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QUANTIFICATION OF TAMOXIFEN AND N-DESMETHYLTAMOXIFEN IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, PHOTOCHEMICAL REACTION AND FLUORESCENCE DETECTION, AND ITS APPLICATION TO BIOPHARMACEUTIC INVESTIGATIONS

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SUMMARY

The anticancer drug tamoxifen and its major metabolite N-desmethyltamoxifen are quantified in human plasma down to subnanogram amounts by high-performance liquid chromatography. Plasma is alkali-buffered and extracted with hexane. The organic solvent is evaporated and the residue dissolved in the mobile phase. An aliquot is sampled automatically and chromatographed. The analytes are detected after postcolumn UV irradiation and rearrangement to substituted phenanthrenes by their intense fluorescence. Precise handling of exact volumes facilitates external calibration. Statistical data for precision and accuracy are given and illustrate reliable quantification. The method is applied to the samples of a clinical study, and the pharmacokinetic parameters of both analytes are presented. The novel design of the photochemical reactor is discussed, with respect to peak broadening and photochemical recovery. The measured peak broadening is smaller than theoretically predicted, owing to non-helical coiling.

INTRODUCTION

Tamoxifen, *Z*-1-(4-*p*-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene, is a well established drug in the treatment of certain kinds of breast tumour. As antioestrogen it has found widespread use against tumour containing oestrogen-receptor proteins. The drug is well tolerated during chronic treatment; its side-effects are mostly related to its antioestrogenic properties. Tamoxifen forms several metabolites in the body, the primary one being N-desmethyltamoxifen. Other metabolites are hydroxylated at one aromatic ring (4-hydroxytamoxifen) or are the result of oxidative dealkylation at the tertiary amine function. These metabolites are primarily excreted as conjugates in the bile. Both tamoxifen and N-desmethyltamoxifen are present in low nanogram amounts in plasma following

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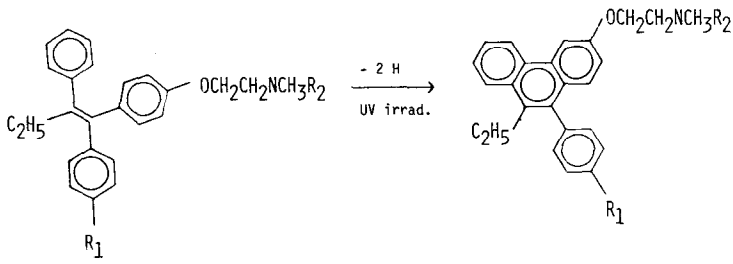

Substance	R_1	R_2
Tamoxifen	H	CH_3
N-Desmethyltamoxifen	H	H
Hydroxytamoxifen	OH	CH_3

Fig. 1. Chemical structure of tamoxifen, its metabolites and their photocyclization products.

single oral doses, and accumulate markedly during chronic therapy. Following an initial distribution phase, the half-life of tamoxifen has been shown to average between four and seven days. Obviously enterohepatic recycling plays a major role in contributing to the long half-life of the compound.

The analysis of tamoxifen is difficult owing to the low plasma concentrations and the extreme light-sensitivity of the compound. Former plasma assays described used gas chromatography–mass spectrometry [1–6]. They required laborious sample work-up prior to analysis with very limited sample throughput. Refined methods use high-performance liquid chromatography (HPLC) with precolumn [7–9] or postcolumn [10,11] UV irradiation to convert the analytes into the respective photocyclization products, i.e. substituted phenanthrenes (Fig. 1). These phenanthrenes exhibit very intense fluorescence, which can be used for quantification. The precolumn photocyclization suffers from the technical problem that the chromatographed analytes are different from the original and not available as pure substances. The postcolumn photocyclization implies the problem of the photochemical reactor. The designs described for the analysis of tamoxifen [10,11] are not optimized in terms of low peak dispersion in the postcolumn reactor and selectivity of irradiation wavelength. Theoretical treatments of peak broadening in postcolumn reactors and practical designs to minimize it were published for other analytes by several authors [12,13]. We describe here a selective assay for tamoxifen and N-desmethyltamoxifen, which was applied to the samples of a pharmacokinetic study.

EXPERIMENTAL

Solvents and reagents

A pure standard of tamoxifen and N-desmethyltamoxifen was provided by Teva Pharmaceuticals (Kfar Saba, Israel). The purity, based on the USP XXI-procedure, was more than 99.5% by thin-layer chromatography (TLC). All other reagents, analytical or HPLC grade, were purchased from E. Merck (Darmstadt, F.R.G.). Mobile phase components (water, methanol, propionic acid, ammonia)

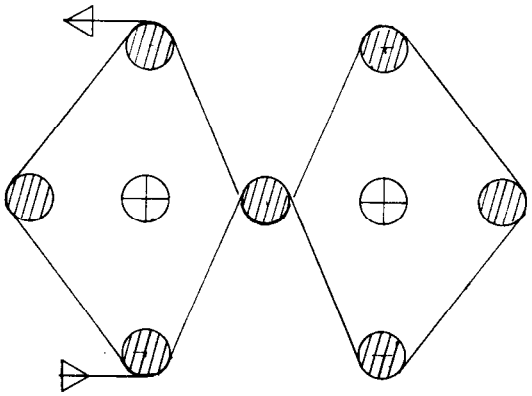

Fig. 2. Schematic drawing of the photochemical reactor. The front view of the non-helical coil is shown. Shaded circles are metal rods and the circles with a cross are UV lamps.

were separately filtered prior to mixing, then degassed with helium after mixing. Stock standard solutions were prepared in methanol and kept in the dark. Working standards were prepared by dilution of the stock solutions with 0.001 *M* hydrochloric acid.

Apparatus and chromatographic conditions

An automated HPLC system consisting of modular components was used. The components were a Gynkotek M 600 solvent-delivery pump, a Waters Model 710 B automatic sample processor, a laboratory-built photochemical reactor and a spectrofluorometer (Kratos SF 970) operated at an excitation wavelength of 265 nm with an emission cut-off filter of 340 nm. The detector output (peak area) was quantitated using a Shimadzu CR 1 B integrator. Data regression and concentration calculations were performed on a Commodore CBM 8032 computer using the LAB CAL software program.

The column used was a normal-phase Waters Porasil 10 (300×4 mm I.D.). The mobile phase consisted of water (100 g), methanol (1000 g), propionic acid (15 g) and 1% ammonia (20 g). The flow-rate was 2.0 ml/min. All analyses were performed at room temperature. The column life under these conditions was at least two months or 1500 injections of 100 μl of plasma extract.

Preparation of samples

The light-sensitivity of the analytes required that all samples were handled under red darkroom lamps. To prepare the samples, 1.00 ml of plasma, 0.10 ml of saturated borate buffer (pH 9.5) and 5.00 ml of *n*-hexane were added to 10-ml round-bottomed culture tubes equipped with PTFE-lined screw-top caps. The samples were treated in the overhead-shaker for 15 min. After centrifugation at 2000 *g*, exactly 4.00 ml of the organic phase were transferred to a 10-ml tapered glass tube. The organic phase was evaporated under dry nitrogen on a water-bath at 30°C. The dry residue was redissolved in 250 μl of mobile phase, and 100 μl were injected onto the chromatograph.

RESULTS AND DISCUSSION

Design of the photochemical reactor

The reactor consists of a PTFE capillary (200 cm × 0.2 mm I.D., 0.5 mm O.D., Labomatic, Sinsheim, F.R.G.) coiled around two low-pressure mercury UV lamps (Pen-Ray ultraviolet lamps, Ultraviolet Products, San Gabriel, CA, U.S.A.). These lamps emit more than 90% of their irradiance in the 254-nm spectral line, and very little heat is produced. Fig. 2 is a schematic drawing of the reactor. The irradiation wavelength of 254 nm is close to the absorbance maximum of the analytes (Fig. 3), and a quantitative rearrangement can be expected. Even with only one burning lamp no difference to the described conditions could be found (Fig. 4). The absorption at 260 nm of the cyclization product is more intense than that of tamoxifen by a factor of 1.5. However, the fluorescence intensities at the (different) emission maxima differ by a factor of more than 25. As the fluorescence detector operates with an emission cut-off filter transparent above 340 nm, in contrast to the emission monochromator of the spectrofluorometer used for spectra recording, the sensitivity difference for tamoxifen and its cyclization product under HPLC conditions can be estimated only roughly. Injection of 1 ng of N-desmethyltamoxifen and 5 ng of tamoxifen (Fig. 4) gave marked peaks for the irradiated products, but no visible peaks without irradiation. Therefore the sensitivity ratio of irradiated tamoxifen to tamoxifen is more than a factor of 30. The dead-volume in the coil was calculated to 63 mm^3 and measured by weighing to 59 mm^3 without connection fittings. From the bottom of the separation column to the flow-cell of the fluorescence detector the system dead-volume is ca. 90 mm^3.

The peak variance σ_{iV}^2 [14]:

$$\sigma_{iV}^2 = \left[2\pi^3 \frac{D_{im} r^6}{V'} + \frac{\pi}{24} \frac{r^4 V'}{D_{im}} \right] \cdot L$$

where r is the radius of the tube, L is the length of the tube, D_{im} is the coefficient of diffusion of the analyte i in the phase m (usually of the order of 10^{-9} m^2/s) and V' is the velocity in ml/s.

As the first term of the equation reaches only very small values, it can be neglected. The second term, with the actual data cited above, gives a value of σ of ca. 120 μl. This would be crucial for HPLC separations, if early eluted peaks should be separated. As there is some evidence [14] that peak dispersion in tubes caused by wall-effects can be reduced by knitting the tube to improve radial mixing, the real peak variance in the reactor was investigated. The peak widths of several phenanthrene derivatives were determined in the described chromatographic system with and without the reactor between column and detector. Table I lists the results: compounds with capacity factors (k') < 2 suffer a symmetric peak broadening, and the elution volumes were enhanced by 50–100 μl. Compounds eluted with $k' > 2$ were much less affected; the elution volumes were enhanced by no more than 25 μl. The fact that the real peak dispersion is lower than theoretically calculated is caused by the special design of the coil, where the

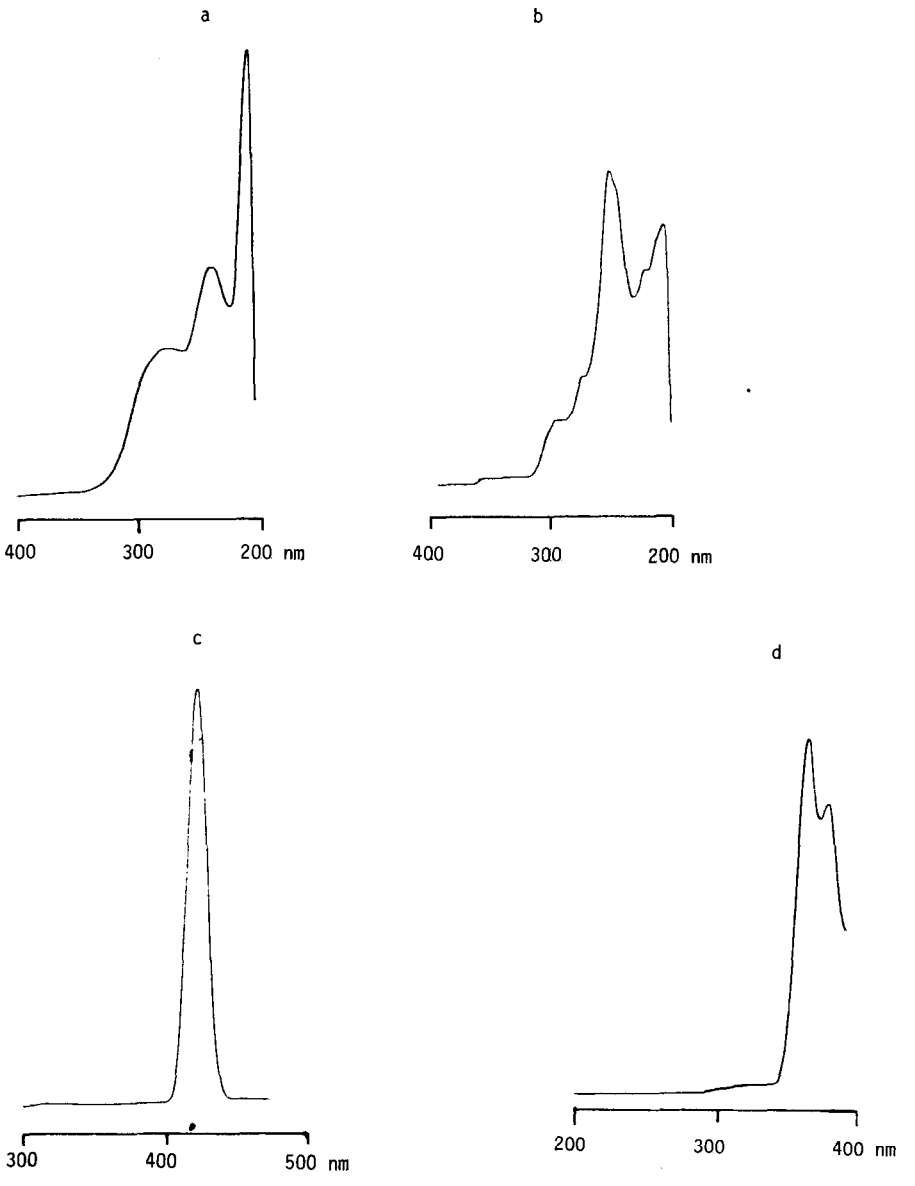

Fig. 3. Absorption and fluorescence emission spectra of tamoxifen and its photocyclization product. (a) Absorption spectrum of tamoxifen in methanol (10 μl/ml). (b) Absorption spectrum of the cyclization product in methanol (10 μg/ml). (c) Fluorescence emission spectrum of tamoxifen in methanol (10 μg/ml); instrumental conditions: excitation wavelength, 260 nm; current amplification factor, 1000. (d) Fluorescence emission spectrum of the cyclization product in methanol (10 μg/ml); instrumental conditions: excitation wavelength, 260 nm; current amplification factor, 10.

flow vector of the convection dispersion changes after short distances. Simple coiling in helixes, as described for other photochemical reactors [8,10], amplifies the convection dispersion and leads not only to peak dispersion, but also to excessive peak tailing. For compounds with $k' < 2$, the same capillary coiled as a helix

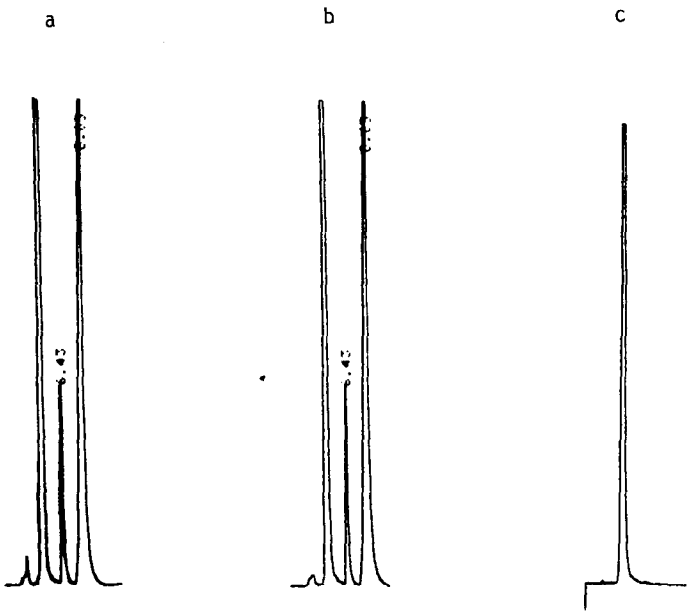

Fig. 4. Evaluation of the photochemical yield. Injection of 1 ng of N-desmethyltamoxifen (peak at 6.43 min) and 5 ng of tamoxifen (peak at 8.83 min) directly on-column under different operating conditions of the photoreactor: (a) two lamps burning; (b) one lamp burning; (c) lamps off. Operation of only one lamp is sufficient for quantitative photochemical rearrangement. Without irradiation the native fluorescence of the unreacted analytes is too weak to be detected.

induced an increase in the peak elution volumes of 150–200 μl with marked peak tailing. Peaks with higher k' values were less influenced, but the tailing tendency remained, however.

One problem with the reactor is the nature of the PTFE capillary. Although

TABLE I

MEASURED PEAK BROADENING IN THE PHOTOCHEMICAL REACTOR WITH NON-HELICAL AND HELICAL COILING

$W_{1/2}$ is the peak width measured at half peak height. The PTFE capillary was 200 cm × 0.2 mm I.D., with a measured dead-volume of 59 mm^3. The shape of the non-helical coil is shown in Fig. 2; the helical coil had a diameter of 10 cm with six parallel coils. The lamps of the photochemical reactor were not operated, and connection fittings remained the same for all three measuring conditions.

Analyte	k'	$W_{1/2}$ (μl)		
		Without coil	With helical coil	With non-helical coil
1-Methylphenanthrene	0.9	265	475	345
Phenanthrene	1.1	310	490	405
Aminophenanthrene	1.7	330	500	375
Diaminophenanthrene	2.1	340	495	365
Phenanthrolene	3.2	510	665	525
Harmol	4.9	690	775	715

TABLE II

RESULTS OF THE TAMOXIFEN CALIBRATION AFTER $1/x$ WEIGHTED REGRESSION

Calculation No.	Concentration found (ng/ml)					
	0.45 ng/ml	0.90 ng/ml	5.50 ng/ml	11.0 ng/ml	25.0 ng/ml	50.0 ng/ml
1	0.45	–	5.03	11.0	25.8	48.2
2	0.46	0.80	5.66	11.5	26.6	46.4
3	0.47	0.88	5.44	10.9	26.3	47.5
4	0.43	0.95	5.25	11.3	26.7	46.9
5	0.50	0.90	5.24	10.7	25.4	48.7
Mean	0.46	0.88	5.33	11.1	26.2	47.6
S.D.	0.024	0.062	0.237	0.319	0.550	0.93
Inaccuracy (%)	2.31	−1.83	−3.20	0.73	4.64	−4.92
Error (%)	5.31	6.98	4.46	2.88	2.18	1.96

Mean slope: $1.04 \pm 0.02\%$
Mean intercept: $-0.03 \pm 0.03\%$
Mean r^2: $0.997 \pm 0.002\%$

chemically inert, the capillary became porous after ca. two weeks of continuous operation because of the conglomerate character of the polymer. For routine maintenance, several coiled capillaries were prepared in advance and exchanged periodically after ca. 240 h of UV irradiation.

Evaluation of the chromatographic conditions

Both analytes were poorly eluted from reversed-phase materials of different chain lengths. Mobile phases containing water completely failed, as the analytes

TABLE III

RESULTS OF THE N-DESMETHYLTAMOXIFEN CALIBRATIONS AFTER $1/x$ WEIGHTED REGRESSION

Calculation No.	Concentration found (ng/ml)					
	0.30 ng/ml	0.50 ng/ml	0.85 ng/ml	2.00 ng/ml	5.00 ng/ml	10.0 ng/ml
1	0.31	–	0.82	1.83	4.97	10.3
2	0.28	0.45	0.93	2.13	4.93	10.0
3	0.29	0.47	0.85	2.23	4.64	10.5
4	0.27	0.53	0.89	1.97	4.76	10.3
5	0.30	0.51	0.89	2.12	5.24	9.90
Mean	0.29	0.49	0.88	2.06	4.91	10.2
S.D.	0.018	0.036	0.043	0.157	0.228	0.245
Inaccuracy (%)	−4.00	−1.80	3.53	3.00	−1.80	2.00
Error (%)	6.14	7.29	4.83	7.61	4.65	2.40

Mean slope: $0.98 \pm 0.01\%$
Mean intercept: $0.023 \pm 0.02\%$
Mean r^2: $0.999 \pm 0.003\%$

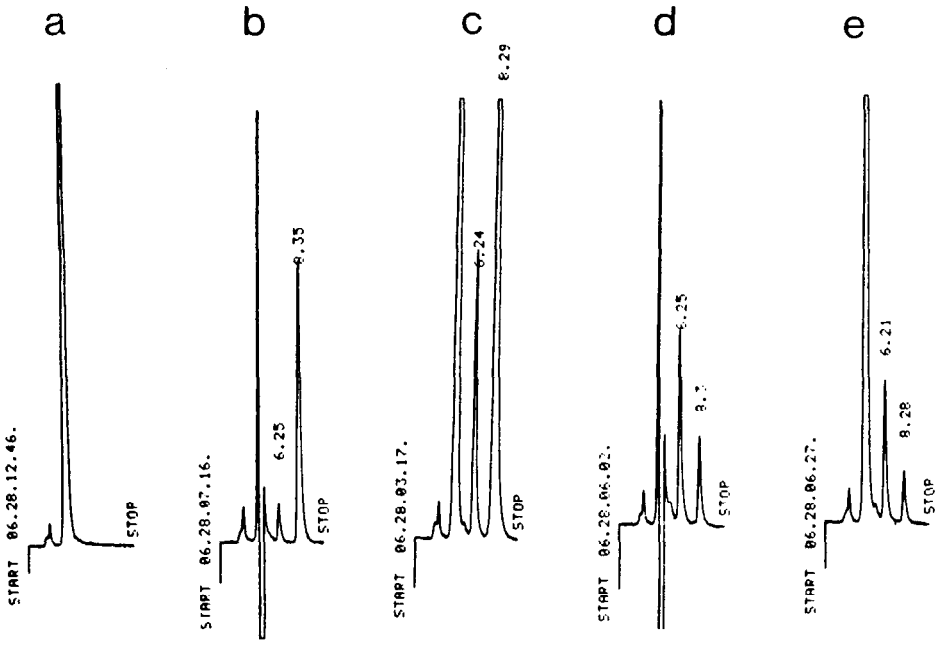

Fig. 5. Typical chromatograms from the pharmacokinetic study. (a) Predose plasma of a subject. (b) Plasma 1 h after administration; the peak at 6.25 is ca. 0.8 ng/ml N-desmethyltamoxifen, the one at 8.35 is 10.0 ng/ml tamoxifen. (c) Plasma 6 h after administration; the peak at 6.24 is ca. 7.2 ng/ml N-desmethyltamoxifen, the one at 8.29 is 36.7 ng/ml tamoxifen. (d) Plasma 14 days after administration with 4.68 ng/ml N-desmethyltamoxifen and 2.63 ng/ml tamoxifen. (e) Plasma 21 days after administration with 2.43 ng/ml N-desmethyltamoxifen and 1.56 ng/ml tamoxifen.

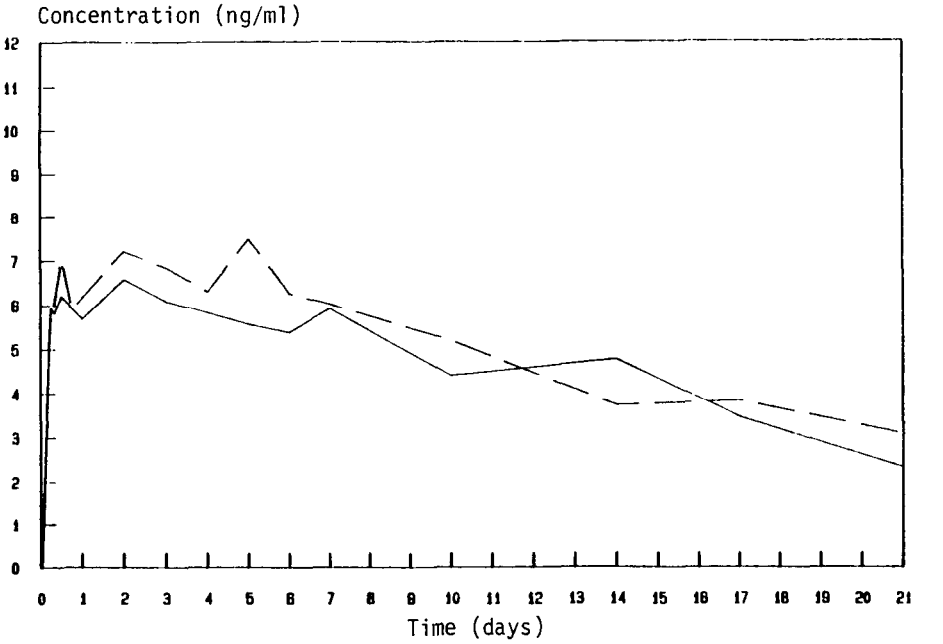

Fig. 6. Plasma concentration-time profile for N-desmethyltamoxifen (mean over ten subjects). The solid line is the measured mean concentration of N-desmethyltamoxifen after treatment with formulation A; the dashed line is the measured mean concentration of N-desmethyltamoxifen after treatment with formulation B.

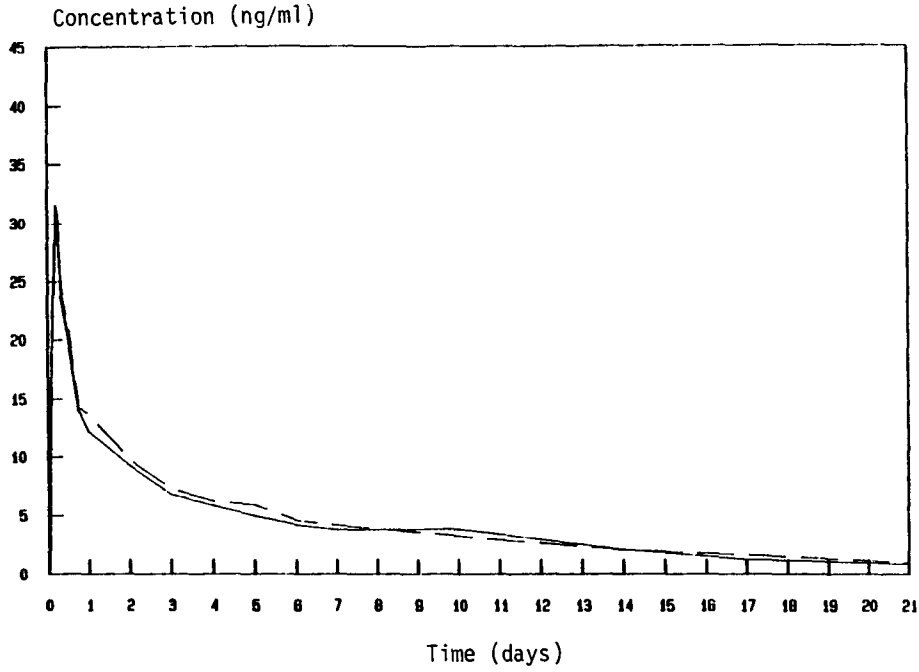

Fig. 7. Plasma concentration–time profile for tamoxifen (mean over ten subjects). The solid line is the measured mean concentration of tamoxifen after treatment with formulation A; the dashed line is the measured mean concentration of tamoxifen after treatment with formulation B.

were irreversibly adsorbed on the column packings. The conditions cited in ref. 11, with a nitrile stationary phase and acidified aqueous acetonitrile, could not be reproduced. Adequate separation of the analytes and plasma constituents was achieved with a silica column (Waters Porasil, 10 μm particle size, 30×0.4 cm I.D.) eluted isocratically with methanol–water containing ammonium propionate buffer. Under these conditions tamoxifen eluted at 8.3 min and N-des-

TABLE IV

CALCULATED PHARMACOKINETIC PARAMETERS FOR TAMOXIFEN AND N-DESMETHYLTAMOXIFEN AFTER SINGLE DOSE OF A 20-mg TABLET

Data given are the mean (±S.D.) values from ten subjects. C_{max} is the maximal concentration; T_{max} is the time when the maximal concentration is reached; AUC^1 is area under the curve from predose to the last sampling time; AUC^3 is area under the curve from predose extrapolated to infinity; k is the mean terminal rate constant; $t_{1/2}$ is the mean terminal half-life.

Parameter	Tamoxifen	N-Desmethyltamoxifen
C_{max} (ng/ml)	32.99 ± 6.61	9.00 ± 3.68
T_{max} (days)	0.217± 0.03	3.38 ± 2.33
AUC^1 (days·ng/ml)	93.47 ±25.9	104.45 ±31.1
AUC^3 (days·ng/ml)	102.7 ±31.6	107.83 ±33.0
k (1/days)	0.154± 0.035	0.050± 0.033
$t_{1/2}$ (days)	4.721± 1.03	22.42 ±16.87

methyltamoxifen at 6.3 min. In real samples a minor peak occurred at 5.3 min, which may have been monohydroxytamoxifen. The maximal concentration of this peak rarely exceeded 2 ng/ml, based on the same response as for tamoxifen.

It was necessary to readjust the buffer composition after column changes to preserve peak separation and retention times. An increase of the ammonia content increased the retention times of both analytes, whereas an increase of the propionic acid decreased retention.

The mobile phase is very sensitive to minor deviations in the described amounts of its components. Volumetric composition of the mobile phase permanently led to variances in retention times. Substitution of methanol by acetonitrile resulted in almost complete loss of separation between both analytes.

Validation of the plasma method

Tables II and III show the results of the method validation from five sequences. Tamoxifen was calibrated from 0.45 to 50.0 ng/ml and N-desmethyltamoxifen from 0.30 to 10.0 ng/ml. The peak areas were processed by $1/x$ weighting; from the weighted results the slope and intercept of the calibration curve were determined. The recovery was checked between both limits of quantification by comparison of the peak areas of the extracted concentrations and the injected amounts, corrected for the handled volumes. For all concentrations of both analytes it was almost quantitative. At the lower limit of quantification (LLQ), the inaccuracy (percentage deviation of the calculated from the nominal values) for both analytes was below 5%, with an imprecision (percentage standard deviation of the calculated values) below 7%. At the upper limit of quantification (ULQ), the inaccuracy for both analytes was less than 5%, with imprecisions lower than 3%.

The calibration curves had excellent linearities with regression coefficients (r^2) greater than 0.995, constant slopes and intercepts near to zero. The signal-to-noise ratio at the LLQ was ca. 70, and the lowest determinable concentrations of both analytes were well below 0.1 ng/ml.

The specificity of the assay was tested with nicotine, caffeine, salicylic acid, propranolol, testolacton and drostanolon. None of these drugs interfered with the assay.

The statistical data prove that the external standardization procedure delivers accurate and precise results.

Application to a clinical study

With this improved method more than 500 samples from a comparative bioequivalence study with two different formulations have been measured. In a randomized cross-over design, ten healthy, male volunteers were treated with a single dose of 20 mg of tamoxifen in tablets. Blood samples were drawn directly before administration and thereafter at distinct times till day 21. After a wash-out period of eight weeks the next single dose followed. Treatment A was a 20-mg tamoxifen tablet from Teva Pharmaceuticals; treatment B was a 20-mg tamoxifen tablet (Nolvadex®) from ICI Pharmaceuticals, U.K.

Fig. 5 shows chromatograms of plasma samples from one subject. In routine analysis no interferences were observed. Irradiated and therefore destroyed sam-

ples were easily discovered, as the cyclization products were eluted at the void volume in a typical chromatogram.

Figs. 6 and 7 show the mean plasma levels of N-desmethyltamoxifen and tamoxifen for ten subjects and both treatments. For tamoxifen, no difference between the two preparations could be found. The differences for N-desmethyltamoxifen were likely to occur by chance, as the inter-individual dispersion is about ten times higher than for tamoxifen.

Table IV presents the pharmacokinetic parameters evaluated for treatment B. A single dose of 20 mg of tamoxifen produces a mean maximal concentration (C_{max}) of 33 ng/ml after 5 h, and 21 days after this single dose concentrations above the LLQ were found in 70% of the subjects.

N-Desmethyltamoxifen reaches a mean C_{max} of 9 ng/ml ca. 81 h after drug administration and 21 days later the subjects showed a mean analyte concentration of 3 ng/ml. The mean terminal half-life of the metabolite was calculated to 22.4 ± 16.9 days, clearly indicating the strong enterohepatic reabsorption of this substance, which is less marked in the case of tamoxifen.

The sophisticated design of the on-line photochemical reactor, as well as the careful evaluation of the assay, allowed for the first time the monitoring of the plasma levels of tamoxifen and N-desmethyltamoxifen after a single dose of 20 mg over a period of 21 days.

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