ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1998) 847–852

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Optimised separation of E- and Z- isomers of tamoxifen, and its principal metabolites using reversed-phase high performance liquid chromatography

J.E. Manns, S. Hanks, J.E. Brown *

Pharmaceutical Chemistry, School of Pharmacy, University of Bradford, Bradford, West Yorkshire BD7 1DP, UK

Received 22 April 1997

Abstract

A reversed phase isocratic high-performance liquid chromatographic method is reported in which a formal structured procedure, the solvent selectivity triangle, was applied to predict the mobile phase composition giving baseline resolution of the clinically important triphenylethylene antioestrogenic agent (Z)-tamoxifen, its principal (Z)-metabolites, and also the clinically relevant (E)-geometric isomers of tamoxifen and 4-hydroxytamoxifen. The technique of solvent selectivity triangle was used to select the optimal organic modifier parameter for use with a Hichrom ODS 1 column, to achieve baseline separation of six triphenylethylene solutes. The detection system utilised post-column ultraviolet irradiation to convert solutes into their respective photocyclisation products, followed by fluorescence detection ($\lambda_{ex} = 254$ nm, $\lambda_{em} = 360$ nm), and the low detection limit for tamoxifen in serum of 0.1 μM. The optimal mobile phase composition was determined to be methanol–acetonitrile–water–trichloroacetic acid (50:31:18.9:0.1, v/v, pH 2.9). A single stage liquid–liquid extraction method for determination of triphenylethylene drugs in serum was developed. Reproducible recoveries for the (Z)-geometric isomers of tamoxifen ($84 \pm 3\%$) and its principal metabolites including Metabolite Y ($94 \pm 3\%$), *N*-desmethyltamoxifen ($94 \pm 3\%$) and 4-hydroxytamoxifen ($92 \pm 3\%$) were achieved, though more variable results were obtained for their corresponding (E)-geometric isomers ($71 \pm 7\%$ and $70 \pm 10\%$, respectively). © 1998 Elsevier Science B.V.

Keywords: Tamoxifen; 4-Hydroxytamoxifen; Triphenylethylene; Liquid–liquid extraction; Formal statistical procedures

1. Introduction

The nonsteroidal antioestrogen tamoxifen (Fig. 1), a triphenylethylene (TPE) antitumour agent, is currently the adjuvant drug of choice in the treatment of oestrogen receptor (ER) positive breast cancer [1]. Tamoxifen and other TPE analogues exist as geometric isomers, which may differ markedly in their agonistic or antagonistic activity [2,3]. The (Z)- or *trans* isomer of tamoxifen is antioestrogenic, whereas (E)- or *cis*-tamoxifen is a full oestrogen agonist [4]. One clinical metabolite of tamoxifen, 4-hydroxytamoxifen has a much higher ER binding affinity than tamoxifen, and

* Corresponding author.

PII S0731-7085(97)00116-7

although both geometric isomers of this metabolite are antioestrogenic, the E-isomer is only weakly antioestrogenic [5]. These E- and Z-geometric isomers of tamoxifen and 4-hydroxytamoxifen can undergo interconversion [6,7], and hence the antioestrogenic activity of (E)-4-hydroxytamoxifen may reflect some conversion of this isomer to the potent (Z)-form in biological fluids [8]. Hence analyses of TPE derivatives in biological fluids and tissues should determine not only tamoxifen and metabolites (Z-isomers), but also (E)-isomers of each TPE analogue.

Current literature methods for the determination of tamoxifen, and its principal metabolites *N*-desmethyltamoxifen and 4-hydroxytamoxifen are largely based on the pre- and post-column conversion of these TPE analogues to their fluorescent phenanthrene derivatives which are then determined by high performance liquid chromatography (HPLC [9–13]). However these methods do not distinguish between the geometric isomers of tamoxifen and its metabolites.

Novel bioanalytical techniques in anthracycline separations were previously reported by this group [14], and recently the application of formal statistical procedures resulted in baseline resolution of eight anthracycline solutes [15]. In these studies the formal optimisation procedure of solvent selectivity triangle was used to determine the effect of changes in the mobile phase composition necessary to resolve four clinically important triphenylethylene solutes including the (E)- and (Z)-isomers of tamoxifen and 4-hydroxytamoxifen, and the (Z)-isomers of *N*-desmethyltamoxifen and Metabolite Y. The HPLC system developed was used to determine the rate of interconversion of TPE homologues in tissue culture medium and mouse serum to assess the stability of TPE isomers in biological fluids.

2. Experimental

2.1. Materials

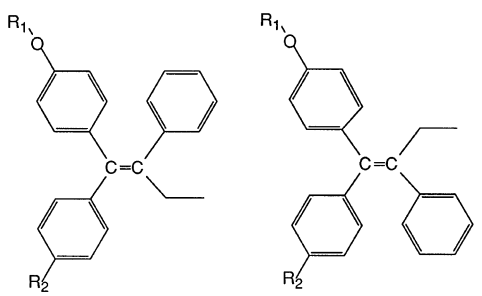
trans-(Z)-Tamoxifen and related compounds were kindly donated by Zeneca (Macclesfield, UK). Acetonitrile, methanol and tetrahydrofuran (THF) were HPLC grade, and all other chemicals were of analytical grade. Water was double distilled in glass. All analytical glassware in contact with drug solutions was silanised with Repelcote (BDH) and rinsed with methanol prior to use.

2.2. Sample preparation

Stock solutions of *trans*-(Z)-tamoxifen and metabolites in methanol (all 10^{-4} M) were prepared, protected from light, stored at -20°C, and thawed immediately prior to use. These were diluted to produce solutions of drug and metabolites in methanol (4.0 μg ml^{-1}, 2.7×10^{-5} M).

2.3. Liquid chromatography

The HPLC system consisted of a LKB 2150 pump with a Hewlett Packard 1046A fluorescence detector (Hewlett Packard, Waldbron, Germany) using excitation and emission wavelengths of 254 and 360 nm, respectively. A Rheodyne 7125 injection valve (20 μl injection loop) was used with a 5



COMPOUND	R_1	R_2
Tamoxifen	$O(CH_2)_2N(CH_3)_2$	H
4-Hydroxytamoxifen	$O(CH_2)_2N(CH_3)_2$	OH
N-Desmethyltamoxifen	$O(CH_2)_2NHCH_3$	H
Metabolite Y	$O(CH_2)_2OH$	H

Fig. 1. Structures of (E) and (Z)-geometric isomers of tamoxifen and its principal metabolites *N*-desmethyltamoxifen, 4-hydroxytamoxifen, and metabolite Y.

μm ODS 1 Hichrom 250 × 4.6 mm i.d. column (Anachem, Luton, UK). Postcolumn UV irradiation was achieved using a laboratory built reactor: PTFE tubing (200 cm × 0.2 mm i.d., 0.5 mm o.d.) was 'french knitted' around a mercury UV lamp as described elsewhere [16]. Initial mobile phase composition comprised methanol–water–trichloroacetic acid (85:14.9:0.1 v/v/w, pH 2.5), with a flow rate of 1.8 ml min^{-1} (mobile phase I). In mobile phases II and III methanol was replaced by acetonitrile (77:23 v/v) and THF (61:39 v/v), respectively. Binary mixtures (1:1 v/v) of mobile phases I and II, I and III, and II and III produced mobile phases IV, V, VI, respectively. Mobile phase VII contained mobile phases I, II and III (1:1:1 v:v:v).

2.4. Recoveries

Recoveries were determined by analyses of five independently prepared horse serum samples containing all four TPE compounds at concentrations in the range 0.8–12 μg ml^{-1}. The recoveries were calculated by comparison with standard solutions of the same drug concentration in elution solvent.

2.5. Formal optimisation procedure—solvent selectivity triangle

A total of seven experiments were performed using three solvents from different selectivity classes—acetonitrile, methanol and tetrahydrofuran. Retention data were analysed using a statistical mixture design strategy to obtain a resolution map.

The seven mobile phases used for construction of the solvent selectivity triangle were prepared in water and contained organic modifier with equivalent solvent strength to 85% methanol. In order to utilise isoeluotropic acetonitrile/water and THF/water binary mixtures, the transfer rules of De Galen [17] were used where:

$$\Phi_{THF} = -0.420\Phi^3_{MeOH} + 0.702\Phi^2_{MeOH} + 0.423\Phi_{MeOH}$$

$$\Phi_{MeCN} = -0.490\Phi^3_{MeOH} + 0.953\Phi^2_{MeOH} + 0.447\Phi_{MeOH}$$

and Φ is the volume fraction of the organic modifier. The proportions of organic modifier equivalent to 85% methanol were 77% acetonitrile, and 61% THF.

The stationary phase (5 μm Hichrom ODS 1 250 × 4.6 mm i.d.) was selected following preliminary studies which indicated the suitability of this column for TPE separations. Mobile phases I to VII were each eluted through the column at 1.8 ml min^{-1} and the eluate irradiated via the UV lamp and the phenanthrene derivatives monitored using fluorescence detection as described above. Aliquots (20 μl) of a mixture containing all four TPE compounds (100.0 μg ml^{-1}) were injected onto each system.

The calculated response (R_p) for any position on the solvent selectivity triangle was derived using the equation [18]:

$$R_p = B_1X_1 + B_2X_2 + B_3X_3 + B_4X_1X_3 + B_5X_1X_2 + B_6X_2X_3 + B_7X_1X_2X_3$$

where $X_{1,2,3}$ refers to the position within the plane of the solvent selectivity triangle and B_1–B_7 are the constants, calculated from the retention data as follows:

$$B_1 = R_1,\ B_2 = R_2,\ B_3 = R_3$$

$$B_4 = 4R_4 - 2(R_1 + R_3)$$

$$B_5 = 4R_5 - 2(R_1 + R_2)$$

$$B_6 = 4R_6 - 2(R_2 + R_3)$$

$$B_7 = 27R_7 - 12(R_4 + R_5 + R_6) + 3(R_1 + R_2 + R_3)$$

R_i refers to the resolution (R_s) between peak pairs for seven experiments using mobile phases I–VII, calculated from the equation:

$$R_s = 1.176(t_{R2} - t_{R1})/(w_{1/2_1} + w_{1/2_2})$$

where t_R = retention time for each peak, and $w_{1/2}$ = peak width at half-height.

2.6. Serum extraction procedures

Samples (2 ml) of horse serum containing known amounts of (Z)-tamoxifen, (Z)-*N*-desmethyltamoxifen, (Z)-4-hydroxytamoxifen and (Z)-metabolite Y (0.8–12.3 μg ml^{-1}) were trans-

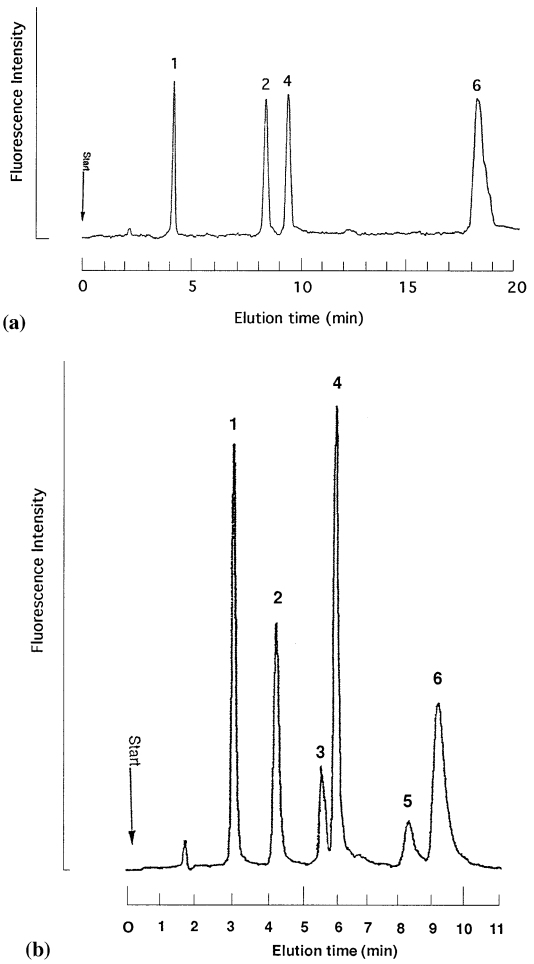


Fig. 2. HPLC separation of tamoxifen and its principal metabolites; (a) initial HPLC chromatogram of tamoxifen and its principle metabolites using a 250×4.6 mm i.d. 5 μm Hichrom ODS 1 column eluting with methanol:water: trichloroacetic acid (85:14.9:0.1); and (b) after the application of formal optimization procedures. The optimal mobile phase composition was methanol:acetonitrile:water:trichloroacetic acid (50:31:18.9:0.1, pH 2.9), flow rate 1.8 ml min^{-1} using fluorescence detection at excitation 254 and emission 360 nm. The key to the chromatographic peaks is: 1, (Z)-metabolite Y; 2, (Z)-4-hydroxytamoxifen; 3, (E)-4-hydroxytamoxifen; 4, (Z)-*N*-desmethyltamoxifen; 5, (E)-tamoxifen; and 6, (Z)-tamoxifen.

ferred to centrifuge tubes and extracted with diethyl ether (2×20 ml). The pooled diethyl ether extract was evaporated to dryness using compressed air and the residue reconstituted in 1.0 ml methanol (HPLC Grade, Rathburn, Scotland). This solution was then centrifuged at 3000 rpm for 3 min to separate any precipitated proteins. An aliquot (20 μl) was then analysed by HPLC using the conditions described above.

2.7. Chromatographic separation of the E- and Z- isomers of tamoxifen, 4-hydroxytamoxifen and tamoxifen derivatives

Conversion of pure Z- forms of tamoxifen and 4-hydroxytamoxifen into corresponding E-isomers was as described elsewhere [19]. Confirmation of the presence of E-isomers was achieved by using a mobile phase of acetonitrile and tetrahydrofuran in water and ammonia (300:75:125:2 v/v, pH 3.0).

3. Results and discussion

The photochemical reactor consisted of a PTFE capillary tube coiled around a low pressure mercury UV lamp in such a way as not to cause band broadening. The lamp emitted more than 90% of its irradiance in the 254 nm spectral line, and very little heat was produced. The irradiation wavelength of 254 nm is close to the absorption maximum of the analytes. The absorption at 254 nm of the cyclisation product is more intense than that of tamoxifen by a factor of 2.0. However, the fluorescence intensities of the phenanthrene derivative at its emission maxima (374 nm) is more intense than that of tamoxifen by a factor greater than 25.0.

Methods for the separation of E- and Z- geometric isomers of tamoxifen and its metabolites were previously described [20–22]. Here the formal statistical procedure of the solvent selectivity triangle, originally developed by Snyder [23] was used to determine the most suitable type of organic modifier for the optimal separation of the TPE compounds. In this technique, solute retention is modelled for each peak and is used to determine resolution between each peak pair over the whole triangle. Plotting the worst case at each position gives the overlapping resolution map. Four compounds were used for these studies: (Z)-Tamoxifen, (Z)-*N*-desmethyltamoxifen, (Z)-4-hydroxytamoxifen and (Z)-metabolite Y. Seven

Table 1
Retention data obtained from solvent selectivity experiments

Compound	Retention time (min)[a]						
	I	II	III	IV	V	VI	VII
Metabolite Y	5.0	4.3	12.3	4.3	7.4	10.8	5.1
4-Hydroxytamoxifen	10.2	11.9	37.2	9.4	13.6	27.9	11.9
N-desmethyltamoxifen	11.5	20.2	64.0	14.5	19.3	53.8	19.1
Tamoxifen	22.7	28.1	87.3	20.2	28.5	65.4	25.0

[a] Numbers I–VII refer to mobile phases I–VII in the solvent selectivity triangle (see text).

experiments were carried out with the different mobile phases (I–VII) and the mobile phase composition giving best resolution for the worst separated peak pair in the initial chromatogram (Fig. 2a) was identified. The retention data (Table 1) were then used to calculate the response surface of the solvent selectivity triangle. The resulting response surfaces for each peak pair were then overlayed to produce an overlapping triangular resolution map (Fig. 3). This shows the combinations of solvents which give the best solute resolution, and this indicated an area of high resolution including the acetonitrile apex, however this is not the case for most combinations of acetonitrile and THF. The optimum organic modifier composition determined was methanol–acetonitrile–water–trichloroacetic acid (50:31:18.9:0.1, pH 2.9), determined by adjusting the apparent pH of the mixture. The final chromatogram (Fig. 2b) obtained by using this mobile phase shows baseline separation of the four Z-isomers in a run time of just over 12 min.

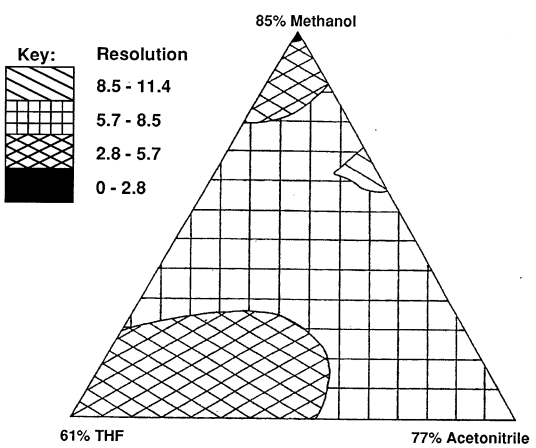


Fig. 3. Overlapping resolution map for triphenylethylene separation from solvent selectivity triangle experiments.

The Z- and E- geometric isomers of tamoxifen, 4-hydroxytamoxifen, (Z)-metabolite Y and (Z)-*N*-desmethyltamoxifen were next extracted from horse serum by a liquid/liquid extraction with diethyl ether. Fig. 4 shows the chromatograms obtained from extraction of Z-isomers from horse serum, and blank horse serum, respectively. The recoveries from this extraction are shown in Table 2. It was shown that the recoveries of the E isomer of tamoxifen ($70.7 \pm 7.2\%$) and 4-hydroxytamoxifen ($70.0 \pm 9.6\%$) were lower than the corresponding Z-isomers ($83.5 \pm 3.2\%$ and $91.9 \pm 2.8\%$, respectively).

Table 2
Triphenylethylene recoveries using liquid–liquid extraction from horse serum spiked with drug in the range 0.8–12 μg ml^{-1} (mean ± RSD, $n = 5$)

Compound	Concentration μg ml^{-1}	Absolute recovery %
(Z)-metabolite Y	4.0	94.2 ± 2.7
(Z)	3.0	91.9 ± 2.8
-4-hydroxytamoxifen		
(E)	0.8	70.0 ± 9.6
-4-hydroxytamoxifen		
(Z)	6.7	93.7 ± 3.3
-4-*N*-desmethylta-moxifen		
(E)-tamoxifen	1.1	70.7 ± 7.2
(Z)-tamoxifen	12.3	83.5 ± 3.2

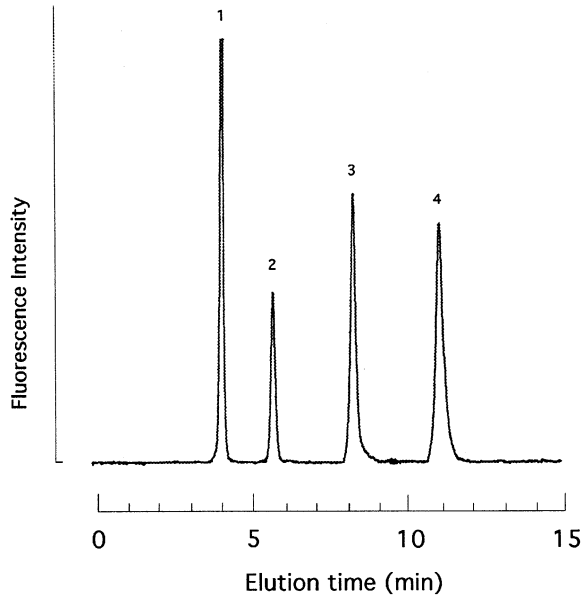


Fig. 4. HPLC chromatogram of tamoxifen and its principal metabolites (400 ng ml^{-1}, 2.7×10^{-6} M) extracted from horse serum. Chromatograph conditions as in Fig. 2b. The key to the chromatographic peaks is: 1, (Z)-metabolite Y; 2, (Z)-4-hydroxytamoxifen; 3, (Z)-N-desmethyltamoxifen; 4, (Z)-tamoxifen.

4. Conclusions

This study has utilised formal statistical techniques in the HPLC analysis of tamoxifen and its metabolites. The application of these formal statistical procedures provided data which determined the optimal mobile phase composition giving baseline resolution of Z- isomers of tamoxifen and analogues, and this system also resolved E-isomers of tamoxifen and 4-hydroxytamoxifen; this comprised methanol–acetonitrile–water–trichloroacetic acid (50:31:18.9:0.1, v/v, pH 2.9). It was also shown that tamoxifen and its metabolites can be efficiently extracted from biological matrices by a simple extraction with diethyl ether.

Acknowledgements

The authors would like to thank the SERC for their financial support of this research programme, and Zeneca for donation of tamoxifen and metabolites.

References

[1] V.C. Jordan, Breast Cancer Res. Treat. 15 (1990) 125–136.
[2] M.J.K. Harper, A.L. Walpole, J. Reprod. Fertil. 13 (1967) 101–119.
[3] L. Terenius, Acta Endocrinol. 66 (1971) 431–438.
[4] V.C. Jordan, C.S. Murphy, Endocrine Rev. 11 (1990) 578–610.
[5] V.C. Jordan, B. Haldemann, K.E. Allen, Endocrinology 108 (1981) 1353–1361.
[6] B.S. Katzenellenbogen, M.J. Norman, R.L. Eckert, S.W. Pletz, W.F. Mangel, Cancer Res. 44 (1984) 112–119.
[7] J.A. Katzenellenbogen, K.E. Carlson, B.S. Katzenellenbogen, J. Steroid Biochem. 22 (1985) 589–596.
[8] V.C. Jordan, R. Koch, S. Langan, R. McCague, Endocrinology 122 (1988) 1449–1454.
[9] Y. Golander, L.A. Sternson, J. Chromatogr. 181 (1980) 41–49.
[10] R.R. Brown, R. Bain, V.C. Jordan, J. Chromatogr. 272 (1983) 351–358.
[11] D. Stevenson, R.J. Briggs, D.J. Chapman, D. De Vos, J. Pharm. Biomed. Anal. 6 (1988) 1065–1068.
[12] C.K. Lim, Z.-X. Yuan, J.H. Lamb, I.N.H. White, F. De Matteis, L.L. Smith, Carcinogenesis 15 (1994) 589–593.
[13] K.M. Fried, I.W. Wainer, J. Chromatogr. B 655 (1994) 261–268.
[14] J.E. Brown, P.A. Wilkinson, J.R. Brown, J. Chromatogr. 226 (1981) 521–525.
[15] G. Nicholls, B.J. Clark, J.E. Brown, J. Pharm. Biomed. Anal. 10 (1993) 949–957.
[16] M. Nieder, H. Jaeger, J. Chromatogr. Biomed. Appl. 413 (1987) 207–217.
[17] L. DeGalan, D.P. Herman, H.A.H. Billiet, Chromatographia 24 (1987) 108–114.
[18] J.C. Berridge, In: Techniques for the Automated Optimization of HPLC Separations, Wiley-Interscience, Chichester, UK, 1985, pp. 55–152.
[19] R. McCague, J. Chem. Soc. Perkin Trans. I (1897) 1011–1015.
[20] R.D. Armstrong, T.J. Ward, N. Pattabiraman, C. Benz, D.W. Armstrong, J. Chromatogr. 414 (1987) 192–196.
[21] H.G. Jalonen, J. Pharm. Sci. 77 (1988) 810–813.
[22] C.K. Osborne, V.J. Wiebe, W.L. McGuire, D.R. Ciocca, M.W. Degregorio, J. Clin. Oncol. 10 (1992) 304–310.
[23] L.R. Snyder, J. Chromatogr. Sci. 16 (1978) 223–224.