

Fast and Adequate Liquid Chromatography–Tandem Mass Spectrometric Determination of Z-endoxifen Serum Levels for Therapeutic Drug Monitoring

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Background: Z-endoxifen (further referred to as endoxifen, unless stated otherwise) is proposed as the most important metabolite of tamoxifen. Patients receiving adjuvant tamoxifen treatment with endoxifen levels below the threshold of 5.9 ng/mL may have an increased risk of breast cancer recurrence. Several factors, such as genetic polymorphisms, drug interactions, and (non)adherence, lead to large interpatient variability in endoxifen exposure, resulting in a substantial number of patients showing subtherapeutic levels. As genotyping and phenotyping are not able to adequately predict endoxifen exposure, therapeutic drug monitoring (TDM) seems to be the best approach for tailored tamoxifen therapy.

Methods: To support TDM services, a rapid and sensitive high-performance liquid chromatography–tandem mass spectrometry assay for the quantification of endoxifen in human serum was developed and validated. Validation was performed according to the latest US FDA and EMA guidelines on bioanalytical method validation.

Results: The successfully validated serum assay quantifies endoxifen with a linear regression calibration model (weighted $1/x^2$) in the concentration range from 1.00 to 25.0 ng/mL. The assay was validated with an inaccuracy of $\pm 7.7\%$ and an imprecision of $\leq 3.9\%$, obtained with an IS normalized matrix factor of 0.925 and a signal-to-noise ratio of >66 .

Conclusions: All validation parameters fulfilled their acceptance criteria, and the developed assay is now successfully being used to support TDM services. Thus far, 32.7% of the more than 500 determined endoxifen serum levels were below the threshold of 5.9 ng/mL.

Key Words: endoxifen, therapeutic drug monitoring, LC-MS/MS
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INTRODUCTION

At the moment, tamoxifen is the drug of choice, besides aromatase inhibitors, in postmenopausal women for the treatment of estrogen receptor–positive breast cancers. The efficacy of this drug is mainly due to its active metabolites 4-hydroxytamoxifen and particularly endoxifen (4-hydroxy-*N*-desmethyldoxifen), which are generated by metabolism through cytochrome P450 (CYP) enzymes 2D6 and 3A. These metabolites show comparable antiestrogenic activity, which is 30- to 100-fold more potent than tamoxifen itself.^{1–3}

In 2011, Madlensky et al stated that the level of the plasma concentration of endoxifen (Fig. 1), but not tamoxifen or other metabolites, was associated with the risk of breast cancer recurrence or the development of another breast cancer in patients receiving adjuvant tamoxifen treatment. An increased risk was observed in those with an endoxifen concentration in the bottom quintile of the distribution of the identified threshold of 5.9 ng/mL.⁴ Saladores et al observed data in line with these results in premenopausal women. Patients with low endoxifen concentrations had higher risk of distant relapse or death compared with those with high concentrations.⁵

Polymorphisms in CYP2D6, and both inhibitors and inducers of this enzyme, can lead to interindividual differences in endoxifen formation. Therefore, research has been devoted to reveal the influence of CYP2D6 genotypes on breast cancer outcome. The results are, however, inconsistent and the exposure to endoxifen can only be partly ascribed to these polymorphisms.^{6,7}

Phenotyping of CYP2D6 and CYP3A using dextromethorphan as a probe drug was able to better predict the endoxifen exposure than CYP2D6 genotyping.^{8–10} Administration of dextromethorphan and blood or breath sampling are needed to perform the phenotyping. Using these methods, the endoxifen exposure can only be partly predicted because the contribution of other factors, such as patient compliance and drug interactions, is excluded.

As can be expected from the previously mentioned reasons, a recent study showed that 44 of 197 patients (22%) on standard 20 mg tamoxifen treatment had endoxifen serum concentrations below the predefined threshold of 5.9 ng/mL.¹¹

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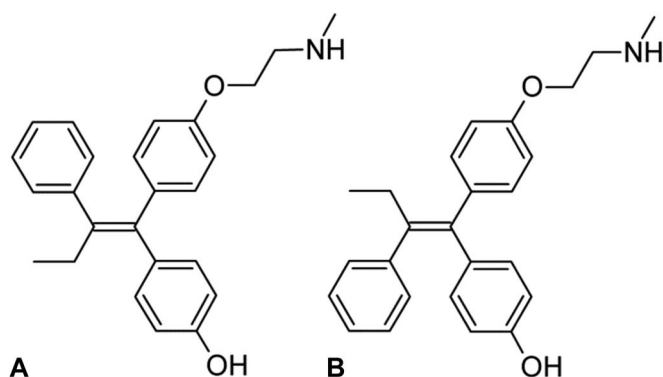


FIGURE 1. Chemical structures of (A) Z-endoxifen (endoxifen, 4-hydroxy-*N*-desmethyltamoxifen, CAS number: 112093-28-4) and (B) E-endoxifen (CAS number: 114828-90-9).

These findings support the rationale for therapeutic drug monitoring (TDM) of endoxifen serum concentrations in patients receiving tamoxifen treatment.^{12,13} As concluded by Jager et al,¹⁴ a highly sensitive and selective analytical assay for the quantification of endoxifen is needed for TDM.

To monitor endoxifen serum levels, a fast and accurate analytical TDM assay was developed based on our previously published bioanalytical assay for the quantitative determination of tamoxifen and 5 of its phase I metabolites.¹⁵ As an increase in sensitivity and robustness was observed using electrospray ionization, the ionization technique was changed from atmospheric-pressure chemical ionization to electrospray ionization. In the routine application of the assay, only 4 calibrators and 3 QC samples are included in each analytical batch, leading to a more rapid turnaround time to report endoxifen levels.

MATERIALS AND METHODS

Reagents and Chemicals

Endoxifen and endoxifen-d5 (both as racemic mixture) were obtained from Toronto Research Chemicals (North York, ON, Canada). Acetonitrile (Supra-Gradient grade), methanol (Supra-Gradient grade), and water (UPLC grade) were obtained from Biosolve Ltd (Valkenswaard, the Netherlands). Ammonium formate (>99%) was obtained from Fluka (Barneveld, the Netherlands). Formic acid (98%) was obtained from Merck (Darmstadt, Germany). Control drug-free human serum was obtained from the MC Slotervaart (Amsterdam, the Netherlands).

Stock Solutions, Calibrators, and Quality Control Samples

Separate 2 mg/mL stock solutions for calibrators and quality control samples (QC samples) were prepared in methanol. Two milligrams per milliliter of endoxifen (racemic mixture) is equivalent to ± 1 mg/mL E-endoxifen and ± 1 mg/mL Z-endoxifen. The Z-endoxifen concentration was calculated based on the data obtained from the certificates of analysis of the supplier. These stock solutions, stored at -70°C , were further diluted with methanol to obtain working

solutions. The working solutions were also stored at -70°C . A stock solution of the internal standard endoxifen-d5 was also prepared at a concentration of 2 mg/mL in methanol. Two milligrams per milliliter of endoxifen-d5 (racemic mixture) is equivalent to ± 1 mg/mL E-endoxifen-d5 and ± 1 mg/mL Z-endoxifen-d5 (endoxifen-d5). The internal standard working solution contained 5.0 ng/mL Z-endoxifen-d5 in methanol-acetonitrile (1:1000 vol/vol). Internal standard stock and working solutions were stored at -70 and -20°C , respectively.

Calibrators were prepared by adding 20 μL of calibrator working solution to 1980 μL of control human serum. Calibrators with the following concentrations were prepared: 1.0, 5.0, 10.0, and 25.0 ng/mL. Calibrators were stored in aliquots of 50 μL at -70°C .

The QC samples were prepared by spiking control human serum with the appropriate QC working solution. Final concentrations at the QC lower limit of quantification (QC LLOQ), QC threshold, and QC upper limit of quantification (QC ULOQ) contained 1.0, 6.0, and 25.0 ng/mL endoxifen, respectively. QC samples were stored in aliquots of 50 μL at -70°C .

Sample Preparation

Serum samples were thawed, vortex-mixed, and centrifuged (5 minutes at 900g) before processing, and a 50- μL aliquot was used for analysis. One hundred fifty microliters of internal standard working solution (5.0 ng/mL) was added to induce protein precipitation. After 10 seconds of vortex-mixing, the samples were centrifuged for 10 minutes at 23,100g. One hundred twenty-five microliters of the clear supernatant was transferred to an amber autosampler vial containing 250 μL of 4 mmol/L ammonium formate buffer, pH adjusted to 3.5. After vortex-mixing for 10 seconds, a volume of 5 μL was injected into the HPLC column.

Liquid Chromatography–Tandem Mass Spectrometry

The chromatographic separation was performed using an 1100 series binary pump, degasser, column oven, and autosampler from Agilent Technologies (Santa Clara, CA). An API4000 triple quadrupole equipped with Turbo ion-spray interface operating in positive ion mode and configured in multiple reaction monitoring was used. The liquid chromatography–tandem mass spectrometry data were acquired and processed with Analyst software from Sciex (Framingham, MA). The autosampler temperature was kept at 7°C and the column oven at 60°C . Mobile phase A consisted of 4 mmol/L ammonium formate buffer, pH 3.5 and mobile phase B was acetonitrile. Gradient elution was applied at a flow rate of 0.4 mL/min through a Kinetex C18 column (150 \times 2.1 mm internal diameter, particle size 2.6 μm), Phenomenex (Torrance, CA). The following mobile phase gradient was applied: 30%–52.5% B (0.0–6.0 minutes), 52.5%–80% B (6.0–6.1 minutes), 80% B (6.1–7.0 minutes), 80%–30% B (7.0–7.1 minutes), and 30% B (7.1–10.0 minutes). Table 1 summarizes the MS operating parameters.

TABLE 1. Mass Spectrometric Parameters of the Analysis of Endoxifen With the Internal Standard Endoxifen-d5

General Settings	API4000	
Run duration, min	10.0	
Ionspray voltage, kV	+5.0	
Turbo gas temperature, °C	700	
Gas 1, psi	70	
Gas 2, psi	50	
Curtain gas, psi	10	
Collision gas, psi	7	
Analyte Specific Parameters	Endoxifen	Endoxifen-d5
Parent mass, m/z	374.2	379.3
Product mass, m/z	58.2	58.2
Dwell time, ms	30	30
Collision energy, V	49	49
Collision exit potential, V	11	11
Declustering potential, V	60	60
Entrance potential, V	10	10
Typical retention time, min	5.7	5.7

Validation Procedures

A full validation, including calibration model, inaccuracy and imprecision, selectivity, lower limit of quantification, matrix effect, carryover, and stability under various conditions, was performed according to the latest US FDA and EMA guidelines on bioanalytical method validation, with the exception of dilution integrity.^{16,17} The determination of dilution integrity was deemed not applicable for the purpose of TDM because the validated range of the assay covers a broad range around the threshold level of endoxifen. Because the determination of higher serum levels is not of clinical relevance and expansion of the validated range will result in a decrease in sensitivity around the threshold, 25.0 ng/mL was chosen as ULOQ. Intra- and inter-assay systematic error was expressed as the bias in percentage and intra- and inter-assay imprecision as the coefficient of variation (CV). An overview of the validated parameters is shown in Table 2.

Clinical Application

Routine patient serum samples (n = 597) were obtained from The Netherlands Cancer Institute, Amsterdam, the Netherlands, where TDM is offered to patients receiving tamoxifen treatment. The received tamoxifen dose was 20 mg in 76.38%, 5 mg in 0.34%, 10 mg in 4.35%, 30 mg in 4.19%, 40 mg in 14.57%, and 50 mg in 0.17% of the patients. Use of routine patient samples for clinical studies is authorized within the institute. Clinicians were advised to discuss the possibility of dose escalation with the patient if the endoxifen concentration was below the threshold.

RESULTS

Calibration Model

Calibrators were prepared and analyzed in duplicate during 3 analytical runs. The linear regression of the peak area ratio versus the concentrations (x) was weighted $1/x^2$ to

TABLE 2. Summary of Validation Results

Validated Parameter	Result
Validated range, ng/mL	1.00–25.0
Calibration model	Linear regression, weighted $1/x^2$
Inaccuracy (bias) intra-assay (at LLOQ)	$\pm 7.7\%$ ($\pm 16.0\%$)
Inaccuracy (bias) interassay (at LLOQ)	$\pm 5.7\%$ ($\pm 11.6\%$)
Imprecision (CV) intra-assay (at LLOQ)	$\leq 3.9\%$ ($\leq 5.2\%$)
Imprecision (CV) interassay (at LLOQ)	$\leq 2.0\%$ ($\leq 3.9\%$)
Selectivity (endogenous and cross-analyte)	0.0%
Lower limit of quantification (S/N)	>66 (n = 3)
Instrument carryover	0.0%
IS normalized MF (mean, CV)	0.975, 2.9%
Final extract stability (2–8°C)	At least 7 d
Reinjection stability (2–8°C)	At least 7 d
Serum stability (–20°C, 2–8°C, and RT)	At least 7 d*
Plasma stability (–20°C, 2–8°C, and RT)	At least 7 d*
Whole blood stability (2–8°C and RT)	At least 7 d*

*When samples are kept longer than 1 day at room temperature, they should be protected from light.

RT, room temperature (20–25°C); IS, Internal standard.

obtain the lowest total bias and most constant bias across the range. The calibration range of endoxifen in serum was 1.00–25.0 ng/mL. Calibration curves were accepted if 75% of the nonzero calibrators (including at least 1 LLOQ and ULOQ) were within $\pm 15\%$ of the nominal value (or $\pm 20\%$ for the LLOQ). All calibration curves met these criteria and correlation coefficients (R^2) of 0.9966 or better were obtained.

Inaccuracy and Imprecision

To assess the inaccuracy and imprecision of the assay, 5 replicates of QC LLOQ, QC threshold, and QC ULOQ in serum were analyzed during 3 analytical runs. Table 3 summarizes the intra- and inter-assay inaccuracy and imprecision of the assay. The biases and CVs were within the acceptance criteria (i.e., $\pm 20\%$ and $\leq 20\%$, respectively at the LLOQ level and $\pm 15\%$ and $\leq 15\%$ at threshold and ULOQ).

Selectivity

Six different batches of control serum were spiked at the LLOQ level with endoxifen to investigate the selectivity. The mean deviation from the nominal concentration for the serum assay was -16.5% , with a CV value of 5.7%. There were no peaks observed with areas $>20\%$ of the LLOQ in the double-blank samples of these batches, and also no interferences were detected at the retention time of the internal standard. Selectivity was therefore considered acceptable.

Cross-analyte and internal standard interferences were tested by spiking control human serum at ULOQ level with endoxifen and the internal standard separately, at the nominal concentration (1 level). The cross-analyte and internal standard interference at the retention time of endoxifen were less than the allowable 20% of the peak of the LLOQ level. For the internal standard, the interference was less than 5% and fulfilling the acceptance criteria.

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TABLE 3. Assay Performance Data for Endoxifen

Nominal Concentration (ng/mL)	n	Intra-assay		Interassay	
		Bias* (%)	CV* (%)	Bias (%)	CV (%)
1.00	15	−16.0; −8.6	2.9–5.2	−11.6	3.9
6.01	15	−7.7; −3.4	1.7–3.9	−5.7	2.0
25.0	15	−2.2; −1.2	0.7–2.6	−1.8	—†

*The range of inaccuracy and imprecision during 3 validation runs is presented.

†Inter-run imprecision could not be calculated (mean square between groups is less than mean square within groups).

n, number of replicates.

Lower Limit of Quantification

The analyte responses at the LLOQ were at least 5 times the response of a blank in 3 validation runs. The signal-to-noise ratio was at least 66. Figure 2 shows representative chromatograms of endoxifen in a QC LLOQ sample and a patient serum sample.

Carryover

Carryover was investigated by injecting 2 double-blank samples after a ULOQ sample in 3 analytical runs. Eluting peaks with areas >20% of the LLOQ were not observed in the blank samples injected directly after ULOQ samples, and therefore the criteria for carryover were fulfilled.

Matrix Factor

The matrix factor (MF) was determined in 6 serum batches at threshold level of endoxifen. Single determinations were performed. Processed blank samples were spiked with neat solutions and compared with matrix-free neat solutions. The MF was determined using the following equation:

$$MF = \frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}}$$

In addition to the MF, the internal standard–normalized MF was calculated by dividing the MF of the analyte by the MF

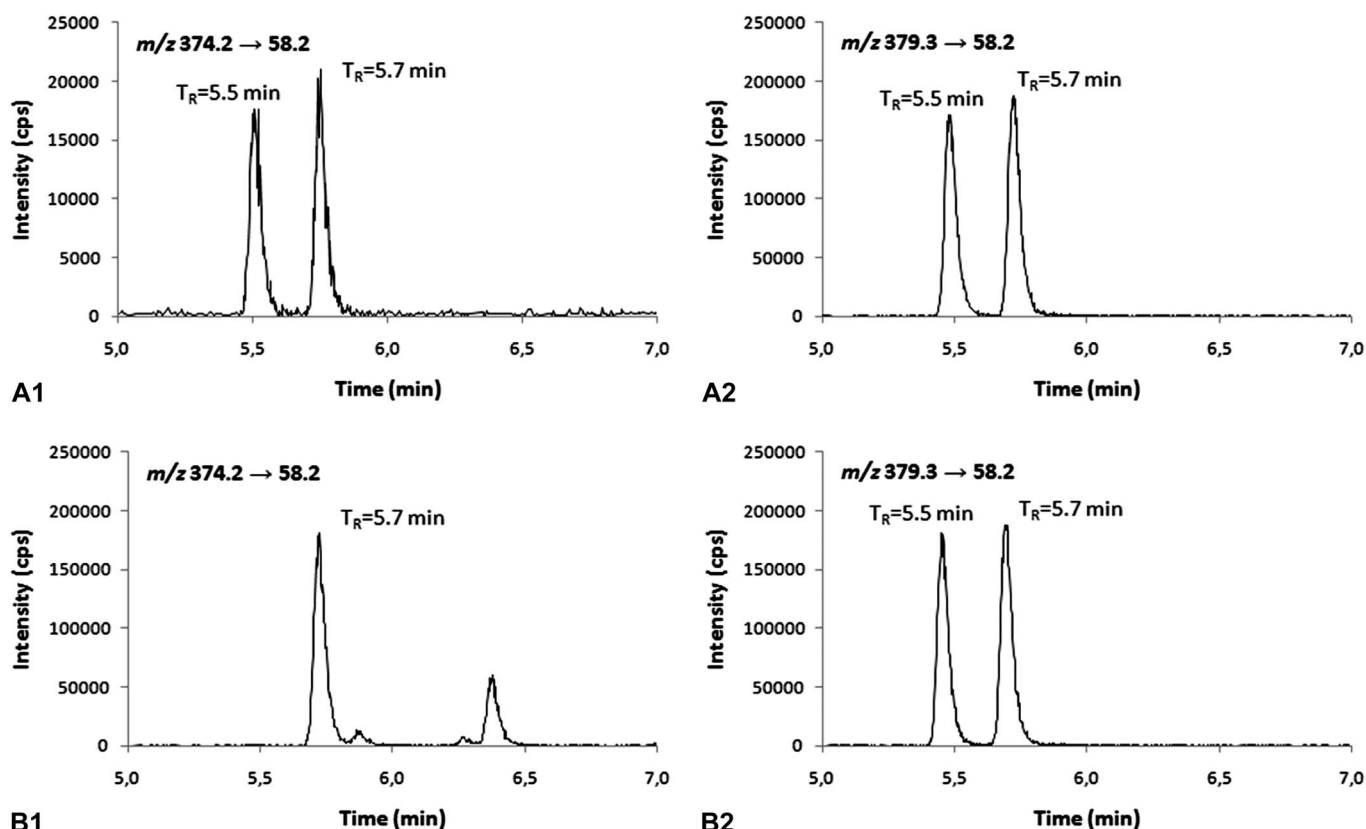


FIGURE 2. MRM chromatograms of endoxifen (E-endoxifen T_R = 5.5 minutes and Z-endoxifen T_R = 5.7 minutes) in serum at LLOQ level (1.0 ng/mL) (A1), a representative patient sample (B1), and the corresponding internal standard signals of endoxifen-d5 (E-endoxifen-d5 T_R = 5.5 minutes and Z-endoxifen-d5 T_R = 5.7 minutes) at 15 ng/mL (A2, B2).

TABLE 4. Stability Data for Endoxifen in Serum, Whole Blood, and Plasma

Conditions	Matrix	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Bias (%)	CV (%)	n
Serum						
−20°C, 7 d	Biomatrix	8.88	9.38	5.6	5.0	3
2–8°C, 7 d	Biomatrix	8.88	9.09	2.4	3.7	3
RT, dark, 7 d	Biomatrix	8.88	9.27	4.4	3.6	3
RT, light, 7 d	Biomatrix	8.88	7.61	−14.3	1.7	3
Reinjection						
2–8°C, 7 d	Final extract	1.00	0.923	−7.7	5.1	5
	Final extract	6.01	5.58	−7.2	1.9	5
	Final extract	25.0	24.7	−1.2	0.8	5
Processed sample						
2–8°C, 7 d	Final extract	1.00	1.01	0.8	4.9	5
	Final extract	6.01	6.18	2.8	1.9	5
	Final extract	25.0	27.3	9.0	0.9	5
Whole blood						
2–8°C, 7 d	Biomatrix	8.88	9.18	3.3	7.3	3
RT, dark, 7 d	Biomatrix	8.88	8.72	−1.8	2.6	3
RT, light, 7 d	Biomatrix	8.88	7.53	−15.2	3.8	3
Plasma						
−20°C, 7 d	Biomatrix	8.88	8.29	−6.6	5.3	3
2–8°C, 7 d	Biomatrix	8.88	8.56	−3.6	4.9	3
RT, dark, 7 d	Biomatrix	8.88	8.58	−3.3	2.3	3
RT, light, 7 d	Biomatrix	8.88	9.10	2.5	5.1	3

RT, room temperature (20–25°C).

of the internal standard. The CV of the internal standard–normalized MF calculated from the 6 serum batches was 2.9%, fulfilling the acceptance criteria of $\leq 15\%$.

Stability

The results of the stability experiments are shown in Table 4. Whole blood, plasma, and serum samples were received from patients after tamoxifen treatment. Endoxifen was stable in whole blood, plasma, and serum at −20, 2–8°C, and at room temperature (20–25°C) protected from light for at least 7 days.

A deviation of −15.2% and −14.3% from the nominal concentration for whole blood and serum samples, respectively, was measured after 7 days at room temperature without protection from light. These results demonstrate that endoxifen is susceptible to degradation when exposed to daylight. Samples should therefore be stored in the dark.

Processed sample and reinjection reproducibility experiments demonstrated that processed serum samples can be successfully (re)injected after 7 days when stored at 2–8°C.

DISCUSSION

The purpose of the validated assay was to support TDM of tamoxifen by assessing the blood levels of the active metabolite endoxifen using a method that is especially designed for daily TDM services. So far, more than 500 blood samples of tamoxifen-treated patients were analyzed with the presented assay. The average determined endoxifen serum concentration

was 8.23 ng/mL (SD 4.23; range 1.07–24.0 ng/mL), resulting in 32.7% of the determined blood serum levels being below the threshold of 5.9 ng/mL. Patient-specific dose optimization was performed based on these endoxifen serum concentrations. Figure 2B1 shows that using this method, endoxifen is still separated from its metabolites in patient samples as shown before with previous methods.¹⁵

CONCLUSIONS

The liquid chromatography–tandem mass spectrometry assay has been developed and validated for the routine TDM analysis of endoxifen in human serum. The validated range is from 1.00 to 25.0 ng/mL using 50 μ L serum aliquots. This fast and adequate bioanalytical assay is considered fit to support TDM of tamoxifen and is now successfully used to support TDM services in which patient-specific dose optimization is performed based on serum concentrations of endoxifen.

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