Journal of Chromatography B, 879 (2011) 1677–1685

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Development and validation of a quantitative assay for the determination of tamoxifen and its five main phase I metabolites in human serum using liquid chromatography coupled with tandem mass spectrometry

S.F. Teunissen[a,*,1], N.G.L. Jager[a,1], H. Rosing[a], A.H. Schinkel[b], J.H.M. Schellens[c,d], J.H. Beijnen[a,d]

[a] Department of Pharmacy & Pharmacology, Slotervaart Hospital/The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands
[b] Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[c] Department of Clinical Pharmacology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[d] Department of Pharmaceutical Sciences, Faculty of Science, Utrecht University, 3508 TB Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 24 November 2010
Accepted 3 April 2011
Available online 16 April 2011

Keywords:
Endoxifen
Liquid chromatography
Mass spectrometry
Metabolites
Tamoxifen

ABSTRACT

A sensitive bioanalytical assay for the quantitative determination of tamoxifen and five of its phase I metabolites (N-desmethyltamoxifen, N-desmethyl-4-hydroxytamoxifen, N-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen) in serum is described. The method has been fully validated at ranges covering steady-state serum concentrations in patients receiving therapeutic dosages of tamoxifen. The bioanalytical assay is based on reversed phase liquid chromatography coupled with tandem mass spectrometry in the positive ion mode using multiple reaction monitoring for drug (-metabolite) quantification. The sample pretreatment consists of protein precipitation with acetonitrile using only 50 μL of serum. In the past, numerous assays have been developed by other groups for the quantification of tamoxifen and its phase I metabolites. However, the number of metabolites included in these studies is very limited and only very few of these assays have been fully validated. A liquid chromatography tandem mass spectrometry assay for the quantification of tamoxifen and four phase I metabolites in human serum that was previously developed by our group is now explicitly improved and described herein. Time of analysis has been reduced by 50% and sensitivity was increased by a reduction of the lower limit of quantification from 1.0 to 0.2 ng/mL for 4-hydroxytamoxifen and 4′-hydroxytamoxifen. Additionally, two phase I metabolites that have never been quantified in human serum hitherto, namely 4′-hydroxytamoxifen and N-desmethyl-4′-hydroxytamoxifen, were included in this assay. Validation results demonstrate an accurate and precise quantification of tamoxifen, N-desmethyltamoxifen, N-desmethyl-4-hydroxytamoxifen, N-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen in human serum. The applicability of the assay was demonstrated and it is now successfully used to support clinical studies in which patient-specific dose optimization is performed based on serum concentrations of tamoxifen metabolites.

1. Introduction

The selective estrogen receptor modulator tamoxifen is widely applied in estrogen receptor positive breast cancer treatment [1,2]. During phase I metabolism, metabolites of tamoxifen are formed that are more active than tamoxifen itself. Tamoxifen is therefore considered a prodrug. Among the bio-activated metabolites, 4-hydroxytamoxifen has been shown to be a potent anti-estrogen (30 to 100-fold more potent than tamoxifen itself) [3,4].

N-desmethyl-4-hydroxytamoxifen (endoxifen) is equipotent to 4-hydroxytamoxifen in estrogen receptor (ER)-alpha and ER-beta binding, as well as in the inhibition of 17β-estradiol induced proliferation in human breast cancer cells [5–7]. Endoxifen, however, is on average present at a much higher steady-state concentration in serum of patients than 4-hydroxytamoxifen [5,7,8]. It is known from the literature that tamoxifen and its metabolites are subject to large inter-patient variation in steady-state serum concentrations [7,9,10]. Numerous studies reported a relationship between *in vivo* metabolite concentrations and patient survival, single nucleotide polymorphisms (SNPs) and cytochrome P450 activity [11–16]. It is of pivotal importance to be able to quantify tamoxifen metabolites *in vivo* as the serum concentrations of active metabolites vary widely among individuals, due to a large

* Corresponding author. Tel.: +31 20 5124073; fax: +31 20 5124753.
E-mail address: Bas.Teunissen@slz.nl (S.F. Teunissen).
[1] These authors contributed equally.

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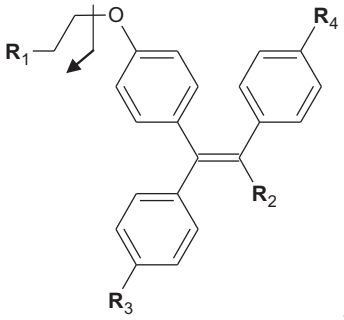
doi:10.1016/j.jchromb.2011.04.011

Table 1
Trivial names, chemical structures, molecular masses and selected fragmentation position of tamoxifen, five of its metabolites and its stable isotope labelled internal standards.



Trivial name	R_1	R_2	R_3	R_4	Mol. formula	Mol. mass
Tamoxifen	$N(CH_3)_2$	$CH_2–CH_3$	H	H	$C_{26}H_{29}NO$	371.5
Tamoxifen-$^2H_5$	$N(CH_3)_2$	$C^2H_2–C^2H_3$	H	H	$C_{26}H_{24}{}^2H_5NO$	376.5
N-desmethyltamoxifen	NH–CH_3	$CH_2–CH_3$	H	H	$C_{25}H_{27}NO$	357.5
N-desmethyltamoxifen-$^2H_5$	NH–CH_3	$C^2H_2–C^2H_3$	H	H	$C_{25}H_{22}{}^2H_5NO$	362.5
N-desmethyl-4-hydroxytamoxifen	NH–CH_3	$CH_2–CH_3$	OH	H	$C_{25}H_{27}NO_2$	373.5
N-desmethyl-4-hydroxytamoxifen-$^2H_5$	NH–CH_3	$C^2H_2–C^2H_3$	OH	H	$C_{25}H_{22}{}^2H_5NO_2$	378.5
N-desmethyl-4′-hydroxytamoxifen	NH–CH_3	$CH_2–CH_3$	H	OH	$C_{25}H_{27}NO_2$	373.5
4-Hydroxytamoxifen	$N(CH_3)_2$	$CH_2–CH_3$	OH	H	$C_{26}H_{29}NO_2$	387.5
4-Hydroxytamoxifen-$^2H_5$	$N(CH_3)_2$	$C^2H_2–C^2H_3$	OH	H	$C_{26}H_{24}{}^2H_5NO_2$	392.5
4′-Hydroxytamoxifen	$N(CH_3)_2$	$CH_2–CH_3$	H	OH	$C_{26}H_{29}NO_2$	387.5

variation in metabolism. This variation might be caused by genotype and co-medication that influences the CYP450 system (e.g. SSRIs) [7,9,10]. Numerous assays have been developed for the quantification of tamoxifen and its phase I metabolites [17]. However, the number of metabolites included in these studies is very limited and only very few of these assays have been fully validated according to FDA guidelines [18]. We have previously designed and fully validated a liquid chromatography tandem mass spectrometry assay for the quantification of tamoxifen and four phase I metabolites in human serum [8]. This assay now requires improvement to optimally support clinical studies for which large numbers of samples need to be analyzed. Therefore, the speed of analysis was reduced by 50%, the sensitivity was increased by a reduction of the lower limit of quantification from 1.0 to 0.2 ng/mL, additional tamoxifen metabolites were included and the validated range was reduced to improve accuracy. The range was chosen based solely on steady-state serum concentrations of women receiving the commonly used daily dose of 20 or 40 mg tamoxifen. The dynamic range in the previously developed assay was much wider, as that method was developed to support both clinical studies in patients as well as toxicokinetic studies in mice receiving high doses of tamoxifen. Besides reducing the validated ranges, the lower limit of quantification (LLOQ) was lowered significantly (1.0–0.2 ng/mL) for 4-hydroxytamoxifen and 4′-hydroxytamoxifen to ensure accurate *in vivo* quantification. This required a mass spectrometer capable of detecting analytes with a concentration as low as 0.2 ng/mL. Additionally, the phase I metabolites selected for quantification were changed. This is the result of profound new insight into the phase I metabolism of tamoxifen obtained from supporting clinical studies using the previously developed assay. In all serum samples obtained from these clinical studies, in which the patients received 20 mg tamoxifen once daily, two additional tamoxifen metabolites were consistently observed. These were 4′-hydroxytamoxifen and N-desmethyl-4′-hydroxytamoxifen (Fig. 1). Their structure and steady-state serum concentration is very similar to 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen (endoxifen), respectively; two metabolites that are acknowledged to be the active metabolites of tamoxifen. To the best of our knowledge, these two metabolites have never been quantified in human specimens hitherto. We included them in this assay for quantification in human serum. Other tamoxifen phase I metabolites were not included in this assay as their steady-state serum concentration was low; around the detection limit of the MS detector (or no reference standard was available). A full validation of the developed assay was performed according to the FDA guidelines [18]. Additionally, the applicability of the assay in clinical samples was demonstrated.

2. Experimental

2.1. Reagents and chemicals

Tamoxifen, tamoxifen-d5, 4-hydroxytamoxifen, 4-hydroxytamoxifen-d5, 4′-hydroxy-tamoxifen, N-desmethyltamoxifen-HCl, N-desmethyltamoxifen-d5, N-desmethyl-4-hydroxytamoxifen (1:1, *E*/*Z* mixture), N-desmethyl-4-hydroxytamoxifen-d5 (1:1, *E*/*Z* mixture) and N-desmethyl-4′-hydroxytamoxifen were purchased from Toronto

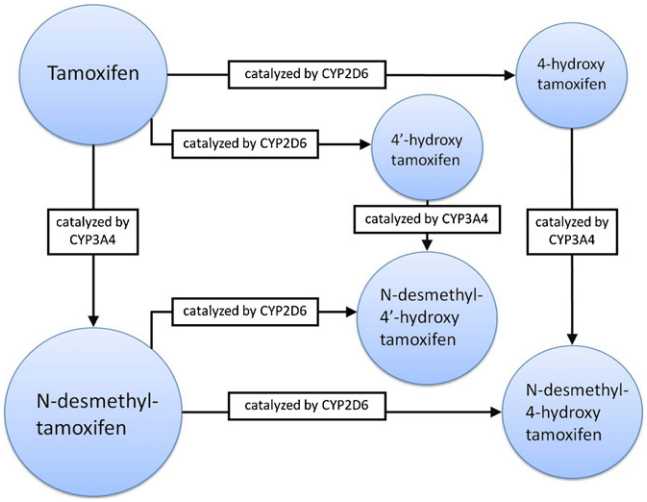


Fig. 1. Metabolism of tamoxifen. The enzymes mentioned in the figure are responsible for the primary metabolism.

Table 2
HPLC gradient parameters used for the separation of tamoxifen and its metabolites using a Kinetex C18 column (150 mm × 2.1 mm I.D., 2.6 μm) thermostatted at 60 °C.

Time (min)	Flow rate (mL/min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0.00	0.4	70	30
6.00	0.4	47.5	52.5
6.01	0.4	20	80
7.00	0.4	20	80
7.01	0.4	70	30
10.0	0.4	70	30

[a] Mobile phase A: 5.0 mM ammonium formate buffer pH 3.5.
[b] Mobile phase B: acetonitrile.

Research Chemicals (North York, ON, Canada). The chemical structures of the analytes and internal standards are shown in Table 1. Acetonitrile and methanol were obtained from Biosolve Ltd. (Amsterdam, The Netherlands). Ammonium formate was purchased from Acros Organics (Geel, Belgium). Formic acid and LiChrosolv water for HPLC were purchased from Merck (Darmstadt, Germany). Small (1 mL) volumes of control drug-free human serum, obtained from Slotervaart Hospital (Amsterdam, the Netherlands), were pooled and used for validation purposes.

2.2. Instrumentation

2.2.1. HPLC

An Agilent HPLC system was used consisting of an 1100 series binary pump, column oven, on-line degasser and autosampler (Agilent Technologies, Palo Alto, CA, USA).

Mobile phase A was prepared by adjusting a 5.0 mM ammonium formate solution to pH 3.5 with a 98% formic acid solution. Mobile phase B consisted of 100% acetonitrile. Mobile phases A and B were pumped through a Kinetex C18 100 Å column (150 mm × 2.1 mm I.D., 2.6 μm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.4 mL/min using a gradient as shown in Table 2. The analytical column was protected by a KrudKatcher inline filter (Phenomenex, Torrance, CA, USA). The separation was performed at 60 °C. Volumes of 15 μL were injected using the autosampler thermostatted at 7 °C. The column was equilibrated for 3 min before the next injection, leading to a total run time of 10 min. The autosampler needle was rinsed with acetonitrile before and after each injection. During the first and last 1.0 min the eluate was directed to waste using a divert valve to prevent the introduction of endogenous compounds into the mass spectrometer.

2.2.2. MS

An API 4000 triple quadrupole mass spectrometer equipped with an Atmospheric Pressure Chemical Ionization (APCI) source (AB Sciex, Foster City, CA, USA) operating in the positive ion mode was used as a detector. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired and processed using Analyst® software (AB Sciex). The quadrupoles were operating at unit resolution (0.7 Da). APCI-MS/MS operating parameters and mass transitions are listed in Table 3.

2.3. Preparation of calibration standards, quality controls and internal standard solutions

Two separate stock solutions of all analytes (1 mg/mL) and internal standards (1 mg/mL) were prepared. Approximately 1 mg was accurately weighed (Sartorius Micro MC5 balance, Sartorius Instrumenten BV, Nieuwegein, the Netherlands) and dissolved in 1 mL methanol. For the analytes, one stock solution was used for the preparation of calibration standards and the other stock solution was used for the preparation of quality control (QC) standards. The preparation of the two stock solutions for each

Table 3
APCI-MS/MS operating parameters.

Parameter	Setting
Run duration	10 min
Ion spray voltage	5500 V
Collision gas	8 arbitrary units
Curtain gas	10 arbitrary units
Turbo gas	45 arbitrary units
APCI temperature	400 °C
Tamoxifen	
Q1 mass	372 amu
Q3 mass	72 amu
Collision energy	45 V
Declustering potential	91 V
Collision exit potential	14 V
N-desmethyltamoxifen	
Q1 mass	358 amu
Q3 mass	58 amu
Collision energy	45 V
Declustering potential	71 V
Collision exit potential	10 V
N-desmethyl-4-hydroxytamoxifen	
Q1 mass	374 amu
Q3 mass	58 amu
Collision energy	47 V
Declustering potential	81 V
Collision exit potential	10 V
N-desmethyl-4′-hydroxytamoxifen	
Q1 mass	374 amu
Q3 mass	58 amu
Collision energy	47 V
Declustering potential	81 V
Collision exit potential	10 V
4-Hydroxytamoxifen	
Q1 mass	388 amu
Q3 mass	72 amu
Collision energy	45 V
Declustering potential	91 V
Collision exit potential	14 V
4′-Hydroxytamoxifen	
Q1 mass	388 amu
Q3 mass	72 amu
Collision energy	45 V
Declustering potential	91 V
Collision exit potential	14 V
Tamoxifen-d5	
Q1 mass	377 amu
Q3 mass	72 amu
Collision energy	45 V
Declustering potential	70 V
Collision exit potential	12 V
N-desmethyltamoxifen-d5	
Q1 mass	363 amu
Q3 mass	58 amu
Collision energy	45 V
Declustering potential	70 V
Collision exit potential	12 V
N-desmethyl-4-hydroxytamoxifen-d5 (*Z*/*E*)	
Q1 mass	379 amu
Q3 mass	58 amu
Collision energy	45 V
Declustering potential	70 V
Collision exit potential	12 V
4-Hydroxytamoxifen-d5	
Q1 mass	393 amu
Q3 mass	72 amu
Collision energy	45 V
Declustering potential	70 V
Collision exit potential	12 V

Table 4
Assay performance data ($n = 5$ per run, $n = 15$ for total).

Analyte (concentration range)	Nominal concentration (ng/mL)	Inter-assay accuracy	Inter-assay precision (% CV)
Tamoxifen	5.12	0.117	8.92
(5.00–1000 ng/mL)	12.8	−2.34	7.13
	400	−3.90	4.75
	800	1.15	3.67
N-desmethyltamoxifen	5.12	−2.40	12.0
(5.00–1000 ng/mL)	12.8	−0.420	6.08
	400	−1.20	3.85
	800	−0.110	4.17
N-desmethyl-4-hydroxytamoxifen	1.02	−4.46	11.1
(1.00–200 ng/mL)	2.56	−1.61	7.94
	80.0	−0.120	4.97
	160	−1.42	5.01
N-desmethyl-4′-hydroxytamoxifen	1.02	0.360	11.1
(1.00–200 ng/mL)	2.56	4.01	8.61
	80.0	0.670	4.62
	160	3.46	5.56
4-Hydroxytamoxifen	0.410	−1.01	6.50
(0.400–80.0 ng/mL)	1.02	−0.950	7.13
	32.0	−1.71	4.70
	64.0	−1.02	5.78
4′-Hydroxytamoxifen	0.205	−2.70	10.5
(0.200–40.0 ng/mL)	0.512	−0.810	9.62
	16.0	−5.00	4.40
	32.0	0.0200	6.33

compound was checked and in all cases deviations were less than ± 5%. The *E*/*Z*-ratio of the reference standard of N-desmethyl-4-hydroxytamoxifen was determined by liquid chromatography with ultraviolet absorption detection. The ratio was determined to be one.

The stock solutions were further diluted with methanol to obtain separate working solutions, each containing all of the six analytes at a 200-fold concentration of the corresponding serum samples. Calibration samples were prepared freshly for every run by spiking a volume of 10 μL working solution to 2.0 mL of control human serum. QC samples were prepared in batches and stored at −70 °C until analysis.

A mixture of internal standard stock solutions was prepared and diluted with acetonitrile to obtain a working solution that was used for sample pretreatment. This internal standard working solution contained: tamoxifen-d5, N-desmethyltamoxifen-d5, N-desmethyl-4-hydroxytamoxifen-d5 (1:1 *E*/*Z* mixture) and 4-hydroxytamoxifen-d5 at concentrations of: 75, 75, 16.5 and 4 ng/mL, respectively.

2.4. Sample preparation

A volume of 150 μL internal standard working solution in acetonitrile was added to a 50 μL serum aliquot. The mixture was vortex mixed for 10 s followed by centrifugation for 10 min at 11,300 × *g*. 120 μL of the clear supernatant was transferred to a 1.5 mL empty amber colored eppendorf tube and evaporated at 30 °C under a gentle stream of nitrogen to form a dried extract. This extract was subsequently reconstituted in 60 μL acetonitrile–4 mM ammonium formate buffer pH 3.5 (3:7, v/v) by vortex mixing for 15 s to form the final extract. The sample was transferred to an amber colored autosampler vial and stored at 2–8 °C until analysis.

3. Validation procedures

A full validation of the assay was performed according to the FDA guidelines [18].

3.1. Regression models

Eight non-zero calibration standards were prepared freshly in duplicate for each run and analyzed in three independent runs. For N-desmethyl-4-hydroxytamoxifen, N-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen linear least-squares regression was applied (area ratio with the internal standard versus the nominal concentration). The calibration standard data for tamoxifen and N-desmethyltamoxifen were fitted quadratic. For all analytes, the reciprocal of the squared concentration ($1/x^2$) was used as a weighting factor. Deviations from the mean calculated concentrations over three runs should be within 85–115% of nominal concentrations. At the LLOQ, a deviation of 20% was permitted and the response of the analyte should be at least five times higher than the response of a blank sample. For four of the six analyzed compounds deuterated internal standards were commercially available. The stable isotope labelled internal standards 4-hydroxytamoxifen-d5 and (*Z*)-N-desmethyl-4-hydroxytamoxifen-d5 were used to correct for signal fluctuations of 4′-hydroxytamoxifen and N-desmethyl-4′-hydroxytamoxifen, respectively.

3.2. Accuracy and precision

Intra- and inter-assay accuracies and precisions of the method were determined by assaying five replicates of each of the QC samples at the LLOQ, low, mid and high concentration level in three separate runs. The concentration of each QC sample was calculated using the calibration standards that were analyzed in duplicate in the same run. The differences between the nominal and the measured concentration were used to calculate the accuracies. The accuracy should be within 85–115% except at the LLOQ, where an accuracy of 80–120% is allowed. The precision should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20%.

The ability to dilute samples originally above the upper limit of quantification (ULOQ) was demonstrated by analyzing QC sam-

Table 5
Results of the stability experiments ($n = 3$).

Matrix	Conditions	Compound	Nominal conc. (ng/mL)	Dev (%)	CV (%)
Methanol	15–25 °C, 2 h	Tamoxifen	400	−3.55	3.53
		N-desmethyltamoxifen	400	−0.123	1.68
		N-desmethyl-4-hydroxytamoxifen	80.0	−2.21	2.27
		N-desmethyl-4′-hydroxytamoxifen	80.0	0.661	0.661
		4-Hydroxytamoxifen	32.0	−0.135	2.62
		4′-Hydroxytamoxifen	16.0	0.287	3.44
Serum	15–25 °C, 6 h	Tamoxifen	12.8	−7.03	4.45
			800	−1.25	2.34
		N-desmethyltamoxifen	12.8	−4.17	2.62
			800	−1.38	1.43
		N-desmethyl-4-hydroxytamoxifen	2.56	−9.11	2.37
			160	−4.58	0.378
		N-desmethyl-4′-hydroxytamoxifen	2.56	−12.2	1.85
			160	−0.625	1.66
		4-Hydroxytamoxifen	1.02	−13.4	4.62
			64.0	−4.69	1.43
		4′-Hydroxytamoxifen	0.512	−13.0	0.934
			32.0	−5.94	0.879
Serum	−70 °C, 1 month	Tamoxifen	12.8	−7.81	3.06
			800	7.17	8.05
		N-desmethyltamoxifen	12.8	−8.07	5.46
			800	9.79	9.42
		N-desmethyl-4-hydroxytamoxifen	2.56	−14.5	2.85
			160	4.79	6.99
		N-desmethyl-4′-hydroxytamoxifen	2.56	−8.85	4.70
			160	13.3	9.40
		4-Hydroxytamoxifen	1.02	−11.5	2.84
			64.0	5.63	9.78
		4′-Hydroxytamoxifen	0.512	−13.8	2.63
			32.0	5.00	8.58
Serum	3 Freeze (−70 °C)/thaw cycles	Tamoxifen	12.8	−5.73	3.74
			800	−1.38	2.25
		N-desmethyltamoxifen	12.8	−1.82	5.30
			800	−0.0833	1.39
		N-desmethyl-4-hydroxytamoxifen	2.56	−5.99	3.33
			160	−4.17	4.72
		N-desmethyl-4′-hydroxytamoxifen	2.56	−0.781	2.19
			160	0.833	2.79
		4-Hydroxytamoxifen	1.02	−8.92	0.812
			64.0	−5.10	2.24
		4′-Hydroxytamoxifen	0.512	−4.75	0.427
			32.0	−3.96	0.939
Dried extract	2–8 °C, 4 days	Tamoxifen	12.8	−8.85	1.31
			800	−2.38	1.66
		N-desmethyltamoxifen	12.8	−2.34	1.39
			800	−3.00	0.670
		N-desmethyl-4-hydroxytamoxifen	2.56	−13.3	3.40
			160	−10.4	0.403
		N-desmethyl-4′-hydroxytamoxifen	2.56	−0.521	3.81
			160	−1.25	0.00
		4-Hydroxytamoxifen	1.02	−5.63	4.09
			64.0	−7.08	0.541
		4′-Hydroxytamoxifen	0.512	−6.12	4.56
			32.0	−5.52	1.56
Final extract	2–8 °C, 7 days	Tamoxifen	12.8	−4.95	3.32
			800	3.50	0.483
		N-desmethyltamoxifen	12.8	−5.47	3.79
			800	4.92	0.793
		N-desmethyl-4-hydroxytamoxifen	2.56	−13.9	2.50
			160	−8.54	1.97
		N-desmethyl-4′-hydroxytamoxifen	2.56	−1.17	2.09
			160	2.92	1.53
		4-Hydroxytamoxifen	1.02	−6.45	5.23
			64.0	−5.94	0.879
		4′-Hydroxytamoxifen	0.512	−4.43	1.94
			32.0	−4.48	1.36
Final extract	2–8 °C, 24 h	Tamoxifen	12.8	−11.3	0.881
			400	−1.63	1.10
			800	−6.46	0.772
		N-desmethyltamoxifen	12.8	−4.95	6.64
			400	−1.00	1.16
			800	−0.417	1.34

Table 5 (*Continued*)

Matrix	Conditions	Compound	Nominal conc. (ng/mL)	Dev (%)	CV (%)
		N-desmethyl-4-hydroxytamoxifen	2.56	−12.0	3.27
			80.0	0.792	3.29
			160	−3.96	0.376
		N-desmethyl-4′-hydroxytamoxifen	2.56	−7.94	1.91
			80.0	−0.458	2.64
			160	−1.67	1.32
		4-Hydroxytamoxifen	1.02	−7.16	2.00
			32.0	0.417	0.359
			64.0	−1.46	2.54
		4′-Hydroxytamoxifen	0.512	−12.8	3.18
			16.0	−2.29	2.06
			32.0	−5.94	3.45

ples containing 10 times the concentration of the high QC sample. These samples were prepared in 5-fold and analyzed after a 10-fold dilution in control human serum.

3.3. Matrix factor and carry-over

To determine the matrix factor, the following samples were prepared in triplicate: (i) a QC sample at a QC mid concentration level in serum processed to final extract according to the procedure described in Section 2.4, (ii) a sample in acetonitrile–4 mM ammonium formate buffer pH 3.5 (3:7, v/v) at a QC mid concentration level processed to final extract according to the procedure described in Section 2.4, (iii) a blank serum sample processed to dried extract according to the procedure described in Section 2.4, followed by reconstitution with a processed sample obtained at (ii). Carry-over was determined by injecting a processed control human serum sample after an ULOQ sample. Areas of peaks in the blank processed sample should be less than 20% of the peak area of the LLOQ sample.

3.4. Specificity and selectivity

Six individual batches of control human serum were used to assess specificity and selectivity of the method. To determine whether endogenous constituents interfere with the assay, a double blank and a sample spiked at the LLOQ were processed from these batches. The samples were subsequently analyzed according to the procedures described above. Areas of peaks co-eluting with analytes should be less than 20% of the peak area of the LLOQ sample in each of the six batches of control human serum. Peak areas co-eluting in the double blank samples with the internal standards should be less than 5% of the peak area of the mean IS response. For the LLOQ, sample accuracies should be within ±20% boundaries of the nominal concentration in four out of six samples. To ensure that other tamoxifen phase I metabolites would not interfere in the assay, the chromatographic behaviour of tamoxifen metabolites with equal precursor and product masses (e.g. 3- and α-hydroxytamoxifen, N-desmethyl-3- and N-desmethyl-α-hydroxytamoxifen) was studied. These metabolites were all baseline separated from the metabolites quantified in this assay.

3.5. Stability

The stability of the analytes was investigated in the stock solutions at ambient temperature. Furthermore, stability was tested in human serum for 6 h at room temperature, during 1 month of storage at −70 °C and after three freeze (−70 °C)–thaw cycles with a minimum interval of 24 h. In-process (dried extract) and final extract stability was investigated during 4 days and 7 days of storage at 2–8 °C, respectively. The QC stability samples were quantified based on a freshly prepared calibration curve. The re-injection reproducibility was determined in the final extract after 24 h at nominally 2–8 °C. All stability experiments were performed in triplicate. Analytes are considered stable in stock and working solutions when 95–105% of the original concentration is found. Analytes are considered stable in the biological matrix or extract when 85–115% of the initial concentration is recovered.

Stability of the deuterated internal standards was assumed to be equal to the corresponding undeuterated analytes. Isotopic purity of deuterated analytes (i.e. internal standard) was investigated during each analytical run by spiking control human serum with internal standard working solution. The peak area in the non-deuterated analyte window should be less than 20% of the peak area of the analytes at their LLOQ level.

Table 6
Steady-state concentrations of tamoxifen and five of its metabolites in serum collected from ten breast cancer patients receiving 20 mg tamoxifen once daily.

Patient	Tamoxifen (ng/mL)	N-desmethyltamoxifen (ng/mL)	N-desmethyl-4-hydroxytamoxifen (ng/mL)	N-desmethyl-4′-hydroxytamoxifen (ng/mL)	4-Hydroxytamoxifen (ng/mL)	4′-Hydroxytamoxifen (ng/mL)
1	123	175	14.5	4.58	1.94	2.23
2	183	336	8.74	9.51	1.61	3.07
3	147	400	11.5	23.0	2.42	6.74
4	82.7	133	7.15	4.06	1.36	1.74
5	144	307	6.22	19.3	1.23	5.21
6	136	197	12.8	5.64	2.32	2.78
7	155	256	15.0	8.15	2.30	3.06
8	85.4	124	11.7	4.93	1.79	2.07
9	104	192	10.2	7.33	1.34	2.99
10	76.2	130	5.81	4.75	1.04	1.66
Mean	124	225	10.4	9.13	1.74	3.16
RSD (%)	28.8	42.6	31.9	72.7	28.7	51.2

4. Results and discussion

4.1. HPLC–MS/MS

The previously developed assay for the analysis of tamoxifen and its metabolites required improvements with regard to the overall time of analysis and sensitivity [8]. A Kinetex C18 column was capable of providing fast, high resolution separations. Thereby the total run time could be reduced drastically from 19 to 10 min [8]. This Kinetex C18 column was superior over other tested HPLC columns (C18, Synergi Hydro (Phenomenex, Torrance, CA, USA)) in terms of resolution and speed. The high column efficiencies are due to the halo – pellicular particles in the Kinetex column. As various tamoxifen metabolites have the same precursor and product mass, baseline separation of these metabolites was required. Reference standards of N-desmethyl-4-hydroxytamoxifen were provided as racemic mixtures of zusammen (*Z*) and entgegen (*E*) isomers. As tamoxifen is administered to patients as a pure *Z*-enantiomer [19], only the (*Z*)-form of metabolites are formed *in vivo*. Therefore, (*Z*)- and (*E*)-N-desmethyl-4-hydroxytamoxifen had to be baseline separated for an accurate and precise quantification of the separate isomers. The Kinetex HPLC column consists of stationary phase particles with a size of 2.6 μm. The relatively high flow rate of 400 μL/min at which the column is operated resulted in a backpressure of ±350 bar. By increasing the column temperature to 60 °C, the backpressure was reduced to ±300 bar, while the resolution increased simultaneously as a result of increased mass transfer rate [20]. An eluent consisting of acetonitrile provided superior peak symmetry compared to methanol. The separation was further optimized by buffering the eluent at pH 3.5 (Fig. 2).

Protonated molecules ($[M+H]^+$) were used as precursor ions to generate product-ion spectra. The most intense product-ions were optimized and used as MRM transitions to ensure high sensitivity and selectivity. In Tables 1 and 3, the optimized mass transitions and proposed fragmentation pathways are presented for tamoxifen and its metabolites. The peak width of eluting metabolites was very small (±6 s) and required fast scanning of the mass spectrometer to ensure enough (>20) data points over the peak. To facilitate sufficient data collection per peak, the chromatogram was divided in two sections of 7.5 and 2.5 min, respectively. The measured mass transitions per section were defined based on the analytes and internal standards eluting therein.

4.2. Regression models

All calibration curves were constructed using a weighting factor of $1/x^2$ and fitted either linearly or quadratically. In the previously developed assay, electrospray ionization (ESI) was used to promote the liquid–gas conversion [8]. To improve the sensitivity of the current method, a different mass spectrometer was used (API 4000 (AB Sciex, Foster City, CA, USA) instead of a Quantum Ultra (Thermo Fisher Scientific, Waltham, MA, USA)). When using an API 4000 equipped with an ESI source, quadratically fitted calibration curves for the majority of analytes were obtained. This is most likely due to the differences in source configuration between both types of mass spectrometers, which might result in, e.g. saturation of the electrospray above a specific analyte concentration. Quadratically fitted calibration curves result in a high risk of deviations in the upper concentration region. To improve the linearity of the calibration curves, the ESI source was replaced by an APCI source. This resulted in linear calibration curves for the majority of the compounds, with the exception of tamoxifen and N-desmethyltamoxifen. However, the calibration curves of these compounds show only a minor deflection in the upper concentration region. The CVs were in all cases less than 15%. Accuracies were in all cases within 85–115%. Mean correlation coefficients (r^2) were ≥0.996.

4.3. Accuracy and precision

Assay performance data (intra-assay and inter-assay accuracy and precision) of all analyzed compounds are summarized in Table 4. Intra-assay and inter-assay accuracies were within 85–115% and precisions were less than 15% for all compounds.

4.4. Matrix factor and carry-over

The total recovery of all analytes was determined at one concentration level and was in all cases near 100%. The total recovery is defined as the analyte recovery after sample pretreatment plus the effect of the matrix on the measured MS signal. As the total recovery is near 100%, the effect of the matrix is negligible. Carry-over was determined by injecting a processed control human serum sample after an ULOQ sample. Areas of peaks in the blank processed sample were less than 20% of the peak area of the LLOQ sample.

4.5. Specificity and selectivity

MRM chromatograms of six batches of control human serum contained no co-eluting peaks larger than 20% of the area at the LLOQ level of all analytes, and no co-eluting peaks larger than 5% of the area of all internal standards. The influence of different control human serum batches on the accuracy and precision at LLOQ level was investigated. The accuracies of analytes at LLOQ level were in all six batches of control human serum within ±20% boundaries of the nominal concentration.

4.6. Stability

In Table 5 the results of the investigated stability parameters are presented. N-desmethyltamoxifen is sensitive to light. To prevent possible degradation under the influence of light and temperature, all stock solutions and samples were stored at nominally −70 °C and samples were processed in amber colored vials.

Results show that stock solutions in methanol appeared to be stable for at least 2 h at room temperature, sufficient for the preparation of working solutions and subsequent spiking of serum. Serum samples are stable for at least 6 h at room temperature and for 1 month at −70 °C. The calibration standards and QC samples were aliquoted, stored at −70 °C and thawed directly before processing to keep the number of freeze/thaw cycles to a minimum. However, patient samples may require re-analysis, which results in extra freeze/thaw cycles. Stability of serum samples is guaranteed during at least three freeze (−70 °C)/thaw cycles. During processing of serum samples, dried extracts are obtained which appeared to be stable for at least 4 days when stored at 2–8 °C in amber colored glassware. Final extracts obtained from serum samples are stable for at least 7 days when stored at 2–8 °C in amber colored glassware.

When required, a full analytical run consisting of calibration standards, QCs and patient samples can be re-injected after 24 h. After 1 month of storage of an internal standard working solution at −70 °C, peak areas of analytes in a processed control human serum sample spiked with the internal standard working solution were less than 20% of the peak areas of the analytes in an LLOQ sample (data not shown).

5. Application of the method

The applicability of the assay was demonstrated by the analysis of steady-state serum concentrations of

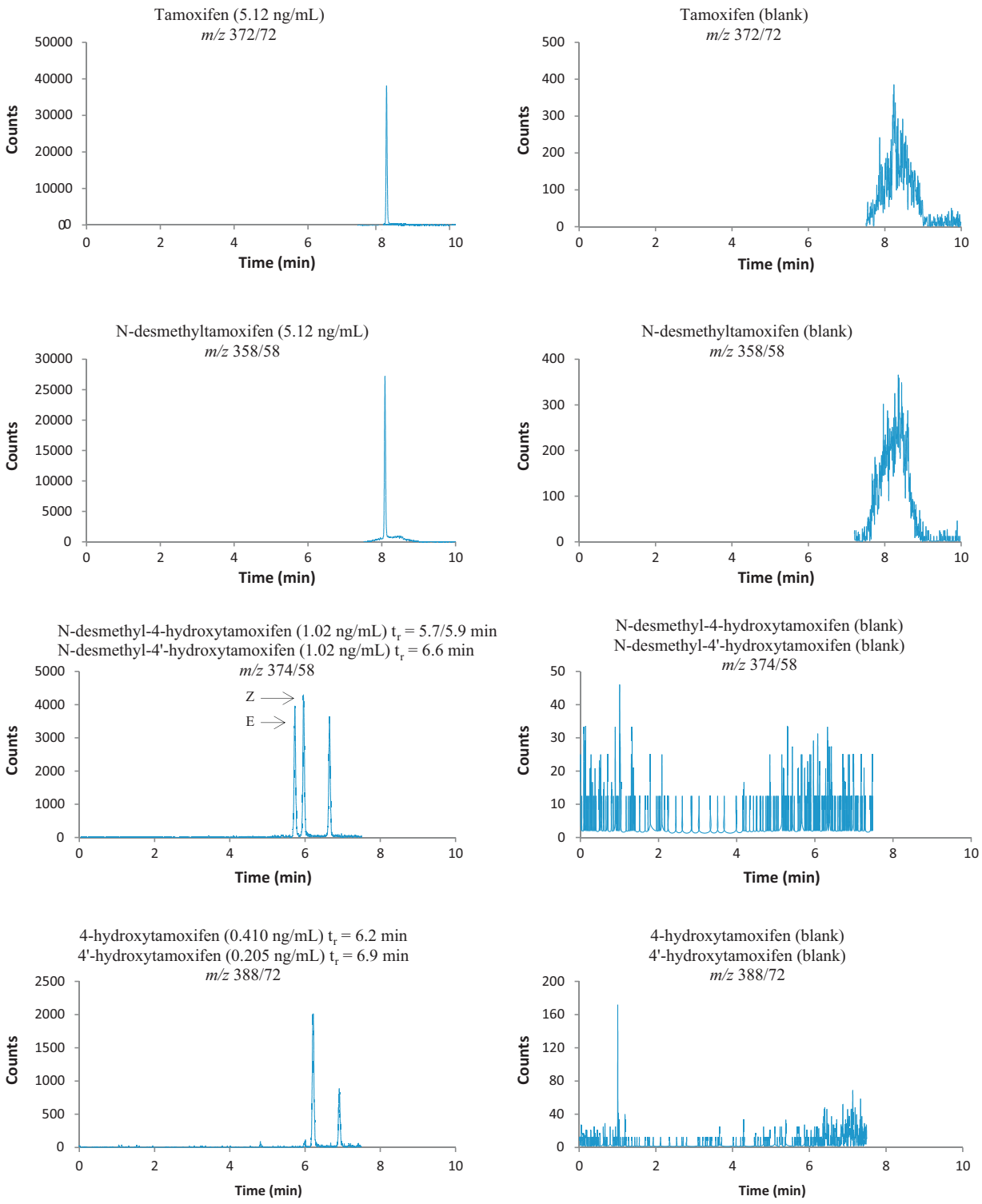


Fig. 2. MRM chromatograms of QC samples at the lower limit of quantification of the analytes and their respective blanks. Concentrations are given in the figure.

patients receiving the regular prescribed dose in breast cancer treatment of 20 mg tamoxifen once daily. The measured concentrations are shown in Table 6. The serum concentrations of tamoxifen, N-desmethyltamoxifen, N-desmethyl-4-hydroxytamoxifen, N-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen were all within the validated range of the developed assay.

6. Conclusion

An HPLC–MS/MS assay has been developed and validated for the simultaneous analysis of tamoxifen, N-desmethyltamoxifen, N-desmethyl-4-hydroxytamoxifen (endoxifen), N-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen in human serum. The assay shows clear improvements with regard to the previously developed assay in terms of time of analysis, sensi-

tivity, resolution and the number of tamoxifen phase I metabolites included in the assay [8].

The LLOQ of tamoxifen and metabolites is well below the lowest concentrations measured in patients at steady-state serum concentrations who receive the commonly used dose of 20 mg tamoxifen once daily. The method is now successfully used to support clinical studies in which patient-specific dose-optimization is performed based on serum concentrations of tamoxifen and its metabolites.

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