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An UPLC–MS/MS method for separation and accurate quantification of tamoxifen and its metabolites isomers

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ABSTRACT

A selective and accurate analytical method is needed to quantify tamoxifen and its phase I metabolites in a prospective clinical protocol, for evaluation of pharmacokinetic parameters of tamoxifen and its metabolites in adjuvant treatment of breast cancer. The selectivity of the analytical method is a fundamental criteria to allow the quantification of the main active metabolites (Z)-isomers from (Z)′-isomers. An UPLC–MS/MS method was developed and validated for the quantification of (Z)-tamoxifen, (Z)-endoxifen, (E)-endoxifen, Z′-endoxifen, (Z)′-endoxifen, (Z)-4-hydroxytamoxifen, (Z)-4′-hydroxytamoxifen, N-desmethyl tamoxifen, and tamoxifen-N-oxide. The validation range was set between 0.5 ng/mL and 125 ng/mL for 4-hydroxytamoxifen and endoxifen isomers, and between 12.5 ng/mL and 300 ng/mL for tamoxifen, tamoxifen N-desmethyl and tamoxifen-N-oxide. The application to patient plasma samples was performed.

1. Introduction

Tamoxifen (Z-1-(p-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene; TAM) is a non-steroidal selective estrogen receptor modulator (SERM). TAM is a competitive antagonist of estrogen receptor activity, which inhibits the estrogen-dependent growth and proliferation pathway in epithelial breast cancer cells. TAM is the main drug used to treat women with estrogen receptor (ER)-positive tumors. As an adjuvant, TAM provides significant clinical benefits in pre- and postmenopausal patients with early-stage breast cancer, prolonging survival and significantly reducing the incidence of recurrences. The most commonly used administration schedule is 20 mg TAM daily for 5 years. However a significant number of patients (30–50%) experience disease recurrence or progression during TAM therapy and despite a good overall tolerability profile subsequently died of disease highlighting the individual differences in response to TAM.

TAM is extensively metabolized by the human cytochrome P450 enzymes into several metabolites resulting from its N-demethylation by the cytochrome *CYP3A4/5* enzyme and hydroxylation by the cytochrome *CYP2D6* enzyme. The Z-endoxifen and Z-4-hydroxytamoxifen (4-HOTam) resulting metabolites have been shown to have antiestrogenic activities which are 30- to 100-fold more potent than TAM [1–4]. Several publications report that poor functionality/activity of the human cytochrome *CYP2D6* leads a decrease in the plasmatic concentration of TAM and its active metabolites (Z-endoxifen and Z-4-HOTam), and it was suggested that genetic polymorphisms of cytochrome *CYP2D6* increase the risk of breast cancer recurrence in patients receiving TAM as an adjuvant therapy [5,6]. However, the relationship between *CYP2D6* genotype and TAM treatment efficiency is still the subject of debate and will not be definitively established until results of a properly prospective clinical trial are obtained [7–9].

Methods for the quantification of TAM and its related metabolites during clinical studies and therapeutic monitoring have been largely developed in the past decade. Teunissen et al. [10] have reported an overview of the LC–MS, LC–MS/MS and UPLC–MS/MS methods developed for quantification of TAM and its metabolites in a biological matrix during metabolism studies or pharmacokinetic applications. Most of these studies focus on the quantification of the more therapeutically active metabolites: 4-HOTam and Z-endoxifen without verification of their method selectivity for the 4′-hydroxytamoxyfen (4′-HOTam) and Z′-endoxifen isomers [11–13]. Therefore, this lack of selectivity could introduce a bias in

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the plasmatic concentrations obtained for these metabolites. The use of LC or UPLC tandem mass spectrometry theoretically offers a highly specific technique for quantification. Nevertheless, the resolution of TAM metabolites showing the same ionization and transition pattern has to be verified as done by Jaremko et al. [14]. Jager et al. [15] report that the lack of selectivity could induce large discrepancies in the reported concentrations average for endoxifen and 4-HOTam. And yet, even in the more recent LC–MS/MS reports [16], this selectivity criterion is not always completely satisfied. Murdter et al. [17] recently reported the concentration levels of a panel of phase I and II metabolites of TAM observed after 6 months of treatment determined with HPLC–MS/MS method but analytical results and validation were not reported in detail. Our aim was to combine plasmatic concentrations of TAM and its phase I metabolites with the pharmacogenetic characteristics of a large number of patients (i.e. 1000 inclusions). To this end, we developed and validated a rapid and selective UPLC–MS/MS method for the quantification of TAM, Z-endoxifen, 4-HOTam, Z′-endoxifen, 4′-HOTam, N-demethyltamoxifen (N-DMTam), including also tamoxifen-N-oxide (Tam-N-ox). The method was then applied to clinical samples.

2. Experimental

2.1. Chemicals

Z′-endoxifen, Z-4-HOTam, Z-4′-HOTam, Z-Tam-N-oxide, and Z-N-DMTam were obtained from Toronto Research chemicals (North York, Canada), d^5 tamoxifen was purchased from Alsachim (Illkisch; France). Endoxifen (E:Z, 1:1 mixture), acetonitrile (HPLC grade), isopropanol and hexane (chromasolv for HPLC quality), dichloromethane and formic acid were purchased from Sigma–Aldrich (St-Quentin Fallavier, France) and methanol from Sharlau (Barcelona, Spain). Ultrapure water was prepared with a Milli-Q System (Millipore Corporation, Molsheim, France). Human plasma was obtained from "Etablissement Français du Sang" (CHU Purpan, Toulouse, France). Minispike filters (EDGE, 13 mm, nylon 0.22 μm) were purchased from Waters (St-Quentin en Yvelines, France).

2.2. UPLC–MS/MS quantification

2.2.1. MS detection

A Waters Acquity UPLC MS/MS composed of UPLC Sample Manager coupled to a Waters TQ Detector (Waters, St Quentin en Yvelines, France) was used for the quantitative analysis.

The UPLC system consisted of an Acquity UPLC® separation module (Waters, Milford, Connecticut, USA) controlled by MassLynx 4.1 software and the QuantiLynx application was used for quantification. Detection was performed by the mass spectrometer Acquity detector with electrospray ionization (ESI) in positive ion mode. The mass spectrometer was used in the multiple-reaction monitoring (MRM) mode, MS collision parameters were summarized in Table 1. The temperature of the ESI source during the run was respectively set at 148 °C (for the source) and 349 °C (for the desolvation gas). The gas flow of the cone was set at 1 L/h and the gas flow of the desolvation was set at 649 L/h.

2.2.2. LC analysis

The chromatographic separations were performed on an UPLC BEH C18 1.7 μm of 2.1 × 100 mm Column (Waters, Milford, MA, USA) thermostated at $T = 50$ °C. LC eluent consisted in a gradient of phase A (2 mM ammonium formate acidified with formic acid (0.1%, v/v) and phase B (acetonitrile acidified with formic acid (0.1%, v/v)) at a flow rate of 0.3 mL/min. Phase B, initially set at 35% increased linearly to 65% over 3.5 min, then phase B was decreased to the initial conditions over 0.5 min and the system was re-equilibrated for 2 min before the following injection. The autosampler was thermostated at 10 °C, volumes of 5 μL were injected into the UPLC with a run time duration of 6 min.

A needle wash solution containing a mix of methanol, isopropanol, water, acetonitrile (1/1/1/1, v/v/v/v) and 0.1% formic acid (v/v) was used to ensure needle cleanup in between each point of analysis; the seal wash solution was a combination of acetonitrile/water (1/3, v/v) solution.

Table 1
MS collision parameters.

Compounds	Parent (*m*/*z*)	Daughter (*m*/*z*)	Collision (V)
E-endoxifen	374.22	57.99	32
Z-endoxifen	374.22	57.99	32
Z′-endoxifen	374.22	57.99	32
4-HOTam	388.22	71.97	38
4′-HOTam	388.22	71.97	38
Tam-N-ox	388.22	71.97	38
TAM	372.22	71.97	50
N-DMTam	358.22	57.99	30
d^5-TAM	377.22	72.04	32

Abbreviations: HOTam: hydroxytamoxifen, Tam-N-ox: tamoxifen-N-oxide, TAM: tamoxifen, N-DMTam: N-desmethyltamoxifen, d^5-TAM: deutered tamoxifen.

2.3. Standard solution for calibration

Standard solutions were prepared from stock solutions (stored at −20 °C) of TAM or metabolites in methanol. The concentrations of stock solutions were 5 mg/mL for TAM, N-DMTam, Tam-N-ox, 4-HOTam and endoxifen (Z/E); 2.5 ng/mL for 4′-HOTam and 1 ng/mL for Z′-endoxifen and 5 mg/mL for d^5 tamoxifen (d^5-TAM).

These stock solutions were diluted to prepare pooled calibration solutions in plasma at 1250 ng/mL and 20 ng/mL for 4-, 4′-HOTam and E-, Z-, Z′-endoxifen and 500 ng/mL for TAM, N-DMTam and Tam-N-ox. Calibration solutions were then diluted in blank plasma to prepare calibration points with concentrations ranging from 0.5 to 125 ng/mL for E-, Z-, Z′-endoxifen and 4-, 4′-HOTam and from 12.5 to 300 ng/mL for N-DMTam, Tam-N-ox and TAM. A working solution of d^5-TAM at 500 ng/mL was prepared in methanol, and added at a final concentration of 25 ng/mL as an internal standard (IS) in each plasma sample before extraction for the quantification of all compounds.

2.4. Validation protocol

Calibration samples and quality control (QC) points at low, medium, and high concentrations in the validation intervals were prepared in blank plasma from independent dilutions of TAM or metabolites in plasma and used for determination of linearity, precision and accuracy of the method. Calibration curves were constructed by correlating peak area ratio for each compound (*versus* d^5-TAM used as internal standard) as a function of the concentration of the spiked standard solutions. Calibration points were set to 0.5, 1, 5, 10, 20, 31.25, 62.5 and 125 ng/mL for E-, Z-, Z′-endoxifen, 4-HOTam, and 4′-HOTam and 12.5, 25, 50, 75, 150, 225 and 300 ng/mL for TAM, N-DMTam, and Tam-N-ox. Regression analysis was performed with weighting $1/X$ which gave the best fitting.

Calibrations started from 0.5 ng/mL for endoxifen and HOTam isomers, which can be taken as the LLOQ, and 12.5 ng/mL for other compounds, which is much higher than the LLOQ (signal to noise ratio respectively higher than 5000 for TAM and NDM-Tam and higher than 1000 for Tam-N-ox), this range was chosen according to the levels of concentration expected in patients.

The choice of the number of QC levels was determined by the range of the concentrations used for calibration for each analyte.