Development and validation of UPLC–MS/MS assay for the determination of phenelzine in plasma using Solid Phase Extraction

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Abstract

A fast and highly sensitive ultra-high performance liquid chromatography (UPLC) method for the determination of phenelzine in human plasma have been developed using tandem mass spectrometry (MS/MS) detection. Hydroxyzine was used as an internal standard(ISTD). The extraction of the phenelzine from human plasma was performed using solid phase extraction. ACE-C18 (5.0µm, 100 x 4.6mm) reverse phase column was employed for chromatographic separation of analyte and internal standard for MS/MS detection at 0.9 ml/min flow. Detection was performed at transitions of m/z 137.258→ 106.906 for phenelzine and m/z 376.022→ 202.006 for hydroxyzine by positive electro-spray ionization (ESI+) in multiple reaction monitoring (MRM) mode using tandem mass spectrometry. The developed method was compared in the terms of validation parameters including linearity, sensitivity, precision and accuracy. The analysis was carried out in 3.0 min and the matrix matched calibration curves in the range of 0.508 ng/mL to 25.144 ng/mL were used for quantification with the correlation coefficients demonstrating good linearity (0.996-0.999). The mean extraction recoveries for phenelzine and IS from plasma were 96.5 % and 88.3% respectively. Matrix based samples were stable at room temperature for 12 hrs, processed samples were stable at least for 28 hrs and also stable at six freeze-thaw cycles. This method was successfully applied for determination of phenelzine in human plasma for pharmacokinetic study.

Materials and Methods

Chemicals and reagents :

Phenelzine sulphate, Hydroxyzine dihydrochloride and Ammonium Acetate were purchased from Sigma-Aldrich Chemicals. Methanol was obtained from JT Baker (LC-MS grade). Ortho Phosphoric Acid was obtained from Progressive Laboratories. Hydrochloric acid (35% Pure, AR grade) obtained from continental chemicals. Pentafluoro benzaldehyde(HPLC grade) obtained from R&D chemicals. Strata-X SPE cartridges obtained from Phenomenex Inc.) and water (LC-MS grade) were purchased from Fisher Chemicals.

Standard solutions preparation

Stock solution preparation

Approximately 5 mg of Phenelzine / 2 mg of hydroxyzine (ISTD) working standard is weighed and transferred to 10.0 mL volumetric flask, to this 5.0 mL of methanol: water (5:5) is added and sonicated to aid dissolution and the final volume is made up with methanol: water (5:5).

Preparation of internal standard dilution

The Hydroxyzine internal standard (ISTD) dilution of about 25 ng/mL from the ISTD stock solution (ISTD stock) using (methanol: water (5:5)) as the diluent is prepared.

Preparation of calibration curve (CC) standards and quality control (QC) samples

Appropriate dilutions of the stock solutions with diluent were made subsequently in order to prepare the working standard solution in the range of 25.185 ng/mL to 1257.246 ng/mL for Phenelzine. All the solutions were stored in a refrigerator between 2°C and 8°C. Calibration standards and quality control samples, in the range of 0.508 ng/mL to 25.144 ng/mL were prepared for calibration. Accuracy and precision, quality control and stability assessment was done by spiking 0.5mL of drug free plasma with appropriate volume of working solution.

Solutions used for UPLC chromatographic separation

Pure methanol was used in pump A, 5 mM ammonium acetate buffer was used in pump B, and washing solution in the ratio of 80:20 Methanol: Water was employed in pump C.

Sample preparation

Retrieved the frozen CC, QC and subject samples from the deep freezer and thawed in water bath maintained at room temperature, vortexed to mix. Removed the caps from the polypropylene tubes. An aliquot of 100 µL of CC, QC and subject samples sample in a ria vial tube was spiked with 25 µL of the ISTD solution (25 ng/mL) and vortex mixed for 30secs. Then 25 µL of 2% (v/v) Pentafluoro benzaldehyde solution was added and vortex-mixed for 30 secs, followed by addition of 200 µL of 0.1N HCL solution and vortex mixed for 1 min. SPE cartridges(strata-X) were conditioned with 1mL methanol, equilibrated with 1mL water and loaded the sample into SPE cartridges(strata-X). The SPE cartridges were washed twice with 1mL of 5% Methanol and 1mL of Water (Milli-Q/HPLC grade). The samples were eluted with 1mL methanol into pre labeled Ria vials. Then evaporated the samples to dryness under the nitrogen pressure with 50°C of temperature. The residual was reconstituted in 100 µL of a mobile phase (A-methanol and B-5mM ammonium acetate (A:B=80:20)) and centrifuged at 4,000rpm for 5 min. Then, 10 µL aliquot was injected on to the LCMS/MS system. For optimal stability, the auto-sampler temperature was set at 5 ˚C.

Data processing :

A Waters Acquity UPLC system (Solvent manager, Degasser and Auto sampler) with a column oven was coupled with an API-4000 mass spectrometry (Applied Bio Systems). Chromatograms were acquired on a API-4000 tandem mass equipped with Electrospray ionization (ESI) and connected to a PC run with the standard software Analyst -1.5.1. Mass spectroscopic detection was performed on a Triple quadrapole instrument.

The calibration curve is constructed by weighted 1/x2 least-square linear regression analysis of the peak area ratio (drug/ISTD) vs. the concentration of drug.

Results

Chromatographic and mass spectrometric conditions :

UPLC separation was carried out on a ACE C18 analytical column (5.0 µm, 100×4.6 mm) with mobile phase A-methanol and B-5mM Ammonium Acetate (A:B=80:20) at a flow rate of 0.9 mL/min and the column temperature was maintained at 35˚C. The sample injection volume was 10 µL and the analytical run time was 3.0 min. The eluent from the analytical column was introduced directly to the MS/MS system using ESI source in the positive ion mode. Source specific and compound specific mass spectrometric parameters are given in Table-1. The precursor $[M{\cdot}H]^+$ ions at m/z 137.258, 376.022 for Phenelzine and hydroxyzine were selected by the first quadrupole (Q1). After collision-induced fragmentation in Q2, the product ions at m/z 106.906, 202.006 for Phenelzine and hydroxyzine were monitored in Q3. A resolution of one unit (at half peak height) was used for both Q1 and Q3. The full scan of parent and product ion spectra for Drug and ISTD is shown in Fig. 1.

Method Validation:

Specificity and selectivity

Six human plasma samples from six individual healthy donors receiving no medication were extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent response at the Retention time of drug and internal standard were compared to the response at the lower limit of quantification (LLOQ) for drug to the response at the working concentration for internal standard. Observed Retention times were about 0.73 min (Phenelzine) and 2.08 min (Hydroxyzine) respectively. No additional peak due to endogenous substances that could have interfered with the detection of the compounds of interest was observed. Representative chromatograms of extracted human blank plasma and extracted human blank plasma spiked with Drug and ISTD are shown in Fig.2.

Linearity

The mean accuracy and precisions for back calculated concentrations of each standard calculated from calibration curves are tabulated as Table 2.

Recovery

The recoveries of Phenelzine and Hydroxyzine were evaluated with 6 replicates at three different concentration levels. In our method we got 96.5% and 88.3% recovery for Phenelzine and Hydroxyzine, which are within the acceptance criteria.

Precision and accuracy

The accuracy and precision for inter day and intra day was tabulated for drug in Table 3.

Stability

In our study quality control plasma samples were used subject to bench top (12h), Auto injector (10–84 h), freeze–thaw (-80 to +20 °C) cycles, wet extract (28 h) at room temperature, wet extract at 2-8°C (28h) and long term (90 days) at deep freezer (at -80 °C) tests are performed. The values obtained for present stability studies are tabulated (Table 4), which are within the acceptance criteria.

Application of the method

The present method was applied for a randomized cross-over bioequivalence study of two different Phenelzine preparations in 24 healthy male volunteers. After single oral administration of the drug blood samples were collected at a suitable time intervals up to 48 hours. This method was successfully used to measure the Plasma concentrations of Phenelzine.

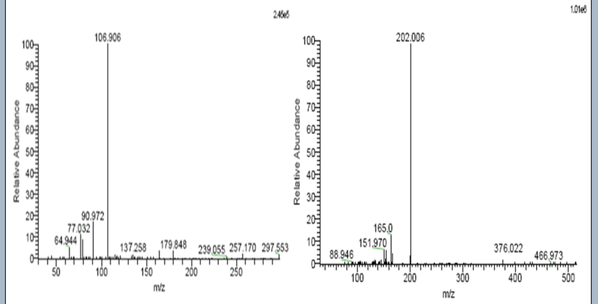

Fig.1. Full scan parent and product ion spectra's for for Phenelzine and Hydroxyzine

Parameters	Phenelzine	Hydroxyzine
Declustering potential	82.00	82.00
Entrance potential	15.00	15.00
Collision energy	28.00	30.00
Collision cell exit Poten.	13.00	10.00
Polarity	Positive	Positive
Curtain gas (CUR)	26.00	
CAD	7.00	
Ion spray voltage (ISV)	4800.00	
Heater temperature (TEM)	400.00°C	
Nebulizer gas (GS1)	45.00	
Heater gas (GS2)	45.00	
Dwell time	200 msec	
Ihe	ON	

Table-1:Source specific and compound specific mass spectrometric parameters

Nominal concentration (ng/mL)	Mean Accuracy (%)	Precision (% RSD)
25.144	90.6	12.6
20.115	96.4	7.3
16.092	95.3	2.1
12.230	97.7	6.4
6.359	100.1	6.0
2.518	92.4	7.6
1.007	98.6	3.8
0.508	94.4	7.1

Table 2: Back calculated concentrations from calibration curves

Nominal Conc. (ng/mL)	0.520	1.270	10.585	18.250
Intra-day accuracy(%)(day1)	98.8	102.4	103.4	97.7
Intra-day precision(%)(day1)	3.7	2.5	5.2	7.6
Intra-day accuracy(%)(day2)	89.1	99.4	97.8	95.2
Intra-day precision(%)(day2)	5.6	4.6	2.2	9.3
Intra-day accuracy(%)(day3)	94.8	95.5	95.8	94.6
Intra-day precision(%)(day3)	9.2	6.5	7.4	4.1
Overall accuracy (%)	94.2	99.1	99.0	95.8
Overall Precision (%)	6.2	4.5	4.9	7.0
Number of determinations	18	18	18	18

Table 3.Assessment of Accuracy and precision of the method

	18.250		1.270	
	Precision	Accuracy	Precision	Accuracy
Freeze thaw stability	5.7	96.1	9.5	98.4
Bench top stability	4.3	102.2	3.5	104.2
Wet stability at RT	2.1	109.4	6.0	93.5
Wet stability at 2-8°C	6.9	97.4	4.8	102.7
Auto sampler stability	3.9	107.7	2.5	108.7
Long term stability	4.7	105.8	7.0	93.1
Interim stability	3.0	104.1	7.7	104.0

Table 4.Stability results

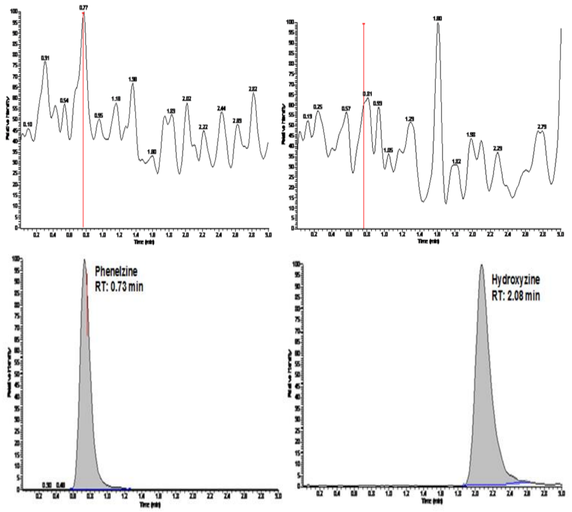

Fig.2. Representative chromatograms of extracted human blank plasma and extracted human blank plasma spiked with Drug and ISTD

Conclusion

The method was applied successfully to the analysis of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence study after therapeutic doses of Phenelzine. The established LC-MS/MS method is sensitive and suitable for the study of Phenelzine in human plasma. Because of the relative short chromatographic runtime (3.0 min), the method is easy to follow and can be adopted for clinical drug monitoring.

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