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Research Article

Determination of phenelzine in human plasma sample using SPE- UPLC–MS/MS assay

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Abstract

In this paper 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 (IS). The extraction of the phenelzine from human plasma was performed using solid phase extraction. ACE-C18 (5µm, 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 (0.994-0.999). The mean extraction recoveries for phenelzine and IS from plasma were 96.5 % and 95.3% 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.

Keywords: Phenelzine, human plasma, UPLC, LC-MS/MS, solid Phase extraction, validation.

1. Introduction

Phenelzine is a potent, long-term non-selective monoamine oxidase inhibitor (MAOI) [1-4]. Phenelzine is a hydrazine derivative and clinically used in the treatment of social phobia, panic disorder and depression, particularly depression associated with anxiety [5-8]. It has a molecular weight of 136.19 g/mol and is chemically described as C8 H12 N2 .

Phenelzine increases brain levels of dopamine, norepinephrine, and serotonin [1,2]. Interestingly, Phenelzine also produces large increases in brain levels of the amino acid GABA [2–4, 9, 10], an effect that appears to be related to the anxiolytic properties of PLZ [11].

A mean peak plasma concentration (Cmax) of 19.8 ng/mL occurred at a time (Tmax) of 43 minutes post dose after a single 30 mg dose of phenelzine sulfate(2 X 15 mg tablets) [12-13]. phenelzine sulfate is extensively metabolized, primarily by oxidation via monoamine oxidase. After oral administration of 13C6-phenelzine, 73% of the administered dose was recovered in urine as phenylacetic acid and parahydroxyphenylacetic acid within 96 hours [12-13]. The mean elimination half-life after a single 30 mg dose is 11.6 hours. Multiple dose pharmacokinetics has not been studied in humans [12-13].

Analytical methods so far reported for quantification of phenelzine, employing electron-capture gas chromatography in brain tissue and urine[14], chromatography—mass spectrometry in human plasma[15], Gas Chromatography with Nitrogen Specific Detection in human fluids[16], capillary gas chromatography with nitrogen specific detection for human plasma[17], High-Performance Liquid Chromatography with Diode Array Detection in

Urine[18], fluorescence detection for Brain tissue [19] and LC-MS/MS detection in Hair samples [20] . To the best of our knowledge, no LC-MS/MS methods have been reported for quantification of phenelzine in human plasma yet. To achieve the goal, the sensitive LC-MS/MS method was developed and validated for determination of phenelzine in human plasma. The method was successfully applied to analyze human plasma samples collected from a pharmacokinetic study.

This paper describes the development and validation of selective and sensitive reverse-phased UPLC method with tandem mass spectrometry (MS/MS) detection for determination of phenelzine in human plasma after solid phase extraction by using hydroxyzine dihydrochloride as internal standard. The applicability of this method for health human pharmacokinetic study was evaluated as well.

2. Materials And Methods

2.1 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.

2.2 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) [20-25]. Chromatograms were acquired on a API-4000 tandem mass spectrometry equipped with Electrospray ionization (ESI) and connected to a PC run with the standard Analyst software. Mass spectroscopic detection [26-30] was performed on a Triple quadrapole instrument (API-4000, Applied Bio Systems). The calibration curve is constructed by weighted $1/x^2$ least-square linear regression analysis of the peak area ratio (drug/ISTD) vs. the concentration of drug [31-32].

2.3. Standard solutions preparation

2.3.1. Stock solution preparation

Approximately 2 mg of Phenelzine / 2 mg of hydroxyzine (IS) 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).

2.3.2. Preparation of internal standard dilution

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

2.3.3. 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.

2.4. Solutions used for UPLC chromatographic separation

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

2.5. 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 in a ria vial tube was spiked with 25 µL of the IS 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 20 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=90:10)) and centrifuged at 4,000rpm for 5 min. Then, 10 µL aliquot was injected on to the LC-MS/MS system. For optimal stability, the auto-sampler temperature was set at 5 °C.

3. Results and discussion

3.1. 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=90:10) 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 are 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 are monitored in Q3. A resolution of one unit (at half peak height) is used for both Q1 and Q3. The full scan of parent and product ion spectra is shown in Figs. 1–2.

3.2 Method Validation [20-39] :

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose" (International Conference on Harmonization Guideline Q2A)[38] "Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use" (US Food and Drug Administration Draft Guidance for Industry, 2013) [39].

3.2.1. 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 IS are shown in Fig.3.A and 3.B.

Table 1: Source specific and compound specific mass spectrometric parameters

Parameters	Phenelzine	Hydroxyzine
Declustering potential(DP)	82.00	82.00
Entrance potential (EP)	15.00	15.00
Collision energy (CE)	28.00	30.00
Collision cell exit potential (CXP)	13.00	10.00
Polarity	Positive	Positive
Curtain gas (CUR)	26.00	
Collision associated dissociation(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	

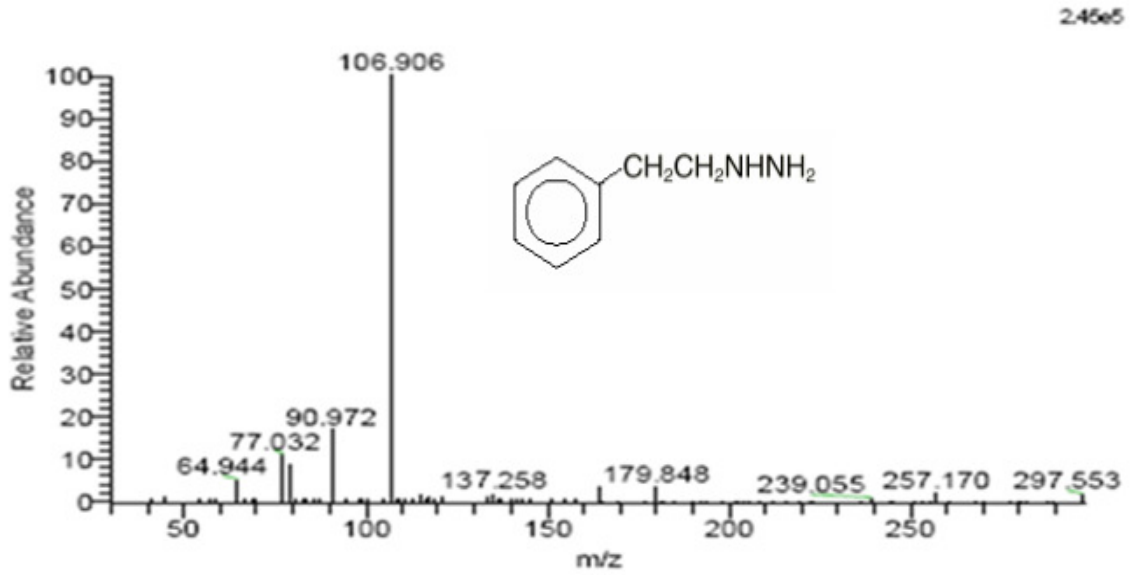

Fig. 1. Mass spectra of the Phenelzine precursor (137.258m/z) and major fragment (106.906m/z).

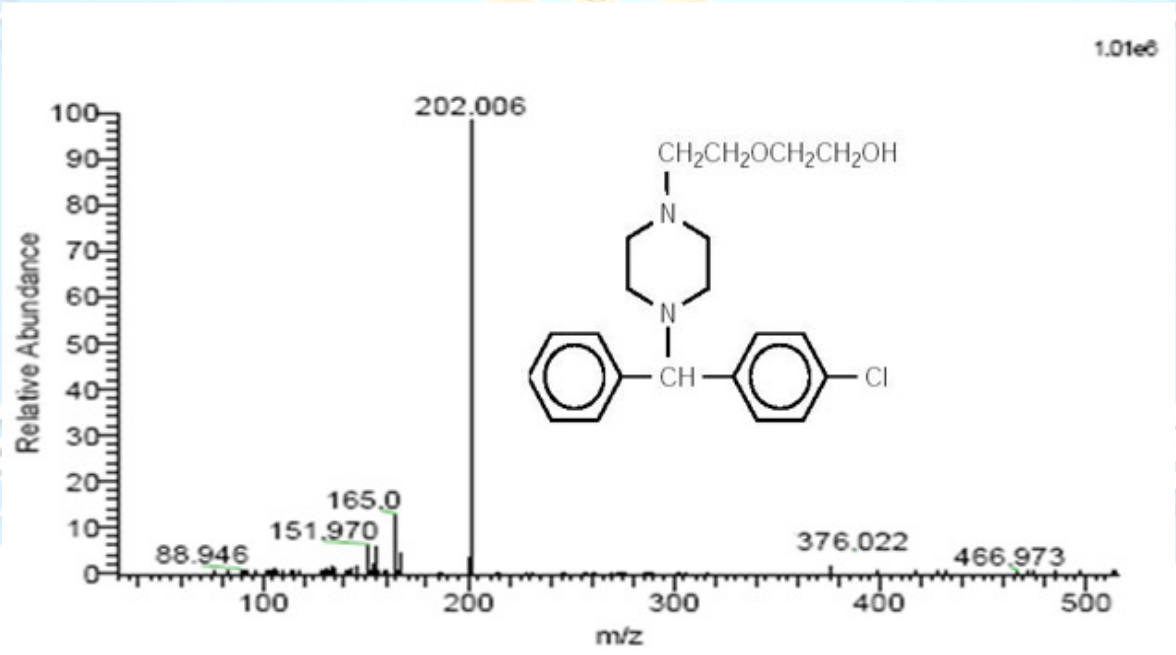

Fig. 2. Mass spectra of the Hydroxyzine precursor (376.002m/z) and major fragment. (202.006 m/z)

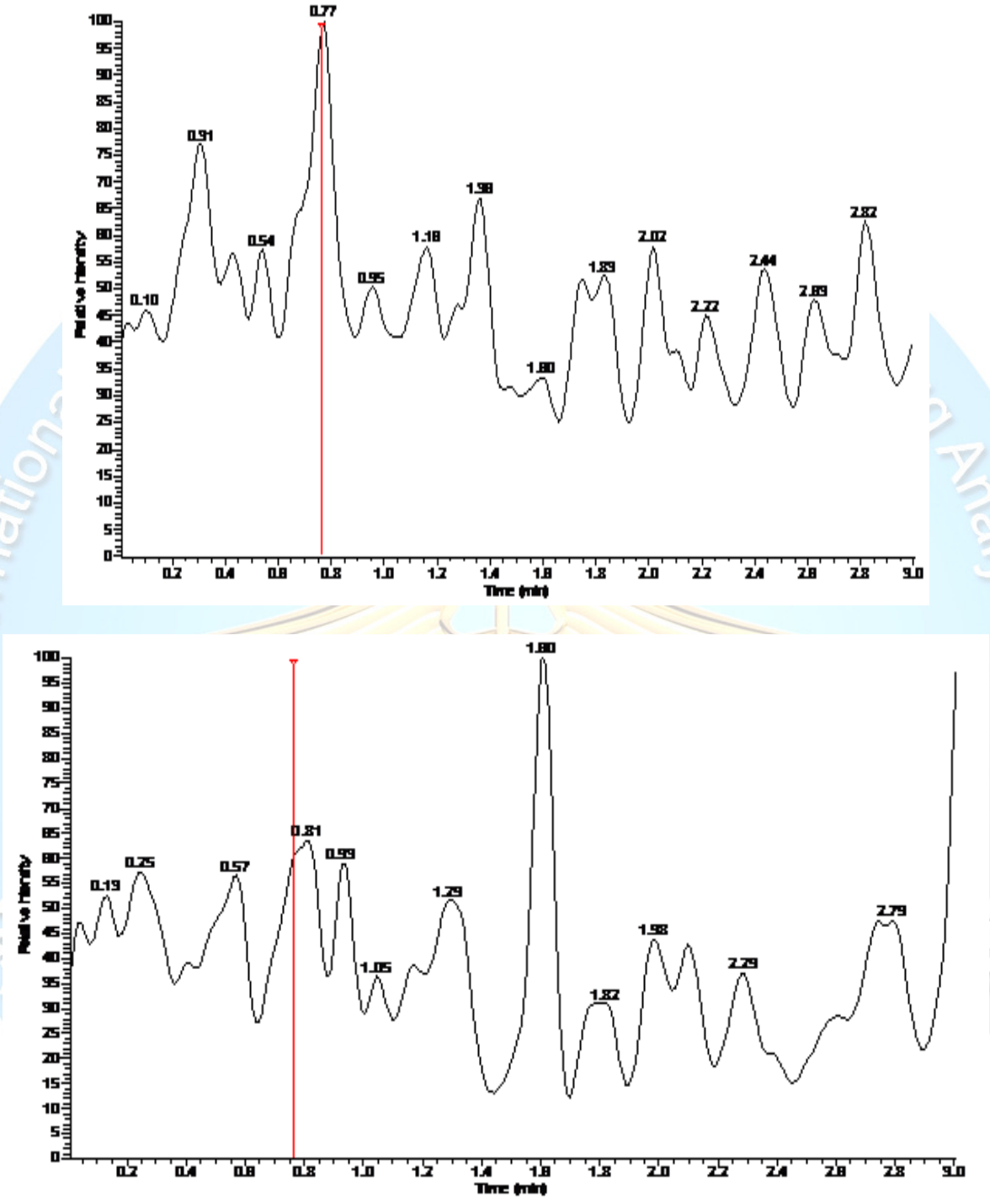

Fig. 3.(A) Representative Chromatograms (Phenelzine & Hydroxyzine) from an extract of Human blank plasma

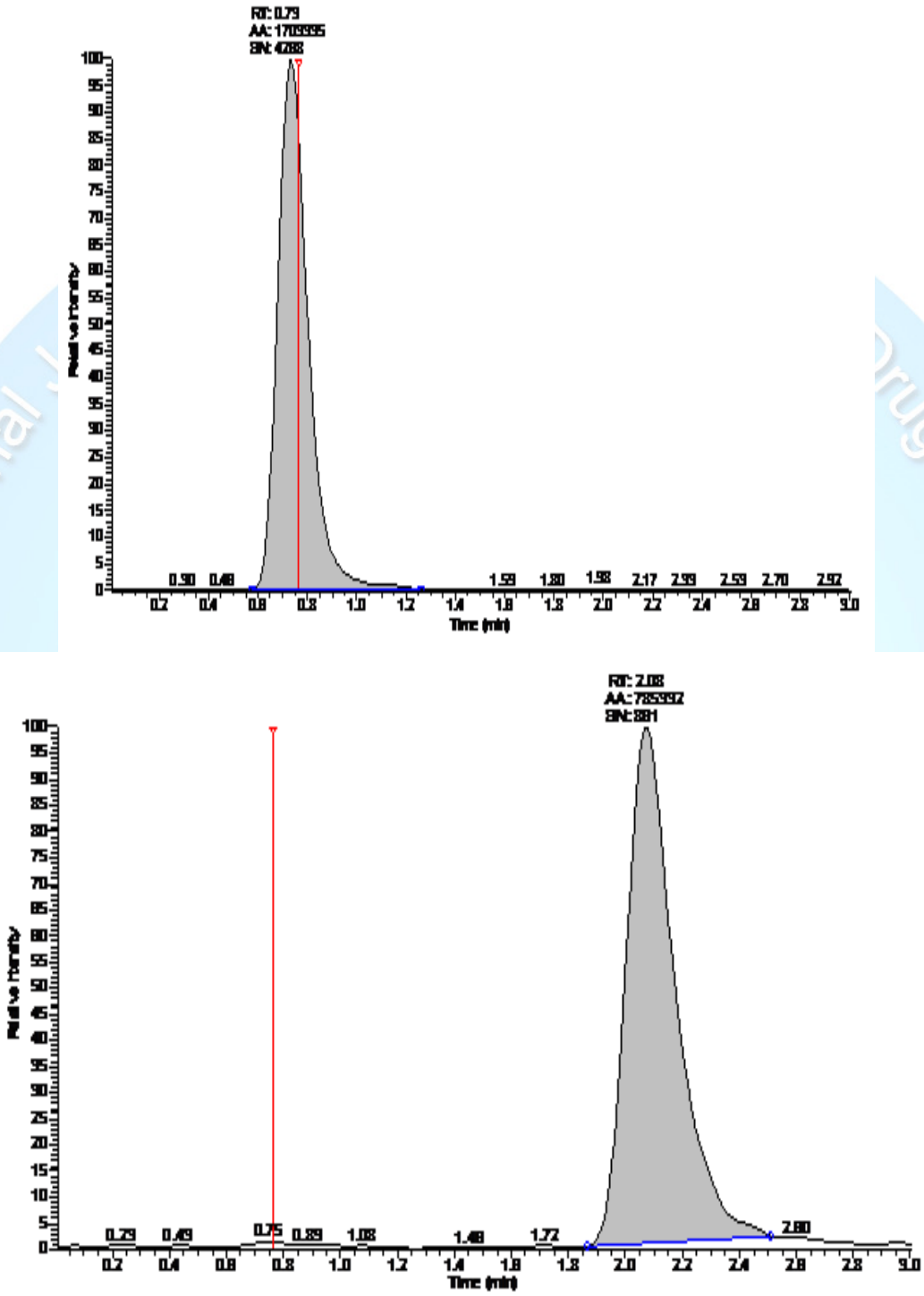

Fig. 3.(B) Representative Chromatograms (Phenelzine & Hydroxyzine) from an extract human blank plasma spiked with Phenelzine and Hydroxyzine (as IS)

3.2.2. Linearity

Linearity means that the assay provides test results that are proportional to the concentration of the analyte in the sample with directly or via a mathematical transformation. The relationship between the experimental response value and known concentrations of the analyte is referred to as calibration curve. In our study calibration curve is

constructed by weighted $1/x^2$ of the peak area ratio (drug/IS) with the above calibration standards to generate a calibration curve. Linear calibration curves were obtained with a coefficient of correlation (r^2) usually higher than 0.994. For each calibration standard level, the concentration was back calculated from the linear regression curve equation. The mean accuracy and precisions for back calculated concentrations of each standard calculated from calibration curves were tabulated as Table 2.

Table 2.Back calculated concentrations from calibration curves

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

Accuracy: 100% measured concentration/nominal concentration.

Precision: coefficient of variation (100% standard deviation/mean).

3.2.3. Recovery

Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations with unextracted standards that represent 100% recovery. Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible. The recoveries of Phenelzine and Hydroxyzine were evaluated with 6 replicates at 3 different concentration levels. In our method we got 96.5% and 95.3% recovery for Phenelzine and Hydroxyzine, which are within the acceptance criteria.

3.2.4. Precision and accuracy

Intra-day accuracy and precision were evaluated by analysis of quality control samples at 4 different levels (n=6 at each level) on the same day. These levels were chosen to demonstrate the performance of the method and to determine the lower limit of quantification of the method. The upper limit of quantification was given by the highest level of the calibration curve. Samples with concentration above this upper limit of quantification should be diluted prior to reanalysis. To assure the interday accuracy and precision, the intraday assays were repeated on 3 different days. The overall performance was calculated. The results were found to be quite comfortable as per international guidelines. The accuracy and precision for inter day and intra day was tabulated for drug in Table 3.

Table 3.Assessment of Accuracy and precision of the method

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 4.Stability results

Nominal Conc. (ng/mL)	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 extract stability at room temperature	2.1	109.4	6.0	93.5
Wet extract stability at 2-8ºC	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 storage stability at -25 °C	3.0	104.1	7.7	104.0

Table 5.Pharmacokinetic parameters of phenelzine

Parameters	Phenelzine			
	Test (T)		Reference (R)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
C_{max} (ng/mL)	8.600±0.529	6.2	12.367 ± 0.321	2.6
AUC_t(hr.ng/mL)	41.577 ± 1.723	4.1	50.034±1.044	2.1
AUC_{inf}(hr.ng/mL)	42.270 ±1.466	3.5	52.315±0.944	1.8
T_{max} (hr)	0.833 ± 0.144	17.3	1.000 ± 0.000	0.0
$t_{1/2}$ (hr)	2.852± 0.736	25.8	2.922 ± 0.119	4.1
k_{el}(1/hr)	0.256 ± 0.077	29.9	0.238 ± 0.010	4.0

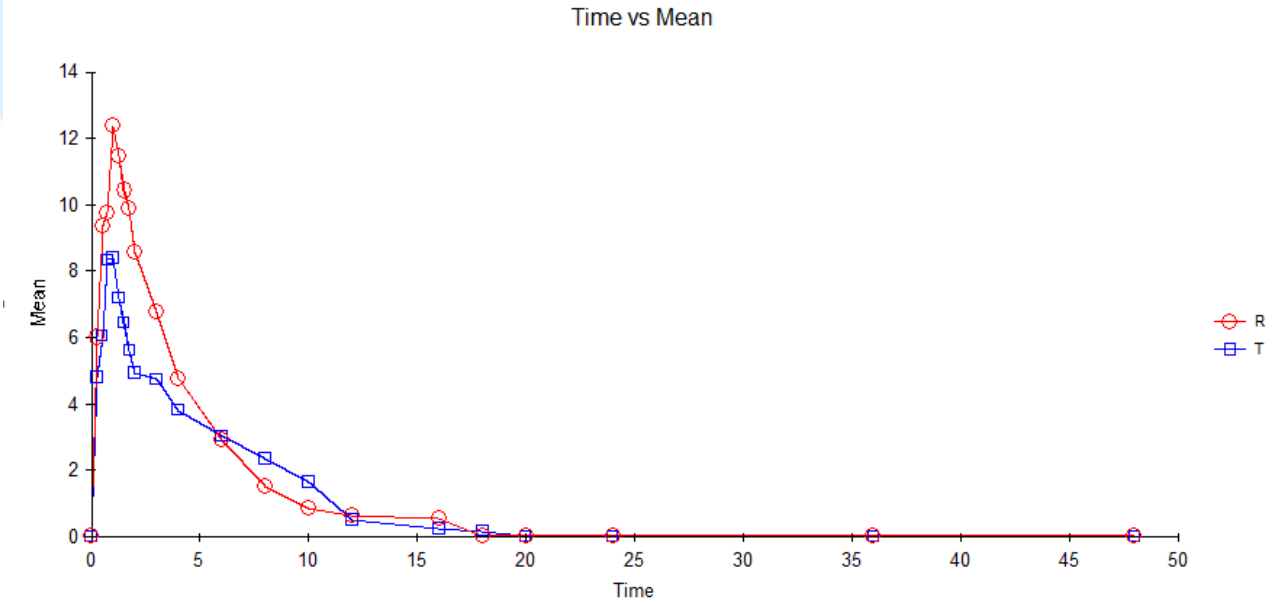

Fig. 4. Mean plasma concentration – time profiles of Phenelzine

3.2.5. Stability

According to FDA guidelines for industry effect of freeze–thaw, bench top, short-term, long-term, stock solution and post preparative stability assessments are evaluated as a part of bioanalytical method validation. In our study quality control plasma samples are 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 (48h) 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.

3.3. 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. Various Pharmacokinetic parameters established and compared for the both of the preparations were given in Table 5. Plasma concentration- time profiles were given as graph (Figure 4.).

4.0 Conclusion

To our knowledge, this is the first fully validated LC–MS/MS method for quantification of phenelzine in human plasma. 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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