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Note

High-performance liquid chromatographic analysis of tamoxifen and major metabolites in human plasma

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Tamoxifen (ICI.46,474, Nolvadex), a synthetic non-steroidal antiestrogenic compound, is currently being used in metastatic breast cancer therapy [1]. The primary metabolic route involves ring hydroxylation yielding the 4-OH derivative (Ib) and hepatic demethylation to Ic. Both compounds Ib and Ic have some antitumor activity [2—5].

The analytical methods proposed to determine tamoxifen (Ia) and metabolites in biological fluids are high-performance liquid chromatography (HPLC) (or thin-layer chromatography) with fluorimetric detection after photocyclisation to phenanthrenes [6—9], and gas chromatography—mass spectrometry with high-resolution, single ion monitoring [10].

We found several problems in the routine use of the analytical methods reported in the literature, and therefore modified the HPLC analysis proposed by Golander and Sternson [6] in the following way: (1) internal standard (Id) added to the plasma samples; (2) extraction of Ia—d by Sep-Pak cartridges; (3) post-column on-line photocyclisation to the corresponding phenanthrenes (IIa—d).

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MATERIALS AND METHODS

Analytical standards of Ia—c were kindly supplied by Imperial Chemical Industries (Macclesfield, Great Britain) and used without further purification. The internal standard (Id) was synthesized by acetylation of Ic with acetic anhydride. Other chemicals were supplied by E. Merck (Darmstadt, G.F.R.) or Carlo Erba (Milan, Italy) and were of the highest analytical grade.

Sample preparation

Plasma samples were obtained by adding to the blood bank plasma known amounts of Ia—c, or by centrifugation of heparinised venous blood samples from advanced cancer patients undergoing tamoxifen therapy.

To the plasma samples (1 ml) were added 100 μl of a stock solution of the internal standard (Id) in methanol, and 2 ml of water—methanol (1:1). After vortexing (20 sec), the mixture was centrifuged at 5000 *g* for 5 min and the supernatant was filtered through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) previously washed with methanol (5 ml) and distilled water (5 ml).

The cartridge was then eluted with water (5 ml), water—acetonitrile (1:1) (1 ml) and acetonitrile (0.5 ml). These eluates did not contain Ia—d and were discarded.

The cartridge was finally eluted with 5 ml of 0.3 *M* phosphoric acid in acetonitrile. The eluate was concentrated under vacuum to 0.5 ml in a Vortex evaporator (Buchler Instrument, Fort Lee, NJ, U.S.A.). After the addition of 0.5 ml of 10 m*M* KH_2PO_4, 100 μl of this sample were injected into the chromatographic system.

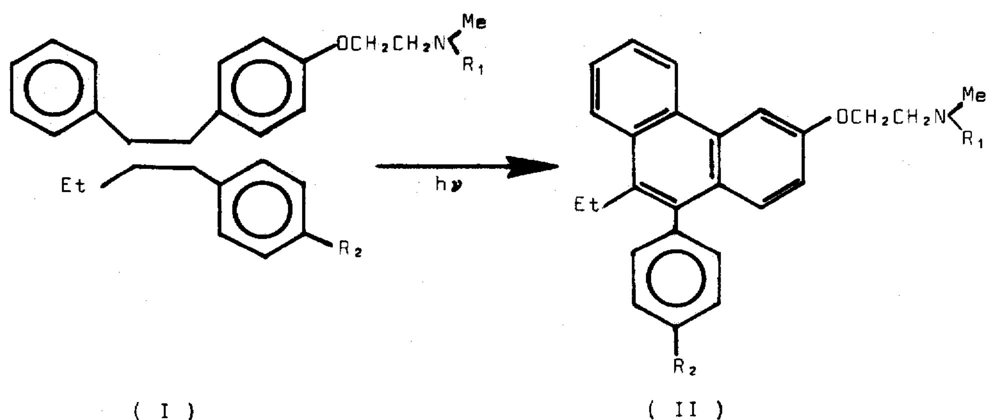
During the preliminary study of the photolysis conditions, a known amount of desipramine (10,11-dihydro-N-methyl-5H-dibenz[*b*,*f*] azepine-5-propanamine), which is naturally fluorescent in the detector conditions, was added to the plasma samples as an additional internal reference.

Chromatography and photocyclisation

The chromatographic system consisted of a Rheodyne Model 7105 injector or a Perkin-Elmer Model 420 Autosampler, a Perkin-Elmer series 3B pump, a Model 650-10S spectrofluorimeter and a Sigma-10 data system.

To the end of the chromatographic column (μBondapak CN, reversed-phase, 10 μm, 30 cm × 3.9 mm, Waters Assoc.) was connected a PTFE capillary tube (Angst+Pfister AGW-28, 0.3 mm I.D.) 5 m long, wound 10 cm away from a Philips HPK-125W high-pressure mercury lamp, mounted inside a 28 × 22 × 22 cm housing. The outlet was directly connected to the fluorescence detector set at λ_{ex} = 260 nm (slit = 10 nm), λ_{em} = 375 nm (slit = 10 nm). This setting guarantees a more favourable signal-to-noise ratio; under these conditions the minimum detectable quantity of 2a—d is less than 0.1 ng.

Photocyclisation of tamoxifen and metabolites (Ia—c), as well as of internal standard (Id), to the corresponding phenanthrene derivatives (IIa—d) (Fig. 1) and complete chromatographic separation of the fluorescent products was achieved with a mobile phase of acetonitrile—0.3 *M* H_3PO_4—10 m*M* KH_2PO_4 (190:50:280) at a flow-rate of 1.5 ml/min.



R_1	R_2	
CH_3	H	Ia, IIa
CH_3	OH	Ib, IIb
H	H	Ic, IIc
$COCH_3$	H	Id, IId

Fig. 1. Photocyclisation of tamoxifen, its metabolites and the internal standard.

Alternatively, the photocyclisation to the phenanthrenes (IIa—d) was carried out by irradiation of the samples obtained in the extraction procedure directly in the Autosampler mini-vials (borosilicate glass, Supelco). In this case, a photochemical reactor was used, consisting of a Philips HPK-125W lamp (placed in the middle of a housing, 80 × 80 × 100 mm, with a 10 × 20 mm slit) and a rotating sample holder (5 cm diameter, 6 vial holders, 60 rpm) with the centre placed 15 cm away from the slit.

RESULTS

Figs. 2a—d are typical chromatograms obtained under different photolysis conditions. Without previous photolysis and with the on-line photoreactor switched off, no peaks were observed, with the exception of desipramine (Fig. 2a), naturally fluorescent and added as internal reference. With 60 min of off-line photolysis and injection of the photocyclisation products, chromatogram 2b was obtained; retention times corresponded to the phenanthrenes (IIa—d). With no previous off-line photolysis and the on-line photoreactor on, chromatogram 2c was obtained. Chromatogram 2d corresponds to the analysis of a sample only partially photolysed off-line (20 min photolysis) and injected into the chromatographic system with the on-line photoreactor switched on. In this case, both phenanthrenes (II) and stilbenes (I) were injected.

The on-line photoreactor gives only partial conversion of Ia—d to phenanthrenes (Ia → IIa, 81.2%, S.D. = 0.45; Ib → IIb, 95.7%, S.D. = 1.8; Ic → IIc, 89.9%, S.D. = 1.3; Id → IId, 81.2%, S.D. = 0.7; mean of five determinations calculated using desipramine as the internal standard). This notwithstanding,

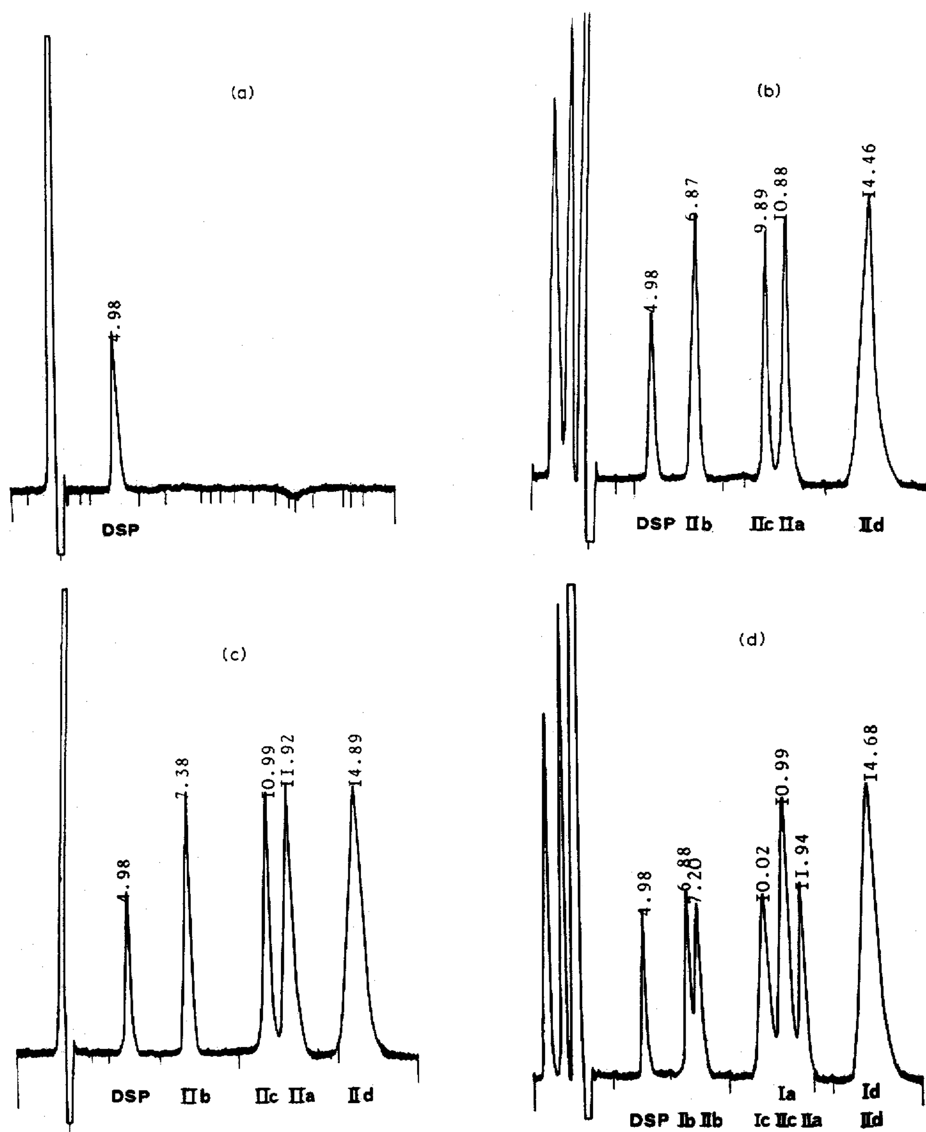


Fig. 2. HPLC analysis of tamoxifen and major metabolites: (a) without on-line photolysis; (b) without on-line photolysis (the sample was previously photolysed off-line); (c) with on-line photolysis; (d) with on-line photolysis and partial off-line photolysis. DSP = desipramine.

the reproducibility is quite satisfactory (Table I) and the calibration factor does not depend on the flow-rate within range 0.7—2.5 ml/min.

Fig. 3 shows the effect of the pH of the mobile phase on the capacity factor k' of tamoxifen (Ia), its metabolites (Ib and Ic), and internal standard (Id). As expected, an increase in pH leads to an increase in the retention time of the basic products (Ia—c), whereas k' for the neutral internal standard (Id) slightly decreases. The optimum operating pH range was, in our experience, 2.0—2.5. It is to be noted that by using reversed-phase C_{18}

TABLE I

REPRODUCIBILITY OF THE ANALYTICAL METHOD

Ia			Ib			Ic		
Added (ng)	Found (ng)	Recovery (%)	Added (ng)	Found (ng)	Recovery (%)	Added (ng)	Found (ng)	Recovery (%)
0.91	1.08	118.7	1.00	1.25	125.0	1.05	0.95	90.5
9.14	10.10	110.5	10.04	10.40	103.6	10.49	12.10	115.3
18.28	17.40	89.7	20.10	20.60	102.5	20.98	18.30	87.2
45.70	43.20	94.6	50.20	52.10	103.8	52.45	52.70	100.5
91.39	91.90	100.6	100.40	93.70	93.3	104.90	107.10	102.1
182.80	172.80	94.5	200.80	208.40	103.7	209.80	210.80	100.4
Mean		102.8			105.6			99.1
S.D.		11.8			11.7			11.1
Mean coefficient of variation (%)		11.5			11.0			11.0

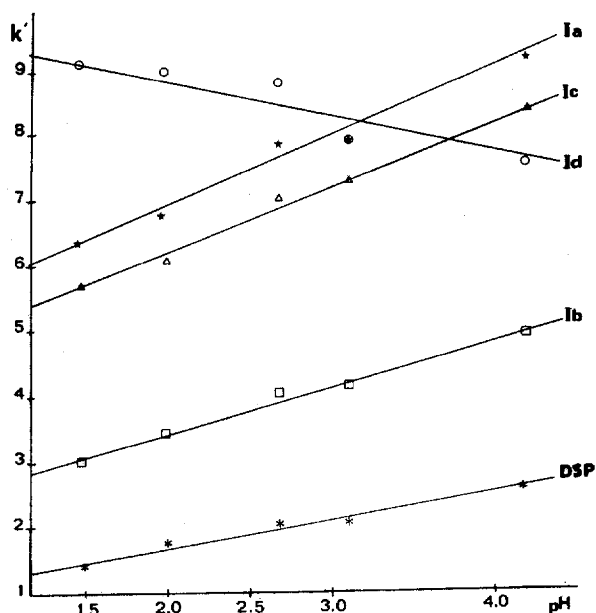


Fig. 3. Influence of the pH of the mobile phase on the capacity factor k'.

or C_8 columns, Id is not a good choice as internal standard because of its rather low retention time.

Fig. 4 reports the plasma levels of tamoxifen and its metabolites determined in one patient treated with a single oral dose of 20 mg. This is the daily dosage currently used in breast cancer management; as can be seen, plasma levels of tamoxifen and metabolites are easily monitored, and well above the sensitivity limit of the analytical method (< 1 ng/ml).

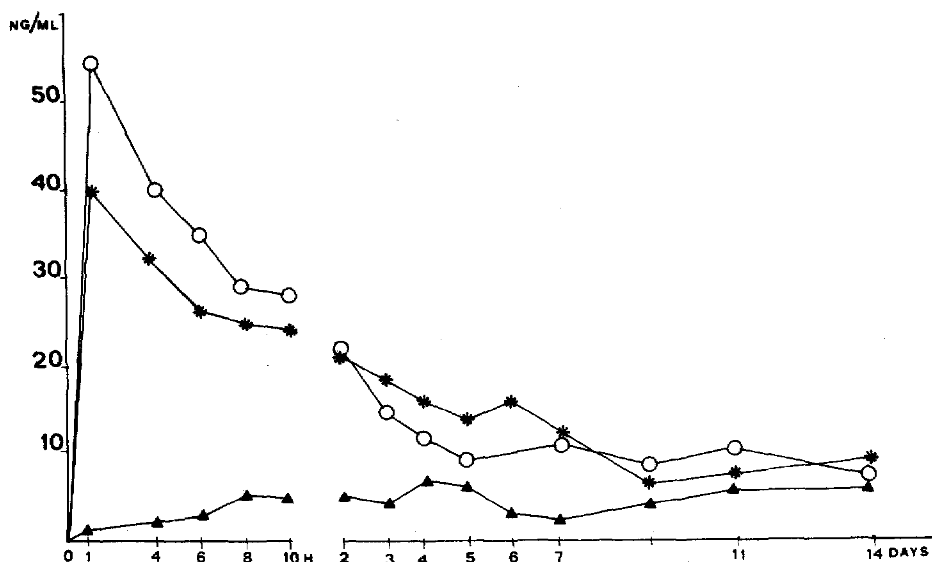


Fig. 4. Plasma levels of tamoxifen (○) and metabolites Ib (▲) and Ic (*) after a single oral 40-mg dose. Patient D.M.G., normal renal and liver functions.

DISCUSSION

Diethyl ether extraction of the plasma samples, redissolving in the HPLC mobile phase after evaporation of the organic solvent, and photocyclisation — as suggested in the literature — led to erratic results when applied to plasma routinely obtained from advanced cancer patients, who were rather heavily treated with antineoplastic therapies. In addition, under the conditions reported in the literature, in our hands, only the hydroxylated derivative (II) had an acceptable chromatographic behaviour.

Surprisingly enough, with two commercially available reversed-phase columns (Supelcosil LC_{18}, 15 cm × 4.6 mm I.D., 5 μm particle size, and the Merck LiChrosorb RP-18, 25 cm × 4.6 mm I.D., 5 μm particle size) we were not able to elute tamoxifen (or its corresponding phenanthrene derivative) even by using 95% methanol plus 2.5% acetic acid and 2 m*M* sodium pentanesulphonate as the mobile phase.

With these stationary phases, the analysis was successful only by using the stronger acid trifluoroacetic acid, instead of acetic acid. Better results were obtained with a Supelcosil LC-8 column [mobile phase acetonitrile—water (55:45) plus 2.5 m*M* sodium pentanesulphonate and 0.1% trifluoroacetic acid) but the column life was too short to allow routine use of the method.

Solid-phase extraction with C_{18} reversed-phase cartridges, on-line photolysis and use of internal standard, led to a drastic improvement in the reliability of the analysis. In addition, the reduction of the work-up time allows a trained operator to process a batch of 20 samples in about 1 h.

This method is currently used in our institute for the routine analysis of plasma levels of tamoxifen and metabolites in advanced cancer patients.

About 400 samples have been processed up to the present without any analytical troubles.

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