Research article

Biomedical Chromatography

Received 2 April 2011, Revised 2 May 2011, Accepted 3 May 2011 Published online in Wiley Online Library: 8 June 2011

(wileyonlinelibrary.com) DOI 10.1002/bmc.1658

Hydrophilic interaction chromatography–electrospray ionization tandem mass spectrometric analysis of anastrozole in human plasma and its application to a pharmacokinetic study

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ABSTRACT: A hydrophilic interaction chromatography–electrospray ionization tandem mass spectrometric method was developed for determination of anastrozole in human plasma. Anastrozole and irbesartan (internal standard) were extracted from human plasma with a mixture of dichloromethane and methyl *tert*-butyl ether (30:70, v/v). Analysis of the analytes was performed on a Luna HILIC column with the mobile phase of acetonitrile–10 mM ammonium formate (95:5, v/v) and detected by electrospray ionization tandem mass spectrometry in the selected reaction monitoring mode. The standard curve was linear ($r^2 = 0.9992$) over the concentration range of 0.10–50.0 ng/mL using 200 µL of plasma sample. The coefficient of variation and relative error for intra- and inter-assay at four QC levels were 1.2–10.0% and −7.2–3.2%, respectively. The present method was applied successfully to the pharmacokinetic study of anastrozole after oral administration of 1 mg anastrozole tablet to healthy male volunteers. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: HILIC/MS/MS; anastrozole; human plasma

Introduction

Anastrozole, 2-[3-(cyano-dimethyl-methyl)-5-[1,2,4]triazol-1-yl-methyl-phenyl]-2-methyl-propionitrile (Fig. 1), is a third-generation, highly potent and selective nonsteroidal aromatase inhibitor used for treatment of breast cancer in postmenopausal women. Anastrozole has been shown to block estrogen synthesis both peripherally and within the breast in postmenopausal women with large, operable breast cancers (Geisler *et al.*, 2002; Geisler, 2003).

Administration of a low therapeutic dose (1 mg) of anastrozole resulted in low plasma concentrations of anastrozole, and the use of a sensitive analytical method is necessary for determination of anastrozole in biological fluids. Capillary gas chromatography with electron capture detection (Bock *et al.*, 1997; Yuan *et al.*, 2001; Duan *et al.*, 2002) and reversed-phase high-performance liquid chromatographic methods with tandem mass spectrometry (RPLC/MS/MS) using atmospheric pressure chemical ionization (Mareck *et al.*, 2006; Apostolou *et al.*, 2008a), electrospray ionization (ESI; Mazzarino and Botre, 2006; Beer *et al.*, 2010; Jangid *et al.*, 2010; Yu *et al.*, 2011) or photospray ionization (Mendes *et al.*, 2007) have been reported. Liquid–liquid extraction using diethyl-ether, methyl *tert*-butyl ether, or a mixture of diethyl-ether and dichloromethane as the extraction solvent (Bock *et al.*, 1997; Yuan *et al.*, 2001; Duan *et al.*, 2002; Mareck *et al.*, 2006; Mazzarino and Botre, 2006; Mendes *et al.*, 2007; Yu *et al.*, 2011), protein precipitation with acetonitrile followed by liquid–liquid extraction (Apostolou *et al.*, 2008a) and solid-phase extraction (Beer *et al.*, 2010; Jangid *et al.*, 2010) have been used as sample clean-up procedures.

Hydrophilic interaction chromatography (HILIC) has been used increasingly in analysis of polar or hydrophilic drugs and metabolites for bioanalysis and metabonomic studies (Apostolou *et al.*, 2008b; Hsieh, 2008; Nguyen and Schug, 2008; Cai *et al.*, 2009; Lee *et al.*, 2009; Cubbon *et al.*, 2010; Ji *et al.*, 2010; Lang *et al.*, 2010; Spagou *et al.*, 2011). HILIC uses polar stationary phases, such as bare silica, amino and sulfobetaine-type zwitterionic columns, and low aqueous/high organic mobile phase. It has been shown to be a valuable tool for use in MS analysis of polar or hydrophilic compounds, particularly ESI technique, compared with RPLC (Nguyen and Schug, 2008). Owing to more favorable desolvation and ionization conditions, the high organic mobile phases in HILIC offer increased sensitivity and can provide direct injection of organic extracts obtained from protein precipitation, liquid–liquid extraction, or solid-phase extraction, resulting in the removal of time-consuming evaporation and reconstitution steps during sample preparation.

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Abbreviations used: HILIC, hydrophilic interaction chromatography.

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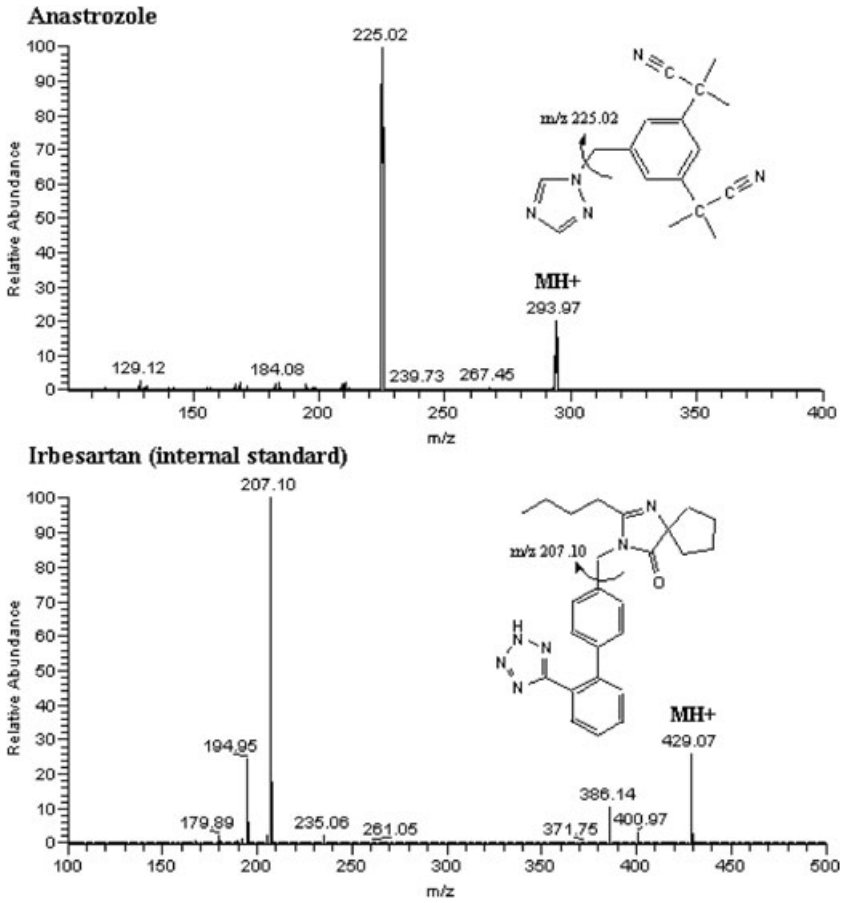

Figure 1. Product ion mass spectra of anastrozole and irbesartan (internal standard).

To the best of our knowledge, the use of the HILIC/MS/MS method for determination of anastrozole in human plasma has not been reported. A simple, rapid, selective and sensitive HILIC/ESI-MS/MS method is described and validated for determination of anastrozole in human plasma. The present method was applied successfully to the pharmacokinetic study of anastrozole after oral administration of anastrozole (1 mg tablet) in male volunteers.

Experimental

Materials and reagents

Anastrozole (purity, 99.8%) and irbesartan (purity, 100.6%; internal standard) were supplied by Dong-A Pharmaceutical Co. (Seoul, Korea). Acetonitrile, dichloromethane and methyl *tert*-butyl ether (HPLC-grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of the highest quality available. Drug-free human plasma containing sodium heparin as the anticoagulant was obtained from healthy volunteers.

Preparation of calibration standards and quality control samples

Primary stock solutions of anastrozole and irbesartan (1 mg/mL) were prepared in dimethylsulfoxide. Standard working solutions of anastrozole were prepared by dilution of the primary stock solution with acetonitrile. The internal standard working solution (25 ng/mL irbesartan) was prepared by dilution of an aliquot of stock solution with acetonitrile. All standard solutions were stored at ca. 4 °C in a 20 mL scintillation vial in the dark, when not in use.

Human plasma calibration standards of anastrozole, i.e., 0.10, 0.20, 0.50, 1.0, 5.0, 10.0, 20.0 and 50.0 ng/mL, were prepared by spiking the standard working solutions into a pool of drug-free human plasma. Quality control (QC) samples at 0.10, 0.40, 8.0 and 40.0 ng/mL were prepared in bulk by addition of 50 µL of the appropriate standard working solutions (20, 80, 1600 and 8000 ng/mL) to drug-free human plasma (9950 µL). Bulk samples were aliquoted (200 µL) into polypropylene tubes and stored at −80 °C until analysis.

Sample preparation

Aliquots of 200 µL of human blank plasma, calibration standards and QC samples were mixed with 10 µL of 25 ng/mL irbesartan in acetonitrile solution (internal standard) and 500 µL of 0.2 M ammonium acetate. The mixtures were extracted with 1000 µL of the mixture of dichloromethane and methyl *tert*-butyl ether (30:70, v/v) in 2.0 mL polypropylene tubes by vortex-mixing for 2 min at high speed and centrifuged at 13,000 ***g*** for 5 min at 4 °C. A 900 µL aliquot of the organic layer was transferred and evaporated to dryness for 10 min at 30 °C using a vacuum concentrator (Ez-2 plus, Genevac, Ipswich, UK). The residue was dissolved in 70 µL of acetonitrile in 10 mM ammonium formate (95:5, v/v) by vortex-mixing for 2 min, sonicated for 5 min, and centrifuged. The aliquot (2 µL) was injected into the LC/MS/MS system.

HILIC/MS/MS analysis

The LC/MS/MS system consisted of a Nanospace SI-2 pump, SI-2 column oven, an SI-2 autosampler (Shiseido, Tokyo, Japan) and a tandem

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quadrupole mass spectrometer (TSQ Quantum Access, ThermoFisher Scientific, CA, USA). The separation was performed on a Luna HILIC column (3 µm, 100 × 2.0 mm i.d., Phenomenex, Torrance, CA, USA) using 95% acetonitrile in 10 mM ammonium formate at a flow-rate of 0.2 mL/min. The column and autosampler tray were maintained at 50 and 6 °C, respectively. The analytical run time was 3.0 min. The electrospray ionization source settings for analysis of anastrozole and irbesartan were as follows: spray voltage, 4.5 kV; vaporizer temperature, 250 °C; capillary temperature, 330 °C; sheath gas pressure, 35 psi; and auxiliary gas pressure, 10 psi. The tube lens offsets for anastrozole and irbesartan were 79 and 71 V, respectively, in order to produce a strong protonated molecular ion ($[M+H]^+$) without formation of adduct and fragment ions. Fragmentation of the $[M+H]^+$ ion for anastrozole and irbesartan was performed at a collision energy of 23 V by collision-activated dissociation with argon gas as the collision gas at a pressure setting of 1.5 on the instrument. Selected reaction monitoring (SRM) mode was employed for the quantification: *m/z* 293.97 → 225.02 for anastrozole and *m/z* 429.07 → 207.10 for irbesartan. Xcalibur® software (ThermoFisher Scientific) was used for LC/MS/MS system control and data processing.

Method validation

Analysis of batches, consisting of three replicate calibration standards at each concentration, was performed on five different days in order to complete the method validation. In each batch, QC samples at 0.10, 0.40, 8.0 and 40.0 ng/mL were assayed in sets of five replicates for evaluation of intra- and inter-day precision and accuracy. The relative error (RE), percentage deviation of the mean from true values and the coefficient of variation (CV) serve as measures of accuracy and precision, respectively.

The matrix effect and recovery of anastrozole were assessed by analysis of three sets of standards at four concentrations, i.e. 0.10, 0.40, 8.0 and 40.0 ng/mL (Matuszewski *et al.*, 2003; Trufelli *et al.*, 2010). The absolute matrix effect for anastrozole was assessed by comparison of the mean peak areas of the analyte at four concentrations spiked after extraction into plasma extracts originating from five different humans (set 2) with the mean peak areas for neat solutions of the analyte in 95% acetonitrile (set 1). The relative matrix effect was evaluated as a measure of variability in the peak areas of the analyte spiked post-extraction into five different plasma extracts (set 2) expressed as CV (%). Recovery of anastrozole and irbesartan was determined by comparison of mean peak areas of analytes spiked before extraction into the same five different plasma samples as set 2 (set 3) with those of the analytes spiked post-extraction into different blank plasma lots at four concentrations (set 2).

For evaluation of process stability, the five replicates of QC samples at low and high concentrations (0.40 and 40.0 ng/mL) were re-injected after 24 h storage in the autosampler.

Clinical application

This method was applied to a pharmacokinetic study after oral administration of anastrozole to male volunteers. The protocol was approved by an institutional review board at the Clinical Trial Center, Pusan Paik Hospital (Pusan, Korea) and the informed consents were obtained from subjects after explaining details of the nature and purpose of the study. Twenty-nine healthy volunteers, who fasted for 10 h, received a single oral dose of anastrozole (Arimidex® tablet 1 mg, AstraZeneca Korea, Seoul, Korea) with 240 mL of water. Blood samples (3 mL) were withdrawn from the forearm vein at 0.33, 0.67, 1, 1.33, 1.67, 2, 3, 4, 6, 8, 12, 24, 48, 96 and 144 h post dosing, transferred to Vacutainer™ tubes (sodium heparin; BD, NJ, USA), and centrifuged at 3000 ***g*** for 10 min. Plasma samples were transferred to polypropylene tubes and stored at 80 °C prior to analysis.

The maximum concentration (C_{max}) and the time to C_{max} (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time curve for anastrozole. A noncompartment analysis (WinNonlin, Pharsight, Mountain View, CA, USA) was used for calculation of area under the plasma concentration–time curve (AUC) from 0 to 144 h and terminal elimination half-life ($t_{1/2}$).

Results and discussion

HILIC/MS/MS

Electrospray ionization of anastrozole and irbesartan produced abundant $[M+H]^+$ ion at *m/z* 293.97 and 429.07, respectively, without evidence of fragmentation and adduct formation. The $[M+H]^+$ ion of anastrozole and irbesartan was selected as the precursor ion and was subsequently fragmented in MS/MS mode in order to obtain the product ion spectra, yielding useful structural information (Fig. 1). Anastrozole produced the major product ion at *m/z* 225.02 by loss of [1,2,4]triazole from $[M+H]^+$ ion, and irbesartan showed a prominent product ion at *m/z* 207.10 (2-butyl-3-methyl-1,3-diaza-spiro[4.4]non-1-en-4-one radical). The SRM mode was used for quantification of analytes due to the high selectivity and sensitivity of SRM data acquisitions, where the transition of the precursor ion to a product ion is monitored as follows: *m/z* 293.97 → 225.02 for anastrozole and *m/z* 429.07 → 207.10 for irbesartan (internal standard).

HILIC operated with a silica column and low aqueous–high organic mobile phase is appropriate for retention of polar anastrozole with MS/MS detection. Primary HILIC chromatographic parameters include column chemistry, the type and composition of organic solvent, buffer pH and ionic strength (Apostolou *et al.*, 2008b; Hsieh, 2008; Nguyen and Schug, 2008; Cai *et al.*, 2009; Lee *et al.*, 2009; Cubon *et al.*, 2010; Lang *et al.*, 2010; Spagou *et al.*, 2011). Anastrozole showed better retention and peak shape, and less matrix effect on a Luna HILIC column, compared with an Atlantis HILIC silica column (Waters Co., Milford, MA, USA). Use of 10 mM ammonium formate itself resulted in better retention and sensitivity of anastrozole, compared with pH 3.0 and 4.5 ammonium formate buffers on a Luna HILIC column. The high acetonitrile content (95%) in the mobile phase increased electrospray ionization efficiency for MS/MS detection and resulted in high sensitivity of anastrozole. The sensitivity for anastrozole using this HILIC/MS/MS method was 0.10 ng/mL plasma, and it was 0.30 ng/mL plasma using RPLC/MS/MS methods with the ESI technique (Apostolou *et al.*, 2008a; Jangid *et al.*, 2010).

In analysis of blank plasma samples from 29 volunteers, no interference peak was observed at the retention times of anastrozole (1.48 min) and irbesartan (1.63 min), indicating the selectivity of the present method (Fig. 2a). The retention times of anastrozole and irbesartan were reproducible throughout the experiment and no column deterioration was observed after analysis of 500 human plasma samples. The sample carryover effect was not observed.

Method validation

Calibration curves were obtained over the concentration range of 0.10–50.0 ng/mL of anastrozole in human plasma. Linear regression analysis with a weighting of 1/concentration gave the optimum accuracy (RE, −3.0–2.0%) and precision (CV, ≤10.0%) of the corresponding calculated concentrations at each level (Table 1). The low CV value (5.3%) for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-day precision and accuracy data for QC samples containing anastrozole. Both

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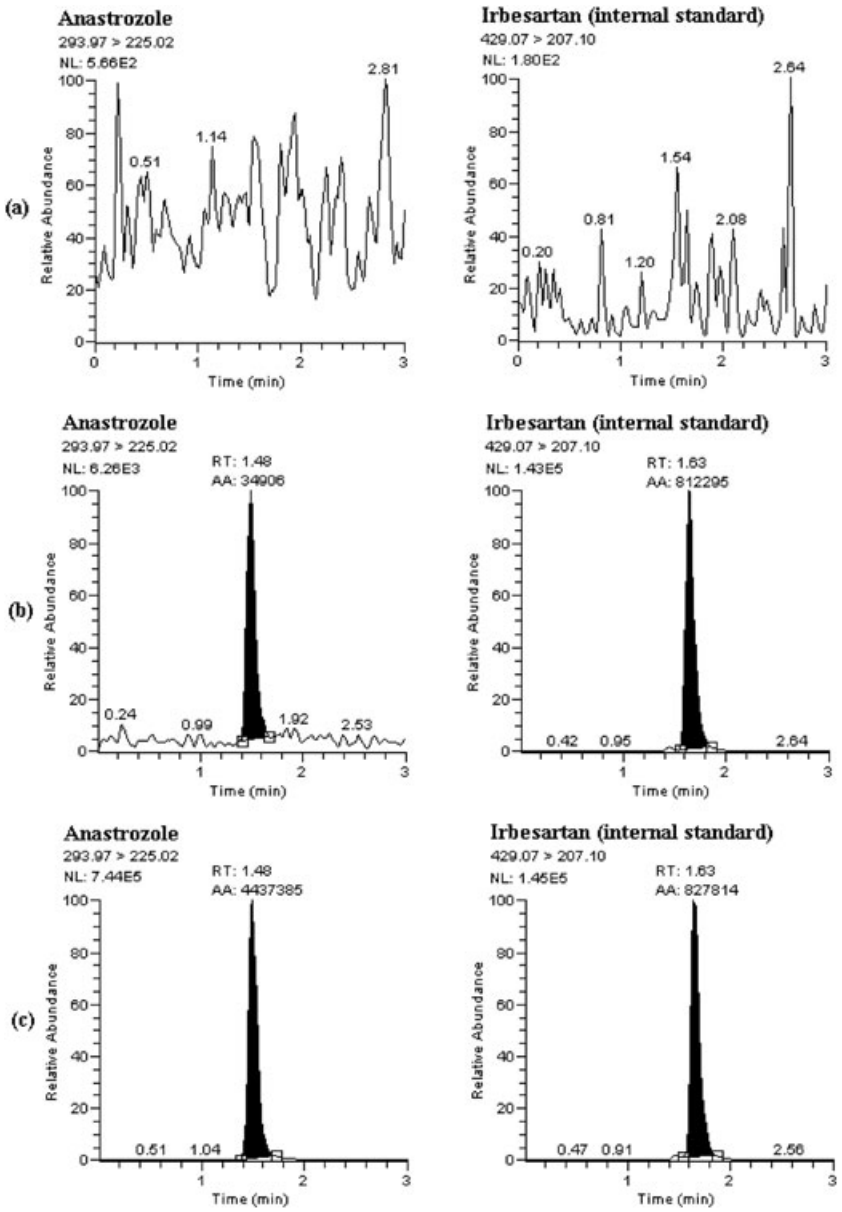

Figure 2. SRM chromatograms of (a) a human blank plasma, (b) a human plasma sample spiked with 0.10 ng/mL of anastrozole and (c) a human plasma sample obtained 1 h after oral administration of anastrozole (1 mg tablet) to a male volunteer.

Table 1. Calculated concentrations of anastrozole in calibration standards prepared in human plasma ($n = 5$)

Statistical variable	Theoretical concentration (ng/mL)								Slope	Intercept	r^2
	0.10	0.20	0.50	1.0	5.0	10.0	20.0	50.0			
Mean (ng/mL)	0.10	0.20	0.49	1.00	5.1	9.7	19.8	50.4	0.3653	0.0053	0.9992
CV (%)	10.0	5.0	4.1	5.0	2.9	3.2	3.3	1.3	5.3		0.1
RE (%)	0.0	0.0	−2.0	0.0	2.0	−3.0	−1.0	0.8			

intra-and inter-assay CV values ranged from 1.2 to 10.0% at four QC levels. Intra-and inter-assay RE values were −7.2–3.2% at four QC levels. These results indicate acceptable accuracy and precision of the present method. The lower limit of quantitation (LLOQ) was set at 0.10 ng/mL for anastrozole using 200 µL of human plasma and a representative chromatogram of the LLOQ is shown in Fig. 2b; the signal-to-noise ratio for anastrozole was higher than 70.

Liquid–liquid extraction using a mixture of methyl-*tert*-butyl ether and dichloromethane (70:30, v/v) at neutral pH showed

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Table 2. Precision and accuracy of anastrozole in human plasma quality control samples

Statistical variable	Intra-day ($n = 5$)				Inter-day ($n = 5$)			
QC (ng/mL)	0.10	0.40	8.0	40.0	0.10	0.40	8.0	40.0
Mean (ng/mL)	0.10	0.38	7.8	41.3	0.10	0.39	7.6	37.1
CV (%)	10.0	2.6	1.2	1.7	10.0	7.7	4.3	6.4
RE (%)	0.0	−5.0	−2.5	3.2	0.0	−2.5	−5.0	−7.2

better recovery of anastrozole, with lower interference and little matrix effect, compared with methyl-*tert*-butyl ether, ethylacetate and their mixtures at acidic and basic pH. Use of ammonium acetate for adjustment of pH to neutral pH in the sample preparation procedure resulted in less matrix effect compared with phosphate buffer. The overall extraction recovery of anastrozole was 81.6%, which was consistent over the concentration range of 0.10–40.0 ng/mL, and recovery of irbesartan (internal standard) was 44.8% (Table 3).

The absolute matrix effects, the mean peak area ratios of set 2 to set 1 multiplied by 100, were 106.8% for anastrozole and 105.5% for irbesartan (Table 3), indicating a slight ionization enhancement for anastrozole and irbesartan. A value of 100% indicated that the response in the solvent and in plasma extracts was the same and that no absolute matrix effect was observed. A value of <100% indicated ionization suppression and a value of >100% indicated ionization enhancement. Assessment of the relative matrix effect was based on direct comparison of the peak areas of anastrozole and irbesartan spiked post-extraction into plasma extracts originating from five different human plasma samples (set 2). The CVs of the determination of set 2 at different concentrations were 2.7–4.6% for anastrozole and 1.4–2.7% for irbesartan (Table 3). This variability appeared to be comparable to CVs of peak areas of standards injected directly in 95% acetonitrile (set 1) (1.0–8.2%), indicating that there was no relative matrix effect for anastrozole and irbesartan. These data suggest that the matrix effects for anastrozole and irbesartan have little effect on determination of anastrozole in human plasma samples.

Reanalysis of the reconstituted extracts stored for 24 h at 4 °C showed acceptable accuracy (RE: 2.5–8.9%) and precision (CVs: ≤ 4.7%) for QC samples at 0.40 and 40.0 ng/mL. According to Jangid *et al.* (2010), anastrozole was found to be stable in plasma for five freeze and thaw cycles and at least 6 h at room temperature.

Clinical application

This method was applied successfully to analysis of 500 plasma samples in a pharmacokinetic study of anastrozole. Representative chromatograms of the extract of a plasma sample obtained 1 h after oral administration of anastrozole at a dose of 1 mg to humans are shown in Fig. 2(c). Figure 3 shows mean plasma concentration profiles of anastrozole obtained after a single oral dosing of anastrozole (1 mg tablet) administered to 29 healthy male volunteers. The C_{max}, T_{max}, AUC_{0-144h} and $t_{1/2}$ of anastrozole were 16.3 ± 6.6 ng/mL, 1.9 ± 1.4 h, 623.7 ± 242.9 ng h/mL and 41.3 ± 13.7 h, respectively.

Conclusion

A rapid, selective, sensitive and reliable HILIC/ESI-MS/MS method for quantification of anastrozole in human plasma was successfully developed. Anastrozole and irbesartan were extracted from 200 µL of plasma samples using a mixture of dichloromethane and methyl *tert*-butyl ether (30:70, v/v) for sample preparation. Use of this method demonstrated acceptable sensitivity (LLOQ 0.10 ng/mL), precision, accuracy, selectivity and matrix effect. The method was applied successfully to determination of anastrozole in human plasma samples for a pharmacokinetic study of anastrozole.

Table 3. Absolute matrix effect and recovery of anastrozole and irbesartan (internal standard) in five different lots of human plasma

Nominal concentration (ng/mL)	Absolute matrix effect[a] (CV[b]) (%)		Recovery[c] (%)	
	Anastrozole	Irbesartan	Anastrozole	Irbesartan
0.1	108.5 (4.6)	105.0 (2.0)	85.5	42.7
0.4	104.3 (3.1)	107.2 (2.7)	78.9	44.8
8.0	105.0 (2.7)	102.8 (1.4)	76.7	43.2
40.0	109.3 (3.1)	106.9 (2.4)	85.4	48.8
Mean	106.8	105.5	81.6	44.8

[a]Absolute matrix effect expressed as the ratio of the mean peak area of an analyte spiked post liquid–liquid extraction (set 2) to the mean peak area of the same analyte standards (set 1) multiplied by 100.
[b]Relative matrix effect expressed as CV of the peak areas of set 2 at different concentrations.
[c]Recovery calculated as the ratio of the mean peak area of an analyte spiked before liquid–liquid extraction (set 3) to the mean peak area of an analyte spiked after liquid–liquid extraction (set 2) multiplied by 100.

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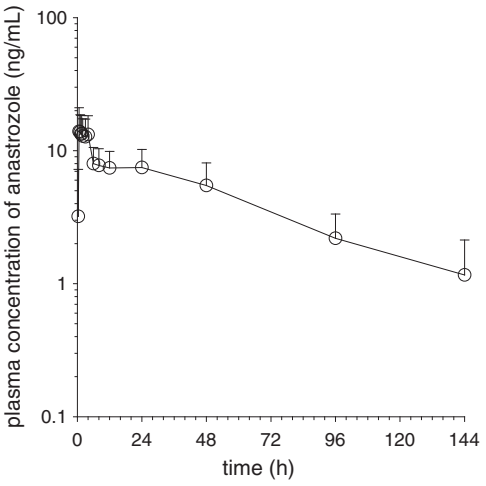

Figure 3. Mean plasma concentration-time plot of anastrozole after administration of a single oral dose of anastrozole (1 mg tablet) to 29 male volunteers. Each point represents the mean ± SD.

Acknowledgment

This study was supported by a National Research Foundation of Korea grant funded by the Korean Government (MEST; 2008–0060156) and The Catholic University of Korea, 2011.

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