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Research Article

VALIDATION AND APPLICATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY ASSAY FOR LETROZOLE IN HUMAN PLASMA

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ABSTRACT

A simple, rapid, sensitive and specific liquid chromatography–tandem mass spectrometry method was developed and validated for quantification of letrozole (I), non-steroidal aromatase inhibitor for the treatment of hormonally-responsive breast cancer after surgery. The analyte and internal standard, letrozole d4 (II), were extracted by liquid–liquid extraction with tert-butyl methyl ether using a Heidolph vibramax 110 shaker. The chromatographic separation was performed on a reversed-phase Hypersil BDS C18 column with a mobile phase of 10mM ammonium acetate–acetonitrile (10:90, v/v, pH adjusted to 3.0 with acetic acid). The protonated analyte was quantitated in negative ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions *m/z* 284.10 → 242.10 and *m/z* 288.00 → 246.00 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 0.2–100.0 ng/mL for Letrozole in human plasma. The lower limit of quantitation was 200 pg/mL with a relative standard deviation of less than 15%. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. A run time of 3 min for each sample made it possible to analyze a throughput of more than 360 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies.

Keywords: Letrazole, hormonally-responsive breast cancer , Liquid-liquid extraction, Bioavailability, Bioequivalence

INTRODUCTION

Letrozole (4-[(4-cyanophenyl)-(1,2,4-triazol-yl)methyl]benzonitrile , see Fig. 1. is an oral non-steroidal aromatase inhibitor for the treatment of hormonally-responsive breast cancer after surgery. Estrogens are produced by the conversion of androgens through the activity of the aromatase enzyme. Estrogens then bind to an estrogen receptor, which causes cells to divide. Letrozole prevents the aromatase from producing estrogens by competitive, reversible binding to the heme of its cytochrome P450 unit. The action is specific, and letrozole does not reduce production of mineralo- or corticosteroids. In contrast, tamoxifen interferes with the estrogen receptor. Tamoxifen is also used to treat hormonally- responsive breast cancer. However, letrozole is effective only in post-menopausal women, in whom estrogen is produced predominantly by the adrenal glands. In pre-menopausal women, estrogen is produced in the ovaries, and letrozole is ineffective. In the BIG 1-98 Study, of post-menopausal women with hormonally-responsive breast cancer, letrozole improved the recurrence of cancer, but not survival, compared to tamoxifen.

Separation and determination of letrozole was achieved by liquid chromatography – tandem mass spectrometry (LC-MS/MS) with gradient elution. letrozole was well separated between 1 and 2 min at pH 3.0 respectively. The sensitivity of this method for letrozole is 0.2 ng/mL. For purification of the plasma sample, it was pre-treated by liquid-liquid extraction and followed by reversed-phase HPLC using a Hypersil BDS column.

It is well known that HPLC tandem MS (MS-MS) further enhances specificity and provides an improved signal-to-noise ratio as compared with single-stage MS (Willoughby *et al.,* 2002). The purpose of this work was to exploit the high selectivity and sensitivity of the triple quadrupole MS system operated in MS-MS mode with an ESI interface for the development and validation of a robust reversed-phase LC-MS-MS method for letrozole quantification in human plasma. It was essential to establish an assay capable of quantifying letrozole at concentrations down to 0.2 ng /mL. At the same time, it was expected that this method would be efficient in analyzing a large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of letrozole.

In recent years, a number of laboratories have reported the use of high-throughput bioanalytical procedures using LC-MS/MS (Jemal, 2000; Ramakrishna *et al.,* 2004a–e). Our method is simple, rapid, robust, specific and sensitive, which makes it an attractive procedure in high-throughput bioanalysis.

EXPERIMENTAL

Chemicals

Letrozole reference standard (99.1% pure, concentrations hereafter are reported as free base corrected for purity unless otherwise stated) was obtained from VARDA Biotech (Mumbai, India). Letrozole-d4 was employed as an internal standard (IS) and obtained from BDG Synthesis (Willington, New Zealand). Chemical structures are presented in Fig. 1. Drug-free human plasma, containing K_2/K_3EDTA as an anticoagulant, was obtained from the Hima bindu blood bank (Hyderabad, India). Stock solutions of letrozole (1 mg/mL) and IS (1 mg/mL) were separately prepared in 10 mL volumetric flasks with methanol. HPLC-grade Labscan methanol and Rankem acetonitrile were from Merck (Darmstadt, Germany). Tert-butyl methyl ether was from Merck(Worli, Mumbai, India). HPLC type I water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC-MS/MS instrument and conditions

The HPLC, Shimadzu HTC (Shimadzu corporation Japan) was equipped with a G1312A binary pump, a G1379A degasser, a G1367A autosampler equipped with a G1330B thermostat, a G1316A thermo statted column compartment and a G1323B control module. The chromatography was on a Hypersil BDS, C_{18} column (5.0 µm, 50 x 4.6 mm i.d.) at 40°C temperature. The mobile phase composition was a mixture of 10mm ammonium acetate (pH 3.0 with acetic acid) –acetonitrile (10:90, v/v), which was pumped at a flow-rate of 0.5 mL/min.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo ion spray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.4.4 software package (SCIEX).

Table 1: Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature, °C	450
Dwell time per transition, ms	200
Ion source gas (gas 1), psi	35
Ion source gas (gas 2), psi	40
Curtain gas, psi	25
Collision gas, psi	5
Ion spray voltage, V	-5000
Entrance potential, V	-10
Declustering potential (DP), V	-70 (Analyte) and IS
Collision energy, V	-30 (Analyte) and -30(IS)
Collision cell exit potential, V	-5 (Analyte) and -11 (IS)
Mode of analysis	Negative
Ion transition for letrozole, *m/z*	284.10 / 242.10
Ion transition for letrozole-d4, m/z	288.00 / 246.00

Table 2: Precision and accuracy data of back-calculated concentrations of calibration samples for letrozole in human plasma

Concentration added (ng/mL)	Concentration found (mean ± SD, *n* = 4; ng/mL)	Precision (%)	Accuracy (%)
0.2	0.19559 ± 0.008	3.205	96.950
0.4	0.39535 ± 0.013	1.877	100.575
2.0	2.0792 ± 0.029	2.734	101.175
8.0	8.2537 ± 0.136	2.572	101.400
25.0	24.867 ± 0.353	3.3.28	100.850
50.0	49.161 ± 0.564	0.785	98.825
75.0	75.723 ± 0.929	1.618	99.950
100.00	99.690 ± 0.488	0.863	100.275

Table 3: Precision and accuracy of the LC-MS/MS method for determining letrozole concentrations in plasma samples

Concentration added (ng/mL)	Within-batch precision (*n* = 6)			Between-batch precision (*n* = 3)		
	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.2	0.190 ± 0.008	4.4	92.9	0.186 ± 0.005	4.0	90.7
0.5	0.552 ± 0.061	11.1	107.8	0.520 ± 0.024	3.7	99.9
30	31.042 ± 0.940	3.2	103.1	29.984 ± 0.418	2.8	99.5
70	71.779±1.483	2.2	102.5	70.207 ± 0.779	2.0	100.7

Table 4: Stability of human plasma samples of letrozole

Sample concentration (ng/mL; n=6)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short – term stability for 7h in plasma			
0.5	0.51119	2.2	96.8
70.0	69.930	1.3	100.48
In Process sample Stability (2hr 9 min)			
0.5	0.51119	2.3	103.45
70.0	69.930	2.6	103.02
Post Processing Sample stability 70h			
0.5	0.51119	1.3	105.65
70.0	69.930	1.1	107.57
Three freeze-thaw cycles -70°C ± 15°C			
0.5	0.51119	5.4	97.98
70.0	69.930	2.1	100.65
Three freeze-thaw cycles -20°C ± 10°C			
0.5	0.51119	3.1	100.82
70.0	69.930	3.7	100.87
Re - injection Reproducibility 32hrs at 10°C			
0.5	0.51119	1.3	105.65
70.0	69.930	1.1	107.57
Long – term Stability for 472 days at -70° C ± 15°C			
0.5	0.51119	2.7	103.65
70.0	69.930	1.3	102.27

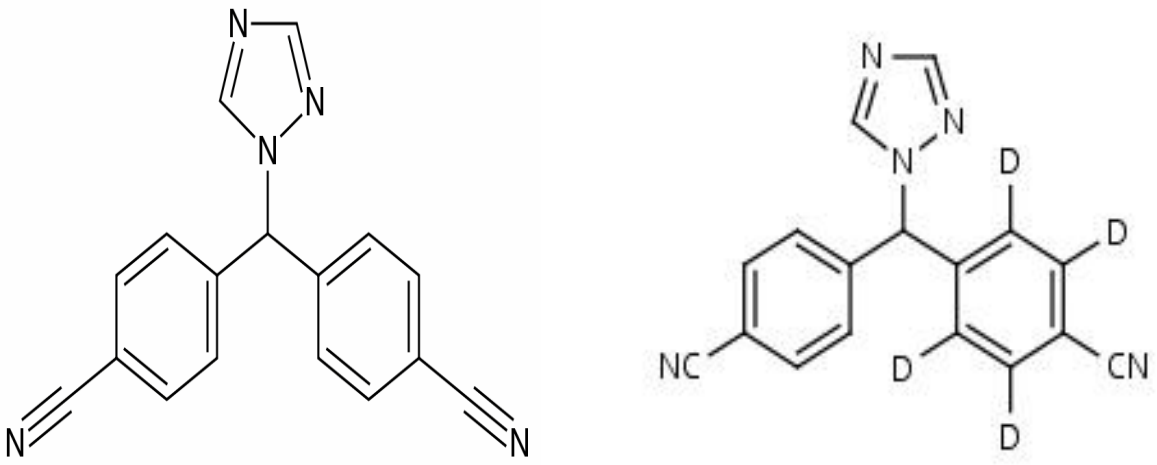


Fig. 1: Litrozole, Litrozole d4

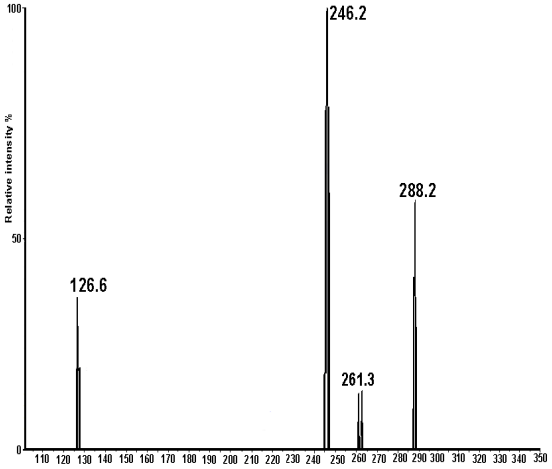


Fig. 2: Full-scan positive-ion turboIonspray (A) Q1 mass spectra and (B) product ion mass spectra of IS (letrozole-d4).

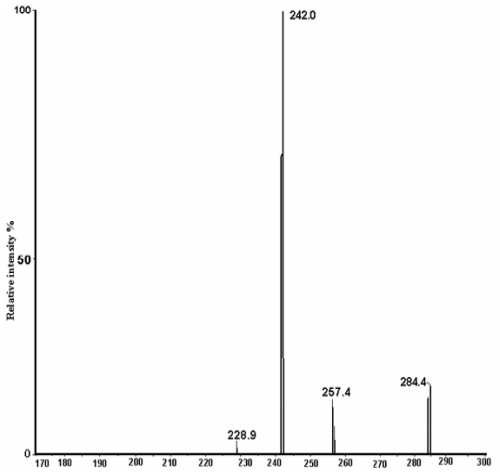
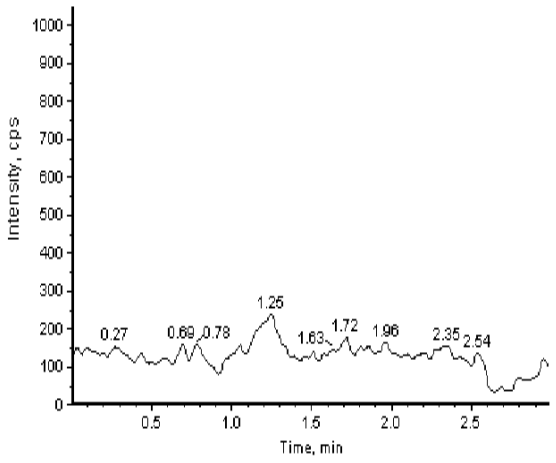


Fig. 3: Full-scan positive-ion turbo Ionspray (A) Q1 mass spectra and (B) product ion mass spectra of IS (letrozole-d4)



Extracted Blank Plasma Letrozol

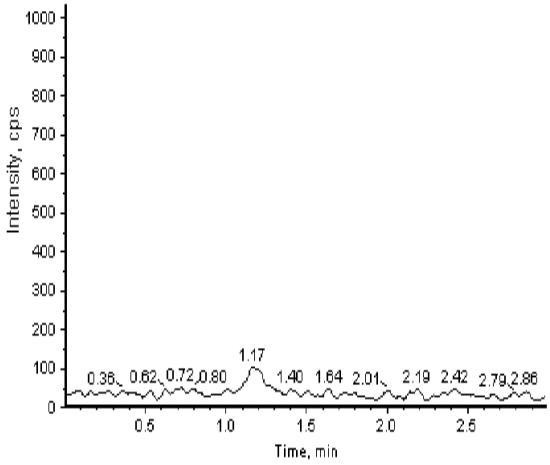
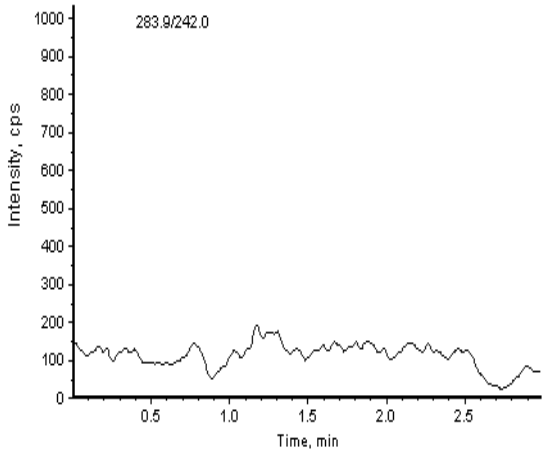


Fig. 4: Extracted Blank Plasma at IS



Blank Letrozole at Zero standard

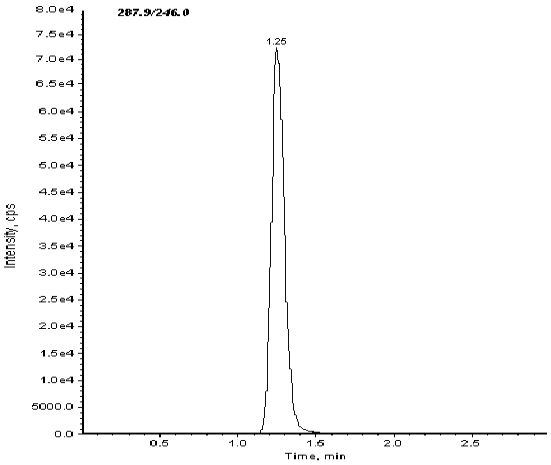


Fig. 5: IS at Zero Standard

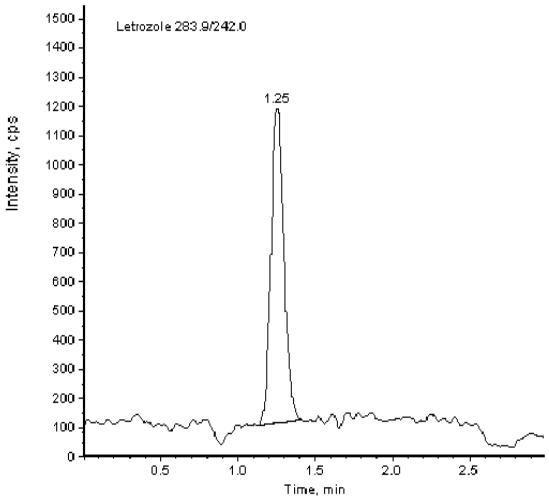


Fig. 6: Extracted LLOQ

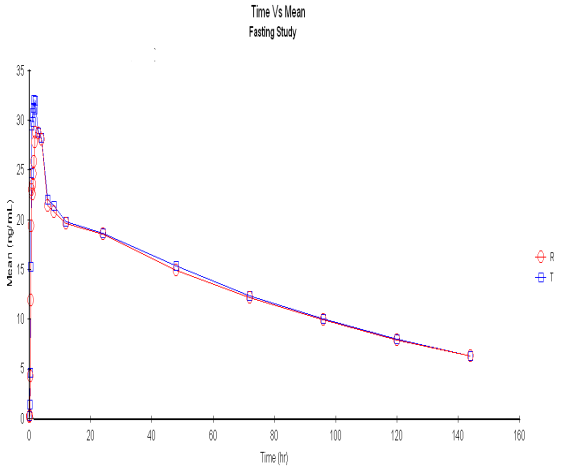
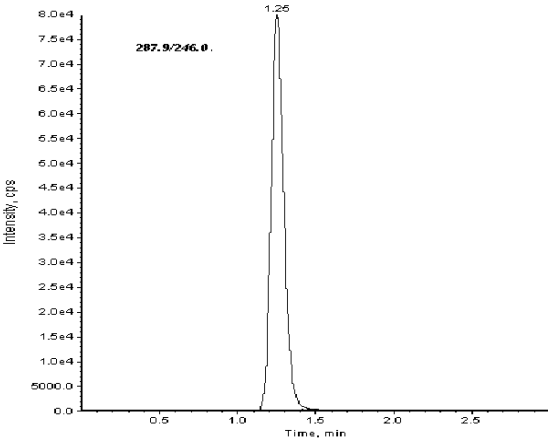


Fig. 8:



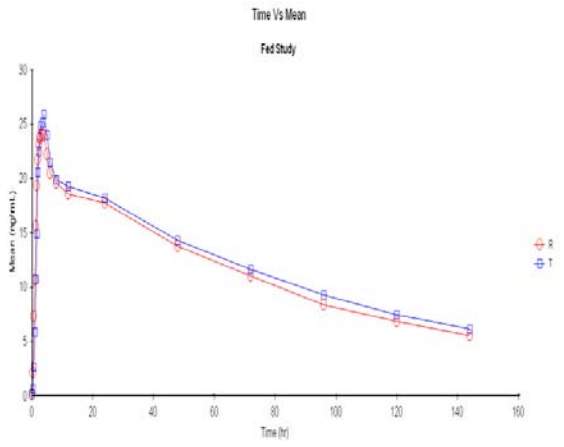


Fig. 7: IS at LLOQ level

Sample processing

A 250 µL volume of plasma sample was transferred to a 15 mL plastic test tube, and then 50 µL of IS working solution (200 ng/mL) was spiked. After vortexing for 30 s, a 3 mL aliquot of extraction solvent, tert-butyl methyl ether was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortex-mixed for 3 min using a Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The organic layer (2.4 mL) was transferred to a 5 mL plastic

tube and evaporated to dryness using TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 500 µL of diluent (10mm ammonium acetate (pH 3.0 with acetic acid) –acetonitrile (10:90, v/v) and a 10 µL aliquot was injected into chromatographic system.

Bioanalytical method validation

Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol (1:1). The IS working solution (200 ng/mL) was prepared by diluting its stock solution with water–methanol (1:1). Aliquots of 250 µL plasma containing 5 µL of working solution. letrozole concentration levels of 0.2, 0.4, 2.0, 8.0, 25.0, 50.0, 75.0 and 100.0 ng/mL. Quality control (QC) samples were prepared in bulk, at concentrations of 0.2 (LLOQ), 0.5 (low), 30.0 (medium) and 70 ng/mL (high).

A calibration curve was constructed from a blank sample (a plasma sample processed without an IS), a zero sample (a plasma processed with IS) and eight non-zero samples covering the total range (0.2–100.0 ng/mL), including the lower limit of quantification (LLOQ). Such calibration curves were generated on four consecutive days. Linearity was assessed by a weighted ($1/x$) least squares quadratic regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

The within-batch precision and accuracy were determined by analyzing six sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing six sets of quality control samples on three different batches. The acceptance criteria of within- and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations and for accuracy were 100 ± 20% or better for LLOQ and 100 ± 15% or better for the rest of concentrations.

Recovery of letrozole from the extraction procedure was determined by a comparison of the peak area of letrozole in spiked plasma samples (six low and high quality controls) to the peak area of letrozole in samples prepared by spiking extracted drug-free plasma samples with the same amounts of letrozole at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted quality control samples (n = 6) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

RESULTS AND DISCUSSION

Electro spray MS-MS was used to analyze letrozole, as it is beneficial in developing a selective and sensitive method. The positive ion Turbo Ion spray Q1 mass spectrum and product ion mass spectrum of letrozole and the IS are shown in Figs2 and 3, respectively. The most sensitive mass transition was from m/z 284.10 to 242.10 for letrozole and from m/z 288.00 to 246.00 for the IS. LC-MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity, selectivity and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

Specificity

The specificity of the method was examined by analyzing blank human plasma extract (Fig. 4) and an extract spiked only with the internal standard (Fig. 5). As shown in Fig. 4, no significant interference in the blank plasma traces was seen from endogenous substances in drug-free human plasma at the retention time of the analyte. Figure 5 shows the absence of interference from the internal standard to the MRM channels of the analyte. Figure 6 depicts a representative ion-chromatogram for the lower limit of quantitation (LLOQ, 0.2 ng/mL) of the calibration curve. Excellent sensitivity was observed for a 10 µL injection volume (LLOQ) corresponding to ca. 0.2 ng on-column.

The product ion chromatogram obtained from an extracted plasma sample of a healthy subject who participated in a bioequivalence study conducted on 72 (Fasting & Fed) subjects. Letrozole was unambiguously identified and was quantified as 0.2 ng/mL.

Owing to the components of the sample matrix, signal suppression or enhancement may occur. These matrix effects in the LC-MS/MS method were evaluated by spiking blank plasma extracts with low and high QC samples. The resulting chromatograms were compared with chromatograms of pure samples equally concentrated. Six independent plasma lots were used with six samples from each lot. The results (data was not shown) showed that there was no significant difference for peak responses between these samples. This effect was most likely due to the sample cleanup with LLE.

The extraction recovery of letrozole was 65.737% on average, and the dependence on concentration is negligible. The recovery of the IS was 66.847% at the concentration used in the assay (200 ng/mL). Recovery of the analyte and IS were low, but it was consistent, precise and reproducible. With the consistency in the recovery of letrozole and IS, the assay has proved to be robust in high-throughput bioanalysis.

Lowest concentration

The lower limit of quantitation (LLOQ) of letrozole in human plasma assay was 0.2 ng/mL. The between- batch precision at the LLOQ was 4.0%. The between-batch accuracy was 90.7% (Table 3). The within-batch precision was 4.4% and the accuracy was 86.0% to 92.9% for letrozole.

Middle and upper concentrations

The middle and upper quantitation levels of letrozole ranged from 0.5 to 70 ng/mL in human plasma. For the between-batch experiment, the precision ranged from 2.0 to 3.7 and the accuracy ranged from 99.5 to 100.7 (Table 3). For the within-batch experiment, the precision and accuracy for the analyte met the acceptance criteria (±15%) and precision was below 12% at all concentrations tested.

Stability

The stability of the analytes in human plasma under different temperature and timing conditions, as well as the stability of the analytes in stock solution, was evaluated as follows.

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during the routine sample preparation (around 7 h). Samples were extracted and analyzed as above. Results are given in Table 4. Short-term stability indicated reliable stability behavior under the experimental conditions of the regular batches.

For In process stability determination, stored plasma aliquots thawed, vortexed, aliquoted and buffer were added as applicable and kept at ambient / respective storage conditions in certain period. Samples analyzed under fresh calibration curve standards. In process stability indicated any delay in sample processing also samples having reliable stability behavior under the experimental conditions of the regular batches.

The post-preparative stability (autosampler stability) of QC samples kept in the autosampler for 70 h was also assessed. The results indicate that letrozole and internal standard can remain at the autosampler temperature for at least 70 h, without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table 4).

The data representing the stability of letrozole plasma samples at two QC levels over three cycles of freeze and thawing are given in Table 4. The performed tests indicate that the analyte is stable in human plasma for three cycles of freeze and thaw, when stored at −70°C ± 15°C and thawed to room temperature.

For Re injection reproducibility determination, lower and higher extracted QC samples are subjected to an initial analysis and the injected samples kept at auto sampler temperature for the time

period that assesses sample extract reanalysis stability. The samples are then reanalyzed and quantified under the initial calibration curve. Sample extract reanalysis stability indicated to determine whether it is possible to reinject / reanalyze processed samples in the event that their initial analysis is interrupted because of, for instance, instrument failure. Table 4 summarizes also the long-term stability data of letrozole in plasma samples stored for a period of 472 days at −70°C ± 15°C.

The stability study of letrozole in human plasma showed reliable stability behavior as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicated that storage of letrozole plasma samples at −7°C ± 15°C s adequate, and no stability-related problems would be expected during the routine analysis of samples for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of stock solutions was tested and established at room temperature for 2 and 40 h and under refrigeration for 12 days. The recoveries for letrozole and letrozole-d4 were 66.34 (CV 10.9%), 71.34 (CV 5.8%), 59.53 (CV 2.7%) and 67.12 (CV 11.1%), 73.02 (CV 5.3%), 60.40 (CV 2.3%), respectively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Application

The validated method has been successfully used to quantitate letrozole concentration in human plasma samples after the administration of a single 2.5 mg oral dose of letrozole. The mean concentration vs time profile of 72 (Fasting & Fed) subjects receiving a single dose of letrozole is presented in Fig. 8.

CONCLUSION

In summary, this is the first method described for the quantification of letrozole from human plasma by LCMS/MS in positive ionization mode using MRM. The current method has shown acceptable precision and adequate sensitivity for the quantification of letrozole in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. Furthermore, it was utilized for the analysis of hundreds of subject samples. The method described is simple, rapid, sensitive, specific and fully validated according to commonly accepted criteria (Shah *et al.,* 1991). The cost-effectiveness, simplicity and speed of liquid–liquid extraction and sample turnover rate of 2 min per sample make it an attractive procedure in high-throughput bioanalysis of letrozole. The validated method allows quantification of letrozole in the 0.2–100.0 ng/mL range.

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