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# DETERMINATION OF TAMOXIFEN AND METABOLITES IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN FLUORESCENCE ACTIVATION

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## SUMMARY

Sensitive and reproducible analyses were developed for assaying tamoxifen, monohydroxytamoxifen, N-desmethyltamoxifen, metabolite E [*trans*-1(4-hydroxyphenyl)1,2-diphenylbut-1-ene] and a new metabolite, metabolite Y [*trans*-1(4-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene] in human serum using high-performance liquid chromatography (HPLC). Three different systems were developed for specific purposes. All chromatography was performed using serum extracts made with hexane—butanol. Detection was by fluorimetry of phenanthrene derivatives, formed by on-stream UV irradiation with a newly described device for post-column irradiation of the HPLC stream. This device may be of use in other HPLC systems requiring post-column photochemical reactions.

## INTRODUCTION

Tamoxifen is a nonsteroidal triphenylethylene anti-estrogen used routinely in the treatment of breast cancer and in basic endocrinology studies in the laboratory [1]. Consequently it is important to have convenient, sensitive and specific methods for assay of the parent drug and its metabolites in order to study pharmacokinetics, drug interactions, and the metabolism of tamoxifen in different species. Currently available methods use thin-layer (TLC) or high-performance liquid chromatographic (HPLC) methods [2—4] with detection based upon measurement of fluorescence developed by UV photochemical conversion of the triphenylethylene nucleus to fluorescent phenanthrenes (Fig. 1). The previously reported liquid chromatographic methods [3, 4] are based upon UV irradiation of extracted metabolites followed by chromatographic separation of the resulting phenanthrenes. While this method has

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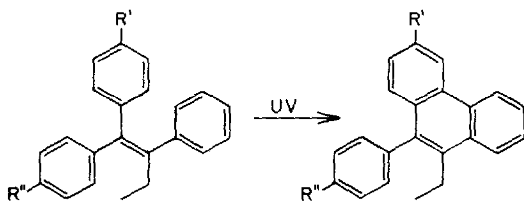


| | | | |
|---|---|---|---|
| (1) | TAMOXIFEN | R'= $OCH_2CH_2N(CH_3)_2$ | R"= H |
| (2) | N-DESMETHYLTAMOXIFEN | R'= $OCH_2CH_2NHCH_3$ | R"= H |
| (3) | 4-HYDROXYTAMOXIFEN | R'= $OCH_2CH_2N(CH_3)_2$ | R"= OH |
| (4) | METABOLITE E | R'= OH | R"= H |
| (5) | METABOLITE Y | R'= $OCH_2CH_2OH$ | R"= H |

Fig. 1. The chemical structure of tamoxifen and some of is metabolites, and the general reaction forming fluorescent derivatives of phenanthrene when these compounds are exposed to UV light.

generated useful information [5], the published chromatograms [3] exhibited broad and irregular peaks, probably the result of further photochemical degradation of the phenanthrenes. In an attempt to improve upon this system, we have developed an assay based upon on-stream activation of the fluorescence after chromatographic separation on HPLC columns. This paper describes a new on-stream post-column UV irradiation device and three HPLC systems for resolution of tamoxifen, several of its established metabolites, and a new metabolite, metabolite Y, from serum samples.

## MATERIALS AND METHODS

### *Solvents and reagents*

Solvents were HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Diethylamine and triethylamine were from Aldrich (Milwaukee, WI, U.S.A.).

Tamoxifen, N-desmethyltamoxifen, ICI 99,311 [1-(4-β-dimethylamino-ethoxyphenyl)-2,2-diphenylacrylonitrile], [$^3$H]monohydroxytamoxifen (42 Ci/mmole), metabolite E [*trans*-1(4-hydroxyphenyl)1,2-diphenylbut-1-ene], and metabolite Y [*trans*-1(4-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene] (Fig. 1) were gifts from ICI (Pharmaceuticals Division), Macclesfield, Great Britain. Stock standard solutions were prepared in appropriate solvents (hexane or methanol), and were kept dark and refrigerated. Working standards were prepared by dilution of the stock solutions in the chromatographic solvent.

### *Apparatus and chromatographic conditions*

A schematic representation of the system is shown in Fig. 2. A Perkin-Elmer Model 601 pumping system (Perkin-Elmer, Norwalk, CT, U.S.A.) was used. Flow from the column passed directly into a Rheodyne injector (Model 7010, Rheodyne, Cotati, CA, U.S.A.) used as a valve to direct the stream either to the fluorescence activation unit or to by-pass this unit. Thus, examination of replicate chromatograms with and without photochemical activation permitted

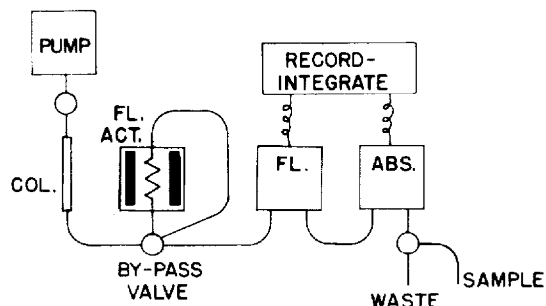


Fig. 2. Schematic representation of the HPLC system for analysis of tamoxifen metabolites using post-column activation of fluorescence by on-stream irradiation with UV light. Abbreviations: FL.ACT., fluorescence activator; COL., HPLC column; FL., fluorimeter with flow cell; ABS., spectrophotometer with flow cell. Components are described in the text.

distinction between fluorogenic components and those with native fluorescence.

The fluorescence activator consisted of a quartz capillary coil (approximately 70 cm × 0.2 mm I.D., 6 mm O.D.) interposed closely between two mercury UV lamps (Mineralite shortwave mercury lamps rated at 1200 $\mu W/cm^2$ at 15 cm) all in an air-cooled housing. Aluminum foil reflectors were placed in the housing to provide maximum luminosity on the quartz coil. Ozone generated from this unit was conducted to an exhaust hood. Each mercury lamp was separately powered so that illumination intensity could be varied. The chromatographic stream from this activator was directed to a filter fluorimeter (Fluorichrom, Varian Instruments) fitted with a 220- or 254-nm interference filter in the primary and a 360-nm filter in the secondary light path. The stream from the fluorimeter in some experiments was passed through a flow spectrophotometer (Model LC-55, Perkin-Elmer) set to measure absorption at 260 nm. A three-way valve in this exit stream allowed diversion of the stream to waste or to a fraction collector from which specific fractions could be recovered for further study such as mass spectrometry. The fluorimeter signal was recorded using an electronic recording integrator (Hewlett-Packard Model 3390A) which recorded peak areas and retention times.

*Sample preparation*

This procedure for the extraction of metabolites was suitable for both HPLC and TLC. All glassware used was washed with sulfuric acid—dichromate, distilled water, and rinsed with methanol prior to use. The serum sample was pipetted into conical centrifuge tubes (15 ml capacity) having PTFE-lined caps. Internal standard (ICI 99,311, [$^3$H]monohydroxytamoxifen, or metabolite E) in 10 $\mu$l of methanol, was added to serum samples, mixed and allowed to stand for at least 5 min before extraction with hexane containing 2% butanol. Serum volumes of 0.01—0.1 ml were extracted once with 1.0 ml of hexane–2% butanol; serum volumes larger than 0.1 ml were extracted once with 10 volumes of hexane—butanol. Tubes were mixed on a vortex mixer for 15 sec, centrifuged for 10 min and a portion of the supernatant fluid was transferred to 2.0-ml conical vials (Reacti-vials, Pierce Chemicals, Rockford, IL, U.S.A.) and evaporated at 55°C under nitrogen. Dried samples were redissolved

in 50 or 100 μl of HPLC solvent and aliquots were used for injection. All manipulations were carried out under subdued light or under yellow fluorescent light to minimize exposure to UV light.

*System I: tamoxifen, N-desmethyltamoxifen, and monohydroxytamoxifen*

Resolution of the major serum components, tamoxifen, N-desmethyltamoxifen, and monohydroxytamoxifen was achieved using a $C_{18}$ reversed-phase column (Whatman ODS-2, 10 μm particle size, 25 × 0.46 cm) eluted isocratically with absolute methanol containing 0.04% diethylamine acetate pumped at 2.0 ml/min. Using these conditions monohydroxytamoxifen has a retention time of 3.3 min, tamoxifen 5.4 min and desmethyltamoxifen 7.1 min, with baseline resolution (Fig. 3). Since it was not possible to find a suitable internal standard for this system, [$^3$H]monohydroxytamoxifen was used in tracer amounts to determine extraction and procedural recoveries. The appropriate fraction was collected from the HPLC stream and counted by liquid scintillation techniques. The quantity added was below fluorescence detection levels. Metabolite E, reported to be a metabolite of tamoxifen in the dog [6], was eluted at the void volume.

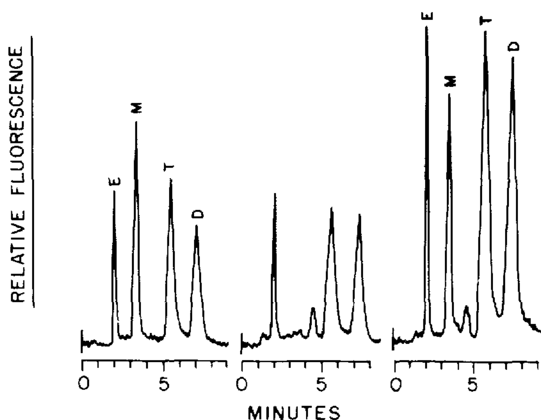


Fig. 3. Chromatography of tamoxifen metabolites on a $C_{18}$ reversed-phase column (System I). The first panel shows the resolution of a standard mixture of 25 ng each of metabolite E, monohydroxytamoxifen (M), tamoxifen (T), and N-desmethyltamoxifen (D). The second panel is a chromatogram of an extract equivalent to 20 μl of serum from a patient (I.J.) receiving 300 mg/m$^2$ of tamoxifen daily. In the third panel is shown a chromatogram of the same serum fortified with the four standard compounds. Chromatographic conditions are described in the text.

*System II: tamoxifen and desmethyltamoxifen*

Since a suitable internal standard for the reversed-phase system (System I) could not be found, a normal-phase system was developed using a silica column (Alltech 600-Si, 10 μm particle size, 25 × 0.46 cm, Alltech, Deerfield, IL, U.S.A.) eluted isocratically with methanol–water–triethylamine–acetic acid (98:2:0.03:0.3, v/v), and pumped at 3.0 ml/min. Using these conditions, N-desmethyltamoxifen had a retention time of 2.4 min, tamoxifen 3.7 min, and compound ICI 99,311, as an internal standard, eluted at 4.5 min. In this system monohydroxytamoxifen is not resolved from tamoxifen. Since monohydroxy-

tamoxifen is a very minor serum component in patients receiving usual doses of tamoxifen, it does not interfere significantly with the tamoxifen assay.

*System III: metabolites E and Y*

Since fluorogenic material including metabolite E was eluted with the void volume of the above system, a third system was developed which would retain this possible metabolite. This system used a silica column (Alltech 600-Si, 10-$\mu$m particle size, 25 $\times$ 0.46 cm), eluted isocratically with hexane containing 1.3% isopropyl alcohol. At a flow-rate of 3.0 ml/min, this system resolved metabolite E (retention time 3.9 min) and a second fluorogenic metabolite, identified as metabolite Y (Fig. 1) by co-chromatography and mass spectra, eluting at 8.0 min. Structural identification and biological properties of metabolite Y are reported elsewhere [7].

## RESULTS AND DISCUSSION

Fig. 1 shows the structures of the compounds studied and depicts the UV-catalyzed formation of fluorescent phenanthrenes which provides a sensitive method for detection. The fluorescence of these phenanthrenes was enhanced in acidic or neutral solvents, hence the diethylamine or triethylamine used in the solvents in Systems I and II was neutralized with acetic acid to improve sensitivity.

A schematic representation of the chromatographic system is shown in Fig. 2. The sample stream, by means of the Rheodyne valve, may be directed to the fluorescence activator, or may by-pass the activator. In this way duplicate injections can be compared with and without fluorescence activation and fluorogenic peaks thus identified. When it is desired to collect a given peak without photochemical alteration, the by-pass mode can be used and peaks collected either by elution time, or by detection of UV absorption in the flow spectrophotometer. Thus, unaltered fractions may be recovered for other studies without significant photochemical degradation.

The resolution of a mixture of standard compounds containing metabolite E, monohydroxytamoxifen, tamoxifen and desmethyltamoxifen using the $C_{18}$ reversed-phase system (System I) is illustrated in the first panel of Fig. 3. The second panel is a chromatogram of a serum extract from a patient who had received 300 mg/m$^2$ of tamoxifen daily for 32 days. The third panel is a chromatogram of the same serum to which were added 25 ng of each of the four standard compounds. Extraction efficiency of internal standard ([$^3$H]-monohydroxytamoxifen) or of added tamoxifen and N-desmethyltamoxifen averaged 85% and the limit of detection of each compound was about 0.2 ng per injection.

Since monohydroxytamoxifen was virtually undetectable in serum extracts using this technique, and since compound ICI 99,311 was found to be useful as an internal standard in our silica column system (System II), it has been more convenient to use System II (Fig. 4) for routine measurements of tamoxifen and its major metabolite, N-desmethyltamoxifen, in sera. The third and fourth panels of Fig. 4 show resolution of standards and of metabolites from serum from a patient receiving the usual dosage of tamoxifen (20 mg/m$^2$/

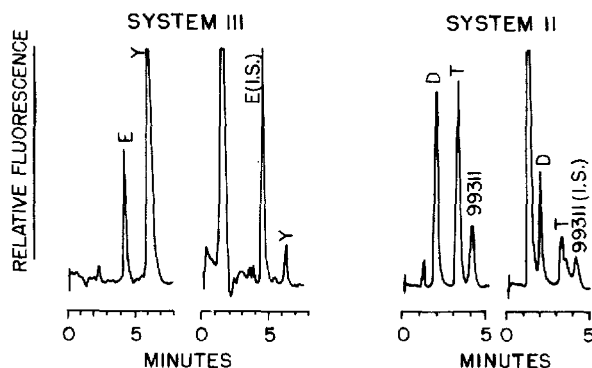


Fig. 4. Chromatograms of extracts from 0.5 ml of serum from a patient receiving normal dosages of tamoxifen (20 mg/m$^2$/day), resolved on System III (silica column eluted with 1.3% isopropyl alcohol in hexane), and in System II (silica column eluted with methanol—water—triethylamine—acetic acid described in the text). Compound ICI 99,311 was used as an internal standard (I.S.) in System II and metabolite E was used as an internal standard in System III. Metabolite E was not present in patient sera without the internal standard spike.

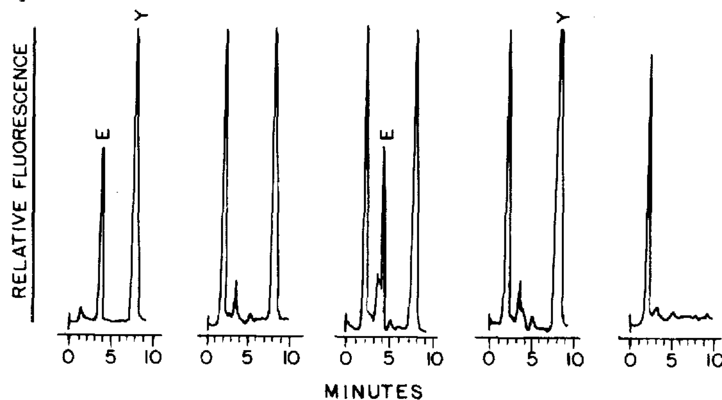


Fig. 5. Chromatography of extracts of serum from a high-dose tamoxifen patient (300 mg/m$^2$/day) using System III and showing the presence of a peak (retention time 8.0 min, panel 2) which co-chromatographs with metabolite Y (panel 4). Panel 2 shows that metabolite E is not detectable in this sample and that E is readily resolved from a minor peak (panel 3). Note that fluorescence disappears when the fluorescence activator is off (bypassed) in the last panel.

day). The limit of detection of tamoxifen and N-desmethyltamoxifen was about 0.2 ng per injection.

Metabolite E, a metabolite in the dog and a potential metabolite in humans, was eluted along with other fluorogenic material in the void volume in Systems I and II. To resolve such possible metabolites in this void volume, a silica system (eluted with hexane–isopropyl alcohol) was developed (System III) which retained metabolite E for a reasonable time (Figs. 4 and 5). Under these conditions, tamoxifen, N-desmethyltamoxifen and monohydroxytamoxifen were not eluted. In a high-dose patient (Fig. 5), there was no convincing evidence of the occurrence of metabolite E. However, in this high-dose patient and also in a normal-dose patient (Fig. 4, panel 2) there was a second fluorogenic peak which co-chromatographed with the compound designated metab-

olite Y (Fig. 5, panel 4). The last panel of Fig. 5 indicates that, except for material with native fluorescence in the void volume, no fluorescence is detectable when the column stream by-passes the UV activator.

Identification of peak Y was by co-chromatography with authentic metabolite Y (kindly provided by Dr. Adam, ICI) and by gas chromatography and mass spectral data of appropriate derivatives [7].

The three systems described gave linear standard curves and were reproducible from day to day within limits of column and equipment stability. Standard curves of tamoxifen, N-desmethyltamoxifen and metabolite E recovered from control or patient sera assayed using System II and System III, were linear in the range from 2—200 ng/ml of serum with correlation coefficients, $r$, of 0.988—0.999. Triplicate analyses using System II carried out on three separate days had a coefficient of variation of 6.8%. Within-day variation was similar. Using System III with an internal standard of metabolite E, recovery of metabolite Y standards added to blank serum averaged 97.4% of the theoretical recovery with an average coefficient of variation of 5.6% for additions in the range 12.5—100 ng for triplicate analyses carried out on two different days. Observed values were highly correlated with theoretical values ($r = 0.9986$).

It would be desirable to have a single system for the analysis of all tamoxifen metabolites. However, we have not been able to develop such a system probably because of the marked differences in properties between metabolites with and without the dimethylaminoethoxy sidechain.

There are several advantages of post-column on-line irradiation for development of fluorescence over previous methods of irradiating manually before chromatography. First, the exposure to UV is dependent only on the stability of the flow-rate and the UV lamp intensity; geometry of the system is fixed. Second, most UV-absorbing materials are resolved from the compounds of interest before irradiation, hence cannot quench their fluorescence development or react photochemically with them. Third, photochemical degradation products of the pre-irradiated sample undoubtedly produce a more complex sample which leads to a more complex chromatogram and poorer resolution, whereas post-column irradiation of the already resolved peaks does not.

The HPLC methods described here for the analysis of tamoxifen, its established metabolites, and the new component, metabolite Y, will be of considerable aid in the further study of the pharmacology, metabolism and endocrinology of this anti-estrogen in patients and in animal model systems.

The device described here for on-stream post-column UV irradiation may well have applications for other HPLC systems requiring photochemical reactions.

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