BET and Internal Standard prepared from the freshly prepared stock solutions (comparison) against stock dilutions of Internal Standard prepared from the stock solutions stored at ambient temperature (stability). Short term stock solution stability was evaluated by comparing the mean response of stability samples against mean response of comparison samples. The precision and accuracy for the stability samples must be within ≤15 and ±15%, respectively, of their nominal concentrations.

Long term stock solution stability

Long term stock solution stability for BET and BETD4 (internal standard) were performed at the stock concentration by using six consecutive injections of aqueous standard equivalent to ULOQ concentration and working concentration respectively after storage of at least 4 days in the refrigerator at 2-8°C. Stability was assessed by comparing the stock dilutions of BET and BETD4 (internal standard) prepared from the freshly prepared stock solutions (comparison) against stock dilutions of BET and BETD4 (internal standard) prepared from the stock solutions stored at 2-8°C (stability). Long term stock solution stability was evaluated by comparing the mean response of stability samples against mean response ratios of comparison samples.

Working solution stability

Short term stability (at least 6 hours at ambient temperature) and long term stability (at least 04 days at 2-8°C) for working solutions of drug (stock solution ULOQ and LLOQ) and Internal Standard were performed by using six consecutive injections of equivalent aqueous standards prepared from fresh and stored solutions. Short term stability and long term stability of working solution were evaluated by comparing the mean response of stability samples against mean response of comparison samples.

Stability of Drug in Biological Matrix

Perform the matrix stability experiment by using freshly prepared calibration curve standard and three replicates of freshly prepared batch qualifying quality control samples at HQC and LQC levels. The precision and accuracy for the stability samples must be within ≤15 and ±15%, respectively, of their nominal concentrations. Stability studies in biological matrix were conducted in the various conditions at LQC and HQC levels as described below:

Freeze thaw stability

Freeze thaw stability of the spiked quality control samples were determined after 1st and 3rd freeze thaw cycles stored at -20±5°C. Six replicates of each HQC and LQC samples were used for assessing each freeze thaw experiment (for first and third cycle at both the freezing temperatures). The first freeze-thaw cycle was of at least 24 hours followed by minimum of 12 hours for subsequent cycles. Process and analyze freeze thaw stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the freeze thaw stability on the basis of % change of LQC and HQC samples. The % Accuracy and % CV of LQC and HQC should be within ± 15.00 and ≤ 15.00 respectively.

Bench top stability

Spiked quality controlled samples (6 replicates of each LQC and HQC) were stored in deep freezer at temperature -20±5°C, which was retrieved after minimum 12 hours of freezing and was kept at ambient temperature on working bench for recommended period of at least 24 hours. Six replicates of each HQC and LQC samples were used for assessing the bench top stability experiment. Upon the completion of recommended period, process and analyze bench top stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the bench top stability on the basis of % Accuracy and % CV of LQC and HQC samples.

Autosampler re-injection reproducibility

Autosampler re-injection reproducibility was evaluated by re-injecting accepted precision & accuracy batch, which were stored preferably in either autosampler or in refrigerator for at least 55 hours or as per requirement. Autosampler re-injection reproducibility was evaluated by % Accuracy and % CV of LQC and HQC samples.

Long Term Stability in Biological Matrix

The long-term stability samples of LQC, MQC and HQC samples were kept frozen in vials at -20±5^{0}C for 55 days were assessed along with freshly processed calibration and comparison samples (six samples each of LQC, MQC, HQC). The initial BET concentration freshly after sample treatment preparation was assumed to be 100%. The selection of the stability duration on the basis of the characteristic of the analyte(s).

RESULTS AND DISCUSSIONS

Method Development

LC-MS/MS has been used as one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The goal of this work is to develop and validate a simple, sensitive, rapid, rugged and reproducible assay method for the quantitative determination of BET from human plasma samples. Chromatographic conditions, especially the composition and nature of the mobile phase, usage of different columns, different extraction methods such as solid phase, Precipitation, Liquid-liquid extraction methods were optimized through several trials to achieve the best resolution and increase the signal of BET and BETD4. The MS optimization was performed by direct infusion of solutions of both BET and BETD4 into the ESI source of the mass spectrometer. The critical parameters in the ESI source include the needle (ESI) voltage, Capillary voltage, source temperature and other parameters such as nebulizer gas, heater gas and desolvation gases were optimized to obtain a better spray shape, resulting in better ionization of the protonated ionic BET and BETD4 molecules. Product ion spectrum for BET and BETD4 yielded high-abundance fragment ions of *m/z 94.0* and *m/z* 94.1 respectively **(**Figure. 2).

After mass spectrometer parameters optimized, chromatographic conditions such as mobile phase optimization, column optimization, extraction method optimization was performed to obtain a fast and selective LC method. A good separation and elution were achieved using Acetontrile :0.1%formic acid (80:20, v/v) as the mobile phase, at a flow-rate of 0.5 mL/min and injection volume of 5 µL.

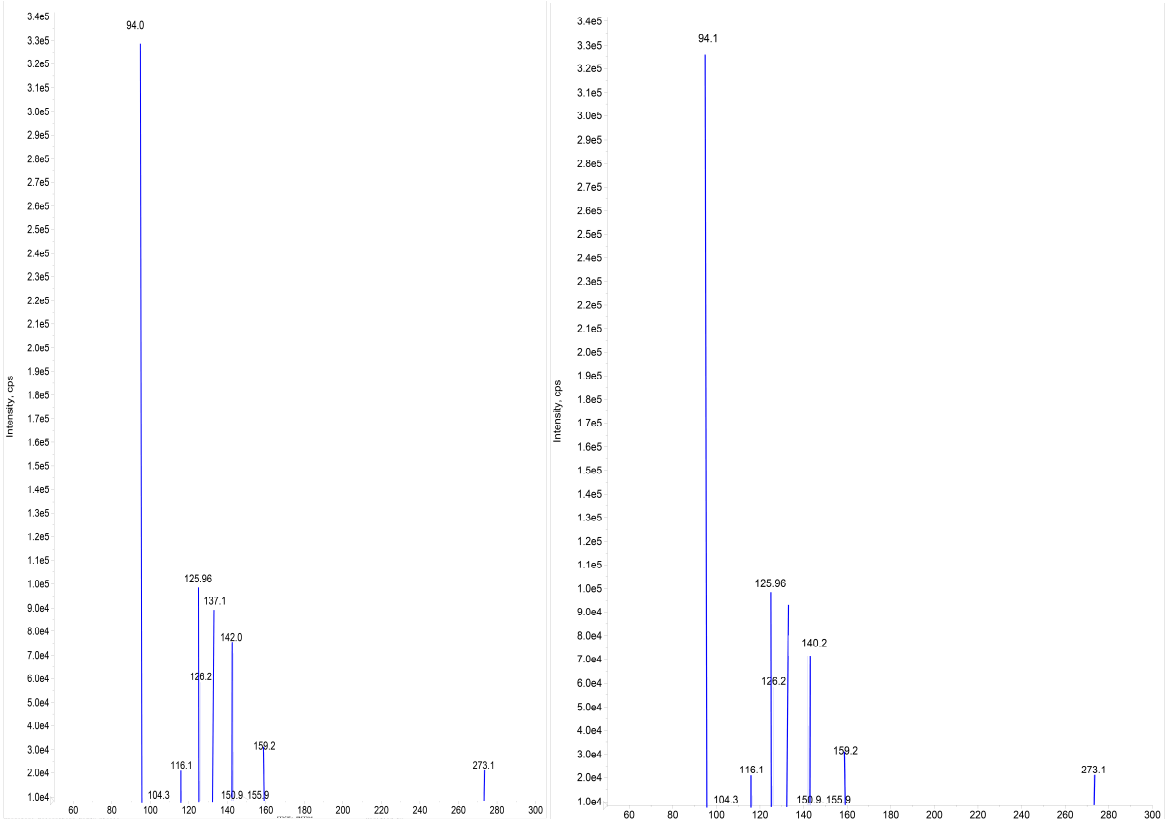


Fig. 2 Mass fragmentation pattern of Betahistine (BET) Betahistine-D4 (BETD4). a) Parent & Product ion mass spectra (Q1&Q3) of BET b) Parent & Product ion mass spectra (Q1 & Q3) of BETD4

Zorbax Eclipse XDB -C18 (150 x 4.6 mm, 5 µ) column and Liquid-liquid extraction method was optimized for the best chromatography.

Method Validation

System Suitability

System performance experiment was performed by injecting six consecutive injections at the beginning of analytical batch. %CV was 1.86.

Carryover Test

For carryover test two samples of upper limit of quantification (ULOQ) and 4 samples of blank plasma were processed. These samples were injected in the following sequence.

a) 2 blank samples b) 2 ULOQ samples c) 2 blank samples. The step (b) and (c) were repeated 2 times. The results demonstrate that there was no interference from the previous injection.

Selectivity and specificity

The analysis of BET and BETD4 using MRM (Multiple reaction monitoring) function was highly selective with no interfering compound. Chromatograms obtained from plasma spiked with EP (10.2 pg/mL) and NP (100 pg/mL).

Limit of Detection (LOD) and Quantification (LOQ)

The limit of detection was used to determine the instrument detection levels for BET even at low concentrations. 5 µL of a 0.5 pg/mL solution was injected and estimated LOD was 2.5 fg with S/N values ≥ 3-5. The limit of quantification for this method was proved as lowest concentration of the calibration curve which was proved as 0.5 pg/ml.(Fig.4)

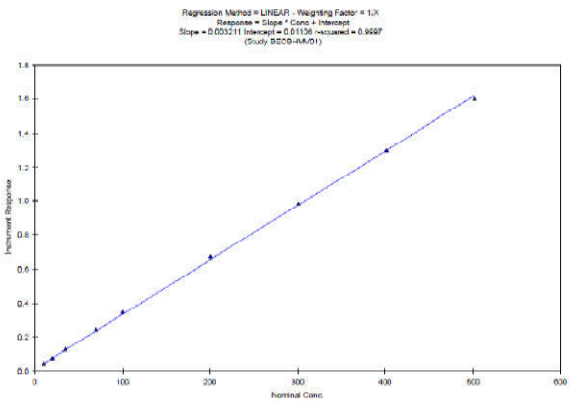
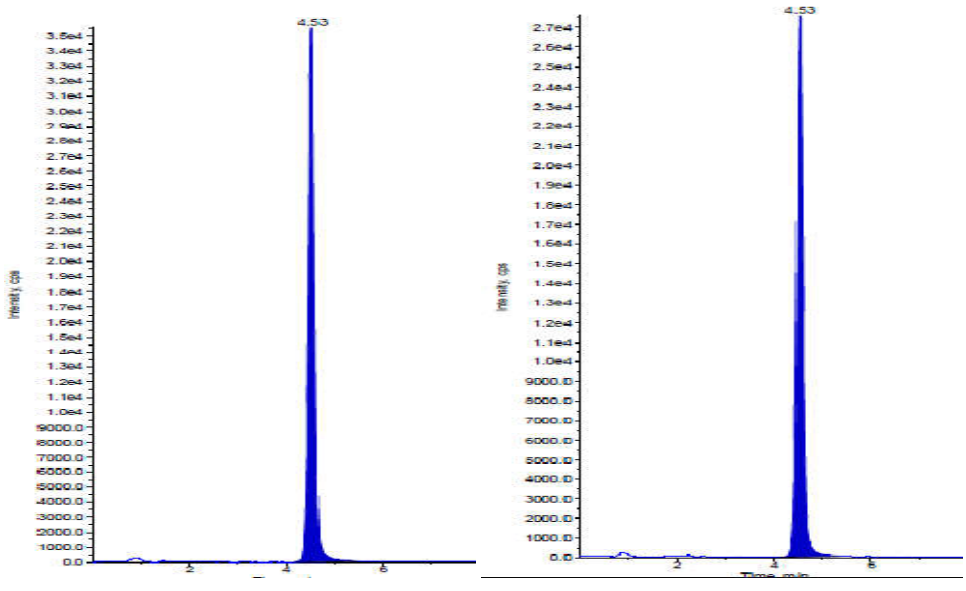


Figure 3 Calibration curve of BET

Table 1 Calibration curve

Spiked plasma Concentration (ng/ml)	Concentration measured (pg/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
10.20	8.651±2.1	1.1	86.506
20.10	17.099±7.5	2.6	85.072
35.10	34.109±0.9	1.7	97.177
70.23	69.937±1	1.5	99.625
100.32	102.283±2.7	2.8	101.978
200.60	204.666±1.9	3.6	102.027
301.00	304.057±0.7	3.2	101.015
401.31	401.807±0.4	3.2	100.126
501.61	497.59±1.4	2.7	99.201

As shown in Table. 2, the intra-batch CV% was less than 5% and the Accuracy ranged from 98 to 101.0%. The inter-batch CV% was less than 5% and the Accuracy ranged from 98.0 to 101.3%. These results indicate the adequate reliability and reproducibility of this method within the analytical range.



A B

Fig 4 Representative chromatograms of BET in plasma a) Chromatogram of BET b) Chromatogram of BETD4

Calibration curve standards, Precision and Accuracy

Calibration curves were plotted as the peak area ratio (BET/BETD4) versus (BET) concentration. Calibration was found to be linear over the concentration range of 10.2- 501.60 pg/mL. The CV% was less than 5 % and the Accuracy ranged from 85 to 102%. The determination coefficients (r^2) were greater than 0.9997 for all curves (Figure.3 & Table. 1).

Recovery

The recovery following the sample preparation using Liquid-liquid extraction with Methyl tertiary butyl ether was calculated by comparing the peak area of EP in plasma samples with the peak area of solvent samples and was estimated at control levels of EP. The recovery of EP was determined at three different concentrations 30.1, 250.0 and 461.0 pg/mL, were found as 94.43, 93.95 and 83.03%

respectively (Table. 3). The overall average recovery of BET and BETD4 were found to be 93.81 and 87.18% respectively.

Table 2 Precision and accuracy

Spiked Plasma Concentration (pg/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Concentration measured (n=6;pg/ml;mean±S.D)	%CV	%Accuracy	Concentration measured (n=6;pg/ml;mean±S.D)	%CV	%Accuracy
30.10	28.91	1.6	98.04	29.32	0.54	98.03
250.80	248.54	1.1	101.14	249.12	0.31	101.14
461.60	462.97	1.3	101.85	461.60	0.20	101.01

Table 3 Recovery of spiked plasma

Spiked concentration of BET (pg/mL)	Peak height ratio (mean ± SD), n=6		% Recovery
	Extracted BET response (n=6;mean±S.D)	Un-extracted BET response (n=6;mean±S.D)	
HQC (461.60)	153564.20 ± 2359.76	162475.42 ± 2809.32	94.52
MQC1(250.80)	61337.14 ± 849.27	65899.19 ± 889.45	93.08
LQC (30.10)	4024.14 ± 251.46	4788.19 ± 253.35	83.83

Working solution each of BET at working curve standard level and internal standard solution at working internal standard level were stable for 165 days. For BET and BETD4 (internal standard) the % Accuracy was 98.34 and 96.32 respectively.

Stability (Freeze - thaw, Auto sampler, Bench top, Long term)

Quantification of the BET in plasma subjected to 3 freeze-thaw (-30^{o}C to room temperature) cycles show the stability of the analyte. The concentrations ranged from 93 to 101.12 % of the theoretical values. No significant degradation of the BET was observed even after 55 hr storage period in the autosampler tray and the final concentrations of BET was between 94.0 to 101.2% of the theoretical values. No significant degradation of the BET was observed even after 24 hr storage period in the room temperature and the final concentrations of BET was between 99.0 to 102.12% of the theoretical values. In addition, the long-term stability of EP in QC samples after 40 days of storage at -20^{o}C was also evaluated. The concentrations ranged from 98 to 101.0 % of the theoretical values. These results confirmed the stability of BET in human plasma for at least 40 days at -20^{o}C. (Table-4).

Table 4 Stability studies

Spiked Plasma concentration (pg/ml)	Room temperature Stability		Autosampler Stability		Long term stability		Freeze and thaw stability	
	24h		55h		40 days		Cycle (48h)	
	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)
30.10	31.21±0.11	1.3	29.87±1.10	1.3	31.08±1.04	0.8	31.49±1.12	1.6
461.70	460.98±0.41	0.9	460.41±1.33	1.5	461.44±0.12	0.4	462.22±2.05	0.7

Matrix effect

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with neat standards of same concentration in alternate injections. The overall precision of the matrix factor is 5.67 for BET. There was no ion-suppression and ion- Enhancement effect observed due to IS and analyte at respective retention time.

Short Term Stock Solution Stability

Short term stock solution stability at room temperature

Stock solution each of BET and internal standard were stable after approximately 0.6 Hrs and 30 Min at room temperature. For BET and BETD4 (internal standard) the % Accuracy was 98.34 and 96.32 respectively.

Short term stock solution stability at refrigerator (2-8^{0}C)

Stock solution each of BET and internal standard were stable after approximately 08 Hrs and 30 Min at refrigerated temperature 2-8^{0}C. For BET and BETD4 (internal standard) the % Accuracy was 98.34 and 96.32 respectively.

Long Term Stock and Working Solution Stability

The long-term stock and working solution stability experiment were completed after completion of the study sample analysis.
Long term *stock solution stability in refrigerator between 2-8^{0} C*

Solution each of BET at working curve standard level and internal standard solution at working internal standard level were stable for 165 days. For BET and BETD4 internal standard) the % Accuracy was 98.34 and 96.32 respectively.

Long term working solution stability in refrigerator between 2-8^{0}C

CONCLUSION

The LC–MS/MS validated method has proved to be very simple, sensitive and reliable and successfully applied for the pharmacokinetic study in human plasma. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The major advantage of this method is the use of deuterated betahistine-d4 as an internal standard. The run time is within 8 min and only 0.200 mL of plasma was required for each determination of Betahistine, and thus the stress to volunteers or patients in clinical studies was greatly reduced. This method is very suitable and convenient for pharmacokinetics and bioavailability study of the drug Betahistine.

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