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

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Abstract

A sensitive (200 ng/g) and selective reversed-phase high-performance liquid chromatography separation has been developed to determine the levels of tamoxifen, 4-hydroxytamoxifen (4-OH) and desmethyltamoxifen (DMT) in tumour tissue taken from patients undergoing tamoxifen therapy. A μBondapak C_{18} 10 μm column (30 cm×3.8 mm I.D.) was used, with a mobile phase of methanol–1% triethylamine at pH 9 (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 followed in individual extracts using a [^{3}H]TAM radiolabelled spike (10 000 dpm), with a range of 60–90%. Accuracy and precision (standard deviation) as determined from tumour spiked with radioinert tamoxifen and its metabolites ranged from 83.4–92.3% (±23–33%) at 20 μg/g; 85.2–87.7% (±18–23%) at 2 μg/g; 88–101% (±15–50%) at 0.2 μg/g and 63–94% (±13–24%) at 0.02 μg/g. Results from seventy-two patients show mean values (±S.D.) of 174±203 ng/g for 4-OH; 783±1326 ng/g for DMT and 410±458 ng/g for TAM, variations reflecting heterogeneity in levels between patients. This methodology can be routinely applied to the determination of tamoxifen and its metabolites in tumour tissues from patients undergoing tamoxifen therapy. © 1997 Elsevier Science B.V.

Keywords: Tamoxifen; 4-Hydroxytamoxifen; Desmethyltamoxifen

1. Introduction

Tamoxifen (TAM) is frequently used in the endocrine therapy of human breast cancer [1–3]. However, although the drug is effective in many, the majority of patients appear resistant, and most patients who respond to therapy eventually relapse [4–11].

This resistance to TAM therapy may be due to; down-regulation or loss of oestrogen receptors [12–17]; altered production of growth factors such as TGFβ [18,19]; altered signal transduction and increased expression of anti-oestrogen binding sites (AEBSs), which may directly mediate anti-oestrogen actions [20–22]. In addition, pharmacological mechanisms such as a shift in metabolism, producing isomers [11] or oestrogenic metabolites, and changes in levels of drug accumulation within tumour tissues [9,10,23] may also be important.

Although significant differences in levels of circu-

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lating TAM and metabolites have not been reported between responding and resistant tumours [24,25] TAM resistance and stimulation of growth may be associated with a reduction in tumoral TAM levels [7,9,10], with stimulation of growth on increasing TAM concentrations in a dose-dependant manner [8]. In breast cancer patients TAM and its metabolites accumulate in tumour tissue compared to serum or plasma [26–28], and tumour levels do not necessarily reflect those in the circulation. It is therefore important to measure tumour levels of TAM.

Most methods for the determination of TAM and its metabolites from tumour tissues use organic extraction [9,29]. Our aim was to develop a rapid, solid-phase extraction (SPE) method and high-performance liquid chromatography (HPLC) detection system to determine levels of TAM and its metabolites, 4-hydroxytamoxifen (4-OH) and desmethyltamoxifen (DMT). This was based on methodology developed for the determination of TAM and metabolites in plasma [25].

2. Experimental

2.1. Materials

TAM base (*z*-type isomer), *cis*-TAM (CIS, *e*-type isomer) and metabolites 4-OH and DMT were generously supplied by Zeneca Pharmaceuticals (Macclesfield, UK). [N-methyl-^{3}H]TAM was obtained from Amersham International (Little Chalfont, 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); triethylamine (TEA) and sodium chloride (NaCl) were obtained from Sigma (Poole, UK) and dimethylsulfoxide (DMSO) was analytical-grade (Fisons, Loughborough, UK). Water was deionised and bi-distilled in a Milli-U10 water purification system (Millipore, Harrow, UK). Breast tumour tissue was obtained from post-menopausal patients at surgery.

2.2. HPLC

The chromatography system consisted of a Merck–Hitachi L-6000 solvent delivery system (Lutterworth, UK); a Spark-Holland Basic marathon autosampler (set to inject 20 μl); an ICT Beam Boost post-column photochemical reaction unit (both from Crawford Scientific, Strathaven, UK); a Waters Associate Model 440 absorbance detector (set to detect 265 nm; Waters, Watford, UK) and a Hewlett-Packard HP3394 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) and the mobile phase consisted of 1% TEA (in dH_2O, pH 9.0)–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.

2.3. Sample preparation

The sample preparation technique developed is shown schematically in Fig. 1. SPE was carried out on Bond-Elut C_2 3 cm^2 columns (Varian sample preparation products, Phenomenex, Macclesfield, UK). An optimal sample size of 1 ml (in DMSO) was applied to each column. Activation and pre-sample washing were carried out using 2 ml volumes, and sample loading and post-sample washes used 1 ml volumes. Final elution was with 1 ml of 1 *M* NaCl–MeOH (5:95, v/v). Eluents were dried down under nitrogen and resuspended in 400 μl of MeOH, 200 μl of which was placed into 0.3 ml, 8 mm glass inserts (Anachem, Luton, UK) for HPLC analysis; 100-μl aliquots were placed into scintillation vials containing 10 ml of general purpose liquid scintillant (NE Technology, Edinburgh, UK) for counting of radiolabelled [^{3}H]TAM.

2.4. Analysis of tumour tissue

Tumour tissue taken from post-menopausal breast cancer patients was placed in biofreeze vials (Costar, UK) and stored in liquid nitrogen until assay. Prior to extraction, the tumour tissues (2×50 mg aliquots) were dismembrated (Mikro-dismembrator, Braun, Germany) in liquid N_2 and resuspended in DMSO (1 ml). At this point a spike of radiolabelled [^{3}H]TAM (approximately 10 000 dpm) was added to each tube to monitor extraction efficiency. Suspensions were

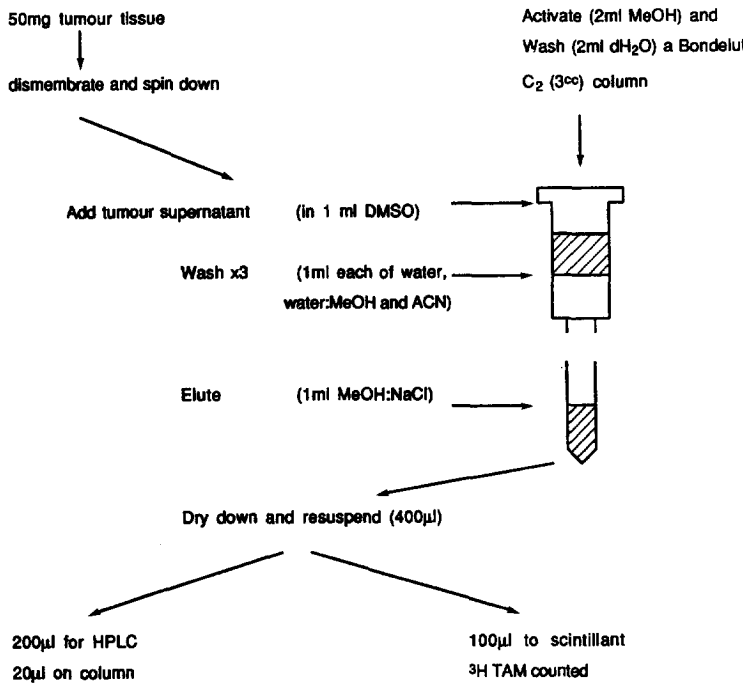

Fig. 1. Schematic representation of solid-phase sample preparation used for the isolation of TAM, 4-OH and DMT from breast tumour tissues (50 mg).

then centrifuged for 10 min at 3000 rpm to pellet particulate matter, and the supernatants were removed for SPE.

3. Results

3.1. HPLC and sample preparation technique

HPLC and sample preparation were based on methods detailed by MacCallum et al. [25]. Tumour homogenisation was initially carried out in PBS with metabolite recoveries of 20–30%. The use of MeOH improved recoveries to between 30–50%, whilst extraction in DMSO increased recoveries to 50–70%. This was improved yet further when tumour samples were dismembrated and then resuspended in DMSO prior to extraction (60–80%). Samples were applied to the extraction column in 1 ml DMSO and, after washing, eluted with 1 ml of elution buffer. A µBondapak C_{18} column was chosen after testing with various other columns (see MacCallum et al. [25]), and the mobile phase was altered to pH 9 to give optimal peak separation of tumour extracts, free from interfering peaks. At this pH, column life was approximately 6 months.

3.2. Validation

Non-extracted standard curves were prepared as detailed by MacCallum et al. [25], for TAM and each of the metabolites (Table 1). Blank tumour samples obtained from post-menopausal women not exposed to TAM treatment, were spiked with a mixture of metabolites to a concentration of 20, 2, 0.2 or 0.02 µg/g (see Fig. 2. for example). Extraction of replicate tumour samples was carried out on at least four occasions (n=4–10), to determine recovery using an external *cis*-TAM standard (Table 2) and a non-interfering [^{3}H]TAM spike. There was good correlation between calculated recoveries in any individual sample, and both methods were routinely applied to monitor individual recoveries.

Extraction efficiencies and between-day variation shown in Table 2 were calculated from spiked tumour samples (combined results from several

Table 1
HPLC of TAM, CIS and major metabolites 4-OH and DMT

Metabolite	Retention time		Linearity of calibration curve 2–2000 ng in MeOH (r^2)	Detection limit on-column (pg)
	(min)	C.V.(%)		
4-OH	6.85	3.9	0.969	40
DMT	8.74	5.0	0.977	40
TAM	11.00	6.2	0.986	40
CIS	11.82	6.7	0.997	40

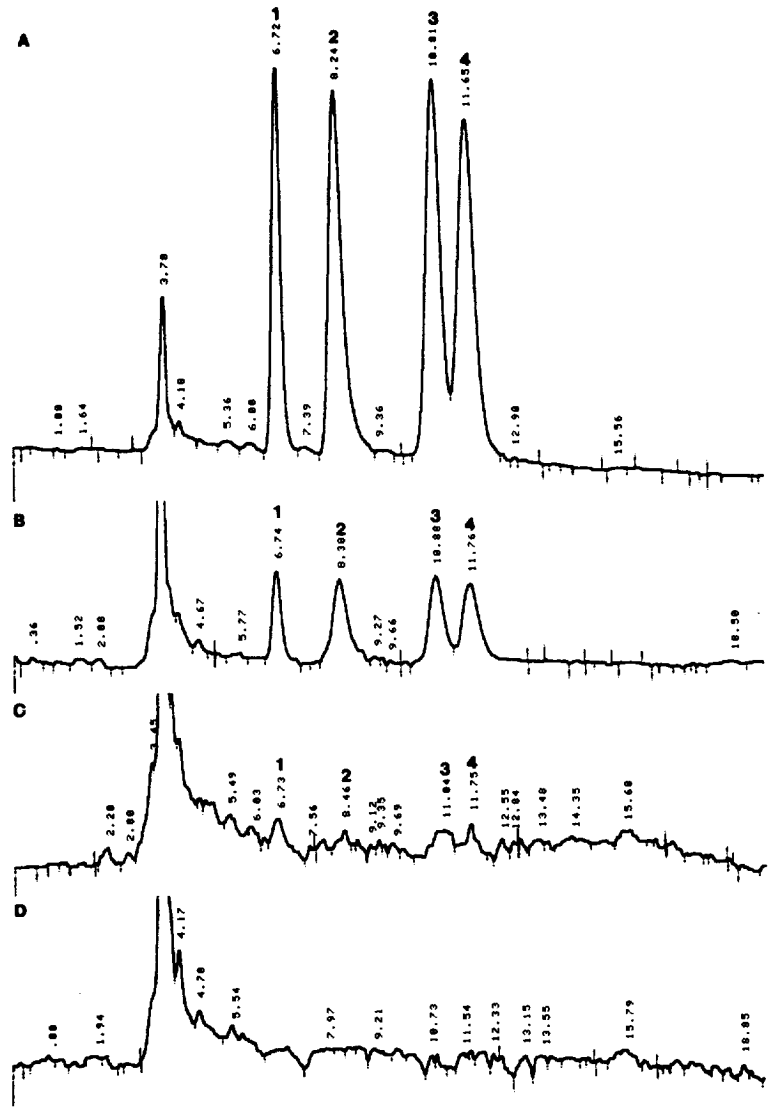

Fig. 2. Reversed-phase isocratic elution HPLC of tumour tissue samples spiked with metabolite mixture at (A) 20 μg/g, (B) 2 μg/g, (C) 0.2 μg/g and (D) blank tumour tissue. Main peaks are 1=4-OH; 2=DMT; 3=TAM and 4=CIS.

Table 2
Extraction efficiencies and detection limits for breast tissue specimens

Metabolite	Between day (n=4–10)		Linearity of calibration curve-spiked tissues (r^2)	Tumour detection limit after extraction (ng/g)
	Extraction efficiency (%)	Variation (%) (0.02–20 μg/g spike)		
4-OH	64.0	18	0.944	20
DMT	64.3	20	0.997	20
TAM	60.8	18	1.000	20
CIS	60.4	16	1.000	20

experiments). Using these values, accuracy and precision (n=4–10) were calculated at 20, 2, 0.2 and 0.02 μg/g for each of the metabolites (Table 3). Accuracy and precision for the [^{3}H]TAM spiked samples was as follows; 91.4±24% at 20 μg/g; 96.4±26% at 2 μg/g; 92.5±6% at 0.2 μg/g and 99.2±9% at 0.02 μg/g.

3.3. Clinical sample analysis

Preliminary analyses were carried out for duplicate portions of a number of tumours (n=72), in the presence of blank tumour samples, spiked tumour samples and standards in MeOH as controls. Concentration were calculated from the peak areas measured by HPLC, and normalised to 100% using individual extraction efficiencies ([^{3}H]TAM recovery). Values were then compared against the appropriate standard curve (spiked tumour samples), taking into account day-to-day alterations (monitored via spiked tumour samples), and finally expressed in terms of ng/g or tissue. An example of an HPLC trace from tumour of a breast cancer patient receiving TAM is shown in Fig. 3. Duplicate extracts of each sample were prepared±CIS as a standard. Concentration ranges in tumour tissue taken after administration of 20 mg TAM/day (3–90 months) were as follows; 0–1440 ng/g for 4-OH; 0–9230 ng/g for DMT and 0–3330 ng/g for TAM.

Table 3
Accuracy and precision of the extraction procedure from breast tissues

Metabolite	Accuracy (%)±precision (%) (n=4–10)			
	20 μg/g	2 μg/g	0.2 μg/g	0.02 μg/g
4-OH	92.3±23	85.2±23	97.0±15	78.9±26
DMT	83.5±31	87.1±18	90.2±30	62.6±13
TAM	83.4±33	87.7±22	82.8±26	89.8±24
CIS	83.8±33	85.2±23	94.9±42	93.8±21

4. Discussion

Our aim was to develop an assay for the determination of TAM and its major metabolites in tumour tissues, with emphasis on speed and simplicity. Our resulting method is quick, free from interference and easy to perform, with good sensitivity (20 ng) and selectivity. The SPE compares favourably with more labour-intensive organic extraction methods [9,29–31], and has reliable efficiency (60–80% monitored via a radioinert CIS, and radiolabelled [^{3}H]TAM spike).

Tumour levels of TAM detected were similar to those obtained using other methods [29,30], although DMT and 4-OH concentrations were in general higher than previously reported, and patterns were similar to those shown by plasma extracts. This may be caused by differences in patient populations, or may reflect the length of time for which patients were treated with TAM (data not shown). Indeed, in the present study many patients have been treated for and extended period of time (up to 90 months).

In conjunction with our method for the determination of TAM and its major metabolites from patient plasma samples [25], we have developed an HPLC assay for the determination of these compounds in tumour tissue taken from patients with breast cancer undergoing TAM therapy. It is hoped to correlate both plasma and tumour levels of TAM and its metabolites with responses in individual patients, in order to gain insight into the development of resistance to TAM therapy.

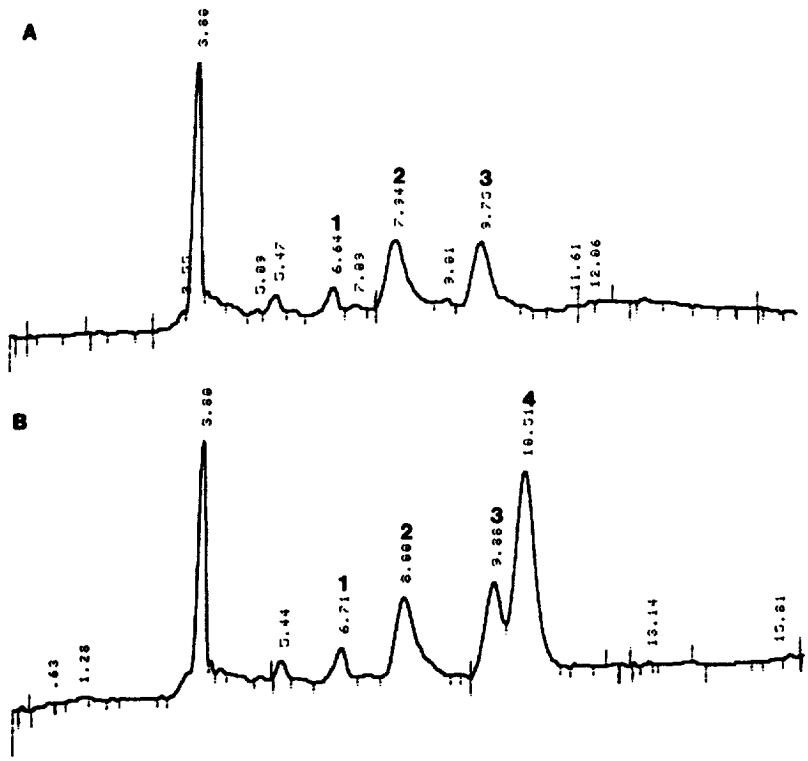

Fig. 3. Example of reversed-phase isocratic elution HPLC of tumour tissue taken from a breast cancer patient undergoing TAM therapy. (A) No CIS spike added; (B) CIS spike added to follow recovery. Corresponding peaks are 1=4-OH (94 ng); 2=DMT (265 ng); 3=TAM (312 ng) and 4=CIS. Recovery was 76.9% and 74.7% as monitored via a CIS spike and a [^{3}H]TAM, respectively.

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