Bioanalytical Method Development and Validation of Letrozole by LC-ESI-MS/MS in Human Plasma

Abstract

Letrozole (CAS Number-112809-51-5) is widely used as an oral non-steroidal aromatase inhibitor for the treatment of hormonally-responsive breast cancer after surgery. For quantitation of letrozole, negative polarity was used to achieve adequate response because this was highly sensitive for compounds with high electron affinity. It was applied to fragment the analytes and to obtain intense and consistent product ions. The deprotonated precursor ions [M-H]⁻ at m/z 284.1 was observed in Q1 (MS) for letrozole. Characteristic product ions found in Q3 (MS) was at m/z 242.0, 102.1, 126.7, 140.7, 215.1, 240.3 for letrozole. Most stable and consistent fragment ion selected m/z 242.0 having the triazole ring. For the internal standard (tolbutamide) the fragment at Q1 and Q3 having m/z 269.1 and 170.1 was responsible for possessing the methoxethyl-phenoxy group. In the present study LC-MS/MS-ESI method was attempted to develop for detection and quantification of letrozole in human plasma.

Therefore, it can be concluded from the present study findings that developed method by using LC-MS/MS API 2000 and liquid-liquid extraction technique for letrozole in human plasma was found to be simple, specific, sensitive, reproducible and fully validated in accordance to EMA and USFAD guidelines. The present method was found superior enough to be applied to comparative pharmacokinetic studies in future.

Keywords: Letrozole; LC-MS/MS-ESI; Human plasma; Gradation; Breast cancer

Introduction

Letrozole (CAS Number-112809-51-5) is an oral non-steroidal aromatase inhibitor for the treatment of hormonally-responsive breast cancer after surgery [1]. 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. In the previously published literature the bio-analytical methods for determining letrozole in human plasma was found to be simple, specific, sensitive, reproducible and fully validated in accordance with EMA and USFAD guidelines.

Chromatographic separation with gradation technique

Letrozole is a weak acid with pKa value of 4.4 corresponding to the acidic nitrogen protons in the triazole ring, and pKa value of 5.4 confirms the presence of dibenazonitrile group [5]. For quantitation of letrozole, negative polarity was used to achieve adequate response. Moreover, negative ionization mode is selective and highly sensitive for compounds with high electron affinity. Thus, negative ionization mode was selected to fragment the analytes and to obtain intense and consistent product ions. Chromatographic analysis with gradation technique was performed on a Shimadzu HPLC system equipped with LC-20AD binary pump, SIL-20A autosampler, CTO-10ASvp Oven and CBM-20A lite system control compartment. Separation was carried out on a Phenomenex Kinetex 5µ C18 100A 50*3mm (Phenomenex Inc., Torrance, CA, USA) column.

Detection and quantification of letrozole by LC-MS/MS

Mass spectrometric detection was performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, ON, Canada) equipped with a Turboelectrospray...
ionization (ESI) interface. The mass spectrometric parameters for the detection of letrozole and internal standard (IS, tolbutamide) were optimized by carrying out full scans in negative ion detection mode.

The deprotonated precursor ions [M-H]⁻ at m/z 284.1 was observed in Q1 (MS) for letrozole. Characteristic product ions found in Q3 (MS) was at m/z 242.0, 102.1, 126.7, 140.7, 215.1, 240.3 for letrozole. However, the most stable and consistent fragment ion selected was m/z 242.0 having the triazole ring. For the internal standard tolbutamide the fragment at Q1 and Q3 having m/z 269.1 and 170.1 respectively was responsible for possessing the methoxyethyl-phenoxy group.

Superior signal to noise ratio (≥22) and baseline resolution was obtained for the analyte by replacing buffer with 0.1% (v/v) formic acid together with Milli Q water having apparent pH 3.2 at a flow rate of 0.6000mL/min. The chromatographic retention time for letrozole and IS were 1.79 and 1.82min respectively, in a total run time of 4.0min. The analysis was carried out by gradation method in which 10% organic solvent (0.01min to 0.70min run time), 90% organic solvent (0.70min to 2.70min run time) and 90% aqueous solvent (2.70 min to 4.0 min) was used for washing purpose.

The conditions optimized for detection and quantification were summarized in Table 1. Data acquisition and quantitation were carried out using Analyst software version 1.5 (Applied Biosystems/MD SCIEX). Full scans in negative ion mode of letrozole and IS were presented in Figure 1-3. Optimal conditions of the gradation were represented in the Figure 4.

**Table 1: Mass spectrometric conditions.**

| Parameter(s)                     | Value                               |
|----------------------------------|-------------------------------------|
| Ionization mode                  | MRM (-ve)                           |
| Source temperature (ºC)          | 400                                 |
| Dwell time per transition (msec) | 200                                 |
| Curtain gas (psi)                | 10                                  |
| CAD gas (psi)                    | 6                                   |
| Ion spray voltage (V)            | -4500                               |
| Ion source gas 1 (psi)           | 55                                  |
| Ion source gas 2 (psi)           | 45                                  |
| Focussing potential (V)          | -400                                |
| Declustering potential (V)       | -40 (letrozole) and -30 (IS)        |
| Entrance potential (V)           | -10                                 |
| Collision energy (V)             | -25 (analyte and IS)                |
| Collision cell exit potential (V)| -5 (analyte) and -4 (IS)            |
| Transition pair of letrozole     | 284.10/242.00                       |
| Transition pair of tolbutamide   | 269.10/170.10                       |

**Figure 1: Q1 and Q3 scans of Letrozole.**

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Figure 2: Q1 and Q3 scans of IS (tolbutamide).

Figure 3: Representative chromatograms of the blank plasma.

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Plasma extraction and sample preparation

Plasma extraction was performed by liquid-liquid extraction technique [6]. A 250µl volume of plasma sample was transferred to a 15ml plastic tarson tube. 50µl of IS working solution (5µg/ml) was spiked into it. After vortexing for 30sec, 3ml TBME was added. After that the sample was vortex-mixed for 3min and then centrifuged at 5000rpm for 15min. Then the organic layer 2.5ml was transferred to a 15ml plastic tarson tube and evaporated at 40°C under inert atmosphere (nitrogen). Then the dried extract was reconstituted in 200µl of diluents methanol: water (50:50). 10µl aliquot was injected into chromatographic system.

Bio-analytical method validation

Validation of the developed LC-MS/MS-ESI method for quantification of letrozole in human plasma was carried out in accordance with EMA and USFDA guidelines [7,8].

Specificity, selectivity and linearity

The specificity and selectivity of the assay was illustrated by the chromatograms of mobile phase run and extract of blank plasma recorded for samples near the C_{max} for 2 to 2.5 hour. The linearity of the calibration curve was determined by an unweighted least square regression analysis. Representative calibration curve of letrozole from human plasma was depicted in the linearity graph.

Precision and accuracy

Between – run precision and accuracy were determined from the low, medium and high QC samples (LQC, MQC and HQC). A total of 5 replicates of each QC concentration were assayed on day 1 and a total of 5 replicates each QC concentration were assayed on day 2 and 3. The QC samples concentrations were determined from three different calibration curves that were assayed with QC samples.

Within-run precision and accuracy were determined from a total of 5 replicates of each QC concentration. The low, medium and high QC samples (LQC, MQC and HQC) were assayed on day 1. The QC samples concentrations were determined from calibration curves LIN1. Precision was expressed as percent variation (%CV), while accuracy was measured as the percent nominal.

Stability

In the present study the freeze thaw, short term (ST), long term (LT) and auto sampler (AS) stability had been performed as per the regulatory guidelines (EMA and USFDA). As per guidelines the freeze thaw stability should be within 80 – 120%. As per guidelines the both the ST and LT stability should be within 90 – 110% and the AS stability should be within 85 – 115%.

Matrix effect and recovery

In the present study, the matrix effect for the internal standard (tolbutamide) and analyte (letrozole) were also carried out. The matrix effect should be within 85-115% as per EMA and US-FDA guidelines.

The percentage recovery was determined by measuring the peak areas of the analyte and IS from the prepared plasma low, medium and high quality control samples. The peak areas of the plasma low, medium and high quality control samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the letrozole and tolbutamide.
Results and Discussion

Bioanalytical method validation

Specificity, selectivity and linearity: Following plasma calibration standards (ng/ml) were prepared - 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 and quality control samples were prepared as 1.56ng/ml (LLOQ), 4.68ng/ml (LQC), 75ng/ml (MQC) and 150ng/ml (HQC). The proposed assay was linear in the range of 1.56ng/ml to 200ng/ml in plasma. The representative calibration curve (code no. LIN 1) was presented in linearity graph (Figure 5). Back calculated concentrations of the calibrant samples of the linearity and their statistical analysis were also represented in Table 2. Representative chromatograms of the quality control samples (LLOQ, LQC, MQC and HQC) were given as Figure 6. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were found 0.43ng/ml and 1.56ng/ml, respectively.

Precision and accuracy: Obtained between-run precision values (%CV) ranged from 1.016% to 1.755%. Between-run accuracy values (% nominal) were 100.67% for LLOQ, 100.95% for low QC (LQC), 100.62% for medium QC (MQC) and 101.43% for high QC (HQC) samples. Within-run precision values (%CV) were ranged from 0.962% to 2.311%. Within-run accuracy values (% nominal) were 101.03% for LLOQ, 100.98% for low QC (LQC), 99.63% for medium QC (MQC) and 102.00% for high QC (HQC) samples. The both precision and accuracy data were represented in Table 3.

Stability: The freeze thaw stability of letrozole ranged between 97.57% to 99.20% after three cycles. The LT and ST stability were ranged between 98.90% to 100.68% and 99.12% to 101.60%, respectively after three cycles. The found range of the auto sampler stability (AS) of letrozole was 98.69% to 99.95% after three cycles. The obtained values were within the limit as specified in the EMA and USFDA guidelines. So the values were acceptable (Table 4).

Matrix effect and recovery: The matrix effect of the internal standard (tolbutamide) were found between 95.12% - 97.61% and for analyte (letrozole), it ranged between 90.58 % to 93.83 % after three cycles (Table 5). The values were within the limit and hence were acceptable. The recovery results were presented in Table 6. Recovery after extraction was found between 93.07% - 98.22% and was satisfactory.

Table 2: Pre-study linearity of detector response.

| Linearity | Concentration (ng/ml) | Statistics |
|-----------|-----------------------|------------|
|           | 1.56 | 3.12 | 6.25 | 12.5 | 25 | 50 | 100 | 200 | Slope (m) | Intercept (c) | R²          |
| LIN 1     | 1.55 | 3.21 | 6.13 | 12.44 | 24.68 | 49.78 | 101.55 | 200.92 | 0.0047 | 0.00072 | 0.9998     |
| LIN 2     | 1.54 | 3.18 | 6.4 | 12.41 | 25.12 | 49.5 | 99.04 | 198.55 | 0.00385 | 0.00098 | 0.9998     |
| LIN 3     | 1.57 | 3.11 | 6.19 | 12.8 | 24.6 | 47.4 | 103 | 204 | 0.00644 | 0.00312 | 0.9995     |
| Avg       | 1.55 | 3.16 | 6.24 | 12.55 | 24.8 | 48.89 | 101.19 | 201.15 | 0.005 | - | 0.9979     |
| ± SD      | ±0.01 | ±0.05 | ±0.14 | ±0.21 | ±0.28 | ±1.30 | ±2.00 | ±2.73 | ±0.001 | - | ±0.0002    |
| % CV      | 0.98 | 1.62 | 2.27 | 1.72 | 1.129 | 2.66 | 1.98 | 1.35 | 26.422 | - | 0.017      |
| % Nominal | 99.57 | 101.5 | 99.84 | 100.4 | 99.2 | 97.79 | 101.2 | 100.58 | - | - |            |

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Figure 6: Representative chromatograms of the quality control samples (LLOQ, LQC, MQC and HQC).
Table 3: Between run and within run precision and accuracy.

|                  | Between Run |                   | Within run |                   |
|------------------|-------------|------------------|------------|------------------|
|                  | Mean ± SD   | C.V.%            | Absolute bias (%) | Mean ± SD      | C.V.%            | Absolute bias (%) |
| LLOQ (1.56 ng/ml)| 1.570±0.016 | 1.016            | 100.67     | 1.576±0.015     | 0.962            | 101.03           |
| LQC (4.68 ng/ml) | 4.724±0.060 | 1.274            | 100.95     | 4.726±0.083     | 1.749            | 100.98           |
| MQC (75 ng/ml)   | 75.46±0.906 | 1.2              | 100.62     | 74.72±0.804     | 1.077            | 99.63            |
| HQC (150 ng/ml)  | 152.14±2.670| 1.755            | 101.43     | 153.00±3.536    | 2.311            | 102              |

Table 4: Long term stability (LT), short term stability (ST) and auto sampler stability (AS) study data.

|                  | Inj No. | LQC (4.68 ng/ml) | MQC (75 ng/ml) | HQC (150 ng/ml) |
|------------------|---------|------------------|----------------|-----------------|
| Freshly thawed   |         |                  |                |                 |
| 1                | 4.81    | 74.1             | 153            |
| 2                | 4.82    | 74               | 154            |
| 3                | 4.65    | 74.9             | 147            |
| 4                | 4.69    | 74.6             | 156            |
| 5                | 4.66    | 76               | 155            |
| Mean             | 4.73    | 74.72            | 153            |
| After 3 cycle    |         |                  |                |                 |
| 1                | 4.72    | 74.2             | 149.36         |
| 2                | 4.61    | 74.88            | 148.48         |
| 3                | 4.55    | 73.74            | 151.3          |
| 4                | 4.72    | 73.84            | 148.23         |
| 5                | 4.63    | 73.96            | 149.05         |
| Mean             | 4.65    | 74.12            | 149.28         |
| After 7 days in freezer |         |                  |                |                 |
| 1                | 4.65    | 75.81            | 152.21         |
| 2                | 4.72    | 75.91            | 154.37         |
| 3                | 4.63    | 74.01            | 151.76         |
| 4                | 4.69    | 75.58            | 150.48         |
| 5                | 4.68    | 74.84            | 150.03         |
| Mean             | 4.67    | 75.23            | 151.77         |
| % Stability      | 98.31   | 99.2             | 97.57          |
| After 7 days in freezer |         |                  |                |                 |
| 1                | 4.67    | 75.48            | 150.96         |
| 2                | 4.66    | 76.85            | 152.21         |
| 3                | 4.65    | 76.08            | 150.94         |
| 4                | 4.78    | 75.8             | 151.56         |
| 5                | 4.74    | 75.35            | 152.59         |
| Mean             | 4.7     | 75.91            | 151.65         |
| % Stability      | 98.9    | 100.68           | 99.2           |
| After 24 hrs     |         |                  |                |                 |
| 1                | 4.67    | 75.48            | 150.96         |
| 2                | 4.66    | 76.85            | 152.21         |
| 3                | 4.65    | 76.08            | 150.94         |
| 4                | 4.78    | 75.8             | 151.56         |
| 5                | 4.74    | 75.35            | 152.59         |
| Mean             | 4.7     | 75.91            | 151.65         |
| % Stability      | 99.45   | 101.6            | 99.12          |
| Auto-sampler after 24 hours |         |                  |                |                 |
| 1                | 4.76    | 73.28            | 152.49         |
| 2                | 4.63    | 73.86            | 153.97         |
| 3                | 4.66    | 74.28            | 149.67         |
| 4                | 4.64    | 74.3             | 155.06         |
| 5                | 4.63    | 75.17            | 153.39         |
| Mean             | 4.66    | 74.18            | 152.92         |
| % Stability      | 98.69   | 99.27            | 99.95          |
Table 5: Matrix effect (IS and analyte).

| Sample (n) | Extracted Blank Plasma | Aqueous | Matrix Effect % | Matrix Factor |
|------------|------------------------|---------|----------------|--------------|
| **Mean ± SD** |                       |         |                |              |
| LQC        | 21847.31 ± 1889.08     | 22380.31 ± 1855.19 | 97.61 ± 1.46  | 0.97 ± 0.01  |
| C.V.%      | 8.65                   | 8.29    | 1.49           | 1.51         |
| MQC        | 19766.08 ± 1491.83     | 20677.02 ± 1759.95 | 95.6±1.66    | 0.96 ± 0.02  |
| C.V.%      | 7.55                   | 8.51    | 1.74           | 1.72         |
| HQC        | 21516.02±2327.62       | 22683.30 ± 3150.74 | 95.1±2.83   | 0.95 ± 0.03  |
| C.V.%      | 10.82                  | 13.89   | 2.98           | 3.01         |

**Table 6: Recovery data.**

| Diluent Sample | Plasma Sample |
|----------------|---------------|
| **LQC** (4.68 ng/ml) | **MQC** (75 ng/ml) | **HQC** (150 ng/ml) |
| LQC | MQC | HQC | LQC | MQC | HQC |
| 872.45 | 10694.82 | 21973.74 | 836.01 | 10259.09 | 21429.55 |
| 792.12 | 10858.95 | 18835.63 | 711.08 | 10628.71 | 17282.27 |
| 683 | 10476.45 | 18183.49 | 602.66 | 10396.29 | 17955.28 |
| 903.31 | 10423.63 | 18291.24 | 838.63 | 10368.5 | 17729.46 |
| 653.22 | 10465.02 | 18912.47 | 645.35 | 10322.51 | 17149.28 |
| 780.82 | 10583.77 | 19239.31 | 726.75 | 10395.02 | 18309.17 |

% Recovery | 93.07 | 98.22 | 95.17

### Conclusion

In the present study a LC-MS/MS-API method was developed for detection and quantification of letrozole in human plasma. This method was also validated as per the EMA and USFDA guidelines. Letrozole is a weak acid. The retention of this drug observed only in acidic pH. Therefore if the elution was carried out in isocratic method, the response found was less. Hence in the present study, a gradient method was applied with the use of 90% aqueous solvent for washing at about 2 min of total 4 min run time. Matrix effect and the recovery considered as the critical phase in the analysis of plasma samples. From the data of the present method, it was evident that the plasma matrix effect was minimized more than 90%. The recovery was also found more than 93%, which signifies the plasma extraction procedure.

All the available organic solvents like chloroform, methyl tert-butyl ether, ethyl acetate, ACN, ethanol was applied for liquid-liquid extraction of letrozole from human plasma. The optimal solvent was found to be TBME with methanol. Also the width of the chromatograms was very short which indicates the higher resolution and selectivity.

Therefore, it can be concluded from the present study that the developed method by using LC-MS/MS API 2000 and liquid-liquid extraction technique for letrozole in human plasma was found to be simple, specific, sensitive, reproducible and fully validated per EMA and USFDA guidelines. The present method was hence found superior enough to be applied to comparative pharmacokinetic studies in future.
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