Journal of Chromatography, 565 (1991) 482–487
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5772

Short Communication

High-performance liquid chromatographic method for the determination of toremifene and its major human metabolites

LORRAINE K. WEBSTER*

Department of Haematology and Medical Oncology, Experimental Chemotherapy Unit, Peter MacCallum Cancer Institute, 481 Little Lonsdale Street, Melbourne, Victoria 3000 (Australia)

NICHOLAS A. CRINIS

Department of Pathology, Peter MacCallum Cancer Institute, Melbourne, Victoria 3000 (Australia)

and

KERRIE H. STOKES and JAMES F. BISHOP

Department of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, Melbourne, Victoria 3000 (Australia)

(First received August 21st, 1990; revised manuscript received December 4th, 1990)

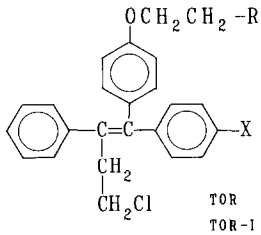
ABSTRACT

A high-performance liquid chromatographic method has been developed for the measurement of toremifene and its major human metabolites in plasma and urine. We have simplified other published methods, such that our assay uses protein precipitation in place of organic extraction, and ultraviolet detection instead of photochemical activation followed by fluorescence detection. In a stability study toremifene and metabolites remained unchanged for up to seven weeks at −70°C. This simple and specific assay allowed toremifene and three metabolites to be quantitated for pharmacokinetic analyses in a high-dose Phase I trial.

INTRODUCTION

Toremifene (Fig. 1) is a new non-steroidal triphenylethylene anti-estrogen compound with significant anti-tumour activity in breast cancer patients [1,2]. At high doses it appears to be active against estrogen receptor-negative tumours, but its exact mechanism of action is not yet known [3]. Structurally, toremifene differs from tamoxifen only by the substitution of a chloride ion for a hydrogen atom on the ethylene alkyl side-chain. Two major metabolites of toremifene, N-desmethyltoremifene (TOR-I) and (deaminohydroxy)toremifene (TOR-III) are present in human plasma in significant quantities. The former at least is biologically active [4–6].

0378-4347/91/$03.50



	R	X
TOR	$-N(CH_3)_2$	H
TOR-I	$-NHCH_3$	H
TOR-II	$-N(CH_3)_2$	OH
TOR-III	$-OH$	H
TOR-IV	$-NHCH_3$	OH
TOR-X	$-NH_2$	H
I.S.	pyrrole	H

Fig. 1. Structures of toremifene, its metabolites quantified by this assay and the internal standard.

High-dose toremifene is now under evaluation for the treatment of breast cancer. For our Phase I pharmacokinetic study of high-dose toremifene, a simple high-performance liquid chromatographic (HPLC) assay was needed. The only published method [5] used an organic extraction and photochemical activation followed by fluorescence detection. The following assay involves protein precipitation and ultraviolet detection and is sufficiently sensitive to measure toremifene and several metabolites in our high-dose trial. A stability study of these compounds has also been conducted and is reported here.

EXPERIMENTAL

Chemicals

Toremifene citrate, N-desmethyltoremifene citrate (TOR-I), (deaminohydroxy)toremifene (TOR-III) and the internal standard (I.S., Fc-1226a) were kindly provided by Farmos Group (Oulu, Finland). HPLC-grade acetonitrile and methanol were obtained from Mallinckrodt, ammonium acetate and acetic acid were from BDH, and triethylamine was from Ajax (all companies in Melbourne, Australia).

Apparatus and chromatography

The analyses were performed on a Waters HPLC system equipped with a Model 501 solvent delivery system, a U6K manual injector, a Model 490E programmable UV–visible spectrophotometric detector and a Baseline 810 chromatography workstation run on an NEC Powermate 1 Plus computer. The reversed-phase column was a Waters NovaPak C_{18} Rad-Pak (10 cm × 8 mm I.D., particle size 4 μm) housed in a Z-module at ambient temperature, and was protected by a μBondapak C_{18} Guard-Pak. The mobile phase consisted of acetonitrile–100 m*M* ammonium acetate–triethylamine (65:35:0.05) adjusted to pH 6.4 with acetic acid. The flow-rate was 2.0 ml/min and UV detection was at 277 nm at 0.005–0.01

a.u.f.s. Peak-height ratios of the drug or metabolite to the internal standard were automatically calculated.

Sample preparation

Stock solutions (1.0 mg/ml) of toremifene, TOR-I, TOR-III and I.S. were prepared in methanol and stored in the dark at −20°C. Standard solutions were prepared in bulk and consisted of mixtures of the three drugs at identical concentrations ranging from 1.0 to 100 μg/ml dissolved in methanol. A solution of the internal standard at 2.0 μg/ml was prepared by dissolution in acetonitrile.

Blank plasma was obtained in bulk during plasmapheresis. Heparinised venous plasma samples from cancer patients in the Phase I toremifene trial were stored at −70°C. Spiked plasma samples for the calibration curve were prepared by diluting the appropriate standard solution with blank plasma in a ratio of 1:9. The concentrations of drug and metabolites for the calibration curves were 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 μg/ml. Samples for the stability study were prepared in bulk at 0.2, 1.0 and 5.0 μg/ml and aliquots stored at −70°C. All other samples were freshly prepared.

Acetonitrile (200 μl) containing the internal standard (2.0 μg/ml) was added to the spiked or patient plasma samples (100 μl) and the samples were vortex-mixed (10 s). The precipitated protein was pelleted by centrifugation (5 min, 13 000 *g*), the supernatants were transferred to a clean tube, and 100 μl were injected onto the HPLC column.

RESULTS AND DISCUSSION

Fig. 2 depicts typical chromatograms of blank plasma, plasma from a patient in the clinical trial and mobile phase spiked with each of the compounds. The mean (±S.D., $n = 20$) retention times (min) for the separated components are:

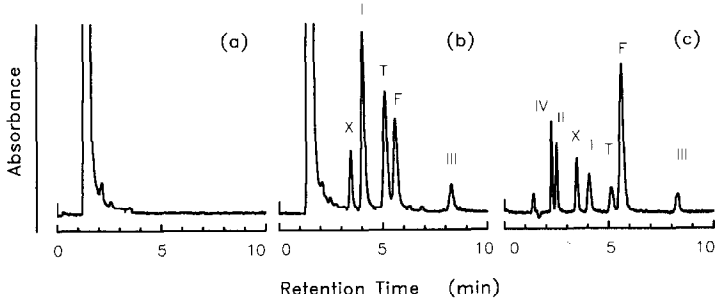


Fig. 2. Chromatograms of toremifene and metabolites on a reversed-phase C_{18} column. Chromatographic conditions as in Experimental. (a) Blank plasma from a patient in the toremifene Phase I trial. (b) The same patient 3 h after taking 560 mg of toremifene. (c) Mobile phase spiked with 1 μg/ml of each of the compounds. Peaks: T = toremifene; I = N-desmethyltoremifene; II = 4-hydroxytoremifene; III = (deaminohydroxy)toremifene; IV = 4-hydroxy-N-desmethyltoremifene; X = didemethyltoremifene; F = internal standard.

toremifene, 5.4 ± 0.2; TOR-I, 4.2 ± 0.2; TOR-III, 8.2 ± 0.3; I.S., 6.1 ± 0.3. Another metabolite was frequently noted at 3.6 min and this metabolite co-eluted with a pure sample of TOR-X (didemethyltoremifene). Two other metabolites, TOR-II (4-hydroxytoremifene) and TOR-IV (4-hydroxy-N-desmethyltoremifene) eluted at 2.5 and 2.3 min, respectively; however, the shoulder on the solvent front frequently prevented their measurement. Since the assay was initially not intended to measure TOR-II or TOR-IV, and because small changes in the methodology were found to unacceptably increase the retention time of TOR-III, the assay was not altered. The possible interference from endogenous compounds in human plasma or from other concurrent medication was evaluated. Plasma samples from ten patients who were typical of those entered in the trial (*i.e.* female breast cancer patients) were assayed. No interfering peaks were seen in plasma from these patients or from patients on the trial. Standard curves were drawn for toremifene, TOR-I and TOR-III by plotting peak-height ratio against concentration, and linearity was assessed by least-squares linear regression analysis. The calibration curves were linear for toremifene and its metabolites within the range 0.1–10.0 μg/ml, with coefficients of determination (r^2) greater than 0.996 ($n = 9$).

The intra-assay precision was determined by preparing and analysing six replicate samples of toremifene, TOR-I and TOR-III at three different concentrations. The intra-assay coefficient of variation ($n = 6$) was generally less than 6% for all three drugs at 0.2, 1.0 and 5.0 μg/ml, the exception being TOR-I at 0.2 μg/ml (Table I). The inter-assay coefficient of variation (C.V.) for aliquots of the same sample analysed eleven times between days 1 and 48 was 6% or less for all three drugs at 1.0 and 5.0, and 16% or less at 0.2 μg/ml (Table I). There was no trend for the concentrations of any of the drugs to either increase or decrease with time, as can be seen in the results of the stability study at 1.0 μg/ml (Fig. 3). It is therefore evident that toremifene, TOR-I and TOR-III are stable for up to seven weeks when stored at −70°C in the dark.

The mean recovery ($n = 5$) was estimated by comparing the peak-height ratios of spiked plasma following protein precipitation to a standard curve made up in methanol and acetonitrile (1:2). Recovery ranged from 97 to 110% for all three compounds at 1.0 and 5.0 μg/ml and from 86 to 118% at 0.2 μg/ml. The limit of quantitation of toremifene, TOR-I and TOR-III was about 200 ng/ml of plasma.

Toremifene is extensively metabolised, and over twenty metabolites have been identified [7,8]. Two of these, TOR-I and TOR-II, are probably active [4–6], and N-desmethylation is the major route of toremifene metabolism in the rat [9]. In humans TOR-I is present at concentrations up to four times higher than toremifene steady-state concentrations [6]. In contrast, TOR-II plasma concentrations are only at 30% of the toremifene levels. No reference could be found to indicate the anti-tumour activity of the other metabolites.

Although the assay can be applied to measurements of toremifene and its metabolites in urine, there have been no quantifiable levels in the urine of any of the patients, indicating that urinary excretion is not a major route of elimination.

TABLE I

INTRA- AND INTER-ASSAY VARIATION FOR HPLC ASSAY OF TOREMIFENE AND TWO METABOLITES IN HUMAN PLASMA

Replicate samples were assayed on the same day (n = 6) or aliquots of the same sample were analysed on different days (n = 11).

Concentration added (μg/ml)	Intra-assay		Inter-assay	
	Concentration found (mean) (μg/ml)	C.V. (%)	Concentration found (mean) (μg/ml)	C.V. (%)
Toremifene				
0.2	0.25	6.2	0.25	15.8
1.0	1.04	2.9	0.95	5.0
5.0	5.42	1.9	4.96	2.1
TOR-I				
0.2	0.22	18.6	0.24	12.4
1.0	1.09	3.3	0.97	3.9
5.0	5.48	2.1	5.03	2.9
TOR-III				
0.2	0.22	4.8	0.24	10.6
1.0	1.15	4.1	1.02	6.2
5.0	5.53	2.2	5.02	2.0

This assay is currently in use for a Phase I high-dose toremifene clinical trial. Patients at the starting dose received 200 mg/m^2 as a daily oral dose, with weekly blood samples and a 24-h pharmacokinetic study at day 42. Toremifene, TOR-I and TOR-III are easily quantitated and no interfering peaks have been noted in pre-dose plasma. Fig. 4 displays the plasma levels of toremifene and two metabolites over seven weeks in a representative patient. In the data from six patients analysed thus far, TOR-I concentrations are consistently higher than those of

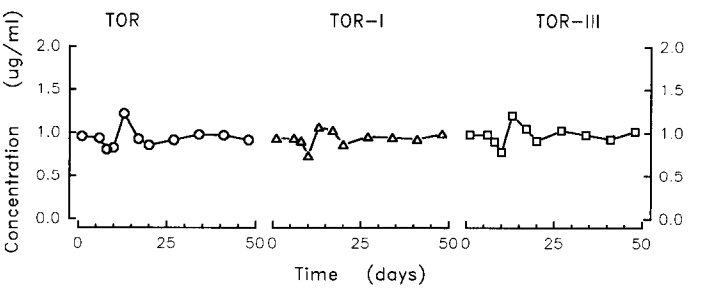


Fig. 3. Stability study for toremifene (TOR), TOR-I and TOR-III. Aliquots of the same sample at 1 μg/ml were measured eleven times between days 1 and 48.

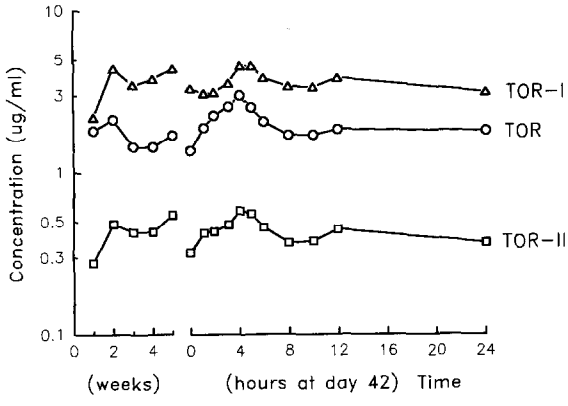


Fig. 4. Plasma levels of toremifene, TOR-I, and TOR-III in a representative patient in the high-dose Phase I clinical trial. Blood samples were drawn weekly until day 42, when a 24-h pharmacokinetic study was performed.

toremifene, in agreement with the data from other Phase I pharmacokinetic studies [6,10]. This successful method of analysis of toremifene and metabolites is therefore simple, reliable and selective, and will allow correlation of concentrations of toremifene and metabolites with toxicity and efficacy.

ACKNOWLEDGEMENT

This study was supported by Farmitalia Carlo Erba.

REFERENCES

1 R. Valavaara, S. Pyrhönen, M. Heikkinen, P. Ressanen, G. Blanco, E. Thölix, E. Nordman, P. Taskinen, L. Holsti and A. Hajba, *Eur. J. Cancer Clin. Oncol.*, 24 (1988) 785.
2 G. Horvath, U. Stendahl, M. Kalling, M. Ferno, A. Himmelmann and A. Hajba, *Anticancer Res.*, 10 (1990) 323.
3 L. Kangas, A. Nieminen, G. Blanco, M. Gronroos, S. Kallio, A. Karjalainen, M. Perila, M. Sodervall and R. Toivola, *Cancer Chemother. Pharmacol.*, 17 (1986) 109.
4 M. W. DeGregorio, J. M. Ford, C. C. Benz and V. J. Wiebe, *J. Clin. Oncol.*, 7 (1989) 1359.
5 W. M. Holleran, S. A. Gharbo and M. W. DeGregorio, *Anal. Lett.*, 20 (1987) 871.
6 V. J. Wiebe, C. C. Benz, I. Shemano, T. B. Cadman and M. W. DeGregorio, *Cancer Chemother. Pharmacol.*, 25 (1990) 247.
7 H. Sipilä, L. Kangas, L. Vuurilehto, A. Kalapudas, M. Eloranta, M. Södervall, R. Toivola and M. Anttila, *J. Steroid Biochem.*, 36 (1990) 211.
8 Farmos Group, Turku, personal communication.
9 S. P. Robinson, D. A. Mauel and V. C. Jordan, *Eur. J. Cancer Clin. Oncol.*, 24 (1988) 1817.
10 M. Anttila, R. Valavaara, S. Kivinen and J. Mäenpää, *J. Steroid Biochem.*, 36 (1990) 249.