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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 methoxyethyl-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 according 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

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Abbreviations: CAN, acetonitrile; TBME, ter butyl methyl ether; HPLC, high performance liquid chromatography; QC, quality control; ST, short term; LT, long term; AS, auto sampler; LLOD, lower Limit of detection; LLOQ, lower limit of quantification

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 were found tedious, lengthy process and also was not cost effective.[2–4] In the present study a LC-MS/MS-ESI method was developed for detection and quantification of letrozole in human plasma.

Material and methods

Chemical and reagents

Acetonitrile (ACN), methanol, chloroform, Ter butyl methyl ether (TBME) and isopropyl alcohol were purchased from Merck (MERCK India Ltd., Mumbai). All solvents used during the analysis were of HPLC grade. Other chemicals and reagents of analytical grade were used throughout the study. HPLC grade water with a resistivity of 18MΩ was obtained from a Milli-Q gradient system of Millipore (Elix, Milli-Q A10, USA). Blank human plasma samples were obtained from the M/S, TAAB Biostudy Services, Kolkata, India.

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 dibenzonitrile 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 an Turbo electrospray 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 (2.70min to 4.0min) 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.

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.

Table 1 Mass spectrometric conditions

Parameter(s)	Value
Ionization mode	MRM (-ve)
Source temperature (°C)	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 (analyte)	284.10/242.00
Transition pair of tolbutamide (IS)	269.10/170.10

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	-	0.9997
± 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	-		

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)	75.461±0.906	1.2	100.62	74.720±0.804	1.077	99.63
HQC (150 ng/ml)	152.140±2.670	1.755	101.43	153.000±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
Freeze thaw stability	After 3 cycle	1	4.72	74.2	149.36
		2	4.61	74.88	148.48
		3	4.55	73.74	151.3

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Table 4 Continued...

		Inj No.	LQC (4.68 ng/ml)	MQC (75 ng/ml)	HQC (150 ng/ml)
		4	4.72	73.84	148.23
		5	4.63	73.96	149.05
		Mean	4.65	74.12	149.28
	% Stability		98.31	99.2	97.57
Long term stability (LT)	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		98.9	100.68	99.2
Short term stability(ST)	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 stability (AS)	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)

Internal Standard (Tolbutamide)					
	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
Mean ± SD	MQC	19766.08 ± 1491.83	20677.02 ± 1759.95	95.66±1.66	0.96 ± 0.02
C.V.%		7.55	8.51	1.74	1.72
Mean ± SD	HQC	21516.02±2327.62	22688.30 ± 3150.74	95.12±2.83	0.95 ± 0.03
C.V.%		10.82	13.89	2.98	3.01
Analyte (Letrozole)					
Mean ± SD	LQC	612.09 ± 42.66	671.82 ± 52.58	91.24 ± 3.82	0.91 ± 0.04
C.V.%		6.97	7.83	4.19	4.45
Mean ± SD	MQC	8826.93 ± 600.48	9743.77 ± 533.35	90.58 ± 3.31	0.90 ± 0.03
C.V.%		6.8	5.47	3.65	3.78
Mean ± SD	HQC	18801.74 ± 1578.49	20039.31 ± 1685.81	93.83 ± 1.07	0.93 ± 0.01
C.V.%		8.4	8.41	1.14	0.96
n=5 replicates for each LQC, MQC and HQC					

Table 6 Recovery data

Diluent Sample			**Plasma Sample**		
LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
(4.68 ng/ml)	(75 ng/ml)	(150 ng/ml)	(4.68 ng/ml)	(75 ng/ml)	(150 ng/ml)
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

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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]

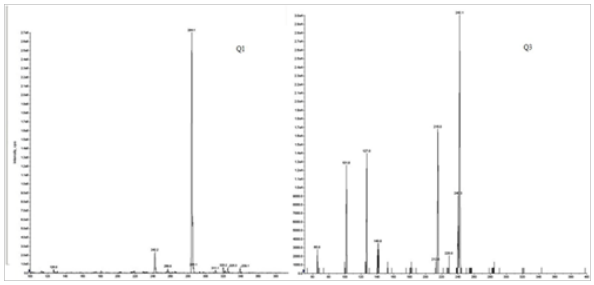

Figure 1 Q1 and Q3 scans of Letrozole.

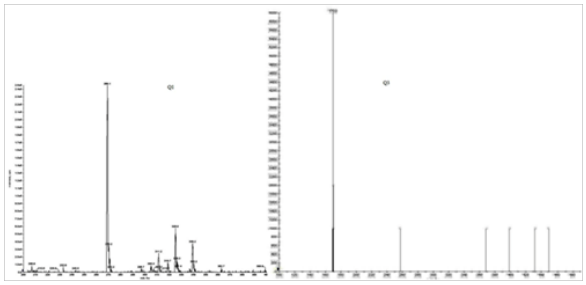

Figure 2 Q1 and Q3 scans of IS (tolbutamide).

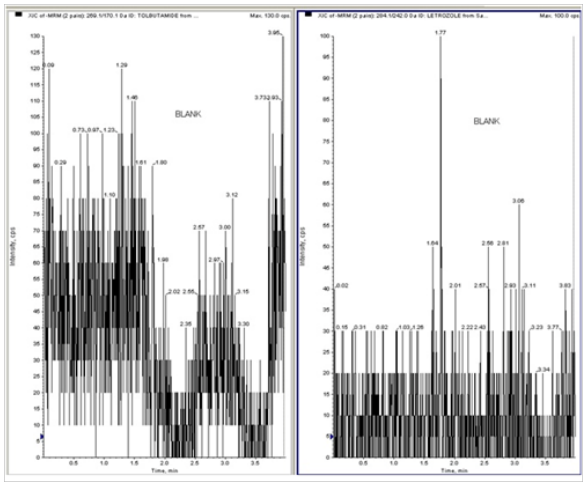

Figure 3 Representative chromatograms of the blank plasma.

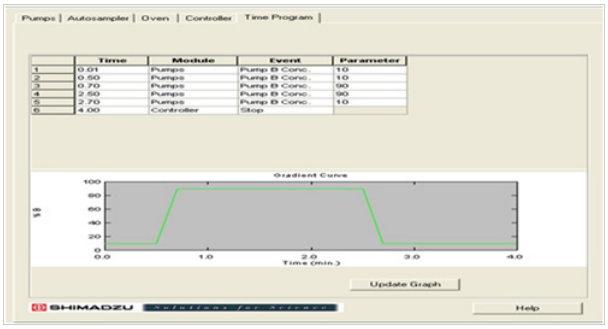

Figure 4 Gradation.

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 Cmax for 2 to 2.5hour. 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.

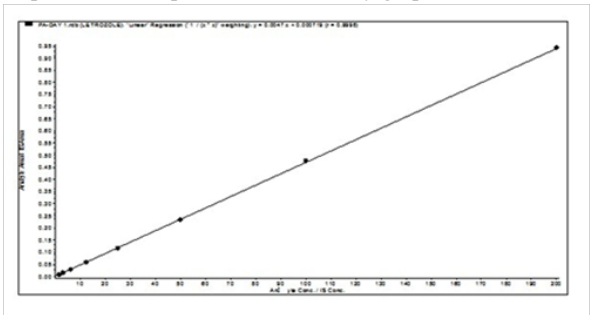

Figure 5 Plasma Calibration Curve of Letrozole.

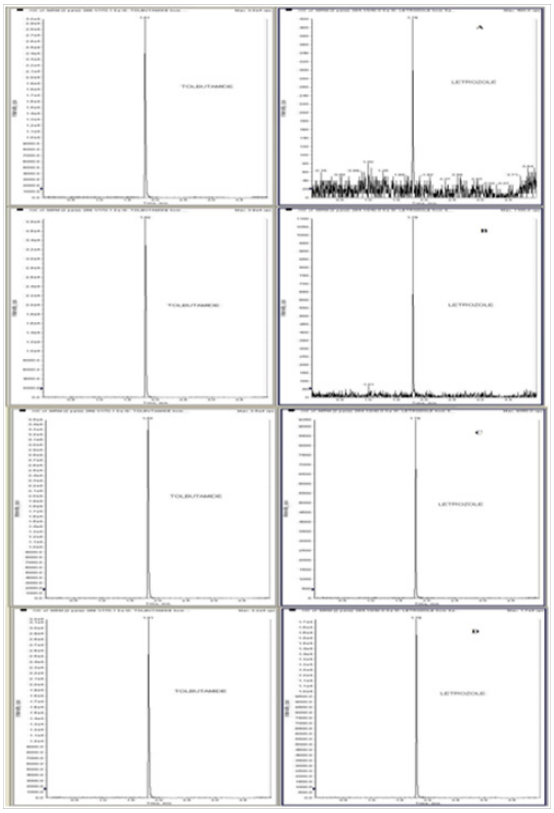

Figure 6 Representative chromatograms of the quality control samples (LLOQ, LQC, MQC and HQC).

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.

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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 I) was presented in linearity graph (Figure 5). Back calculated concentrations of the calibrant samples of the linearities 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 100.6 % 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.

Conclusion

In the present study a LC-MS/MS-ESI method was developed for detection and quantification of letrozole in human plasma. This developed 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 2min of total 4min 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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Conflicts of interest

Author declares there are nno conflicts of interest.

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References

1. Filipe A, Almeida S, Spínola AC, et al. Bioequivalence study of two letrozole tablet formulations. *Arzneimittelforschung.* 2008;58:419–422.
2. Sioufi A, Gauducheau N, Pineau V, et al. Absolute bioavailability of letrozole in healthy postmenopausal women. *Biopharm Drug Dispos* . 1997;18(9):779–789.
3. Dyderski S, Grześkowiak E, Szałek E, et al. Comparative bioavailability study of two preparations of letrozole in healthy subjects. *Arzneimittelforschung.* 2005;55(9):514–519.
4. Zarghi A, Foroutan SM, Shafaati A, et al. HPLC determination of letrozole in plasma using fluorescence detection: application to pharmacokinetic studies. *Chromatographia.* 2007;66(9):747–750.
5. http://www.fda.gov/ohrms/dockets/ac/03/briefing/3995B1_07_GFI-BioAvail-BioEquiv.pdf
6. Dan S, Halder D, Pal TK, et al. Bio-analytical method development and validation of tadalafil with a special emphasis on pharmacokinetic study in healthy Indian subjects for the ODS formulation. *Current Analytical Chemistry*. 2015;11(3):175–183.
7. Liu Y, Zhang Y, Wang O, et al. Bioequivalence and safety study of letrozole tablet in healthy Chinese postmenopausal women volunteers. *Journal of Chinese Pharmaceutical Sciences*. 2013;22(2):190–196.
8. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf.