ELSEVIER

International Journal of Pharmaceutics 177 (1999) 259–270

international journal of pharmaceutics

Short communication

Studies on the stability of *trans*-α-acetoxytamoxifen in Sprague–Dawley female rat liver slices, homogenate and subcellular fractions including microsomes and mitochondria

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Received 20 May 1998; received in revised form 10 October 1998; accepted 20 October 1998

Abstract

This work examined the stability of trans α-acetoxytamoxifen in Krebs–Henseleit buffer (pH 7.4), in the presence of Sprague–Dawley female rat liver slices, rat liver homogenate and hepatic subcellular fractions, including microsomes and mitochondria, at pH 7.4 and at 37°C over 300 min. The rate of hydrolysis was determined using high-performance liquid chromatography, and degradation profiles were obtained from which the rate and order of degradation were both evaluated. By applying zero-, first-, second- and third-order models of drug disappearance and the generation of by-products, first- and second-order appeared to produce the best fit. *trans*-α-Acetoxytamoxifen degraded rapidly in buffer and more slowly in the biological systems, probably due to the fact that the agent partitions into the hydrophobic component of the biological tissue and hence degrades at a much slower rate. The principal degradation products were *trans*-α-hydroxytamoxifen and, to a lesser extent, *cis*-α-hydroxytamoxifen. Another peak could not be identified. The production of *trans*-α-hydroxytamoxifen was enhanced in the presence of biological enzymes, whereas the concentration of the cis isomer remains relatively constant in buffer only (pH 7.4) and in the presence of biological enzymes. Therefore, the formation of identical adducts with DNA is consistent, because it has been shown that α-acetoxytamoxifen breaks down to form α-hydroxytamoxifen in vitro. The percentage of *trans*-α-acetoxytamoxifen remaining after 300 min was 40% in mitochondria and 32% in homogenate. The half-life ($t_{1/2}$) was calculated for each condition by applying zero-, first- and second-order rate kinetics.

Keywords: *trans*-α-Acetoxytamoxifen; Stability; Kinetics; Liver slices; Homogenate; Hepatic subcellular fractions

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0378-5173/99/$ - see front matter
PII: S0378-5173(98)00344-5

1. Introduction

Tamoxifen is an anti-oestrogen, which is clinically used and requires metabolic activation before binding to DNA, and this activation occurs both in vitro and in vivo by activation with liver microsomes from rats (Hard et al., 1993; Pathak and Bodell, 1994; Osborne et al., 1996) or man (Hard et al., 1993; Pathak and Bodell, 1994). It has been proposed that α-hydroxytamoxifen [(E)-4 - {4 - [2 - (dimethylamino) - ethoxy]phenyl} - 3, 4 - diphenyl-3-buten-2-ol], which has been identified as a metabolite in rat hepatocytes (Pathak et al., 1995), human liver (Phillips et al., 1994a), and in plasma of patients treated with tamoxifen (Phillips et al., 1994a), is an intermediate in this activation. α-Hydroxytamoxifen reacts with DNA in vitro, giving low levels of adducts that are indistinguishable from those found in rat hepatocytes treated with tamoxifen (Pathak et al., 1995).

The α-hydroxy group requires either protonation or conjugation before it can act as a leaving group and can generate the reactive carbocation. A tamoxifen derivative with a good leaving group at the α position should give a high level of adducts on reaction with DNA. Therefore, α-acetoxytamoxifen [(E)-4-{4-[2-(dimethylamino)eth-oxy]phenyl}-3,4-diphenyl-2-acetoxy-3-butene] was prepared by Osborne et al. (1996) and, on examining the reaction of this compound with DNA; they found that this compound reacted with DNA in vitro to a much greater extent than α-hydroxytamoxifen. These authors also stated that the products of *trans*-α-acetoxytamoxifen, when analysed by reverse-phase high-performance liquid chromatography (HPLC) were identical to those isolated from DNA treated with α-hydroxytamoxifen and to those found in the liver DNA of rat hepatocytes treated with tamoxifen or of the livers of rats treated with tamoxifen.

Here, the stability of *trans*-α-acetoxytamoxifen was examined in Krebs–Henseleit buffer (pH 7.4), female rat liver slices, rat liver homogenate and hepatic subcellular fractions including microsomes and mitochondria at pH 7.4. Four different models of drug degradation were applied, zero-, first-, second- and third-order rate kinetics, and it was found from the linearity of the plots (Fig. 2a–d) that first- and second-order appeared better than zero- or third-order. It is not easy to determine the order of degradation in the presence of liver slices or subcellular fractions, because the order is difficult to validate and may be rate-limited by several factors (enzymes, co-factors, tissue degradation, etc.). However, an investigation of the stability and kinetics of this compound had not been previously investigated and, from the results of our experiments, it is shown to be unsuitable for metabolism studies due to its instability.

2. Materials and methods

2.1. Chemicals

trans-(*Z*)-Tamoxifen, NaCl, $CaCl_2 \cdot 6H_2O$, KCl, $MgSO_4$, KH_2PO_4, Tris buffer, $MgCl_2$, sucrose, bovine serum albumin, fraction 5 (BSA) and NADPH were purchased from Sigma Chemical Co. (Poole, UK). The bicinchoninic acid (BCA) Protein Assay Reagent Kit was obtained from Pierce (Rockford, IL 61105). All solvents used were HPLC grade and were obtained from BDH Chemical (Poole, UK). *trans*-α-Acetoxytamoxifen and *cis*- and *trans*-α-hydroxytamoxifen were synthesised according to published procedures described in Osborne et al. (1996) and Phillips et al. (1994b), respectively, in this laboratory. Female Sprague–Dawley rats and mice were obtained from breeders within the UK.

2.2. Preparation of liver slices

Preparation of female rat liver slices, and liver slice incubations were carried out in Krebs–Henseleit buffer (pH 7.4) from a concentrate which was stable at 4°C for up to 3 months. The composition of the concentrate was as follows: NaCl (118 mM), $CaCl_2 \cdot 6H_2O$ (2.2 mM), KCl(4.75 mM), $MgSO_4$ (1.2 mM) and KH_2PO_4 (1.2 mM). Buffer solutions were prepared daily by adding 20.00 ml of the concentrate and 1.05 g of sodium hydrogen carbonate to 450 ml of distilled water. The pH was adjusted to 7.4 with 0.1 M HCl and the buffer volume diluted to 500 ml with distilled water. One

Sprague–Dawley rat (250–350 g) was killed by cervical dislocation, the liver was immediately excised and placed in ice-cold Krebs–Henseleit buffer (pH 7.4), which was continuously purged with 95% oxygen/5% carbon dioxide. The liver slices were prepared using a manual slicer and each slice was approximately 0.3 μm thick. The first and the last slices from each core were discarded because they contained the liver capsule. Slices were placed in Krebs–Henseleit buffer (pH 7.4) and saturated with oxygen/carbon dioxide until slice preparation was complete. Slices were then immediately used for incubations.

2.3. Preparation of hepatic subcellular fractions (microsomes and mitochondria) and liver homogenate

Hepatic subcellular fractions (microsomes and mitochondria) were prepared in this laboratory according to the procedure described in Critchley et al. (1992).

Rat liver homogenate was produced when approximately 10 g liver was homogenised in 35.00 ml of 1.15% KCl solution containing 0.1 mM EDTA. Approximately 0.5 ml homogenate is equivalent to four liver slices.

2.4. To determine the protein content in the hepatic subcellular fractions

A stock solution of Bovine Serum Albumin, fraction 5 (BSA) (Sigma) (2 mg/ml) and EDTA (0.1 mM) was prepared. Protein standards were prepared from the stock BSA giving the concentrations 200, 400, 600, 800 and 1000 μg/ml. A BCA working reagent (WR) was prepared when 50 parts of BCA Reagent A was mixed with 1 part of BCA Reagent B. When Reagent B was initially added to Reagent A, a turbidity was observed that quickly disappeared upon mixing. Appropriate dilutions of the hepatic subcellular fractions were prepared. Test tubes were labelled and 2 ml of the WR was added to each tube. Each standard and subcellular fraction dilution (100 μl) was added, then mixed and incubated at 37°C for 30 min. The absorbance of each solution was measured at 562 nm, and a calibration curve for protein concentration was drawn and, hence, the protein content of each fraction determined.

2.5. Preparation of standard solutions of *trans*-α-acetoxytamoxifen, *cis*- and *trans*-α-hydroxytamoxifen

A standard solution of *trans*-α-acetoxytamoxifen was made up (200 μM) and a series of dilutions was made from this (0–200 μM). The calibration curve produced was found to be linear within this range ($r^2 = 0.999$). *trans*-α-Acetoxytamoxifen was insoluble in aqueous solvent such as Krebs buffer (pH 7.4), therefore an appropriate amount (mg) was weighed out and dissolved in a small volume (1 ml) of dimethylsulphoxide (DMSO) or methanol. A volume of this solution (μl) was then added to the incubation. It was very important to keep the percentage of organic solvent as low as possible ($< 1\%$) in the incubation, as this may poison the enzyme systems present. Degradation studies were carried out on a range of substrate concentrations (50–200 μM), and it was found that the concentration of degradates produced remained relatively constant above 155 μM, so therefore 155 μM was chosen for *trans*-α-acetoxytamoxifen degradation studies.

cis- and *trans*-α-Hydroxytamoxifen compounds produced linear calibration curves within the range 0–200 μM ($r^2 = 0.999$). Like *trans*-α-acetoxytamoxifen, the above-mentioned solutions were insoluble in aqueous solvent so, therefore, they were made up in methanol or DMSO.

The compounds were synthesised in the laboratory and they were purified by column chromatography. Their purity was then checked by HPLC using diode array detection and found to be 100%. Their structural identity was confirmed by spectroscopic analysis, i.e. nuclear magnetic resonance and mass spectrometry.

2.6. Stability studies using *trans*-α-acetoxytamoxifen

trans-α-Acetoxytamoxifen (155 μM) was incubated in Krebs–Henseleit buffer (pH 7.4) to a total volume of 3 ml at 37°C for 300 min. Incubations were performed in 10 ml closed vials which

had holes drilled in their caps for the purpose of oxygenation at regular intervals throughout the incubation period. Oxygen is needed for oxidative metabolism. These were then placed in a shaking water-bath and pre-warmed to 37°C before initiation of the reaction by the addition of four liver slices or 0.5 ml of homogenate or subcellular liver fraction. The vials were wrapped in tin foil and the water-bath was protected from light throughout the incubation period, since *trans*-α-acetoxytamoxifen is photosensitive. A separate vial was used for each time point in the case of the liver slices, this was done because some degradates may adhere to the slices. This problem was overcome by homogenising the slices at each time point, centrifuging and then sampling. Aliquots (100 μl) were removed at 0, 5, 10, 20, 60, 120, 180 and 300 min time intervals using a Gilson micropipette. Incubations containing liver homogenate or subcellular liver fractions were carried out in the presence of NADPH (0.5 mM), while incubations containing liver slices had intact NADPH. Control incubations were carried out along with normal incubations, these contained no *trans*-α-acetoxytamoxifen, no NADPH and no tissue, respectively. These were absolutely necessary to validate the results obtained with normal incubations. Standards of *trans*-α-acetoxytamoxifen, *cis*- and *trans*-α-hydroxytamoxifen were also run with each set of incubations. Aliquots (100 μl) were removed at specified time intervals and added to 200 μl of 5% DMSO in methanol to terminate the reaction. Samples were centrifuged in a Beckman bench-top microcentrifuge at $9000 \times g$ for 5 min, and the supernatant was subsequently analysed by HPLC. This technique was used to monitor the appearance of degradation product and the disappearance of the parent compound.

2.7. HPLC analysis

HPLC analysis was carried out using a system supplied by Waters Associates (Northwich, Cheshire), which consisted of a 717 plus Autosampler, UV 486 tunable Absorbance Detector and a 510 pump, all controlled by Millennium software. All samples were analysed by HPLC using a Spherisorb 5ODS1, 5 μm (25 cm × 4.6 mm i.d.) column with a (Bondapak C18 Guard-Pak insert. The mobile phase consisted of methanol/acetonitrile/water/trichloracetic (pH 2.9) (50:31:19:0.1% v/v) at a flow rate of 1.0 ml/min. This mobile phase was developed in our laboratory (Manns et al., 1993). The reactions were monitored at 280 nm and room temperature. The concentration (μM) of each compound was calculated with respect to standards synthesised in the laboratory. Each compound had the following retention times: *trans*-α-acetoxytamoxifen, 10.0 min; *trans*-α-hydroxytamoxifen, 7.0 min; and *cis*-α-hydroxytamoxifen, 8.0 min. Another peak, which appeared consistent with *cis*-α- acetoxytamoxifen, had a retention time of 11.5 min.

2.8. Peak purity analysis

Peak purity analysis was carried out using a system which consisted of a Severn Analytical Autosampler, a Gynkotek UVD-340S Variable Wavelength Diode Array Detector and a LC-9A Shimadzu Liquid Chromatograph pump, all controlled by GynkoSoft software. The chromatographic conditions used were similar to those already described in the HPLC analysis section.

3. Results and discussion

The rate of degradation of *trans*-α-acetoxytamoxifen and the appearance of the degradation products was monitored using HPLC. The purpose of these degradation studies was to measure the stability of the *trans*-α-acetoxytamoxifen in various biological media at pH 7.4 and at 37°C, and also to monitor the rate of formation of the products of degradation and to confirm their identity. It was essential that these studies be carried out in order to check the stability of the *trans*-α-acetoxytamoxifen before metabolism studies could be performed. The results obtained proved that metabolism studies could not be carried out due to the unstable nature of the compound. Degradation profiles were prepared showing the appearance of the degradates and the

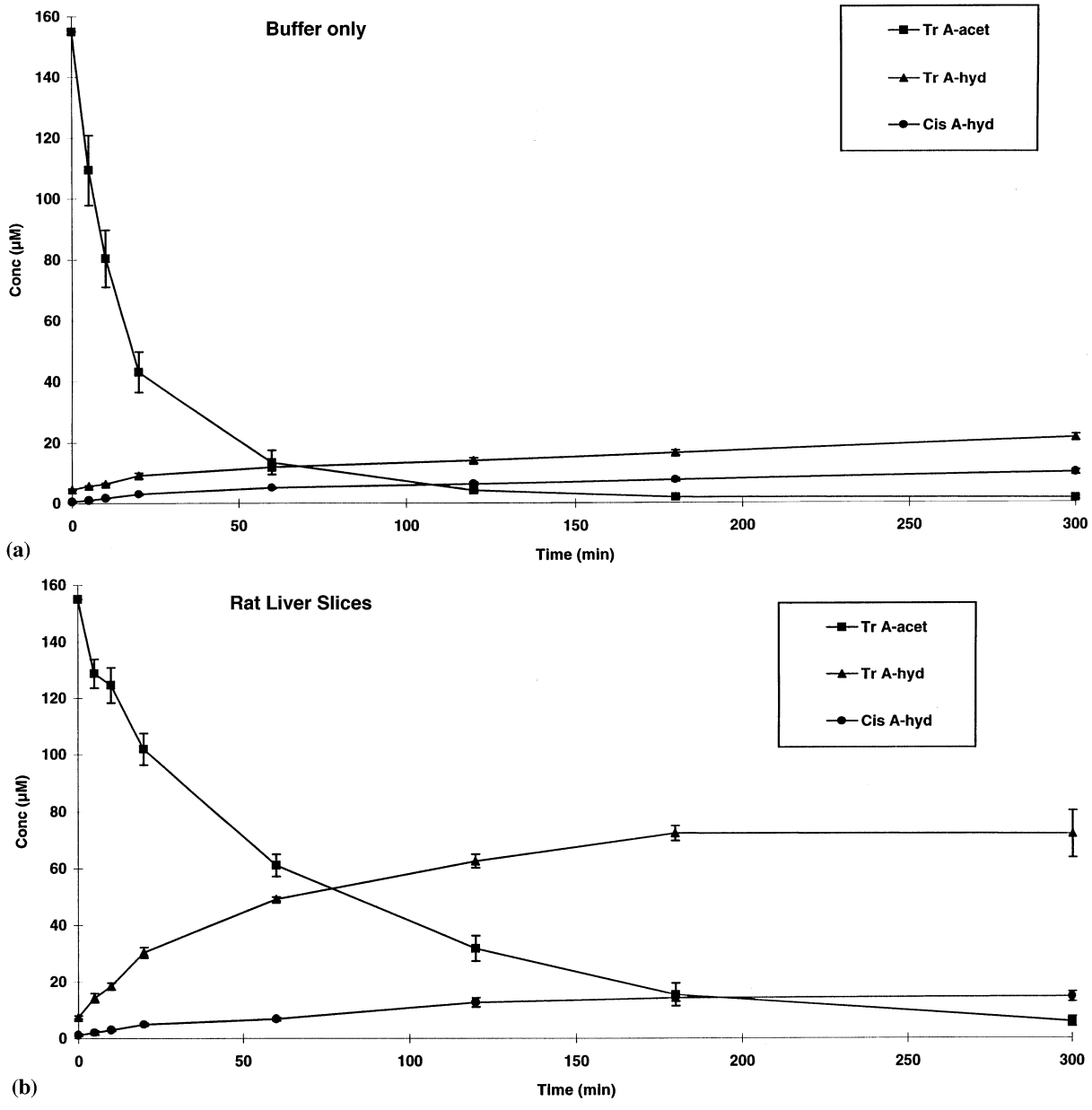
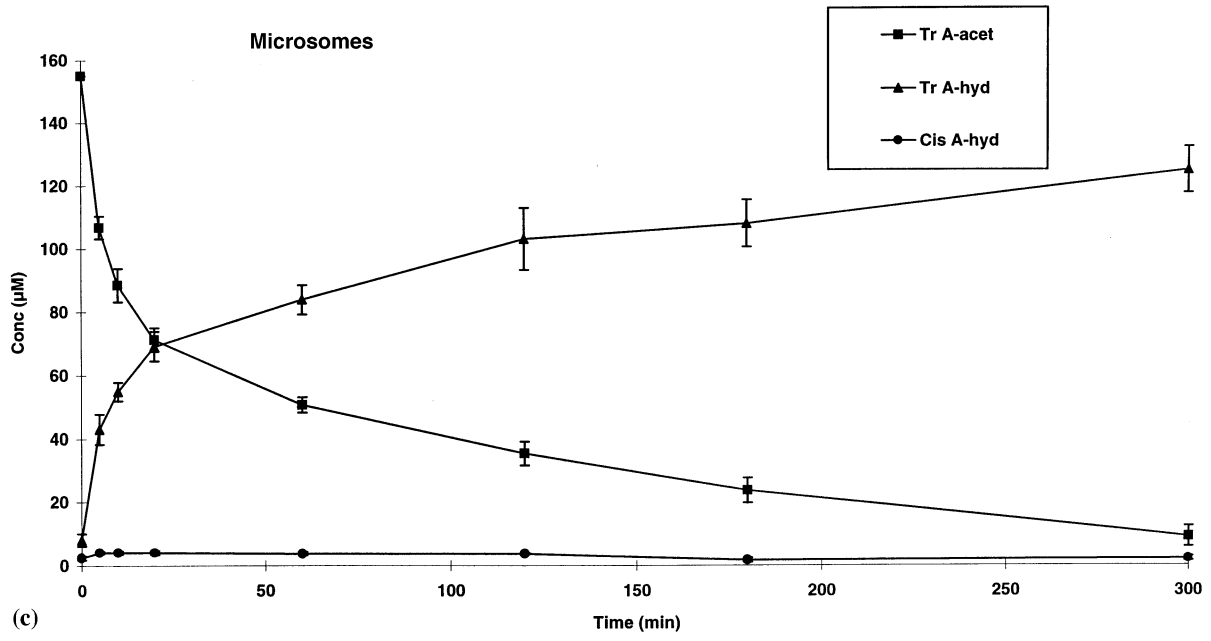
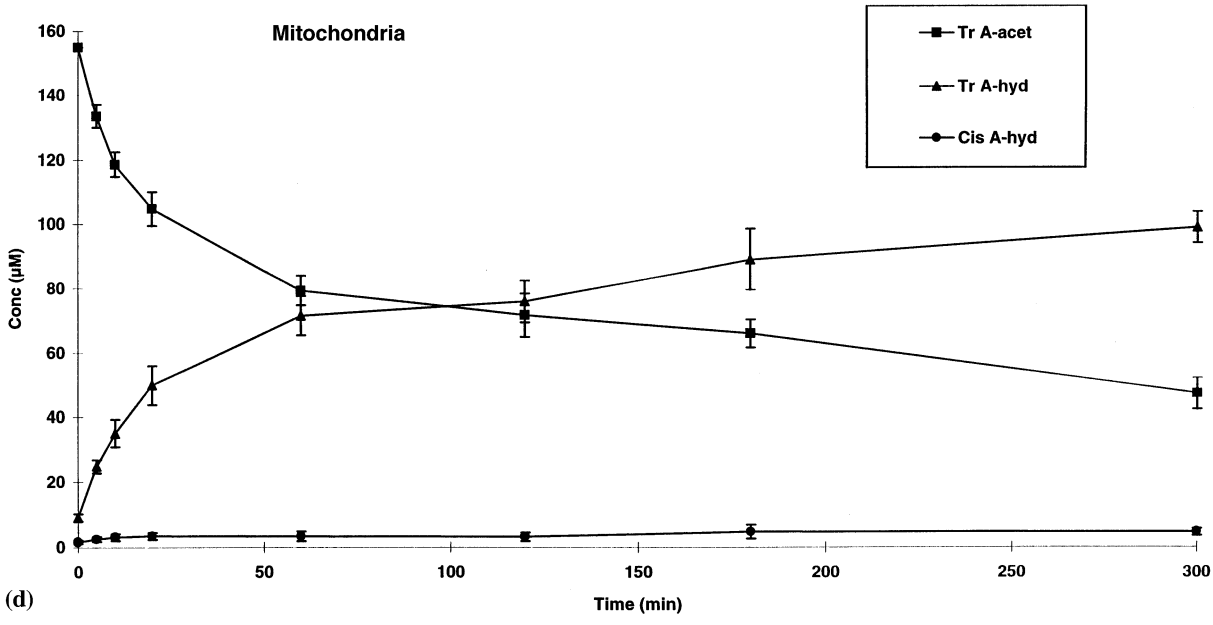


Fig. 1. (a)–(d). Profiles showing the disappearance of *trans*-α-acetoxytamoxifen and the formation of *cis*- and *trans*-α-hydroxytamoxifen in Krebs–Henseleit buffer only, female rat liver slices and female rat liver subcellular fractions at pH 7.4 and 37°C over 300 min. Mean ± S.E.M. ($n = 4$), mean of four separate animals.

disappearance of the parent compound in Krebs–Henseleit buffer (pH 7.4), female rat liver slices, homogenate and subcellular fractions including microsomes and mitochondria at pH 7.4 over 300 min and at 37°C (Fig. 1(a)–(d)). By applying the graphic method, plots of zero-, first-, second- and third-order degradation rates were drawn (Fig. 2(a)–(d)). From the graphs, there appeared no absolutely best order for all the experiments. First- and second-order (Fig. 2(b) and (c), respec-



(c)



(d)

Fig. 1. (*Continued*)

tively) appeared better than zero- or third-order (Fig. 2(a) and (d), respectively). By modelling, differential equations were used to predict concentration of *trans*-α-acetoxytamoxifen as a function of time. This was based on the mean concentrations. The differential equations used were simple:

$$\text{Zero-order} \quad dc/dt = -k \tag{1}$$

$$\text{First-order} \quad dc/dt = -k \times c \tag{2}$$

$$\text{Second-order} \quad dc/dt = -k \times c \times c \tag{3}$$

$$\text{Third-order} \quad dc/dt = -k \times c \times c \times c \tag{4}$$

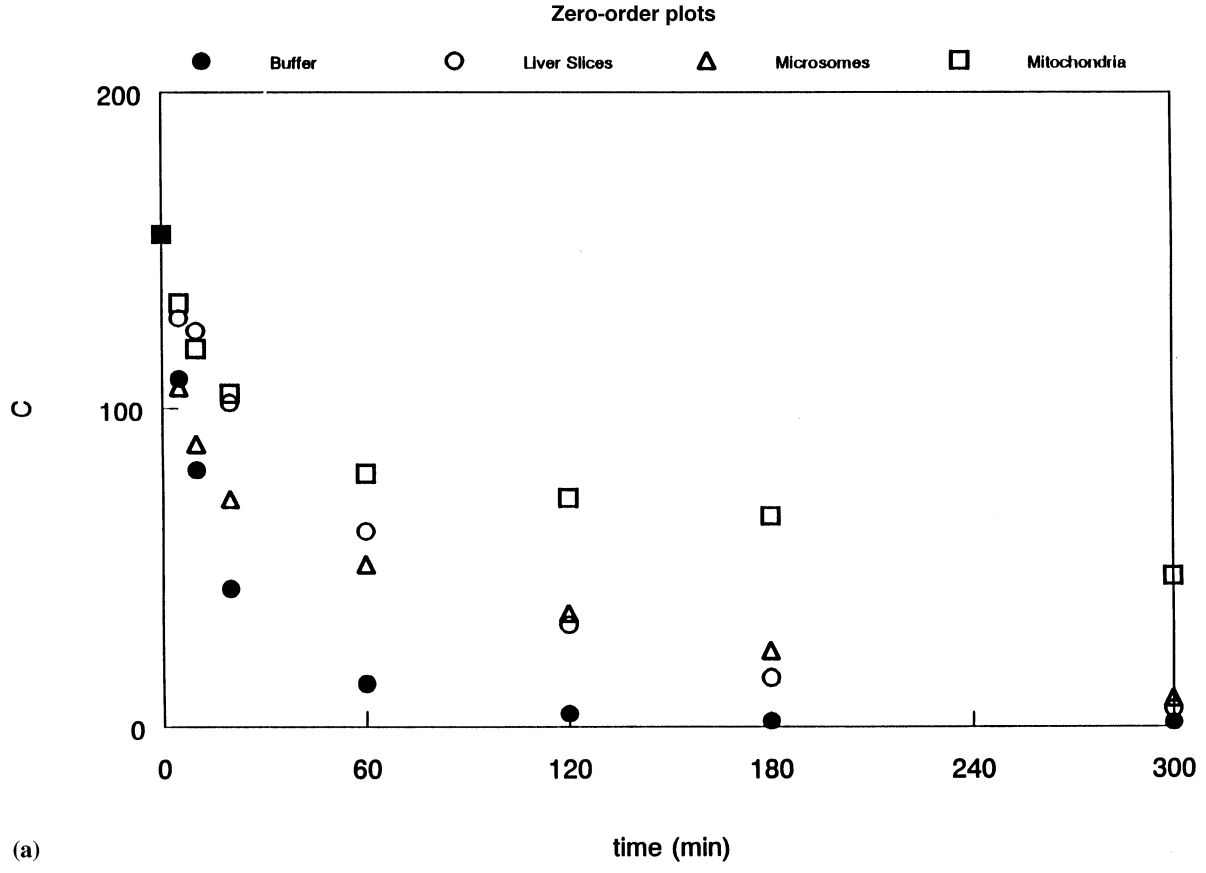


Fig. 2. (a)–(d). Zero-, first-, second- and third-order plots for the degradation of *trans*-α-acetoxytamoxifen in the various media at pH 7.4 and 37°C over 300 min.

None of the models were perfect for the data but first- and second-order appeared better than zero- or third-order. The rate constants (k) and correlation coefficients for c versus time are shown in Table 2. To achieve a better distribution of residuals, a weight of $1/y$ was used in the modelling. Although some r values are high, there still is an uneven distribution of residuals. Thus, the order of the process is not clear. Since the higher rates shown for buffer and liver slices (Table 2) examine the process over a greater concentration range, the first-order model seems most appropriate in this instance. The half-lives are calculated differently for each reaction order as follows (C_0 is the initial concentration, 155 μM):

$$\text{Zero-order} \quad t_{1/2} = C_0/(2k) \tag{5}$$

$$\text{First-order} \quad t_{1/2} = 0.693/k \tag{6}$$

$$\text{Second-order} \quad t_{1/2} = 1/(C_0 k) \tag{7}$$

$$\text{Third-order} \quad t_{1/2} = 3/(2C_0 \times C_0 \times k) \tag{8}$$

The predicted half-lives ($t_{1/2}$) are calculated in Table 2. It is not easy to identify the order of degradation with liver slices and microsomes because the order is difficult to validate and may be rate-limited by several factors such as enzyme, co-factor and tissue degradation. One would not necessarily expect the rate order to be the same with the different cellular matrices, this is due to the presence of different enzymes in each matrix. The K_m and V_{max} values are different for each

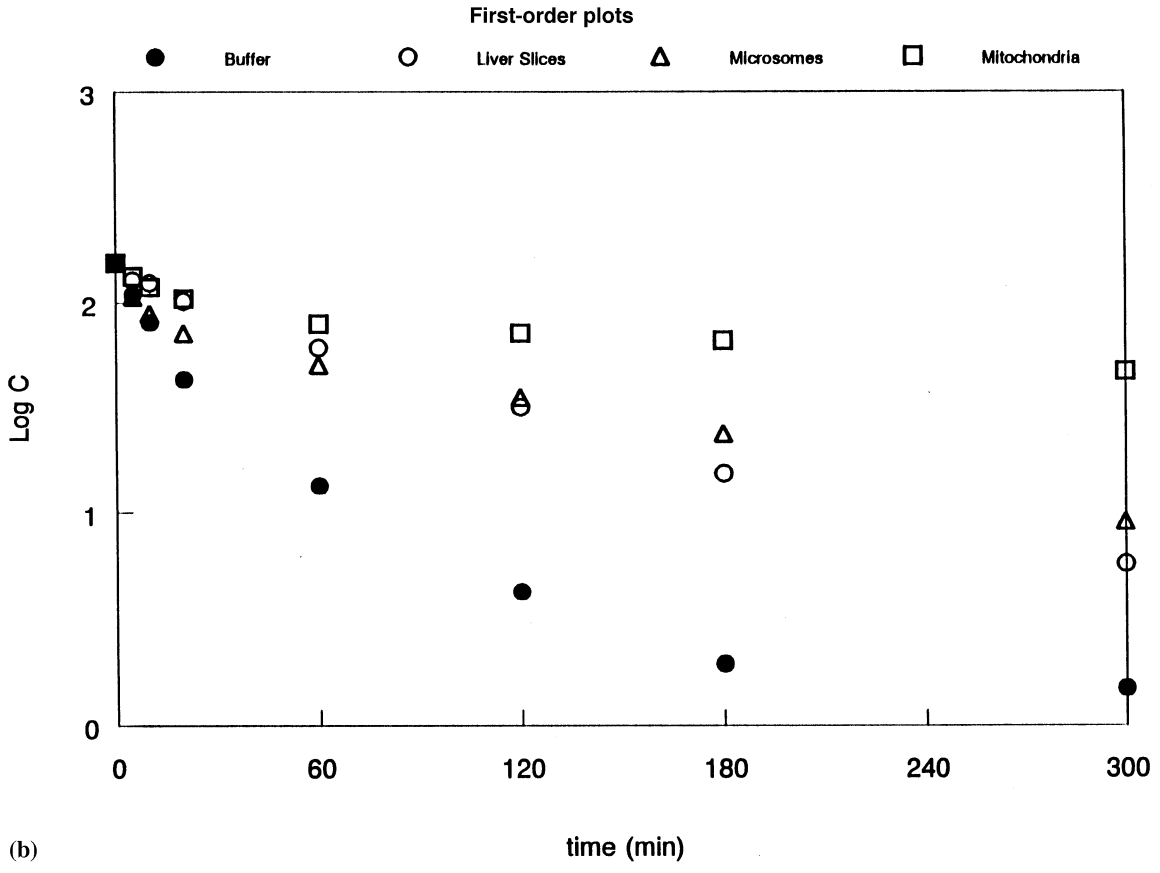


Fig. 2. (*Continued*)

enzyme, and tissue degradation occurs at a different rate in each case. All these variable factors result in each cellular matrix obeying different rate-order kinetics. In some cases, more than one rate-order may be obeyed, one may obtain complicated mixed-order kinetics. In contrast, in the presence of buffer only, first- and second-order kinetics is definitely obeyed, this is due to the absence of biological tissue which complicates reaction kinetics.

The principal degradation products for *trans*-α-acetoxytamoxifen were *trans*-α-hydroxytamoxifen and, to a lesser extent, *cis*-α-hydroxytamoxifen; there was also the appearance of another peak which could not be identified. Therefore, the formation of identical adducts with DNA is consistent because α-acetoxytamoxifen breaks down to form α-hydroxytamoxifen in vitro (Osborne et al., 1996). The identification of the principal degradation products was confirmed by running an authentic standard of each compound as synthesised in this laboratory. Another compound appeared which had a longer retention time than the parent compound and this was probably consistent with the cis isomer being formed from the trans isomer via geometric isomerisation during hydrolysis (Osborne et al., 1996). However, this could not be confirmed since an authentic standard was not available. All peaks were very pure (peak purity index of 999) when analysed using a Gynkotek Variable Wavelength Diode Array Detector.

trans-α-Acetoxytamoxifen is unstable in aqueous solution and has a low $t_{1/2}$ value in Krebs–Henseleit buffer at pH 7.4 (Table 2). However, in the presence of biological systems, it displayed much higher $t_{1/2}$ values (Table 2); this is

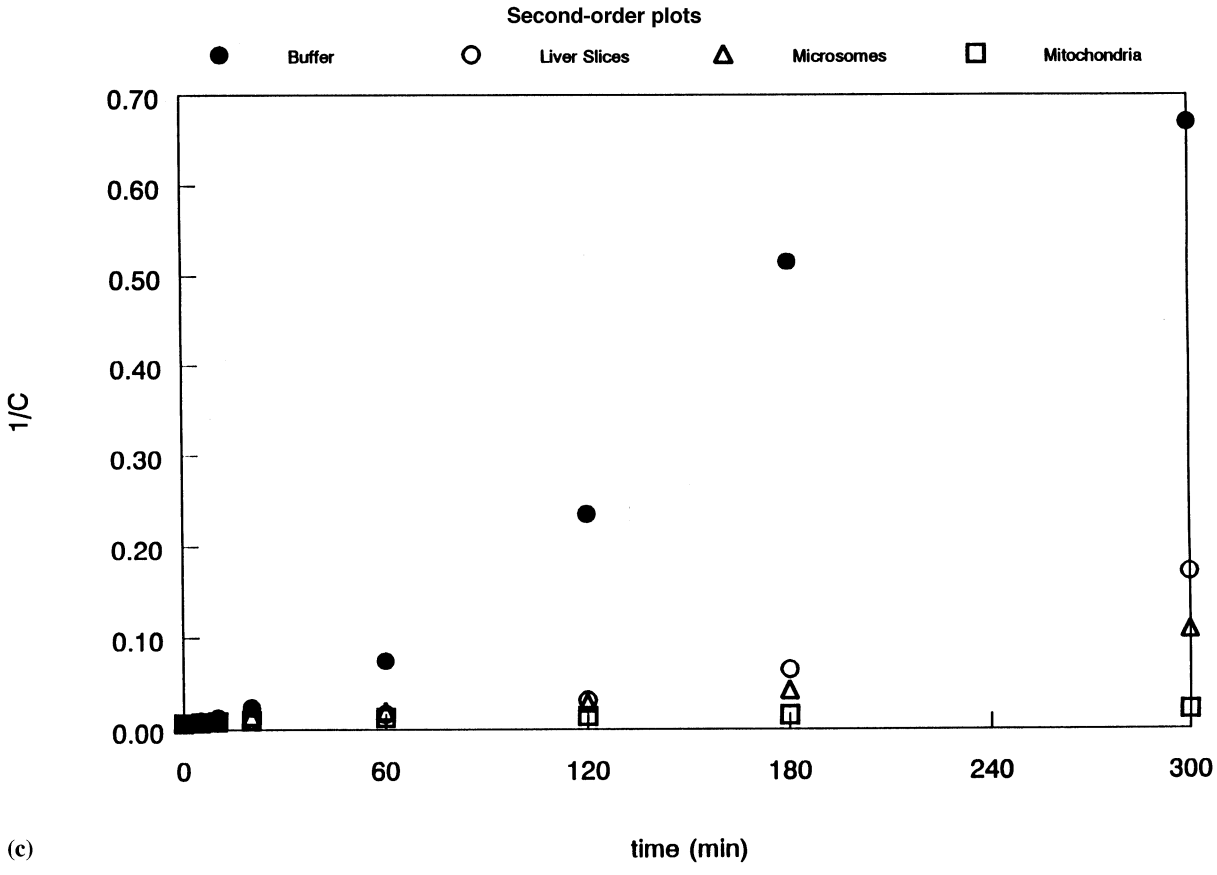


Fig. 2. (*Continued*)

probably because it partitions into the hydrophobic component of the biological tissue and hence degrades at a slower rate. The percent of *trans*-α-acetoxytamoxifen remaining after 300 min is shown in Table 1.

Fig. 1(a)–(d) shows that the concentration (μM) of *trans*-α-hydroxytamoxifen produced is much greater than that of the cis isomer. This phenomenon is true in the presence of all biological media, due to the hydrolysis of the acetoxy group (Sykes, 1965).

It has been reported that when explants of human endometrium were incubated with tamoxifen, there was no evidence of tamoxifen–DNA adduct formation (Carmichael et al., 1996). However, when they were incubated with α-hydroxytamoxifen, there were adducts formed (Phillips et al., 1994a). According to the results of our work, we found that *trans*-α-acetoxytamoxifen breaks down to produce *trans*-α-hydroxytamoxifen, therefore one would expect adducts to be produced with DNA if *trans*-α-acetoxytamoxifen were incubated with human endometrium explants.

Strong evidence implicates α-hydroxylation as a major pathway leading to DNA damage. α-Hydroxylation produces 25–49 times as many DNA adducts in rat liver cells as tamoxifen does, and these adducts are identical to those from tamoxifen (Phillips et al., 1994b). [Ethyl-D_5] tamoxifen, in which metabolism at the α-position is suppressed (Jarman et al., 1995), gives lower levels of DNA adducts in rat liver in vivo and induces fewer micronuclei in a human cell line (Phillips et al., 1994a). Toremifene, α-chlorotamoxifen, induces fewer tumors in rat liver than tamoxifen

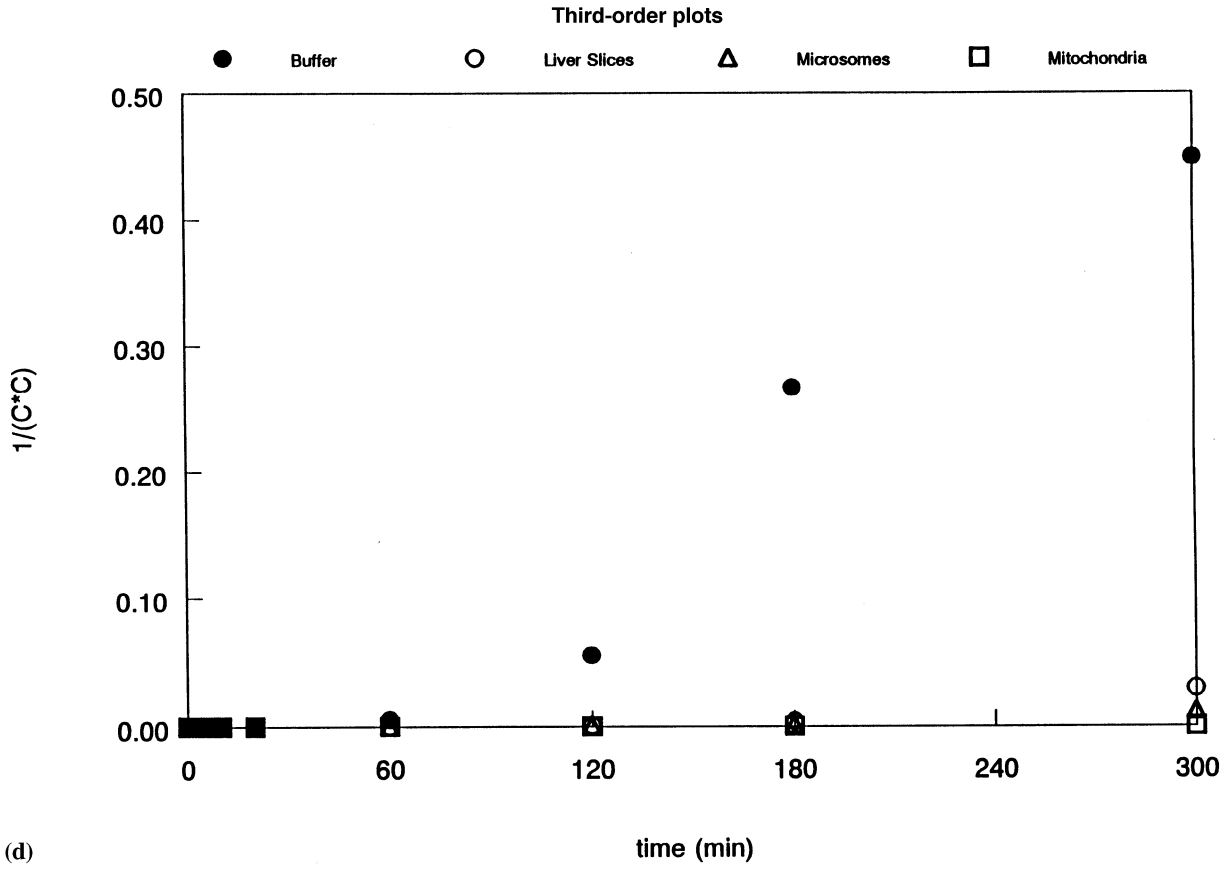


Fig. 2. (*Continued*)

(Hard et al., 1993), and its binding to rat liver DNA was barely detectable (White et al., 1992); this implies again the involvement of the ethyl group in metabolic activation. It is unlikely, however, that α-hydroxytamoxifen is ultimately responsible for the genotoxic effects of tamoxifen; although it reacts with DNA, the extent of this reaction is low at neutral pH (Styles et al., 1994).

α-Hydroxytamoxifen undergoes conjugation within the cell to the sulphate, phosphate, glucuronide, or other ester; this then forms a highly reactive carbocation, which attacks DNA (Osborne et al., 1996). Therefore, *trans*-α-acetoxytamoxifen was prepared as a useful model for such an intermediate. It has been shown that the cis isomer of hydroxytamoxifen does not form any adducts and shows much less activity than the trans, hence it is of much less importance (Osborne et al., 1996).

Table 1
Percentage of *trans*-α-acetoxytamoxifen remaining after 300 min in various media at pH 7.4 and 37°C

Media (pH 7.4)	*trans*-α-Acetoxytamoxifen (% remaining after 300 min)
Buffer only	1.00
Liver slices[a]	7.00
Homogenate[b]	32.00
Microsomes[c]	13.00
Mitochondria[d]	40.00

[a] Four liver slices/3 ml incubation (four liver slices ≡ 0.5 ml tissue).
[b] 0.5 ml tissue/3 ml incubation.
[c] 12 mg protein/ml incubation (0.5 ml used in inc.).
[d] 8 mg protein/ml incubation (0.5 ml used in inc.).

Table 2
Physico-chemical parameters of *trans*-α-acetoxytamoxifen for the different reaction orders in the various media at pH 7.4 and 37°C[a]

Order	Condition	K[b]	SEM	r	$t_{1/2}$ (min)
Zero	Buffer	0.624	0.092	0.734	124.20
	Liver slices[c]	0.548	0.014	0.904	141.42
	Microsomes[d]	0.544	0.010	0.907	142.46
	Mitochondria[e]	0.428	0.014	0.898	181.07
First	Buffer	0.0588	0.0037	0.997	11.79
	Liver slices	0.0138	0.0002	0.999	50.22
	Microsomes	0.0181	0.0053	0.980	38.29
	Mitochondria	0.0056	0.0012	0.953	123.75
Second	Buffer	0.001006	0.000091	0.998	6.41
	Liver slices	0.000245	0.000019	0.987	26.33
	Microsomes	0.000300	0.000025	0.985	21.51
	Mitochondria	0.000069	0.000001	0.978	93.50

[a] K, rate constant; SEM, standard mean error; r, correlation coefficient ($n = 4$); $it_{1/2}$ = half-life (min).
[b] K units: zero-order, μM/min; first-order, /min; second = order, /(μM min).
[c] Four liver slices/3 ml incubation (four liver slices ≡ 0.5 ml tissue).
[d] 12 mg protein/ml incubation (0.5 ml used in inc.).
[e] 8 mg protein/ml incubation (0.5 ml used in inc.).

In conclusion, our work examines the stability of *trans*-α-acetoxytamoxifen in Krebs–Henseleit buffer (pH 7.4) and in different biological media at pH 7.4 and at 37°C. Our studies show that its degradation best obeys first- or second-order rate kinetics, even though this is difficult to validate due to rate limiting factors such as enzyme, co-factor or tissue degradation. The production of *trans*-α-hydroxytamoxifen is enhanced by the presence of enzymes in the biological tissues, particularly the mitochondria and microsomes, but the concentration (μM) of the cis isomer remains relatively constant in buffer only (pH 7.4) and in biological tissue. The inability of the cis isomer to increase in concentration (μM) in the presence of biological enzymes is another indication of its low activity compared with the trans isomer (Osborne et al., 1996). The results obtained from these studies would also have a lot of relevance to in vivo data. This would be particularly evident in the case of the mitochondria where *trans*-α-acetoxytamoxifen degrades slowly, hence reaching its target organ more slowly, possibly with lower incidence of side-effects.

Acknowledgements

The authors gratefully acknowledge financial support from the Biotechnology and Biological Sciences Research Council (BBSRC) and Yorkshire Cancer Research Campaign (YCRC) for this research.

References

Carmichael, P.L., Ugwumadu, A.H.N., Neven. P., Hewer, A., Poon, G.K., Phillips, D.H., 1996. Lack of genotoxicity of tamoxifen in human endometrium. Cancer Res. 56.

Critchley, J.P., Rance, D.J., Beedham, C., 1992. Subcellular localisation of guinea pig hepatic molybdenum hydroxylases. Biochem. Biophys. Res. Commun. 185, 54–59.

Hard, G.C., Iatropoulos, M.J., Jordan, K., Radi, L., Kaltenberg, O.P., Imondi, A.R., Williams, G.M., 1993. Major differences in the hepato-carcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD (BR) rats. Cancer Res. 53, 4534–4541.

Jarman, M., Poon, G.K., Rowlands, M.G., Grimshaw, R.M., Horton, M.N., Potter, G.A., McCague, R., 1995. The deuterium isotope effect for the α-hydroxylation of tamoxifen by rat liver microsomes accounts for the reduced

genotoxicity of [D_5-ethyl] tamoxifen. Carcinogenesis (Lond) 16, 683–688.

Manns, J.E., Hanks, S., Brown, J.E., 1993. Methods for liquid chromatographic analysis of tamoxifen tamoxifen metabolites and their geometric isomers in biological samples. Anal. Proc. 30, 161–163.

Osborne, M.R., Hewer, A., Hardcastle, I.R., Carmichael, P.L., Phillips, D.H., 1996. Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. Cancer Res. 57, 66–71.

Pathak, D.N., Bodell, W.J., 1994. DNA adduct formation by tamoxifen with rat and human liver microsomal activation systems. Carcinogenesis (London) 15, 529–532.

Pathak, D.N., Pongracz, K., Bodell, W.J., 1995. Microsomal and peroxidase activation of 4-hydroxytamoxifen to form DNA adducts: Comparison with DNA adducts formed in Sprague–Dawley rats treated with tamoxifen. Carcinogenesis (London) 16, 11–15.

Phillips, D.H., Potter, G.A., Horton, M.N., Hewer, A., Crofton-Sleigh, C., Jarman, M., Venitt, S., 1994a. Reduced genotoxicity of [D_5-ethyl]-tamoxifen implicates α-hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. Carcinogenesis 15, 1487–1492.

Phillips, D.H., Carmichael, P.L., Hewer, A., Cole, K.J., Poon, G.K., 1994b. Hydroxytamoxifen a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. Cancer Res. 54, 5518–5522.

Styles, J.A., Davies, A., Lim, C.K., de Matteis, F., Stanley, L.A., White, I.N.H., Yuan, Z.-X., Smith, L.L., 1994. Genotoxicity of tamoxifen tamoxifen epoxide and toremifene in human lymphoblastoid cells containing human cytochrome P450s. Carcinogenesis 15, 59.

Sykes, P., 1965. Nucleophilic substitution at a saturated carbon atom. A Guidebook to Mechanism in Organic Chemistry. Longman Group, London, pp. 57–75.

White, I.N.H., de Matteis, F., Davies, A., Smith, L.L., Crofton-Sleigh, C., Venitt, S., Hewer, A., Phillips, D.H., 1992. Genotoxic potential of tamoxifen and analogues in female Fischer F344/n rats DBA/2 and CS7 B1/6 mice and in human MCL-5 cells. Carcinogenesis (London) 13, 2197–2203.