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Solid-phase extraction and high-performance liquid chromatographic determination of tamoxifen and its major metabolites in plasma

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Abstract

Tamoxifen (TAM) is a triphenylethylene anti-oestrogen, commonly used in the treatment of breast cancer. Patients receiving tamoxifen therapy may experience both de novo and acquired resistance. As one of the mechanisms for this may be extensive peripheral bio-transformation of tamoxifen, there has been considerable interest in the pharmacokinetics and metabolism of tamoxifen. A reversed-phase high-performance liquid chromatography separation has been developed to determine the levels of tamoxifen and its major metabolites in human plasma. The method is highly sensitive (2 ng/ml) and selective for tamoxifen, *cis*-tamoxifen (CIS), 4-hydroxytamoxifen (4-OH) and desmethyltamoxifen (DMT). A μBondapak C_{18} 10 μm column (30 cm × 3.9 mm I.D.) was used, with a mobile phase of methanol–1% triethylamine at pH 8 (89:11, v/v). Sample preparation was carried out using a C_2 (500 mg sorbent, 3 ml reservoirs) solid phase extraction method, and extraction efficiencies were approximately 60% for TAM and its metabolites. Accuracy and precision, as determined by spiking plasma samples with a mixture of tamoxifen and its metabolites, ranged from 85–110% (± 5–10%) at 1 μg/ml, 101–118% (± 8–20%) at 0.1 μg/ml and 111–168% (± 43–63%) at 0.01 μg/ml. Results from 59 patients show mean values of 54 ng/ml for 4-OH; 190 ng/ml for DMT; 93 ng/ml for TAM and 30 ng/ml for CIS (detected in three patients only). This methodology can be applied routinely to the determination of TAM and its metabolites in plasma from patients undergoing therapy.

Keywords: Tamoxifen; Hydroxytamoxifen; Desmethyltamoxifen

1. Introduction

Tamoxifen (TAM) is a non-steroidal anti-oestrogen used in the endocrine therapy of human breast cancer [1–3]. Treatment with tamoxifen is beneficial in approximately one third of breast cancer patients [4,5], and when given as an adjuvant, tamoxifen prolongs disease-free survival and overall survival [6,7]. Although the drug is effective in many, the majority of patients appear resistant, and most patients who respond to therapy eventually relapse [8–15].

Tamoxifen is extensively metabolised, producing demethylated and hydroxylated derivatives [16–18] that can be detected in the bloodstream of treated patients [19–22]. Whilst certain metabolites are biologically active and, as in the case of 4-hydroxytamoxifen (4-OH), may have higher affinities for the

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oestrogen receptor than tamoxifen itself [23,24], others are inert and some may even have oestrogenic activity. It is thus potentially important to monitor the level of tamoxifen and its metabolites during treatment.

However, the analysis of tamoxifen is difficult due to low plasma concentrations and light instability of the target compounds [25]. To enhance sensitivity, TAM and its metabolites can be detected after photochemical activation, by which TAM, a triphenylethylene, is converted by UV radiation to a phenanthrene [26]. Thus, levels can be estimated by UV fluorescence after conversion to phenanthrene derivatives, either before, or after, thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). HPLC has been used most extensively in the detection of tamoxifen and its metabolites [13,14,27–34]. Most methods use time-consuming and labour-intensive organic extraction methods prior to HPLC [14,26,27], with efficiencies of 60–90%. In addition, some solid phase extraction methods have been employed [29,35], but efficiencies have proven to be very low (approximately 30%). On-line extraction has also been shown to be a possibility for TAM analysis [25,33]. Thus, we have developed an HPLC assay for tamoxifen, its isomer *cis*-tamoxifen and for two of its major metabolites (4-OH and DMT, Fig. 1), in plasma samples from breast cancer patients. This includes a rapid and simple solid phase extraction method that is both sensitive and selective.

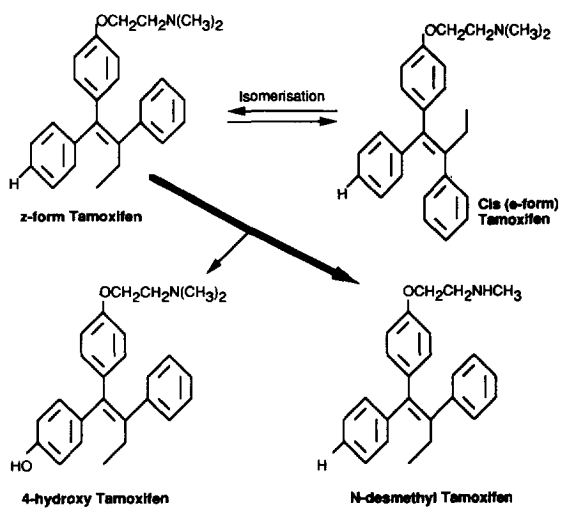

Fig. 1. Metabolism of tamoxifen to its major metabolites, 4-hydroxytamoxifen and desmethyltamoxifen, and its isomerisation to *cis*-tamoxifen.

2. Experimental

2.1. Materials

Tamoxifen base (TAM)(z-type isomer), *cis*-tamoxifen (e-type isomer, CIS) and metabolites [4-hydroxytamoxifen (4-OH) and desmethyltamoxifen (DMT)] were generously supplied by Zeneca Pharmaceuticals (Macclesfield, UK). Standard solutions were prepared in methanol (MeOH) and stored at 4°C. MeOH and acetonitrile (ACN) were of HPLC reagent grade (Rathburn Chemicals, Walkerburn, UK) and triethylamine (TEA) and sodium chloride (NaCl) were obtained from Sigma (Poole, UK). Water (dH_2O) was deionised and bi-distilled in an Analyst HP Select still (Purite, Thame, UK). Human plasma for control experiments, from post-menopausal patients presenting with breast cancer, was provided by Mr. J.M. Dixon at the Edinburgh Breast Unit (Western General Hospital, Edinburgh, UK).

2.2. HPLC

The chromatography system consisted of a Merck Hitachi L-6000 solvent delivery system (Merck, Lutterworth, UK); a Spark-Holland Basic Marathon autosampler (set to inject 20 μl); an ICT Beam Boost photochemical reaction unit (both from Crawford Scientific, Strathaven, UK); a Waters Associate Model 440 absorbance detector (set to detect 265 nm - Millipore UK, Harrow, UK) and a Hewlett-Packard HP 3394 A integrator (Bracknell, UK). The stationary phase was μBondapak C_{18} (125 Å, 10 μm) packed in a 30 cm × 3.9 mm I.D. stainless steel column with a 1-cm C_{18} guard column (Waters, Millipore UK) and the mobile phase consisted of 1% TEA (in dH_2O, pH 8.0) in MeOH (11:89, v/v). Mobile phase components were passed through a 0.22-μm filter and degassed prior to use. Elution was isocratic at a flow-rate of 1.2 ml/min at ambient room temperature, and the column life was approximately 6 months.

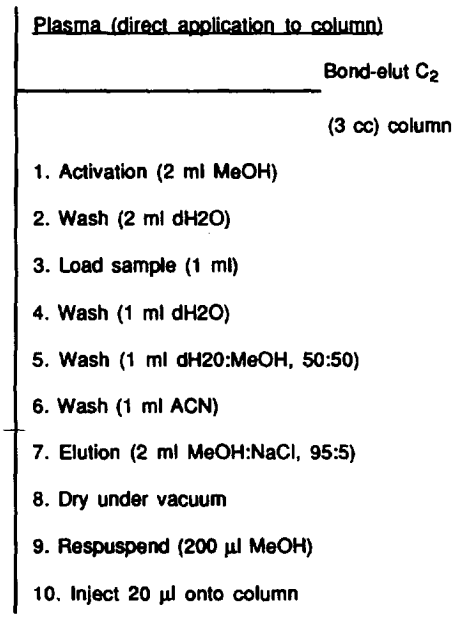

Fig. 2. Schematic representation of the solid-phase sample preparation technique used for the isolation of tamoxifen and its major metabolites from plasma (up to 1 ml).

2.3. Sample preparation

The sample preparation technique developed is shown schematically in Fig. 2. Solid phase extraction was carried out on Bond-elut C_2 3 cm^3 columns (Varian sample preparation products, Phenomenex, Macclesfield, UK). An optimum sample size of 1 ml was applied to each column. Activation and pre-sample washing were carried out using 2-ml volumes, after which, sample loading and post-sample washes were carried out using 1-ml volumes. Final elution was with 2 ml of 1 *M* NaCl in MeOH (5:95, v/v). Eluents were dried down in a Uniscience Univap centrifuge (Banbury, UK), then resuspended in 200 µl of MeOH and finally placed into 0.3 ml, 8 mm glass insert vials (Anachem, Luton, UK) for analysis.

2.4. Analysis of plasma

Blood samples taken from post-menopausal breast cancer patients were placed into Heparin-Lithium vials and spun at 834 *g* for 15 min to separate the plasma fraction. The plasma samples were then wrapped in foil and stored at −40°C or −80°C until use. Prior to extraction the plasma samples were thawed and the precipitated protein was removed by centrifugation at 469 *g* for 5 min. Preliminary analysis of patient plasma samples only was carried out, in order to compare concentration ranges with those determined by other workers. No data on tumour biological or pathological status, or response to tamoxifen therapy, was examined for correlation at this stage.

3. Results

3.1. HPLC

Method development was carried out using non-extracted standard concentrations of TAM and its metabolites, prepared in MeOH. The initial mobile phase consisted of MeOH–1%TEA (89:11, v/v), pH 12. By decreasing the pH, separation was improved, with pH 8 being chosen as the optimal pH for resolution of the four components of interest and for their separation from interfering peaks (Fig. 3.). On testing various columns; Inertsil ODS 2: 150 × 4.6 mm I.D., 5 µm; Primesphere C_{18}-MC300: 250 × 4.6 mm I.D., 5 µm (Phenomenex) and Optimus Spherisorb ODS 2: 250 × 4.6 mm I.D. (Anachem,

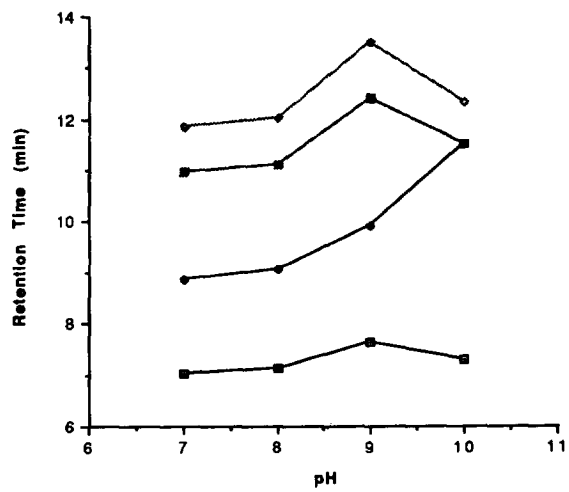

Fig. 3. Retention times (*y*-axis) for the four components 4-OH (□), DMT (◇), TAM (■) and CIS (◇) under varying mobile phase pHs (*x*-axis).

Table 1
High-performance liquid chromatography of TAM, CIS and the major metabolites 4-OH and DMT

Metabolite	Retention time		Linearity of calibration curve	Detection limit on column
	(min)	C.V. (%)	2–2000ng in MeOH (r^2)	(pg)
4-OH	6.77	1.8	0.986	40
DMT	8.20	2.9	0.999	40
TAM	10.45	2.7	0.972	40
CIS	11.32	3.1	0.952	40

Luton, UK); a μBondapak C_{18} column was found to give the best peak separation, symmetry and area to height ratios (0.4–0.6).

3.2. Sample preparation technique

The preliminary extraction method chosen resembled that detailed in Section 2, except for the elution step, which was initially carried out using MeOH. Other eluting buffers were tested, containing various counter ions (ammonium acetate, acetic acid, calcium chloride, lithium chloride and sodium chloride), with optimal elution from a 3 cm^3 C_2 Bond-elut column being achieved using MeOH–1 *M* NaCl (95:5, v/v). After experimenting with different volumes, the method was further optimised for volume of plasma extracted and for the final volume of resuspended extract. The lowest signal-to-noise ratio was achieved for extraction of 1 ml of plasma, with 200 μl of sample loaded onto the HPLC column.

3.3. Validation

Non-extracted standard curves were prepared for TAM and each of the metabolites 4-OH, DMT and CIS using seven concentrations over a range of 0.002–2 μg/ml in MeOH (0.04–40 ng on column) (Table 1). Standard curves of plasma samples spiked with mixed metabolite standards were also prepared for the same range of concentrations (Table 2). Standard curves showed good linearity with a limit of detection for all the metabolites of 2 ng/ml (40 pg on column) at a signal-to-noise ratio of 3:1.

Blank plasma samples obtained from various postmenopausal women were spiked (10 μl of mixture added to 990 μl of plasma) with a mixture of metabolites at 0.01, 0.1 and 1 μg/ml (Fig. 4). Extraction of several replicate plasma samples was carried out on four separate occasions (n=4–7) in order to calculate the extraction efficiency for each of the four components being examined (extracted amounts were compared to expected values from standard curves in methanol); between-day variation in extraction efficiency, and accuracy and precision of the assay (Table 2 and Table 3). Extraction efficiencies of approximately 60% were accomplished for each of the four species, irrespective of the concentration of the spike (Table 2).

Between-day variation in extraction efficiencies for 4-OH, TAM and CIS were consistently less than 10%, although DMT showed a higher variation (14.5%). At 1 μg/ml accuracy was between 85–

Table 2
Extraction efficiencies and detection limits for blood plasma specimens

Metabolite	Between-day		Linearity of calibration curve - spiked plasmas (r^2)	Plasma detection limit after extraction (ng/ml)
	Extraction efficiency (%)	Variation (%)		
4-OH	63	7.8	0.922	2
DMT	61	14.5	0.867	2
TAM	59	2.3	0.950	2
CIS	64	2.4	0.996	2

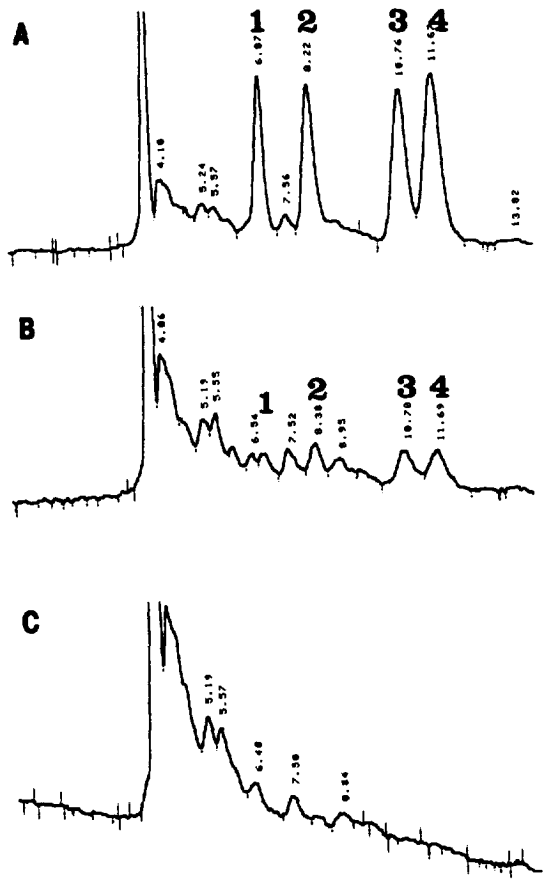

Fig. 4. Reversed-phase isocratic elution HPLC of plasma samples spiked with a metabolite mixture at (A) 0.1 μg/ml, (B) 0.01 μg/ml or (C) blank plasma. Main peaks visible are 1 = 4-OH; 2 = DMT; 3 = TAM and 4 = CIS.

109% (± 5–10%), 101–118% (± 8–20%) at 0.1 μg/ml and 111–168% (± 43–63%) at the lowest concentration (0.01 μg/ml) (Table 3). At very high detector sensitivities only, small additional peaks, with early retention times, were noted, but these did

Table 3
Accuracy and precision of the extraction procedure from blood plasma

Metabolite	Accuracy (%) ± precision (%)		
	1.0 μg/ml	0.1 μg/ml	0.01 μg/ml
4-OH	98.4±10	114±8.5	131±43
DMT	84.7±5.2	118±20	168±63
TAM	95.9±8.2	101±8.2	121±52
CIS	109.0±5.5	104±9	111±46

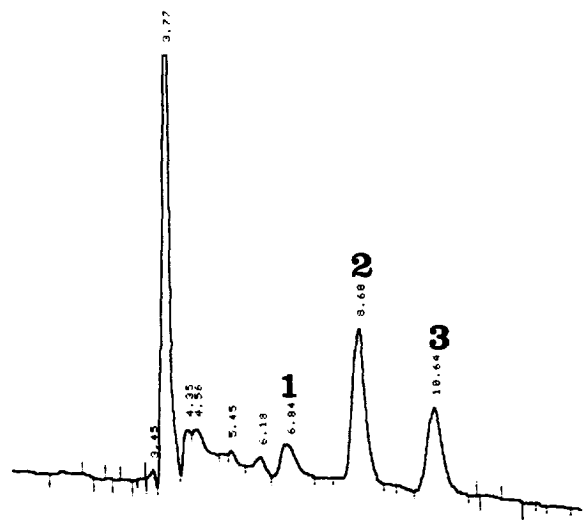

Fig. 5. An example of reversed-phase isocratic elution HPLC of plasma from a breast cancer patient undergoing tamoxifen therapy. Corresponding peaks are 1 = 4-OH (44.8 ng); 2 = DMT (119.4 ng) and 3 = TAM (67 ng).

not appear to interfere directly with any of the TAM species eluting early from the column (4-OH and DMT) (Fig. 4), although there was a tendency to overestimate extracted concentrations at these lower concentations. Stability of the stored patients' plasma samples at −40°C was checked by assaying stored, spiked samples at regular intervals (1, 2, 4, 8, 12 months and ongoing) and showed no detectable degradation.

3.4. Clinical sample analysis

Preliminary analyses were carried out, in duplicate, on two separate occasions, in the presence of blank plasma samples, spiked plasma samples and with standards in MeOH, as controls. An example of a patient's sample trace is shown in Fig. 5. Clear peaks were obtained for 4-OH, DMT and TAM, with CIS only detectable in three of the plasma samples assayed. Concentration ranges in plasma at section, from 59 patients after administration of 20 mg per day of TAM were; 4-OH = 5–200 ng/ml; DMT = 25–530 ng/ml; TAM = 20–289 ng/ml and CIS = 5–75 ng/ml (n = 3). No correlations with response to treatment, tumour biology or histology were made

at this stage, but these will be carried out in the future.

4. Discussion

Our aim in the present study was to develop an HPLC assay for the detection of TAM and its metabolites, with emphasis on speed and simplicity. To achieve this, we developed a solid-phase extraction method that was quick, noise-free and easy to use, comparing favourably with the more time- and labour-intensive organic extraction methods. Using this method, TAM and its metabolites were extracted with reliable efficiency (approx. 60%). These extraction efficiencies were superior to other solid-phase methods (approx 30%) [29,36], but were less than those achievable by traditional organic extraction methods (60–90%) [14,26–28], or by on-line extraction (80–100%) [25,33]. The limit of detection was low at 40 pg on column, equivalent to that previously achieved by various other methods [25,27,29]. The levels of TAM and DMT detected in plasma using the present method were similar to those found previously [21,36,37], although we routinely detected higher concentrations of 4-OH within our patient population. This could be due to accumulation of 4-OH over time, as many of the patients were on long-term therapy. Alternatively, these differences may simply reflect a difference in the levels of 4-OH within our patient population, although there was a tendency to overestimate metabolite levels at the lower concentrations at which 4-OH would be expected to be present. Whilst small amounts of CIS have been detected in only three of the plasma samples examined, this may represent a good opportunity for internal standardisation of our method, as opposed to the external standard method presently adopted.

In conclusion, we have developed a reversed-phase HPLC method for the detection of TAM, CIS and two major metabolites (4-OH and DMT) in plasma from patients undergoing TAM therapy for breast cancer. The methodology developed is sensitive, simple to employ, and compares favourably with other available methods. Solid-phase extraction has the advantage of speed over traditional organic extraction techniques, and achieves consistently good efficiency of extraction. It is our intention to modify this method for the detection of TAM and its metabolites in tumour tissue taken from these breast cancer patients.

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