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Determination of tamoxifen and five metabolites in plasma*

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Introduction

Tamoxifen is an anti-estrogenic agent widely used in the treatment of breast cancer. It is usually administered at a dose of 20–40 mg daily. Methods for the determination of tamoxifen (TX) and its major metabolite *N*-desmethyltamoxifen (DMTX) in plasma have usually been based on the conversion of TX and DMTX to their fluorescent phenanthrene derivatives followed by HPLC [1] or TLC [2]. The reaction has been carried out both pre- [1, 3] and post- [4] column. Gas liquid chromatography–mass spectrometry has also been used [5].

The majority of previous work has been carried out on TX and DMTX only. Several other metabolites of TX have been identified [6] and attention has recently focused on these. Several thousand plasma samples have been assayed for TX and DMTX in the Authors' laboratories [7, 8] using a modification of the method of Sternson. The aim of the present work was to adapt this method to allow the determination of four further metabolites of tamoxifen: 4-hydroxytamoxifen (B); *N*-desdimethyltamoxifen (Z); 4-hydroxyethoxytriphenylethylethylene (Y); and 4-hydroxytriphenylethylethylene (E): the designations B, Z, Y and E are from the literature [6]. The structures are shown in Fig. 1. The method was used to determine plasma levels of these metabolites from patients at steady state taking 30 mg TX daily and also in a study of patients taking a loading dose (160 mg) of TX before taking 30 mg daily.

Experimental

Reagents

Tamoxifen and all metabolites were gifts from Pharmachemie B.V. Ammonium hydroxide and trifloroacetic acid were AR grade, methanol was HPLC grade. Diethylether (AR grade) was redistilled before use.

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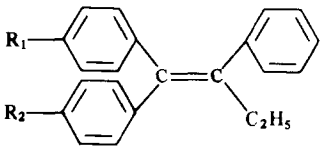

Figure 1
Structure of tamoxifen and metabolites.

Compound	R_1	R_2
Tamoxifen	$(CH_3)_2N(CH_2)_2O$	H
N - desmethyltamoxifen	$CH_3NH(CH_2)_2O$	H
Metabolite B	$(CH_3)_2N(CH_2)_2O$	OH
Metabolite Z	$H_2N(CH_2)_2O$	H
Metabolite Y	$HOCH_2CH_2O$	H
Metabolite E	HO	H

Sample preparation

A 1.0 ml aliquot of plasma was taken, made alkaline by the addition of 0.5 ml of ammonium hydroxide (1 M) and shaken for 1 h with 10.0 ml diethyl ether. The samples were centrifuged and exactly 8.0 ml of the upper ether layer was taken and evaporated to dryness under nitrogen with heating at approx. 40°C. Samples were redissolved in 300 μl of HPLC eluent. Tubes were then irradiated for exactly 15 min using a bactericidal UV source (original Hanau sterilising lamp). A 100 μl aliquot was injected onto the HPLC. A standard curve in pooled plasma over the range 5–200 ng ml^{-1} of each metabolite was run with each sample batch.

Chromatography

The column was Spherisorb ODS (10 cm). The pump was an Altex 110A; injection was either manual (Rheodyne 7125) or automated (Waters WISP). The mobile phase was methanol–water–trifluoracetic acid (80:20:0.1) at 3.0 ml min^{-1}. The detector was a Schoeffel FS970 adjusted to 256 nm (excitation) with a 340 emission cut-off filter. If only TX and DMTX were to be measured, the procedure was identical, except that 25 mM sodium pentane sulphonate was added to the HPLC eluent and acetic acid used instead of TFA.

Patient studies

Two studies were undertaken on patients with advanced breast cancer. In the first study 50 patients (who had not received TX) were given a loading dose of 160 mg, followed by a daily dose of 30 mg. In the second study 12 patients received 30 mg daily of TX for at least three months ("steady state" study). Blood samples were always taken prior to administration of tamoxifen.

Results

The method described for metabolites B, E, Y and Z has been used for over 200 samples. Good standard curves have been obtained for more than 12 sample batches. Day-to-day precision was assessed using pools of spiked control plasma. The day-to-day relative standard deviation (RSD) for metabolites E, Y, Z and B was ±9.3%, ±9.8%, ±7.5% and ±7.7%, respectively, at levels ranging from 10–40 ng ml^{-1}. The mean

plasma levels of tamoxifen and of each of the five metabolites in the two studies is shown in Table 1. Tamoxifen and DMTX were measured separately.

Discussion

Under the conditions used routinely for TX and DMTX assay the four metabolites coeluted as one early peak. It was thus necessary to expand the early part of the chromatogram. Lowering the concentration of organic modifier in the eluent gave poor peak shape. Tamoxifen has previously been shown to decrease in retention with the addition of a pairing-ion to the mobile phase [9] (the exact opposite of conventional reversed-phase ion-pair theory). All the metabolites followed the same pattern. Omission of pentane sulphonate from the eluent allowed separation of metabolites B, Z, Y and E, as well as of DMTX and TX. Metabolites E and Y were only partially resolved, but this was sufficient for quantitation at the very low levels found in patient samples. Specimen chromatograms are shown in Fig. 2. Tamoxifen and DMTX are present in much greater concentration than the metabolites and the TX peak is very broad under the chromatographic conditions used for metabolites. TX and DMTX have thus been

Table 1
Mean tamoxifen and metabolite levels (ng ml^{-1}) from patient studies

Time	TX	DMTX	E	Y	Z	B
1. Loading dose study						
1 day	91	46	<5	<5	<5	<5
1 week	92	91	<5	5	6	<5
4 weeks	114	207	14	20	27	<5
2. Steady state study	113	242	<5	26	33	<5

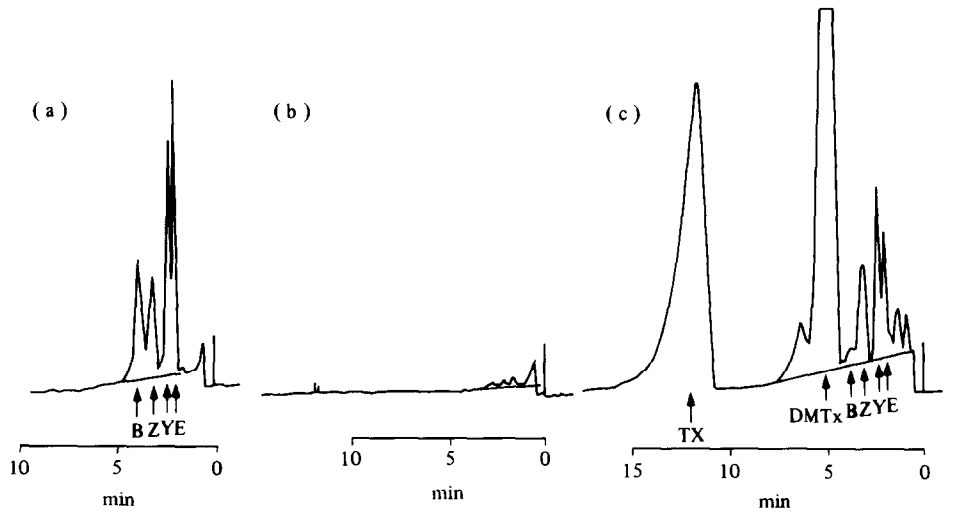

Figure 2
Specimen chromatograms (conditions in text). (a) 20 ng ml^{-1} standard; (b) blank plasma; (c) sample from patient.

assayed using the previous procedure [7, 8] and the metabolites E, Y, Z and B using the proposed HPLC conditions.

When using a new UV lamp it must be calibrated for production of phenanthrene derivatives with time. The time to maximum production of each metabolite has always been very similar. In addition, experiments to determine the yield of metabolites at different TX and DMTX concentrations showed that these did not vary significantly over the range of 0–500 ng ml^{-1}.

The new procedure has proved suitable for the assay of these four metabolites down to 5 ng ml^{-1} plasma. After 4 weeks of treatment metabolites Y and Z have reached levels of 20 and 27 ng ml^{-1}, metabolite E has reached levels of 14 ng ml^{-1}, but 4-hydroxytamoxifen (metabolite B) is still present at levels below 5 ng ml^{-1}.

The method will therefore allow further investigation of the metabolism of this important anti-cancer drug.

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