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Journal of Chromatography B, 674 (1995) 253–260

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Sensitive method for the quantitation of droloxifene in plasma and serum by high-performance liquid chromatography employing fluorimetric detection

David A. Tess, Roderic O. Cole, Steven M. Toler*

Drug Metabolism Department, Pfizer Inc., Central Research Division, Eastern Point Road, Groton, CT 06340, USA

First received 2 May 1995; revised manuscript received 11 July 1995; accepted 12 July 1995

Abstract

A simple and highly sensitive reversed-phase fluorimetric HPLC method for the quantitation of droloxifene from rat, monkey, and human plasma as well as human serum is described. This assay employs solid-phase extraction and has a dynamic range of 25 to 10 000 pg/ml. Sample extraction (efficiencies >86%) was accomplished using a benzenesulfonic acid (SCX) column with water and methanol rinses. Droloxifene and internal standard were eluted with 1 ml of 3.5% (v/v) ammonium hydroxide (30%) in methanol. Samples were quantitated using post-column UV-photochemical cyclization coupled with fluorimetric detection with excitation and emission wavelengths of 260 nm and 375 nm, respectively. Relative ease of sample extraction and short run times allow for the analysis of approximately 100 samples per day.

1. Introduction

Droloxifene (Fig. 1) is an estrogen agonist/antagonist currently in development for the treatment of breast cancer and osteoporosis. Previously published methods for the quantitation of droloxifene from biologic matrices have lower limits of quantitation (LLOQ) of ⩾1 ng/ml [1,2]. At clinically relevant doses, droloxifene concentrations may decline below 1 ng/ml before a complete dispositional profile may be obtained. We have developed a simple high-performance liquid chromatographic method using fluorimetric detection that allows for the quantitation of droloxifene from rat, monkey, and human plasma as well as human serum with an LLOQ of 25 pg/ml. Ease of sample preparation and short retention times of both droloxifene and internal standard allow for the quantitation of approximately 100 samples per day in addition to the standard curve and quality control samples.

Although droloxifene has little or no fluorescent properties, it can be easily photocyclized to a highly fluorescent phenanthrene derivative [3]. This assay employs post-column UV-photochemical derivatization of droloxifene to droloxifene-phenanthrene (Fig. 2). Droloxifene-phenanthrene is then detected via fluorimetry with excitation and emission wavelengths of 260 nm and 375 nm, respectively.

* Corresponding author.

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SSDI 0378-4347(95)00305-3

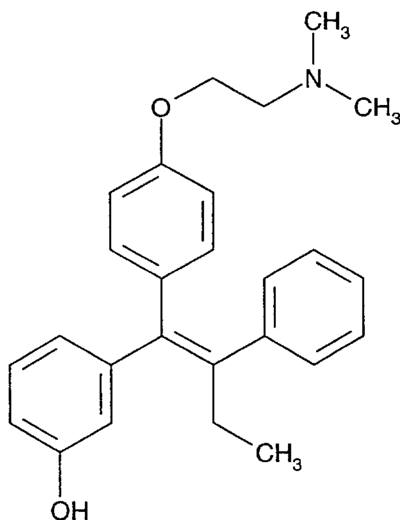

Fig. 1. Structure of droloxifene.

2. Experimental

2.1. Equipment

The HPLC equipment consisted of an F-1080 fluorimeter (Hitachi, Tokyo, Japan), constaMetric 3500 solvent delivery system (Thermo Separation Products, Riviera Beach, FL, USA), Waters 717 plus autosampler (Millipore, Milford, MA, USA), membrane degasser (Thermo Separation Products), Model LC-22C column heater (BAS, West Lafayette, IN, USA), 0.25 mm I.D. × 3.1 m Teflon reaction coil (made in house), Beam Boost 254 nm photochemical reaction lamp (ICT, Frankfurt, Germany) and Beam Boost photochemical reaction unit (ICT). The detector output was digitized by Fisons VG chromatographic A/D converter (Fisons Instruments, Beverly, MA, USA) and data processed using VAX Multichrom 2 V1.0 system (Fisons Instruments). Analytes were extracted from plasma and serum using Bond Elut (Varian, Harbor City, CA, USA) or BakerBond (J.T. Baker, Phillipsburg, NJ, USA) 100 mg benzenesulfonic acid (SCX) solid-phase extraction cartridges (both SPE cartridges were found to be equivalent) and a Baker spe-12G vacuum manifold (J.T. Baker). A C_{18} 3 μm 100 × 4.6 mm column (Rainin Instrument, Woburn, MA, USA) was used as the analytical column.

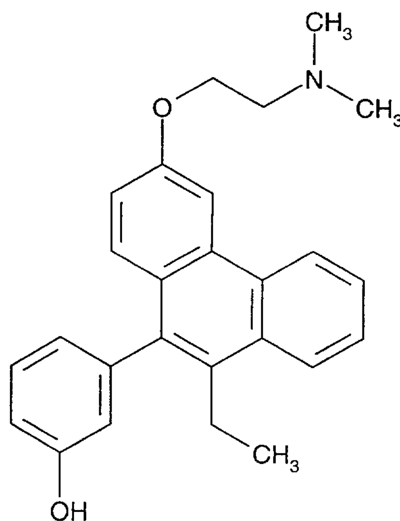

Fig. 2. Structure of droloxifene-phenanthrene.

2.2. Reagents

Droloxifene, internal standard (K 21.089 E Base) and the predominant plasma/serum metabolites of droloxifene (Fig. 3) were provided by Klinge Pharma (Munich, Germany). Acetonitrile and methanol were HPLC grade (J.T. Baker). Reagent grade ammonium hydroxide (30%), glacial acetic acid and phosphoric acid (85%) were obtained from J.T. Baker. Reagent grade sodium phosphate monobasic was obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.3. Extraction procedure

Column conditioning solutions were aspirated through the SCX solid-phase extraction columns by vacuum at an approximate rate of 1 ml/min [ca. 10 in. Hg (ca. $3 \cdot 10^4$ Pa)]. The solid-phase columns were conditioned with 1 ml of 3.5% (v/v) ammonium hydroxide (30%) in methanol (prepared by adding 3.5 ml of 30% ammonium hydroxide to 96.5 ml of methanol), followed by 1 ml of methanol, and finally with 1 ml of 1% (v/v) acetic acid. Sample extraction was achieved by

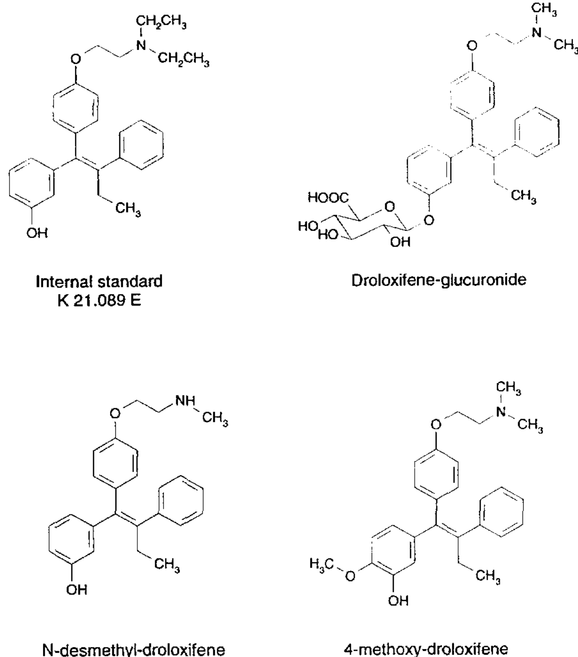

Fig. 3. Structures of K 21.089 E (internal standard) and predominant plasma metabolites.

first applying 0.5 ml of a 1% acetic acid solution to the SCX column (still damp from conditioning) followed by 200 μl of internal standard solution (2 ng/ml in mobile phase) and finally 200 μl of sample matrix. This mixture was then aspirated through the column. The column was washed with 1 ml of water, followed by 1 ml of methanol. The analytes were eluted from the column with 1 ml of 3.5% (v/v) ammonium hydroxide in methanol and collected in a 10-ml glass conical tube. The eluate was evaporated to dryness under vacuum at 75°C or a stream of nitrogen at 50°C (both evaporation methods were found to be equivalent) and reconstituted in 100 μl of mobile phase, vortex-mixed, centrifuged at 1000 *g* for 30 s, transferred to HPLC injection vials, and 80 μl was injected onto the column.

2.4. Chromatographic conditions

The mobile phase consisted of 55% 50 m*M* sodium phosphate buffer and 45% acetonitrile (adjusted to a pH′ of 3.5 with phosphoric acid). Prior to use, the mobile phase was sparged with helium for 5 min/l. The mobile phase was delivered to the column at a flow-rate of 2 ml/min. The analytical column was maintained at a constant temperature of 40°C with a column heater. Fluorimetric excitation and emission wavelengths were set at 260 nm and 375 nm, respectively. Droloxifene and the internal standard had retention times of 3.1 and 4.8 min, respectively.

2.5. Calibration standards and quality control samples

A stock solution of droloxifene was prepared from droloxifene citrate in methanol at a concentration of 69.4 μg/ml droloxifene base equivalent. The stock solution was serially diluted daily with control plasma or serum to provide droloxifene solutions at 1000 and 100 ng/ml. The 100 ng/ml solution was serially diluted with control plasma or serum to prepare the standard solutions for the calibration curve daily (25, 50, 100, 500, 1000, 5000, and 10 000 pg/ml).

Rat, monkey and human quality control samples were prepared by diluting droloxifene stock solution in 1 ml of the corresponding plasma to produce 1000 ng/ml substocks. From these substocks, quality control samples were prepared at concentrations of 7500 pg/ml, 750 pg/ml, and 75 pg/ml. Quality control samples were assayed on the day of preparation and aliquots stored at −70°C in 2 ml polypropylene vials for future analysis.

2.6. Quantitation

Quality control samples (75, 750, and 7500 pg/ml) and calibration standards were assayed during each analytical run. Calibration curves were generated from weighted linear regression analysis ($1/\text{concentration}^2$) of the peak height ratios of droloxifene/internal standard to known concentrations in the calibration standards. Droloxifene concentrations in the quality control and unknown samples were derived from this curve.

2.7. Analytical variables

Control rat plasma was fortified with 2000 pg/ml of internal standard and droloxifene at concentrations of 75, 750, and 7500 pg/ml. These samples were then extracted as in Section 2.3, analyzed and compared to recovery standards (non-extracted) prepared at the same theoretical concentrations in mobile phase. Recovery of analyte is calculated as the ratio of the mean response for processed standards to the mean response for recovery standards based on peak height ratios of droloxifene/internal standard.

This assay was first validated in Sprague–Dawley rat plasma. Cross species matrix compatibility was investigated for cynomolgus monkey plasma, human plasma, and human serum. No significant interfering peaks were observed within the detection windows of droloxifene or the internal standard in five individually obtained control samples from each matrix. The mean assay accuracy ± precision ($n = 5$) was investigated at 75, 750, and 7500 pg/ml and assayed against a standard curve prepared in rat plasma.

Intra-assay accuracy and precision were investigated in control rat plasma samples fortified with droloxifene at concentrations of 75, 750, and 7500 pg/ml with seven replicates of each concentration. Inter-assay accuracy and precision were investigated in control rat plasma samples fortified with droloxifene at concentrations of 75 pg/ml, 750 pg/ml, and 7500 pg/ml on seven separate occasions.

The stability of droloxifene was evaluated at concentrations of 75, 750, and 7500 pg/ml in rat plasma following three freeze/thaw cycles, over a 61-day period of storage at −70°C, and extracted samples at room temperature in the autosampler for 48 h to simulate the longest anticipated time per analysis set.

3. Results and discussion

3.1. Method

This assay employs the photocyclization of triphenylethylenes (droloxifene) to produce fluorescent phenanthrenes (droloxifene-phenanthrene) as previously reported [3]. We have made numerous alterations from previously reported fluorimetric assays for the quantitation of droloxifene [1,2]. These alterations have resulted in an increase in assay sensitivity of approximately 40-fold and decreased assay run times by more than 50%. This assay does not use column switching as previously reported and employs a shorter analytical column which jointly reduces assay run times and cost. We found the Hitachi F-1080 fluorimeter more sensitive than the Shimadzu RF-10A fluorimeter (Kyoto, Japan) with a 10-fold increase in the signal-to-noise ratio. The shorter and less expensive analytical column (100 mm vs. 250 mm) coupled with the increased flow-rate produced less band spreading. The highly selective extraction procedure produced very clean eluates and allowed for a 2-fold increase in sample concentration. Direct application of the internal standard solution to the cartridge produced higher extraction efficiencies

Table 1
Recoveries of droloxifene from rat plasma using solid-phase extraction

Peak-height ratio of droloxifene/I.S.		
75 pg/ml	750 pg/ml	7500 pg/ml
Extracted samples		
0.037	0.465	4.278
0.047	0.423	3.779
0.040	0.476	3.817
0.046	0.473	4.527
0.047	0.485	4.528
0.045	0.461	4.485
0.045	0.465	4.517
Mean ± S.D.:		
0.044 ± 0.004	0.464 ± 0.020	4.276 ± 0.338
Recovery samples		
0.051	0.517	4.812
0.051	0.510	4.792
0.052	0.508	4.718
0.050	0.510	4.692
0.052	0.510	4.725
0.051	0.515	4.734
0.051	0.512	4.729
Mean ± S.D.:		
0.051 ± 0.001	0.512 ± 0.003	4.743 ± 0.043
Recovery (%):		
86	91	90

for droloxifene relative to pre-mixing the internal standard solution with plasma/serum. Collectively, these changes resulted in an assay with a lower limit of quantitation of 25 pg/ml, a lower limit of detection of 10 pg/ml ($S/N = 4$), a dynamic range of 25 pg/ml to 1000 pg/ml and assay run times of 7 min.

3.2. Recovery

The mean extraction efficiencies of droloxifene from rat plasma using SCX solid-phase extraction at concentrations of 75, 750, and 7500 pg/ml were 86%, 91% and 90%, respectively (Table 1). Prewashing the SCX columns with 3.5% ammonium hydroxide in methanol removed interferences inherent in the extraction columns themselves. Droloxifene was tightly retained on the SCX column allowing for a two-step sample clean up. This extraction procedure produces extremely clean eluates and allows for approximately 90% extraction efficiency.

3.3. Assay performance

All control matrices investigated yielded clean chromatographic baselines. Cross species matrix compatibility accuracy and coefficients of variation were maintained within 15% (Table 2).

Table 2
Cross species matrix compatibility of droloxifene in cynomolgus monkey plasma, human plasma and human serum

	Concentration (mean ± S.D.) (pg/ml)	*n*	Accuracy (%)	C.V. (%)
Concentration 75 pg/ml				
Monkey plasma	80 ± 3	5	107	4
Human plasma	86 ± 7	5	115	8
Human serum	85 ± 2	5	113	2
Concentration 750 pg/ml				
Monkey plasma	783 ± 38	5	104	5
Human plasma	839 ± 42	5	112	5
Human serum	824 ± 15	5	110	2
Concentration 7500 pg/ml				
Monkey plasma	7690 ± 200	5	103	3
Human plasma	8120 ± 130	5	108	2
Human serum	8170 ± 100	5	109	1

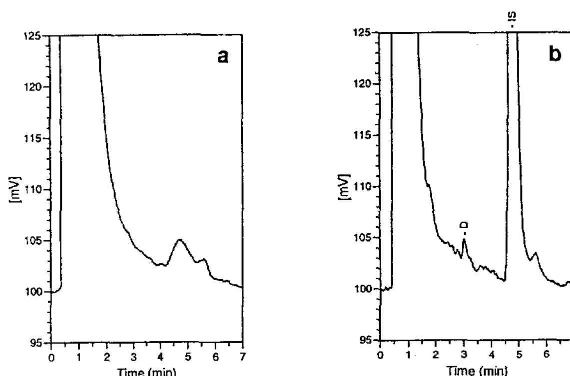

Fig. 4. Chromatogram of (a) blank rat plasma and (b) 25 pg/ml droloxifene in rat plasma. D = droloxifene. IS = internal standard.

Both droloxifene and the internal standard yield sharp, demarcated peaks with retention times of 3.1 and 4.8 min, respectively, with baseline separation. Calibration curves were generated over a range of concentrations from 25 to 10 000 pg/ml. The calibration curves were linear with a mean (±S.D.) coefficient of determination of 0.9962 ± 0.0038 ($n = 7$). The lower limit of quantitation was 25 pg/ml (accuracy of 104% with an associated coefficient of variation of 8%, $n = 7$, Fig. 4). The upper limit of quantitation was 10 000 pg/ml (accuracy of 95% with an associated coefficient of variation of 4%, $n = 7$). Intra- and inter-assay accuracy and coefficients of variation were maintained within 10% (Table 3). These data indicate excellent assay precision and accuracy. The three

Table 3
Intra- and inter-assay accuracy and precision of droloxifene

	Concentration (mean ± S.D.) (pg/ml)	*n*	Accuracy (%)	C.V. (%)
Concentration 75 pg/ml				
Intra-assay	80 ± 7	7	107	9
Inter-assay	79 ± 3	7	105	4
Concentration 750 pg/ml				
Intra-assay	809 ± 35	7	108	4
Inter-assay	770 ± 37	7	103	5
Concentration 7500 pg/ml				
Intra-assay	7430 ± 590	7	99	8
Inter-assay	7640 ± 370	7	102	5

Table 4
Retention times of droloxifene, internal standard and predominant plasma/serum metabolites of droloxifene

Compound	Retention time (min)	$w_{1/2}$ (min)	R_s from droloxifene
t_0	0.59	–	–
Droloxifene-glucuronide	0.86	0.12	8.6
N-Desmethyl-droloxifene	2.59	0.15	1.6
Droloxifene	3.05	0.18	–
4-Methoxy-droloxifene	3.48	0.24	1.2
Internal standard	4.77	0.25	4.7

Table 5
Stability of droloxifene at −70°C

	Concentration (mean ± S.D.) (pg/ml)	n	Time stored frozen at −70°C (days)	Initial concentration (%)
Concentration 75 pg/ml				
Initial	80 ± 7	7		
Assay 2	82 ± 4	3	4	103
Assay 3	81 ± 1	3	11	101
Assay 4	82 ± 3	3	13	103
Assay 5	77 ± 8	3	18	96
Assay 6	77 ± 3	3	34	96
Assay 7	74 ± 2	5	61	93
Concentration 750 pg/ml				
Initial	809 ± 35	7		
Assay 2	806 ± 30	3	4	100
Assay 3	756 ± 16	3	11	93
Assay 4	768 ± 20	3	13	95
Assay 5	742 ± 14	3	18	92
Assay 6	798 ± 6	3	34	99
Assay 7	708 ± 13	5	61	88
Concentration 7500 pg/ml				
Initial	7430 ± 590	7		
Assay 2	8090 ± 130	3	4	109
Assay 3	7670 ± 170	3	11	103
Assay 4	8010 ± 130	3	13	108
Assay 5	7750 ± 540	3	18	104
Assay 6	7000 ± 90	3	34	94
Assay 7	7550 ± 80	5	61	102

primary plasma/serum droloxifene metabolites (droloxifene-glucuronide, N-desmethyldroloxifene, and 4-methoxydroloxifene) were well resolved from droloxifene and their retention times and resolution from droloxifene are listed in Table 4.

3.4. Droloxifene stability

Droloxifene extracted from prepared sample was stable at 75, 750, and 7500 pg/ml for at least 48 h when stored in injection vials in the autosampler at room temperature (final concentra-

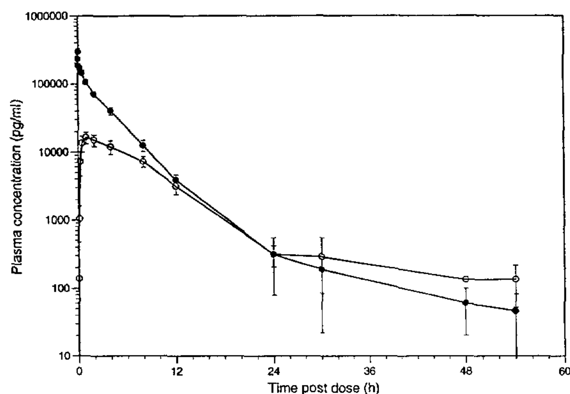

Fig. 5. Mean (±S.D., $n = 6$) plasma concentrations of droloxifene in female Sprague–Dawley rats after a single intravenous (●) or oral (○) administration of 1 mg/kg droloxifene base equivalents.

tions within 15% of initial concentrations) and stable for at least 61 days when stored in the dark at −70°C (Table 5). In addition, droloxifene was stable when subjected to three freeze/thaw cycles conducted over 72 h (final concentrations within 15% of initial concentrations).

3.5. Assay application

This analytical method has been successfully applied to pharmacokinetic studies in the Sprague–Dawley rat. A representative disposition profile is shown in Fig. 5. The high degree of sensitivity produced by this assay allowed for the detection of a biexponential disposition profile in the rat. Had the lower limit of detection been 1 ng/ml, only the first exponential phase would have been characterized. Sensitivity obtained in the current assay is not a function of large matrix volume extraction; only 200 μl of plasma or serum is required to quantitate droloxifene concentrations as low as 25 pg/ml. Circulating metabolites do not produce assay interference. However, a metabolite present in feces, 3-methoxy-4-hydroxydroloxifene, could interfere with the present assay. Therefore, we do not recommend that this assay be employed for analysis of fecal samples. In addition, it should not be employed for metabolite quantitation since most circulating metabolites of droloxifene exist as polar conjugates which elute in the solvent front. A typical chromatogram from a pharmacokinetic study conducted in Sprague–Dawley rats is shown in Fig. 6. Samples collected prior to 12 h post-dose were diluted as much as 1:49 with control rat plasma to produce concentrations within the dynamic range. Quality control and dilution quality control samples were within 15% of known concentrations.

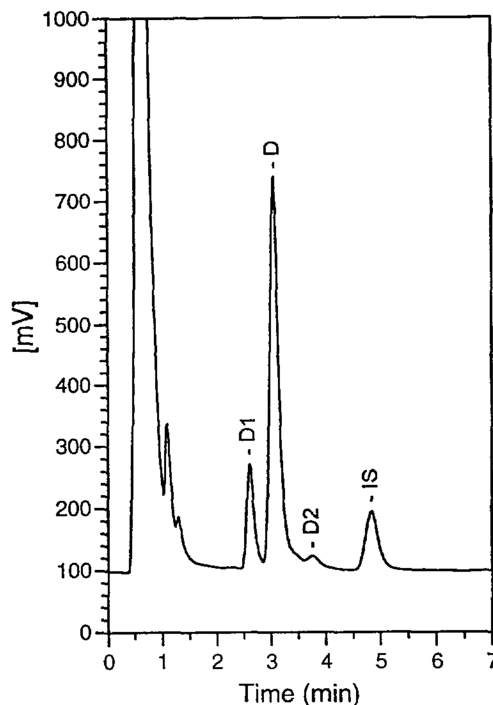

Fig. 6. Chromatogram of rat plasma 4 h post-dose after receiving an oral administration of 1 mg/kg droloxifene base equivalents. D = droloxifene; D1 = N-desmethyl-droloxifene; D2 = 4-methoxy-droloxifene; IS = internal standard.

4. Conclusions

A highly sensitive, specific, and rapid fluorimetric HPLC assay has been developed to quantitate trace droloxifene concentrations in rat, monkey, and human plasma as well as human serum. An easy two-step solid-phase extraction procedure yields high recovery and removes essentially all plasma interferences. This method has been successfully applied to pharmacokinetic studies in the Sprague–Dawley rat.

Acknowledgement

The authors wish to thank D.F. Nickerson for his analysis of droloxifene concentrations in rat plasma following oral and intravenous administration.

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