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Identification of products formed during UV irradiation of tamoxifen and their use for fluorescence detection in high-performance liquid chromatography

JAROSLAV ŠALAMOUN*
Institute of Analytical Chemistry, Kounicova 82, 611 42 Brno (Czechoslovakia)
MIROSLAV MACKA and MILOŠ NECHVÁTAL
Research Institute of Pure Chemicals, Lachema, Karásek 28, 621 33 Brno (Czechoslovakia)
MILOŠ MATOUŠEK
Central Testing and Checking Institute of Agriculture, Zemědělská 1a, 658 37 Brno (Czechoslovakia)
and
LUBOMÍR KNESEL
Regional Hygiene Station, Hygiene Laboratories, Cornovova 68, 618 00 Brno (Czechoslovakia)
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ABSTRACT

During the UV irradiation of tamoxifen, isomerization of the *trans* to the *cis* isomer takes place and consequently corresponding highly fluorescent phenanthrene derivatives are formed. Their formation can be used for the sensitive and selective detection of tamoxifen in high-performance liquid chromatography (HPLC). The structure of photoproducts was identified by ^{1}H NMR spectroscopy, HPLC, gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry. Owing to the variety of products formed and the higher selectivity and fluorescence response, on-line postcolumn photocyclization is preferred to the precolumn mode. A chromatographic system for the separation of isomers and photoproducts is suggested.

INTRODUCTION

Tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene] is a non-steroidal antiestrogen used in the treatment of breast cancer[1]. The parent compound is synthesized as a mixture of the *cis* and *trans* isomers, although it is the *trans* isomer that acts as an estrogen antagonist.

Recently, several liquid chromatographic (LC) methods[2–10] have been published for the determination of tamoxifen in human plasma. All the methods involve photochemical conversion of tamoxifen to the fluorescent phenanthrene derivative before or after chromatography. Mendenhall *et al.*[2] identified the main fluorescent

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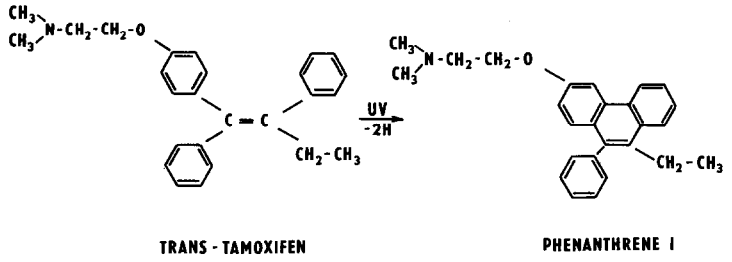

Fig. 1. Photocyclization reaction scheme of *trans*-tamoxifen.

photooxidation product (Fig. 1) by thin-layer chromatography (TLC), NMR spectroscopy and mass spectrometry and (MS). They found, however, that some NMR data are not unequivocal owing to the apparent purity of the isolated products.

The photochemistry of stilbenes, of which tamoxifen is a derivative, has been intensively studied (see ref. 11 and papers cited therein). Most workers accept a reaction mechanism in which stilbene is converted to 4*a*,4*b*-dihydrophenanthrene prior to dehydrogenation. However, dihydrophenanthrene was difficult to isolate, not only because of its oxidation to phenanthrene but also because of the reverse reaction to stilbene.

In none of the papers cited[2–10] was it mentioned whether *cis*- and *trans*-tamoxifen were separated. The only described separations were effected by the use of phenyl[12] or cyclodextrin[13] columns. Both methods have drawbacks, such as poor resolution of isomers on the phenyl column and a poor efficiency of the cyclodextrin column.

In this paper, an improved chromatographic system for the separation of tamoxifen isomers is suggested; the structure of the photooxidation products was identified by ^{1}H NMR spectroscopy, LC with fluorimetric and diode-array photometric detection, gas chromatography (GC)–MS and LC–MS.

EXPERIMENTAL

Chemicals

trans-Tamoxifen [99% purity by high-performance LC (HPLC) and *cis*-tamoxifen (91%), were prepared in the Research Institute of Pure Chemicals (Lachema, Brno, Czechoslovakia). Acetonitrile (LiChrosolv) and tetrahydrofuran (LiChrosolv) were supplied by Merck (Darmstadt, F.R.G.) and 1-octanesulphonic acid by Janssen Chimica (Beese, Belgium). All other chemicals were of analytical-reagent grade.

Equipment and methods

HPLC separations were performed on a Hewlett-Packard (Vienna, Austria) Model HP 1090 liquid chromatograph with a Model HP 1040 diode-array detector, a Model HP 1046 programmable fluorescence detector and a Model HP 310 Chemstation. Fluorescence was measured by using a 280-nm cut-off filter, a 1 × 1 mm slit on the excitation side (12 nm) and a 2 × 2 mm slit on the emission side (25 nm).

Stainless-steel analytical columns (25 × 0.4 cm I.D.) were packed with Silasorb fenyl, d_p = 7.5 μm, and Silasorb SPH C_{18}, d_p = 7.5 μm (Lachema).

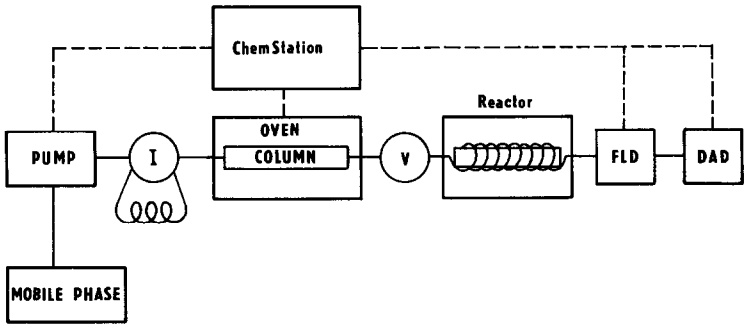

Fig. 2. Schematic diagram of the chromatographic apparatus. Solid lines, liquid connections; dashed lines, electrical connections. I = Injection valve; V = stop-flow valve; FLD = fluorescence detector; DAD = diode-array detector.

Photooxidation was accomplished in a PTFE capillary (400 cm × 0.25 mm I.D. × 1.59 mm O.D.) which was coiled around a tubular 8-W low-pressure mercury lamp (GTE, Sylvania G8T5) inserted in a MINUVIS viewer for TLC (Desaga, Heidelberg, F.R.G.). This light source emits the known mercury spectrum including the most intense line in the UV region at 254 nm. Because of the low power rating of the light source, no active cooling in the photoreactor was necessary. A schematic diagram of the chromatographic apparatus is shown in Fig. 2. A Rheodyne (Cotati, CA, U.S.A.) Model 7125 six-way valve was inserted between the analytical column and the capillary in order to apply the stop-flow technique for the measurement of fluorescence spectra.

The mobile phase for the separation of the two isomers consisted of acetonitrile–water–tetrahydrofuran–glacial acetic acid (68:27:8:0.2, v/v); 0.25 g of sodium octanesulphonate salt was added to 250 ml of this mixture. Acetonitrile–25% ammonia solution (500:0.7, v/v) was used for the separation of the photooxidation products on Silasorb C_{18}.

^{1}H NMR spectra were obtained on a Bruker (Karlsruhe, F.R.G.) WP 80SY spectrometer with a working frequency of 80.13 MHz.

GC–MS measurements were effected with a Hewlett-Packard system consisting of a Model HP 5890 gas chromatograph, an HP 5970 mass-selective detector and an HP 310 workstation.

The LC–MS system consisted of a Model HP 1090 liquid chromatograph, a HP 59888A mass spectrometer with a thermospray interface and a HP 310 workstation.

Photoproducts of *cis*- or *trans*-tamoxifen were extracted from the first of the above mobile phases into benzene after alkalinization in accord with GC–MS requirements. The extraction yield of all the compounds of interest was higher than 95% and was checked by HPLC.

RESULTS AND DISCUSSION

Separation

The separation of *cis*- and *trans*-tamoxifen was carried out on silica gel modified with phenyl groups, in a system similar to that described by Doyle *et al.*[11]

TABLE I

INFLUENCE OF THE ADDITION OF TETRAHYDROFURAN ON THE NUMBER OF THEORETICAL PLATES (N) AND RESOLUTION (R_s) OF ISOMERS

Tetrahydrofuran in mobile phase (%)	N		R_s[a]
	cis-Tamoxifen	*trans-Tamoxifen*	
0	2600	2800	0.5
2	3500	2800	1.25

[a] Peak capacity factor $k = 3$.

Tetrahydrofuran was added to the mobile phase mentioned above to improve the resolution and peak symmetry. The influence of the addition of tetrahydrofuran on the number of theoretical plates (N) and resolution (R_s, calculated by published LC nomenclatures[14]) of isomers is shown in Table I.

The second system with a Silasorb C_{18} packing was used to give a much better resolution of the two main fluorescent photoproducts of tamoxifen. However, all the analytes were poorly eluted from this reversed-phase material. A mobile phase consisting of only pure methanol or acetonitrile completely failed as the solutes were irreversibly adsorbed on the residual silanol groups. Subsequent addition of a small amount of ammonia resulted in the elution of all peaks and an acceptable resolution of all the main compounds present in the reaction mixture. Some workers[10] have previously used precolumn photoderivatization and detected the two main photoproducts as one broad or double peak.

Characteristics of photoproducts

The ^{1}H NMR spectrum (Fig. 3) confirms that the photocatalysed isomerization

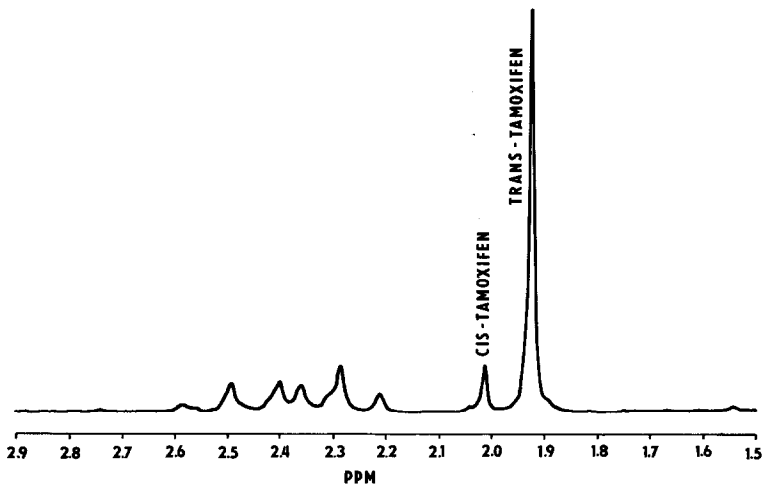

Fig. 3. ^{1}H NMR spectrum of *trans*-tamoxifen solution (0.5 mg/ml) exposed to daylight for 10 h. Solvent, deuterobenzene; spectrum width, 1200 Hz; digital resolution, 0.07 Hz per point; pulse duration, 2.5 μs.

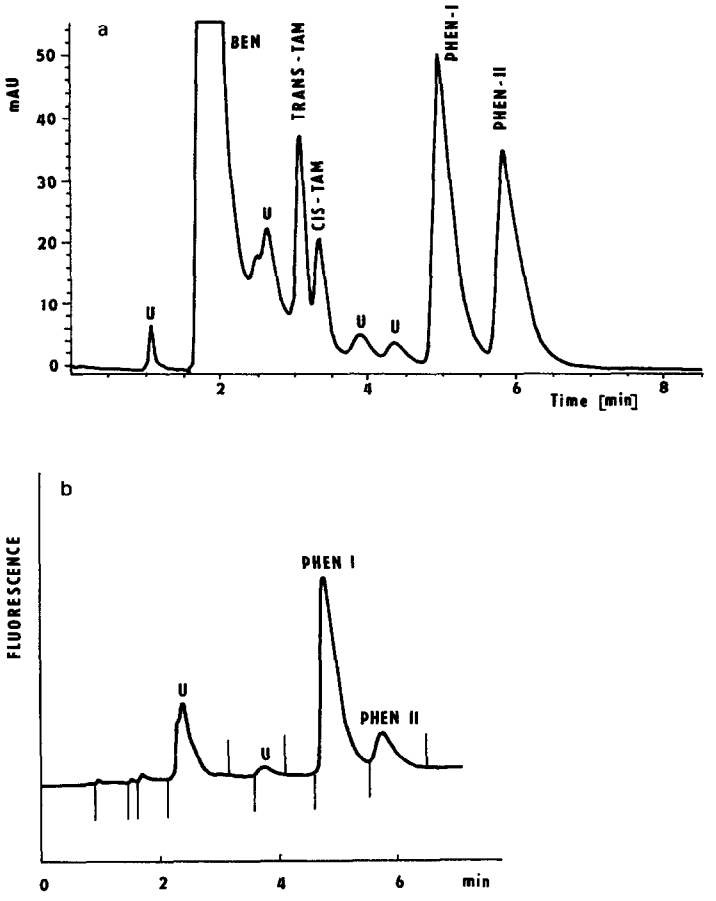

Fig. 4. HPLC of photoproducts of tamoxifen. BEN = benzene; TRANS-TAM = *trans*-tamoxifen; CIS-TAM = *cis*-tamoxifen; PHEN I = phenanthrene I; PHEN II = phenanthrene II; U = unknown. Flow-rate, 1 ml/min; sample, effluent fraction from irradiated PTFE capillary after extraction into benzene; detection, (a) UV (diode-array), 254 nm and (b) fluorescence, λ_{ex} = 245 nm, λ_{em} = 385 nm.

of one isomer to the other occurs with daylight. During irradiation for 10 h in an aqueous solution, 11% of the *trans* isomer was converted to the cis-isomer. This result explains the strict instruction that tamoxifen solution should be stored in the dark[8].

HPLC with diode-array and fluorescence monitoring of the effluent from the PTFE capillary confirmed that this isomerization paralleled the appearance of two other highly fluorescent peaks (Fig. 4). Photochemical cyclization of both isomers appeared to proceed through almost identical reaction sequences and no difference in reaction behaviour was noted when either *cis*- or *trans*-tamoxifen was irradiated. Tamoxifen isomerizes and the corresponding phenanthrenes are produced (Fig. 5). This assumption is supported by the existence of an absorption band at 236 nm in the UV spectra of *trans*-tamoxifen and phenanthrene I (Fig. 6). The UV spectra of both phenanthrene derivatives are similar to that of a photocyclization product published by Mendenhall *et al.*[2] and Nieder and Jaeger[8].

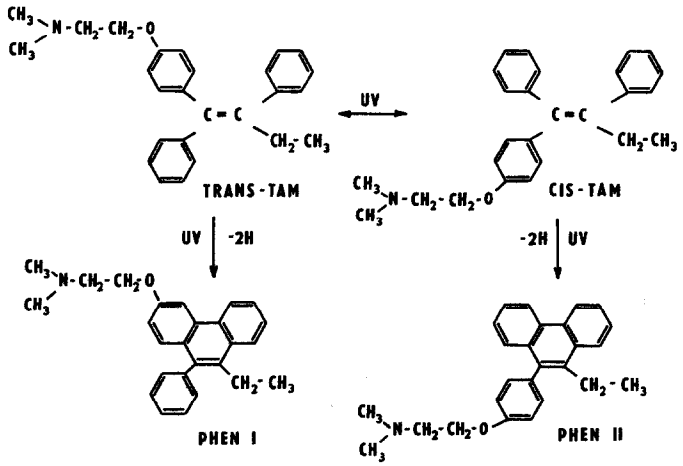

Fig. 5. Reaction scheme derived from identified products and their behaviour.

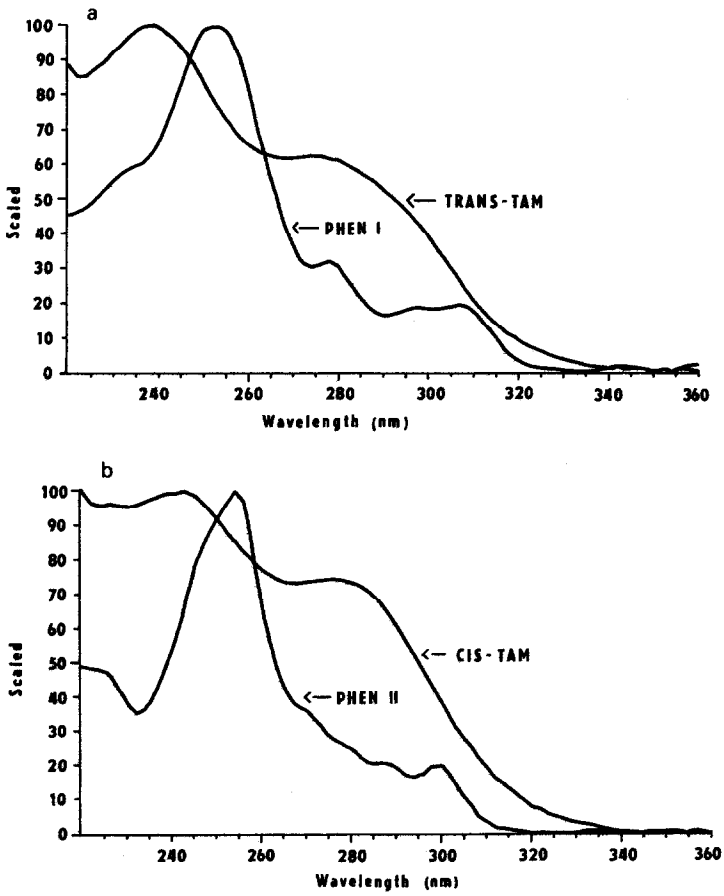

Fig. 6. UV spectra of (a) *trans*-tamoxifen and phenanthrene I and (b) *cis*-tamoxifen and phenanthrene II For abbreviations, see Fig. 4.

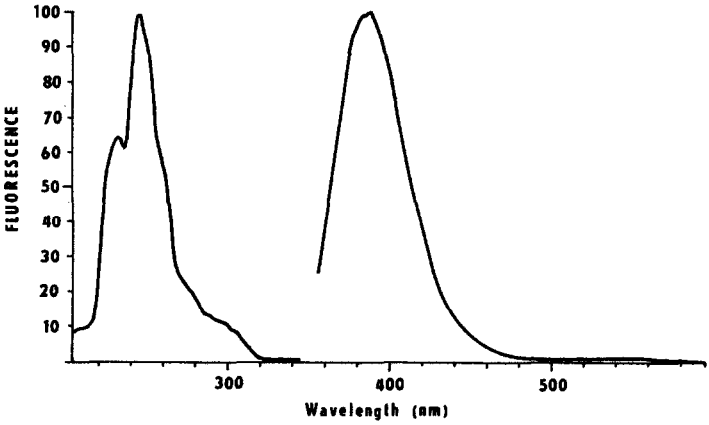

Fig. 7. Fluorescence spectrum of phenanthrene I. λ_{ex} = 245 nm, λ_{em} = 385 nm.

The fluorescence response of phenanthrene I is three times higher than that of phenanthrene II. This difference is probably caused by the presence and the position of the electron-donating group $-OCH_2CH_2N(CH_3)_2$, which tends to enhance the fluorescence by increasing the electron density of the phenanthrene aromatic system. The molecule of phenanthrene I could form a more rigid and planar structure, which is usually favourable for fluorescence.

Both *cis*- and *trans*-tamoxifen exhibit virtually no native fluorescence. The irradiation of effluent containing these compounds results in a rapid increase in the fluorescence response when measured with λ_{ex} = 245 nm and λ_{em} = 385 nm. The

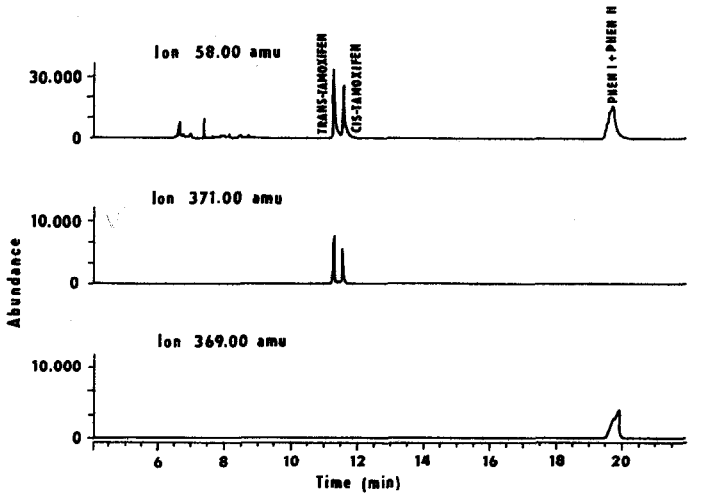

Fig. 8. GC–MS of photoproducts of *trans*-tamoxifen. For abbreviations, see Fig. 4. Chromatographic conditions: column, ULTRA 1 HP (12 m × 0.2 mm I.D.), film thickness 0.33 μm; carrier gas, helium; flow-rate, 0.4 ml/min; temperature programme, 60 to 260°C at 40°C/min; injection port temperature, 280°C. For sample, see Fig. 4.

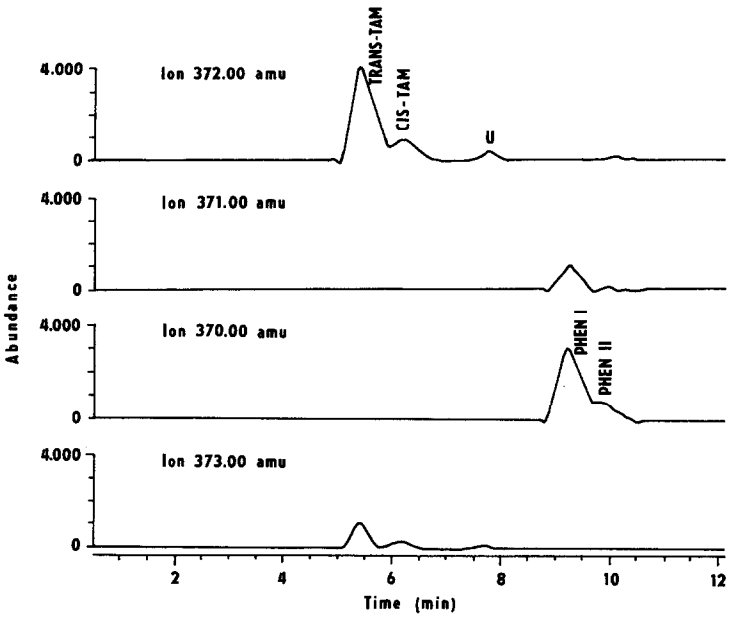

Fig. 9. HPLC–MS of *trans*-tamoxifen photoproducts. Chromatographic conditions: column, packed with Silasorb C_{18}; mobile phase, methanol–water–amonia (1000:50:7, v/v/v) plus 0.2 g/l of ammonium acetate; flow-rate, 1.2 ml/min; stem temperature, 120°C. For sample, see Fig. 4.

published excitation maxima vary from 251 to 266 nm and emission from 320 to 360 nm, depending on the type and the construction of the fluorescence detector[2–10]. The fluorescence spectra (Fig. 7) of the two phenanthrene derivatives are nearly identical when measured with a programmable fluorescence detector in the stop-flow mode. The spectra have maxima at λ_{ex} = 245 nm and λ_{em} = 385 nm. The fluorescence emission spectrum of a cyclization product published by Nieder and Jaeger[8] has two maxima at *ca.* 365 and 390 nm. The difference could be caused by the differences in the slits used for spectral scanning.

The identification of photoproducts was confirmed by GC–MS. All the main photoproducts contain the most intensive dimethylaminoethylene ion at *m/z* 58 produced by cleavage of the side-chain (Fig. 8, top). Although the separation of the two phenanthrene derivatives is not sufficient, it is evident that their mass is 369 u, whereas the separated tamoxifen isomers are detected at mass of 371 u.

Better resolution of all the photoproducts was achieved by LC–MS (Fig. 9). Mass spectra were measured by the use of the thermospray technique and ammonium acetate in the mobile phase so that M + 1 ions were produced. Peaks detected at 372 u are M^{+1} ions of tamoxifen isomers and those at 370 u are M + 1 ions of phenanthrene derivatives. Additionally, other smaller peaks at 371 and 373 u were found to be co-eluting phenanthrene peaks. The origin of these ions is not clear and an explanation would require further research. Unstable dihydrophenanthrene derivatives were not confirmed, although on the chromatogram (Fig. 8), in addition to the two peaks for the tamoxifens, other smaller peaks of mass 372 u were found. However, the disappearance of the yellow colour of irradiated effluents containing higher concen-

trations of tamoxifen could indicate the presence of dihydrophenanthrene derivatives[11].

The time of irradiation strongly influences the fluorescence yield and the formation of reaction products. At shorter times (1–5 s) isomerization mainly takes place and, *e.g.*, from *trans*-tamoxifen more phenanthrene I than II is formed. Prolonged irradiation resulted in a higher fluorescence response with the maximum at 9 s. The longer the irradiation of *trans*-tamoxifen, the more phenanthrene II was found in the reaction mixture and the total fluorescence response decreased.

CONCLUSIONS

This work has clarified that the tamoxifen cyclization used in LC makes it easier to detect trace amounts of tamoxifen, *e.g.*, in biological fluids. The variety of fluorescent products formed by UV irradiation reduces the advantages of precolumn derivatization.

The on-line postcolumn photocyclization of solutes in a PTFE or quartz capillary has the following advantages over precolumn derivatization: a higher fluorescence signal-to-noise ratio, which summarizes the contributions of all fluorescent products formed during photoreactions; and a less selective separation system may be required if the postcolumn reaction is used because a smaller number of peaks are separated.

Generally, this example takes advantage of an on-line and on-the-fly reaction system, permitting a more selective determination of compounds of interest in a biological matrix.

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