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Sensitive HPLC–PDA determination of tamoxifen and its metabolites N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in human plasma

Marina Venzon Antunes[a,b,*], Daniela Dornelles Rosa[b,c], Tamyris dos Santos Viana[a], Huander Andreolla[c], Tiago Ozelame Fontanive[b], Rafael Linden[a]

[a] Instituto de Ciências da Saúde, Universidade Feevale, Novo Hamburgo, Brazil
[b] Programa de Pós-graduação em Ciências Médicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[c] Hospital Fêmina de Porto Alegre, Grupo Hospitalar Conceição, Porto Alegre, Brazil

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ABSTRACT

A highly sensitive HPLC–UV method for the simultaneous determination of tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in human plasma samples was developed and validated. The method employs a two step liquid–liquid extraction and a reversed phase separation on a Hypersil Gold® C18 column (150 mm × 4.6 mm, 5 μm) with isocratic elution. Mobile phase was a mixture of triethylammonium phosphate buffer 5 mM pH 3.3 and acetonitrile (57:43, v/v). Total analytical run time was 16 min. Precision assays showed CV % lower than 10.53% and accuracy in the range of 93.0–104.2%. The lower limits of quantification (0.75–8.5 ng ml^{-1}) are adequate to measure clinically relevant concentrations in plasma samples. The method was successfully applied to 110 clinical plasma samples. Median plasma levels and interquartile range were: tamoxifen 55.77 ng ml^{-1} (38.42–83.69 ng ml^{-1}), N-desmethyltamoxifen 124.83 ng ml^{-1} (86.81–204.80 ng ml^{-1}), 4-hydroxytamoxifen 1.09 ng ml^{-1} (0.76–1.53 ng ml^{-1}) and endoxifen 6.18 ng ml^{-1} (4.17–8.22 ng ml^{-1}). The procedure has adequate analytical performance and can be employed in therapeutic drug monitoring of tamoxifen or pharmacokinetics studies.

1. Introduction

Tamoxifen (TAM) [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene] is a non-steroidal anti-estrogen drug widely used in the treatment of breast carcinoma and, to a lesser extent, to the prevention of breast cancer [1]. TAM therapy has a significant impact on the survival rates of women with breast cancer, reducing mortality by approximately 31% [1] and recurrences by 50% [2].

TAM is a prodrug since some of its metabolites are more biologically active than the parent compound [3]. Cytochrome P450 enzymes CYP 3A4/3A5 metabolize TAM to N-desmethyltamoxifen (NDT), which is further transformed, mainly by CYP2D6, to the most abundant active metabolite, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen, EDF). Another important product of TAM metabolism is 4-hydroxy-tamoxifen (HTF) [4]. HTF and EDF have about 100-fold higher antiestrogenic effects than TAM. However EDF is considered the major active metabolite since its plasma concentrations are approximately 6 times higher than of HTF [3,5,6]. The structures of TAM and its major metabolites are presented in Fig. 1.

Besides the wide use of TAM in breast cancer treatment, there is substantial inter-patient variability in both the development of resistance to TAM therapy and the occurrence of adverse reactions. One of the possible explanations for this phenomenon is that activity and side effects of TAM may be attributed not only to the concentration of the parent drug but also to the levels of its metabolites and their accumulation in plasma [3]. Due to their high affinity to estrogen receptors, HTF and EDF are believed to give a major contribution to the effects of TAM. Side effects, however, may be caused either by TAM and its demethylated metabolite NDT, which are present in plasma in concentrations 50–135 times higher than HTF and EDF [7].

Plasma concentrations of EDF are highly variable among patients, mainly due to the polymorphic distribution of CYP2D6 activity, resulting in a range of metabolic activity phenotypes, and to the effect of enzyme activity modulators [8]. Several studies suggested that reduced levels of EDF are related to worse prognosis, including higher recurrence rates and mortality [4,9,10]. Thus, periodic monitoring of EDF plasma levels could be considered as a useful alternative in the assessment of TAM therapy.

The widespread use of TAM and the interindividual variability in therapeutic response stimulated efforts to develop routine assays

* Corresponding author at: Rodovia RS 239, n. 2755, CEP 93352-000, Novo Hamburgo-RS, Brazil. Tel.: +55 51 35868800; fax: +55 51 35868800.
E-mail address: marinaantunes@feevale.br (M.V. Antunes).

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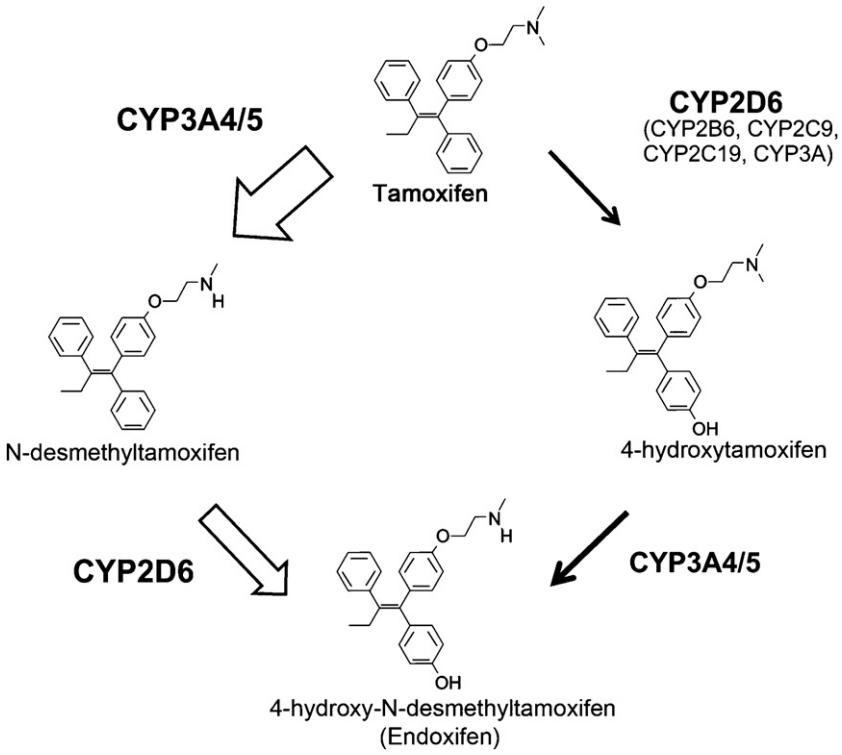

Fig. 1. Main metabolic pathways of tamoxifen, including formation of 4-hydrohytamoxifen, N-desmethyltamoxifen and endoxifen.

for quantitation of the drug and its metabolites in human plasma. Various publications reported analytical methods for the analysis of TAM and its phase I metabolites [3,11–20]. Gas chromatography with mass spectrometry detection (GC–MS) is highly specific, however time consuming since TAM and metabolites are non volatile and a derivatization step is necessary [11,20]. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is a sensitive and highly specific method that has been successfully using the measurement of TAM and metabolites in biosamples [3,8,12,18,21,22]. However, LC–MS/MS requires expensive equipment that is generally not available in many routine laboratories, especially in developing countries.

A cost-effective and more accessible approach is the use of high performance liquid chromatography (HPLC) coupled to fluorescence (FL) or ultra-violet (UV) detection [13,14,16,17,19,23]. These systems are free from matrix ionization effects observed in LC–MS/MS, however requiring complete chromatographic separation of the analytes and matrix components [24]. Fluorescence detection involves pre- or post-column photochemical conversion of TAM and its metabolites to fluorescent phenanthrene derivatives. Besides the high sensibility obtained in this approach, the method is labor intensive and requires special instrumentation [16,17,19,23,25]. HPLC–UV is a relatively low cost system, which allows robust and reproducible measurements [24] at acceptable sensitivity once proper sample preparation is employed. However, currently there is no report of a HPLC–UV method for the simultaneous determination of TAM, NDT, HTF and EDF at the concentration levels usually found in clinical samples. Additionally, the specificity of the UV detection can be further improved with the use of a photodiode array detector (PDA), allowing spectral comparisons and peak purity evaluation, what is especially useful in the presence of other drugs [26].

In the present work we developed and validated a sensitive HPLC–PDA assay for TAM and its main metabolites NDT, HTF and EDF, with acceptable performance to support clinical studies and therapeutic drug monitoring of these drugs in breast cancer patients.

2. Experimental

2.1. Chemicals

Tamoxifen, 4-hydroxytamoxifen and verapamil hydrochloride were purchased from Sigma (Saint Louis, USA). N-desmethyltamoxifen hydrochloride and N-desmethyl-4-hydroxytamoxifen were obtained from Toronto Research Chemicals (North York, Canada). Triethylammonium phosphate buffer 1 M pH 3.0 was purchased from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane was purchased from Nuclear (Diadema, Brazil). Phosphoric acid 85%, reagent grade, was obtained from Merck (Darmstadt, Germany). Methanol, acetonitrile, *n*-propanol and hexane (60% *n*-hexane), all HPLC grade, were also obtained from Merck. Ultrapure water was obtained through an Elga Purelab Ultra® apparatus from Elga Labwater (High Wycombe, UK).

2.2. Preparation of solutions and standards

Individual methanolic stock solutions of TAM, NDT, EDF, and verapamil (IS) were prepared by powder dissolution in order to obtain a concentration of 1 mg ml^{-1}. Stock methanolic solution of HTF was prepared at the concentration of 0.1 mg ml^{-1}. Working solutions were prepared combining aliquots of each stock solution and proper volumes of methanol to obtain 6 standard solutions containing the following concentrations of TAM, NDT, HTF and EDF, respectively: (1) 50, 85, 7.5 and 20 ng ml^{-1}, (2) 100, 170, 10 and 40 ng ml^{-1}, (3) 500, 850, 20 and 80 ng ml^{-1}, (4) 1000, 1700, 50 and 100 ng ml^{-1}, (5) 2000, 3400, 100 and 200 ng ml^{-1}; (6) 3000, 5100, 200 and 400 ng ml^{-1}.