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METABOLISM OF TAMOXIFEN BY ISOLATED RAT HEPATOCYTES

IDENTIFICATION OF 1-[4-(2-HYDROXYETHOXY)PHENYL]-1-(4-HYDROXYPHENYL)-2-PHENYL-1-BUTENE AND THE DEPENDENCE OF *N*-OXIDATION ON OXYGEN AVAILABILITY

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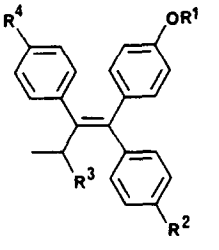
Abstract—Metabolism of tamoxifen by rat hepatocytes and hydrolysis of the resulting polar metabolites corresponding to conjugates with β-glucuronidase gave a major component which was identified as 1-[4-(2-hydroxyethoxy)phenyl]-1-(4-hydroxyphenyl)-2-phenyl-1-butene by comparison of mass spectral properties with those of synthetic material. This compound, which was not observed as a phase I metabolite, is believed to have been found previously in rat bile and in human faeces (metabolite F) but its structure had been incorrectly assigned. Its binding affinity for the estrogen receptor was greater than that of tamoxifen but less than that of 4-hydroxytamoxifen, and it possessed a corresponding degree of antitumour activity against the MCF-7 breast cancer cell line. By carrying out the hepatocyte incubation separately under oxygen and air, it has been shown that the *N*-oxidation of tamoxifen is favoured by a high concentration of oxygen during *in vitro* metabolism but that the rate of 4-hydroxylation is not dependent on oxygen availability.

Metabolism may play an important role in modulating the biological activity of tamoxifen {*Z*-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butene} (**1**), a synthetic antiestrogen currently in clinical use for the treatment of estrogen-dependent breast cancer [1]. Serum metabolites which have been detected are 4-hydroxytamoxifen (**2**) (metabolite B), *N*-desmethyltamoxifen (**3**) (metabolite X), *N*,*N*-didesmethyltamoxifen (**4**) (metabolite Z) and the primary alcohol (**5**) (metabolite Y) [2–6]. In addition, hepatic microsomal metabolism studies have revealed tamoxifen *N*-oxide (**6**), 4′-hydroxytamoxifen (**7**), and α-hydroxytamoxifen *N*-oxide (**8**) as further metabolites [7–9]. Of these metabolites, 4-hydroxytamoxifen (**2**) has attracted considerable interest since it binds to the estrogen receptor much more strongly than tamoxifen and has high antiestrogenic potency *in vitro* [10, 11]. However, 4-hydroxytamoxifen has a lower antitumour potency than tamoxifen *in vivo* since only low plasma levels are attained, attributable to rapid metabolite clearance [12, 13].

In order to develop the use of hepatocytes as a model for metabolism *in vivo* we have studied the metabolism of tamoxifen. Incubations were carried out separately under air and oxygen because of our suspicion that *N*-oxidation could be an artefact of high oxygen availability during *in vitro* experiments. In order to gain an understanding of the pathways of the metabolic clearance of tamoxifen, particular attention was paid to the fraction of polar metabolites corresponding to conjugates. After hydrolysis of this conjugate fraction using β-glucuronidase, a major component of the hydrolysate had properties corresponding to a metabolite observed previously in rat bile [14] and in human faeces [2] (Metabolite F). This metabolite had been assigned the structure (**9**) [14]. We report that this previous assignment was



	R¹	R²	R³	R⁴
(1)	—$CH_2CH_2NMe_2$	—H	—H	—H
(2)	—$CH_2CH_2NMe_2$	—OH	—H	—H
(3)	—CH_2CH_2NHMe	—H	—H	—H
(4)	—$CH_2CH_2NH_2$	—H	—H	—H
(5)	—CH_2CH_2OH	—H	—H	—H
(6)	—$CH_2CH_2N(\rightarrow O)Me_2$	—H	—H	—H
(7)	—$CH_2CH_2NMe_2$	—H	—H	—OH
(8)	—$CH_2CH_2N(\rightarrow O)Me_2$	—H	—OH	—H
(9)	—CH_2CH_3	—OH	—OH	—H
(10)	—CH_2CH_2OH	—OH	—H	—H
(11)	—CH_2CO_2Et	—H	—H	—H
(12)	—$CH{=}CH_2$	—OH	—H	—H
(13)	—$CH_2CH_2N(\rightarrow O)Me_2$	—OH	—H	—H
(14)	—CH_2CH_2NHMe	—OH	—H	—H
(15)	—CH_2CH_2Cl	—OH	—H	—H

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incorrect; the metabolite is the phenolic primary alcohol (**10**).

By analogy with 4-hydroxytamoxifen the metabolite (**10**), by virtue of its 4-hydroxy function, would be expected to bind strongly to estrogen receptors. We have assessed its binding affinity to estrogen receptors both in calf uterine cytosol and in MCF-7 whole cells. In addition the compound was assayed for growth inhibitory activity against the MCF-7 cell line in culture.

MATERIALS AND METHODS

Materials. *Z*-[ring-^{14}C]tamoxifen citrate (specific activity 21 mCi/mmol and 97% chemically pure) was purchased from Amersham International, U.K. Unlabelled tamoxifen and β-glucuronidase (*Escherichia coli* K12; 200 U/ml) were purchased from Sigma Chemical Co., U.K., and Boehringer (Mannheim) respectively. Potential metabolites (**2**), (**3**), (**6**) and (**18**) of tamoxifen and the synthetic intermediates (**11**) and (**12**) were synthesised by published procedures [9, 15, 16]. Small samples of metabolites (**13**) and (**14**) (*Z*-*E*-mixtures) used as standards for t.l.c. and mass spectrometry were prepared by oxidation of 4-hydroxytamoxifen with hydrogen peroxide to give (**13**) and by treatment of the chloroethoxy compound (**15**) with methylamine to give (**14**) [16].

In vitro *metabolism.* Male adult Wistar rats fed *ad libitum* and given phenobarbital in their drinking water (0.5 g/l) for 10–14 days were used to prepare hepatocytes by a 2-step perfusion method as described previously [17]. The freshly isolated cells (5 ml of 6–8 × 10^6/ml and 90% viable) were incubated under an atmosphere either of oxygen or air at 37° with the mixture of [^{14}C] tamoxifen citrate (0.8 × 10^6 dpm) and unlabelled tamoxifen citrate (100 μmol/l) in 25 ml conical flasks in a phosphate buffered saline medium at pH 7.5 [18] containing Hepes (20 mmol/l) and glucose (5 mmol/l). After 60 min, the flasks were cooled in ice, the hepatocytes disintegrated by ultrasonication and the protein precipitated with 3 vol. of acetone. Acetone was removed from the supernatant under vacuum and after adjustment to pH 9.5, phase I non-polar metabolites and non-utilised tamoxifen were extraced with ethyl acetate. Conjugates remaining in the aqueous fraction were concentrated on Sep-pak cartridges and eluted with methanol. Aliquots of this latter fraction were hydrolysed by incubation with β-glucuronidase (70 μl of solution as purchased) in 0.1 M phosphate buffer at pH 6.5 for 44 hr. The metabolite fractions were subjected to normal phase chromatography on silica gel 60F-254 (Merck) plates in chloroform:methanol:25% aqueous ammonia [75:25:0.5]. A Berthold automatic TLC analyser was used to locate the radioactive peaks and to determine the proportion of the label in individual bands. Isolated metabolites were subjected to electron impact (E.I.) mass spectrometry.

Binding studies: cytosol assay [19]. Calf uterine cytosol was incubated at 18° for 30 min with 5 × 10^{-9} M [^{3}H]-estradiol in the absence and presence of increasing amounts (10^{-9} to 10^{-5} M) of 4-hydroxytamoxifen (**2**), the primary alcohol (**10**) or unlabelled estradiol (control). Unbound compounds were then removed using dextran-coated charcoal and the amounts of estrogen receptor bound [^{3}H]-estradiol were measured. The relative concentration of estradiol and test compound required to achieve 50% inhibition of [^{3}H]-estradiol binding is the relative binding affinity (RBA); i.e. RBA = ([I_{50}] estradiol/[I_{50}] test compound) × 100.

Whole cell assay [20, 21]. MCF-7 cells were incubated at 37° for 50 min with 10^{-9} M [^{3}H]-estradiol in the absence or presence of increasing amounts (10^{-10} to 10^{-5} M) of 4-hydroxytamoxifen (**2**), the primary alcohol (**10**) or unlabelled estradiol (control). Bound compounds were then extracted with ethanol and the amounts of estrogen receptor bound [^{3}H]-estradiol were measured. The RBA values were calculated as for the cytosol assay.

*Effect of compound (**10**) on MCF-7 cell growth* [22]. MCF-7 cells were plated in a 96-multiwell dish (Falcon; well volume 200 μl, plating density 5000 cells/ml). After 24 hr of culture, compound (**10**) was added to various wells of the dish to give final concentrations of 10^{-8}, 10^{-7} and 10^{-6} M; a part of the dish remained free of compound (control). After 2 days, the medium was replaced by a fresh medium and the culture maintained for three additional days. The monolayer was then fixed with 90% ethanol and coloured with hematoxylin. The intensity of the coloration was determined with a multiscan spectrophotometer at 540 nm (Flow Laboratories Inc.). Previous experiments have shown that values of optical density thus obtained have an approximately linear relationship with the number of cells as evaluated by cell counting, and from a calibration graph the number of cells can be estimated [22].

Instrumentation. E.I. mass spectra (70 eV) were recorded using a VG7070H spectrometer with a direct insertion probe and VG2235 data system. The proton NMR spectrum of compound (**10**) was recorded on a solution in DMSO-d_6 by courtesy of the University of London Intercollegiate Research Service.

SYNTHESIS

The primary alcohol (metabolite Y) (**5**) was prepared by reduction of the ester (**11**) [15] with lithium aluminium hydride, and its 4-hydroxy analogue (**10**) by hydroboration of the *O*-vinyl compound (**12**) [16] followed by hydrogen peroxide oxidation of the resulting alkylborane. In each case the starting material and product was of pure *Z*-configuration as determined by proton NMR spectrometry.

Z-1-[4-(2-*Hydroxyethoxy*)*phenyl*]-1,2-*diphenyl*-1-*butene* (**5**)

A solution of methyl *Z*-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]ethanoate (**11**; 850 mg, 2.2 mmol) [13] in dry ether (5 ml) was added to a stirred suspension of lithium aluminium hydride (200 mg, 5.3 mmol) in ether (10 ml) at 5°. After 10 min, excess reagent was destroyed by the addition of 2% water in tetrahydrofuran (2 ml) and the mixture partitioned between ether (100 ml) and dilute hydrochloric acid (1 M; 100 ml). The ether solution was washed with

water (100 ml), dried with anhydrous sodium sulphate and concentrated. The residue was recrystallised from light petroleum (b.p. 80–100°) to give the title compound (**5**), yield 573 mg (76%), m.p 105–106° lit. m.p. 110.5–111.5° [23], mass spectrum: *m/z* 344 (M^+, 100%), 329 (M^+—CH_3, 6%), 299 (M^+—CH_2CH_2OH, 4%), 284 (M^+—CH_3—CH_2CH_2OH, 8%), 252 (6%), 227 (8%), 207 (14%), 191 (13%), 165 (12%), 91 (21%) and 45 (CH_2CH_2OH, 22%).

Z- 1- [4- (2- *Hydroxyethoxy)phenyl*]- 1- (4- *hydroxyphenyl*)-2-*phenyl*-1-*butene* (**10**)

Z-1-(4-Ethenoxyphenyl)-1-(4-hydroxyphenyl)-2-phenyl-1-butene (**12**; 1.49 g, 4.35 mmol) [16] was dissolved in a solution of borane in tetrahydrofuran (1 M; 10 ml, 10 mmol) at 20°. After 15 min, excess reagent was destroyed by the addition of water (1 ml). Aqueous hydrogen peroxide (100 vol; 10 ml) and sodium hydroxide (3 M, 10 ml) were then added, the mixture stirred for 2 hr, then poured into saturated aqueous sodium hydrogen carbonate (100 ml), and the product extracted with either (100 ml). The ether extract was concentrated and the crude product recrystallised from chloroform-light petroleum 1:1 (b.p. 80–100°) to give the *title compound* (**10**) (926 mg, 59%), m.p. 183–185°, NMR: δ 0.85 (t, *J* 7.3 Hz, CH_3CH_2), 2.41 (q, *J* 7.3 Hz, 2H, CH_3CH_2), 3.66 (m, 2H, OCH_2CH_2OH), 3.83 (t, *J* 4.9 Hz, 2H, OCH_2CH_2OH), 4.79 (t, *J* 5.4 Hz, 1H, OCH_2CH_2OH), 6.57 (d, *J* 8.7 Hz, 2H, Ar*H ortho* to OCH_2), 6.70 (d, *J* 8.7 Hz, 2H, Ar*H meta* to OCH_2), 6.75 (d, *J* 8.5 Hz, 2H, Ar*H ortho* to OH), 6.98 (d, *J* 8.5 Hz, 2H, Ar*H meta* to OH), 7.04–7.22 (m, 5H, Ph) and 9.41 (s, 1H, ArO*H*); mass spectrum: *m/z* 360 (M^+, 100%), 345 (M^+—CH_3, 13%), 315 (M^+—CH_2CH_2OH, 3%), 301 (6%) 300 (M^+—CH_3–CH_2CH_2OH, 8%), 299 (6%), 243 (8%), 223 (8%), 207 (20%), 165 (8%), 107 (20%), 91 (12%) and 45 (CH_2CH_2OH, 18%); Anal. Calc. for $C_{24}H_{24}O_2$: C, 80.0; H, 6.7. Found: C, 79.65; H, 6.7%.

RESULTS

Identification of metabolites

The extent of metabolism of tamoxifen using our hepatocyte system was 76% when the incubation was carried out under oxygen and 48% when carried out under air. Phase I metabolites identified by comparison of their chromatographic mobilites and mass spectra with those of samples synthesised previously (see Table 1) were 4-hydroxytamoxifen (**2**), desmethyltamoxifen (**3**), tamoxifen *N*-oxide (**6**), α-hydrotamoxifen *N*-oxide (**8**) and 4-hydroxytamoxifen *N*-oxide (**13**). Percentages of the metabolites identified were obtained from the automatic TLC analyser and are given in Table 2. *N*-Oxidation was substantially inhibited by a reduction in oxygen availability but the rate of 4-hydroxylation was unaffected. The rate of demethylation appeared to be slightly reduced when oxygen was deficient. In control experiments using only buffer, tamoxifen underwent *N*-oxidation to an extent of 3% whether under air or oxygen. A proportion of the radioactivity (17% after incubation under oxygen, 8% under air) was not extracted from aqueous solution by ethyl acetate and therefore corresponded to conjugates. After hydrolysis of this conjugate fraction with β-glucuronidase, 45% (oxygen experiment) or 61% (air experiment) of this fraction was now extractable into ethyl acetate. From these extracts we identified 4-hydroxytamoxifen, 4-hydroxytamoxifen *N*-oxide, 4-hydroxydesmethyltamoxifen (**14**), and metabolites giving base peaks at highest mass corresponding to molecular ions in their mass spectra at *m/z* 344 and at *m/z* 360. The availability of oxygen had no significant influence on the relative proportions of conjugated metabolites. The percentages of metabolites from the hydrolysate calculated as a proportion of the original amount of tamoxifen are given in Table 3.

The metabolite of molecular weight 344 gives a mass spectral fragmentation pattern that is consistent with that reported for the primary alcohol (**5**) [6]. This identification was confirmed by comparison with a synthesised sample. The metabolite of molecular weight 360 gave a mass spectrum with a similar overall appearance to that of (**5**) but with peaks 16 mass units higher, consistent with a hydroxylated derivative of compound (**5**). Our suspicion that this metabolite was the dihydroxy compound (**10**) was confirmed by comparison of its mass spectrum with that of synthetic material (Fig. 1). The small peak seen at *m/z* 316 in the sample of the metabolite is probably due to contamination by the *bis*-phenol 1,1-di(4-hydroxyphenyl)-2-phenyl-1-butene, a known metabolite of 4-hydroxytamoxifen in human breast tissue [24].

We found that the metabolites (**5**) and (**10**) could be separated cleanly on normal phase t.l.c. from those bearing a basic side chain by using a solvent system of dichloromethane-ether (70:30). Basic metabolites then remained on the base line. Using this chromatography system we reinvestigated the composition of phase I metabolites and could detect a trace of metabolite (**5**) but could not detect metabolite (**10**).

*Biological activity of metabolite (**10**)* in vitro

The ability of the synthesised metabolite (**10**) to inhibit the binding of [^{3}H]-estradiol to estrogen receptors was compared with that of 4-hydroxytamoxifen (**2**). In the cytosol assay, the metabolite (RBA = 6) binds much more weakly than 4-hydroxytamoxifen (RBA = 100). Nevertheless, its binding is significantly stronger than that of tamoxifen (RBA = 1). The whole cell assay gives reduced binding affinity values for antiestrogens of the triarylethylene category when compared with cytosol binding data, a feature which according to previous studies [21] might be indicative of a low estrogenicity of these compounds. Using this assay method, the metabolite (RBA = 1.7) still bound with an affinity between those of tamoxifen (RBA = 0.06) and 4-hydroxytamoxifen (RBA = 2.9). However, the reduction in RBA observed in the whole cell assay is less for metabolite (**10**) than it is for 4-hydroxytamoxifen. This relatively high whole cell RBA of the metabolite could be explained either by its having a relatively high estrogenicity or by its having an increased rate of entrance into cells compared with

Table 1. Chromatographic mobilities and principal mass spectral fragments for tamoxifen and metabolites observed

Compound	R_f values* Solvent I	Solvent II	Mass spectral fragments
(1)	0.82	0.03	371 (M^+, 4%), 72(25), 58(100)
(2)	0.71	0.01	387 (M^+, 4%), 72(22), 58(100)
(3)	0.61	—	357 (M^+, 10%), 300(22), 58(29), 44(100)
(5)	0.84	0.45	344 (M^+, 100%), 329(6), 299(4), 284(8)†
(6)	0.33	—	371 (M^+–0, 3%), 326(100), 58(84)
(8)	0.29	—	387 (M^+–0, 2%), 342(1.5), 209(28), 72(17), 58(100)
(10)	0.79	0.25	360 (M^+, 100%), 345(13), 300(8)‡
(13)	0.23	—	341 (M^+–Me_2NOH, 60%), 72(28), 58(100)
(14)	0.39	—	373 (M^+, 6%), 316 (9%), 58(6), 44(100)

* Using silica F-254 plates. Solvent systems used were: I $CHCl_3$:MeOH:25% aq. ammonia 75:25:0.5; II CH_2Cl_2:$(C_2H_5)_2O$ 70:30.
† Complete mass spectral data given in the experimental.
‡ See Fig. 1.

Table 2. Percentages of phase I metabolites obtained (as proportion of radiolabelled tamoxifen)

Compound		Incubation under oxygen %	Incubation under air %
Recovered tamoxifen	(1)	24	52
4-Hydroxytamoxifen	(2)	6	8
N-Desmethyltamoxifen	(3)	15	12
Tamoxifen *N*-oxide	(6)	15	5
α-Hydroxytamoxifen *N*-oxide	(8)	2	0.6
4-Hydroxytamoxifen *N*-oxide	(13)	2	0.9
Unidentified metabolites		10	8

Table 3. Percentages of metabolites obtained by hydrolysis of conjugates with β-glucuronidase (as proportion of original amount of radiolabelled tamoxifen)

Compound		Incubation under oxygen %	Incubation under air %
4-Hydroxytamoxifen	(2)	1.5	0.9
Metabolite Y (M.W. 344)	(5)	0.3	0.4
Metabolite F (M.W. 360)	(10)	0.9	0.7
4-Hydroxytamoxifen *N*-oxide	(13)	0.8	0.8
4-Hydroxydesmethyltamoxifen	(14)	0.4	0.1
Unidentified metabolites		3.3	2.1

tamoxifen. However, only the former explanation is consistent with the reported *in vivo* observation that metabolite Y (**5**) has a somewhat lower anti-estrogenicity than tamoxifen as determined in a rat uterine weight test [25].

The anti-tumour potency of metabolite (**10**) was determined by measuring its inhibition of the rate of growth of MCF-7 human mammary tumour cells in monolayer culture. Under the control conditions, cell numbers increased by approximately 70-fold. The metabolite (**10**) inhibited this growth with a potency that was in accordance with the cytosol binding data and showing that it had significant anti-estrogenic activity. Thus from the data in Table 4, it can be deduced that the metabolite gives 50% growth inhibition, $I_{50} = 10^{-8}$–10^{-7} M only slightly lower than the figure for 4-hydroxytamoxifen ($I_{50} \sim 10^{-8}$ M) and better than that of tamoxifen which gives no inhibition at 10^{-8} M and only *ca.* 20% inhibition at 10^{-7} M.

This result clearly shows that replacement of the dimethylamino group by a hydroxyl group does not abolish the antitumour activity.

DISCUSSION

The metabolite of molecular weight 360 identified as 1-[4-(2-hydroxyethoxy)phenyl]-1-(4-hydroxyphenyl)-2-phenyl-1-butene (**10**) gives mass spectral data very similar to those quoted by Fromson *et al.* for a metabolite observed in rat bile and subsequently

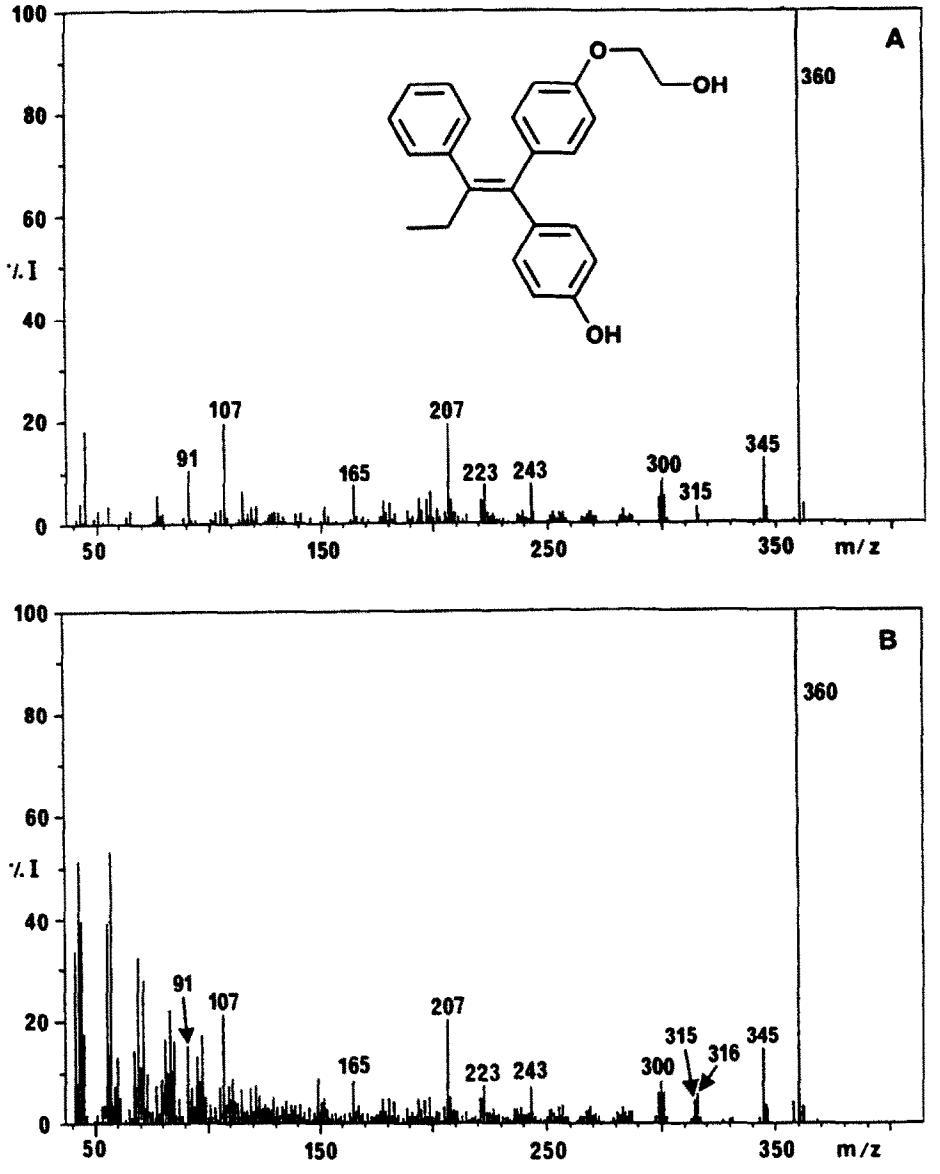

Fig. 1. Electron impact mas spectra of 1-[4-(2-hydroxyethoxy)phenyl]-1-(4-hydroxyphenyl)-2-phenyl-1-butene; A = synthesised compound, B = isolated metabolite.

in human faeces which was denoted metabolite F [14]. Thus, in addition to a base beak at m/z 360, fragments were reported at m/z 315, 301, 300 and 299, as found with our metabolite, although we observed a fragment at m/z 207 rather than at m/z 208 as recorded by Fromson *et al.* Fromson had discounted the structure (**10**) on the basis that by comparison of the spectrum of 2-phenoxyethanol, a loss of a fragment of 44 mass units was expected but was not observed. The alternative structure (**9**) was consequently suggested. This assignment was later criticised by Kemp *et al.* who observed that the metabolite (**5**) did not undergo loss of a fragment in the mass spectrum of 44 mass units, and that a compound of structure (**9**) would have been expected to give a fragment due to loss of water [6]. This latter prediction is consistent with our reported observation that a loss of 18 mass units is found in the mass spectrum of α-hydroxytamoxifen *N*-oxide [9]. Kemp *et al.* suggested the structure (**10**) for metabolite F but were unable to obtain an authentic sample of the compound. We therefore confirm that the suggestions of Kemp *et al.* were correct.

Since a significant proportion of the metabolites not extractable into ethyl acetate could be hydrolysed by β-glucuronidase, glucuronidation represents a major pathway of phase II metabolism of tamoxifen in our system. This observation is consistent with the report of Fromson *et al.* that some (45%) of the biliary metabolites of tamoxifen in the rat are hydrolysable by β-glucuronidase. Interestingly, glucuronides were formed not only from those metabolites bearing a phenolic function but also from the aliphatic primary alcohol (**5**).

Since the metabolite (**10**) was not observed prior to hydrolysis of the glucuronides, it is likely that

Table 4. Growth inhibition of the MCF-7 cell line

Compound	Concn.	Optical density* (mean ± SD)	Cells/ml (/10³)†
Metabolite (10)	Control	0.344 ± 0.008	305 (100)‡
	10^{-8} M	0.242 ± 0.034	200 (66)
	10^{-7} M	0.143 ± 0.032	113 (37)
	10^{-6} M	0.102 ± 0.029	81 (27)
Tamoxifen (1)§	Control	0.450 ± 0.037	427 (100)
	10^{-8} M	NI‖	
	10^{-7} M	0.367 ± 0.050	329 (77)
	10^{-6} M	0.246 ± 0.032	205 (48)
4-Hydroxytamoxifen (2)§	Control	0.453 ± 0.014	430 (100)
	10^{-8} M	0.218 ± 0.061	177 (41)
	10^{-7} M	0.171 ± 0.032	135 (31)
	10^{-6} M	0.067 ± 0.020	53 (12)

* Each figure corresponds to the mean of the optical density measurements obtained from four separate samples of culture.

† Estimated by reference to reported calibration data [22].

‡ Percentage of control value.

§ Data obtained previously using an identical experimental protocol. Values obtained by this method are in total agreement with those established by measuring the cellular DNA content [27].

‖ NI = No inhibition.

glucuronidation of this compound is very rapid. In contrast, 4-hydroxytamoxifen was observed both before and after the hydrolysis. Since metabolite (**10**) was a major component of the hydrolysate it would seem that this compound is conjugated more rapidly than 4-hydroxytamoxifen. It follows that a major route for excretion of tamoxifen, at least in the rat, probably involves formation of metabolite (**10**) either via 4-hydroxytamoxifen or metabolite (**5**) or both, followed by its rapid conjugation and then presumably elimination into the bile.

Comparison of the proportions of metabolites obtained in the presence of air or oxygen has shown that *N*-oxidation is strongly dependent on oxygen availability. Although a small proportion of the tamoxifen underwent non-enzymatic chemical *N*-oxidation, this does not account for the amounts of *N*-oxide formed, especially when the incubation was carried out under oxygen. Since it would be envisaged that access to oxygen will be somewhat restricted *in vivo*, *N*-oxidation may only be a significant process during *in vitro* experiments and may be considered an artefact of these experiments. Additionally, it is likely that *N*-oxidation is reversible, evidence for which is that tamoxifen *N*-oxide has been shown to undergo some de-*N*-oxygenation during hepatic microsomal metabolism [9], and it is likely that this reverse reaction will be more significant when oxygen is deficient. Demethylation appeared to be slightly reduced when using air rather than oxygen. The slight increase in 4-hydroxytamoxifen obtained is most probably a consequence of less tamoxifen having been consumed by other metabolic pathways. As was the case for metabolite Y (**5**), the 4-hydroxy metabolite (**10**) was formed both in the presence of air and oxygen, so that unlike tamoxifen *N*-oxide, we have no evidence that compound (**10**) is an *in vitro* artefact. Although the extent of tamoxifen metabolism is much reduced when carried out under air rather than oxygen, we suggest that *in vitro* metabolism experiments are better carried out under air if the results are to reflect metabolism *in vivo*.

When taking into account metabolites recovered from glucuronides, we observe using our hepatocyte system metabolites identified in human serum [i.e. desmethyltamoxifen, 4-hydroxytamoxifen and metabolite Y (**5**)]. We therefore suggest that the hepatocyte system described herein is, at least in the case of tamoxifen, an excellent model for metabolism in patients. Although our study has revealed tamoxifen being metabolised to comparable amounts of desmethyl and 4-hydroxy metabolites there is no reason to believe that the figures are inconsistent with *in vivo* data where *N*-desmethyltamoxifen maintains much the higher plasma concentration [26]. The low plasma levels of 4-hydroxytamoxifen then observed are likely to be due to its rapid conjugation rather than to a low rate of 4-hydroxylation of tamoxifen.

It has been suggested that the primary alcohol metabolite Y (**5**) would be expected to contribute to the antineoplastic activity of tamoxifen during therapy despite it having only weak activity with an RBA to ER only one twelfth that of tamoxifen [25]. The 4-hydroxylated metabolite (**10**) has a much higher potency, greater than that of tamoxifen and it would seem reasonable therefore that this metabolite could also make a significant contribution to the activity of tamoxifen. However, in view of the rapid conjugation of this metabolite, its plasma levels would be very low and its contribution is probably less significant than that of 4-hydroxytamoxifen. Nevertheless significant concentrations of metabolite (**10**) could become present within the target tissues where conjugation would not be expected.

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