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Specific high-performance liquid chromatographic analysis of tamoxifen and its major metabolites by "on-line" extraction and post-column photochemical reaction

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Abstract A new and simplified high-performance liquid chromatographic (HPLC) method for the determination of the antiestrogenic drug tamoxifen (TAM) and its desmethylated and hydroxylated metabolites in human plasma is described Specific and sensitive fluorescence detection is achieved by "on-line" photochemical conversion of the TAM structure to a highly fluorescent phenanthrene product using a newly developed post-column photoreactor included in the HPLC system A highly selective chromatographic separation was established by using unmodified silica with aqueous mobile phase for separation, with the sample preparation step on a small CN-propyl pre-column, included in the HPLC system Due to the high specificity of the separation and detection system, even small volumes of deproteinized plasma sample can be injected directly without prior sample extraction The described method permits a very fast and reproducible determination of TAM and its two major metabolites in plasma on a routine basis, down to 100 pg ml^{-1} concentration

Keywords *HPLC, fluorescence detection, tamoxifen, "on-line" photochemical reaction, pre-column sample preparation, blood, plasma*

Introduction

Tamoxifen (TAM) (Z)-2-(*p*-(1,2-diphenyl-1-butenyl)phenoxy)-*N,N*-dimethylethylamine (Fig 1) has become a widely used antiestrogenic drug in the treatment of female breast cancers and other endocrine tumors Concerning the pharmacology and the clinical effects of this drug, a sensitive and reliable method by which TAM and its major metabolites in human plasma can be measured, is of great interest

Many different analytical approaches, in which thin-layer chromatography [1] and high-performance liquid chromatography (HPLC) are used, have been published [2–7] Some of these HPLC methods refer to the unique property of TAM's molecular structure to react under UV-irradiation, to form a highly fluorescent compound [2–4, 6, 7] Including the photoconversion of TAM in the chromatographic separation system, by

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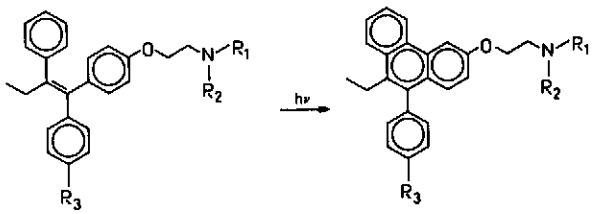

Figure 1
Chemical structure of TAM, its two major metabolites and their photocyclization products TAM, R1 = CH_3, R2 = CH_3, R3 = H, DMT, R1 = CH_3, R2 = H, R3 = H, 4-OH TAM, R1 = CH_3, R2 = CH_3, R3 = OH

using an "on-line" photoreactor [6, 7], proved to be a useful approach, resulting in higher reproducibility and increased sensitivity

This paper describes a new and reliable HPLC analysis of TAM and its two major metabolites, desmethyltamoxifen (DMT) and 4-hydroxytamoxifen (4-OH TAM), especially applicable for routine work It includes the optimization of the "on-line" photochemical conversion and the development of a new HPLC separation system In order to avoid irreversible adsorption of TAM and its metabolites onto glass surfaces and to reduce sample manipulation, "on-line" solid phase sample preparation was used

Experimental

Reagents, chemicals and standards

TAM (Nolvadex™), DMT and 4-OH TAM were a gift of Imperial Chemical Industries (Macclesfield, UK). All reagents used were analytical grade or better and were purchased from E Merck (Darmstadt, FRG) or Baker Chemicals (Deventer, The Netherlands) Solvents used in chromatography were obtained in HPLC-grade (water and methanol) and HPLC-far UV-grade (acetonitrile) from Rathburn Chemicals (Walkerburn, UK)

Stock solutions of TAM and its metabolites were prepared in 80% methanolic solution and kept in the dark at −70°C Generally, to avoid photochemical degradation, all sample containers with solutions of TAM or its metabolites were wrapped with aluminium foil

Instruments and equipment

An isocratic HPLC system consisting of the following components was used a Gilson solvent delivery pump M 302, a Valco CW-10 syringe-loading injection valve equipped with a 20 µl loop or, alternatively, with a 25 × 2 mm i d pre-column, a Merck–Hitachi F 1000 fluorometric detector, and a LDC CI-10 integrator HPLC injections and "on-line" sample preparation steps were carried out by using gas-tight Hamilton syringes

The "on-line" photochemical reactor used in this study was designed and built in the authors' laboratory It consists of a 30 cm long, 8 W low-pressure mercury lamp (GTE, Sylvania G8) having an intensive UV-band at 254 nm, built in a small plastic container The reaction capillary made of 1/16″ o d , and 0 01″ i d Teflon tubing is crocheted in a 3-D configuration and is fixed by a stainless steel net arranged very close in a half circle around the light tube

Construction of the photochemical reactor

The reactor configuration was obtained by crocheting approx 15 m Teflon tubing using a crochet hook No 4 Following the initial steps similar to the reactor construction described by Poulsen *et al* [8] the Teflon tubing was crocheted as a rectangular piece of 10 × 5 cm It is connected to the HPLC system by steel fittings and Teflon ferrules to avoid compressing the Teflon tubing

Chromatographic system

TAM and DMT were separated on a 110 × 4 6 mm i d cartridge, packed with Partisil Si (Whatman, New Jersey, USA) having a mobile phase consisting of methanol–5 mM ammonium acetate buffer (90 10% v/v)

In cases of additional analysis of 4-OH TAM, a 125 × 4 mm i d column, packed with Lichrosorb RP-2 (Research Center Seibersdorf, Austria) with a mobile phase mixture of methanol–water–acetic acid–*N,N*-dimethylhexylamine (73 27 0 5 0 2% v/v/v/v) was used

For "on-line" sample preparation a small stainless steel 25 × 2 mm i d pre-column (Knauer, Berlin, FRG) filled dry with 40 μm Sepralyte CN-propyl modified silica (Analytichem Int , California, USA) was substituted for the sample loop in the HPLC injection valve

Pre-column extraction of blood samples

Until analysis, plasma samples were stored in aluminium foil-wrapped containers at −70°C After thawing, 0 8 ml of a plasma sample (blank or patient plasma) was mixed with 0 2 ml of 0 1 N HCl and centrifuged at approx 12,000 *g* for 2 min to remove particulate material Supernatants were used for further analysis

The "on-line" extraction was started by conditioning the pre-column in a washing cycle with 0 5 ml methanol followed by 0 7 ml water and 0 3 ml of 0 1 N HCl Then 0 5 ml of sample supernatant was loaded onto the pre-column The pre-column was then washed with 1 0 ml water before being switched into the HPLC stream

For direct sample injection, plasma samples were deproteinized by precipitation with equal amounts of acetonitrile After centrifugation, the supernatant was injected into the HPLC system using a 20 μl injection loop

Results and Discussion

"On-line" photochemical conversion of the TAM structure

UV-irradiation of TAM induces the formation of an intensely fluorescent product with $\lambda_{ex(max)}$ = 256 nm and $\lambda_{em(max)}$ = 380 nm A structural study shows that in a first step the molecular TAM structure is converted by photochemical reaction through ring fusion into a corresponding phenanthrene [3] (Fig 1) In Fig 2 chromatograms obtained (a) with and (b) without photochemical irradiation are compared

In "off-line" illumination experiments, the complete photochemical reaction of TAM has been described by a two-step kinetic, forming multiple products in the reaction mixture, not all of them fluorescently active This is one of the main reasons why analytical approaches, in which "off-line" photoconversion prior to the chromatographic separation is used, may lead to unreproducible results The photoreaction can be coupled in "on-line" mode into an HPLC system [6, 7], the eluate flowing from the chromatographic column through an UV-transparent reaction capillary, illuminated by

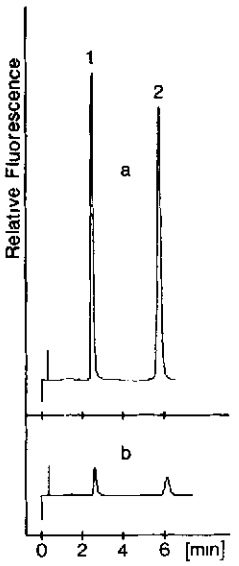

Figure 2
Comparison between chromatograms obtained (a) with, 2 ng TAM (1) and DMT (2) each in 20 µl, and (b) without photochemical reaction, 220 ng TAM and DMT each in 20 µl Chromatographic conditions as in Fig 6

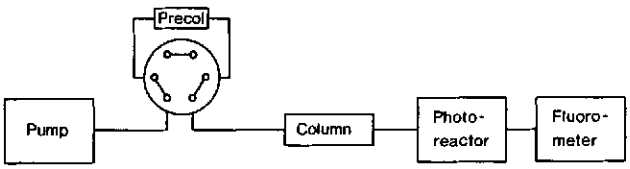

Figure 3
Flow scheme of the HPLC system used

an UV-light source before entering the fluorescence detector (Fig 3) Previous experiments with a self-constructed photoreaction system with 150 W mercury light source, similar to systems described in the literature [9], revealed that the "on-line" formation of TAM's photochemical product(s) was only slightly dependent on the power rating of the light source

Considering this result, a new and simplified version of an "on-line" photoreactor was constructed (see Experimental) Figure 4 shows the 3-D configuration of the crocheted Teflon reaction tubing This photoreactor design has some advantages over other systems described in the literature due to the low power rating of the Hg-lamp, additional cooling of the reaction capillary is unnecessary, because the eluate's temperature will never exceed approx 40°C Because of the longitudinal geometry of the UV-lamp, up to 20 m of the post-column reaction capillary can be illuminated, which allows reaction times up to 90 s at a flow of 1 ml min^{-1} Furthermore, it is possible to vary illumination periods continuously by covering different fractions of the crocheted net By using a crocheted geometry of the reaction capillary instead of coiling it directly around the light bulb, secondary flow within the capillary is enforced, thus minimizing peak dispersion induced by the photoreactor [10]

This newly developed photoreactor was used to study the rate of the "on-line" photochemical reaction of TAM and its metabolites in dependence of the irradiation time (Fig 5) To reach a maximum, photochemical conversion of TAM and 4-OH TAM

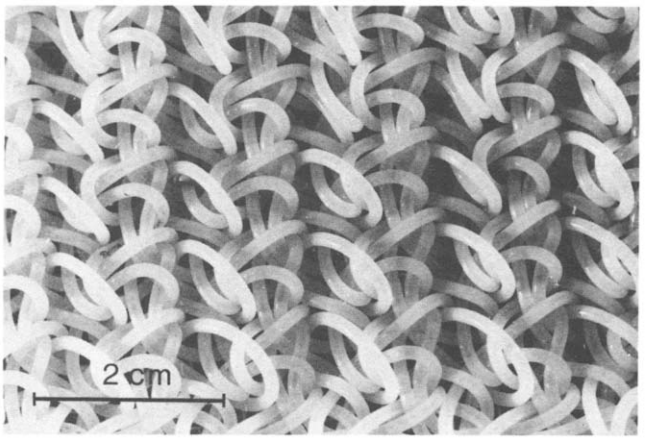

Figure 4
Configuration of the crocheted PTFE reaction tubing

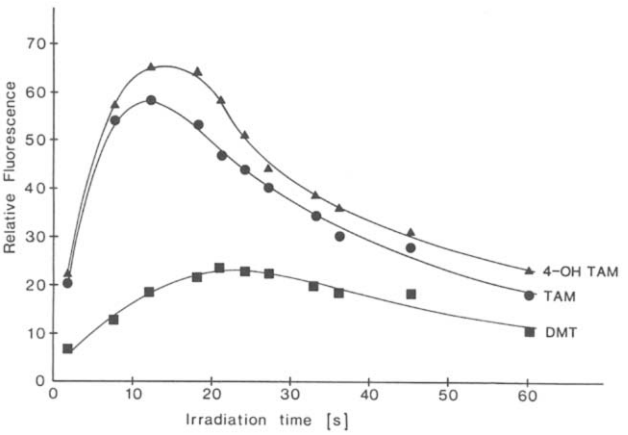

Figure 5
Time course of fluorescence change for "on-line" UV-irradiated TAM, DMT and 4-OH TAM in 90% methanolic solution

takes an illumination period of only 12 s at a flow of 1 4 ml min^{-1}, which corresponds to 4 m of illuminated reaction coil Increasing the illumination period leads to a decrease in the formation of the fluorescent product For DMT, a longer illumination time, thus an increased length of reaction capillary (7–8 m, corresponding to 22–24 s illumination) is necessary to reach maximum fluorescence In the analysis of TAM and DMT in plasma samples, a reaction period of 18 s (6 m reaction coil) was chosen These results indicate that "on-line" photochemical conversion of TAM and both metabolites seems to be more efficient than in "off-line" photoreaction systems, for which optimal illumination periods of up to 25 min have been reported [3]

Chromatographic separation of TAM and metabolites

Different liquid chromatographic systems have been described for the determination of TAM and its metabolites in plasma Using reversed-phase columns and mobile phase

systems with high percentage of organic modifier [2, 5–7], these separation systems show strong peak tailing of TAM and its metabolites It can be minimized, but not eliminated, by adding an organic amine [2, 6] or an ion-pairing substance [4, 5] to the mobile phase This chromatographic behaviour of TAM indicates that the retention of TAM is based not only on a pure reversed-phase mechanism, but additionally on interactions with the polar silica surface Considering this observation and our experience with polar separations of amino containing drugs on unmodified silica [11], a silica column with aqueous mobile phase system was tested for separation of TAM and metabolites In the separation system using a Partispher Si cartridge with a methanol–5 mM ammonium acetate buffer (90 10% v/v), TAM and DMT are well separated (α for TAM and DMT = 3 8) and better peak symmetry and performance than using a C18-separation, can be observed (Fig 6a)

Using the equation, $N_{eff} = N\ (k'/1 + k)^2$ the following values were calculated N_{eff} (TAM) = 10,824/m and N_{eff} (DMT) = 25,215/m 4-OH TAM is not well resolved from TAM in this chromatographic system Therefore, to analyse 4-OH TAM in the same sample, the polar silica column was replaced by a Nucleosil RP-2 column with different mobile phase composition, by which a different selectivity for 4-OH TAM was achieved Thus 4-OH TAM can be fully separated from TAM and the other metabolite in this system But as in case of other described reversed-phase separation systems, this system offers only mediocre peak performance and symmetry per TAM

When plasma samples of patients treated with TAM were analysed, no hydroxylated metabolite, 4-OH TAM, could be detected although such a result has been described in one previous study [4] Therefore, because of the more efficient separation, the silica system with aqueous eluents was used for all further measurements of plasma samples

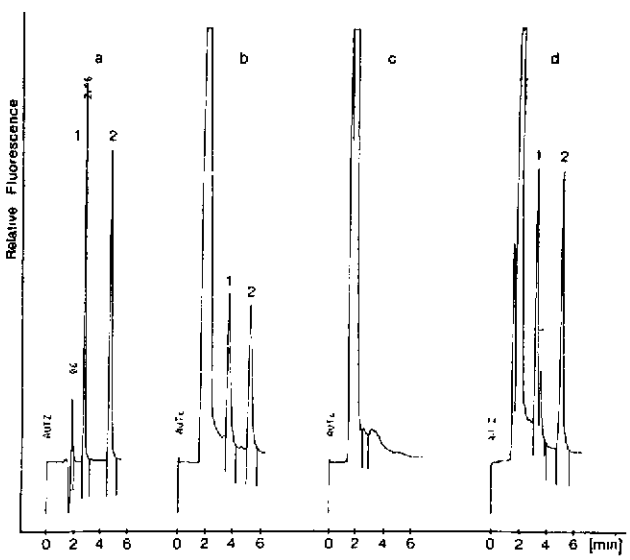

Figure 6
Chromatograms obtained by loop injection and after pre-column enrichment (a) loop injection of standards 88 ng TAM (1) and 79 ng DMT (2) in 20 µl, (b) 0 5 ml spiked plasma after pre-column sample extraction 88 ng TAM and 79 ng DMT/ml plasma, (c) 0 5 ml blank plasma, (d) 0 5 ml patient plasma receiving 20 mg Nolvadex™ daily, 16 h after last medication Plasma level 199 ng TAM and 210 ng DMT/ml Chromatographic conditions Partisphere Si, 110 × 4 6 mm i d Mobile phase methanol–5 mM ammonium acetate buffer (90 10% v/v), flow, 1 4 ml min^{-1} Fluorometric detection λ_{ex} = 256 nm, λ_{em} = 380 nm

Direct injection of deproteinized plasma samples

Because of its increased chromatographic performance as a chromatographic system for the separation of TAM and DMT, the polar silica system with aqueous eluents offers several other advantages, due to the high specificity of the polar retention on silica almost no compound with k'-value over one, originating from the sample matrix, can be observed in the chromatogram, when an extracted plasma is injected

Selective separation together with specific fluorescence detection make the direct injection of small volumes of unextracted plasma samples onto the HPLC system possible Figure 7b shows a chromatogram after injection of 20 µl deproteinized plasma from a patient receiving 20 mg Nolvadex™/day Larger injection volumes than 40 µl of deproteinized plasma samples may lead to clogging of the column frit and will decrease column lifetime and should be avoided

This method of direct sample injection offers a very fast and simple procedure for routine analysis of TAM in plasma, as long as plasma levels to be measured are not below the range of 10 ng ml^{-1}, defined by the detection limit of the procedure Using the silica separation system direct injection of plasma samples give comparable results as for a system using "on-line" extraction and reversed-phase separation (see Experimental) (Table 1)

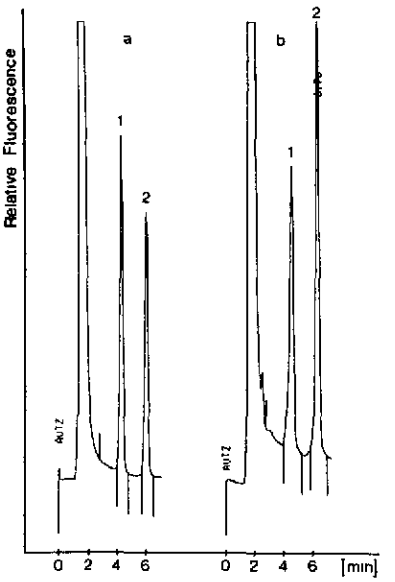

Figure 7
Chromatograms obtained by direct injection of 20 µl plasma samples after precipitation with acetonitrile (a) Calibration plasma spiked with 125 ng ml^{-1} TAM (1) and 113 ng ml^{-1} DMT (2) (b) Patient plasma 146 ng TAM and 206 ng DMT/ml Chromatographic conditions as in Fig 6

Table 1
Calibration curves in plasma linear regression of the peak area (Y) vs the applied plasma volume onto the pre-column (X), number of replicates = 5

	Mean values ± SD		
	Slope	Intercept	R^2
TAM	2 816 ± 0 050	0 094 ± 0 021	0 995 ± 0 005
DMT	4 732 ± 0 091	0 185 ± 0 072	0 995 ± 0 004

Volume applied 0 1–1 0 ml plasma corresponding to 30–300 ng TAM and DMT, respectively

"On-line" sample preparation of TAM plasma samples

For a successful measurement of very small concentrations of TAM in blood, TAM has to be isolated from 500–1000 μl of plasma sample All methods for the extraction of TAM described in the literature are based on one- (or more)-step liquid–liquid extraction procedures with organic solvents [6, 7] The sample preparation step in the analysis of TAM and its metabolites proved to be of crucial importance due to the photochemical reactivity of the TAM structure, prolonged exposure of the sample to light during sample extraction leads to photochemical conversion of TAM or its structural analogues Additionally the strong interaction between TAM and the polar silica surface may cause irreversible adsorption of TAM and its metabolites on any glass surface of sample containers In order to avoid these problems and to reduce sample manipulation to a minimum, the sample preparation step was included in the chromatographic system Therefore, the injector loop of the HPLC system is replaced by a small pre-column (25 × 2 mm i d) packed with 40 μm CN-propyl modified silica, which offers a moderately polar surface

Due to their lipophilic and basic properties, TAM, DMT and 4-OH TAM are retained equally well and quantitatively on the CN-propyl modified silica from plasma provided the sample size applied to the pre-column does not exceed 1000 μl Reproducibility of the "on-line" extraction (Table 2) and accuracy of the overall analytical procedure is very acceptable (Table 3) In total, up to 6 ml of plasma (or 6–12 samples) can be applied before the CN-propyl silica in the pre-column has to be exchanged By this pre-column enrichment very low detection limits for TAM and its metabolites down to 100 pg ml^{-1} can be attained

Table 2
Comparison of TAM and DMT determinations in plasma samples using two different chromatographic systems

Sample	TAM (ng ml^{-1}) System A	System B	% Diff	DMT (ng ml^{-1}) System A	System B	% Diff
1	72 0	69 5	−3 5	201 1	194 0	−3 5
2	134 2	135 0	+0 8	229 9	228 0	+0 5
3	207 0	203 5	+1 7	312 0	310 1	−0 5
4	97 5	94 0	−3 6	86 5	84 0	−2 9
5	20 5	18 7	−8 7	17 1	16 1	−5 9

System A direct injection, fluorescence detection, Partisphere Si, 110 × 4 mm, methanol–5 mM ammonium acetate (90 10% v/v)

System B "on-line" extraction, UV-detection, Nucleosil RP2, 125 × 4 mm, methanol–water–acetic acid–*N,N*-dimethyl hexylamine (73 27 0 5 0 2% v/v/v/v)

Table 3
Precision and accuracy [(conc found/conc added) × 100%] of the determination of TAM and DMT in spiked human plasma using "on-line" extraction and fluorescence detection Values in parentheses are coefficient of variation (%), $n = 5$

Sample	TAM (ng ml^{-1}) Added	Found	Acc (%)	DMT (ng ml^{-1}) Added	Found	Acc (%)
1	20 0	20 4	102 (2 6)	18 0	18 2	101 (1 8)
2	50 0	49 4	98 (4 1)	45 0	46 1	102 (2 4)
3	100 0	97 0	97 (3 2)	90 0	89 9	100 (2 7)
4	150 0	153 9	103 (1 2)	135 0	139 9	103 (1 4)
5	300 0	311 1	104 (0 9)	270 0	279 5	104 (0 6)

By the described methods plasma levels from approx 50 female patients receiving chronic TAM therapy were measured The plasma levels were in the range of 68–391 ng ml^{-1} for TAM and 93–446 ng ml^{-1} for DMT, both well corresponding to values cited in the literature [12]

Conclusions

The fast and efficient photoconversion of the molecular TAM structure in "on-line"-mode and fluorescence detection are pre-conditions for high sensitivity in the analysis of TAM and its major metabolites in plasma samples down to subnanogram levels A separation system with high selectivity and chromatographic performance was obtained by polar separation on unmodified silica with methanolic buffer eluent Inaccuracy in the analysis due to photolytic degradation of TAM and metabolites in the sample during preparation was reduced by including the extraction step in the chromatographic system by means of a small pre-column packed with CN-propyl modified silica in place of the sample loop Due to the high specificity of separation and detection, small volumes of deproteinized plasma samples can be injected directly without further sample preparation, thus offering an ideal chromatographic system for the fast routine measurement of TAM and its major metabolite DMT in plasma of patients under TAM therapy

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