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Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection: application to a clinical trial

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Abstract

A sensitive and reproducible assay employing liquid–liquid extraction and high-performance liquid chromatography with fluorescence detection for the quantification of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen, and *Z*-4-hydroxy-*N*-desmethyltamoxifen in human plasma is described. The compounds and internal standard, propranolol, were separated with a cyano column and a mobile phase of acetonitrile–20 m*M* potassium phosphate buffer (pH 3; 35:65, v/v) then detected with fluorescence using a modified version of a method originally described by Fried and Wainer [J. Chromatogr. B 655 (1994) 261]. The coefficients of variation for the midpoint of the standard curve for each compound were less than 10%. This method was applied to a pharmacokinetic study of tamoxifen disposition in breast cancer patients.
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Keywords: Tamoxifen; *N*-Desmethyltamoxifen; 4-Hydroxytamoxifen; *Z*-4-Hydroxy-*N*-desmethyltamoxifen

1. Introduction

Tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene], a non-steroidal triphenylethylene, is currently the endocrine therapeutic agent of choice for all stages of breast cancer and has also been approved in the United States for use as a chemopreventive agent in women at high risk for the disease. The metabolism of tamoxifen could play an important role in modulating the biological activity of the drug because active metabolites may interact with the parent drug at the estrogen receptors. It has been known for many years that tamoxifen is extensively metabolized by hepatic cytochrome P450 (CYP) in humans [2–9]. The structures of tamoxifen and its major metabolites are illustrated in Fig. 1 [10].

The widespread use of tamoxifen has stimulated efforts to develop routine assays for this drug and its metabolites in human plasma. Procedures based on gas chromatography with mass spectrometry are highly specific, but require derivatization of sample and involve equipment not generally available [11]. Thin-layer and high-performance liquid chromato-

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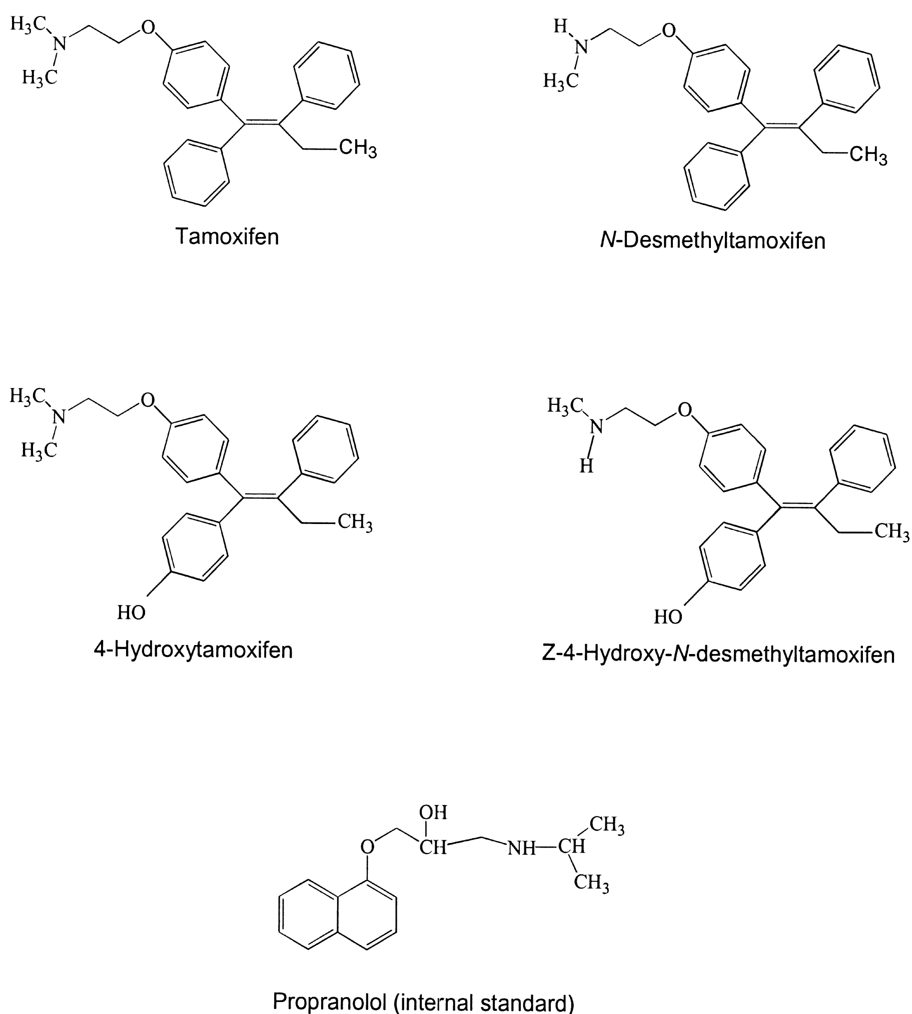

Fig. 1. Chemical structures of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen, 4-hydroxy-*N*-desmethyltamoxifen, and propranolol (internal standard).

graphic (HPLC) methods [10,12,13] involve photochemical conversion of tamoxifen and its metabolites to fluorescent phenanthrene derivatives [14]. In 1994, Fried and Wainer [1] reported a HPLC assay that incorporates post column fluorescent activation and that avoids problems related to the variable photochemical degradation of the phenanthrenes. This method applies solvent to deproteinate the sample, an expeditious procedure that prevents degradation of parent compound and metabolites, that is mixed and spun prior to injection into the HPLC [1]. The method we report here represents an evolution of the Fried and Wainer [1] method with three major differences; the incorporation of an internal standard in the assay, the use of a liquid–liquid extraction procedure to further purify and allow concentration of the sample, and the quantification of 4-hydroxy-*N*-desmethyltamoxifen, a metabolite that may have anti-cancer activity. Thus, a sensitive and reproducible HPLC assay with fluorescence detection using

propranolol as the internal standard for the quantification of tamoxifen and three metabolites in human plasma is described in this paper.

2. Experimental

2.1. Chemicals and reagents

Tamoxifen, 4-hydroxytamoxifen, and propranolol were purchased from Sigma (St. Louis, MO, USA). *N*-Desmethyltamoxifen was a gift from Dr Irving W. Wainer (Department of Pharmacology, Georgetown University, Washington, DC, USA). 4-Hydroxy-*N*-desmethyltamoxifen (approximately 20 mg) was synthesized in our laboratory according to a previously published method with slight modifications using *Z*-4-hydroxytamoxifen (purity >98%) as a starting material [15]. The *Z*-isomer of 4-hydroxy-*N*-desmethyltamoxifen, the only one quantified with this method, was further purified from any potential contamination of the *E*-isomer by additional HPLC.

HPLC-grade acetonitrile, hexane, isopropyl alcohol, ethanol, methanol, and ACS-grade orthophosphoric acid (85%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals and reagents used were of the highest commercially quality available. Glycine (electrophoresis purity reagent) was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

The 1 *M* NaOH–glycine buffer (pH 11.3) was prepared by mixing equal volumes of 1 *M* glycine and 1 *M* NaCl. The pH of the buffer was adjusted to 11.3 by the addition of either 1 *M* glycine solution or 1 *M* NaCl solution. All buffers were washed with hexane prior to use.

2.2. Stock solutions

Standard solutions of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen, and *Z*-4-hydroxy-*N*-desmethyltamoxifen were prepared by dissolving 10 mg free base of each compound in 10 ml of ethanol. Dilutions of the standard stock solutions were made in ethanol from 0.005 to 100 μg/ml to prepare the standard curve and quality control samples. A stock solution of propranol, the internal standard, was prepared by dissolving 10 mg free base in 10 ml of ethanol and a working solution (2.5 μg/ml) was prepared by diluting the stock solution with ethanol. All solutions were stored at −20 °C.

2.3. Standard curve

Standard curves were prepared for each compound by adding diluted standards of each compound to drug-free plasma. The range of standard concentrations for each compound is listed below: *Z*-4-hydroxy-*N*-desmethlytamoxifen (0.5–20 ng/ml), 4-hydroxytamoxifen (0.25–10 ng/ml), *N*-desmethyltamoxifen (15–600 ng/ml), and tamoxifen (15–600 ng/ml). Quality control (QC) samples were prepared in duplicate each time standard curve samples were prepared. The concentrations of the QC samples for each compound are listed below: *Z*-4-hydroxy-*N*-desmethlytamoxifen (low QC, 2 ng/ml; high QC, 15 ng/ml), 4-hydroxytamoxifen (low QC, 1.25 ng/ml; high QC 7.5 ng/ml), *N*-desmethyltamoxifen and tamoxifen (low QC, 60 ng/ml; high QC 450 ng/ml). The standard curve for each compound was deemed acceptable if the low quality control samples were within the error listed below: *Z*-4-hydroxy-*N*-desmethlytamoxifen (low QC, 25%), 4-hydroxytamoxifen (low QC, 38%), *N*-desmethyltamoxifen (low QC, 20%) and tamoxifen (low QC, 24%), and within 10% of the high QC for each compound.

2.4. Assay accuracy and precision

Inter-day accuracy and precision were assessed for each compound. Standard curves and quality control samples were prepared each day and assayed. Then the concentration of each standard was estimated from the standard curve run that day. An average (±SD) of each standard for each compound was calculated and used to estimate accuracy and precision. Accuracy (%) was calculated by the following equation: Accuracy (%)=100%−(100*((|standard concentration−observed concentration|)/standard concentration). Precision, coefficient of variation (C.V.), was estimated by the following equation: C.V. (%)=100*(standard deviation of observed concentration/average of observed concentration).

2.5. Extraction efficiency

A non-extracted sample containing one concentration of each compound was injected into the HPLC. This sample qualified as the 100% sample. Then, three samples of each concentration were prepared for each compound, extracted and injected into the HPLC. The peak height of each compound was compared to the peak height of the non-extracted sample to estimate the extraction efficiency. The average (±SD) extraction efficiency of three samples is reported for each concentration.

2.6. Extraction procedure

One ml of plasma was placed into clean 13-ml screw-cap glass tubes. For standards and quality control samples the appropriate volume of diluted standard was added to each tube. Next, 50 μl of the internal standard solution (containing 2.5 μg/ml propranolol in ethanol) were added to each tube. The mixture was made alkaline by adding 1 ml of 1 *M* NaOH–glycine buffer (pH 11.3) and vortex-mixed. Then, 6 ml of hexane (95%)–isopropyl alcohol (5%) was added to each tube. The tubes were mixed on a shaker for 30 min then centrifuged for 5 min at high speed. The organic phase was transferred to 13×100-mm glass culture tubes and evaporated to dryness. The resulting residue was reconstituted with a mixture of acetonitrile and mobile phase (4:1 v/v, 150 μl), and then injected into the HPLC.

2.7. Chromatography

The HPLC system that we used was modified from that originally described by Fried and Wainer [1] to allow the effective separation of 4-hydroxy tamoxifen from 4-hydroxy-*N*-desmethyl tamoxifen and the use of an internal standard (propranolol) to allow quantitative determinations to be performed without use of an external standard. The system consisted of a Waters Model 600 dual-piston multi-solvent delivery system, a Waters WISP 717 plus autosampler, a Spectrovision FD-300 dual monochromator fluorescence detector (Groton Technology, Concord, MA, USA) and a ICT Beam Boost post-column photochemical reactor supplied with a 5-m reaction coil and a 254-nm UV lamp (Astec, Advanced Separation Technologies, Whippany, NJ, USA) that converted the tamoxifen and its metabolites to highly fluorescent phenanthrene derivatives.

Upon injection of the sample deionized water was switched to the guard column to wash for 2 min at a flow of 1 ml/min. After 2 min the mobile phase was run through the column. The compounds were separated by a semi-permeable surface C_{18} guard column (100×4.6 mm I.D.; Regis Chemical, Morton Grove, IL, USA) and a cyano (5 μm) analytical column (Rexchrom® 250×4.6 mm I.D.; Regis Chemical) with a mobile phase of 35% acetonitrile in 20 m*M* potassium phosphate buffer (pH 3) at a flow-rate of 1 ml/min. The fluorescent detector was set at an excitation wavelength of 256 nm and emission wavelength of 380 nm. Peak heights of each compound were generated through the computerized software (Waters Millenium chromatography software manager).

2.8. Pharmacokinetic study

Subjects who were taking oral tamoxifen (Nolvadex®, AstraZeneca Pharmaceuticals, Wilmington, DE, USA) equivalent to 20 mg of tamoxifen once a day were recruited from Lombardi Cancer Center, Georgetown University Hospital through their physicians. Prior to subject recruitment, the Institutional Review Board of the Georgetown University Medical Center approved the study. Blood samples for the measurement of tamoxifen and its metabolites were collected before tamoxifen therapy and after 1, 4, 8, and 12 months of continuous treatment.

3. Results and discussion

3.1. Chromatography

Z-4-hydroxy-*N*-desmethyltamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, and tamoxifen exhibited good chromatography with baseline resolution of each compound (Figs. 2 and 3). The retention times for *Z*-4-hydroxy-*N*-desmethyltamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, and tamoxifen were 29.9, 32.3, 53.3, and 58.5 min, respectively.

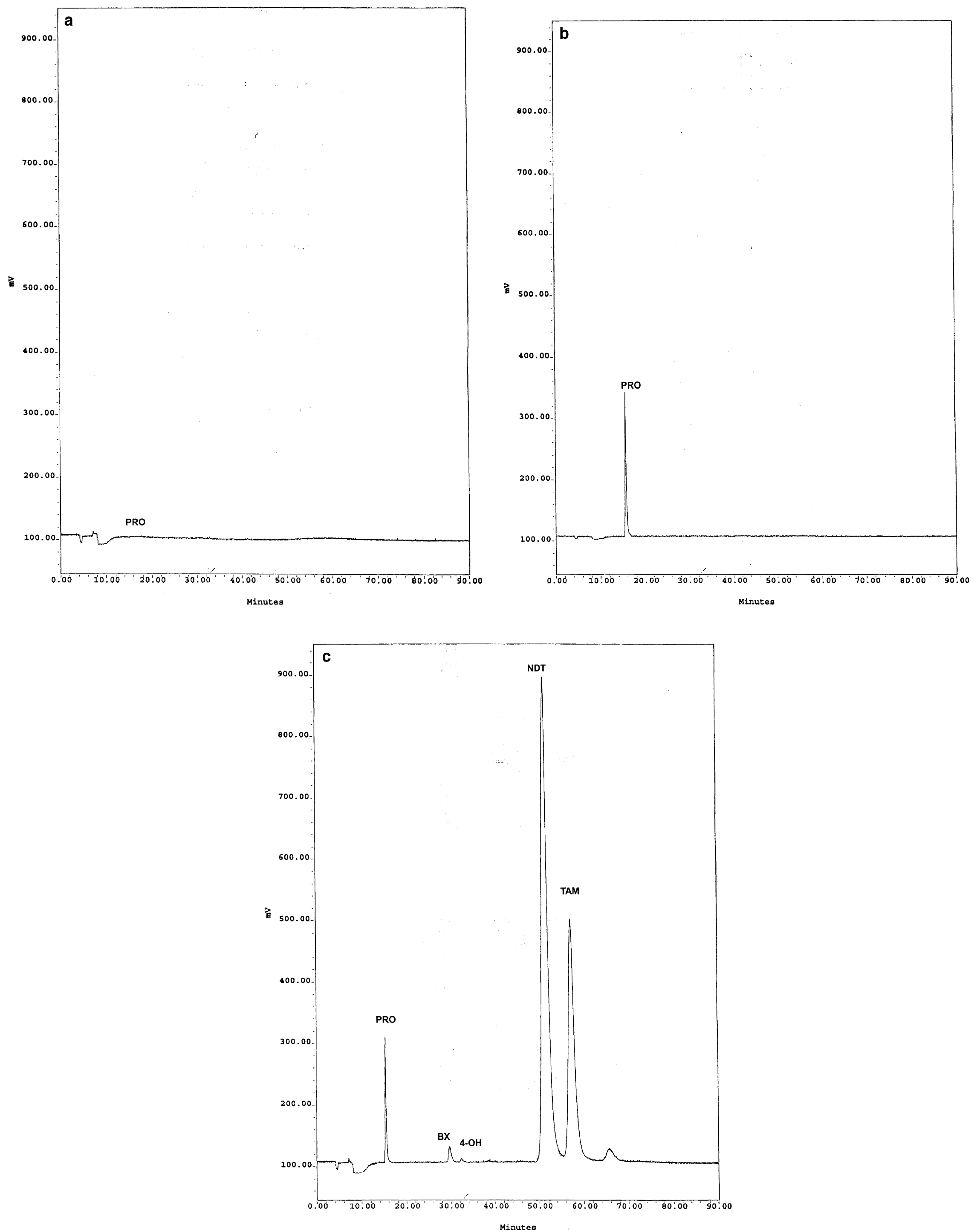

Fig. 2. Representative chromatograms of extracted (A) drug free human plasma (B) plasma standard containing 125 ng/ml internal standard (I.S.), propranolol, and (C) plasma standards containing 5 ng *Z*-4-hydroxy-*N*-desmethyltamoxifen (BX)/ml, 1 ng 4-hydroxytamoxifen (4-OH)/ml, 150 ng *N*-desmethyltamoxifen (NDT)/ml, and 150 ng tamoxifen (TAM)/ml.

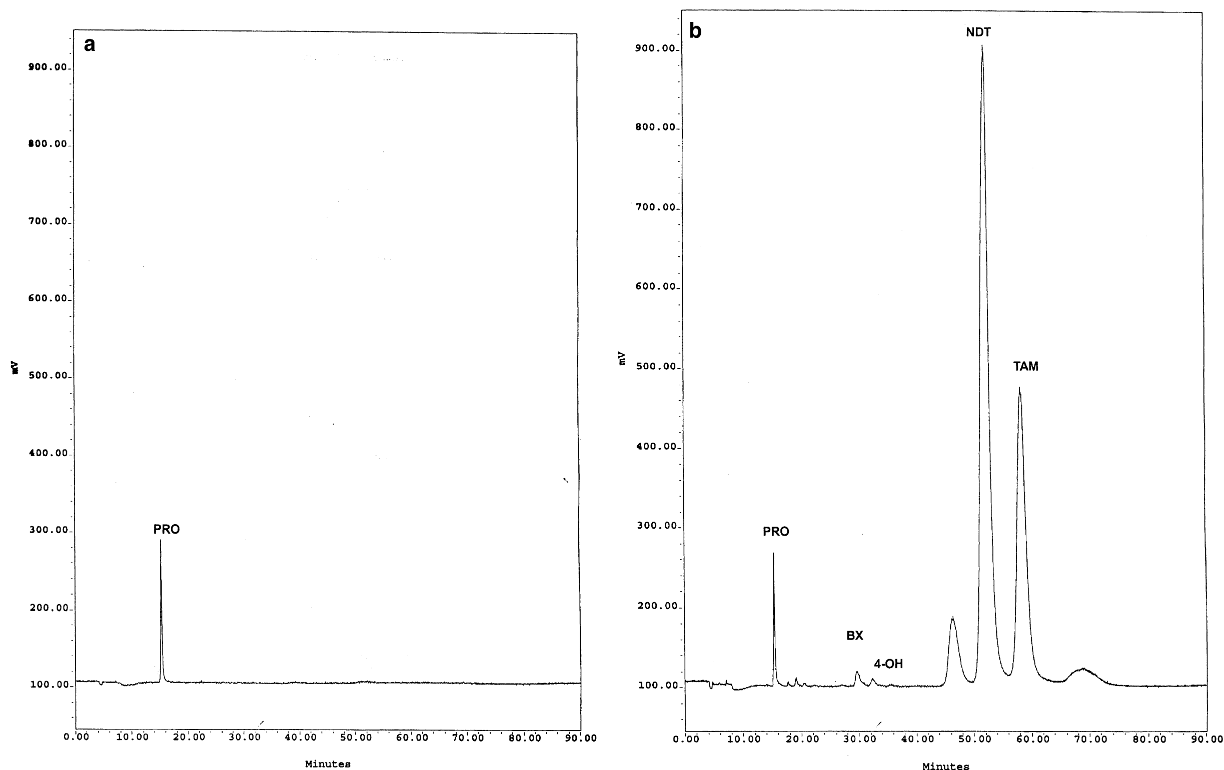

Fig. 3. Representative chromatograms of extracted (A) a predose plasma sample from a subject spiked with the I.S. and (B) a plasma sample obtained from a subject 4 months after administration of a 20-mg daily oral dose of tamoxifen. *Z*-4-hydroxy-*N*-desmethyltamoxifen (BX), 4-hydroxytamoxifen (4-OH), *N*-desmethyltamoxifen (NDT), tamoxifen (TAM).

Many compounds were tested for possible use as an internal standard, e.g. nelfinavir, dextromethorphan, imipramine, and propranolol. Propranolol exhibited the most suitable retention time (15.4 min) and peak shape of all the compounds tested. Thus, it was selected as the internal standard for this assay.

3.2. Linearity

Linear regression calibration curves based on six data points, which includes 0, were constructed for each compound by plotting peak height ratio of the compounds to propranolol versus the concentrations of plasma standards of each compound. The calibration curves were consistently linear from 0.5 to 20 ng/ml for *Z*-4-hydroxy-*N*-desmethyltamoxifen, 0.25–10 ng/ml for 4-hydroxytamoxifen, and 15–600 ng/ml for *N*-desmethyltamoxifen and tamoxifen.

3.3. Extraction efficiency

The extraction efficiency of *Z*-4-hydroxy-*N*-desmethyltamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, and tamoxifen was obtained by comparing the extracted standard curves to an unextracted standard. The extraction efficiency was greater than 84% for each compound at each concentration (Table 1). The high extraction efficiencies indicate there was little to no degradation of each compound during the procedure.

3.4. Assay precision and accuracy

Standards in human plasma were extracted and analyzed to assess the inter-day variability of the method. Accuracy and precision (C.V.) throughout the standard curve are summarized in Table 2a–d. The lowest standard for each compound exhibited

Table 1
Average (±SD) extraction efficiency of tamoxifen and metabolites

BX (ng/ml)	Efficiency (%)	4-OH (ng/ml)	Efficiency (%)	NDT (ng/ml)	Efficiency (%)	TAM (ng/ml)	Efficiency (%)
0.5	96 (±33.9)	0.25	100 (±27.0)	15	115 (±22.6)	12.5	119 (±26.5)
1	99 (±23.5)	0.5	104 (±22.1)	30	110 (±22.9)	25	110 (±16.1)
2	101 (±17.2)	1	111 (±12.7)	120	90 (±3.8)	50	96 (±4.2)
5	99 (±19.3)	2.5	108 (±23.1)	300	87 (±19.0)	125	84 (±20.9)
10	98 (±15.0)	5	105 (±6.1)	600	88 (±2.2)	250	85 (±2.0)

BX: *Z*-4-hydroxy-*N*-desmethyltamoxifen; 4-OH: 4-hydroxytamoxifen; NDT: *N*-desmethyltamoxifen; TAM: tamoxifen.

the largest variation (20–38%). In general, the C.V.s were less than 10% for the standard concentrations for each compound, which illustrates a precise assay. The least accurate assessment for this assay was 120%, which occurred twice, both times at the lower end of the concentration spectrum for *Z*-4-hydroxy-*N*-desmethyltamoxifen and 4-hydroxytamoxifen. In general, the accuracy of the reported method was about 100%, which is excellent for any quantitative assay.

3.5. Limit of quantification

The limit of quantification (LOQ) was the lowest calibration standard for each compound (Table 2). The limit of quantification allowed successful mea-

Table 2
Inter-day accuracy and precision for *Z*-4-hydroxy-*N*-desmethyltamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, and tamoxifen

Standard concentration (ng/ml)	Estimated concentration (ng/ml)	Accuracy (%)	C.V.s (%)
Z-4-Hydroxy-*N*-desmethyltamoxifen			
0.5	0.6 (±0.15)	120	25
1	1 (±0.2)	100	20
5	5 (±0.4)	100	8
10	10 (±0.6)	100	6
20	20 (±0.3)	100	2
4-Hydroxytamoxifen			
0.25	0.24 (±0.09)	96	38
0.5	0.6 (±0.07)	120	12
2.5	2.4 (±0.03)	96	1
5	5 (±0.1)	100	2
10	10 (±0.2)	100	2
N-Desmethyltamoxifen			
15	14 (±2.8)	93	20
30	33 (±2.9)	110	9
150	153 (±9.1)	102	6
300	288 (±15.7)	96	5
600	594 (±17.3)	99	3
Tamoxifen			
15	14 (±3.4)	93	24
30	30 (±2.5)	100	8
150	154 (±10.1)	103	7
300	293 (±14.4)	98	5
600	598 (±6.7)	100	1

Table 3
Mean (±SD) plasma concentrations of tamoxifen and its metabolites in 17 women after chronic administration of 20 mg tamoxifen/day

Sampling period (months)	Tamoxifen (ng/ml)	NDT (ng/ml)	4-OH (ng/ml)	BX (ng/ml)
1	149 (±46)	219 (±111)	2.7 (±0.63)	3.9 (±1.49)
4	119 (±54)	150 (±78)	3.1 (±0.87)	4.6 (±2.35)
8	140 (±67)	218 (±104)	3.7 (±1.07)	5.0 (±2.35)
12	153 (±54)	180 (±71)	2.4 (±1.16)	5.1 (±2.64)

NDT: *N*-desmethyltamoxifen; 4-OH: 4-hydroxytamoxifen; BX: 4-hydroxy-*N*-desmethyltamoxifen.

surement of therapeutic plasma concentrations of tamoxifen and its metabolites in a prospective clinical trial of tamoxifen administration.

3.6. Pharmacokinetic study

The reported method was applied to a clinical study of 17 women who were taking oral tamoxifen (20 mg/day) chronically for the treatment or prevention of breast cancer. The mean plasma concentrations of tamoxifen and its metabolites determined in the subjects after chronic administration of tamoxifen are illustrated in Table 3. It was possible to detect tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen, and 4-hydroxy-*N*-desmethyltamoxifen in all samples. The plasma concentrations that were estimated with this method confirm the range of concentrations originally reported in the literature [16].

4. Conclusions

The method presented here describes a specific, sensitive and reproducible human plasma assay using HPLC with internal standard and fluorescence detection. The major evolutionary changes of this assay relative to its predecessor [1] are the incorporation of an internal standard in the assay, the use of a liquid–liquid extraction procedure to further purify and allow concentration of the sample and the quantification of 4-hydroxy-*N*-desmethyltamoxifen. This method should make it possible to conduct detailed studies of pharmacokinetics and pharmacodynamics of tamoxifen and its active metabolites that will allow better understanding of the contribution of each metabolite to tamoxifen anti-cancer activity and to identify pharmacogenetic and other factors that through modification of the metabolite concentrations influence the safety and efficacy of tamoxifen.

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