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Development of a methodology to quantify tamoxifen and endoxifen in breast cancer patients by micellar liquid chromatography and validation according to the ICH guidelines

Enrique Ochoa Aranda[a,*], Josep Esteve-Romero[a], Maria Rambla-Alegre[a], Juan Peris-Vicente[a], Devasish Bose[b]

[a] Química Bioanalítica, QFA, ESTCE, Campus Riu Sec, Universitat Jaume I, 12071 Castelló, Spain
[b] Criminology and Forensic Science, Dr. H.S. Gour University, Sagar, India

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ABSTRACT

A simple micellar liquid chromatographic procedure is described to determine tamoxifen and endoxifen in plasma. For the analysis, tamoxifen and endoxifen solutions were diluted in water and UV-irradiated for 20 min to form the photocycled derivative with a phenanthrene core which shows intense fluorescence. Samples were then directly injected, thus avoiding long extraction and experimental procedures. The resolution from the matrix was performed using a mobile phase containing 0.15 mol L^{-1} SDS–7% *n*-butanol at pH 3, running at 1.5 mL min^{-1} through a C18 column at 40 °C. Detection was carried out by fluorescence, and the excitation and emission wavelengths were 260 and 380 nm, respectively. The chromatographic analysis time was 20 min. The analytical methodology was validated following the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines. The response of the drugs in plasma was linear in the 0.5–15 μg mL^{-1} range, with $r^2 > 0.99$. Accuracy and precision were <14% in both cases. Limits of detection and quantification (ng mL^{-1}) in plasma were 75 and 250 for endoxifen, and 50 and 150 in tamoxifen. The method developed herein does not show interferences by endogenous compounds. Finally the analytical method was used to determine the amount of tamoxifen and endoxifen in several plasma samples of breast cancer patients from a local hospital.

1. Introduction

Tamoxifen (TAMO; MW = 371 g mol^{-1}) or trans-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene (Fig. 1), a non-steroidal triphenylethylene selective oestrogen receptor modulator, is currently an endocrine therapeutic agent used widely for all stages of breast cancer given the complete inhibition of oestradiol binding to the high-affinity oestrogen receptor [1–3]. It has been approved by US Food and Drug Administration (FDA) for use as a chemopreventive agent in women at high risk of breast cancer [1,2], despite its side effects [4,5]. However, the drug is not effective in certain patients who respond to therapy and who eventually relapse, and side effects also vary depending on the patient [6].

The metabolism of tamoxifen plays a significant role in the drug's activity. The activity and side effects caused by ingesting the drug are due not only to TAMO, but also to its biological active metabolites [1,3,7,8]. Tamoxifen is metabolised by the cytochorome P450 enzymes *in vivo* to several compounds such as *N*-desmethyltamoxifen, 4-hydroxy-tamoxifen and tamoxifen-*N*-oxide, α-hydroxytamoxifen, *N*-didesmethyltamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen) [1,3,7–9]. Endoxifen (ENDO, MW = 373 g mol^{-1}; Fig. 1) is a hydroxylated derivative of tamoxifen which has shown strong anti-oestrogen activity that is 30–100-fold more potent than tamoxifen itself [1,3,9–11]. In fact, it has been proved that the outcomes of women with enzyme variants that cannot produce endoxifen may not be as successful with tamoxifen therapy [3]. The simultaneous determination of tamoxifen and endoxifen can provide information about the drug's activity [3].

Several methods based on HPLC have been developed for the simultaneous quantification of tamoxifen and endoxifen in incubated cells [12], serum [7,13] and plasma [2,14]. Some pharmacokinetic studies have also been performed [13]. The methodologies to study analytes in serum and sample include removing the matrix by liquid/liquid extraction with an organic solvent [2,13,14], or injecting the sample after deproteinisation by precipitation with acetonitrile [7]. Detection of tamoxifen and

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* Corresponding author. Tel.: +34 964728093; fax: +34 964728066.
E-mail address: josep.esteve@qfa.uji.es (E.O. Aranda).

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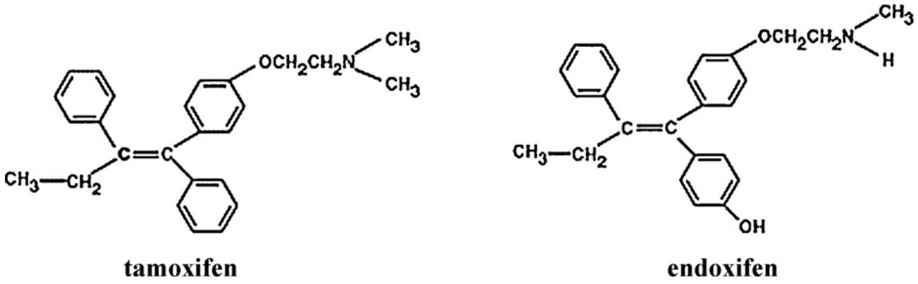

Fig. 1. Structure of tamoxifen and endoxifen.

endoxifen may be performed by mass spectrometry [7,13] and fluorescence [2,14]. In this case, the sample must be previously on-line [2,14] or off-line [1] irradiated by UV at 254 nm in order to convert tamoxifen and endoxifen into the photocyclation product (with a phenanthene core) [15], which shows intense fluorescence.

Micellar liquid chromatography (MLC) is an alternative to these methods for drug determination in physiological fluids. Micelles tend to bind proteins competitively, therefore releasing protein-bound drugs and proteins rather than precipitating into the column to be solubilised and washed away harmlessly to elute with the solvent front. This allows the direct injection of the sample, thus avoiding complex, time-consuming extraction steps. Another advantage is that micellar mobile phases are non-toxic, non-flammable, biodegradable and relatively inexpensive if compared to aqueous-organic solvents [16]. Sodium dodecyl sulphate (SDS) is a widely used anionic surfactant in micellar media given its solubility in water, its low critic micellar concentration and low cost, and it is easily removed from the chromatographic system. Several analytical methods based on micellar media using SDS and an organic modifier have been developed to determine drugs in biological fluids, such as tamoxifen in plasma [15], barbiturates in serum [17], verapamil in serum [18] and catecholamines in plasma [19].

The aim of this work is to perform a fast, easy, accurate and reliable methodology using micellar mobile phases for the simultaneous quantification of tamoxifen and endoxifen in the plasma of breast cancer patient samples. Analytes must be separated from the other compounds of the sample with high sensitivity in a short time. The proposed procedure offers the main advantage of it being associated with the use of micellar media, such as fast and easy preparation and the use of a smaller amount of pollutant organic modifier. This methodology has been validated following the ICH guidelines [20] in terms of linearity, limits of detection (LOD) and quantification (LOQ), intra- and inter-day precisions, selectivity, recovery and robustness.

2. Experimental

2.1. Reagents and solutions

Tamoxifen and endoxifen were supplied by Sigma (St. Louis, MO, USA). The other reagents used were sodium dodecyl sulphate (SDS) (Merck, Darmstadt, Germany), sodium dihydrogenphosphate monohydrate, HCl (Panreac, Barcelona, Spain), NaOH (Scharlab, Barcelona), and methanol (J.T. Baker, Holland). Ultrapure water (Millipore S.A.S., Molsheim, France) was used to prepare the aqueous solutions and mobile phases. Butanol and pentanol were purchased from Scharlau (Barcelona, Spain).

The tamoxifen and endoxifen standard solutions were prepared by solubilising the appropriate amount in ultrapure water, and the mixture was ultrasonicated for 2 min. These solutions were prepared in amber flasks to avoid the extensive photochemical degradation of the drug. Fresh solutions were prepared weekly.

Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK), and were centrifuged for 5 min at 3000 rpm at 4 °C; plasma was kept.

2.2. Apparatus

The chromatograph used was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, a degasifier for the mobile phase, an autosampler, and a temperature controller for the column module coupled to a fluorescence detector. A Kromasil 5 C18 column with a 5 μm particle size and 150 mm × 4.6 mm i.d. (Scharlab) was used. Chromatographic signals were acquired and processed with the HP ChemStation (Rev. B.01.01).

An ultrasonic bath was used to dissolve the standards (model Ultrasons-H, Selecta, Abrera, Spain) and the pH of the solutions was measured with a Crison GLP 22 (Barcelona) potentiometer equipped with a combined Ag/AgCl/glass electrode. The analytical balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland).

Samples were irradiated using a Philips original home solaria UV lamp (Philips, Eindhoven, The Netherlands).

2.3. Chromatographic conditions

Separation and quantification of tamoxifen and endoxifen were performed using a mobile phase containing 0.15 mol L^{-1} SDS and 7% (v/v) n-butanol buffered at pH 3, running at a constant flow of 1.5 mL min^{-1}. The buffer was made adding 0.01 mol L^{-1} sodium dihydrogenphosphate monohydrate, adjusting the pH by using suitable volumes of 0.1 mol L^{-1} HCl. The mobile phase was filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA). The Kromasil 5 C18 column head pressure was 150 bars. The column temperature was kept constant at 40 ± 0.5 °C. The excitation and emission wavelengths were set at 260 and 380 nm, respectively.

2.4. Experimental procedure

A volume of 100 μL of solution containing the analyte (standard or sample) was placed in a small glass vial insert, pulled point glass, 6 mm diameter (Agilent) and 100 μL of ultrapure water were added. The mixture was irradiated at 254 nm at 40 W for 20 min. Then the solution was diluted with 0.3 mL of ultrapure water, and 20 μL were injected into the chromatographic system.

3. Results and discussion

3.1. Optimisation of the experimental conditions

3.1.1. Irradiation time

Tamoxifen and endoxifen show low fluorescence so that they were converted into their corresponding phenanthrene deriva-

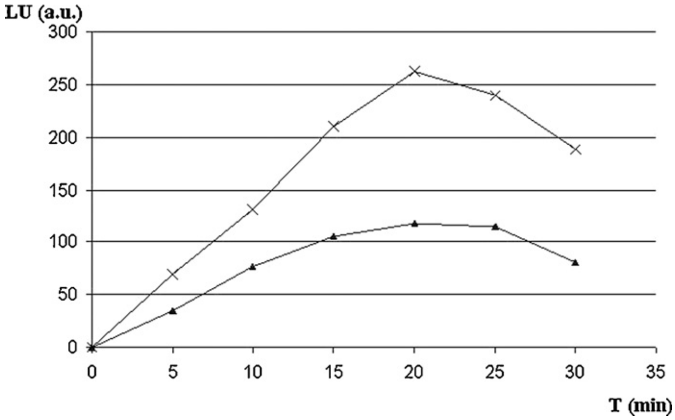

Fig. 2. First-order plots of the fluorescence for tamoxifen (×) and endoxifen (▲) versus the UV-irradiation time.

tives by UV irradiation, showing stronger sensitivity. However, this product is unstable and the irradiation time must be adjusted by considering the final yield of the reaction and the decomposition of the derivative. Tamoxifen and endoxifen standards were irradiated increasing times and the area of the corresponding peak was measured. As seen in Fig. 2, the maximum fluorescence was obtained after a 20-min irradiation.

3.1.2. Mobile phase selection

The mobile phase selection was based on the resolution of compounds and a suitable analysis time.

The octanol–water partition coefficient of tamoxifen and endoxifen are 6.64 and 6.01, respectively [21], which means that it is hydrophobic in nature and can be expected to have high retention in micellar liquid chromatography. An aqueous 0.15 mol L^{-1} SDS mobile phase buffered at pH 3 was first tried; and analytes eluted after a long time with poor resolution. Thus an alcohol was needed to improve the resolution and to lower the retention time. Using *n*-pentanol, tamoxifen and endoxifen elute too quickly and overlap with the protein band. So, *n*-butanol was selected as the organic modifier. Finally, sodium dodecyl sulphate 0.15 mol L^{-1}–7% *n*-butanol (v/v) at pH 3 was selected as the optimum mobile phase in which endoxifen eluted at 10 min and tamoxifen at almost 14 min with an adequate peak shape.

3.2. Method validation

This methodology has been validated according to the ICH Tripartite Guideline [20] which includes linearity, limits of detection (LOD) and quantification (LOQ), intra- and inter-day precisions, selectivity, recovery and robustness.

3.2.1. Selectivity

To study the matrix effects in plasma of the possible co-eluting compounds, ten blank samples were analysed (Fig. 3a). The protein band appeared before 5 min, and there were no peaks near the analytes' time. A plasma sample spiked with 10 μg mL^{-1} of endoxifen and another spiked with 10 μg mL^{-1} of tamoxifen were analysed. The peaks of the two analytes can be observed as being sufficiently separated from other peaks, which avoids overlapping (Fig. 3b).

3.2.2. Linearity and sensitivity

A blank was simultaneously spiked at nine concentration levels of endoxifen and tamoxifen in the range 0.5–15 μg mL^{-1}. The slope and intercept were obtained by a correlation between the areas of the chromatographic peak of each analyte from a triplicate injection vs. the concentration by least square linear regression. Calibration was repeated five times (preparing the sample on each occasion) over a 2-month period, and each calibration level was analysed six times. The LODs and LOQs were set as three and ten times the standard deviation of the blank, respectively. The regression curves and limits were:

$$\text{Endoxifen}: A = (1540 \pm 150)[ENDO] - (130 \pm 250)\ r^2 = 0.990$$
$$(\text{LOD} = 75\,\text{ng mL}^{-1}; \text{LOQ} = 250\,\text{ng mL}^{-1}$$

$$\text{Tamoxifen}: A = (1040 \pm 30)[TAMO] - (320 \pm 50)\ r^2 = 0.9991$$
$$(\text{LOD} = 50\,\text{ng mL}^{-1}; \text{LOQ} = 150\,\text{ng mL}^{-1}$$

where *A* is in arbitrary units and the concentration amount is provided in μg mL^{-1}.

3.2.3. Precision and accuracy

The intra- and inter-day accuracy and precision of the proposed methodology were determined for endoxifen and tamoxifen with plasma samples spiked at three concentration levels (0.5; 1.25; 2.5 μg mL^{-1}). The intra-day analysis was determined by injecting aliquots of these samples six times on the same day, while the inter-day analyses correspond to the average of five measurements of the intra-day values taken over a 3-month period. The results,

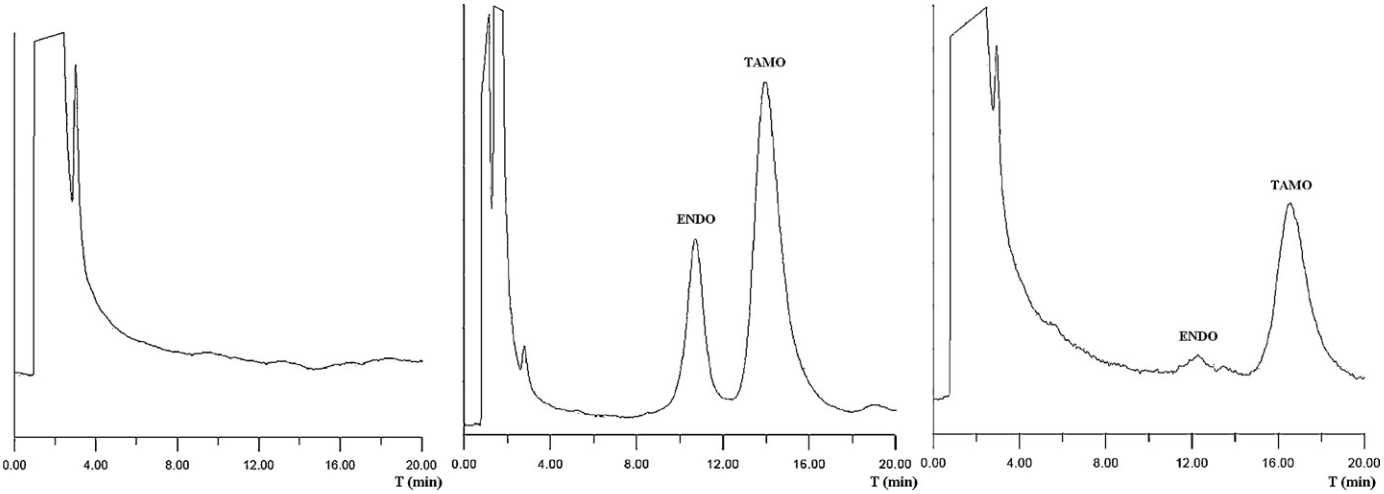

Fig. 3. Chromatograms obtained by analysing tamoxifen samples by the proposed methodology: (a) a plasma blank; (b) plasma spiked with 10 μg mL^{-1} of TAMO and 3.6 μg mL^{-1} of ENDO, (c) plasma from the breast cancer patient labelled T-4, with 3.8 μg mL^{-1} of tamoxifen and 0.15 μg mL^{-1} of endoxifen.

Table 1
Inter- and intra-day precision and accuracy.

Compound	Added concentration (μg/ml)	Found[a] (mean ± SD) (μg/ml)	Accuracy (%)	Intra-day C.V. (%)	Found[b] (mean ± SD) (μg/ml)	Accuracy (%)	Inter-day C.V. (%)
Endoxifen	0.5	0.43 ± 0.03	−14.1	7.3	0.45 ± 0.03	−9.9	8.3
	1.25	1.36 ± 0.04	+9.0	3.3	1.34 ± 0.03	+7.2	2.7
	2.5	2.46 ± 0.12	−1.7	4.9	2.37 ± 0.12	−5.2	5.4
Tamoxifen	0.5	0.478 ± 0.010	−4.3	8.5	0.47 ± 0.01	−5.8	6.1
	1.25	1.28 ± 0.03	+2.8	2.5	1.32 ± 0.05	+5.8	5.2
	2.5	2.49 ± 0.13	−0.5	6.1	2.40 ± 0.12	−3.9	5.8

[a] $n = 6$.
[b] $n = 5$.

Table 2
Evaluation of the robustness of the MLC method.

Compound	Changes in the mobile phase parameters	Level	Retention time (min) (RSD, %)	Area (arbitrary unit) (RSD, %)
Endoxifen	Concentration SDS (M)	0.145–0.155	10.2 ± 0.3 (2.9)	15300 ± 500 (3.3)
	pH	2.9–3.1	9.8 ± 0.4 (4.1)	14900 ± 300 (2.0)
	Flow (mL/min)	1.45–1.55	10.6 ± 0.5 (4.7)	15100 ± 800 (5.3)
	Butanol (%)	6.9–7.1	9.9 ± 0.3 (3.0)	15400 ± 600 (3.9)
Tamoxifen	Concentration SDS (M)	0.145–0.155	13.8 ± 0.6 (4.3)	10200 ± 400 (3.9)
	pH	2.9–3.1	13.9 ± 0.3 (2.2)	9900 ± 300 (3.0)
	Flow (mL/min)	1.45–1.55	14.5 ± 0.8 (5.5)	10400 ± 700 (6.7)
	Butanol (%)	6.9–7.1	14.1 ± 0.2 (1.4)	9800 ± 300 (3.1)

Table 3
Determination of the analyte in plasma of patients, 24 h after intake of tamoxifen pharmaceutical.

Patient	Endoxifen ± C.V. (μg/ml)[a]	Tamoxifen ± C.V. (μg/ml)[a]
T-1	n.d.	n.d.
T-2	n.d.	1.90 ± 0.09
T-3	n.d.	3.38 ± 0.12
T-4	0.15 ± 0.05	3.8 ± 0.3
T-5	0.12 ± 0.06	1.42 ± 0.07
T-6	0.12 ± 0.05	3.20 ± 0.07
T-7	0.14 ± 0.06	1.36 ± 0.05
T-8	0.12 ± 0.04	2.3 ± 0.3
T-9	0.13 ± 0.03	0.92 ± 0.05
T-10	0.41 ± 0.05	n.d.
T-11	n.d.	0.34 ± 0.08
T-12	n.d.	0.32 ± 0.05
T-13	0.27 ± 0.04	0.33 ± 0.05
T-14	0.15 ± 0.04	1.31 ± 0.11

[a] $n = 3$, n.d.: under LOD.

expressed as variation coefficients for accuracy and relative error for precision, are shown in Table 1. The data show good accuracy (<14%) and adequate precision (2.7–8.5%) for both analytes, which are useful for routine analyses.

3.2.4. Robustness

Robustness of the method was examined by replicate injections ($n = 6$) of standard solution at a concentration of 10 μg mL^{-1} of tamoxifen and endoxifen under small changes in the chromatographic parameters (SDS and butanol concentrations, pH and flow rate). Insignificant differences in peak areas and less variability in retention time were observed (see Table 2). Results indicate that the selected factors remain unaffected by small variations in these parameters: RSD was below 6.7%.

3.3. Application of the methodology to patients' plasma

Plasma studies were performed following the oral administration of a single dose of 10 mg of tamoxifen to several breast cancer patients. Samples were taken over a 24-h period. The results are shown in Table 3. Fig. 3c shows the chromatogram obtained by analysing the sample from patient T-4. Endoxifen and tamoxifen could clearly be quantified without interferences.

4. Conclusion

Micellar liquid chromatography has proved a suitable technique to analyse tamoxifen in human plasma. One advantage of this procedure is the possibility of injecting diluted sample into the chromatographic system, thus avoiding long, tedious extractions. After sample irradiation to improve sensitivity, the analyte was satisfactorily resolved using a mobile phase of 0.15 mol L^{-1} SDS–7% n-butanol at pH 3 from the matrix in an analysis time of under 20 min. Validation was performed according to the ICH guidelines with satisfactory results in terms of linearity, selectivity, precision, accuracy and recovery. The limit of detection and the lineal range were sufficient to detect the usual amount of tamoxifen and endoxifen in patients' plasma. Moreover, this method is relatively inexpensive and uses a smaller amount of toxic organic modifiers, thus making it all the more interesting.

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