CLIN. CHEM. 33/9, 1608–1614 (1987)

Determination of Tamoxifen and Four Metabolites in Serum by Low-Dispersion Liquid Chromatography

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In this assay of tamoxifen and four metabolites in human serum, the serum samples are deproteinized with an equal volume of acetonitrile, then injected into a small (0.21 × 2 cm) precolumn packed with 5-μm-diameter octadecylsilane (ODS) particles. The samples are concentrated on-column by equilibrating the column with an equivolume solution of water and acetonitrile containing 3 mmol of acetic acid and 2 mmol of diethylamine per liter. The drugs are then directed into an analytical ODS column (0.21 × 10 cm) by changing the mobile phase followed by column switching. The primary alcohol of tamoxifen ("metabolite Y"), 4-hydroxytamoxifen ("metabolite B"), tamoxifen, *N*-desdimethyltamoxifen ("metabolite Z"), *N*-desmethyltamoxifen ("metabolite X"), and 4-methoxytamoxifen (internal standard) are eluted in this order at a flow rate of 0.3 mL/min with a mobile phase of acetonitrile/water (91/9 by vol) at low ionic strength (1 mmol of acetic acid and 0.67 mmol of diethylamine per liter) and detected by post-column fluorescence activation by passage through a capillary quartz tube exposed to ultraviolet light. Analytical recovery was close to 100%. Within-day precision corresponded to a CV of 1–5% at serum concentrations of tamoxifen or metabolites >10 μg/L; the detection limit of the assay for these compounds was about 1 μg/L. This fully automated assay has the advantage of simple sample processing, high sample output, low solvent consumption, high analytical recovery of tamoxifen and four metabolites in serum, and determination of all these compounds plus an internal standard in a single run.

Additional Keyphrases: *drug assay · chemotherapy · anti-estrogen drug · pharmacokinetics · mass spectrometry*

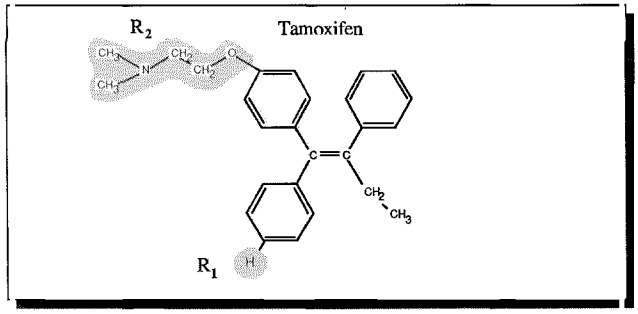
Tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene], a nonsteroidal anti-estrogen, has been used extensively in the palliative treatment of breast cancer for more than a decade (*1*). The biological activity of this drug has been attributed to both the parent compound and its metabolites. *N*-Desmethyltamoxifen ("metabolite X") is the major metabolite of tamoxifen in human serum, whereas only trace amounts of 4-hydroxytamoxifen ("metabolite B") have been detected (*2, 3*). However, interest has been focused on the latter compound because of its high potency as an anti-estrogen (*4*). Other metabolites in humans are *trans*-1-(4-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene ("metabolite Y") (*5, 6*) and *N*-desdimethyltamoxifen ("metabolite Z") (*5*). The structures of these compounds are depicted in Figure 1.

The widespread use of tamoxifen has stimulated efforts to develop routine assays for this drug and its metabolites in human plasma. Several techniques have been published. A procedure based on gas chromatography and mass spectrometry is highly specific, but requires derivatization of sample and involves equipment not generally available (*2*). The thin-layer and "high-pressure" liquid-chromatographic (HPLC) methods (*7–9*) described all involve photochemical conversion of tamoxifen and its metabolites to fluorescent phenanthrene derivatives before chromatography.[5] An HPLC assay with post-column fluorescence activation, developed by Brown et al. (*10*), avoids problems related to the variable photochemical degradation of the phenanthrenes.

The published HPLC methods for tamoxifen in serum do not allow the simultaneous determination of the drug and its major metabolites in serum. Furthermore, most assays require a time-consuming extraction of the compounds into an organic phase, evaporation, and redissolving the samples before injection. Sufficient sample clean-up has also been obtained by passing samples through reversed-phase cartridges (*11*). The assays based on precolumn derivatization (*8, 9*) include even further processing, i.e., constant ultraviolet illumination of the extract. The variable recovery obtained can be compensated for by using an internal standard, but trying to select a suitable compound has presented problems (*10*). Another disadvantage with the established assays is the high consumption of the organic solvent, usually methanol, in the mobile phase, owing to the hydrophobic properties of the analytes and the high flow rates used.

Our efforts to develop an improved assay for tamoxifen

[5] Nonstandard abbreviations: HPLC, "high-pressure" liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; ODS, octadecylsilane.



Identity	Abbreviation	R_1	R_2
4-Hydroxytamoxifen	Metabolite B	HO	CH_3, CH_3 >NCH_2CH_2O-
N-Desmethyltamoxifen	Metabolite X	H	CH_3, H >NCH_2CH_2O-
Primary alcohol	Metabolite Y	H	HO–CH_2CH_2O-
N-Desdimethyltamoxifen	Metabolite Z	H	$H_2NCH_2CH_2O$-

Fig. 1. Structural formulas of tamoxifen and four metabolites

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Received March 26, 1987; accepted June 1, 1987.

and its metabolites were motivated by the need to evaluate the biological effects and pharmacokinetics of the newly discovered metabolites in serum relative to the kinetics of the parent drug. Furthermore, new therapeutic regimens combining the use of tamoxifen with other endocrine therapies in breast cancer (*12–15*) suggest the possibility of pharmacokinetic interaction. Finally, both tamoxifen and its active metabolites in serum must be monitored to optimize dose schedules and to evaluate patient compliance.

Here we describe a simple assay for the determination of tamoxifen and four metabolites in human serum. With this assay, which exploits the recent development of "low dispersion" liquid chromatography (*16*), we obtained on-column concentration and separation of these compounds under conditions characterized by low acetonitrile content and low ionic strength, respectively.

Materials and Methods

Materials

Reagents. Tamoxifen, 4-hydroxytamoxifen, and *N*-desmethyltamoxifen were obtained from Pharmachemie B.V., Haarlem, Holland. Metabolite Y, *N*-desdimethyltamoxifen, and the 4-hydroxytamoxifen used to synthesize the internal standard were gifts from Imperial Chemical Industries PLC, Pharmaceuticals Div., Macclesfield, Cheshire, U.K. "HPLC-grade" acetonitrile was purchased from Rathburn Chemicals, Ltd., Walkerburn, Scotland, U.K. Acetic acid and diethylamine were from Merck AG, Darmstadt, F.R.G. The 0.21 × 10 cm reversed-phase analytical column [packed with 5-μm particles of octadecylsilane (ODS)-Hypersil] and the 0.21 × 2 cm precolumn (same packing) were purchased from Hewlett-Packard, Palo Alto, CA.

The internal standard was synthesized from 4-hydroxytamoxifen by derivatization in the presence of diazomethane (*17*). Diazomethane in ether was added to a solution of 4-hydroxytamoxifen in methanol (400 mg in 10 mL), then stirred for 30 min at room temperature. The reaction mixture was analyzed and purified on a 0.46 × 10 cm reversed-phase column packed with 3-μm particles of ODS-Hypersil, eluted isocratically with a mobile phase of acetonitrile/water (84/16 by vol) containing 3 mmol of acetic acid and 2 mmol of diethylamine per liter. Four ultraviolet-absorbing peaks, including 4-hydroxytamoxifen, were observed; the peak with longest retention time was analyzed in the chromatographic system routinely used for determination of tamoxifen and its metabolites in serum (see below), and was separated from these compounds. Therefore, the material in this peak was regarded as a suitable internal standard. The selected peaks from repeated runs were pooled, evaporated, and subjected to liquid chromatography/mass spectrometry (LC/MS) with a Model 201 mass spectrometer (Vestec, Houston, TX). The mass of the molecular ion $(M + 1)^+$ was 402 *m/z*. We tentatively identified the compound as methoxytamoxifen.

Standards. Tamoxifen, metabolite Y, 4-hydroxytamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen were dissolved in 100% methanol at a concentration of 4 g/L. We diluted these stock solutions to known concentrations, either in acetonitrile/water (1/1 by vol) or in serum.

Procedures

Sample processing. Serum was routinely processed by mixing samples with an equal volume of 100% acetonitrile; we removed the precipitated protein by centrifugation. The supernates were transferred to sample vials, capped, and analyzed.

Chromatography. The HPLC system was programmed to inject 250-μL samples into the 0.21 × 2 cm precolumn packed with 5-μm particles of ODS-Hypersil and equilibrated with acetonitrile/water (1/1, by vol) containing 3 mmol of acetic acid and 2 mmol of diethylamine per liter. Under these conditions the analytes are concentrated on the precolumn. After 0.1 min, we changed the acetonitrile proportion of the mobile phase to 91% and the concentrations of acetic acid and diethylamine to 1 and 0.67 mmol/L, respectively. The mobile phase flows through the precolumn, bypassing the analytical column from time zero to 1.4 min, after which the effluent from the precolumn is directed to the small-bore analytical reversed-phase column, 0.21 × 10 cm, packed with 5-μm particles of ODS-Hypersil, by an automated column-switching valve. The temperature is kept at 40 °C and the flow rate at 0.3 mL/min.

Recovery and precision studies. Tamoxifen, metabolite Y, 4-hydroxytamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen were added to drug-free serum or to an equivolume solution of acetonitrile/water to give a concentration of 4 mg of each compound per liter. From these solutions, we prepared samples containing each compound at 800, 100, or 10 μg/L in either matrix. We then extracted the serum samples with an equal volume of acetonitrile and calculated the recovery as the percentage recovered from serum relative to the amount detected in the acetonitrile/water matrix.

To determine the within-run precision (CV) of the assay, we assayed 10 replicates of serum with added tamoxifen, metabolite Y, 4-hydroxytamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen, each at a concentration of 800, 100, or 10 μg/L. The between-day precision was determined by assaying, on 10 different days, sera from another batch prepared in the same way.

We included the internal standard in all samples by adding this compound to the acetonitrile used for deproteinizing the samples. The amount added was adjusted to give a fluorescence peak corresponding to that produced by about 100 μg of tamoxifen per liter.

Assay standard curve and detection limit. We prepared a standard curve by adding known concentrations of tamoxifen and its metabolites, ranging between 1600 and 0.5 μg/L, to serum. The detection limit was determined by extracting serum samples containing added tamoxifen and its metabolites, at concentrations of 20, 10, 4, 2, 1, or 0.5 μg/L.

Instrumentation

We used a Hewlett-Packard liquid chromatograph (Model HP 1090), equipped with a ternary solvent-delivery system, an HPLC autosampler, a column-switching valve, and a 250-μL injection syringe. The columns were mounted in an oven and the temperature was set at 40 °C. The effluent from the column was connected to a post-column converter with a 0.17 mm (i.d.) stainless-steel tube. The converter (Figure 2) was built at our laboratory, as follows:

We obtained a flexible capillary quartz tube, 1 m × 0.2 mm (i.d.), by removing the coating from a fused silica tubing. We then connected the silica tubing to a stainless-steel 0.17 mm (i.d.) tubing by using a zero-dead-volume butt connector (Supelco, Bellefonte, PA). We made every effort to avoid dead volume, which seriously deteriorates the chromatographic resolution of the low-dispersion LC system. We mounted the fused silica tubing in a reflector chamber,

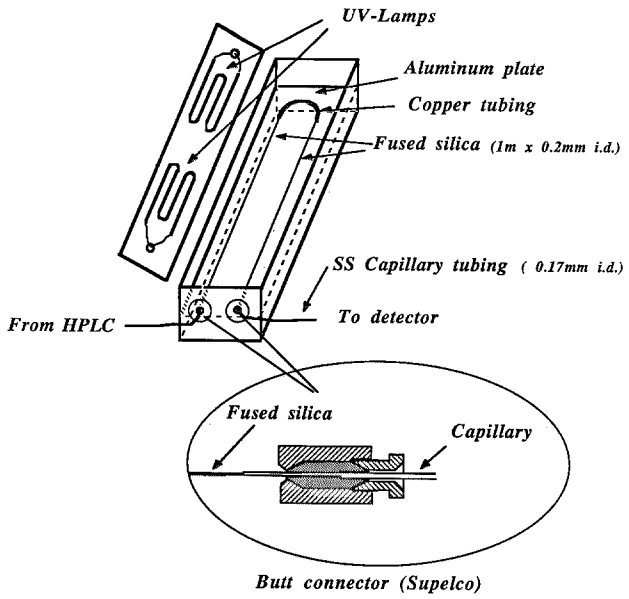

Fig. 2. Construction of the post-column fluorescence converter
SS, stainless steel; *UV*, ultraviolet

supporting the bent portion with copper tubing. The silica tube was illuminated by two ultraviolet-emitting lamps (R-52G; Ultra-Violet Products, Inc., San Gabriel, CA) placed 2 cm from the tube. The chamber was cooled and the ozone produced was removed from the chamber by using a fan. The outlet from the converter was connected to an HPLC fluorescence detector (Model 980; Kratos, Manchester, U.K.). We adjusted the wavelength of the primary light path to 251 nm (10-nm slit width) and fitted the secondary light path with an 360-nm interference filter.

To identify the eluting peaks, we connected the effluent from the column to a LC/MS thermospray system (Vestec). Before it entered the thermospray probe, the effluent was mixed with 0.1 mmol/L ammonium acetate reagent, delivered at a rate of 0.7 mL/min via a zero-dead-volume T-connector.

Results

Extraction procedures. We prepared serum samples to contain 10, 100, or 800 μg each of tamoxifen, metabolite Y, 4-hydroxytamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen per liter. All samples contained the same concentration of internal standard. We obtained complete recovery of these compounds from serum extracted with an equal volume of acetonitrile (Table 1).

Liquid chromatography. Figure 3 shows chromatograms of serum from a patient not taking tamoxifen (blank serum, trace *B*); drug-free serum with added tamoxifen, metabolite Y, 4-hydroxytamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen (20 or 400 μg/L each), and the internal standard (traces *A* and *C*); and serum from a patient who had received 40 mg of tamoxifen twice daily for months (trace *D*).

We found no interfering compounds in blank plasma.

The compounds eluted in the order: metabolite Y, 4-hydroxytamoxifen, tamoxifen, *N*-desdimethyltamoxifen, *N*-desmethyltamoxifen, and the internal standard. This baseline separation depended critically on eluting the analytical column at low ionic strength (1 mmol of acetic acid and 0.67 mmol of diethylamine per liter). When we increased the concentrations of acetic acid and diethylamine in the mobile phase by more than twofold, tamoxifen and *N*-desdimethyltamoxifen co-eluted in all elution systems tested, including variations in acetonitrile concentration (60–100%) and pH (obtained by changing the relative amounts of diethylamine and acetic acid).

We also observed that these two compounds co-eluted when the concentration of acetonitrile deviated markedly from 91%.

Metabolite Y eluted close to the solvent front, such that a serum peak caused some interference at low concentrations of this compound (<4 μg/L). Also, 4-hydroxytamoxifen eluted at a point corresponding to a slight fluctuation in the baseline, which interfered with peak integration at low concentrations. We therefore used peak height instead of peak area for the determination of this compound in the concentration range 1–3 μg/L. The hydrophobic compounds—tamoxifen, *N*-desdimethyltamoxifen, *N*-desmethyltamoxifen, and the internal standard—showed the longest retention times, eluting in a region where the baseline was stable. All compounds, except *N*-desdimethyltamoxifen, showed about the same fluorescence yield (Figure 3).

Identification of chromatographic peaks by LC/MS. Spectra for all reference compounds, except metabolite Y, gave only one major peak corresponding to the $(M + 1)^+$ ion, whereas metabolite Y gave the $(M + 18)^+$ ion, which corresponds to the ammonia adduct.

Figure 4, trace *B*, shows a single-ion monitoring trace of the same serum sample shown in Figure 3*C*. We set the instrument to monitor one ion at a time, at selected times: *m/z* 362 (metabolite Y) from 0 to 5 min, *m/z* 388 (4-hydroxytamoxifen) from 5 to 7.5 min, *m/z* 372 (tamoxifen) from 7.5 to 10 min, *m/z* 344 (*N*-desdimethyltamoxifen) from 10 to 12 min, *m/z* 358 (*N*-desmethyltamoxifen) from 12 to 15 min, and *m/z* 402 (4-methoxytamoxifen) from 15 to 21 min. The response differed markedly between the compounds. 4-Hydroxytamoxifen produced a large peak; tamoxifen, metabolite Y, and *N*-desmethyltamoxifen gave a moderate response; and *N*-desdimethyltamoxifen gave a small response. The internal standard could not be detected.

Table 1. Analytical Recovery of Tamoxifen and Its Metabolites in Serum

Concn, μg/L	% of added that was measured: Without I.S. Mean	SD	CV, %	With I.S. Mean	SD	CV, %
Metabolite Y						
10	102	2.1	2.0	121	2.8	2.3
100	111	1.1	1.0	129	1.5	1.2
800	105	0.7	0.7	132	2.8	2.1
4-Hydroxytamoxifen						
10	106	1.3	1.2	126	2.0	1.6
100	107	0.6	0.6	130	1.5	1.1
800	108	0.7	0.7	138	3.3	2.3
Tamoxifen						
10	102	1.7	1.8	118	2.6	2.2
100	103	0.8	0.7	123	1.9	1.5
800	106	0.8	0.8	125	2.5	2.0
N-*Desdimethyltamoxifen*						
10	103	5.7	5.6	111	6.3	5.7
100	101	1.4	1.4	117	1.4	1.2
800	100	0.9	0.9	125	2.5	2.0
N-*Desmethyltamoxifen*						
10	104	2.1	2.0	124	2.7	2.2
100	105	0.6	0.6	125	1.8	1.5
800	108	1.2	1.1	125	2.9	2.3

n = 10 each. I.S., internal standard.

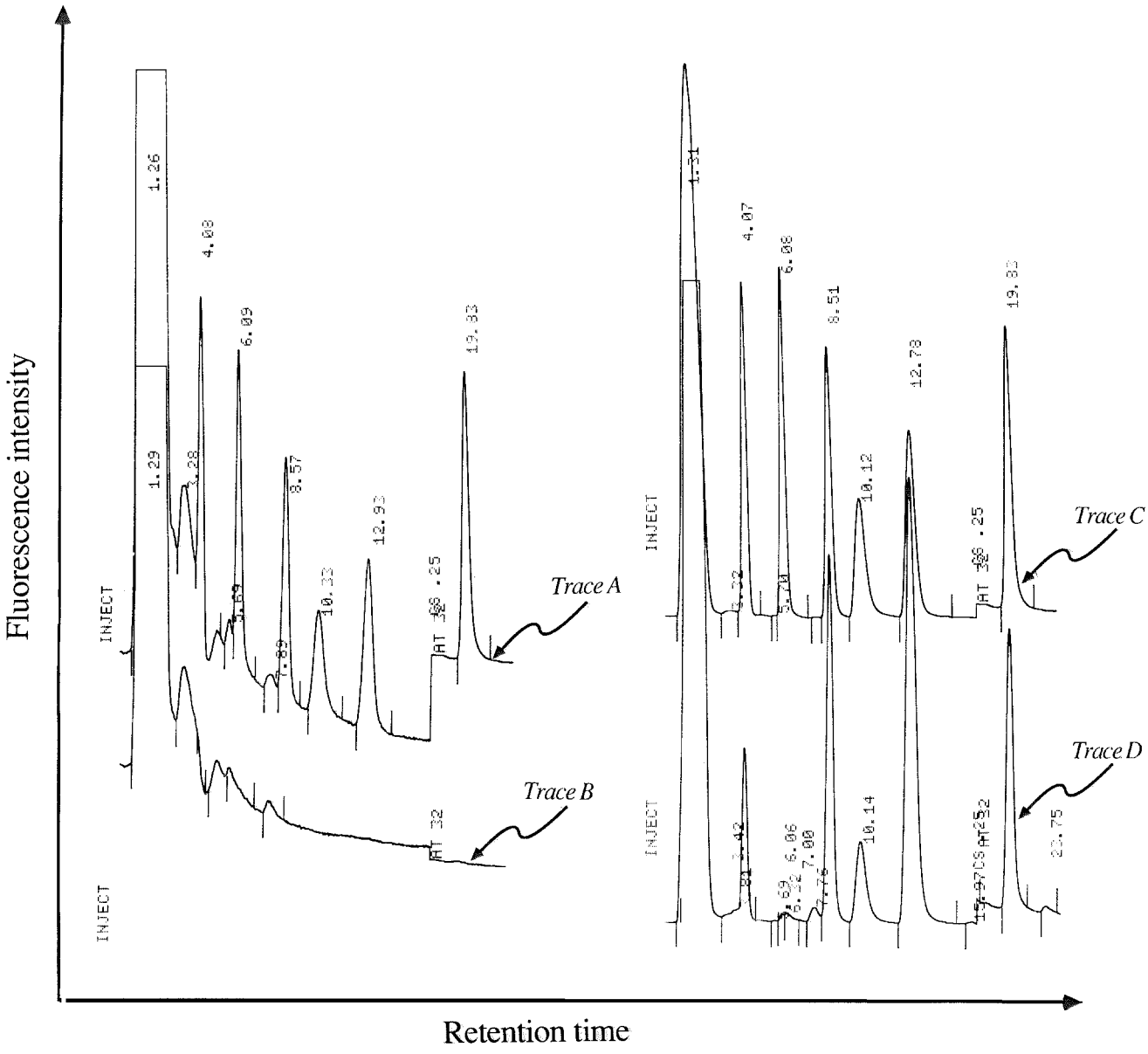

Fig. 3. Chromatograms of tamoxifen and four metabolites plus internal standard in serum, indicating the respective retention times (minutes)

A; serum plus 20 μg/L each of metabolite Y (4.08 min), 4-hydroxytamoxifen (6.09 min), tamoxifen (8.57 min), *N*-desdimethyltamoxifen (10.33 min), and *N*-desmethyltamoxifen (12.78 min). *B;* blank serum recorded at the same attenuation as *A*. *C;* serum with 400 μg of each compound added per liter. *D;* serum from a patient treated with tamoxifen (40 mg twice daily) for 12 months; attenuation as in *C*. All samples except blank serum were supplemented with internal standard to give a fluorescence yield corresponding to that of 100 μg of tamoxifen per liter. The attenuation was automatically adjusted after 16 min, so that the height of the peak did not exceed the range of the chart recorder

Trace *C*, a LC/MS trace of the serum shown in Figure 3*D* for a patient receiving tamoxifen for months, contains distinct peaks corresponding to the retention times of metabolite Y, tamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen. Inspection of Figure 3*C* and *D* and Figure 4*B* and *C* shows that the heights of the separate peaks relative to that of the standards were about the same for both detector systems. This observation confirms the identity of these peaks in chromatograms from the patient treated with tamoxifen.

Standard curves, detection limit, and precision of the method. The standard curves for tamoxifen, metabolite Y, 4-hydroxytamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen are shown in Figure 5. The standard curves were linear over a wide range, from about 1 to 1600 μg/L in serum, except for the curves for metabolite Y and 4-hydroxytamoxifen. These curves leveled off at about 1000 μg/L because the sharp, early-eluting peaks exceeded the range of the fluorescence detector.

The detection limit for tamoxifen and *N*-desmethyltamoxifen was 0.5–1 μg/L of serum. Because of the low fluorescence yield of *N*-desdimethyltamoxifen, its detection limit was somewhat higher, about 2 μg/L (Figure 3). The detection limit of metabolite Y and 4-hydroxytamoxifen was also about 2 μg/L, owing to the chromatographic interference at low concentrations described above.

The within-day precision (CV) of the assay of tamoxifen and its metabolites was about 1% at 800 μg/L, 1% at 100 μg/L, and 3% at 10 μg/L. The corresponding between-day CVs were about 4%, 3%, and 6%, respectively. Notably, the

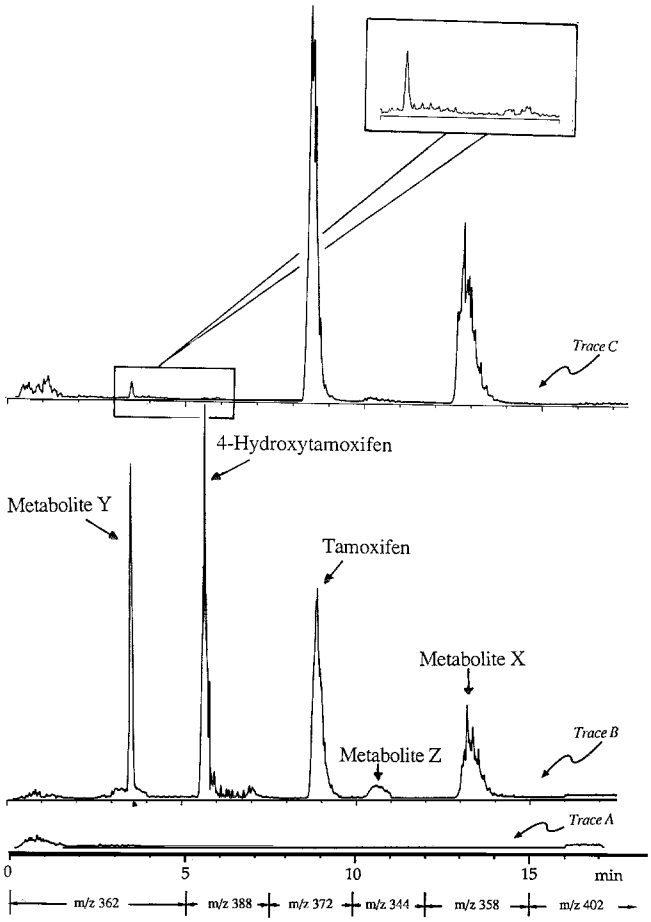

Fig. 4. Identification of tamoxifen and metabolites in serum by LC/MS
A; blank serum. *B;* serum with added drug and metabolites (400 μg/L). *C;* serum from same patient as in Fig. 3*D*. The time-table for the selection of the monitored ions (as described in detail in text) is shown below the time axis, and the abundance of the separate ions is plotted vs retention time

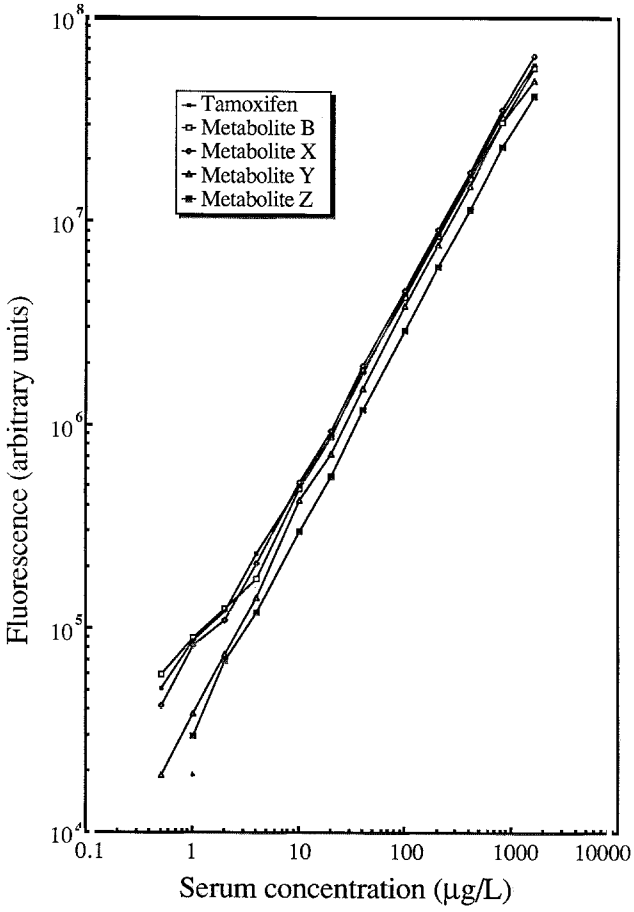

Fig. 5. Standard curves for tamoxifen and four metabolites
Linear regression analysis gave the following curve fits: tamoxifen, $y = 38102x + 522823$, n = 12, $r = 1.00$; 4-hydroxytamoxifen (metabolite B), $y = 36340x + 502079$, n = 12; $r = 1.00$; *N*-desmethyltamoxifen (metabolite X), $y = 41913x + 360545$, n = 12, $r = 1.00$; metabolite Y, $y = 32406x + 650485$; n = 12, $r = 0.99$; *N*-desdimethyltamoxifen (metabolite Z), $y = 26907x + 280646$, n = 11, $r = 1.00$

precision was not significantly improved by including the internal standard and correcting the values accordingly. Details of the precision experiments are given in Table 2.

Interference from various drugs. We tested various drugs for interference with the tamoxifen assay by analyzing serum from patients who were taking therapeutic doses of the following drugs but not tamoxifen: acetaminophen, allopurinol, amitriptyline, chlorpromazine, chlorthalidone, codeine, dexamethasone, diazepam, diflunisal, doxepin, furosemide, flurazepam, glibenclamide, hydrochlorothiazide, ipratropium bromide, isosorbide dinitrate, megestrol acetate, methotrexate, morphine, naproxen, nortriptyline, propranolol, sulfamethoxazole, terbutaline sulfate, theophylline, trimethoprim, and warfarin. None of these drugs caused interference with the assay.

Serum concentration curves. Figure 6 shows the serum profiles of tamoxifen and *N*-desmethyltamoxifen after a single 30-mg dose of tamoxifen. Tamoxifen showed a peak concentration of 70 μg/L in serum 4 h after intake. The serum concentration of *N*-desmethyltamoxifen increased rapidly during the first 4 h, then increased more slowly.

Figure 7 shows the accumulation of tamoxifen and its metabolites in serum during long-term treatment with tamoxifen (30 mg/day). Tamoxifen and *N*-desmethyltamoxifen progressively increased for several weeks, to about 100 and 200 μg/L, respectively. *N*-Desdimethyltamoxifen and metabolite Y also accumulated in serum within this time period, but at much lower concentrations (30 and 15 μg/L, respectively). The concentration of 4-hydroxytamoxifen in serum leveled off at about 3 μg/L within a week.

Discussion

Recent developments in liquid-chromatographic equipment have resulted in pumps that can steadily deliver mobile phase at a flow rate as slow as a few microliters per minute. The small dead volumes and tubing dimensions allow a total change in composition of the mobile phase within a few hundred microliters. These instruments keep extra-column band-broadening low and are compatible with the analytical performance of small-bore columns (*16*).

The method we describe here, based on low-dispersion LC, exploits these instrumental innovations. In addition, post-column activation of the analytes requires the connection of converter to detector to have zero dead volume, to produce minimal additional band-broadening. The details in Figure 2 emphasize the precautions necessary.

Our method is based on a simple processing of serum samples. The one-step protein-precipitation procedure with an equal volume of acetonitrile avoids the laborious extraction procedures, evaporation of organic material, and reconstitution of samples often included in published assays (*6–9, 11, 18*). Simple and reproducible sample processing is impor-

Table 2. Precision of the Assay for Tamoxifen and Its Metabolites in Serum[a]

Concn, μg/L	Within-day: Without I.S. Mean (SD), μg/L	Within-day: Without I.S. CV, %	Within-day: With I.S. Mean (SD), μg/L	Within-day: With I.S. CV, %	Between-day: without I.S. Mean (SD), μg/L	Between-day: without I.S. CV, %
Metabolite Y						
10	10.2 (0.2)	2.0	12.1 (0.3)	2.3	7.9 (0.6)	7.7
100	111 (1.1)	1.0	129 (1.5)	1.2	94 (4.9)	5.2
800	840 (5.5)	0.7	1057 (22)	2.1	782 (37)	4.8
4-Hydroxytamoxifen						
10	10.6 (0.1)	1.2	12.6 (0.2)	1.6	10.5 (0.6)	5.5
100	107 (0.6)	0.6	130 (1.5)	1.1	104 (2.5)	2.4
800	864 (5.6)	0.7	1105 (26)	2.3	865 (19)	2.1
Tamoxifen						
10	10.2 (0.2)	1.8	11.8 (0.3)	2.2	9.8 (0.5)	4.7
100	103 (0.8)	0.7	123 (1.9)	1.5	100 (2.5)	3.8
800	849 (6.4)	0.8	996 (20)	2.0	796 (30)	3.8
N-*Desdimethyltamoxifen*						
10	10.3 (0.6)	5.6	11.1 (0.6)	5.7	9.2 (0.6)	6.6
100	101 (1.4)	1.4	117 (1.4)	1.2	97 (2.7)	2.8
800	800 (6.8)	0.9	996 (20)	2.0	751 (44)	5.9
N-*Desmethyltamoxifen*						
10	10.4 (0.2)	2.0	12.4 (0.3)	2.2	10.3 (0.8)	7.4
100	105 (0.6)	0.6	125 (1.8)	1.5	102 (3.0)	2.9
800	864 (9.8)	1.1	1001 (23)	2.3	795 (31)	3.9

[a] Different samples of drug-supplemented serum were used for determining within-day and between-day precision. n = 10 each. I.S., internal standard.

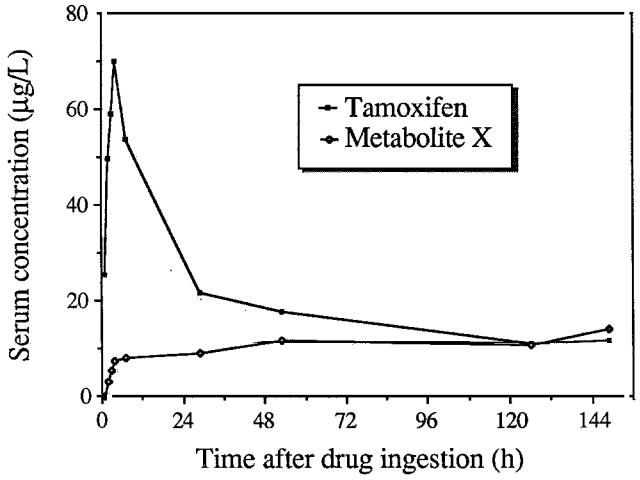

Fig. 6. Concentration curves for tamoxifen and *N*-desmethyltamoxifen in serum after a single ingestion of 30 mg of tamoxifen

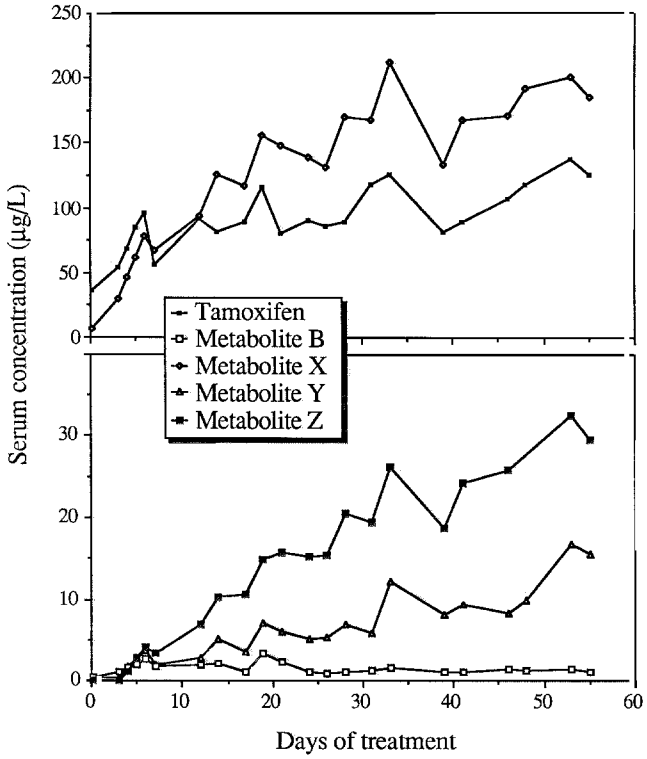

Fig. 7. Concentration curves for tamoxifen and four metabolites in serum during chronic drug treatment (30 mg of tamoxifen per day)

tant for a high sample output, and this, together with automatic injection of a large sample volume, explains the high reproducibility of the method. Notably, the reproducibility of the assay was in fact not increased by including an internal standard (Table 2), so we did not routinely use one. However, the internal standard may become useful if extraction of serum into an organic solvent is necessary for whatever reasons.

We injected 250 μL of sample into a small precolumn having a total volume of 69 μL. The analytes in this sample are retained on the top of the precolumn, which had been equilibrated with an equivolume solution of acetonitrile and water containing acetic acid and diethylamine. This on-column concentration is necessary to achieve the sensitivity necessary to detect small amounts of tamoxifen and its metabolites in serum, as in single-dose studies. We could further decrease the detection limit by extracting large serum samples into diethyl ether, or by increasing the injection volume, but this was not required in monitoring the drug concentrations in serum after ingestion of therapeutic doses of tamoxifen.

We directed the flow-through to waste and the analytes to the analytical column, using an automated column-switch-

ing valve. The column-switching has the following impact on the performance of the present method: (*a*) There is less interference from fluorescent material eluting in or near the solvent front. (*b*) The analytical column is by-passed during sample clean-up, and is not exposed to salts from serum (these serum constituents cause the chromatographic resolution, which is critically dependent on elution at low ionic strength, to deteriorate. (*c*) The analytical column is protected from the material present in the large sample of crude serum extract injected into the column, thereby prolonging column life.

The precolumn and the analytical column have the same internal diameter, 2.1 mm, which is less than that of conventional HPLC columns. These small columns are eluted at a low flow rate, i.e., 0.3 mL/min, in the present assay, which results in low consumption of mobile phase. Because elution of tamoxifen and its metabolites from a reversed-phase column requires a mobile phase containing a high percentage of organic solvent, the low flow rate significantly affects the cost of the assay. In addition, small-bore columns require only low amounts of packing material, for a further cost reduction.

In addition to simple sample processing and high precision, we obtained total recovery of tamoxifen and four serum metabolites. In preliminary experiments about 75%, 68%, and 57% of 4-hydroxytamoxifen, tamoxifen, and *N*-desmethyltamoxifen were recovered after extraction with diethyl ether (data not shown). Moreover, to our knowledge, this is the only tamoxifen assay that determines the parent drug and four metabolites in human serum plus an internal standard in a single run. We obtained this separation by eluting the analytical column at low ionic strength.

The present assay was useful for studying the pharmacokinetics of tamoxifen and some metabolites in serum after single doses (Figure 6), and during various therapeutic regimens including chronic dosing with tamoxifen (Figure 7), alone or in combination with other drugs. We developed this assay for serum samples, but have observed that it works equally well with plasma.

One person can prepare several hundred samples within a few hours. Because of low solvent consumption there is no need to replenish the mobile phase, and prolonged unattended analyses are possible. Thus, the number of samples to be analyzed by this procedure is limited only by the capacity of the sample tray.

In conclusion, our low-dispersion liquid-chromatographic assay for tamoxifen and metabolites in serum exploits recent developments in liquid-chromatographic hardware. This assay is characterized by simple sample processing, high recovery of analytes, low consumption of solvent and packing material, high sample output, high precision, and simultaneous quantification of the parent drug and four serum metabolites. In addition, the assay is fully automated.

We gratefully acknowledge the technical assistance of Mrs. Gry Kvalheim and Audun Høylandskjær. This work was supported by grants from the Norwegian Cancer Society, the Norwegian Society for Fighting Cancer, and the Michael Irgens Flocks Legat. Tamoxifen, 4-hydroxytamoxifen, and *N*-desmethyltamoxifen were generously provided by Nyegaard et Co A/S, Oslo, Norway.

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