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Development of a high performance liquid chromatography-tandem mass spectrometry assay for the analysis of tamoxifen and three metabolites in human serum

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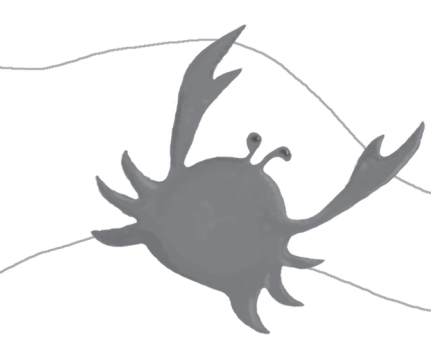

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ABSTRACT

A specific method was developed using high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) aiming to identify and quantify tamoxifen and the metabolites N-desmethyltamoxifen (NDMTam), 4-hydroxytamoxifen (4OHTam), 4-hydroxy-N-desmethyltamoxifen (endoxifen), tamoxifen-N-oxide (TamNox) and the potentially carcinogenic metabolite α-hydroxytamoxifen (αOHTam) in human serum. Relevant metabolites with equal mass-to charge ratio and fragmentation pattern were chromatographically separated using the current LC-MS/MS method. This resulted in accurate detection and quantification of tamoxifen, NDMTam and the main active metabolites 4OHTam and endoxifen. The metabolites αOHTam and TamNox were identified, but could not be accurately quantified. The LC-MS/MS method was successfully applied to several prospective studies in early and metastatic breast cancer patients in The Netherlands and Belgium. In the future this LC-MS/MS method might be used for therapeutic drug monitoring in tamoxifen treated breast cancer patients.

INTRODUCTION

The potent effect of tamoxifen, a selective estrogen receptor modulator, on breast cancer was first discovered in the 1970s.[1] At present, tamoxifen is still an important therapeutic agent for endocrine sensitive breast cancer, although attention has shifted to alternative agents such as aromatase inhibitors. Tamoxifen metabolism is complex and involves the biotransformation into various metabolites by the cytochrome P450 enzyme system.[2, 3] The main metabolites are the result of N-demethylation, hydroxylation and N-oxidation. Both 4-hydroxy-N-desmethyltamoxifen (endoxifen) and 4-hydroxytamoxifen (4OHTam) are considered the most active tamoxifen metabolites. They are equally potent, although endoxifen exhibits plasma concentrations that are 5-10-fold higher than 4OHTam.[4, 5] Since endoxifen is recognized as the most active tamoxifen metabolite, renewed interest has been shown in tamoxifen. The belief that alterations in the metabolism may influence tamoxifen efficacy has led to numerous studies and publications.[5] Most of the research has focused on the cytochrome P450 isoenzyme 2D6 (CYP2D6), which primarily mediates the biotransformation of tamoxifen to endoxifen. Common genetic variants of CYP2D6 leading to low enzymatic activity and low endoxifen concentrations during tamoxifen treatment have been associated with clinical outcome.[6-11] Conflicting data however have caused controversy over the validity and clinical relevance of this association.[12-14] In only one retrospective study endoxifen plasma concentrations have been related to clinical outcome.[15] This study however was not designed for this objective. Prospective studies with the aim to relate endoxifen levels and CYP2D6 genotype to clinical outcome are therefore needed. In The Netherlands and Belgium we initiated a prospective study addressing the relation between CYP2D6 genotype, active metabolite serum concentrations and clinical outcome in tamoxifen treated early breast cancer patients (CYPTAM study: NTR1509).[16] In a separate pharmacokinetic study the influence of tamoxifen dose escalation on endoxifen and other metabolite levels is investigated in early breast cancer patients with a CYP2D6 poor or intermediate metabolizer phenotype.[16] Both studies were approved by the Medical Ethical Committee of the Leiden University Medical Center).In view of these studies we developed a high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay to analyze tamoxifen and several metabolites in human serum. The tamoxifen metabolites were selected for the purposes of both prospective studies and included N-desmethyltamoxifen (NDMTam), 4-hydroxytamoxifen (4OHTam), 4-hydroxy-N-desmethyltamoxifen (endoxifen), tamoxifen-N-oxide (TamNox) and α-hydroxytamoxifen (αOHTam). The selection was made to calculate metabolic ratios (e.g. N-desmethyltamoxifen (NDMTam)/endoxifen ratio), to investigate if alternative metabolic pathways become essential when CYP2D6 activity is limited and to detect

how the concentrations of metabolites including α-hydroxytamoxifen (αOHTam), a genotoxic and potentially carcinogenic metabolite,[17] are increased when the tamoxifen dose is escalated. The metabolites 4'hydroxytamoxifen (4'OHTam), 3-hydroxytamoxifen (3OHTam) and 4'-hydroxy-N-desmethyltamoxifen (4'endoxifen) have the same mass-to charge ratio and fragmentation pattern as 4OHTam and endoxifen respectively. We aimed to chromatographically separate these metabolites, enabling accurate quantification of the active metabolites.

EXPERIMENTAL

Chemicals and reagents

Tamoxifen and 4OHTam were obtained from Sigma-Aldrich (St Louis, MO, USA). NDMTam, endoxifen (approx. 1:1 E/Z mixture), 4'endoxifen, αOHTam, 4'OHTam, 3OHTam, TamNox and the deuterated internal standards (IS) D5-tamoxifen, D5-4-OH-tamoxifen (D5-4OHTam), D5-N-desmethyltamoxifen (D5-NDMTam) and D5-endoxifen (approx. 1:1 E/Z mixture) were all purchased from Toronto Research Chemicals Inc (North York, ON, Canada). Ammonium Acetate, Acetonitrile, Zinc Sulfate, Ethanol and Methanol were obtained from E. Merck (Darmstadt, Germany). Formic Acid was purchased from Fluka AG (Buchs, Switzerland). Demineralized water from a Milli Q water purification system (Millipore, Amsterdam, The Netherlands) was used for preparation of mobile phase solutions. Blank pooled serum from different individual donors was obtained from Sanguin.

Preparation of calibration standards, quality control samples and internal standard solutions

Stock solutions of tamoxifen, metabolites and the available internal standards were prepared at a concentration of 1.00 mg/ml by dissolving an accurately weighed amount of each substance either in methanol (endoxifen, TamNox, D5-endoxifen, D5-4OHTam), ethanol (Tamoxifen, 4OHTam, 4'OHTam, NDMTam, αOHTam, 4'endoxifen, D5-Tamoxifen) or ethyl acetate (D5-NDMTam). The stock solutions were stored at -20°C. Subsequently, substock solution were made by dilution of the stock solutions and stored at -20°C.

The (sub)stock solutions were used to prepare a working solution which was diluted in blank human donor serum on each day of analysis to prepare calibration standards of tamoxifen and 7 metabolites at the following concentrations: Tamoxifen: 0-20-40-100-300-500 µg/L; 4OHTam: 0-1-2-5-15-25 µg/L; 4'OHTam: 0-0,5-1-2,5-7,5-12,5 µg/L; NDMTam: 0-40-80-200-600-1000 µg/L; αOHTam: 0-0,5-

1-2,5-7,5-12,5 µg/L; TamNox: 0-0,5-1-2,5-7,5-12,5 µg/L; Endoxifen E+Z: 0-2-4-10-30-50 µg/L; E- and Z-Endoxifen (assuming an 1:1 E/Z mixture): 0-1-2-5-15-25 µg/L; 4'-endoxifen: 0-1-2-5-15-25 µg/L.

Quality control (QC) samples were prepared from stock solutions and a working solution was made for all substances in the following low (LQC) and high(HQC) concentrations: Tamoxifen: 50 µg/L and 400 µg/L; 4OHTam: 2.5 µg/L and 20 µg/L; 4'OHTam: 1.25 µg/L and 10 µg/L; NDMTam: 100 µg/L and 800 µg/L; αOHTam: 1.25 µg/L and 10 µg/L; TamNox: 2.5 µg/L and 20 µg/L; Endoxifen E+Z: 5 µg/L and 40 µg/L; 4'-endoxifen: 2.5 µg/L and 20 µg/L. Quality controls were stored at -20°C.

A working solution of the 4 deuterated internal standards (4D5-IS) was prepared on each day of analysis by dilution of the substock solutions in acetonitril consisting of the internal standards in the following concentrations: D5-Tamoxifen: 200 µg/L; D5-4OHTam: 10 µg/L; D5-NDMTam: 400 µg/L; D5-endoxifen: 20 µg/L.

Of note, 3OHTam was not included in the calibration standards and quality control samples, because the current LC-MS/MS method did not chromatographically separate 3OHTam from 4OHTam (Results section, paragraph Chromatography and detection).

Sample preparation

Prior to extraction, frozen serum samples were thawed at room temperature and homogenized by vortex mixing. A 0.2 ml aliquot of serum was pipetted into an Eppendorf tube; 0.05 ml 0.1M ZnSO4 and 0.2 ml of the internal standard working solution 4-D5-IS were added. The tube was mixed vigorously for 3 min on a vortex mixer, followed by centrifugation at 13000 rpm for 5 min at ambient temperature. A volume of 20 µl of supernatant was injected onto the HPLC instrument for quantitative analysis using an autosampler operating at room temperature.

Chromatographic and mass spectrometric conditions

Chromatographic analysis was performed using a Waters Micromass Quattro micro API Tandem MS equipped with a Dionex P680A DGP-6 HPLC pump, a Dionex Ultimate 3000 autosampler and a Dionex Thermostated Column Compartment. Separation of the analytes from potentially interfering material was achieved using a Waters X-bridge C18 Column (3.5 µm, 4.6 x 50 mm) with a Spark HySphere C18 HD pre-column (7 µm) in a Phenomenex holder. The temperature-controlled column compartment operated at 20°C. The mobile phase used for the chromatographic separation was composed of 25% of solution A (0.1% formic acid + 2 mM ammonium acetate in H_2O) and 75% of

solution B (0.1% formic acid + 2 mM ammonium acetate in methanol) and was delivered isocratically at a flow rate of 0.4 ml/min. The mass spectrometric detector was equipped with an atmospheric pressure ionization interface, operating in the positive mode, and controlled by MassLynx, version 4.1 software, running under Microsoft Windows XP on a Lenovo personal computer. The samples were analyzed using an atmospheric pressure ionization probe in the positive ionization mode operating at a capillary voltage of 0.5 kV for all substances. Cone voltages and collision energy for all analytes are shown in Table 4.1. Samples were introduced into the interface through a heated nebulized probe (130°C). The spectrometer was programmed to allow the [MH]+ ion to pass through the first quadrupole (Q1) into the collision cell (Q2) at the following mass-to-charge ratios (m/z): 372.1 (tamoxifen); 374.1 (endoxifen E+Z, 4'endoxifen); 388.2 (4OHTam, 4'OHTam, TamNox, αOHTam); 358.1 (NDMTam); 377.1(D5-tamoxifen); 393.2 (D5-4OHTam); 363.1 (D5-NDMTam); 379.1 (D5-endoxifen E+Z). The product ions were monitored through the third quadrupole (Q3) at the following mass-to-charge ratios (m/z): 71.8 (tamoxifen, D5-tamoxifen, 4OHTam, D5-4OHTam, 4'OHTam, TamNox, αOHTam); 57.8 (NDMTam, D5-NDMTam, endoxifen E+Z, 4'endoxifen, D5-endoxifen E+Z). Argon was used as collision gas at a pressure of 3x10-3 mbar, and the dwell time per channel was 0.100 seconds for data collection. The selected analytes with their LC-MS/MS retention times and mass transitions are shown in Table 4.1.

Table 4.1 Selected analytes with LC-MS/MS specifications

Analyte	Retention time (min)	Mass transition (m/z) Parent > daughter	Cone voltage (Volts)	Collision energy (eV)
E-endoxifen	2.51	374 > 58	35.0	20.0
Z-endoxifen	2.75			
Endoxifen E + Z	2.51 + 2.75			
4'-endoxifen	3.53			
D5-endoxifen E + Z	2.51 + 2.75	379 > 58	35.0	20.0
α-OH-tamoxifen	1.92	388 >72	35.0	25.0
4-OH-tamoxifen*	2.72			
4'-OH-tamoxifen	3.46			
Tamoxifen-N-oxide	6.15			
D5-4-OH-tamoxifen	2.70	393 > 72	35.0	25.0
N-desmethyltamoxifen	5.46	358 > 58	30.0	20.0
D5-N-desmethyltamoxifen	5.39	363 > 58	30.0	20.0
Tamoxifen	5.32	372 > 72	35.0	25.0
D5-tamoxifen	5.25	377 > 72	35.0	25.0

* 3-OH-tamoxifen has the same retention time; mass transition (2.72; 388 > 72), but was hardly detectable in patients (n=4); Dwell time for all analytes is 0.10 seconds; Capillary voltage for all analytes is 0.5 kV

Calibration lines

Calibration lines for tamoxifen and metabolites were computed using the ratio of the peak area of analyte and internal standard by using weighted (1/x) linear regression analysis.

The parameters of each calibration line were used to compute back-calculated concentrations and to obtain values for the QC samples and unknown samples by interpolation.

METHOD VALIDATION

Linearity

The linearity of the assay for tamoxifen and all metabolites was tested by injecting three different concentrations of each analyte in duplicate that were all higher than the highest calibration concentration up to twice that concentration. A coefficient of variation (cv) ≤ 15% was considered acceptable. Accuracy was expressed as [(overall mean concentration/ nominal concentration) x 100%] and should be within the range from 90-110%.

Limits of detection and LLQ

The detection limit and lower limit of quantification (LLQ) were established by injection of 6 different low concentrations of each analyte measured on 6 separate days. A coefficient of variation ≤ 20% for inter-assay variation was considered acceptable. Accuracy was expressed as [(overall mean concentration/ nominal concentration) x 100%] and should be within the range from 80-120%.

Accuracy and precision

Intra-assay variation and the accuracy of the method were established by measuring serum samples enriched with analytes at low and high concentrations (6 samples of each concentration in a single run). The inter-assay variation and accuracy were established by measuring the lowQC and highQC and the calibration line on 6 different days. The concentration of each sample was calculated using the calibration lines. A coefficient of variation ≤ 10% for intra-assay variation and ≤ 15% for inter-assay variation was considered acceptable. Accuracy was expressed as [(overall mean concentration/ nominal concentration) x 100%] and should be within the range from 90-110%.

Matrix effect and recovery

The influence of the matrix was tested by determination of the accuracy of tamoxifen and metabolites in serum samples of 6 different donors spiked with low and high concentrations of tamoxifen and metabolites. A coefficient of variation ≤ 10 % was considered acceptable.

The recovery of all analytes (including deuterated internal standards) was determined by comparing the response of replicate (n=6) blank donor serum, spiked with high and low analyte concentrations before extraction with ZnSO4 and acetonitrile, with the response of analytes from post-extracted blank donor serum spiked with equivalent concentrations.

Stability

Three replicate lowQC and three highQC samples were analyzed at different time points after storage at room temperature (bench top) exposed to daylight, at 4 °C and at -20°C at 1, 3, 6, 13, 20, 41 and 65 days, respectively. Analytes were considered stable when 90-110% of the initial concentration was recovered. In-vial stability was tested at 20°C for 24 hours.

Suitability

Suitability of the method was tested by analyzing serum samples of patients using tamoxifen obtained at a time point reflecting trough levels at steady state (> 2 months of tamoxifen use) of tamoxifen and all metabolites.

RESULTS

Chromatography and detection

All metabolites with the same parent and fragment ion m/z detected in multiple-reaction-monitoring (MRM) mode could be separated under the chromatographic conditions described, except for 3OHTam and 4OHTam (retention time = 2.72 min; Table 4.1 and Figure 4.1). It was also possible to discriminate E- and Z- isomers of the approximately 1:1 mixture of E- and Z-endoxifen obtained from Toronto Research Chemicals Inc. As we know that in patients only Z-endoxifen exists because of the administration of pure Z-tamoxifen, the peaks with retention times of 2.51 and 2.75 min could be assigned to E- and Z-endoxifen respectively. Changing the mixture of solutions A and B in the mobile phase to 30% and 70% resulted in different retention times for 3OHTam (3.92 min) and

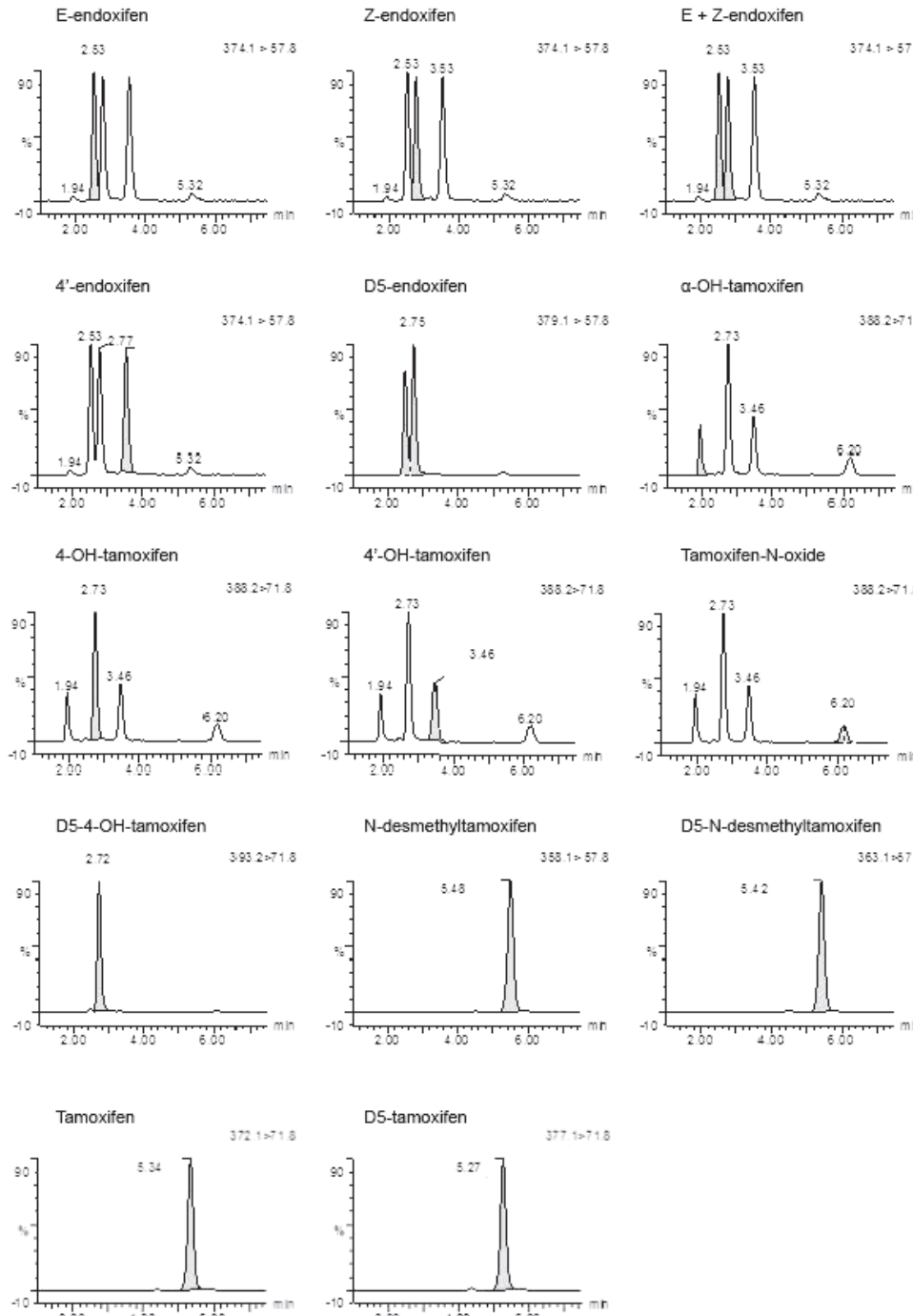

Figure 4.1 Chromatograms for all analytes and internal standards.

4OHTam (3.74 min), but caused elongation of the total retention time needed to elute all analytes and caused co-elution of other metabolites. In the first 4 patient samples analyzed, 3OHTam was hardly detectable and was considered not relevant in patients. We therefore assumed that the peak in the mass chromatogram will only reflect 4OHTam, despite the similar retention time of 3OHTam, and could still be used for accurate quantification.

Linearity

Coefficients of variance of intra-assay variation experiments were all below 15%. Accuracy was within the 90-110% range for tamoxifen and all metabolites except for TamNox (accuracy 69.0 to 85.1%) and 4'endoxifen (40 µg/L: accuracy 89.3%).

Limits of detection and LLQ

The LLQ for all analytes was below the lowest calibration standard except for TamNox. The LLQ were as follows: E-endoxifen: 0.25 µg/L; Z-endoxifen: 0.25 µg/L; E + Z-endoxifen: 0.50 µg/L; 4'-endoxifen: 0.50 µg/L; αOHTam: 0.25 µg/L; 4OHTam: 0.25 µg/L; 4'OHTam: 0.25 µg/L; NDMTam: 10.0 µg/L; tamoxifen: 5.0 µg/L.

Accuracy and precision

Coefficients of variance of intra-assay variation experiments were all below 10% except for TamNox (1.00 mg/L: cv=21.8%); accuracy was within the 90-110% range for tamoxifen and all metabolites except for TamNox (10 µg/L: 83% accuracy). Coefficients of variance of inter-assay variation experiments were all below 15% except for TamNox (2.50 µg/L: cv=22.6%; 20.0 µg/L: cv=19.7%); accuracy was within the 90-110% range for tamoxifen and all metabolites except for TamNox (2.50 µg/L: 83.1% accuracy; 20.0 µg/L: 75.2% accuracy).

Matrix effect and recovery

An acceptable effect of matrix on analytes was found with coefficients of variance below 10% except for TamNox at low and high concentration (1.00 mg/L: cv=26.6%; 10.00 mg/L: cv=11.2%) and αOHTam at low concentration (1.00 mg/L, cv=10.2%).

Recovery of all analytes was between 73.1 and 85.5% with coefficients of variance below 15%, except for TamNox (1.00 mg/L: cv=20.6%, 110% recovery).

Stability

At -20°C all analytes were stable (accuracy within 90-110% range of the initial concentration) up to 65 days, except for αOHTam (lowQC: 87.2%) and TamNox (lowQC: 76.2%; highQC: 77.0%), At room temperature and at -4°C tamoxifen, NDMTam, 4OHTam and endoxifen were stable (accuracy within 90-110% range) up to 41 days. In-vial stability at 20°C for 24 hours was acceptable for all analytes.

Suitability

The suitability of the assay was demonstrated by analysis of steady-state serum through concentrations of 664 early breast cancer patients receiving 20 mg tamoxifen once daily, who were enrolled in the CYPTAM study (NTR1509). The mean serum concentrations (SD) of tamoxifen, NDTam, 4OHTam and Z-endoxifen were 116 µg/L (45); 216 µg/L (80); 2.0 µg/L (0.9); 11.0 µg/L (5.9) respectively and were globally similar to serum concentrations previously reported.[18] The E-isomer of endoxifen was not detected in patients.

DISCUSSION AND CONCLUSION

Here we describe a high performance LC-MS/MS method which accurately detects and quantifies tamoxifen, the main active metabolites 4OHTam and endoxifen and the most abundant primary metabolite NDMTam. All metabolites with equal mass-to charge ratio and fragmentation pattern were chromatographically separated, except for 3OHTam. Nonetheless, this resulted in accurate detection and quantification of 4OHTam, as 3OHTam was hardly detectable in patients and therefore would not largely interfere with the quantification of 4OHTam (Table 4.1 and Figure 4.1). The metabolites αOHTam and TamNox were identified, but could not be adequately quantified, because of lack of linearity (αOHTam, TamNox), lack of accuracy and precision (TamNox), inacceptable matrix effect and bad recovery (TamNox) and analyte instability (αOHTam, TamNox). Suitability of the method was only tested for tamoxifen, NDMTam, 4OHTam and Z-endoxifen. The inactive metabolites 4'endoxifen (lack of linearity) and 4'OHTam were not quantified.

Previously, high performance liquid chromatography with fluorescence detection,[19-22] micellar liquid chromatography[23, 24] and LC-MS/MS methods to detect tamoxifen and its metabolites have been described in both plasma[25-31] and serum.[18, 32] In human serum two LC-MS/MS methods have been previously described to detect and quantify tamoxifen, 4OHTam, NDMTam, tamoxifen-N-oxide (TamNox) and endoxifen.[18, 32] In a previous study the inability to separate endoxifen from 4'endoxifen

and 4OHTam from 4'OHTam resulted in a factor 2-3 overestimation of endoxifen and 4OHTam serum concentrations.[32, 33] This exemplifies the importance of chromatographic separation of metabolites and isomers with the same molecular mass and fragmentation pattern for selective analysis. The mean 4OHTam and endoxifen serum concentrations in our study resemble the mean serum levels of these metabolites found in another study where chromatographic separation from 4'OHTam and 4'endoxifen was also feasible.[18]

This LC-MS/MS is now successfully used in several prospective studies in the Netherlands and Belgium (CYPTAM study: NTR1509 and CYPTAMBRUT-2: NCT00965939and CYPTAMBRUT-3: NCT00966043). If endoxifen serum concentrations will prove to predict tamoxifen efficacy, our LC-MS/MS method may be used for therapeutic drug monitoring.

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