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Development and Validation of LC-MS/MS for the Estimation of Betahistine in Human Plasma

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Praveen Kumar Dasari*, Kumar Raja Jayavarapu, Ch Gopi Krishna, Tanuku Satyanarayana

Mother Teresa Pharmacy College, Sathupally, Khammam, Telangana 507303 India.

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ABSTRACT

A simple, sensitive, and specific Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) quantification of Betahistine in human plasma using deuterated Betahistine-d4 as internal Standard. The analytical method contains liquid-liquid extraction of plasma samples followed by the determination of Betahistine by an LC-MS/MS. The analyte was separated on a Zorbax Eclipse XDB -C18 (150mm x 4.6mm, 5µm) column with an isocratic mobile phase of Acetonitrile: 0.1% formic acid (80: 20 v/v) at a flow rate of 0.7 mL/min. The protonated ions were established by a turbo ion spray in a positive mode was used to detect analyte and internal standard. 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. This method was validated with the correlation coefficients of (r2)≥0.9997 over a linear concentration range of 10.00-501.60 pg/mL. This method demonstrated Intra and inter-day precision within 1.1–1.5% and 0.2–0.54% and accuracy within 99.36-100.29% and 97.40–100.47% for Betahistine.

INTRODUCTION

Betahistine Dihydrochloride (BET) well known Anti Vertigo drug[1] is chemically N-Methyl-2-pyridineethanamine. The chemical formula of Betahistine Dihydrochloride is $C_8H_{12}N_2.2HCl$ and the molecular weight is 136.194. Betahistine is official in Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), European Pharmacopoeia (EP), and the United States Pharmacopoeia (USP). In which, USP[2] and IP[3] describe the Liquid chromatographic method for estimation, while BP[4] and EP[5] describe the 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] for the estimation of Betahistine in the tablet.

A literature survey does not reveal any simple and sensitive LC-MS/MS method for the estimation of Betahistine in human plasma. The main goal of this study is to develop a simple, sensitive, rapid, accurate, and precise LC-MS/MS method for the estimation of Betahistine in human plasma.

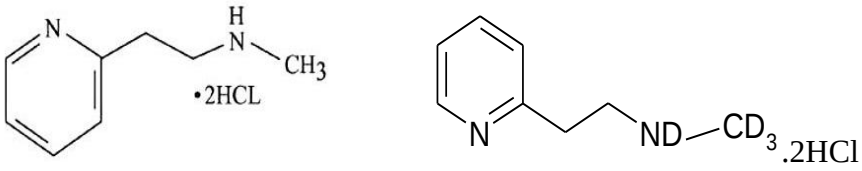
MATERIAL AND METHODS

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 operated. Data processing was carried out on the Analyst 1.4.1 software package (SCIEX).

Reagents / Materials

Betahistine Dihydrochloride (Figure 1A & B) was obtained from Cadila Pharmaceuticals, India. Betahistine Dihydrochloride-D4 (BETD4) as an internal standard was procured from Clear Synth, India. Water (HPLC Grade), Formic Acid (analytical grade) was purchased from Merck, Mumbai, India. Acetonitrile (HPLC Grade), ethyl acetate, and dichloromethane (HPLC grade) were obtained from J.T. Baker, USA. Human plasma was acquired from Navjeevan Blood Blank, Hyderabad. Milli Q water was obtained from the in-house Milli-Q system.



A **B**

Figure No. 1: Chemical structure of A) Betahistine Dihydrochloride and B) Betahistine Dihydrochloride-D4

Detection

Detection was performed by turboionspray (API) positive mode with unit resolution. For Betahistine, mass transitions were obtained from parent ion to production (137.10 m/z to 94.00 m/z). Similarly, BETD4 mass transitions were obtained from parent ion to production (140.20 m/z to 94.10 m/z).

Chromatographic conditions

Chromatographic separation was performed using an Xbridge Zorbax Eclipse XDB - C18 (150mm x 4.6mm, 5µm) at a temperature of 40ºC. The mobile phase was composed of Acetonitrile: 0.1% formic acid (80: 20, v/v) at a flow rate of 0.7mL/min. Deuterated BETD4 (Internal standard-IS) was used as the appropriate IS in terms of chromatography and extractability. BET and BETD4 were eluted at 4.46 min, approximately, with a total run time of 7 min for each sample.

Preparation of standards and quality control samples

Standard stock solutions of Betahistine (10.0 mg/mL) and internal standard BETD4 (10.0 mg/mL) were prepared in Acetonitrile. The IS spiking solution (100.0 pg/mL) was prepared in mobile phase solution (Acetonitrile: 0.1% formic acid (80: 20, v/v) from BETD4 stock solution. Standard stock solutions and internal standard spiking solutions were stored in refrigerator conditions of 2–8ºC until analysis. Standard stock solutions of BET (10.0 mg/mL) were added to the 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 (Acetonitrile: 0.1% formic acid (80:20, v/v) and stored in the refrigerator at 2–8ºC until analysis.

Sample preparation

The LLE method was used to isolate Betahistine and BETD4 from human plasma. For this purpose, 50 µL of BETD4 (10 pg/mL) and 200 µL of plasma samples were added to the labeled polypropylene tubes and vortexed for about 5 min. Thereafter, 50 µL of 0.1M NaOH solution and 3mL of extraction solvent (in the ratio of ethyl acetate: dichloromethane 80:20(v/v)) was added and vortexed for about 10 min. Next, the samples were centrifuged at 4000 rpm for 5 min at ambient temperature. From each, a supernatant sample was transferred into labeled polypropylene tubes and evaporated to dryness of 40ºC, and then reconstituted with a mobile phase solution (Acetonitrile: 0.1% formic acid, 80:20, v/v), and the sample was transferred into autosampler vials and injected into the LC-MS for study.

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, benchtop, short- term, and long-term stock solutions, working solutions and long term stability in the matrix), carryover effects, recovery, matrix effect, autosampler, re-injection reproducibility, and ruggedness experiment[10, 11].

System suitability

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

Selectivity and sensitivity

Selectivity was performed by analyzing human blank plasma samples with an additional hemolyzed group and lipidemic group to test for interference at the retention times of analytes. The sensitivity was collated with the lower limit of quantification (LLOQ) of the analyte with its blank plasma sample. The peak area of blank samples should not be higher

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 the standard curve (Linearity and range)

The linearity of the method was determined by using standard plots associated with a nine-point standard curve including LLOQ and ULOQ. The concentration of calibration curve standards was calculated against the calibration curve and the linearity of the method and they are evaluated by ensuring the acceptance of precision and accuracy of calibration curve standards. Two consecutive calibration curve standards should not exceed 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 the 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 % 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 were determined by comparing the mean peak area of the 6 replicates of extracted plasma quality control samples at high, middle and low concentrations against respective mean peak area of the six replicates of un-extracted quality control samples at high, middle and low concentrations. Recovery of more than 50% was considered requisite to obtain the required sensitivity. The % means internal standard recovery was determined by comparing the mean peak area of the internal standard in the extracted plasma quality control samples at MQC concentration against the mean peak area of the internal standard in the un-extracted quality control samples at MQC concentration.

Stability of Betahistine and Betahistine-D4

Short term stock solution stability

Short term stock solution stability for Betahistine and internal standard were performed at the stock concentration by using six consecutive injections of an aqueous standard equivalent to ULOQ concentration and working concentration respectively after storage of at least 6 hours at ambient temperature. Stability was evaluated by comparing the stock dilutions of Betahistine and internal standard from the freshly prepared stock solutions (comparison) against stock dilutions of an 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 the 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 Betahistine and BETD4 were carried out at the stock concentration by using six consecutive injections of an 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 estimated by comparing the stock dilutions of BET and BETD4 from the freshly prepared stock solutions against stock dilutions of BET and BETD4 prepared from the stock solutions stored at 2-8°C (stability). Long term stock solution stability was assessed by comparing the mean response of stability samples against mean response ratios of contrast samples.

Working solution stability

Short term stability (6 hours at ambient temperature) and long term stability (04 days at 2-8°C) for working solutions of the 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 the working solution were evaluated by comparing the mean response of stability samples against the mean response of comparison samples.

Stability of Drug in Biological Matrix

The matrix stability experiment was performed by using a 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 the 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 was determined after 1st and 3rd freeze- thaw cycles stored at -20±5°C. Six replicates of each sample of HQC and LQC were used for evaluating the freeze-thaw experiment (for the first and third cycle at both the freezing temperatures), the first freeze-thaw cycle was at 24 hours followed by a 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 a screened biological matrix. The freeze-thaw stability based on % change LQC and HQC samples were evaluated. The % Accuracy and % CV of LQC and HQC should be within ± 15.00 and ≤ 15.00 respectively.

Benchtop stability

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

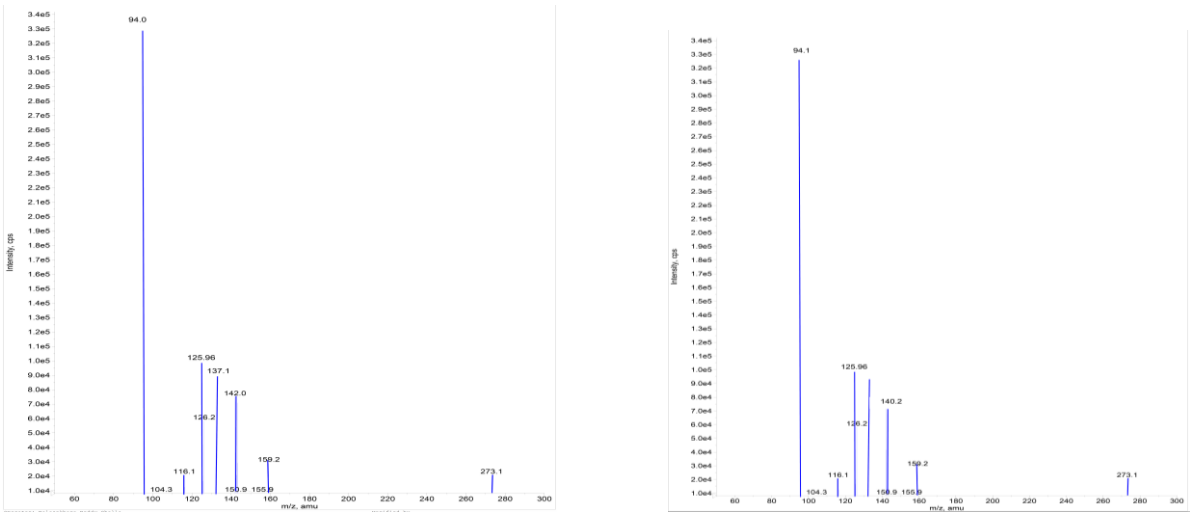
Long Term Stability in Biological Matrix

The long-term stability samples of LQC, MQC and HQC were kept frozen in vials at -20±5ºC for 55 days were assessed along with freshly processed calibration and comparison samples (six samples each of LQC, MQC, HQC). The selection of the stability duration is based on the characteristic of the analyte(s).

RESULTS AND DISCUSSIONS

Method Development

The goal of this work is to develop and validate a simple, sensitive, rapid, rugged, and reproducible assay method for the quantitative determination of Betahistine from a human plasma sample. Chromatographic conditions like 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 Betahistine and internal standard. The MS optimization was performed by direct infusion of solutions of both Betahistine and BETD4 into the ESI source of the mass spectrometer. In the ESI source, the critical parameters include the needle (ESI) voltage, capillary voltage, source temperature, and other parameters such as nebulizer gas, and heater gases were optimized to acquire a better spray shape, resulting in better ionization of the protonated ionic Betahistine and BETD4 molecules. Product ion spectrum for Betahistine and BETD4 produced high-abundance fragment ions of *m*/*z* 94.0 and *m*/*z* 94.1 respectively **(**Figure. 2**).** Good separation and elution were achieved using Acetonitrile: 0.1% formic acid (80:20, v/v) as the mobile phase, at a flow-rate of 0.7 mL/min and injection volume of 5 µL. Zorbax Eclipse XDB -C18 (150mm x 4.6mm, 5µm) column and Liquid-liquid extraction method were optimized for the best chromatography.



A **B**

Figure No. 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.

Method Validation

System Suitability

The system performance experiment was carried out by injecting six consecutive injections at the beginning of the analytical batch. % CV was 1.86.

Carryover Test

For the carryover test, two samples of the upper limit of quantification (ULOQ) and 4 samples of blank plasma were performed. These samples were introduced in the following sequence. 1) 2 blank samples 2) 2 ULOQ samples 3) 2 blank samples. Here the step (2) and (3) were repeated 2 times. The results indicate that there was no interference from the previous injection.

Selectivity and specificity

The analysis of BET and BETD4 using MRM (Multiple reaction monitoring) functions was highly selective with no interfering compounds. Chromatograms obtained from plasma spiked with Betahistine (Figure. 3).

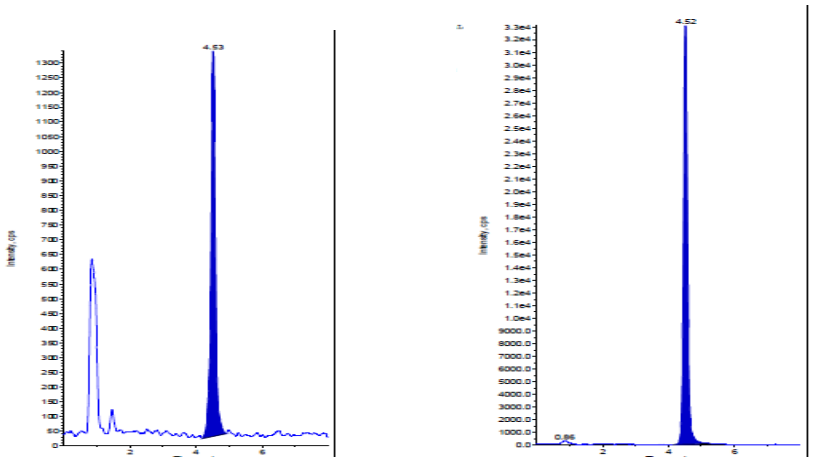


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Figure No. 3: Representative chromatograms of BET in a plasma a) Chromatogram of BET b) Chromatogram of BETD4.

Limit of Detection (LOD) and Quantification (LOQ)

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

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 10.2-501.61 pg/mL. The % CV was less than 5 % and the Accuracy ranged from 84.00 to 101%. The determination coefficients (r^2) were greater than 0.9997 for all curves (Figure.4 & Table. 1). The intra-batch % CV was less than 5% and the % Accuracy ranged from 99.36 to 100.29 %. The inter-batch % CV was less than 5% and the % Accuracy ranged from 97.4 to 100.47 % (Table. 2). These results express the adequate reliability and reproducibility of this method within the analytical range.

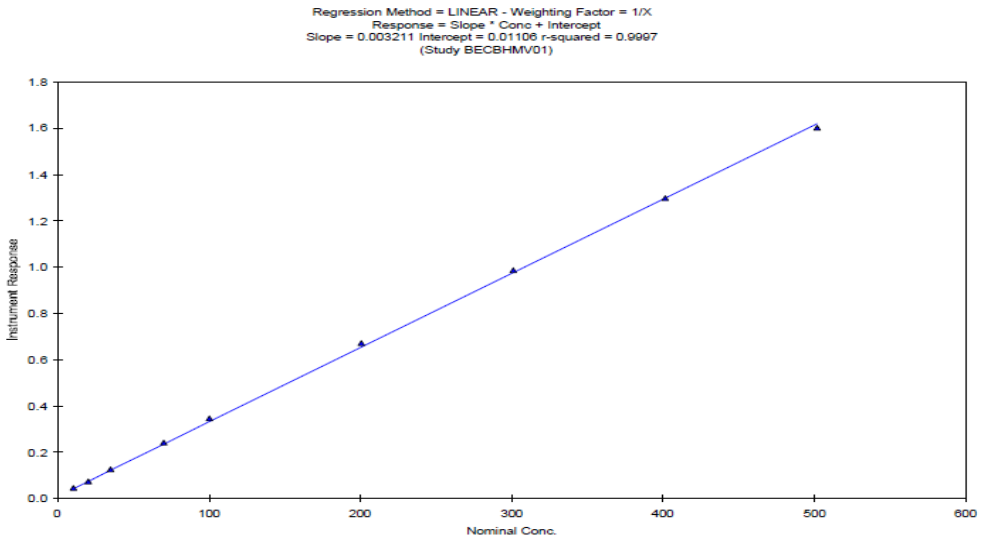

Figure No. 4: Calibration curve of Betahistine

Table No. 1: Calibration curve concentrations

Spiked plasma Concentration (pg/ml)	Concentration measured (pg/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
10.20	8.651±2.1	1.1	84.813
20.10	18.099±2.5	1.6	90.044
35.20	35.109±0.9	1.7	99.741
70.23	69.837±1	1.5	99.440
100.32	101.283±1.7	1.8	100.959
200.60	202.666±1.9	1.6	101.029
301.00	301.057±0.7	1.2	100.018
400.20	402.807±0.4	1.2	100.651
501.60	500.59±1.4	1.7	99.978

Table No. 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	29.91	1.5	99.36	29.32	0.54	97.40
250.80	249.54	1.1	99.49	248.82	0.31	99.21
461.60	462.97	1.3	100.29	463.80	0.20	100.47

Recovery

The sample preparation using Liquid-liquid extraction with Methyl tertiary butyl ether was calculated by comparing the peak area of Betahistine in plasma samples with the peak area of solvent samples and was estimated at control levels of Betahistine. The recovery of Betahistine was determined at three different concentrations 30.1, 250.8, and 461.6pg/mL were found as 83.83, 93.08, and 94.52 % respectively. The overall average recovery of Betahistine and BETD4 was found to be 90.47 and 87.18% respectively.

Short Term Stock Solution Stability

Short term stock solution stability of the sample at room temperature

Stock solutions of Betahistine and internal standard were stable after approximately 6 hours and 30 min at room temperature. For Betahistine and BETD4 the % Accuracy was 98.34 and 96.32 respectively.

Short term stock solution stability at refrigerator (2-8ºC)

Stock solutions of Betahistine and internal standard were stable after 08 hours and 30m in at refrigerated temperature 2-8ºC. For Betahistine and BETD4 the % Accuracy was 90.11 and 89.56 respectively.

Long Term Stock and Working Solution Stability

The long-term stock and working solution stability experiments were completed after completion of the study sample analysis.

Long term stock solution stability in the refrigerator between 2-8ºC

Solutions of Betahistine 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 91.21 and 92.54 respectively.

Long term working solution stability in the refrigerator between 2-8ºC

Working solutions of Betahistine at working curve standard level and internal standard solution at working internal standard level were stable for 165 days. For BET and B E T D 4 (internal standard) the % Accuracy was 95.76 and 97.78 respectively.

Stability of BET in plasma samples (Freeze-thaw, Autosampler, Benchtop, Long term)

Quantification of the BET in plasma subjected to 3 freeze-thaw (-30ºC to room temperature) cycles show the stability of the analyte and % CV was between 0.7 to 1.6, No significant degradation of the Betahistine was observed even after 55 hours storage period in the autosampler tray and the % CV was between 1.3 to 1.5. No significant degradation of the BET was observed even after 24 hours storage period in room temperature and % CV was between 0.9 to 1.3 of the theoretical values. Also, the long-term stability of BET in QC samples after 40 days of storage at -20ºC was also evaluated and % CV was ranged from 0.4 to 0.8. These results confirmed the stability of BET in human plasma for at least 40 days at -20ºC (Table-3).

Table No. 3: 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
461.60	460.98±0.41	0.9	460.41±1.33	1.5	461.44±0.12	0.4	462.22±2.05	0.7

CONCLUSION

The developed and validated LC-MS/MS method has proved to be very simple, sensitive, selective, reliable, and successfully applied for the pharmacokinetic study in human plasma. The assay method was specific due to the intrinsic 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 7 min and the only 200µL of plasma was required for each determination of Betahistine, and thus the stress to volunteers or patients in clinical studies were greatly reduced. This method is very suitable and convenient for pharmacokinetics and bioavailability study of the drug Betahistine.

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