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Direct determination of tamoxifen and its four major metabolites in plasma using coupled column high-performance liquid chromatography

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Abstract

A rapid, rugged and fully automated method has been developed for the determination of tamoxifen and its major metabolites in plasma. The system is based upon an in-line extraction process combined with column switching to a coupled analytical column. The plasma sample is deproteinated by the addition of acetonitrile before injection onto a semi-permeable surface (SPS) cyano guard column (1.0 × 0.46 cm I.D.). After washing the guard column briefly with water, the sample is eluted with a mobile phase composed of 35% acetonitrile in 20 m*M* potassium phosphate buffer (pH 3). The eluent is directed through a cyano analytical column (25 × 0.46 cm I.D.) and a photochemical reactor where the analytes are converted to highly fluorescent phenanthrene derivatives. Tamoxifen, 4-hydroxytamoxifen, N-desdimethyltamoxifen, N-desmethyltamoxifen and tamoxifen-ol are eluted in that order at a flow-rate of 1.0 ml/min. The method has been validated for use in a clinical study utilizing tamoxifen in the treatment of recurrent cerebral astrocytomas.

1. Introduction

Tamoxifen is a nonsteroidal antiestrogen which is used in the treatment of all stages of breast cancer [1,2]. For example, clinical trials of adjuvant tamoxifen therapy for at least two years have demonstrated a reduction in the incidence of cancer in the contralateral breast [3,4] and the potential use of tamoxifen as a chemopreventative for breast cancer in women who are at high risk for the disease is currently being evaluated in the Breast Cancer Prevention Trial, a randomized, placebo-controlled clinical study.

In patients with breast cancer, the standard chronic clinical dose of tamoxifen is 10 mg twice daily administered orally and steady-state plasma levels are reached in 3 to 5 weeks. Tamoxifen is extensively metabolized in the human. Three of the metabolites found in plasma, 4-hydroxytamoxifen, 4-hydroxy-N-desmethyltamoxifen, and tamoxifen-ol are products of hydroxylation and two others are formed by N-demethylation, N-desmethyltamoxifen and N-desdimethyltamoxifen [5–9]. In plasma, N-desmethyltamoxifen is the major species; the ratio of N-desmethyltamoxifen to unchanged tamoxifen at steady state is normally greater than 1.0 [5–9].

Tamoxifen is undergoing evaluation in the

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treatment of patients suffering from recurrent Grade III or IV cerebral astrocytomas. In order to determine if a relationship exists between clinical response in the cerebral astrocytoma protocol and steady-state plasma levels of tamoxifen and its major metabolites, an HPLC method has been developed to rapidly process the clinical samples.

There are several methods described in the literature for the determination of tamoxifen and its metabolites. While these approaches are reliable, they do not overcome some of the problems inherent with the recovery and analysis of the target compounds. In particular, the analysis of tamoxifen is difficult due to the low plasma concentrations and extreme light sensitivity of the target compounds. Marked differences in extraction and elution properties between metabolites with and without hydroxy and/or dimethylaminoethoxy side chains have led to additional difficulties in designing an extraction scheme and a single chromatographic system for all species involved.

The photochemical conversion of tamoxifen and its metabolites to highly fluorescent phenanthrenes decreased the HPLC limits of detection to sub-nanogram levels [9–14,17]. However, when the photolysis was carried out off-line, broad, irregular peaks and irreproducible results caused by degradation of these derivatives affected the HPLC analysis. Brown *et al.* [11] were the first to avoid these problems by using post-column in-line irradiation. Later work optimized post-column treatment by refining the design of the photoreactor unit [12,13].

Nevertheless, the issue of pre-column photodegradation remained a concern since most published methods required time-consuming, labor-intensive sample clean-up either by liquid–liquid or solid-phase extraction before injection [9–11,14]. Kikuta and Schmid [12] reduced sample manipulation by introducing on-line sample preparation by substituting a 25×2 mm I.D. pre-column for the sample loop on the injection valve. A similar approach has been reported by Matlin *et al.* [15,16]. Lien *et al.* [17] successfully addressed the difficulties of sample handling and developing a chromatographic system for the simultaneous determination of tamoxifen and all its known metabolites. However, this assay required special equipment, such as a column oven, custom built post-column reactor, and custom packed, narrow-bore guard and analytical columns. We desired to develop an assay for tamoxifen and all its known metabolites using commercially available equipment and supplies which would allow us to handle large numbers of samples easily and economically.

The assay described in this paper uses a semipermeable surface (SPS) guard column to clean-up and concentrate deproteinated plasma samples in-line. SPS packing material is made up of two phases. A covalently bonded polyoxyethylene polymer forms a hydrophilic outer phase. The inner hydrophobic phase consists of reversed-phase material, in this case nitrile, bonded below the polymer to the silica surface. Off-line sample preparation as well as in-line clean-up is accomplished in minutes, thereby eliminating the problem of photodegradation. The analysis is performed at room temperature with commercially available guard columns, analytical column, and photoreactor. The assay has been validated and is presently in use in our laboratory.

2. Experimental

2.1. Chemicals

Tamoxifen, 4-hydroxytamoxifen, N-desmethyltamoxifen, N-desdimethyltamoxifen, and the primary alcohol tamoxifen-ol were gifts from Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). HPLC-grade acetonitrile was obtained from Anachemia Science (Montreal, Que., Canada). The dibasic potassium phosphate was purchased from BDH (Ville St. Laurent, Que., Canada).

2.2. Standard stock solutions

Methanolic stock solutions of tamoxifen and its four metabolites were each prepared in poly-

propylene tubes at a concentration of 1.0 mg/ml, wrapped in foil, and stored at −20°C.

2.3. Sample loading chromatography

The in-line sample loading chromatography was performed on a modular chromatograph composed of a Spectra-Physics SP2000 binary pump and a Spectra-Physics SP8880 autosampler equipped with a 50-μl sample loop (Spectra-Physics, San Jose, CA, USA). The compounds of interest were retained during the washing process on an SPS CN guard column (Regis Chemical, Morton Grove, IL, USA). Mobile phase B (deionized water passed through a 0.22-μm filter), at a flow-rate of 1.0 ml/min, was used for sample clean-up.

2.4. Separation chromatography

The separation chromatographic system included an ICT Beam Boost post-column photochemical reactor supplied with a 5-m reaction coil and a 254-nm UV lamp (Astec, Whippany, NJ, USA) which converted the compounds of interest to fluorophors. The effluent from the post-column converter was connected to an ABI SF 980 fluorescence detector (Ramsey, NJ, USA) operating at an excitation wavelength of 250 nm with an emission cut-off filter of 370 nm. A Spectra-Physics Datajet integrator acted as an interface for the electronic data collection using Winner on Windows software on a Spectra 386 computer (Spectra-Physics). The separation was performed on a Regis Rexchrom 250 × 4.6 mm I.D. CN (5 μm) column with a Regis C_{18} guard column.

Mobile phase A, used with the analytical system, consisted of 20 m*M* dibasic potassium phosphate (pH 3.1)–acetonitrile (65:35, v/v). The flow-rate was maintained at 1.0 ml/min by the same Spectra-Physics binary pump described in *Sample loading chromatography* when the analytical system was switched in-line.

2.5. Switching system

The two systems were connected through a six-port Rheodyne 7040 switching valve equipped with a Rheodyne 5701 pneumatic actuator. The switches were controlled by electric signals from the external events port of a Spectra-Physics DataJet integrator to a Rheodyne 7163 solenoid valve which then converted those signals to the corresponding pneumatic control signals. The switching-valve positions used during chromatography and the functions of these positions are presented in Table 1. A schematic representation of the system described is shown in Fig. 1.

2.6. Sample preparation

After thawing, the plasma samples were vortex-mixed vigorously for 1 min and 150 μl was transferred to a 0.5-ml amber polypropylene

Table 1
Program of valve positions during chromatography

Time (min)	SPS CN Column	Valve position	Analytical system
Pre-injection	On-line; mobile phase B at 1 ml/min	To waste	Off-line; equilibrated with mobile phase A; no flow through system
0-2	On-line; mobile phase B at 1 ml/min	To waste	Off-line
2-4	On-line; mobile phase A at 1 ml/min	To waste	Off-line
4-75	On-line; mobile phase A at 1 ml/min	To analytical system	On-line; mobile phase A at 1 ml/min to photoreactor and detector

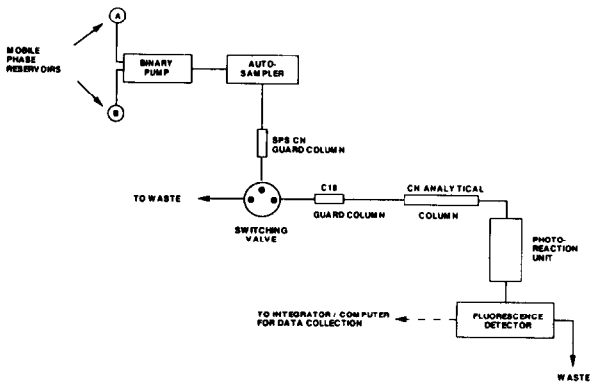


Fig. 1. Schematic representation of the HPLC system described in this paper.

microfuge tube. An equal volume (150 μl) of acetonitrile was added, the mixture was vortex-mixed for 2 min and centrifuged at 13 000 g for 5 min. A portion of the supernatant was transferred to 200-μl polypropylene autosampler vials.

2.7. Standard curves

A 6-point standard curve was prepared by adding known concentrations of tamoxifen and its metabolites covering the range anticipated in this study to drug-free plasma. 4-Hydroxytamoxifen was added at 5, 10, 20, 50, 100, and 200 ng/ml, whereas the concentrations of the other compounds of interest were 50, 100, 200, 500, 1000, and 2000 ng/ml.

2.8. Assay validation

Recovery of all compounds of interest was tested in plasma at two levels. The higher concentration sample contained 4-hydroxytamoxifen at 200 ng/ml and all other components at 2000 ng/ml. For recovery at a lower concentration the samples contained 4-hydroxytamoxifen at 20 ng/ml with the remainder of components at 200 ng/ml.

For intra-day and inter-day validation studies, control samples were prepared from drug-free plasma with tamoxifen and its metabolites added at three different levels. The low control sample contained 5 ng/ml 4-hydroxytamoxifen with 50 ng/ml of all other components, the middle contained 20 ng/ml 4-hydroxytamoxifen with 200 ng/ml of all other components, and the high control sample contained 100 ng/ml 4-hydroxytamoxifen with 1000 ng/ml of all other components.

The control samples used in the validation were stored in the dark at −20°C and have been re-assayed over a six-month period. There were no significant changes in the chromatographic result indicating that the samples are stable for at least this period of time.

2.9. Patient eligibility

Participating patients were 18 years of age or older and had histological diagnoses of recurrent Grade III or IV cerebral astrocytomas.

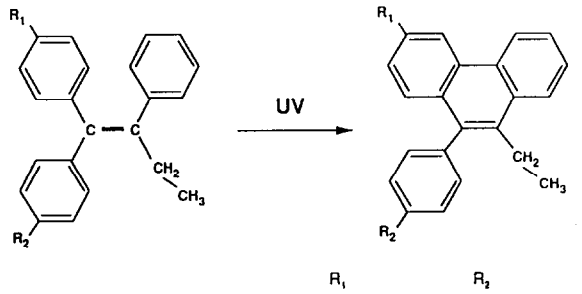
2.10. Study protocol

Patients received 200 mg tamoxifen orally twice daily (≈ 120 mg/m^2) for an 8-week period. Treatment was continued as long as there was evidence of stable disease, partial tumour response, or complete tumour regression. Pre-dose and steady-state samples (3–5 weeks) were collected for determination of tamoxifen and its metabolites. All biological samples were stored in the dark at −20°C until analysis.

3. Results and discussion

3.1. Chromatography

Fig. 2 shows the structures of the known tamoxifen species and their photochemically converted fluorescent phenanthrene derivatives. The compounds were eluted in the order: 4-hydroxytamoxifen, N-desdimethyltamoxifen, N-desmethyltamoxifen, tamoxifen, and tamoxifen-ol. The method is robust, showing little between-run variability in k' values as summarized in Table 2. Under the described chromatographic conditions, baseline separation of tamoxifen and



	R_1	R_2
tamoxifen	$OCH_2CH_2N(CH_3)_2$	H
4-hydroxytamoxifen	$OCH_2CH_2N(CH_3)_2$	OH
N-desmethyltamoxifen	$OCH_2CH_2NHCH_3$	H
primary alcohol	OCH_2CH_2OH	H
N-desdimethyltamoxifen	$OCH_2CH_2NH_2$	H

Fig. 2. Structures of tamoxifen and its metabolites along with their fluorescent phenanthrene derivatives.

its established metabolites was accomplished in biological samples. Towards this end, the addition of a C_{18} guard column to the analytical system was necessary to prevent co-elution of an unidentified peak with tamoxifen in plasma samples from patients in this study. No interfering compounds were found in pre-dose blank plasma. There were, however, several unidentified peaks found in steady-state plasma samples but not in pre-dose plasma blanks. It is possible to conclude that some of these peaks are other metabolites of tamoxifen, such as 4-hydroxydesmethyltamoxifen but without reference samples it was not possible to establish the identities of these compounds. Trace A in Fig. 3 is a chromatogram of spiked blank plasma; trace B is a chromatogram of pre-dose plasma from a patient and trace C is a chromatogram of a steady-state sample from the same patient.

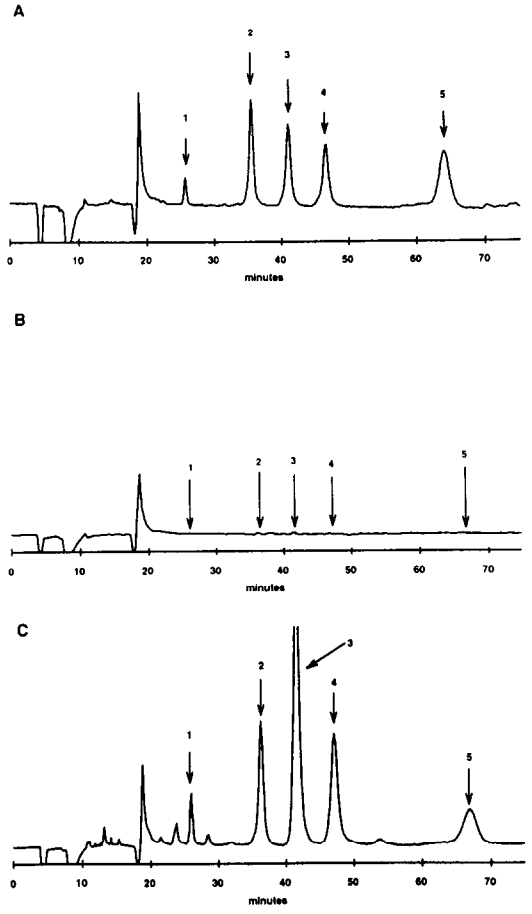


Fig. 3. (A) Chromatogram of drug-free plasma spiked with 50 ng/ml of 4-hydroxytamoxifen and 500 ng/ml each of N-desdimethyltamoxifen, N-desmethyltamoxifen, tamoxifen, and tamoxifen-ol. (B) Chromatogram of drug-free plasma. (C) Chromatogram of plasma from a patient after 4 weeks on a dose of 200 mg twice daily. Peaks: 1 = 4-hydroxytamoxifen; 2 = N-desdimethyltamoxifen; 3 = N-desmethyltamoxifen; 4 = tamoxifen; 5 = tamoxifen-ol.

Table 2
k' values for tamoxifen and its metabolites[a]

Run No.	4-Hydroxytamoxifen	N-Desdimethyltamoxifen	N-Desmethyltamoxifen	Tamoxifen	Tamoxifen-ol
1	2.39	3.32	3.84	4.36	6.00
5	2.39	3.31	3.82	4.34	5.95
40	2.41	3.34	3.85	4.36	6.13
110	2.41	3.36	3.85	4.35	6.16

[a] $T(0)$ was established as the first baseline disturbance after the valve switch.

Table 3
Plasma recoveries ($n = 4$)

Compound	Concentration (ng/ml)	Recovery (%)	C.V.[a] (%)
4-Hydroxytamoxifen	20	96.0	2.9
	200	93.1	2.4
N-Desdimethyltamoxifen	200	94.4	2.4
	2000	89.3	1.5
N-Desmethyltamoxifen	200	99.8	2.0
	2000	91.4	1.7
Tamoxifen	200	107.4	3.7
	2000	93.0	2.1
Tamoxifen-ol	200	84.3	3.1
	2000	88.0	2.1

[a]C.V. = coefficient of variation.

3.2. Validation

Recoveries for all compounds of interest were calculated at two levels between the limits of quantification by comparing peak areas of the in-line extracted concentrations from spiked plasma samples with peak areas from spiked aqueous samples. For all compounds other than tamoxifen-ol, the recoveries were equal to or greater than 89%; For tamoxifen-ol, recoveries were 84% and 88% at 200 and 2000 ng/ml, respectively. These apparent low recoveries were due to a peak shoulder seen on chromatograms of spiked plasma samples; the shoulder did not appear on chromatograms of spiked aqueous or patient plasma samples. C.V. for all recoveries were equal to or less than 3.7%. The results are tabulated in Table 3.

The standard curves, constructed by plotting peak area *vs.* spiked plasma concentrations, were linear over a range of 5–200 ng/ml for 4-hydroxytamoxifen and 50–2000 ng/ml for the remaining compounds. For all compounds studied, the correlation coefficients (r) were in the range of 0.9998 to 1.0000 while the regression coefficients (r^2) ranged from 0.9996 to 1.0000. The equations for the calibration curves including the S.E. values of the slope (a) and the y-intercept (b) with their corresponding correlation coefficients are presented in Table 4. Using a signal-to-noise ratio of 3, the calculated lower limit of detection (LLOD) for 4-hydroxy-

Table 4
Calibration curves

Compound	a (± S.E.)[a]	b (± S.E.)[a]	r
4-Hydroxytamoxifen	32.0 (± 0.13)	0.42(± 11.0)	1.0000
N-Desdimethyltamoxifen	20.2 (± 0.05)	−25.9 (± 47.4)	1.0000
N-Desmethyltamoxifen	17.1 (± 0.16)	48.7 (± 139.5)	0.9998
Tamoxifen	15.4 (± 0.03)	19.7 (± 27.9)	1.0000
Tamoxifen-ol	23.6 (± 0.11)	193.6 (± 91.3)	1.0000

($y = ax + b$): values for the slope and y-intercept (± S.E.) and the correlation coefficient for tamoxifen and its metabolites.
[a]All values = $\times 10^3$.

tamoxifen is 2 ng/ml and for all other compounds of interest ranges between 5 and 8 ng/ml for the method as written. However, since the chromatography resulted in such clean chromatograms, it was anticipated that, if necessary, the sensitivity of the method could be increased by increasing the injection volume.

The results of the intra- and inter-day precision and accuracy are given in Table 5. In all cases, accuracy was within 11% of theoretical and precision, calculated as the coefficient of variation (C.V.), was less than 7%.

3.3. Clinical study

This method is currently in use for the analysis of plasma samples from patients participating in a study for the treatment of cerebral astrocytomas with high doses of tamoxifen. The levels of tamoxifen and its four metabolites recovered varied greatly from individual to individual. The results for eight patients are shown in Table 6.

In order to compare inter-individual differences in metabolism, the concentrations of the metabolites were normalized to the concentration of tamoxifen in the same eight subjects. The resulting ratios are shown in Fig. 4. In all cases, the ratio of N-desmethyltamoxifen to tamoxifen was in the range of 1.3 to 2.1 whereas the ratio of 4-hydroxytamoxifen, the least abundant, to tamoxifen is never more than 0.1. These ratios are consistent with previously reported results [5–8].

4. Conclusion

The assay described in this manuscript is rugged, utilizes commercially available components and can be run at room temperature. Since the method is fully automated, large numbers of

Table 5
Accuracy and precision

Compound	Intra-day validation ($n = 3$)				Inter-day validation (5 days, $n = 3$)			
	Actual concentration (ng/ml)	Measured concentration (mean ± S.D.; ng/ml)	Accuracy (%)	C.V.[a] (%)	Actual concentration (ng/ml)	Measured concentration (mean ± S.D.; ng/ml)	Accuracy (%)	C.V.[a] (%)
4-Hydroxytamoxifen	5	4.6 ± 0.1	92.0	1.4	5	4.6 ± 0.2	92.0	3.9
	20	19.0 ± 0.5	95.0	2.8	20	19.0 ± 0.3	95.0	1.7
	100	99.6 ± 0.5	99.6	1.4	100	95.8 ± 5.4	95.8	5.6
N-Desdimethyltamoxifen	50	47.1 ± 0.9	94.2	1.9	50	45.8 ± 1.7	91.6	3.8
	200	195.2 ± 2.8	97.6	1.4	200	195.0 ± 7.6	97.5	3.9
	1000	990.3 ± 20.6	99.0	2.1	1000	1068.1 ± 66.3	106.8	6.2
N-Desmethyltamoxifen	50	49.1 ± 0.4	98.2	0.8	50	47.3 ± 2.2	94.6	4.7
	200	196.3 ± 0.8	98.0	0.4	200	198.0 ± 4.9	99.0	2.5
	1000	1026.8 ± 16.3	102.7	1.6	1000	1070.0 ± 42.4	107.0	4.0
Tamoxifen	50	48.5 ± 0.8	97.0	1.6	50	47.9 ± 0.8	95.8	1.7
	200	198.3 ± 3.4	99.2	1.7	200	198.4 ± 1.5	99.2	0.8
	1000	1035.8 ± 10.4	103.6	1.0	1000	1052.5 ± 36.7	105.3	3.5
Tamoxifen-ol	50	44.9 ± 1.2	89.8	2.6	50	45.1 ± 0.5	90.2	1.1
	200	198.7 ± 5.2	99.4	2.6	200	202.2 ± 4.1	101.1	2.0
	1000	951.5 ± 18.9	95.2	2.0	1000	1004.8 ± 32.4	100.5	3.2

[a]C.V. = coefficient of variation.

Table 6
Tamoxifen and metabolite levels determined from plasma samples collected 4 weeks after initiation of daily tamoxifen administration

Subject	4-Hydroxytamoxifen (ng/ml)	N-Desdimethyltamoxifen (ng/ml)	N-Desmethyltamoxifen (ng/ml)	Tamoxifen (ng/ml)	Tamoxifen-ol (ng/ml)
1	37.1	323.6	871.3	423.5	192.0
2	58.9	465.4	1288.9	798.4	400.2
3	67.4	639.4	1388.1	849.4	477.3
4	42.7	521.4	1258.4	657.1	537.5
5	69.7	455.0	1713.0	1298.3	496.9
6	40.0	357.3	972.0	585.4	299.9
7	51.2	379.0	1113.5	579.6	205.1

specimens can be processed within a short period of time with a minimal risk of photochemical degradation. The system also protects the analytical column from plasma components through the use of an SPS guard column which can be easily and inexpensively replaced. Thus, this analytical approach overcomes many of the disadvantages of other published methods.

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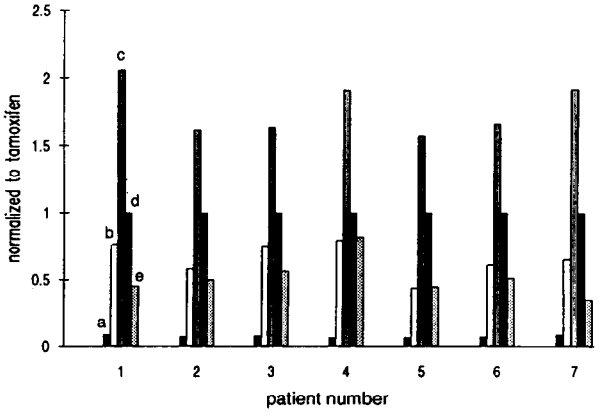

Fig. 4. Analytical results of the steady state plasma samples from 7 patients where: a = 4-hydroxytamoxifen, b = tamoxifen, c = N-desmethyltamoxifen, d = tamoxifen-ol, and e = N-desdimethyltamoxifen. The concentrations (in ng/ml) of the metabolites were normalized to the concentration of tamoxifen.

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