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Method Validation and Quantitative Determination of Anastrozole in Human Plasma by an CMS/MS

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ABSTRACT : A simple, economic, accurate LCMS/MS method was developed for the determination of anastrozole in human plasma was developed and fully validated using dexchlorpheniramine as the internal standard (I.S.) is described herein. The analyte and the I.S. were extracted from 200 µl of human plasma by liquid-liquid extraction using a mixture of diethyl ether: dichloromethane (70:30, v/v) solution. The extracts were analyzed by high performance liquid chromatography coupled with Electrospray Ionization source - tandem mass spectrometry (LCMS/MS). Chromatography was performed isocratically on a Genesis C18, 4 µm analytical column (100 mm × 2.1 mm i.d.). The method had a chromatographic run time of 3.0 min and a linear calibration curve ranging from 0.5-100 ng/ml. The limit of quantification (LOQ) was 0.5 ng/ml.

Keywords : Anastrozole; LCMS/MS; Plasma; liquid-liquid extraction.

INTRODUCTION

Anastrozole, [2,2′–[5–1H–1, 2, 4–triazole–1–y–methyl)–1, 3–phenylene] bis (2–methylpropiononitrile)] is a potent, selective non–steroidal aromatase inhibitor used to treat breast cancer in post–menopausal women. Which is a potent aromatase inhibitor. It is a white crystalline solid, odorless and is freely soluble in methanol, acetone, ethanol and tetrahydrofuran, and very soluble in acetonitrile having melting point 81–82°C (Budavari 1996, wellington 2002). Anastrozole is indicated for the treatment of advanced breast cancer in postmenopausal women with disease progression following tamoxifen therapy and even in patients with ER negative disease (Plourde *et al.* 1994). Anastrozole is a potent, selective non–steroidal aromatase inhibitor used to treat breast cancer in post–menopausal women (Plourde 1994). Anastrozole is not official in IP, USP and BP. Several methods have been used for the determination of anastrozole substance and pharmaceutical preparation Mendes *et al.* [5] reported a novel method to measure anastrozole by HPLC–MS–MS using acetonitrile: methanol: water: acetone (60:20:15:5) as mobile phase. Due to the low dose (1 mg) of anastrozole, the plasma concentration of anastrozole is rather low, which inhibits detection of plasma anastrozole. There are few reports about the determination of anastrozole in biological samples. High–performance liquid chromatography with ultraviolet, fluoresencence or electrochemical detection is not sensitive enough to detect anastrozole in plasma from samples obtained in clinical studies in which standard oral doses have been used. Consequently, anastrazole has been determined in plasma only by capillary gas chromatographic assay with electron capture detection (Bock *et al* 1997, yuan *et al* 2001. Duan *et al.* 2002). In the present work, a fast, sensitive and selective method for measuring plasma anastrozole by liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS), using Electrospray. Ionisation to quantify anastrozole in human plasma, using dexchlorpheniramine as the internal standard. This HPLC–MS–MS procedure was used to assess the pharmacokinetic parameters of the test and reference formulations under comparison in a BA/BE study.

MATERIALS AND METHODS

The chemicals and reagents used are Anastrozole (99.8%), dexchlorpheniramine (99.9%), diethyl ether and hexane (analysis grade). Ultra–pure water. Blank human blood was collected from healthy drug–free volunteers and plasma was obtained by centrifugation of blood treated with sodium heparin as anticoagulant.

Solutions of anastrozole and internal standard (dexchlorpheniramine) were prepared in acetonitrile: water (50 : 50 v/v) at concentrations of 100ppm. Calibration curves of anastrozole were prepared by spiking blank plasma at concentrations of 0.5, 1.5, 5.0, 10.0, 25.0, 50.0, 75.0 and 100.0 ng/ml. The analysis was carried out in duplicate for each concentration. The quality control samples were prepared in blank plasma at concentrations of 1.5, 20.0 and 85.0 ng/ml (LQC, MQC and HQC, respectively). The spiked plasma samples (standards and quality controls) were extracted from each analytical batch along with the unknown samples.

Human plasma samples were previously thawed at room temperature and centrifuged at 2000 × g for 1 min at 4 °C to precipitate solids. Two–hundred microliters of sample human plasma was introduced into glass tubes followed by addition of 50 µl of the internal standard solution (10 ng ml^{-1} of Dexchlorpheniramine in acetonitrile:water 50/50; v/v solution); then, samples were vortex–mixed for approximately 15 s. Diethyl ether/dichloromethane (70/30; v/v) was added (4 µl) to all tubes, and the extraction was performed by vortex–mixing for 40 s. The upper organic phase was transferred to

another set of clean glass tubes and evaporated to dryness under nitrogen at 40°C. The dry residues were dissolved with 200µl of a solution of acetonitrile:water (50 : 50, v/v). Vials were capped and then placed into the autosampler.

Under these conditions, typical standard retention times were 1.59 ± 0.1min for anastrozole and 1.92 ± 0.2 min for dexchlorpheniramine, and back–pressure values of approximately 60 bar were observed. Temperature of the auto–sampler was kept at 6°C and run–time was set to 3.0 min.

Mass spectrometry was performed in a Sciex API 4000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an Electrospray Ionization source operating in positive mode.

The corresponding values for internal standard were 26 (V), 170 (V), 29 (eV) and 22 (V), respectively. Data were acquired by Analyst software (1.4.2, Applied Biosystems, Foster City, CA, USA).

Linearity was determined to assess the performance of the method. Unknown sample peak–area ratios were then interpolated from the calibration curve to provide concentrations of anastrozole.

Blank samples from six different pools of plasma, including one lipemic and one haemolyzed, were tested for interference using the proposed extraction procedure and analytical conditions.

The recovery was evaluated by calculating the mean (and RSD) of the response of each concentration and dividing the extracted sample response by the unextracted sample mean of the corresponding concentration.

Stability quality control plasma samples (1.5 and 85.0 ng/ml) were subjected to short–term (8 hrs) room temperature, three freeze/thaw (–15 to 25°C) cycles, 28 hrs autosampler stability (6°C) and long–term stability at –15 to 25°C (25 days) tests. Subsequently, the anastrozole concentrations were measured compared to freshly prepared samples.

To assess precision and accuracy of the developed analytical method, four distinct concentrations in the range of expected concentrations were evaluated using eight determinations per concentration.

A procedure to assess the effect of ion suppression on the MS/MS was performed. The experimental set–up consisted of an infusion pump connected to the system by a "zero volume tee" before the split and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of anastrozole, i.e. acetonitrile:methanol : water : acetone (60 : 20 : 15 : 5, v/v/v/v) containing 0.1% of acetic acid and 10 mM of ammonium acetate at a flow–rate of 0.450 ml min^{-1}.

Blood samples (4 ml) from a suitable antecubital vein were collected by an indwelling catheter into heparin containing tubes prior to administration and at 20 min, 40 min, 1 h, 1 h 20 min, 1 h 40 min, 2 h, 2 h 20 min, 2 h 40 min, 3 h, 3 h 30 min, 4 h, 4 h 30 min, 5 h, 5 h 30 min, 6 h, 8 h, 10 h, 12 h, 18 h, 24 h, 48 h, 96 h and 120 h after administration the of 1 mg anastrozole tablet formulation. Blood samples were centrifuged at approximately 2000 × g for 10 min at room temperature and the plasma was stored at –20 °C until assayed for anastrozole content.

RESULTS

Plasma calibration curves were prepared and assayed in triplicate on three different days to evaluate linearity, precision, accuracy, recovery, limit of quantitation(LOQ), selectivity and stability.

The method was considered linear at the concentration range between 0.50 ng/mL and 100 ng/mL. Linear regression analysis was determined with correlation coefficients (r^2) greater than 0.99 for the calibration curves. The representative linearity curve is presented in Fig. 1.

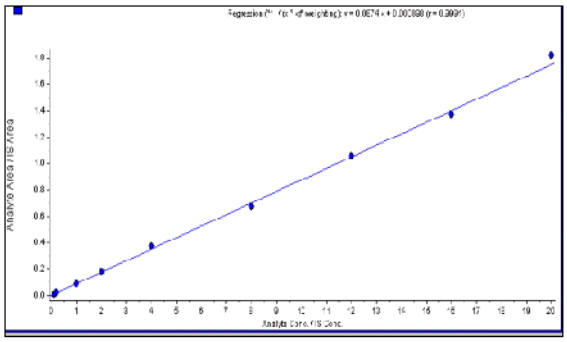

Fig. 1. Linearity Curve for Anastrazole.

The LOQ were estimated and were 0.5 ng/mL. The representative chromatogram for LOQ is presented in Fig. 2.

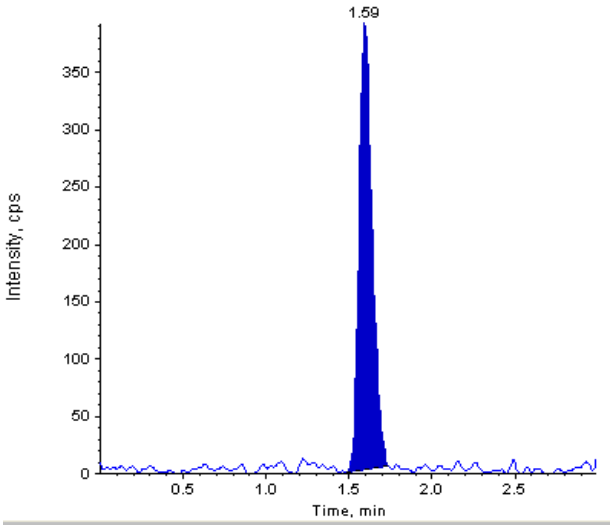

Fig. 2. Chromatogram for LOQ of Anastrazole.

Results of intra– and inter–day precision showed CV% values not exceeding 15%, which mean that the method is precise.

As the calculated values of accuracy were always within 15% of the nominal value, the method could be considered accurate. Results are summarized in Table 1 and 2. Recoveries varied between 85.18% and 87.19% for Anastrazole. Recovery for Dexchlorpheniramine was 63.90%. Results are summarized in Table 3 and 4.

Table 1: Intra–Day Precision and Accuracy for Anastrazole.

File Name	LQC 1.50 ng/mL		MQC 20.0 ng/mL		HQC 85.0 ng/mL	
	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.
P&A_2	1.496	100.27	20.814	96.09	84.556	100.53
P&A_2	1.503	99.80	20.589	97.14	84.869	100.15
P&A_2	1.508	99.47	19.754	101.25	84.987	100.02
P&A_2	1.492	100.54	19.741	101.31	84.566	100.51
P&A_2	1.489	100.74	20.521	97.46	85.902	98.95
N	6	6	6	6	6	6
Mean	1.50	100.16	20.38	98.17	84.98	100.02
SD (±)	0.01	0.47	0.51	2.49	0.49	0.58
CV (%)	0.47	0.47	2.51	2.54	0.58	0.58

Table 2: Inter–Day Precision and Accuracy for Anastrazole.

File Name	LQC 1.50 ng/mL		MQC 20.0 ng/mL		HQC 85.0 ng/mL	
	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.
P&A_1	1.488	100.81	19.898	100.51	84.566	100.51
P&A_2	1.498	100.16	20.384	98.17	84.982	100.02
P&A_3	1.503	99.80	19.786	101.08	84.911	100.10
N	3	3	3	3	3	3
Mean	1.50	100.25	20.02	99.92	84.82	100.21
SD (±)	0.01	0.51	0.32	1.54	0.22	0.26
CV (%)	0.51	0.51	1.59	1.54	0.26	0.26

Table 3: Recovery of analyte from Biological Matrix.

P&A_2	7685	8129	61570	71411	127659	147664
P&A_2	6576	8118	60394	73844	124215	144545
P&A_2	6734	8018	62413	75730	122887	130887
P&A_2	6823	8165	65376	71632	122589	144395
P&A_2	6655	7904	61245	71946	120024	149842
P&A_2	7723	8059	60860	71985	124483	135810

N	6	6	6	6	6	6
Mean	7032.667	8066	61976	72758	123643	142191
SD (±)	526.58	94.94	1799.91	1694.19	2527.48	7312.84
% CV	7.49	1.18	2.90	2.33	2.04	5.14
% Recovery	87.19				85.18	86.96

Table 4: Recovery of Internal Standard From Biological Matrix.

File Name	Internal Standard (235 ng/ml) Test Area	Comparison
P&A_2	284724	429022
	288520	439390
	285366	430489
	276344	432462
	276493	438441
	280084	437372
	272597	435747
	281048	441933
	271617	441661
	286786	444693
	275302	431411
	273961	447844
	286472	435730
	277785	441632
	282364	437946
	276310	435985
	273188	438735
	279736	429380
Mean	279372	437215
SD (±)	5344.12	5288.38
% CV	1.91	1.21
% Recovery	63.90	

Blank samples from six different pools of plasma, including one lipemic and one haemolyzed, were tested for interference using the proposed extraction procedure the results are summarized in Table 5.

Table 5: Blank Matrix Specificity.

Matrix Identification (Number)	Anticoagulant	Interference with Analyte (% of LLOQ)	Interference with Internal Standard (% of IS)
BLK HP/UP/A/374–1	Na. Heparin	*	*
BLK HP/UP/A/378–1	Na. Heparin	*	*
BLK HP/UP/A/382–1	Na. Heparin	*	*
BLK HP/UP/A/388–1	Na. Heparin	*	*
BLK HP/UP/A/276–1(H)	Na. Heparin	*	*
BLK HP/UP/A/272–1(L)	Na. Heparin	*	*
	Na. Heparin	*	
	Na. Heparin	*	

Plasma stability was tested at different conditions such as Bench top, Auto sampler stability and Dry Extract. Results are summarized in Table 6, 7 and 8. Freeze – Thaw stability was evaluated after three cycle and found within acceptance limit. Results are summarized in Table 9.

Table 6: Bench Top Stability.

File Name	LQC 1.50 ng/mL Calculated Conc.		HQC 85.0 ng/mL Calculated Conc.	
	Test	Comparison	Test	Comparison
P&A_2	1.477	1.499	85.006	84.978
P&A_2	1.500	1.509	85.098	85.12
P&A_2	1.509	1.501	84.882	84.888
P&A_2	1.508	1.498	84.578	84.991
P&A_2	1.488	1.478	84.756	85.003
P&A_2	1.469	1.499	85.091	84.833
N	6	6	6	6
Mean	1.492	1.497	84.902	84.969
SD (±)	0.02	0.01	0.21	0.10
CV (%)	1.11	0.69	0.24	0.12
% Changes			–0.37	–0.08

Table 7: Auto Sampler Stability.

File Name	LQC 1.50 ng/mL Calculated Conc.		HQC 85.0 ng/mL Calculated Conc.	
	Test	Comparison	Test	Comparison
P&A_3	1.488	1.502	85.245	84.888
P&A_3	1.508	1.500	85.098	85.868
P&A_3	1.498	1.487	85.111	85.895
P&A_3	1.488	1.494	85.227	84.867
P&A_3	1.449	1.502	84.989	85.871
P&A_3	1.467	1.500	85.001	85.889
N	6	6	6	6
Mean	1.483	1.498	85.112	85.546
SD (±)	0.02	0.01	0.11	0.52
CV (%)	1.45	0.40	0.13	0.61
% Changes		–0.97		–0.51

Table 8: Dry Extract Stability.

File Name	LQC 1.50 ng/mL Calculated Conc.		HQC 85.0 ng/mL Calculated Conc.	
	Test	Comparison	Test	Comparison
P&A_3	1.512	1.502	84.334	84.888
P&A_3	1.491	1.500	85.001	85.868
P&A_3	1.504	1.487	85.098	85.895
P&A_3	1.509	1.494	85.004	84.867
P&A_3	1.489	1.502	84.773	85.871
P&A_3	1.488	1.500	85.081	85.889
N	6	6	6	6
Mean	1.499	1.498	84.882	85.546

SD (±)	0.01	0.01	0.29	0.52
CV (%)	0.72	0.40	0.34	0.61
% Changes			0.09	–0.78

Table 9: Freeze Thaw Stability.

File Name	LQC 1.50 ng/mL		HQC 85.0 ng/mL	
	Test Conc.	Comparison	Test Conc.	Comparison
P&A_3	1.492	1.502	84.645	84.888
P&A_3	1.499	1.500	85.387	85.868
P&A_3	1.478	1.487	85.465	85.895
P&A_3	1.498	1.494	84.657	84.867
P&A_3	1.492	1.502	85.234	85.871
P&A_3	1.500	1.500	84.335	85.889
N	6	6	6	6
Mean	1.493	1.498	84.954	85.546
SD (±)	0.01	0.01	0.47	0.52
CV (%)	0.55	0.40	0.55	0.61
% Changes			–0.29	–0.69

CONCLUSION

The proposed HPLC–MS/MS method can be regarded as selective, accurate, precise, and valid for determination of anastrozole with a total running time of 2.0 min. Through this method it was possible to evaluate, anastrozole quantification in human plasma and offers advantages over methods previously reported. The present method is more sensitive, while the analytical run is shorter, permitting a high throughput.

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