Journal of Chromatography B, 879 (2011) 2845–2851

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Liquid chromatography–mass spectrometry method for the quantification of tamoxifen and its metabolite 4-hydroxytamoxifen in rat plasma: Application to interaction study with biochanin A (an isoflavone)

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ARTICLE INFO

Article history:
Received 1 March 2011
Accepted 27 July 2011
Available online 2 August 2011

Keywords:
Tamoxifen
4-Hydroxytamoxifen
Rat plasma
Validation
LC–MS/MS
Pharmacokinetics

ABSTRACT

Tamoxifen is the agent of choice for the treatment of estrogen receptor-positive breast cancer. Tamoxifen is a substrate of P-glycoprotein (P-gp) and microsomal cytochrome P450 (CYP) 3A, and biochanin A (BCA) is an inhibitor of P-gp and CYP3A. Hence, it could be expected that BCA would affect the pharmacokinetics of tamoxifen. In the present study we have developed and validated a simple, sensitive and specific LC–ESI-MS/MS method for the simultaneous quantification of tamoxifen and its metabolite 4-hydroxytamoxifen with 100 μL rat plasma using centchroman as an internal standard (IS). Tamoxifen, 4-hydroxytamoxifen and IS were separated on a Supelco Discovery C18 (4.6 mm × 50 mm, 5.0 μm) column under isocratic condition using 0.01 M ammonium acetate (pH 4.5):acetonitrile (10:90, v/v) as a mobile phase. The mobile phase was delivered at a flow rate of 0.8 mL/min. The method was proved to be accurate and precise at linearity range of 0.78–200 ng/mL with a correlation coefficient (r) of ≥0.996. The intra- and inter-day assay precision ranged from 1.89 to 8.54% and 3.97 to 10.26%, respectively; and intra- and inter-day assay accuracy was between 87.63 and 109.06% and 96 and 103.89%, respectively for both the analytes. The method was successfully applied to study the effect of oral co-administration of BCA (an isoflavone) on the pharmacokinetics of tamoxifen and 4-hydroxytamoxifen in female rats. The coadministration of BCA caused no significant changes in the pharmacokinetics of tamoxifen and 4-hydroxytamoxifen. However, the peak plasma concentration (C_{max}) of 4-hydroxytamoxifen in BCA pretreated rats was significantly ($P < 0.05$) lower than those from control group.

1. Introduction

Tamoxifen is a non-steroidal anti-estrogenic compound being used in the treatment of breast cancer. Orally administered tamoxifen undergoes extensive CYP450 mediated hepatic metabolism and subsequent biliary excretion [1–3]. Several metabolites of tamoxifen have been identified in human plasma. These include N-desmethyltamoxifen, N-desdimethyltamoxifen, 4-hydroxytamoxifen, etc. [4]. Among the serum metabolites, 4-hydroxytamoxifen has received particular attention since it has higher *in vitro* affinity towards the estrogen receptor than the parent drug. It has been reported to be about 100 times more potent as an estrogen antagonist than tamoxifen and its plasma and tumor concentrations found only about 2% of those of the parent compound [5,6]. Tamoxifen and its metabolite 4-hydroxytamoxifen are substrates of P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) 2 [7,8]. Since, the tamoxifen is a substrate of P-gp and undergo extensive CYP450 mediated metabolism makes it vulnerable for interactions. Several interaction studies have been reported for tamoxifen with flavonoids in rats [9–11].

Till date, several bioanalytical methods have been reported for tamoxifen in human plasma [12–15]. However, there is no validated method available in literature for simultaneous quantification of tamoxifen and its metabolite 4-hydroxytamoxifen in rat plasma. Therefore, the purpose of present study was to develop and validate the LC–MS/MS method for simultaneous quantification of tamoxifen and its metabolite 4-hydroxytamoxifen in rat plasma. This validated method was successfully applied for interaction study of tamoxifen with biochanin A (BCA, an isoflavone) in rats. BCA is a principal component in red clover extracts, such as Promensil (Novogen, Inc., Samford, CT, USA). BCA shown to be a potent inhibitor of the P-gp and BCRP [16,17].

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doi:10.1016/j.jchromb.2011.07.036

2. Experimental

2.1. Chemicals and reagents

Tamoxifen and 4-hydroxytamoxifen were purchased from Sigma Aldrich Ltd. (St. Louis, USA). Centchroman (IS) was synthesized at the Medicinal Chemistry Division of Central Drug Research Institute Lucknow, India. Chemical structure of tamoxifen, 4-hydroxytamoxifen and IS is shown in Fig. 1. HPLC grade acetonitrile and isopropanol were purchased from Sisco Research Laboratories (SRL) Pvt. Limited (Mumbai, India). Dimethyl sulfoxide was purchased from Thomas Baker Chemicals Pvt. Limited (Mumbai, India). Ammonium acetate and glacial acetic acid (GAA) AR were purchased from E Merck Limited (Mumbai, India). Milli-Q Ultra pure water was obtained from a Millipore Elix water purification system purchased from Millipore India Pvt. Ltd. (New Delhi, India). Heparin sodium injection I.P. (1000 IU/mL) was purchased from Gland Pharma (Hyderabad, India). Blank, drug free plasma samples were collected from adult, healthy female Sprague-Dawley rats at Division of Laboratory Animals (DOLA) of Central Drug Research Institute (Lucknow, India). Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcass of animals.

2.2. Instrumentation and chromatographic conditions

HPLC system consists of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin-Elmer instruments, Norwalk, USA) was used to inject 10 μL aliquots of the processed samples on a Supelco Discovery C18 column (4.6 mm × 50 mm, 5.0 μm). The system was run in isocratic mode with mobile phase consisting of 0.01 M ammonium acetate (pH 4.5) and acetonitrile in ratio of 10:90 (v/v). Mobile phase was delivered at a flow rate of 0.8 mL/min. Mobile phase was duly filtered through 0.22 μm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min prior to use. Separations were performed at room temperature.

Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an electrospray ionization (ESI) source. The ion spray voltage was set at 5500 V. The instrument parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 40, 10, 50 and 10, respectively. Compound parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were 95, 35, 10, 10 V, 90, 40, 10, 10 V and 100, 43, 10, 10 V for tamoxifen, 4-hydroxytamoxifen and IS, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada).

2.3. Preparation of stock and standard solutions

Primary stock solutions of tamoxifen, 4-hydroxytamoxifen and IS were prepared by dissolving the compounds in DMSO followed by diluting the solution with methanol to achieve desired concentration of 1 mg/mL. Working standard solutions of tamoxifen and 4-hydroxytamoxifen were prepared by combining the aliquots of each primary stock solution and diluting with methanol. A working stock solution of IS (0.1 μg/mL) was prepared by diluting an aliquot of primary stock solution with methanol. Calibration standards of tamoxifen and 4-hydroxytamoxifen (0.78, 1.56, 3.13, 6.25, 12.50, 25, 50, 100 and 200 ng/mL) were prepared by spiking the pooled drug free rat plasma with the appropriate working standard solution of the analytes. All the stock solutions were stored at 4 °C until analysis. Quality control (QC) samples were prepared at four concentration levels [0.78 ng/mL (lower limit of quantitation,

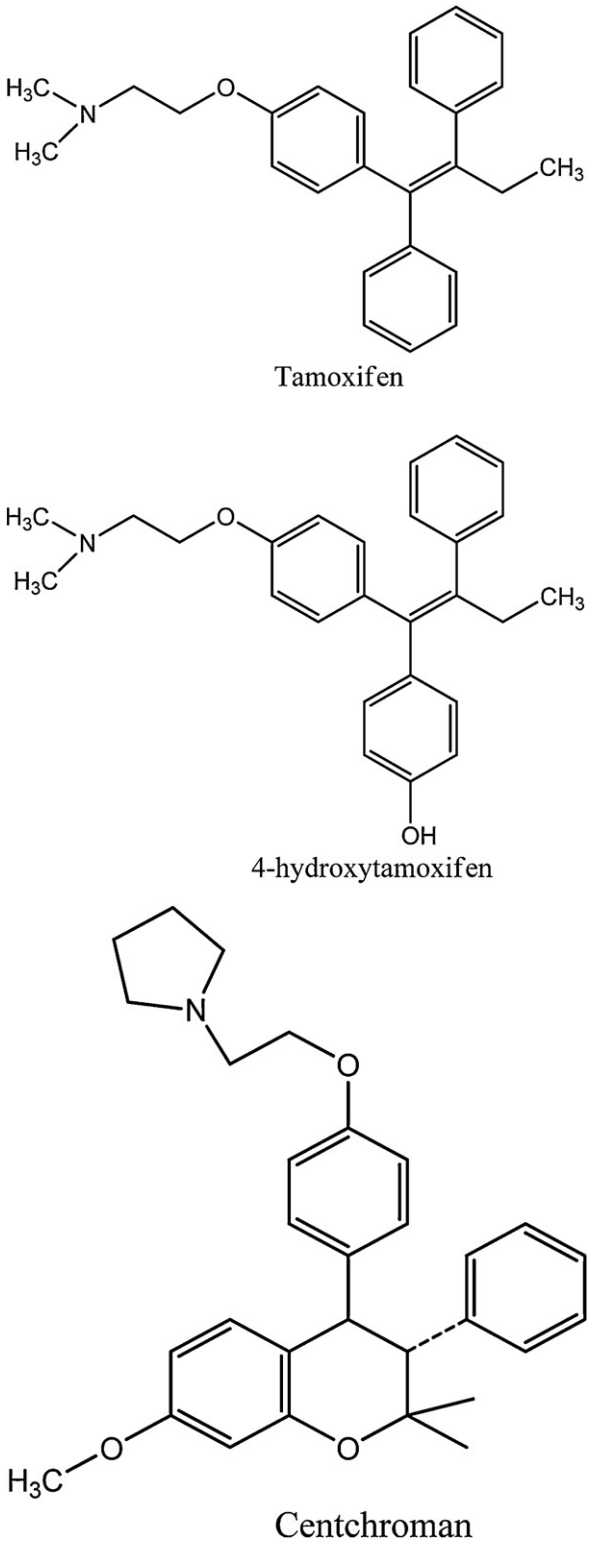

Fig. 1. Structural representation of tamoxifen, 4-hydroxytamoxifen and centchroman (IS).

LLOQ), 2.5 ng/mL (QC low), 80 ng/mL (QC medium) and 160 ng/mL (QC high)] and stored at −70 ± 10 °C until analysis.

2.4. Recovery

The extraction recovery of analytes, through liquid–liquid extraction procedure, was determined by comparing the peak areas of extracted plasma (pre-spiked) standard QC samples ($n = 6$) to those of the post-spiked standards at equivalent concentrations [18,19]. Recoveries of tamoxifen and 4-hydroxytamoxifen were determined at three concentration levels QC low, QC medium and QC high concentrations viz., 2.5, 80, and 160 ng/mL, whereas the recovery of the IS was determined at a single concentration of 5 ng/mL.

2.5. Sample preparation

All QCs, calibration curve and plasma samples of administered drugs were prepared as following processing. To 100 μL of plasma in a tube, 5 μL of IS solution (0.1 μg/mL in methanol), was added and mixed for 15 s on a cyclomixer (Spinix Tarsons, Kolkata, India). Then 2 mL of 2.5% (v/v) isopropanol in *n*-hexane was added and the mixture was vortexed for 3 min, followed by centrifugation for 5 min at 2000 × *g* on Sigma 3-16K (Frankfurt, Germany). The organic layer (1.6 mL) was separated and evaporated to dryness under vaccum in speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200 μL of the mobile phase and 10 μL was injected onto analytical column.

2.6. Validation procedures

A full validation according to the FDA guidelines was performed for the assay in rat plasma [20].

2.6.1. Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples collected from six different rats to investigate the potential interferences at the LC peak region for analytes and IS using the proposed extraction procedure and chromatographic–MS conditions.

2.6.2. Matrix effect

The effect of rat plasma constituents over the ionization of tamoxifen, 4-hydroxytamoxifen and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ($n = 6$) with the response of analytes from neat standard samples at equivalent concentrations [18,19]. The matrix effect for tamoxifen and 4-hydroxytamoxifen was determined at three concentration levels viz., 2.5, 80 and 160 ng/mL whereas the matrix effect over the IS was determined at a single concentration of 5 ng/mL. A value of >100% indicates ionization enhancement and a value of <100% indicates ionization suppression. The post-extracted samples were the drug-free control plasma spiked with working standard stock solutions after extraction.

2.6.3. Calibration curve

The calibration curve was acquired by plotting the peak area ratio of analyte to that of IS against the nominal concentration of calibration standards. The results were fitted to linear regression analysis using $1/X^2$ as weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.995 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [20].

2.6.4. Precision and accuracy

The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 0.78, 2.5, 80 and 160 ng/mL. The inter-day assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% standard deviation (S.D.) from the nominal values and a precision of within ±15% relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed ±20% of accuracy as well as precision [20].

2.6.5. Stability experiments

All stability studies were conducted at two concentration levels, i.e., QC low and QC high, using six replicates at each concentration levels. Replicate injections of processed samples were analyzed up to 18 h to establish autosampler (AS) stability of analytes and IS. The peak areas of analytes and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. The stability of tamoxifen and 4-hydroxytamoxifen in the biomatrix during 6 h exposure at room temperature in rat plasma (bench top, BT) was determined at ambient temperature (25 ± 5 °C). Freeze/thaw (FT) stability was evaluated up to three cycles. In each cycle samples were frozen for at least 24 h at −70 ± 10 °C. Freezer stability of tamoxifen and 4-hydroxytamoxifen in rat plasma was assessed by analyzing the QC samples stored at −70 ± 10 °C for at least 15 days. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% S.D.) and precision (i.e., ±15% R.S.D.).

2.7. Application to interaction study

An interaction study was performed to show the applicability of newly developed and validated bioanalytical method. Study was performed in female Sprague-Dawley rats ($n = 5$, weight range 200–220 g). The rats were fasted overnight (14–16 h) prior to the experiment but given free access to water. Rats were divided into four groups ($n = 5$, each); control group (tamoxifen 10 mg/kg, oral, dissolved in a 0.9% NaCl-injectable solution and Tween 80, 9:1, v/v) and three pretreatment groups (5, 10 and 20 mg/kg of oral BCA (mixed in distilled water). BCA was administered 10 min prior to oral administration of tamoxifen. Blood samples (approximately 0.25 mL) were collected from the retro-orbital plexus into heparinized microfuge tubes at 0.08, 0.50, 1, 3, 5, 7, 9, 24, 30 and 49 h post-dosing and plasma was harvested by centrifuging the blood at 15,000 × *g* for 10 min and stored frozen at −70 ± 10 °C until bioanalysis. Plasma (100 μL) samples were spiked with IS, and processed as described above. Along with the plasma samples, QC samples were distributed among calibrators and unknown samples in the analytical run. The data was accepted based on performance of QCs prepared using rat blank plasma.

2.8. Pharmacokinetic and statistical analysis

The observed maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were obtained by visual inspection of the experimental data. Various pharmacokinetic parameters like area under the curve (AUC), elimination half life ($t_{1/2}$), for each rat were calculated using a non compartmental pharmacokinetic program WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). The metabolic ratio (MR) was defined as the ratio of AUC value for 4-hydroxytamoxifen to that of tamoxifen.

The relative bioavailability was calculated as follows:

$$\text{Relative bioavailability (RB\%)} = \frac{\text{AUC}_{\text{coadmin}}}{\text{AUC}_{\text{control}}} \times 100$$

The mean pharmacokinetic parameters were compared using a one-way analysis of variance, followed by followed by Tukey test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Liquid chromatography

Method development began with the optimization of chromatographic conditions including mobile phase composition and column type. Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH range of 4.0–7.0, along with altered flow rates (in the range of 0.4–1.0 mL/min) were tested to get good peak shape, selectivity and sensitivity for tamoxifen, 4-hydroxytamoxifen and IS (data not shown). Mobile phase comprising 0.01 M ammonium acetate (pH 4.5):acetonitrile (10:90, v/v) was delivered at a flow rate of 0.8 mL/min was found to be suitable during LC optimization and enabled the determination of electrospray response for tamoxifen, 4-hydroxytamoxifen and IS. Versatility, suitability, and robustness of the method was checked with several C18 columns from various manufacturers (data not shown) and found that peak shape, selectivity and sensitivity were good with Supelco Discovery C18 column (4.6 mm × 50 mm, 5.0 μm). The overall analysis time was 5 min.

3.2. Mass spectrometry

In order to optimize ESI conditions for tamoxifen, 4-hydroxytamoxifen and IS, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra for tamoxifen, 4-hydroxytamoxifen and IS revealed peaks at *m*/*z* 372.5, 388.2 and 458.5 respectively as protonated molecular ions $[M+H]^+$. Following detailed optimization of mass spectrometry conditions (provided in Section 2.2), *m*/*z* 372.5 precursor ion $[M+H]^+$ to the *m*/*z* 72 product ion for tamoxifen, *m*/*z* 388.2 precursor ion $[M+H]^+$ to the *m*/*z* 72 product ion for 4-hydroxytamoxifen and *m*/*z* 458.5 precursor ion $[M+H]^+$ to the *m*/*z* 98.1 product ion for IS was used for the quantitation purpose.

3.3. Validation procedures

3.3.1. Specificity, recovery and matrix effect

In the present study, the specificity and selectivity have been studied by using independent plasma samples from six different rats.

Fig. 2 shows a typical chromatogram for the drug-free plasma (Fig. 2a), drug-free plasma spiked with tamoxifen and 4-hydroxytamoxifen at LLOQ and IS (Fig. 2b), and an *in vivo* rat plasma sample after oral administration of tamoxifen (Fig. 2c). As shown in Fig. 2a, there is no significant interference from plasma found at retention time of either the analyte or the IS. Auto-sampler

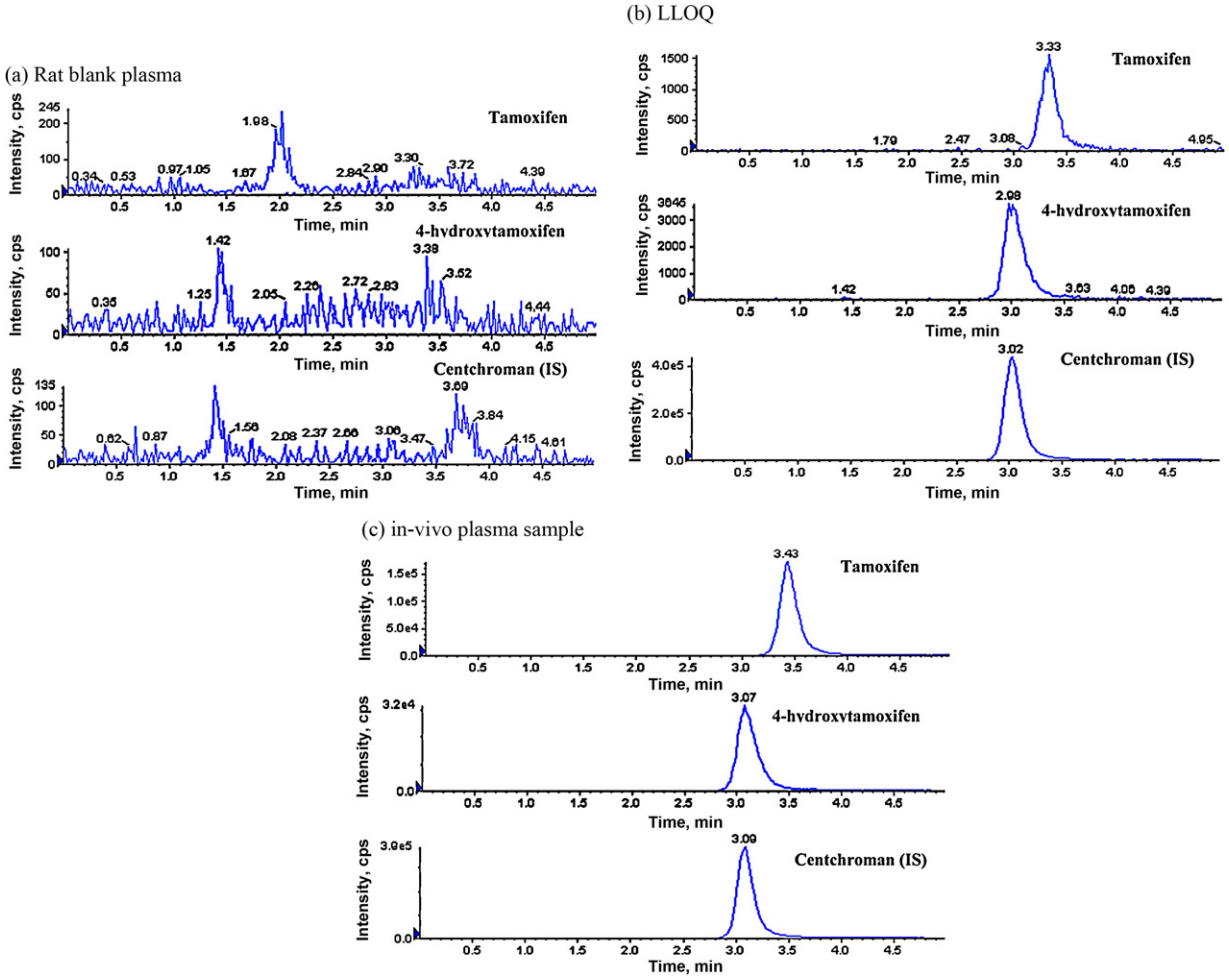

Fig. 2. Typical MRM chromatograms of tamoxifen and 4-hydroxytamoxifen in rat plasma (a) a drug free plasma, (b) drug free plasma spiked with tamoxifen and 4-hydroxytamoxifen at LLOQ (0.78 ng/mL) and IS and (c) an *in vivo* rat plasma sample showing tamoxifen (81.20 ng/mL) and 4-hydroxytamoxifen (7.03 ng/mL) peak obtained following oral coadministration of tamoxifen with BCA.

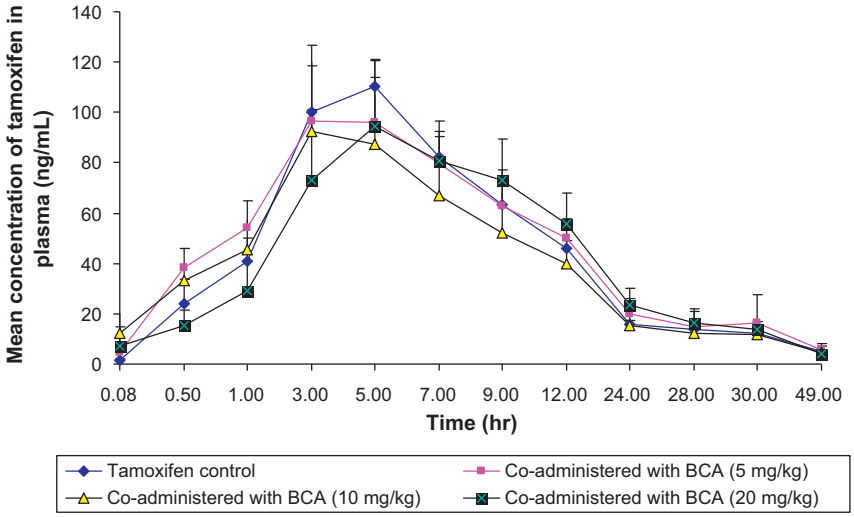

Fig. 3. Mean plasma concentration-time profiles of tamoxifen after the oral administration of tamoxifen (10 mg/kg) with or without BCA (5, 10 and 20 mg/kg) to rats. Bars represent the standard deviation ($n = 5$).

carry-over was determined by injecting the highest calibration standard then a blank sample. No carry-over was observed, as indicated by the lack of tamoxifen, 4-hydroxytamoxifen and IS peaks in the blank sample.

The extraction recovery was determined by comparing the peak areas of pre-spiked standards at 2.5, 80 and 160 ng/mL with those of post-extraction blank plasma standards spiked with corresponding concentrations. The extraction recoveries of the tamoxifen and 4-hydroxytamoxifen ranged from 96.58 to 105.14%, and the extraction recovery of the internal standard was 96.15%.

The ion suppression or enhancement by plasma was less than 8% for the analytes and IS which demonstrated that the matrix effects do not cause quantitation bias. Therefore, matrix effect could be negligible under the experimental conditions.

3.3.2. Calibration curve

The average regression for calibration curve ($n = 3$) was found to be 0.997 for tamoxifen and 0.998 for 4-hydroxytamoxifen. The % accuracy observed for the mean of back-calculated concentrations for three calibration curves was within 90.67–108.68; while the % precision values ranged from 1.23 to 7.35 for both the analytes.

3.3.3. Accuracy and precision

The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits. The intra- and inter-day assay precision ranged from 1.89 to 8.54% and 3.97 to 10.26%, respectively; and intra- and inter-day assay accuracy was between 87.63 and 109.06% and 96 and 103.89%, respectively for both the analytes.

3.3.4. Stability

The predicted concentrations for tamoxifen and 4-hydroxytamoxifen at 2.5 and 160 ng/mL samples deviated within the nominal concentrations in a battery of stability tests. The % precision values for both the analytes were found in the range of 91.58–111.04%; while the % accuracy ranged between 1.94 and 8.38%.

3.3.5. Dilution integrity

Dilution integrity experiments carried out at six replicates by 10 times dilution with blank plasma and assay precision and accuracy were determined in a similar manner as described in Section 2.6.4. The % accuracy of diluted QCs was in the range of 88.41–108.54; while % precision values ranged from 5.76 to 7.08 for both the analytes. The results suggested that samples whose concentrations were greater than the upper limit of calibration curve could be re-analyzed by appropriate dilution.

3.4. Application to interaction study

There has been considerable scientific interest in the role of isoflavones in numerous aspects of human health. Dietary supplements and herbal preparations containing isoflavones, specially derived from red clover are heavily marketed for the treatment of osteoporosis, cancer and for the alleviation of menopausal symptoms [21–23]. BCA is the principal isoflavones in red clover based dietary and herbal supplements. A combination of tamoxifen and BCA may be desirable because they may have synergistic antiproliferative and cytotoxic actions against breast cancer. However, studies concerning their pharmacokinetic interactions have not been reported.

In the present study we are reporting pharmacokinetic interaction between tamoxifen and BCA. The rat plasma samples, generated following interaction study, were analyzed by the newly developed and validated method along with QC samples. The mean plasma concentration–time profiles of tamoxifen administered (10 mg/kg) alone or in combination with BCA (5, 10 and 20 mg/kg) orally in rats, are shown in Fig. 3. Absorption of tamoxifen was rapid; tamoxifen was detected in plasma from the first blood sampling time (5 min) for both without and with BCA. Table 1 summarizes the pharmacokinetic parameters of tamoxifen.

BCA has been shown to inhibit P-gp and CYP3A [16,17] and thus was likely to modulate the plasma levels of tamoxifen, a known substrate of CYP3A/P-gp. However, as shown in Table 1, there are no apparent ($P > 0.05$) changes in the AUC, $t_{1/2}$, C_{max} and T_{max} of tamoxifen by co-administration of BCA with tamoxifen.

Among the reported metabolites of tamoxifen, 4-hydroxytamoxifen has received particular attention since it has higher *in vitro* affinity towards the estrogen receptor than the parent drug. The mean plasma concentration–time profiles of 4-hydroxytamoxifen are shown in Fig. 4, and the pharmacokinetic parameters are summarized in Table 2.

4-Hydroxytamoxifen is produced in humans by CYP2D6, CYP2C9, CYP2E1 and CYP3A4 [1,8]. Since, BCA is known to be metabolized by CYP1A2, CYP2D6 and CYP2E1 [24] and can inhibit CYP3A; it was expected to modulate the formation of

Table 1
Pharmacokinetic parameters of tamoxifen following the oral administration of tamoxifen (10 mg/kg) with or without biochanin A (5, 10 and 20 mg/kg) in rats.

Parameters	Tamoxifen control	Biochanin A coadministration (mg/kg)		
		5	10	20
$AUC_{0-\infty}$ (h ng/mL)	1572.35 ± 90.44	1525.69 ± 150.025	1494.98 ± 323.22	1610.93 ± 148.47
C_{max} (ng/mL)	111.74 ± 14.05	101.06 ± 27.99	98.38 ± 32.97	100.86 ± 8.11
T_{max} (h)	4.6 ± 0.89	4.20 ± 1.10	3.80 ± 1.10	5.00 ± 0.00
$t_{1/2}$ (h)	10.92 ± 1.89	12.09 ± 2.68	11.14 ± 2.18	9.47 ± 0.83
RB (%)	100.00	97.03	95.08	102.45

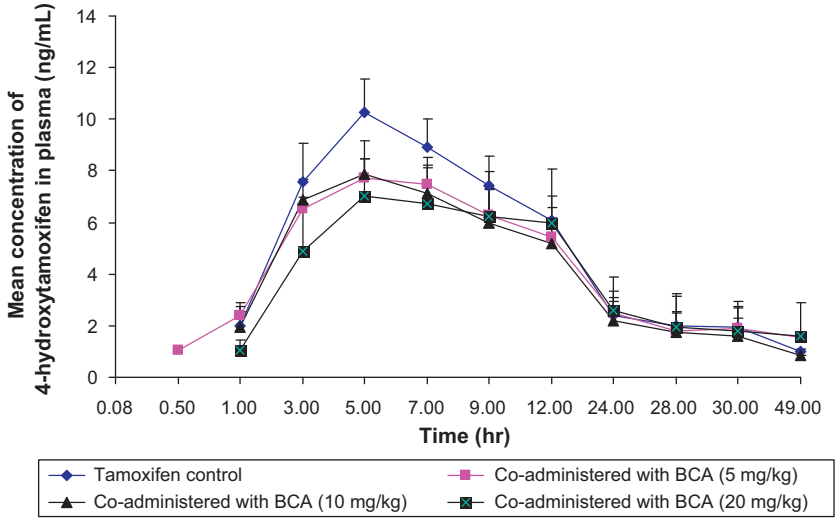

Fig. 4. Mean plasma concentration-time profiles of 4-hydroxytamoxifen after the oral administration of tamoxifen (10 mg/kg) with or without BCA (5, 10 and 20 mg/kg) to rats. Bars represent the standard deviation ($n = 5$).

Table 2
Pharmacokinetic parameters of 4-hydroxytamoxifen following the oral administration of tamoxifen (10 mg/kg) with or without biochanin A (5, 10 and 20 mg/kg) in rats.

Parameters	Tamoxifen control	Biochanin A coadministration (mg/kg)		
		5	10	20
$AUC_{0-\infty}$ (h ng/mL)	177.52 ± 31.90	170.21 ± 68.92	154.36 ± 45.97	158.39 ± 58.44
C_{max} (ng/mL)	10.31 ± 1.25	7.72 ± 1.06*	7.92 ± 0.57*	8.11 ± 0.90*
T_{max} (h)	5.00 ± 0.00	5.00 ± 0.00	5.80 ± 1.10	5.00 ± 1.63
MR	0.11 ± 0.02	0.11 ± 0.05	0.10 ± 0.03	0.10 ± 0.04
RB (%)	100.00	95.88	86.95	89.22

* Statistically significant at $P < 0.05$ when compared with the control.

4-hydroxytamoxifen. However, the MR ratio was found to be comparable in all groups (Table 2), suggesting that BCA has no significant effect on formation of 4-hydroxytamoxifen. There were no significant ($P > 0.05$) changes in the AUC and T_{max} of 4-hydroxytamoxifen in BCA pretreated rats in comparison to control. However, the C_{max} of 4-hydroxytamoxifen in BCA pretreated rats was significantly ($P < 0.05$) lower than those from control group.

4. Conclusion

In this study, we have validated LC–MS/MS method for simultaneous quantitation of tamoxifen and its metabolite 4-hydroxytamoxifen after liquid–liquid extraction from rat plasma. Validation results show that there is no significant matrix effect on analytes and selected IS. The validated method was successfully applied for studying the effect of BCA co-administration on pharmacokinetics of tamoxifen and its metabolite 4-hydroxytamoxifen in rats. No significant effect of BCA was observed on pharmacokinetics of tamoxifen. However, the BCA pretreatment significantly reduced the C_{max} of 4-hydroxytamoxifen upon co-administration of BCA (an isoflavone) at the dose of 5, 10 and 20 mg/kg with tamoxifen.

Acknowledgements

The authors are thankful to Director, CDRI for his constant encouragement and support. We also acknowledge Council of Scientific and Industrial Research (CSIR) for providing research fellowship to S.P. Singh.

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