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Determination of Droloxifene and Two Metabolites in Serum by High-Pressure Liquid Chromatography

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Summary: In this assay of the nonsteroidal antiestrogen droloxifene and two metabolites in human serum, the serum samples were deproteinized with an equal volume of acetonitrile and then injected into an analytical octadecylsilane column. The analytical column was equilibrated with acetonitrile/water (1/1, vol/vol) containing acetic acid and diethyl amine and eluted isocratically with 66% acetonitrile in the same buffer. Droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene were post-column converted to fluorophors by ultraviolet illumination while passing through a 10-m transparent knitted polytetrafluorethylene reaction coil. Analytical recovery was close to 100%. Within- and between-day precision corresponded to a coefficient of variation (CV) of 2–5% at serum concentrations of ≥25 ng/ml, except for 4-methoxydroloxifene (CV 7–10% at a concentration of 25 ng/ml). By increasing the injection volume from 50 to 250 μl, the detection limits could be decreased from ~5 to 1 ng/ml. Conjugated droloxifene could be estimated in a second run after treatment of sample with an enzyme preparation containing β-glucuronidase plus sulphatase. The recovery of droloxifene glucuronide was close to 100%. Sulphate conjugates have not been identified and were not accounted for. **Key words:** Antiestrogens—Droloxifene—Drug assay—Pharmacokinetics.

The nonsteroid antiestrogen tamoxifen is the endocrine treatment modality most widely used in breast cancer (1,2). Tamoxifen is an effective drug both against advanced disease and in the adjuvant setting, but its estrogen agonistic effects may be responsible for some adverse effects, including an increased incidence of endometrial carcinomas (3). In addition, tamoxifen has an extremely long half-life with tissue drug retention for months after terminating treatment (4). Thus, there is much interest in developing alternative antiestrogens with dynamic and kinetic profiles different from those of tamoxifen.

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Recently a new estrogen receptor–blocking drug, droloxifene (3-OH-tamoxifen) (Fig. 1) has been launched for clinical testing.

Droloxifene has an affinity for the estrogen receptor that is 10 to 60 times higher than that of tamoxifen (5), and results from animal and human studies suggest that the drug has less estrogen agonistic properties than does tamoxifen (6).

Sparse data concerning the pharmacokinetics and metabolism of droloxifene have been published. The half-life of droloxifene is shorter than that of tamoxifen (7). Two metabolites (*N*-desmethyldroloxifene and 4-methoxydroloxifene) have been demonstrated in human serum (7).

Due to limited estrogen agonistic effects and short half-life, droloxifene may exhibit fewer side effects than tamoxifen and thus represent an alternative estrogen receptor blocking drug. The drug

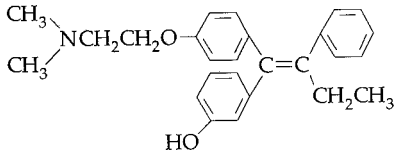

FIG. 1. Structural formula of droloxifene.

that has been evaluated in phase II trials (8) has a response rate similar to that observed during tamoxifen therapy.

The aim of this study was to develop a simple method for the determination of droloxifene and its major metabolites suitable for automated analyses of large numbers of samples.

MATERIALS AND METHODS

Reagents

Droloxifene, *N*-desmethyldroloxifene, 4-methoxydroloxifene, and droloxifene-O-glucuronide were gifts from Klinge Pharma, Munich, Germany. The primary alcohol of tamoxifen, which was a gift from Imperial Chemical Industries PLC, Pharmaceuticals Division, Macclesfield, Cheshire, England, was used as the internal standard.

Glusulase (a preparation of the intestinal juice of the snail helix pomatia, containing 126,000 U β-glucuronidase and 4,000 U sulphatase per milliliter) was from I.E. DuPont, Wilmington, Delaware, U.S.A.

Acetonitrile [high-pressure liquid chromatography (HPLC) grade S] was purchased from Rathburn Chemicals, Ltd., Walkerburn, Scotland. Acetic acid and diethyl amine were from Merck AG, Darmstadt, Germany. The 15-cm reversed-phase analytical column with an internal diameter of 0.46 cm [packed with 3-μm particles of octadecylsilane (ODS)-Hypersil] was purchased from Hewlett Packard, Palo Alto, California, U.S.A.

The standard solutions were prepared by dissolving 4 mg of droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene in 10 ml of 100% methanol. We diluted this stock solution to the actual concentrations either in acetonitrile/water (1/1 vol/vol) or in serum. Standard solutions were stored in the dark at −20°C.

Sample Processing

The blood samples were obtained from patients participating in a study on droloxifene pharmacokinetics. All patients gave their informed consent to participate in the study. The study protocol was approved by the regional ethical committee.

Blood samples were obtained by venous puncture. Each sample was allowed to clot for 30–60 minutes before centrifugation. Serum was removed and stored in darkness at −20°C until analysis.

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

Recovery and Precision Studies

Droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene (2,000 ng/ml) were added to drug-free serum or to a solution of acetonitrile/water (1/1, vol/vol). From these solutions, we prepared samples containing 500, 100, or 25 ng/ml of each compound in both matrices. 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. In a parallel experiment, internal standard (200 ng of this compound per milliliter) was simultaneously added to the samples of serum and acetonitrile/water matrix, and the calculated amount of droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene were corrected for variation in the recovery of internal standard.

To determine the within-run precision [coefficient of variation (CV)] of the assay, we assayed 10 replicates of serum with added droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene, each at a concentration of 500, 100, or 25 ng/ml. The between-day precision was determined by assaying sera (prepared in the same way) on 10 different days. The precision studies were performed with and without internal standard, which was included by adding 200 ng of this compound per milliliter to the acetonitrile used for deproteinizing the samples.

Standard Curve and Detection Limit

The standard curve was prepared by adding known concentrations of droloxifene and its metabolites, ranging between 2,500 and 0.5 ng/ml, to serum. The detection limit was determined by extracting serum samples containing added droloxifene and its metabolites at concentrations of 20, 10, 4, 2, 1, or 0.5 ng/ml.

Deconjugation of Droloxifene Glucuronide

Droloxifene glucuronide (purity 87%) was added to drug-free serum to a concentration of 400 ng/ml and treated with glusulase as described by Bakke and Scheline (9). To 5 ml serum, 4.9 ml 0.2 *M* sodium acetate (pH 5) and 100 μl glusulase were added. The mixture was incubated at 37°C for 0, 1, 2, 4, 8, 20, and 24 h, and then analyzed by HPLC. Droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene were added to drug-free serum and treated with glusulase as described. These samples were analyzed after 0 and 24 h.

Chromatography

Samples of 50 μl were routinely injected into a small-bore analytical reversed-phase column (3-μm particle ODS-Hypersil, 0.46 × 15 cm), equilibrated with acetonitrile/water (1/1, vol/vol) containing 3 mmol of acetic acid and 2 mmol of diethyl amine per liter. The column was eluted isocratically at ambient temperature with 66% acetonitrile containing 3 m*M* acetic acid and 2 m*M* diethyl amine. The flow rate was 1 ml/min.

Instrumentation

We used a Hewlett Packard liquid chromatograph (model HP 1090; Hewlett Pakard Co., Palo Alto, CA, U.S.A.), equipped with a ternary solvent delivery system, HPLC autosampler, column-switching valve, and 50-μl injection syringe. The analytes were post-column converted to fluorophors by ultraviolet illumination (10,11) while passing through a 10-m transparent knitted polytetrafluorethylene reaction coil (12) inside the model Beam Boost (ict Handels GmbH, Frankfurt, Germany). The wavelength of the ultraviolet light was 254 nm, and the internal diameter of the reaction coil was 0.3 mm, which corresponds to a residence time of 42 s.

The fluorescence was monitored using an HPLC detector Model RF-535 from Shimadzu, Kyoto, Japan. The excitation wavelength was 260 nm and the emission wavelength 360 nm. Quantitation was done by estimation of peak area.

RESULTS

Extraction Procedures

The analytical recovery was close to 100% for droloxifene and its metabolites, except for 4-methoxydroloxifene, which had a recovery of 131% and 115% at the concentrations of 25 and 100 ng/ml, respectively (Table 1).

Deconjugation of Droloxifene Glucuronide

The recovery of droloxifene from droloxifene glucuronide was 84.4% after 8 h and 99.7% after 24 h of treatment with glusulase (Fig. 2). Adding glusulase to samples containing unconjugated droloxifene or its metabolites had no influence on recovery, and the glusulase did not interfere with the assay.

Chromatography

Figure 3 shows chromatograms of drug-free serum spiked with 50 ng/ml of droloxifene, *N*-desmethyldroloxifene, and methoxydroloxifene, as well as the internal standard (trace A), serum of a patient not given droloxifene (blank serum, trace B), and serum obtained from a patient after having received a single dose of 100 mg droloxifene and during steady state (traces D and C).

The compounds eluted in the order internal standard, *N*-desmethyldroloxifene, droloxifene, and 4-methoxydroloxifene. 4-Methoxydroloxifene showed a fluorescence yield of ~50% that of droloxifene and *N*-desmethyldroloxifene (Fig. 3).

We tested for interference with the droloxifene assay by analyzing sera from patients taking the following drugs: atenolol, captopril, clodronate, dexamethasone, dextropropoxyphene, diazepam, doxycycline, econazole, enoxaparin, felodipine, flunitrazepam, furosemide, glibenclamide, glyceryl

TABLE 1. *Recovery of droloxifene and its metabolites in serum*

Concentration (ng/ml)	Without IS[a]		With IS[a]	
	Mean	CV	Mean	CV
N-Desmethyldroloxifene				
25	99	7.3	91	4.3
100	99	2.5	91	2.5
500	99	2.2	92	1.7
Droloxifene				
25	98	5.0	90	5.3
100	100	3.3	93	2.9
500	102	2.5	94	2.2
4-Methoxydroloxifene				
25	117	11.1	131	11.9
100	109	3.5	115	4.1
500	101	3.1	93	3.7

IS, internal standard.
[a] Values are percentages of substance recovered. n = 10 each.

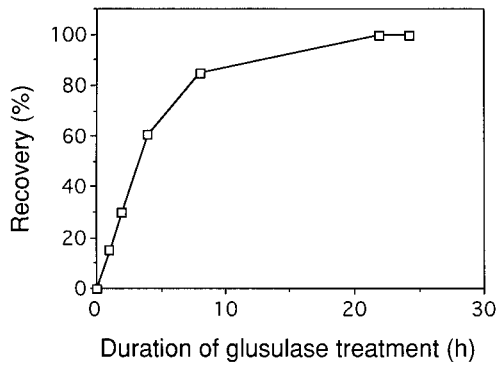

FIG. 2. Recovery of droloxifene from serum supplemented with droloxifene glucuronide and deconjugated with glusulase.

trinitrate, indomethacin, insulin, isosorbide mononitrate, megestrol acetate, metoclopramide, mianserin, morphine, oxazepam, paracetamol, perphenazine, phenytoin, pivmecillinam, prochlorperazine, promethazine, ranitidine, and tamoxifen. With the exception of tamoxifen, which caused a peak close to that of 4-methoxydroloxifene, none of these drugs interfered with the assay.

Standard Curves, Detection Limits, and Precision of the Method

The standard curves for droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene are shown in Fig. 4. The standard curves were linear in the range of 10–2,500 ng/ml ($R > 0.99$ for *N*-desmethyldroloxifene, droloxifene, and 4-methoxydroloxifene)

The detection limit for *N*-desmethyldroloxifene and droloxifene was ~5 ng/ml and 5–10 ng/ml for 4-methoxydroloxifene (signal-to-noise ratio of 2:1). By increasing the injection volume to 250 µl, concentrations down to ~1 ng/ml could be detected.

The within-day precision was <5% for all compounds at 500 ng/ml, 100 ng/ml, and 25 ng/ml, except for 4-methoxydroloxifene at a concentration of 25 ng/ml. At this concentration, the within-day precision for 4-methoxydroloxifene was 10.4% (Table 2).

The between-day precision was <5% for all compounds at 500 ng/ml, 100 ng/ml, and 25 ng/ml, again except for 4-methoxydroloxifene. At a concentration of 25 ng/ml, the between-day precision for 4-methoxydroloxifene was 7.0% (Table 2).

DISCUSSION

Our method is based on a simple processing of serum samples, including one-step protein precipitation with an equal volume of acetonitrile. Laborious extraction procedures, evaporation of organic material, and reconstitution of samples often included in assays for antiestrogens (13) are not required. The sample output is limited by the run time and the equilibration time, and two samples can be analyzed per hour. The number of samples analyzed during 24 h (~50) can be prepared within 60

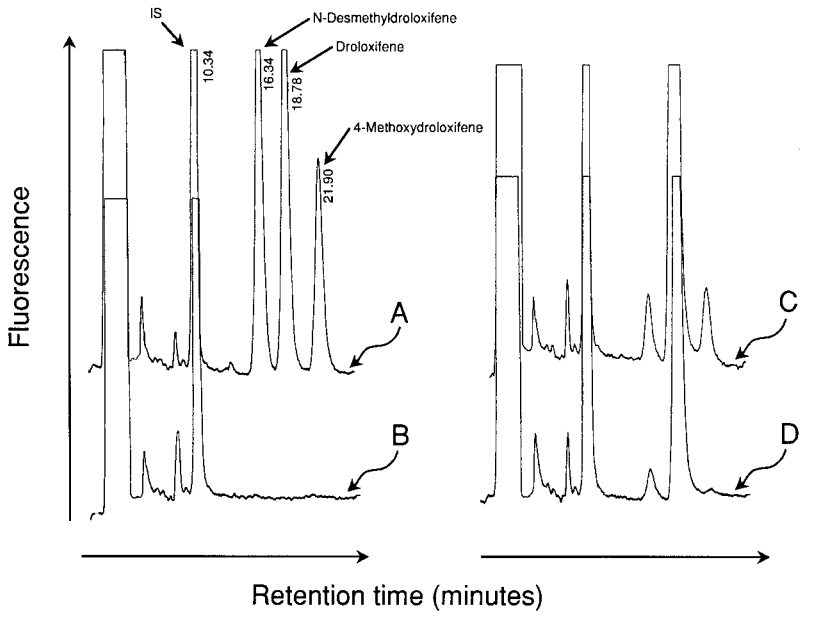

FIG. 3. Chromatograms of droloxifene, *N*-desmethyldroloxifene and 4-methoxydroloxifene in serum. Trace A: blank serum spiked with 50 ng/ml of each compound. Trace B: blank serum spiked with internal standard (IS), which was the primary alcohol of tamoxifen. Trace C: serum obtained during steady state, 4 h after last dose (100 mg). Trace D: serum obtained 3 h after a single dose (100 mg).

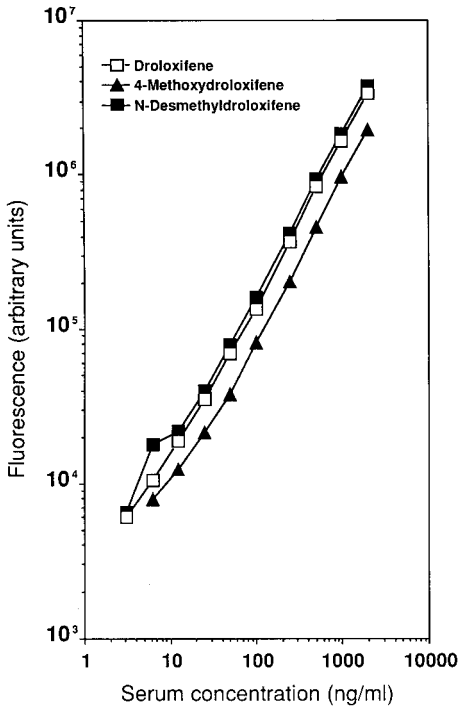

FIG. 4. Standard curves for droloxifene, *N*-desmethyldroloxifene, and methoxydroloxifene. Linear regression analyses gave coefficients of correlation of R > 0.99 for the three compounds.

min. The instrumental and column setup are simpler for our HPLC assay than for the elegant column switching method described by Jank et al. (14).

Simple sample processing, together with the automated injection, explain the high reproducibility of the method, which was in fact only moderately increased by including an internal standard (Table 2). Therefore, the internal standard may be excluded. However, it may become useful if extraction of serum into an organic solvent becomes necessary to further increase the sensitivity of the method.

The recovery was occasionally slightly higher than 100% (Table 1). This may be related to surface adsorption of reference compound dissolved in the acetonitrile-water solution. For tamoxifen we have previously observed that such adsorption is prevented by serum (15).

The injection volume was 50 μl, which corresponds to 25 μl of serum. The small sample requirement might be advantageous in kinetic studies based on repeated sampling, but limits the concentration sensitivity of the assay (~5 ng/ml). In single-dose studies, the levels of *N*-desmethyldroloxifene and methoxydroloxifene may decrease below this level. However, the detection limit can be improved to ~1 ng/ml by increasing the injection volume to 250 μl without loss of chromatographic resolution.

Grill et al. found mean C_{max} concentrations of droloxifene of 25 ± 7, 64 ± 11, and 149 ± 50 ng/ml after single oral doses of 20, 40, and 100 mg of droloxifene, respectively (7). With the present method we found a C_{max} level of 81 ng/ml in a breast cancer patient after a single oral dose of 100 mg (Fig. 5). The assay was useful for studying the pharmacokinetics of droloxifene and two of its major metabolites in serum after a single dose and during chronic dosing (Fig. 5).

We observed no interference with the assay after

TABLE 2. *Precision of the assay for droloxifene and its metabolites in serum*

	Drug measured							
	Within-day				Between-day			
	Without IS		With IS		Without IS		With IS	
Drug added (ng/ml)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)
N-Desmethyltamoxifene								
25	23.2	8.0	26.9	4.6	24.7	3.7	24.9	3.7
100	95.9	1.6	104.6	2.8	101.8	5.0	102.7	3.8
500	553.7	2.3	502.3	1.5	522.1	3.5	530.2	2.5
Droloxifene								
25	22.4	3.9	25.7	4.8	24.5	1.0	24.9	4.7
100	97.8	1.9	105.3	3.9	101.7	5.4	103.3	4.8
500	572.6	2.4	529.6	1.7	533.8	4.0	544.7	1.9
4-Methoxydroloxifene								
25	28.0	7.3	31.3	10.4	22.1	7.2	22.6	7.0
100	112.1	3.3	117.5	4.3	100.6	3.7	102.5	4.7
500	596.8	3.0	550.0	3.3	536.4	3.4	551.2	3.4

n = 10 each.

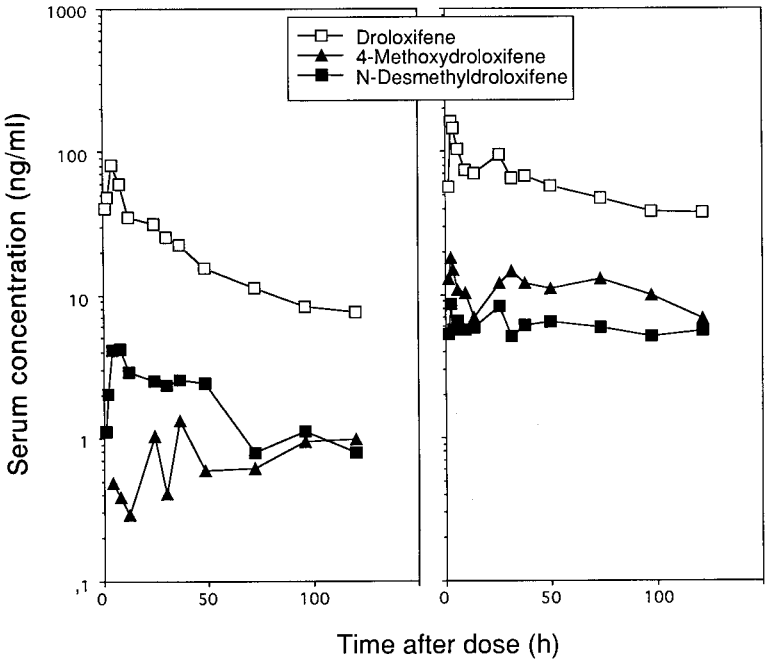

FIG. 5. Serum concentration curves for droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene in a patient with breast cancer given 100 mg droloxifene orally as a single dose (**left panel**) and during steady state (**right panel**).

intake of several other drugs often used concomitantly in cancer patients.

The glucuronides of droloxifene and its metabolites are present in serum in concentrations higher than the nonconjugated compounds (16). No methods for direct assay of the glucuronides of droloxifene or its metabolites have been published, and standards of the conjugates of droloxifene metabolites are not available. However, after deconjugation of droloxifene glucuronide by treatment with glusulase for 8 h, the recovery of droloxifene was close to 100% and did not change after a further 16 h of glusulase treatment (Fig. 2). Nonconjugated droloxifene, *N*-desmethyldroloxifene, and 4-methoxydroloxifene were stable during 24 h of treatment with glusulase (data not shown). Thus, the concentration of droloxifene glucuronide may be calculated after a second analysis of glusulase-treated serum by subtracting the originally unconjugated droloxifene concentration from the total concentration in the hydrolyzed sample.

Significant amounts of sulphate conjugates of droloxifene have not been demonstrated (14). In case trace amounts of such conjugates exist, these are expected to be deconjugated by treatment with glusulase, which contains both β-glucuronidase and sulphatase.

In conclusion, our high-pressure liquid chromatographic assay is characterized by simple sample processing, high precision, and high sample output. Interference with other drugs was not observed. We obtained total recovery of droloxifene and its metabolites *N*-desmethyldroloxifene and 4-methoxydroloxifene. Thus, the present assay is useful for studying the pharmacokinetics of droloxifene and two of its metabolites in serum after a single dose and during various therapeutic regimens including steady-state treatment.

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