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An ultra performance liquid chromatography–tandem MS assay for tamoxifen metabolites profiling in plasma: First evidence of 4′-hydroxylated metabolites in breast cancer patients

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ABSTRACT

There is increasing evidence that the clinical efficacy of tamoxifen, the first and most widely used targeted therapy for estrogen-sensitive breast cancer, depends on the formation of the active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen). Large inter-individual variability in endoxifen plasma concentrations has been observed and related both to genetic and environmental (i.e. drug-induced) factors altering CYP450s metabolizing enzymes activity. In this context, we have developed an ultra performance liquid chromatography–tandem mass spectrometry method (UPLC–MS/MS) requiring 100 μL of plasma for the quantification of tamoxifen and three of its major metabolites in breast cancer patients. Plasma is purified by a combination of protein precipitation, evaporation at room temperature under nitrogen, and reconstitution in methanol/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with formic acid. Reverse-phase chromatographic separation of tamoxifen, *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyl-tamoxifen is performed within 13 min using elution with a gradient of 10 mM ammonium formate and acetonitrile, both containing 0.1% formic acid. Analytes quantification, using matrix-matched calibration samples spiked with their respective deuterated internal standards, is performed by electrospray ionization–triple quadrupole mass spectrometry using selected reaction monitoring detection in the positive mode. The method was validated according to FDA recommendations, including assessment of relative matrix effects variability, as well as tamoxifen and metabolites short-term stability in plasma and whole blood. The method is precise (inter-day CV%: 2.5–7.8%), accurate (−1.4 to +5.8%) and sensitive (lower limits of quantification comprised between 0.4 and 2.0 ng/mL). Application of this method to patients' samples has made possible the identification of two further metabolites, 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen, described for the first time in breast cancer patients. This UPLC–MS/MS assay is currently applied for monitoring plasma levels of tamoxifen and its metabolites in breast cancer patients within the frame of a clinical trial aiming to assess the impact of dose increase on tamoxifen and endoxifen exposure.

1. Introduction

Tamoxifen (*Z* isomer) (Fig. 1) is a standard hormonal therapy currently used for the secondary treatment of hormone-responsive breast cancer [1–6] and for the prevention in women at high risk of developing the disease [7]. Tamoxifen is a non-steroidal selective estrogen receptor modulator (SERM), which competitively binds to estrogen receptors (ERs) and inhibits estrogen-dependent growth and proliferation of malignant breast epithelial cells [1,6]. However, several lines of evidence indicate that the overall anti-proliferative effects of tamoxifen depends notably on the formation of the clinically active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethytamoxifen (endoxifen) (B and E in Fig. 1) which have 100-fold greater affinity to ERs and 30–100-fold greater potency in suppressing breast cancer cell proliferation as compared to the parent drug [8–12].

Tamoxifen can thus be considered a quasi-prodrug that is extensively metabolised by several polymorphic cytochrome P450 (CYP)

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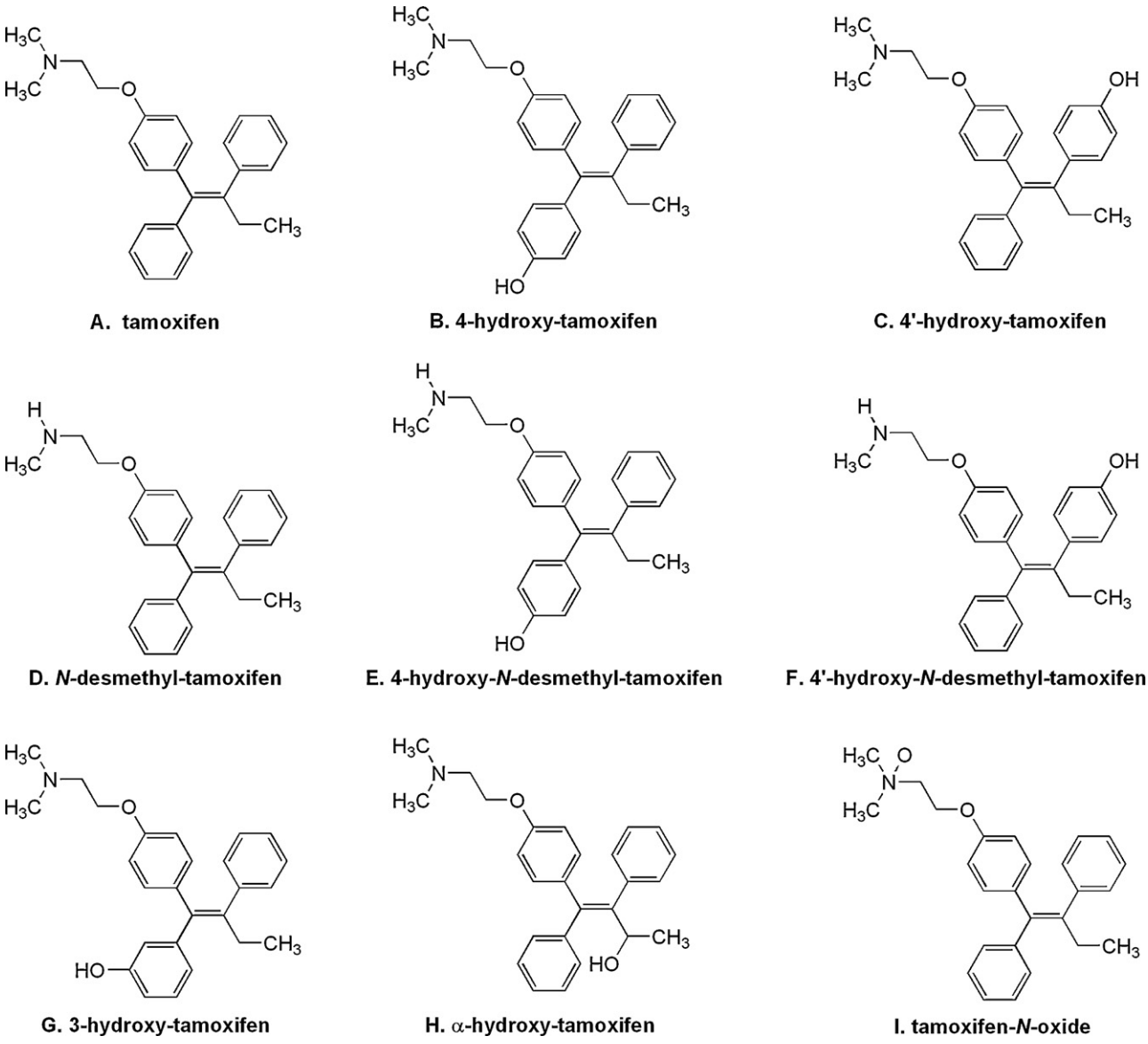

Fig. 1. Chemical structures of the tamoxifen and its three major metabolites studied: (A) tamoxifen; (B) 4-hydroxy-tamoxifen; (C) 4′-hydroxy-tamoxifen; (D) *N*-desmethyl-tamoxifen; (E) 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen); (F) 4′-hydroxy-*N*-desmethyl-tamoxifen; (G) 3-hydroxy-tamoxifen; (H) α-hydroxy-tamoxifen; (I) tamoxifen-*N*-oxide.

enzymes into its active metabolites 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethytamoxifen (endoxifen) [1]. Briefly, tamoxifen is primarily oxidized to *N*-desmethyl-tamoxifen (the most abundant metabolite in human plasma) and 4-hydroxy-tamoxifen predominantly by CYP3A4/5 and CYP2D6, respectively, followed by endoxifen formation from *N*-desmethyl-tamoxifen, exclusively catalyzed by CYP2D6 and from 4-hydroxy-tamoxifen by CYP3A4/5. Tamoxifen and its metabolites undergo further glucuronidation and sulphation [13,14].

Endoxifen is considered to be responsible for an important part of the *in vivo* pharmacological activity of tamoxifen, as endoxifen plasma concentrations are about 5–10-fold higher than those of 4-hydroxy-tamoxifen, with a different mode of action for endoxifen being suggested [8,10,15].

The clinical outcomes of tamoxifen treatment in terms of efficacy and side effects are inconstant, and some patients either fail to respond or become resistant to tamoxifen therapy [14,16,17]. One of the proposed mechanisms explaining the impaired response to tamoxifen therapy is an altered bio-activation into endoxifen by genetic or environmental factors. A polymorphism in CY2D6 enzymes that catalyze this conversion has been reported to influence the blood level of endoxifen [14,18–21] and, in some retrospective studies, to predict clinical outcomes in patients [14,21–25]. This has prompted the consideration of a potential role for CYP2D6 genotype/phenotype testing in patients' management, which remains controversial, however [26–34]. In fact, large inter-patient variability in endoxifen levels still subsists even after correcting for CYP2D6 status [18,27]. The remaining variability may depend on the activity of other cytochromes (CYP3A4/5, 2C9, 2C19), some of them known to be polymorphic, and on the influence of environmental factors such as interacting co-medications, among others. Of importance are some selective serotonin reuptake inhibitors (SSRIs) with strong CYP2D6 inhibiting activity, such as paroxetine and fluoxetine advised formerly to treat tamoxifen-induced hot flashes or depression are known to influence tamoxifen bioactivation [10,28,29].

The plasma concentration of the active metabolites of tamoxifen (mainly endoxifen and 4-hydroxy-tamoxifen) corresponding to the final phenotypic trait, may therefore represent a better predictor of tamoxifen efficacy than patients' CYP2D6 genotype.

However, whether the monitoring of endoxifen plasma concentrations in breast cancer patients would constitute a valid approach to optimize individual dosage remains to be demonstrated. In that context, several analytical methods have been published for the monitoring of tamoxifen and its metabolites in human biological fluids, including GC–MS [35], CE-MS [36], conventional and micellar liquid chromatography methods coupled to fluorescence detection [37–40] and LC–MS/MS methods [41–46]. Reports have also been published describing liquid chromatography method coupled to mass spectrometry or fluorescence detection for the study of tamoxifen metabolism in vitro and in vivo [47–54]. For mass spectrometry techniques, conventional HPLC [42,45,46] and fast liquid chromatography coupled to tandem MS methods using monolithic [41] or small particles (3 μm) packed columns [43,44] have been proposed for the quantification of tamoxifen and/or its metabolites. With the exception of the HPLC-MS/MS methods recently published [42,46], the potential impact of biological matrix effects variability on tamoxifen metabolites quantification was only scarcely addressed, as previous assays were using either no I.S. [45], or only a single labeled I.S. [41,43] as a surrogate I.S. for the quantification of tamoxifen and/or its metabolites.

Herein, we describe the development and validation of an UPLC–MS/MS method for the sensitive quantification in human plasma of tamoxifen, *N*-desmethyl-tamoxifen, and the active metabolites 4-hydroxy-tamoxifen and endoxifen within 13 min. The influence of matrix effects on tamoxifen and its metabolites quantification has been thoroughly investigated. The chromatographic profile of known (tamoxifen-*N*-oxide, α-hydroxy-tamoxifen) and previously unreported tamoxifen metabolites (4′-hydroxy-tamoxifen, 4′-hydroxy-*N*-desmethyl-tamoxifen, 3-hydroxy-tamoxifen) has also been studied in detail to exclude the risk of interferences during the comparatively short duration of the UPLC–MS/MS analysis.

2. Experimental

2.1. Chemicals and reagents

Tamoxifen (Tam) and *Z*-4-hydroxy-tamoxifen (4-OH-Tam) were purchased at Sigma–Aldrich (Schnelldorf, Germany). *N*-desmethyl-tamoxifen (*N*-D-Tam) hydrochloride, 4-hydroxy-*N*-desmethyl-tamoxifen 1:1 *E/Z* mixture (4-OH-*N*-D-Tam), 4′-hydroxy-tamoxifen (4′-OH-Tam), 4′-hydroxy-*N*-desmethyl-tamoxifen (4′-OH-*N*-D-Tam), α-hydroxy-tamoxifen (α-OH-Tam), 3-hydroxy-tamoxifen (3-OH-Tam), tamoxifen-*N*-oxide (Tam-*N*O), and the internal standards (I.S.): tamoxifen-ethyl-d5 (Tam-d5), *N*-desmethyl-tamoxifen-ethyl-d5 (*N*-D-Tam-d5), 4-hydroxy-tamoxifen-ethyl-d5 (4-OH-Tam-d5) and 4-hydroxy-*N*-desmethyl-tamoxifen-ethyl-d5 (endoxifen-d5), were purchased from Toronto Research Chemicals Inc. (North York, Canada).

Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (MeCN) purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate was purchased from Fluka (Buchs, Switzerland). Formic acid (98%) and methanol for chromatography Lichrosolv® (MeOH) were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Different sources of blank plasma used for the assessment of matrix effects and for the preparation of calibration and control samples were isolated (1850 g, 10 min, +4 °C, Beckman Centrifuge, Model J6B) from outdated blood donation units from the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland) or from citrated blood withdrawn from patients with Vaquez's Disease (polycythemia vera).

2.2. Equipment

The liquid chromatography system consisted of Rheos 2200 quaternary pumps, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS software (Flux Instruments, AG, Thermo Fischer Scientific Inc., Waltham, MA). Separations were done on a 2.1 mm × 30 mm Acquity UPLC® BEH C18 1.7 μm analytical column (Waters, Milford, MA, USA) placed in a thermostated column heater at 40 °C (Hot Dog 5090, Prolab, Switzerland). The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fisher Scientific, Inc. equipped with an Ion Max electrospray ionization (ESI) interface and operated with Xcalibur software package (Version 2.0.7, Thermo Fischer Scientific Inc., Waltham, MA).

2.3. Solutions

2.3.1. Mobile phase and extracts reconstitution solutions

The mobile phase used for chromatography was composed of 10 mM ammonium formate in ultrapure water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (FA). A solution of MeOH/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA, was used for the reconstitution of the extracted plasma samples prior to their analysis.

2.3.2. Working solutions, internal standard, calibration standards and quality controls (QCs) solutions

Stock solutions of deuterated internal standards (I.S.) (0.5 mg/mL in MeOH) were diluted with acetonitrile (ACN) to obtain a single working I.S. solution containing 25 ng/mL of tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and 50 ng/mL of endoxifen-d5 (1:1 *E/Z* mixture).

Standard stock solutions of tamoxifen base, *N*-desmethyl-tamoxifen hydrochloride, 4-hydroxy-tamoxifen base and endoxifen (1:1 *E/Z* mixture) base each at 1 mg/mL were prepared in MeOH and stored at −20 °C. Appropriate volumes of stock solutions were serially diluted with H_2O/MeOH (3:1) as indicated in Table 1 to obtain single working solutions of analytes at concentration ranging from to 0.008 to 20 μg/mL. These working solutions were diluted 1:20 with blank citrated plasma to obtain for tamoxifen/metabolites the calibration samples ranging from 0.4 to 1000 ng/mL and their corresponding three quality control (low (L), medium (M) and high (H) QCs) samples ranging from 1.2 to 750 ng/mL. All spiked plasma samples were prepared according to the recommendations for bioanalytical methods validation stating that total added volume must be ≤10% of the biological sample [55]. The calibration and control plasma samples were stored as 100 μL aliquots at −80 °C. Of note, the accuracy of calibration and QC samples is subsequently verified by comparison with another batch of calibration and QCs samples prepared with freshly made stock solutions (at the occasion of plasma calibration batch renewal). The response of both series (i.e. new and previous) of calibration samples are compared, and analytes' levels in the two series of QC samples calculated using the calibration curve established with both series of calibrations samples. Residuals for newly and previous calibration standards and quality controls have to meet the acceptance criteria for precision and accuracy.

2.4. LC–MS/MS conditions

The mobile phase was delivered using the stepwise gradient elution program reported in Table 2. The thermostated column heater was set at +40 °C and the autosampler was maintained at +4 °C. The injection volume was 10 μL.

Table 1
Preparation of working solutions.

Drug	Stock solution solvent	Stock solution concentration	Working solution concentration (obtained by dilution of stock solution with H_2O/MeOH 3:1)	Calibration range (obtained by dilution of working solution with plasma 1/20)	QCs controls
Tam	MeOH	1 mg/mL	0.02–10 μg/mL	1–500 ng/mL	3; 50; 375 ng/mL
4-OH-Tam	MeOH	1 mg/mL	0.008–4 μg/mL	0.4–200 ng/mL	1.2; 20; 150 ng/mL
N-D-Tam	MeOH	1 mg/mL	0.04–20 μg/mL	2–1000 ng/mL	6; 100; 750 ng/mL
E-endoxifen	MeOH	0.5 mg/mL	0.02–10 μg/mL	1–500 ng/mL	3; 50; 375 ng/mL
Z-endoxifen	MeOH	0.5 mg/mL	0.02–10 μg/mL	1–500 ng/mL	3; 50; 375 ng/mL

All stock solutions are mixed together to give single working solutions.

Table 2
Gradient elution program.

Time (min)	Buffer A (%)	Solvent B (%)	Flow rate (μL/min)
0.00	70.0	30.0	300
9.00	48.0	52.0	300
9.01	48.0	52.0	300
9.50	70.0	30.0	350
13.00	70.0	30.0	350

Buffer A: 10 mM NH_4 formate + 0.1% formic acid. Solvent B: acetonitrile + 0.1% formic acid. Temperature (°C): 25. Injection volume (μL): 10.

The MS conditions were as follows: ESI in positive mode, capillary temperature: 350 °C; in source collision induced dissociation): 4 V; tube lens voltages range: 122–126 V; spray voltage: 4 kV; sheath gas pressure: 60 psi and auxiliary gas (nitrogen) pressure: 10 (arbitrary units). The Q2 collision gas (argon) pressure was 1.5 mTorr (0.2 Pa); Q2 collision induced dissociation (CID): 10 V. MS is acquired in selected reaction monitoring (SRM). The optimal parameters and MS/MS transitions were determined by direct infusion of tamoxifen, its metabolites and I.S. solutions separately into the MS/MS detector at a concentration of 1 μg/mL in MeOH/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA. The selected *m/z* transitions and the collision energy for each analyte and I.S. are reported in Table 3.

The first (Q1) and third (Q3) quadrupoles were set at 2.8 amu mass resolution (Full-Width Half-Maximum = 2 Da). Scan time and scan width were 0.02 s and 0.5 *m/z*, respectively. MS acquisitions were done in centroid mode. Two segments of data acquisition were programmed in the positive mode: the first acquisition segment from 0 to 6 min, and the second one from 6 to 12 min.

Chromatographic data acquisition, peak integration and quantification were performed using the QUAL and QUAN browser of Xcalibur software package (version 2.0.7, ThermoQuest, Thermo Fischer Scientific Inc., Waltham, MA).

2.5. Clinical blood samples collection

Blood samples were obtained from consenting breast cancer patients enrolled in the study protocol "Tamoxifen metabolism and the impact of tamoxifen dose on the level of the active metabolites in endocrine sensitive breast cancer patients" (ClinicalTrials.gov Identifier: NCT00963209), approved by the Ethics Committee of the University Hospital. Written informed consent was obtained from all patients. Blood samples (5.5 mL) from breast cancer patients treated with tamoxifen were collected at random time after last drug intake in Monovettes® (Sarstedt, Nümbrecht, Germany) containing K-EDTA as anticoagulant. According to study protocol, blood samples were collected in patients receiving 20 mg tamoxifen once daily, at two occasions at baseline (e.g. on day 0 and day 1, i.e. after inclusion and before dose escalation), and after 1, 3 and 4 months of continuous treatment at a regimen of 20 mg tamoxifen twice daily (BID).

2.6. Plasma sample extraction procedure

A 100 μL aliquot of plasma was mixed with 100 μL of I.S. solution (25 ng/mL of tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5, and 50 ng/mL of endoxifen-d5 1:1 *E/Z* mixture, in ACN) and with acetonitrile (300 μL), carefully vortex-mixed and sonificated for 30 s. (Branson Ultrasonics Corporation, Danbury, CT, USA). The mixture was centrifuged at 4 °C for 10 min at 16,000 × *g* (12,000 rpm) on a benchtop Hettich® Centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). A 400 μL aliquot of the supernatant was transferred into a polypropylene tube and evaporated to dryness under nitrogen at room temperature. Of note, SpeedVac® concentrator may also be used, presenting the advantage of organic solvent recuperation. The solid residue was reconstituted in 600 μL of a solution of MeOH/20 mM ammonium formate 1:1 (v/v), adjusted to pH 2.9 with FA, vortex-mixed and centrifuged again under the above-mentioned conditions. A 400 μL of the supernatant was introduced into 1.5 mL glass HPLC microvials maintained at +4 °C in the autosampler rack during the entire LC–MS/MS analysis.

Table 3
Instrument method for the LC–MS/MS analysis of tamoxifen/metabolites with deuterated analogs as internal standards.

Drug	Parent (*m/z*)	Product (*m/z*)	CE (eV)	Tube lens (V)	Mean RT (min)	Polarity mode
Tamoxifen (Tam)	372.3	72.10	23	122	7.7	Positive
N-desmethyl-tamoxifen (*N*-D-Tam)	358.3	58.10	21	122	7.4	Positive
Z-4-hydroxy-tamoxifen (4-OH-Tam)	388.3	70.10	38	126	4.3	Positive
		72.10	25	126		Positive
		129.10	28	126		Positive
Endoxifen (1:1 *E/Z* mixture)	374.3	58.10	22	122	4.0	Positive
		129.10	28	122		
		223.10	20	122		
Tamoxifen-d5 (Tam-d5)	377.3	72.10	24	122	7.7	Positive
N-desmethyl-tamoxifen (*N*-D-Tam-d5)	363.3	58.10	21	122	7.4	Positive
4-Hydroxy-tamoxifen (4-OH-Tam-d5)	393.3	72.10	25	126	4.3	Positive
Endoxifen-d5 (1:1 *E/Z* mixture)	379.3	58.10	22	122	4.0	Positive

CE, collision energy; RT, retention time; MS acquisition time (min) = 12.00. Q2 Collision gas pressure (mTorr) = 1.5.

2.7. Calibration curves

Quantitative analysis of tamoxifen and its three main metabolites (*N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and *Z*-endoxifen) in plasma was performed using the internal standard method. Deuterated compounds of each target analyte were used as I.S. Each level of the calibration curve was measured with two sets of calibrators: the first at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared in citrated plasma.

Calibration standard curves have been calculated and fitted by quadratic log–log regression [56] of the peak-area ratio of tamoxifen and its metabolites to its respective I.S., versus the nominal concentrations of each analyte in each standard sample. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. The seven-point calibration curves for tamoxifen and its three metabolites were established over the range reported in Table 1. The ranges of calibration were selected to cover the range of concentrations expected in patients according to previously published studies [18–20,42].

2.8. Analytical method validation

The method validation was based on the recommendations published on-line by the Food and Drugs Administration (FDA) [55] as well as on the recommendations of the Workshop/Conference Report "Quantitative Bioanalytical Methods Validation and implementation: Best Practice for Chromatographic and Ligand Bindings Assays" [57] and the Arlington Workshop "Bioanalytical Methods Validation – A Revisit with a Decade of Progress" [58]. Recommendations from Matuszewski to assess matrix effects were also considered [59,60].

2.8.1. Selectivity

The assay selectivity was assessed by analysing plasma extracts from ten batches of blank plasma from different sources.

2.8.2. Accuracy and precision

The concentrations for the quality control (QC) samples were selected to encompass the whole range of the calibration curve corresponding to the drug levels anticipated to occur in most patient samples: low (L), medium (M) and high (H). The concentration selected for the low QC sample corresponds to 3 times the respective lower limit of quantification (i.e. the lowest calibration level) kept in the finalized method, in accordance to the FDA recommendations [55]. Replicate analysis ($n = 6$) of three QC samples was used for the intra-assay precision and accuracy determination. Inter-assay accuracy and precision were determined by duplicate analysis of the three QC repeated on six different days. The precision was calculated as the coefficient of variation (CV %) and the accuracy was calculated as the bias or percentage of deviation between the nominal and measured concentrations.

After the completion of the above validation procedure, for the routine analysis of patient samples, duplicate QC samples at the three concentration levels (L, M and H) were used.

2.8.3. Matrix effects, extraction yield and overall recovery

In the initial step of method validation, matrix effects were examined qualitatively by the simultaneous post-column infusion of tamoxifen/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of 6 different blank plasma extracts. The standard solution of all analytes and their corresponding deuterated I.S. at 5 μg/mL was infused at a flow-rate of 20 μL/min during the chromatographic analysis of blank plasma extracts. The chromatographic signals in each selected MS/MS transition were examined to check for any signal perturbation (drift or shift) at the analytes' retention time (data not shown).

Subsequently, the matrix effects were also quantitatively assessed. Three series of QC samples at L, M and H concentrations were processed as follows:

(A) Pure stock solutions dissolved in the reconstitution solvent (MeOH-buffer (ammonium formate 20 mM, pH adjusted to 2.9 with FA) 1:1) and directly injected onto column.
(B) Plasma extracts samples from 6 different sources, spiked after extraction with tamoxifen/metabolites and I.S. (from pure stock solutions in the reconstitution solvent).
(C) Plasma samples from 6 different sources (same as in B) spiked with tamoxifen/metabolites standard solutions and I.S. before extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of plasma matrix (i.e. matrix effects) was assessed by comparing the absolute peak areas of the analytes either dissolved in the reconstitution solvent: MeOH-buffer 1:1 (A), or spiked after plasma extraction (B) or spiked before plasma extraction (C), using 6 different batches of plasma, based on the recommendations proposed by Matuszewski et al. [59,60].

The *extraction yield* of tamoxifen/metabolites and I.S. was calculated as the absolute peak-area response in processed plasma samples spiked with the standard analytes *before* extraction (C) expressed as the percentage of the response of the same amount of analytes spiked into blank plasma *after* the extraction procedure (B) (C/B ratio in %). The *matrix effect* was assessed as the ratio of the peak areas of the analytes spiked into blank plasma *after* the extraction procedure (B) to the peak areas of the analytes solubilised in MeOH-buffer 1:1 (A) (B/A ratio in %). The *overall recovery* of tamoxifen/metabolites and I.S. was calculated as the ratio of absolute peak-area responses of tamoxifen/metabolites spiked in processed plasma samples *before* extraction (C) to the peak areas of the analytes solubilised in MeOH-buffer 1:1 (A) (C/A ratio %). Recovery studies were performed with plasma from 6 different sources spiked with tamoxifen, its metabolites and their respective I.S. at the concentrations reported in Table 4. The results normalized with the signal of I.S. (i.e. B2 and C2), used as an index of the effective injection volume, are also reported in Table 4.

2.8.4. Carry-over

Memory effect has been investigated by the injection during an analytical run of 2 or 3 blank plasma after the highest calibration standard. Peak area response in the blank plasma sample, at each expected retention time, was compared to the peak area of the corresponding analyte at the lowest limit of quantification (LLOQ).

2.8.5. Dilution effect

During the course of patients' samples analyses, one patient sample was found to have tamoxifen concentration exceeding the highest level of the calibration curve (see Table 1). To ascertain whether the dilution of this sample could affect the accuracy of the drug or its metabolites determination, a blank plasma sample was spiked with pure standards (tamoxifen/metabolites) at a concentration exceeding by two-fold the highest calibration level. The sample was thereafter analysed in duplicate after a three, four, five and six fold dilution to bring the concentration within the calibration range. Dilution was carried out with blank plasma. Calculated and expected concentrations were compared.

Table 4
Matrix effects, extraction yield, overall recovery and process efficiency of tamoxifen/metabolites.

Component	Nominal conc. (ng/mL)	Mean peak area			Mean peak area ratio		ME (%)	CV (%)	extRE (%)	CV (%)	Analysis RE (%)	Mean	CV (%)	PE (%)	CV (%)
		A ($n=6$)	B ($n=6$)	C ($n=6$)	B2	C2	B/A		C/B		C2/B2			C/A	
Tam	3	1,263,441	1,255,380	1,372,710	0.072	0.076	99.4	3.7	109.3	4.3	105.0	96.4	8.1	108.6	1.6
	50	28,878,341	27,938,705	27,393,059	1.606	1.513	96.7	2.0	98.0	4.0	94.2			94.9	3.5
	375	228,978,707	226,034,897	21,1505,317	12.997	11.683	98.7	1.0	93.6	2.3	89.9			92.4	1.8
N-D-Tam	6	804,396	497,408	613,605	0.243	0.304	61.8	18.0	123.4	6.9	124.8	109.9	11.9	76.3	14.2
	100	17,796,321	10,574,145	10,929,594	5.169	5.406	59.4	21.0	103.4	8.9	104.6			61.4	18.0
	750	125,333,845	80,869,169	80,226,329	39.529	39.685	64.5	18.4	99.2	6.7	100.4			64.0	16.2
4-OH-Tam	1.2	537,944	545,444	559,305	0.062	0.067	101.4	2.9	102.5	5.0	107.8	104.1	3.3	104.0	3.5
	20	10,730,921	10,607,311	10,417,567	1.202	1.241	98.8	1.5	98.2	3.0	103.3			97.1	3.4
	150	79,332,011	79,252,260	76,170,959	8.980	9.076	99.9	1.9	96.1	2.1	101.1			96.0	1.1
Z-endoxifen	3	227,307	235,540	230,538	0.059	0.063	103.6	5.2	97.9	8.1	106.5	105.2	1.2	101.4	4.7
	50	4,467,005	4,597,862	4,431,075	1.149	1.205	102.9	0.9	96.4	4.6	104.9			99.2	4.8
	375	32,717,609	33,369,469	31,924,813	8.339	8.682	102.0	1.5	95.7	2.5	104.1			97.6	1.1
E-endoxifen	3	154,699	162,010	160,857	0.055	0.059	104.7	5.7	99.3	2.8	106.6	103.6	2.5	104.0	3.7
	50	3,048,595	3,146,053	2,988,009	1.074	1.095	103.2	2.3	95.0	3.1	102.0			98.0	4.1
	375	22,805,748	23,258,002	22,122,028	7.939	8.109	102.0	1.5	95.1	0.9	102.1			97.0	1.3
Tam-d5	25	17,793,384	17,391,055	18,104,182			96.1	2.8	104.1	1.7				101.7	2.8
N-D-Tam-d5	25	3,404,892	1,959,036	1,937,111			57.5	17.3	98.9	6.8				56.9	15.5
4-OH-Tam-d5	25	8,825,185	8,825,420	8,392,730			100.0	3.0	95.1	3.1				95.1	2.4
Z-endoxifen-d5	25	3,796,772	4,001,590	3,677,241			105.4	2.7	91.9	3.1				96.9	3.8
E-endoxifen-d5	25	2,881,493	2,929,682	2,728,140			101.7	2.0	93.1	2.6				94.7	3.9

A, peak area of standard solutions without matrix and without extraction (MeOH/buffer A 1:1); B, peak area of analytes spiked after extraction; C, peak area of analytes spiked before extraction; B2, ratio of the peak area of the analyte and the I.S. spiked after extraction; C2, ratio of the peak area of the analyte and the I.S. spiked before extraction; ME, matrix effect expressed as the ratio of the mean peak area of the analytes spiked after extraction (*B*) to the mean peak area of the same standard solution without matrix (*A*) multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression; ext RE, extraction procedure recovery calculated as the ratio of the mean peak area of the analytes spiked before extraction (*C*) to the mean peak area of the analytes spiked after extraction (*B*) multiplied by 100; Analysis RE, analysis recovery calculated as the ratio of the mean peak-area ratio of the analytes spiked before extraction (C2) to the mean peak-area ratio of the analytes spiked after extraction (B2) multiplied by 100; PE, process efficiency expressed as the ratio of the mean peak area of the analyte spiked before extraction (*C*) to the mean area of the same analyte standards (*A*) multiplied by 100.

2.8.6. Stability of tamoxifen and its metabolites

Stability studies of tamoxifen and its three metabolites at different storage conditions included:

(a) Stability in plasma spiked with tamoxifen/metabolites (i.e. QCs at L, M and H concentrations) over time at room temperature (RT) and at +4 °C up to 48 h. Variations of tamoxifen/metabolite concentrations were expressed as percentages of the initial concentration measured immediately after preparation, i.e. T_0. Analyses were performed in triplicate at T_0 and at each subsequent time point.
(b) Stability of tamoxifen/metabolites in whole blood at +4 °C and at RT assessed by calculating the percent deviation of the I.S. normalized peak area of each analyte in the collected plasma from the initial peak area ratio measured at T_0. Two batches of whole blood samples spiked with analytes at the L, M and H levels (1 ml final volume) were prepared in triplicate and kept for 0, 1, 2, 4, 8, 24 and 48 h before plasma separation at +4 °C and at RT. All plasma samples collected from centrifuged blood aliquots were stored at −80 °C and subsequently analysed in the same analytical sequence.
(c) Stability in plasma samples after multiple freeze-thaw cycles: plasma QCs at low, medium and high levels of tamoxifen/metabolites underwent three freeze-thaw cycles. Frozen samples were allowed to thaw at RT for 2 h and were subsequently refrozen at −80 °C during approximately 24 h. Tamoxifen/metabolites levels were measured in aliquots from the three consecutive freeze–thaw cycles.
(d) Stability in plasma samples kept frozen at -80 °C: QCs samples at the L, M and H concentrations were stored at–80 °C during 4 months and measured using fresh plasma calibration samples.

2.8.7. Identification of other tamoxifen metabolites

Next to tamoxifen, *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen analysis, additional phase I tamoxifen metabolites were identified in patients samples by comparison of the retention times and product-ion mass spectra of authentic standard compounds spiked into blank plasma, or added to patients' plasma samples. The full-scan mass spectra were acquired over a scan range of 40–400 *m*/*z* at scanning speed of 0.08 s/scan.

In the present analytical work, the concentrations of the newly identified metabolites 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen have also been estimated using 4-OH-Tam-d5 and endoxifen-d5 as I.S. in a separate series of analysis of 20 patients' samples.

3. Results and discussion

3.1. Chromatograms

The proposed ultra performance-liquid chromatography coupled with tandem MS method enables the simultaneous quantification within 13 min of tamoxifen and three metabolites: *N*-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and *Z*-endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen), in 100 μL plasma aliquots. A chromatographic profile of the highest calibration plasma sample containing tamoxifen/metabolites is shown in Fig. 2 in the positive ionization mode, during the two acquisition segments (0–6 and 6–12 min), using the selected reaction monitoring (SRM) detection mode; the proposed gradient program is described in Table 2. Tamoxifen and its metabolites were eluted in less than 9 min, followed by approx. 4 min of column re-conditioning step with 70% of buffer A (ammonium formate 10 mM + 0.1% FA) and 30% of solvent B (acetonitrile + 0.1% FA) at a flow rate of 0.35 mL/min (Table 2). The respective retention times and mass spectrometry conditions for tamoxifen/metabolites and their corresponding stable isotope labeled I.S. are reported in Table 3. Three *m*/*z* transitions were selected for 4-hydroxy-tamoxifen (*m*/*z* 388) with product ions at *m*/*z* 70, 72 and 129, and for endoxifen (*m*/*z* 374) with product ions at *m*/*z* 58, 129 and 223, in order to increase the detection sensitivity for these metabolites. The fragment ions at *m*/*z* 72 and 58 are the major signals visible on the product ion spectrum of 4-hydroxy-tamoxifen and endoxifen, respectively.

A satisfactory separation was achieved for all considered analytes, especially for (*E*-) and (*Z*-) isomers of endoxifen and endoxifen-d5 obtained as a racemic mixture (Fig. 2). Data from blank plasma samples spiked with the deuterated I.S., obtained throughout the method validation procedure and during patients' plasma samples analyses, revealed no interfering "cross-talk" signals arising from the isotopically-labeled I.S. on the transition of the corresponding target analyte, thus testifying the isotopic purity of these isotope labeled I.S.

Moreover, the proposed UPLC method provides an excellent chromatographic separation of tamoxifen-*N*-oxide from tamoxifen, preventing therefore analytical bias due to potential *in-source* dissociation of tamoxifen-*N*-oxide into tamoxifen that would give rise to spuriously elevated levels of tamoxifen. Of note, it was rather unexpected that tamoxifen-*N*-oxide, intuitively more polar, elutes later than tamoxifen on a reverse phase column, in line with previous reports [41,42,49]. Alterations of intra- or inter-molecular bindings, or pH-dependent changes in molecular lipophilicity, (i.e. Log D) [50,52] might be involved.

Fig. 3a shows the chromatographic profile of a plasma sample collected from a hormone sensitive breast cancer patient having received tamoxifen for 1 month at a regimen of 20 mg twice a day. The plasma levels of tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen measured 7.5 h after last drug intake were 666.6, 929.4, 15.2 and 217.9 ng/mL respectively). As reported in the literature, only the (*Z*) isomers of 4-OH-Tam and endoxifen were observed in plasma, thus excluding any *E*-*Z* interconversion of tamoxifen metabolites during sample preparation [42,44,61,62].

Fig. 3b shows the chromatographic profile of a plasma obtained from a hormone sensitive breast cancer patient receiving tamoxifen for 1.5-year at the standard regimen of 20 mg once daily. The plasma levels of tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen and *Z*-endoxifen measured 13.25 h after last drug intake were 207.6, 445.2, 1.4 and 6.2 ng/mL, respectively).

3.2. Method validation

3.2.1. Selectivity

No peaks from endogenous compounds were observed at the drugs retention time in any of the blank plasma extracts. The product ion monitoring was selected, based on its relative abundance, while avoiding possible structural analogies with the other analysed drugs or metabolites. All channels were simultaneously observed, and no selectivity issue as well as no crosstalk were detected across the acquisition channels.

3.2.2. Internal standard and calibration curve

The use of stable isotope-labeled internal standards is considered to be the best approach to minimize the influence of matrix effects on the accuracy and precision of a quantitative method, of particular importance when using electrospray mass spectrometry [59,60,63].

Therefore, deuterated analogs of tamoxifen and the metabolites to be quantified, have been used throughout our analytical method validation procedure (i.e. tamoxifen-d5, *N*-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and endoxifen-d5 1:1 *E*/*Z* mixture). No problems regarding the isotopic purity, *E* to *Z* interconversion,

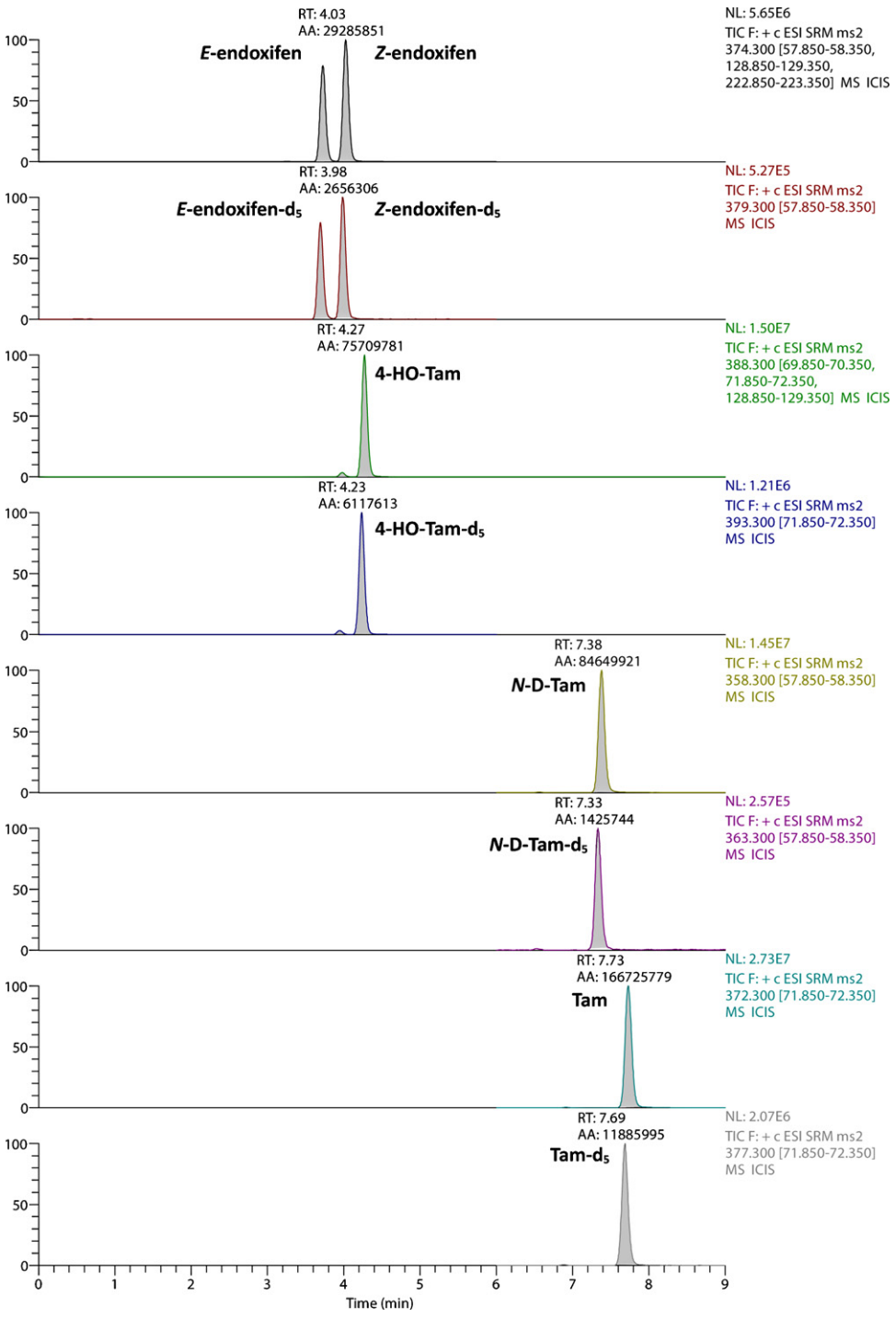

Fig. 2. Chromatogram of the highest calibration sample containing tamoxifen and its three major metabolites. Corresponding deuterated analogs are used as internal standards (details in the text).

and chemical stability of the I.S. (either in stock solution or in biological fluid and during sample processing), neither any "cross-talk" between MS/MS channels used for monitoring tamoxifen and the considered metabolites and the I.S. were identified throughout method validation procedure.

Calibration curves over the entire ranges of concentrations delineated in Table 1 were satisfactorily described by quadratic log-log regression of the peak-area ratio of tamoxifen and its metabolites to their I.S., versus the concentrations of the respective analytes in each standard sample. This model of calibration described by Singtoroj et al. [56] was found well suited to best fit the criteria of homoscedasticity (homogeneity of variance over the entire calibration range) and minimum bias for each single calibrator. The determination coefficients (R^2) of all calibration curves were higher than 0.999 with back-calculated concentrations of the calibration samples within ±15% of nominal values (±20% at LLOQ).

There was originally some concern that the calibration samples prepared with citrated plasma collected from blood from outdated transfusion bags or from Vaquez patients may not fully reflect the plasma matrix from patients collected on EDTA. However, getting blood on EDTA from volunteers solely for the purpose of calibra-

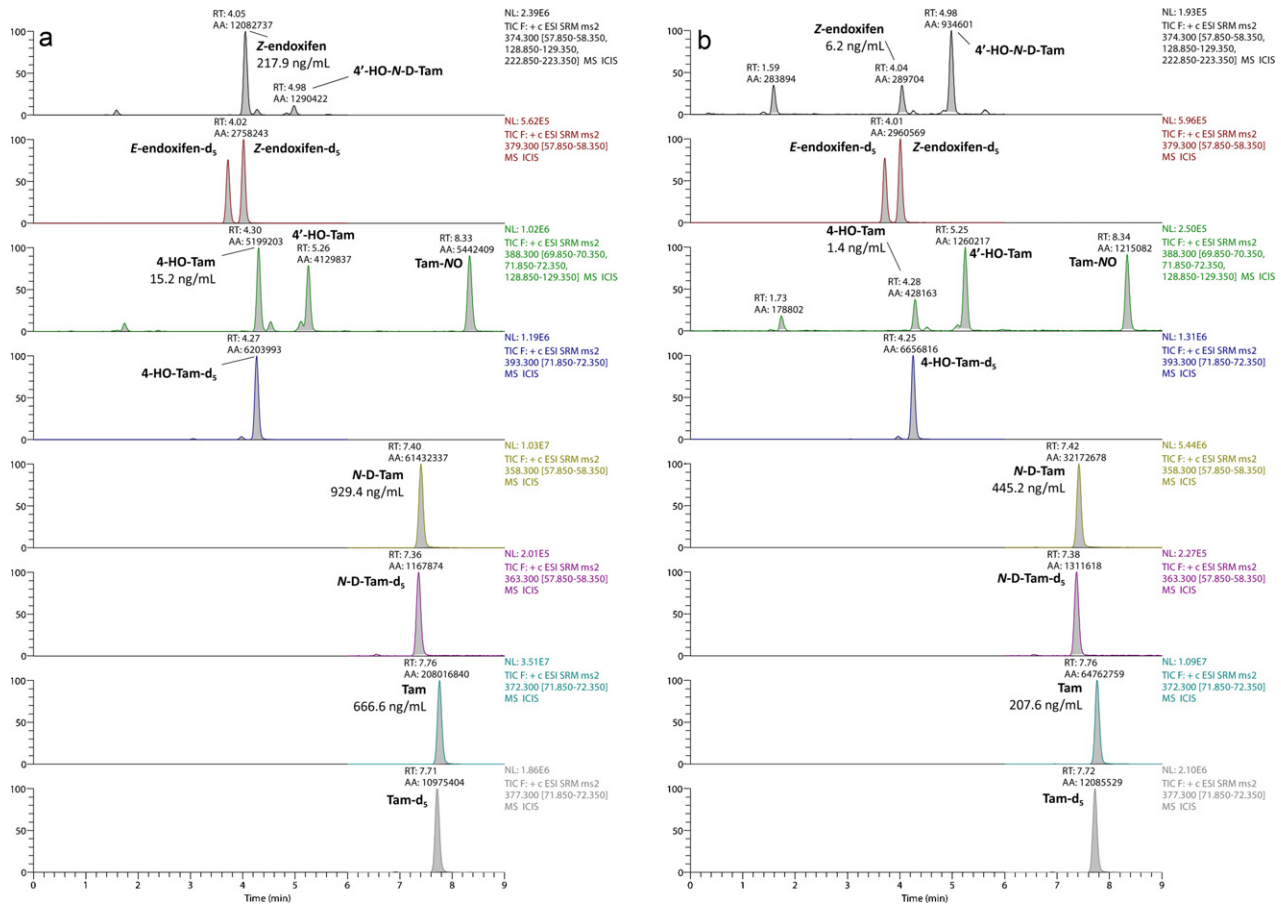

Fig. 3. Chromatographic profiles of plasma samples from two patients (a and b) receiving tamoxifen (details in the text).

tion samples preparation would be unpractical and difficult to justify from an ethical point of view. For the sake of validation, a cross-validation was performed by replicate analysis ($n = 3$) of QC samples at the three levels, prepared both in citrated and in EDTA plasma. The QC samples were assayed using the calibration curve established with citrated plasma samples. Head-to-head comparison shows that the anticoagulant does not influence significantly the analytical results for tamoxifen and its metabolites. No statistically significant differences ($p > 0.05$) in concentrations were found for QCs samples prepared in EDTA and citrated plasma using calibration curves established with citrated plasma (p values comprised within 0.07–0.92 for tamoxifen, *N*-desmethyl-tamoxifen, *Z*-4-hydroxy-tamoxifen, *Z*-endoxifen and *E*-endoxifen (Student *t*-test).

3.2.3. Precision, accuracy, and LLOQ

Precision and accuracy determined with the L, M and H QC samples are summarized in Table A.1 (on-line supplementary data). The mean intra-assay precision was similar over the entire concentration range and always less than 6.8%. Overall, the mean inter-day precision was within 2.5 and 7.8%. The intra-assay and inter-assay deviation (bias) from the nominal concentrations of QCs ranged between −5.3 and +7.4%, and −1.4 and +5.8%, respectively.

Of note, the chosen ranges of calibration were selected initially to cover the clinical range of tamoxifen/metabolites concentrations previously reported in the literature [18–20,42]. In fact, we observed during the method's validation that the responses attained at the LLOQs levels would be sufficient so that it may be possible to validate this method at even lower levels (ca. 0.1–0.75 ng/mL) if desired in the future.

3.2.4. Matrix effects and recovery

Matrix effects were examined qualitatively by the simultaneous post-column infusion of tamoxifen/metabolites and I.S. into the MS/MS detector during the chromatographic analysis of six different batches of blank plasma. During the chromatography of blank matrices, the signals at all the *m/z* transitions selected showed a remarkably similar pattern, with all traces being essentially superimposable. No noticeable matrix effects (no drifts or shifts of the signals) were observed at the respective retention time of tamoxifen and its metabolites and their deuterated I.S. (data not shown).

The inter-subject variations in suppression/enhancement profiles have also been studied quantitatively (Table 4). The results reported in Table 4 (column B/A) indicate that co-eluting plasma matrix components appear to have a minimal effect on the considered analytes, except for *N*-D-Tam whose signal was approximately halved (mean ratio B/A = 62%). As expected, a similar extent of ion suppression was observed with *N*-D-Tam labeled internal standard (*N*-D-Tam-d5) (B/A ratio = 57.5%). Thus overall, the mean B/A ratios for *N*-D-Tam when normalized with those of deuterated I.S. was 1.1 (i.e. at or slightly above unity), demonstrating the value of stable isotope-labeled I.S. use for an efficient control of the relative matrix effect [64]. Plasma matrix does not appear to significantly interfere with Tam, 4-OH-Tam and both endoxifen isomers ionisation (B/A ratio ranged between 96.7 and 104.7%).

Using the proposed protein precipitation, supernatant evaporation and dissolution in appropriate buffer, our plasma extraction procedure provided a good extraction recovery (C/B, column extRE) always higher than 95%, resulting in an excellent sensitivity.

As indicated in Table 4, the *analytical recovery values* were always higher than 89.9%. The *process efficiency* (i.e. overall recovery) was comprised within 92.4-108.6% except for *N*-D-Tam, which

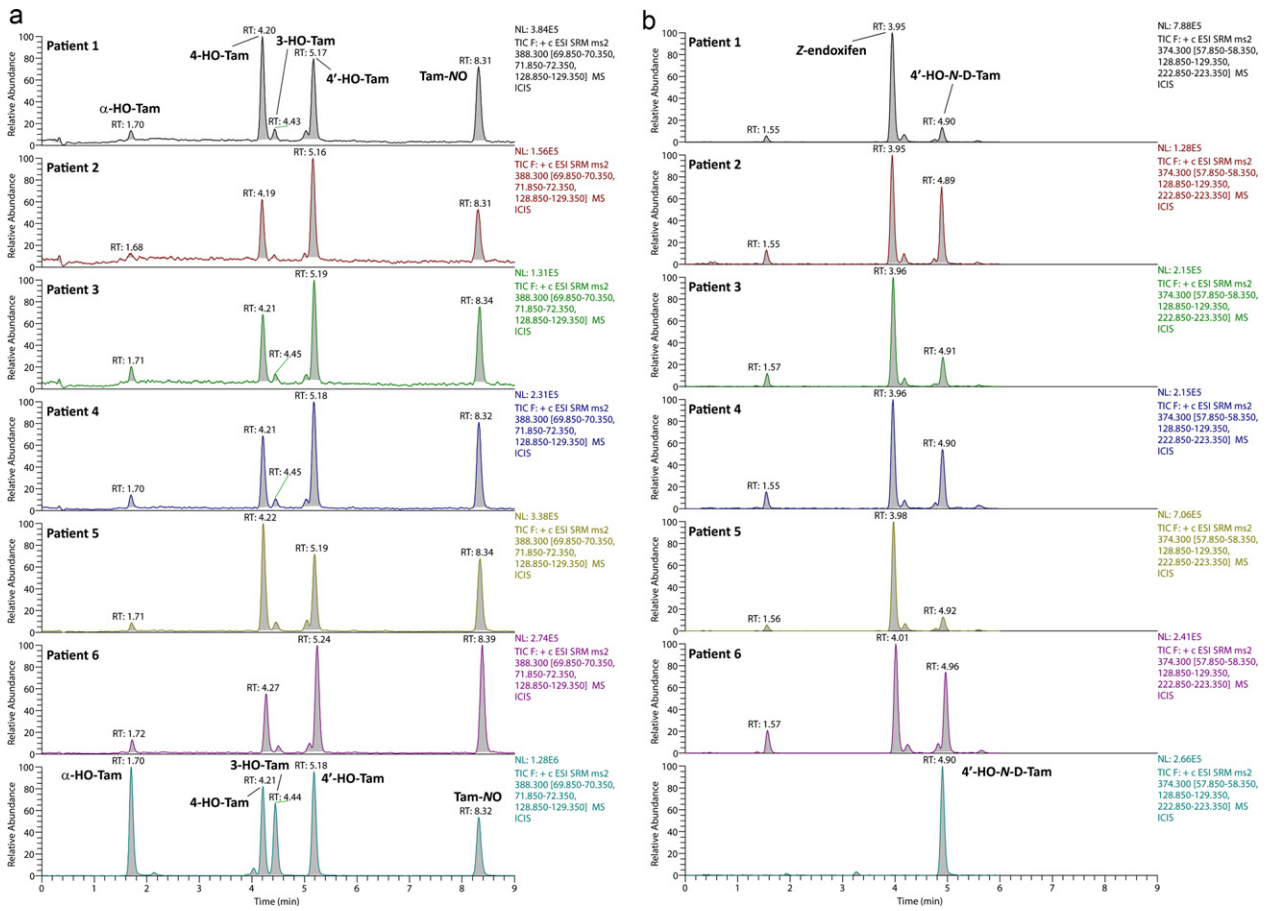

Fig. 4. Chromatographic profiles (a) at SRM transition (m/z 388 → 70, 72, 129) and (b) at SRM transition (m/z 374 → 58, 129, 223) in plasma from 6 unselected patients receiving tamoxifen. Last chromatograms (bottom traces) are blank plasma spiked with: (a) pure standards of α-hydroxy-tamoxifen, 4-hydroxy-tamoxifen, 3-hydroxy-tamoxifen, 4′-hydroxy-tamoxifen and tamoxifen-*N*-oxide and (b) pure standard of 4′-hydroxy-*N*-desmethyl-tamoxifen (details in the text).

gives a process efficiency around 67%. As reported above, matrix components do influence to some extent *N*-D-Tam ionisation and consequently the overall process efficiency, requiring therefore the preparation of calibration and control samples in a plasma matrix reflecting at best the composition of the samples to be analysed. Most importantly, this is not so much the absolute matrix effect, but rather its variability (relative matrix effect) that must be reduced. As shown in Table 4, the variability of the matrix effect in 6 different plasma matrix were close to 20% for *N*-D-Tam at all QCs and never exceeded 5.7% for all other analytes, which indeed demonstrates that the proposed extraction procedure is able at least to normalize these matrix effects, even in the absence of the correcting effect of labeled I.S. In fact, the use of isotope-labelled internal standards in our method seems to effectively control most of the residual relative matrix effect variability. This has been experimentally verified notably for *N*-D-Tam for which the observed matrix effect variability in 6 plasma lots never exceeded 4% when *N*-D-Tam peak areas where normalized to those from its deuterated I.S. (N-D-Tam-d5).

3.2.5. Memory effect

No major carry-over was observed with our method. The highest memory effect was observed for tamoxifen, the most lipophilic analyte. This carry-over effect was successfully eliminated by programming the injection of three blank samples after the highest calibration standard, prior to the analysis of patients' samples. The peak intensity visible in the third blank matrix sample corresponds to less than 20% of that of the LLOQ sample. In fact, during routine plasma analysis, it has prudently been decided to program a single blank plasma injection after each patient's sample which was found sufficient to reduce the memory effect to an extent unlikely to affect the accuracy of tamoxifen and its metabolites measurements in the following patients' plasma samples.

3.2.6. Dilution effect

After the three, four, five and six-fold dilutions of the spiked plasma with tamoxifen/metabolites at a concentration exceeding by two-fold the high calibration level, the deviation (bias) from the expected concentrations of all compounds was less than 8.2%. This indicates that plasma samples containing tamoxifen/metabolites above the highest level of calibration can be adequately diluted with blank plasma prior to the LC–MS/MS analysis, to bring down concentration within the calibration range.

3.2.7. Stability of tamoxifen/metabolites in plasma and whole blood

(a) The stability of tamoxifen/metabolites in human plasma samples was ascertained with QC samples left at room temperature (RT) and at +4 °C up to 48 h. The variation over time of the concentrations of tamoxifen and its metabolites in plasma remained comprised within ±15% of initial (T_0) concentrations (see Table A.2 in on-line supplementary data), indicating that tamoxifen and its metabolites are stable in plasma at RT and at +4 °C.

(b) During the clinical study, which prompted this analytical development, some blood samples had to be stored temporarily at +4 °C before being shipped to our laboratory and centrifuged for plasma collection. Given the absence of information on the stability of tamoxifen and its principal metabolites in blood, we

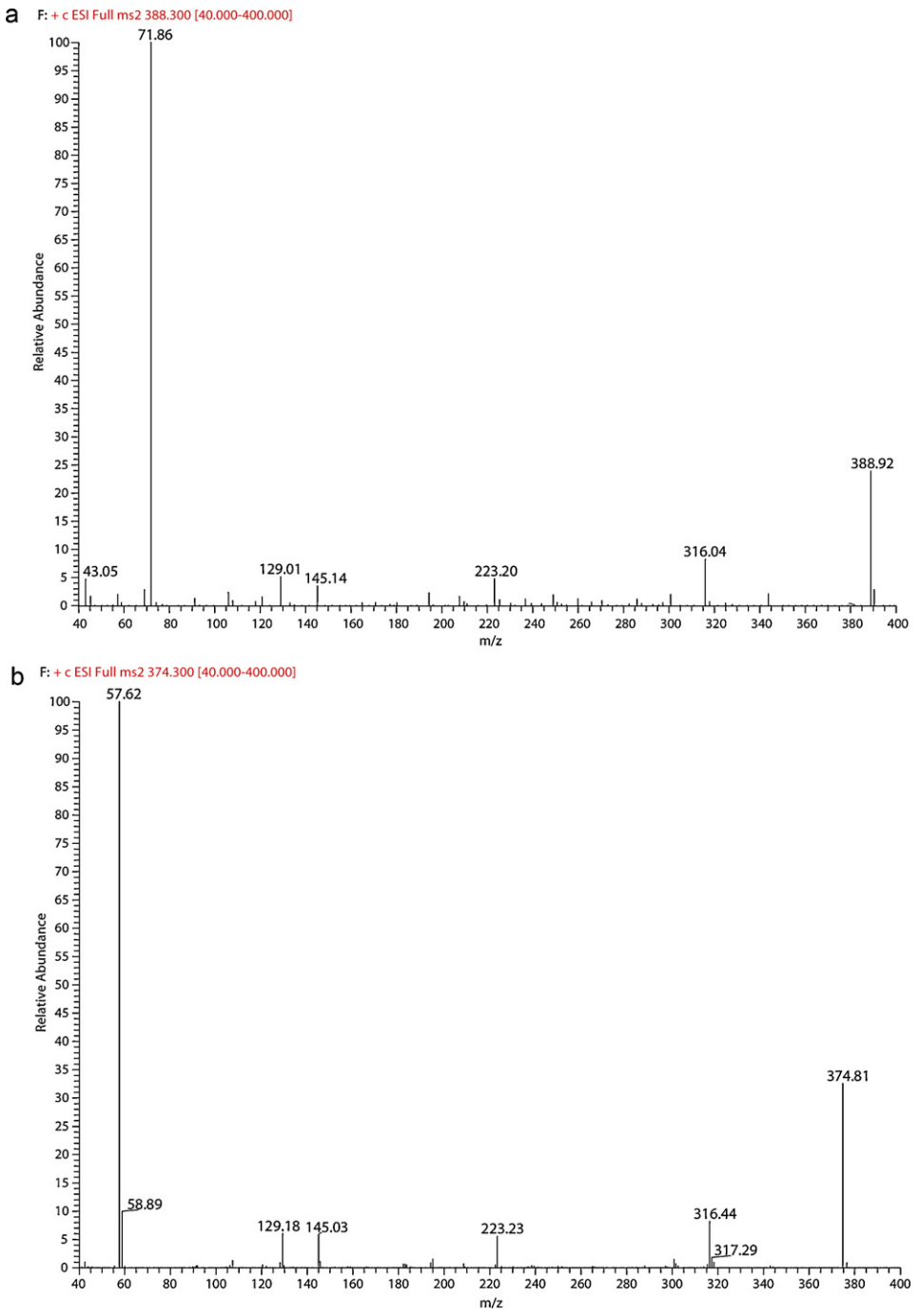

Fig. 5. Product ion spectra of the pure standards (a) 4′-hydroxy-tamoxifen and (b) 4′-hydroxy-*N*-desmethyl-tamoxifen spiked into blank plasma.

have studied the evolution of their concentrations over time in whole blood. The results of stability studies in whole blood are summarized in Table A.3 (on-line supplementary data), indicating that tamoxifen and its metabolites can reliably be considered as stable in whole blood, up to 8 h storage either at +4 °C or at RT.

(c) Variations of tamoxifen/metabolites concentrations were always less than −15% from nominal levels after three freeze-thaw cycles (Table A.2, in on-line supplementary data), indicating no significant loss of drug upon this procedure.

(d) QCs samples prepared in batches, distributed as 100 μL aliquots and stored at −80 °C in 1.5 ml Eppendorf vials were analysed 4 months later. All QCs (L, M and H) were analysed in duplicate. Variations of tamoxifen/metabolites concentrations were less than -11.9% from their nominal concentrations, indicating the long term stability of tamoxifen and its metabolites in plasma samples stored at −80 °C.

3.3. Metabolites profiles studies and metabolites identification

Given the reduced elution time of analytes with UPLC, it was critical for this analytical development to verify that tamoxifen metabolites would not potentially perturb the quantification. The chromatographic elution pattern of reported or putative tamoxifen metabolites was therefore studied thoroughly.

Three additional peaks were observed in patients samples at 1.7, 5.2 and 8.3 min on the SRM transition (*m/z* 388 → 70, 72, 129) selected for 4-hydroxy-tamoxifen (itself eluted at 4.2 min)

(Fig. 3b, third chromatogram from top, and Fig. 4a). These metabolites were identified in patients (Fig. 4a) as α-hydroxy-tamoxifen, 4′-hydroxy-tamoxifen and tamoxifen-*N*-oxide, respectively (H, C, I, respectively in Fig. 1) [13,49,51,65] by comparison to the retention times (Fig. 4a, lower chromatogram) and/or product-ion spectra of authentic standards spiked into blank plasma or added to patients' plasma samples (data not shown). The fragmentation pattern of the 4′-hydroxy-tamoxifen standard spiked into blank plasma (Fig. 5a) was equivalent to that observed for the putative endogenous 4′-hydroxy-tamoxifen. The product ions (72, 129, 145, 223, 316 *m*/*z*) were invariably observed in all product ion scans determined at the retention time of the metabolite observed in patients samples.

Interestingly, the UPLC gradient program also allows the baseline separation of 4-hydroxy-tamoxifen and 3-hydroxy-tamoxifen eluted at 4.2 and 4.4 min, respectively (Fig. 4a, lower chromatogram of standard compounds spiked in plasma). The compound 3-hydroxy-tamoxifen is a metabolite reported to be produced *in vitro* upon incubation of tamoxifen with human liver microsomes (HLMs) [13]. In patients' plasma however, there was only a very small peak, if any, visible at the retention time of 3-hydroxy-tamoxifen. (Fig. 4a, metabolites profiles in patients).

Finally, inspection of the transition (*m*/*z* 374 → 58, 129, 223) selected for monitoring *Z*-endoxifen (eluted at 4.0 min) revealed the presence in patients samples of two additional peaks at 1.5 and 4.9 min (Fig. 3b, upper chromatographic profile, and Fig. 4b). The first eluted peak at 1.5 min was tentatively identified as α-hydroxy-*N*-desmethyl-tamoxifen based on literature (no available reference material). The latest peak visible in this *m*/*z* transition at 4.9 min was identified as 4′-hydroxy-*N*-desmethyl-tamoxifen, which has the same retention time (Fig. 4b, lower trace) and a comparable product-ion mass spectrum as the synthetic compound (Fig. 5b) either spiked into blank plasma or patients' plasma samples. The product ions (58, 129, 145, 223 and 316 *m*/*z*) were observed during the fragmentation of the 4′-hydroxy-*N*-desmethyl-tamoxifen pure compound and were likewise detected in all product-ion scans at the retention time of the putative endogenous metabolite. As recently described, the fragment at 129 *m*/*z* was reported to be indicative of the tamoxifen structure [54] and was detected in product ion spectra of both metabolites 4′-hydroxy-*N*-desmethyl-tamoxifen and 4′-hydroxy-tamoxifen.

The metabolite 4′-hydroxy-tamoxifen, whose formation might be catalyzed by the polymorphic CYP2B6 [13,61], has been previously detected in rat and mouse liver microsomes [13,52,61,65] and in recent *in vitro* studies (using Human Cytochrome P450 Systems) as primary metabolite of tamoxifen [13,51], but its occurrence had never been formally reported in humans. Similarly, 4′-hydroxy-*N*-desmethyl-tamoxifen has been previously detected in mouse liver microsomal incubates [52]. Neither metabolite has yet been identified so far in patients.

This is the first report of the occurrence of 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen in plasma from patients under tamoxifen therapy. Typical metabolites profiles in 6 unselected patients receiving tamoxifen are shown in Fig. 4a and b: 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen are detected in patients' samples at 5.1 and 4.9 min in their respective *m*/*z* transition channel. So far, both metabolites were found in all patients' samples analysed ($n = 70$), with substantial variability in plasma levels.

Although our method has not been formally validated for the quantification of these newly identified metabolites, their plasma levels have been estimated in a separate analysis of 20 unselected patients' samples. The concentrations of 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen ranged between 2.2 to 5.5 ng/mL, and 4.4 to 11.8 ng/mL, respectively, in patients under tamoxifen 20 mg QD, and between 3.3 to 9.5 ng/mL, and 6.2 to 20.6 ng/mL, respectively, in patients under 20 mg BID tamoxifen regimen. The clinical importance of these new metabolites, and their potential contribution to the clinical effects of tamoxifen remain to be determined [13]. Limited data available from the literature suggest that 4′-hydroxy-tamoxifen might have higher affinity for the estrogen receptor than tamoxifen itself [13,66,67].

4. Conclusion

We have developed and validated a specific and sensitive UPLC–MS/MS method enabling reliable and sensitive monitoring of tamoxifen and three clinically relevant metabolites in patients' plasma. Our method provides an excellent chromatographic separation of tamoxifen and seven known and previously unreported metabolites in a relatively short gradient program of 13 min. The method was developed using deuterated I.S. for all target analytes, which further strengthen our analytical assay for selective and sensitive quantification of tamoxifen and its metabolites by electrospray ionisation mass spectrometry.

During the course of these chromatographic investigations, we have been able to identify for the first time the two metabolites 4′-hydroxy-tamoxifen and 4′-hydroxy-*N*-desmethyl-tamoxifen in plasma from breast cancer patients. Our estimation of 4′-hydroxy metabolites plasma levels in a subset of patients indicates that the range of 4′-hydroxy-tamoxifen plasma concentrations was similar to that measured for 4-hydroxy-tamoxifen. Conversely, 4′-hydroxy-*N*-desmethyl-tamoxifen plasma levels were two to three times lower than the endoxifen levels determined in 20 unselected patients. The clinical importance of these previously unreported metabolites and their potential contribution to the clinical effects of tamoxifen has yet to be determined. Finally, we could show that 3-hydroxy-tamoxifen is very limitedly, if not at all, found in the blood of patients on tamoxifen therapy.

In conclusion, This UPLC–MS/MS method has been shown suitable for measuring exposure of tamoxifen and its metabolites in tamoxifen-treated breast cancer patients. In this context, the present analytical methodology is currently applied in a population pharmacokinetic study of tamoxifen and its metabolites, helping us primarily at characterizing the influence of pharmacogenetic and environmental factors (including interacting medications) on plasma concentrations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.10.027.

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