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Quantification of tamoxifen and three of its phase-I metabolites in human plasma by liquid chromatography/triple-quadrupole mass spectrometry

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ABSTRACT

In view of future pharmacokinetic studies, a highly sensitive ultra performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method has been developed for the simultaneous quantification of tamoxifen and three of its main phase I metabolites in human lithium heparinized plasma. The analytical method has been thoroughly validated in agreement with FDA recommendations. Plasma samples of 200 μl were purified by liquid–liquid extraction with 1 ml *n*-hexane/isopropanol, after deproteination through addition of 50 μl acetone and 50 μl deuterated internal standards in acetonitrile. Tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen were chromatographically separated on an Acquity UPLC® BEH C18 1.7 μm 2.1 mm × 100 mm column eluted at a flow-rate of 0.300 ml/min on a gradient of 0.2 mM ammonium formate and acetonitrile, both acidified with 0.1% formic acid. The overall run time of the method was 10 min, with elution times of 2.9, 3.0, 4.1 and 4.2 min for endoxifen, 4-hydroxy-tamoxifen, N-desmethyl-tamoxifen and tamoxifen, respectively. Tamoxifen and its metabolites were quantified by triple-quadrupole mass spectrometry in the positive ion electrospray ionization mode. The multiple reaction monitoring transitions were set at 372 > 72 (*m*/*z*) for tamoxifen, 358 > 58 (*m*/*z*) for N-desmethyl-tamoxifen, 388 > 72 (*m*/*z*) for 4-hydroxy-tamoxifen and 374 > 58 (*m*/*z*) for endoxifen. The analytical method was highly sensitive with the lower limit of quantification validated at 5.00 nM for tamoxifen and N-desmethyl-tamoxifen and 0.500 nM for 4-hydroxy-tamoxifen and endoxifen, which is equivalent to 1.86, 1.78, 0.194 and 0.187 ng/ml for tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, respectively. The method was also precise and accurate, with within-run and between-run precisions within 12.0% and accuracy ranging from 89.5 to 105.3%. The method has been applied to samples from a clinical study and cross-validated with a validated LC–MS/MS method in serum.

1. Introduction

The selective estrogen receptor modulator tamoxifen remains an important drug in the treatment of estrogen receptor (ER) positive breast cancer. In the United States tamoxifen is also approved for the prevention of breast cancer in women at high-risk [1–4]. Tamoxifen reduces the risk of recurrence and the risk of mortality, however, not all women benefit from tamoxifen therapy, and treatment-related adverse reactions also vary greatly between patients. Inter-individual variability in metabolism of tamoxifen, which is influenced by both genetic and environmental factors, contributes to the differences in efficacy and toxicity of tamoxifen [1,2,5–7].

Tamoxifen is a prodrug and undergoes biotransformation into several metabolites, including N-desmethyl-tamoxifen, which is the most abundant metabolite, and its potent metabolites 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen). The cytochrome P450 enzymes CYP3A4 and CYP2D6 play a dominant role in the biotransformation of tamoxifen, with other CYP enzymes (CYP2B6, CYP2C9 and CYP2C19) playing a minor role [7–9]. The anti-estrogenic potency of 4-hydroxy-tamoxifen and endoxifen, regarding ER-binding and suppression of estrogen-dependent proliferation of breast cancer cells, is 30–100-fold higher compared with tamoxifen. As plasma concentrations of endoxifen are 5–10 times higher than of 4-hydroxy-tamoxifen, endoxifen is thought to be of most importance for the pharmacological activity of tamoxifen treatment [7,10,11].

Several studies have shown that genetic variation in CYP2D6 enzymes and the concomitant use of CYP2D6 inhibitors influence

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endoxifen plasma concentrations [7,12–14]. In addition, the activity of other CYP enzymes (CYP3A4/5, CYP2C9, and CYP2C19), which are also affected by genetic polymorphisms and concomitant medication, may also be responsible for the large inter-patient variability in endoxifen plasma concentrations. Therefore, monitoring endoxifen plasma concentrations rather than CYP2D6 genotype testing is suggested to be a better approach to personalize tamoxifen therapy.

To assess the effects of genetic polymorphisms in cytochrome P450 enzymes and influences of co-medication on the plasma concentrations of tamoxifen and its metabolites and for monitoring of endoxifen plasma concentrations, quantification of these compounds with a sensitive and validated analytical method is important. For this purpose, the development of bioanalytical methodologies for the quantification of tamoxifen and its metabolites in human serum, plasma, urine and tissue have been reported in various publications, reviewed by Teunissen et al. [15]. However, not all analytical assays included tamoxifen and its three main metabolites (N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen). In addition, not all assays have been thoroughly validated, which is important for its use in clinical pharmacokinetic studies and clinical practice [15].

Although a few LC–MS/MS assays have been adequately validated and included at least the three main phase I metabolites [16–18], the sensitivity of the methods may not be enough for the determination of low metabolite concentrations. One of these validated LC–MS/MS methods [16] was used for the quantification of tamoxifen and its metabolites in a recent study, in which dextromethorphan was used as a phenotyping probe to predict endoxifen exposure in patients using tamoxifen [19]. In several patients, serum levels of the tamoxifen metabolites 4-hydroxy-tamoxifen and endoxifen were below the lower limits of quantification of 1.13 and 2.69 ng/ml, respectively, and could not be reliable determined.

In view of future pharmacokinetic studies, we developed a highly sensitive and selective ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) assay for tamoxifen and its main phase I metabolites. The method requires only 200 μl plasma and involves a liquid–liquid extraction procedure for the purification of the plasma samples. The method is fully validated according to the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA, with lower limits of quantitation of 1.86, 1.78, 0.194 and 0.187 ng/ml for tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, respectively.

2. Experimental

2.1. Chemicals

Pure Z (*cis*)-isomers of tamoxifen, N-desmethyl-tamoxifen and 4-hydroxy-tamoxifen, the stable labeled deuterated internal standards tamoxifen-d5, N-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and a racemic mixture of the Z- and E-isomers (1:1) of 4-hydroxy-N-desmethyl-tamoxifen-d5 were obtained from Toronto Research Chemicals (North York, ON, Canada). The pure Z (*cis*)-isomer of 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) was kindly provided by Jina Pharmaceuticals Inc. (Libertyville, IL). All chemicals were of analytical grade or higher. Acetonitrile, methanol and water were from Biosolve BV (Valkenswaard, The Netherlands). Dimethylsulphoxide (DMSO), ammonium formate, glycine and *n*-hexane were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands), sodium hydroxide and 2-propanol from Merck (Darmstadt, Germany) and formic acid from J.T. Baker (Deventer, The Netherlands). Blank human lithium heparinized plasma was obtained from Biological Specialty Corporation (Colmar, PA).

2.2. Preparation of stock solutions, calibration standards and quality control samples

Stock solutions containing 1.00 mM free base of tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen in DMSO were prepared individually. Following preparation, stock solutions were stored at $T < -70\,^{\circ}C$. Individual stock solutions of tamoxifen and its metabolites were used for the preparation of a working stock solution, containing 200 μM tamoxifen, 200 μM N-desmethyl-tamoxifen, 20 μM 4-hydroxy-tamoxifen and 20 μM endoxifen in DMSO. The working stock solution was divided into 150 μl aliquots, which were used for the construction of calibration curve standards during the validation. Separate stock solutions (i.e., independent weightings) of tamoxifen and its metabolites were used for the preparation of the pools of quality control samples. The variation between the stock solutions of tamoxifen and its metabolites used for the construction of the calibration standards and QC samples was in all cases <5%.

Deuterated internal standards were dissolved in DMSO separately, to obtain internal standard stock solutions at a concentration of 1 mg/ml free base, which subsequently were aliquotted and stored at $T < -70\,^{\circ}C$. Aliquots of 10 μl of the individual stock solutions were concurrently 10,000-fold diluted in acetonitrile, resulting in an internal standard working solution containing 100 ng/ml tamoxifen-d5, N-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and 4-hydroxy-N-desmethyl-tamoxifen-d5, which was stored at $T < 8\,^{\circ}C$ for a maximum of 3 months.

Calibration curve standards were freshly prepared (in duplicate) for each run, by addition of 10 μl aliquots of appropriate dilutions of the working stock solution in acetonitrile/DMSO (1:1, v/v) to 190 μl aliquots of human lithium heparinized plasma (excepted of calibration standard 7, which was prepared by addition of 45 μl diluted working stock solution to 955 μl plasma) at the following concentrations: 5.00, 10.0, 50.0, 100, 250, 500, 900, and 1000 nM for tamoxifen and N-desmethyl-tamoxifen and 0.500, 1.00, 5.00, 10.0, 25.0, 50.0, 90.0, and 100 nM for 4-hydroxy-tamoxifen and endoxifen.

A total of five pools of quality control (QC) samples were prepared by spiking appropriate dilutions of stock solutions of tamoxifen and its metabolites to human lithium heparinized plasma at concentrations of 5.00 nM (LLQ), 15.0 nM (QC-Low), 400 nM (QC-Middle), 800 nM (QC-High) and 16,000 nM (QC-Diluted) for tamoxifen and N-desmethyl-tamoxifen and at 0.500 nM (LLQ), 1.50 nM (QC-Low), 40.0 nM (QC-Middle), 80.0 nM (QC-High) and 1,600 nM (QC-Diluted) for 4-hydroxy-tamoxifen and endoxifen. QC-Diluted was processed after a 20-fold dilution in blank human lithium heparinized plasma. Pools of QC samples were aliquotted and stored at $T < -70\,^{\circ}C$ until analysis.

2.3. Plasma sample preparation

Aliquots of 50 μl of internal standard working solution and 50 μl of acetone were added to 200 μl of plasma samples in 1.5 ml microcentrifuge tubes and vigorously vortexed for 5 min. The samples were then centrifuged at 18,000 × *g* at ambient temperature for 10 min. Subsequently, the supernatant was transferred into 2 ml microcentrifuge tubes and 100 μl aliquots of glycine buffer (pH 11.5) and 1 ml aliquots of n-hexane/2-propanol (95:5, v/v) were added. Hereafter, the samples were again vortexed and centrifuged under the previously mentioned conditions. Aliquots of 800 μl of the organic phase were transferred into 4.5 ml glass tubes and evaporated to dryness under nitrogen at $T = 60\,^{\circ}C$. The residues were reconstituted in 100 μl aliquots of acetonitrile/water/formic acid

(40:60:0.1, v/v/v) and centrifuged for 30 s at 4000 × *g*. The supernatants were transferred into 350 μl 96-well plates, which were placed into a chilled (*T* = 10 °C) autosampler, from which aliquots of 5 μl were injected onto the UPLC column.

2.4. Equipment

The UPLC–MS/MS system was composed of a Waters Acquity UPLC Sample Manager coupled to a Waters TQ Detector (Waters, Etten-Leur, The Netherlands). The MassLynx V4.1 SCN627 software package was used for the acquisition and processing of data. Quantification was performed using QuanLynx as implemented in the MassLynx software.

2.4.1. Chromatographic conditions

An Acquity UPLC® BEH C_{18} column 1.7 μm, 100 mm × 2.1 mm, (Waters, Etten-Leur, The Netherlands), thermostatted at *T* = 50 °C, was used for the separation of the analytes. Aqueous ammonium formate (0.2 mM) and acetonitrile, both acidified with 0.1% formic acid, were used as mobile phase A and mobile phase B, respectively. Using these mobile phases, a gradient at a flow-rate of 0.300 ml/min was achieved. A linear gradient separation was used with 30–80% of mobile phase B from 0 to 6 min, then 80–30% of mobile phase B over 2 min, which was held for 2 min for re-equilibration of the system. An autosampler (at 10 °C) injected volumes of 5 μl onto the UPLC column. The overall run time was 10 min. The needle of the autosampler was washed using a strong needle wash solvent (water/acetonitrile/2-propanol/methanol/formic acid, 25:25:25:25:0.1 (v/v/v/v/v)) and a week needle wash solvent (30% acetonitrile in water). The column effluent was introduced to the mass spectrometer and monitored.

2.4.2. Mass spectrometry

Tandem mass spectrometry was performed in the positive ion electrospray ionization mode. Mass transitions of *m*/*z* were optimized for tamoxifen, its metabolites and the deuterated internal standards of tamoxifen and its metabolites by infusion of the respective analytes in acetonitrile/water/formic acid (40:60:0.1, v/v/v) via combined infusion. Optimal MS settings were adjusted manually. The desolvation gas was set at 800 L/h, the cone gas at 25 L/h (nitrogen) and the ionspray voltage was kept at 1.50 kV. The cone voltage was kept at 45 V for tamoxifen, endoxifen and their deuterated internal standards, 42 V for N-desmethyl-tamoxifen and N-desmethyl-tamoxifen-d5 and 47 V for 4-hydroxy-tamoxifen and its internal standard, with a source temperature of *T* = 150 °C and desolvation temperature of *T* = 350 °C. The dwell times were set at 50 ms and the inter-channel delay at 10 ms. Multiple reaction monitoring (MRM) mode was applied for the quantitation with the parameters as presented in Table 1. The collision cell pirani pressure was set at ~5e^{-3} mbar (argon).

Table 1
MS/MS settings.

Analyte	Scan window (min)	Parent (*m*/*z*)	Daughter (*m*/*z*)	Collision (V)
Tamoxifen	3.50–5.00	372	72	25
Tamoxifen-d5	3.50–5.00	377	72	25
N-desmethyl-tamoxifen	3.50–5.00	358	58	21
N-desmethyltamoxifen-d5	3.50–5.00	363	58	21
4-OH-tamoxifen	2.50–3.50	388	72	25
4-OH-tamoxifen-d5	2.50–3.50	393	72	25
Endoxifen	2.50–3.50	374	58	23
Endoxifen-d5	2.50–3.50	379	58	23

2.4.3. Quantitation

Calibration curves were constructed by plotting the peak area ratios of the components to internal standards versus the known concentrations with a weight factor of 1/concentration^2.

2.5. Light sensitivity of tamoxifen and its metabolites

An experiment in which tamoxifen and its metabolites were exposed to several light sources, was conducted to investigate the stability during sample handling and preparation. A solution of tamoxifen and its metabolites was prepared by addition of 50 μl working stock solution to 20 ml human lithium heparinized plasma. Subsequently four groups of samples were prepared using this solution. The first group was protected from light for 6 h, the second group was exposed for 6 h to UV-light (254 nm), the third group was exposed for 6 h to daylight (~350–700 nm) in 1.5 ml microcentrifuge tubes and the fourth group was exposed for 6 h to daylight (~350–700 nm) in 1.5 ml amber-colored microcentrifuge tubes. The four groups of plasma samples were analyzed by UPLC–MS/MS using the conditions described in Section 2.

2.6. Method validation

The UPLC–MS/MS method was validated in agreement with the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA (www.fda.gov/downloads/Drugs/ GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf).

Blank human lithium heparinized plasma samples of ten different lots were analyzed to determine the potential presence of endogenous contaminating compounds that may interfere with the

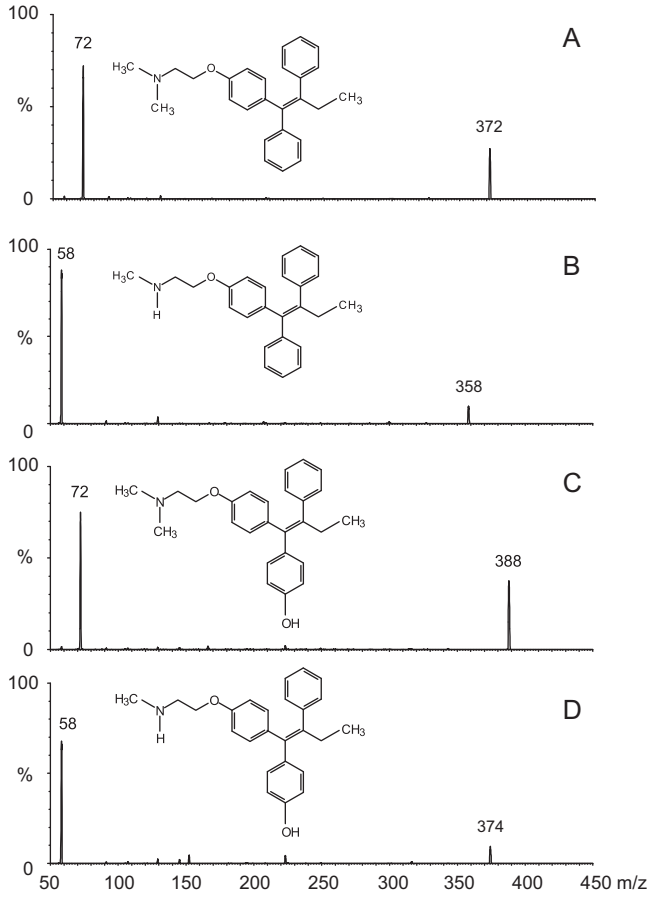

Fig. 1. Mass spectrum and chemical structures of tamoxifen (A), N-desmethyl-tamoxifen (B), 4-hydroxy-tamoxifen (C) and endoxifen (D).

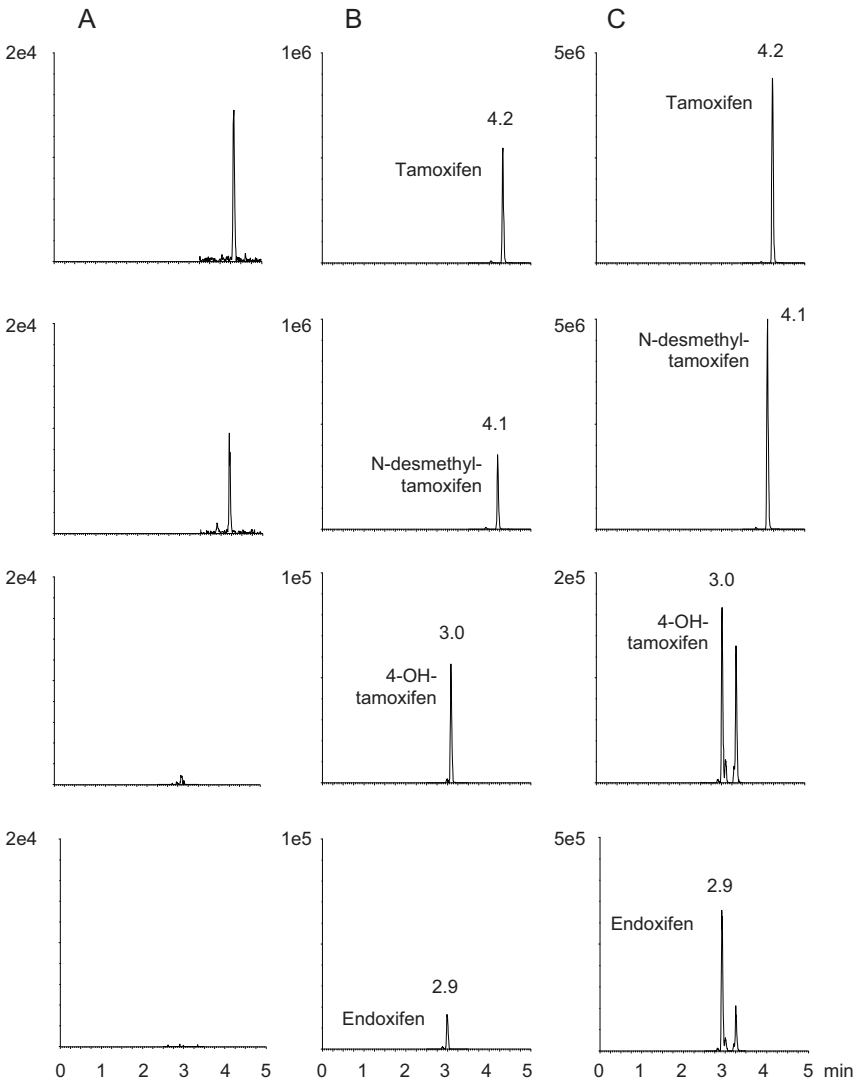

Fig. 2. Representative chromatograms of a double blank processed plasma sample (A), a plasma sample spiked at the concentration of the LLQ (B) and a plasma sample collected 4 h after 20 mg tamoxifen administration on steady state containing 143 nM tamoxifen, 229 nM N-desmethyl-tamoxifen, 4.29 nM 4-hydroxy-tamoxifen and 20.1 nM endoxifen (C).

assay. Potential clinical co-administered drugs were investigated for possible interference with the analytical method, including aprepitant, citalopram, dexamethasone, dextromethorphan, domperidon, ibuprofen, lorazepam, metoclopramide, oxazepam, pantoprazol, paracetamol, paroxetine, ranitidine, rifampicin and venlafaxine. All drugs have been dissolved and/or diluted in water to a concentration of 1 mg/ml and subsequently 200-fold diluted in human lithium heparinized plasma to provide final concentrations of 5 μg/ml. Aliquots of QC-Diluted (i.e., 16,000 nM for tamoxifen and N-desmethyl-tamoxifen and 1600 nM for 4-hydroxy-tamoxifen and endoxifen) have subsequently been diluted in the plasma containing the above mentioned drugs at concentrations of QC-High (in triplicate), which have been processed and compared to equal dilutions of QC-Diluted in blank human lithium heparinized plasma.

For the determination of the LLQ, blank human lithium heparinized plasma samples of 10 different donors were spiked at a concentration of 5.00 nM for tamoxifen and N-desmethyl-tamoxifen and 0.500 nM for the other two metabolites and analyzed during one run. Accuracy (ACC), within-run precision (WRP) and the between-run precision (BRP) were determined by analyzing 5 replicates of pools of LLQ and QC samples independently over a three-run period, with the calibration curve standards processed in duplicate. The ACC, WRP and BRP at the level of the LLQ and QC samples were calculated by one-way analysis of variance, using the run as the variable as earlier described [20,21].

The evaluation of the matrix effect for tamoxifen and its metabolites was tested by comparing the MS/MS response of tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen at a concentration of 25.0 nM and 80.0 nM spiked in triplicate in acetonitrile/water/formic acid (40:60:0.1, v/v/v) to the MS/MS responses of the analytes spiked in triplicate into extracts of blank human lithium heparinized plasma, as described recently [21,22].

Extraction recovery (RE) was determined by comparing the MS/MS response of tamoxifen and its metabolites at 25.0 nM and 80.0 nM spiked in triplicate into six different lots of blank lithium heparinized plasma before extraction, to the MS/MS responses of the analytes spiked in triplicate into extracts of blank human lithium heparinized plasma after extraction, corrected for the evaporated volume of organic phase [21,22].

The stability of tamoxifen and its metabolites in human lithium heparinized plasma was tested in triplicate at the concentrations of QC-Low, QC-High and QC-Diluted during overnight (i.e., ~18 h) incubation at ambient temperature, following three freeze-thaw

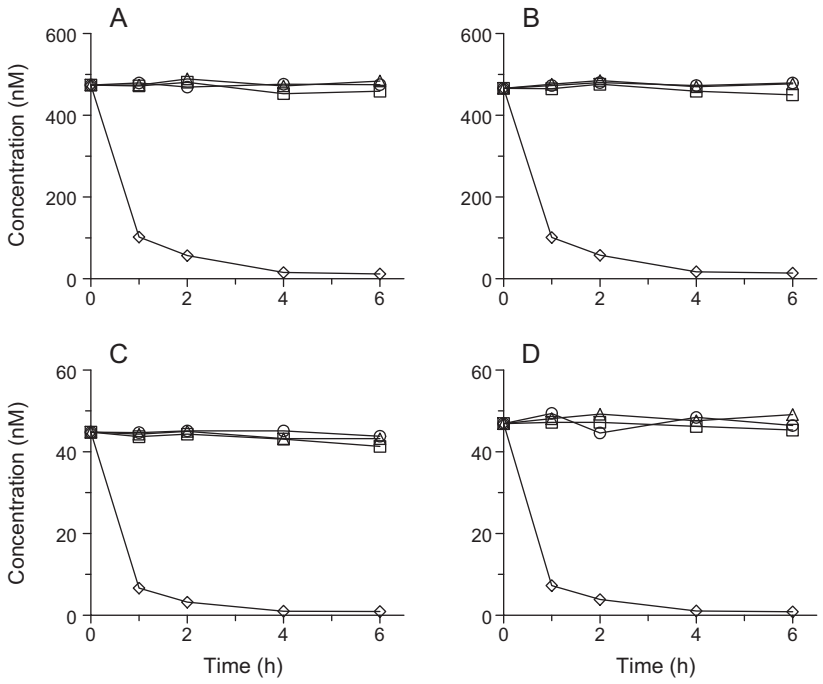

Fig. 3. Exposure of tamoxifen (A), N-desmethyl-tamoxifen (B), 4-hydroxy-tamoxifen (C) and endoxifen (D) to UV-light (lozenges), daylight in transparent microcentrifuge tubes (squares), daylight in amber-colored microcentrifuge tubes (triangles) and protected from light (circles).

cycles, in which the samples were thawed for at least 15 min followed by refreezing for at least 18 h. The storage stability of processed samples in the autosampler was tested using samples at the same concentrations. QC samples were processed in triplicate and repeatedly injected at different time points (within a period of 24 h).

2.7. Application of method to clinical samples

The method has been cross-validated with a validated LC–MS/MS method for the analysis of tamoxifen and its metabolites in serum. A total of 76 samples of patients using tamoxifen (see www.trialregister.nl; NTR Study No. 1751), from which serum samples have been analyzed using the method as published by Teunissen et al. [16] and from which also plasma aliquots were available, were quantitated by the method described here.

The described analytical method has also been applied to pharmacokinetic samples, derived from the previously mentioned clinical study, with serum levels of 4-hydroxy-tamoxifen and endoxifen below the lower limits of quantification of the analytical method (1.13 and 2.69 ng/ml, respectively).

3. Results and discussion

3.1. LC–MS/MS conditions and method development

The tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen product ion spectra (Fig. 1) yield abundant product ions suitable for use in multiple reactions monitoring. The selected product ions and collision energies of tamoxifen, its metabolites and their respective deuterated internal standards are presented in Table 1.

Because of its direct influence on the sensitivity and selectivity of the method, sample pre-treatment is of great importance in the development of an analytical method. As protein precipitation results in less clean extracts, remaining endogenous compounds may cause ion-suppression and thereby negatively affect the sensitivity of the assay. Solid phase and liquid–liquid extraction lead to more purified extracts and are, therefore, appropriate sample pre-treatment procedures. Solid phase extraction has, if not automated, disadvantages including poor reproducibility and is, compared to liquid–liquid extraction, relatively laborious [15,23]. In this method, a liquid–liquid extraction procedure was applied with acetonitrile, acetone and *n*-hexane/isopropanol, which resulted in clean extracts.

By applying a linear gradient, tamoxifen and its three metabolites were adequately base-line separated and separated from early eluting hydrophilic, potentially interfering matrix components, while maintaining a relative short injection to injection time of 10 min with elution times of 2.9 min for endoxifen, 3.0 min for 4-hydroxy-tamoxifen, 4.1 min for N-desmethyl-tamoxifen and 4.2 min for tamoxifen (Fig. 2). Two additional peaks were detected in the chromatograms of 4-hydroxy-tamoxifen and endoxifen, with elution times of approximately 3.3 and 3.2 min, respectively, which are 4′-hydroxy-tamoxifen and 4′-hydroxy-N-desmethyl-tamoxifen [17,18].

3.1.1. Light sensitivity of tamoxifen and its metabolites

It has been reported that tamoxifen is light sensitive and should be protected from light during sample handling and preparation [16]. Data on light sensitivity of tamoxifen and its metabolites are, however, lacking. To investigate the sensitivity of tamoxifen and its metabolites to light, the extent of degradation of tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen under different light source conditions was determined. One group of samples was for 6 h protected from light, the second group was exposed for 6 h to UV-light (254 nm), the third group was exposed for 6 h to daylight (~350–700 nm) in 1.5 ml microcentrifuge tubes and the last group was exposed for 6 h to daylight (~350–700 nm) in 1.5 ml amber-colored microcentrifuge tubes. Samples were analyzed and compared to samples which were immediately stored at $T < -70\,^{\circ}C$ after preparation (reference samples). Tamoxifen and its metabolites were very light sensitive under UV-light (254 nm). No degradation of tamoxifen or its metabolites was observed when the samples were exposed to daylight in 1.5 ml

Table 2
Calculations of the between-run and within-run precisions and the average accuracy of the LLQ and QC samples.[a]

Sample	Spiked (nM)	GM (nM)	ACC (%)	WRP (%)	BRP (%)	n^c
Tamoxifen						
LLQ	5.00	4.55	91.0	6.10	2.01	15 of 15
Low	15.0	13.7	91.3	3.76	2.78	14 of 15
Middle	400	371	92.8	2.97	0.75	15 of 15
High	800	724	90.5	2.70	#[b]	15 of 15
Diluted	16,000	15,588	97.4	3.83	7.72	14 of 15
N-desmethyl-tamoxifen						
LLQ	5.00	4.51	90.2	3.90	5.63	15 of 15
Low	15.0	14.0	93.3	4.81	#[b]	14 of 15
Middle	400	376	94.0	3.90	1.94	15 of 15
High	800	734	91.8	2.48	1.73	15 of 15
Diluted	16,000	15,466	96.7	3.53	4.47	15 of 15
4-OH-tamoxifen						
LLQ	0.500	0.520	104.0	6.08	5.44	15 of 15
Low	1.50	1.52	101.3	3.60	2.63	15 of 15
Middle	40.0	41.1	102.8	3.52	#[b]	15 of 15
High	80.0	80.5	100.6	3.03	#[b]	15 of 15
Diluted	1600	1684	105.3	2.97	5.17	15 of 15
Endoxifen						
LLQ	0.500	0.457	91.4	12.0	8.19	12 of 15
Low	1.50	1.35	90.0	4.06	1.05	13 of 15
Middle	40.0	36.7	91.8	3.54	2.67	14 of 15
High	80.0	71.6	89.5	2.84	2.29	13 of 15
Diluted	1600	1517	94.8	5.00	5.83	15 of 15

Abbreviations: GM, grand mean; WRP, within-run precision; BRP, between-run precision; ACC, average accuracy.
[a] $n = 5$ in 4 separate runs (3 runs at the LLQ).
[b] No additional variation observed by performing the assay in different runs.
[c] Number of individual samples falling within acceptable range of accuracy of 85–115% (80–120% at LLQ).

(transparent) microcentrifuge tubes (Fig. 3). Sample handling and preparation could therefore be conducted under normal laboratory conditions.

3.2. Assay performance

The method results were linear ($r^2 \geq 0.995$) in the concentration range of 5.00 to 1000 nM for tamoxifen and N-desmethyl-tamoxifen and of 0.500 to 100 nM for 4-hydroxy-tamoxifen and endoxifen in human lithium heparinized plasma and none of the blank plasma samples showed potential interference for tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen, endoxifen or any of the deuterated internal standards.

None of the tested, potentially co-administered drugs interferes with the quantitation of tamoxifen or its metabolites.

The LLQ was validated at 5.00 nM for tamoxifen and N-desmethyl-tamoxifen and at 0.500 nM for 4-hydroxy-tamoxifen and endoxifen, which is equivalent to 1.86, 1.78, 0.194 and 0.187 ng/ml for tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, respectively. The LLQ has been validated in separate runs. In one validation run, analytes were spiked to 10 different lots of human lithium heparinized plasma. In three other runs, a pool of LLQ samples was processed as QC-samples. For tamoxifen, measured concentrations in 9 of 10 independently spiked plasma samples fell within the acceptable range of accuracy of 80–120%, with an average measured concentration of 4.78 ± 0.554 nM. The measured concentrations of N-desmethyl-tamoxifen for all 10 independent heparinized plasma samples fell within the acceptable range of accuracy, with an average observed concentration of 4.98 ± 0.454 nM. The average concentration for 4-hydroxy-tamoxifen in the 10 independent samples (8 in acceptable range) was 0.554 ± 0.053. For endoxifen, measured concentrations in 9 of 10 independent samples fell within the acceptable range of accuracy, with an average concentration of 0.496 ± 0.053 nM.

The within-run and between-run precisions and the accuracies at five tested concentrations, including at the level of the LLQ, are summarized in Table 2 and all fell within the accepted ranges as specified by the FDA (www.fda.gov/downloads/Drugs/ GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf).

Table 3
Extraction recovery (RE) and matrix effect (ME) in lithium heparinized plasma from six different lots spiked with all analytes at a concentration of 25.0 nM and 80.0 nM.[a]

Analyte	25.0 nM		80.0 nM	
	ME (%)	RE (%)	ME (%)	RE (%)
Tamoxifen	126 ± 7.4	64 ± 6.8	103 ± 2.2	79 ± 13.6
N-desmethyl-tamoxifen	111 ± 10.0	83 ± 4.5	91 ± 2.4	95 ± 16.0
4-OH-tamoxifen	108 ± 0.7	87 ± 5.3	107 ± 1.3	90 ± 8.4
Endoxifen	96 ± 5.0	76 ± 4.2	97 ± 5.6	81 ± 8.3

[a] Data presented as mean ± SD ($n = 6$).

The extraction recovery (RE) and matrix effect (ME) were determined in six different lots of lithium heparinized plasma, spiked with tamoxifen and its metabolites at a concentration of 25.0 nM and 80 nM. The mean measured extraction efficiencies and matrix effect are shown in Table 3. As shown, no matrix effect was observed for tamoxifen or its metabolites. The recoveries ranged from 64% for tamoxifen to 87% for 4-hydroxy-tamoxifen.

Tamoxifen and its metabolites were stable in lithium heparinized plasma during overnight incubation at ambient temperature, following three freeze-thaw cycles and as processed samples in the chilled ($T = 10\,^{\circ}C$) autosampler for at least 24 h.

3.3. Clinical application

As shown in Fig. 4, concentrations of tamoxifen and its metabolites quantitated in serum using the method of Teunissen et al. [16] and in lithium heparinized plasma by our method are comparable, with random errors across all concentrations for all compounds. Differences in quantitated concentrations of tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen between plasma and serum analysis were determined. Respectively 96%, 91%, 96% and 93% of the samples fell within a range of 30% difference and respectively 92%, 79%, 86% and 80% of the samples even fell within a range of 20% difference.

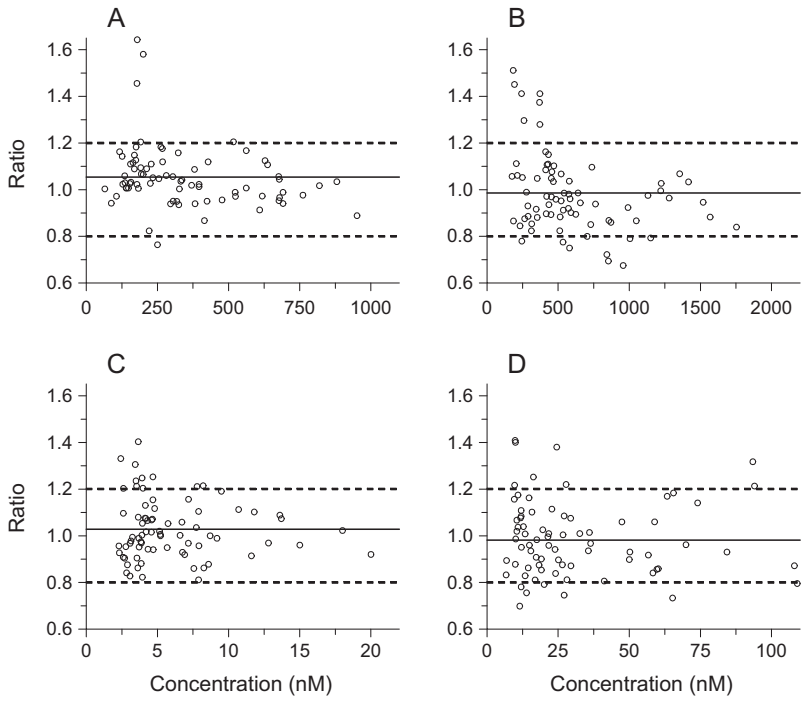

Fig. 4. Cross validation results of the analysis of 76 samples analyzed in serum by the method published recently [16] and the current method in plasma for tamoxifen (A), N-desmethyl-tamoxifen (B), 4-hydroxy-tamoxifen (C) and endoxifen (D). On the *X*-axis, the serum concentrations are plotted and on the *Y*-axis the ratio's C_{plasma}/C_{serum}. The solid line represents the average ratio (i.e., 1.0 is equal), while the dotted lines represents the 20% difference between the plasma and serum analysis.

The described analytical method was also applied to samples from a previous clinical study, with serum concentrations below the lower limits of quantification for 4-hydroxy-tamoxifen and endoxifen (1.13 and 2.69 ng/ml, respectively) [16]. We observed concentrations as low as 1.18 nM (0.46 ng/ml) for 4-hydroxy-tamoxifen and 2.39 nM (0.891 ng/ml) for endoxifen, stressing the need for highly sensitive analytical methods.

4. Conclusion

A highly sensitive, selective, accurate and precise method has been developed and validated for the simultaneous analysis of tamoxifen and its three main phase I metabolites, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, in human heparinized plasma. As tamoxifen and its three main metabolites were stable in daylight in transparent microcentrifuge tubes, sample handling and preparation can be conducted under normal laboratory conditions.

The validation method meets the current requirements of bioanalytical method validation and is one of the most sensitive methods, especially for endoxifen, published so far with lower limits of quantitation of 1.86, 1.78, 0.194 and 0.187 ng/ml for tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, respectively. The analytical method has been successfully cross-validated with a validated LC–MS/MS method for the analysis of tamoxifen and its metabolites in serum.

By using the described analytical method, we were able to quantify low concentrations of 4-hydroxy-tamoxifen and endoxifen. As observed in some patients in a previous clinical study, quantification of low endoxifen concentrations is important in view of future pharmacokinetic studies and for monitoring of endoxifen plasma concentrations.

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