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Metabolism of tamoxifen by rat liver microsomes: formation of the *N*-oxide, a new metabolite

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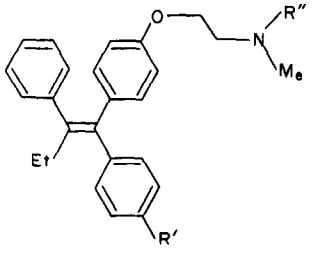
Tamoxifen [1, 2] [compound **1**, Nolvadex, ICI 46,474, *trans*-1-(*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene see Scheme] is a non-steroidal antiestrogen which is in current use for the treatment of breast cancer [3]. Fromson *et al.* [4, 5] reported that in rat, mouse, rhesus monkey and dog, tamoxifen was extensively metabolized and the major route of excretion was via the bile into the faeces. It was concluded [4] that the major pathway of metabolism involved aromatic hydroxylation and that the preponderant faecal metabolite was the glucuronide of 4-hydroxytamoxifen (compound **2**). However, in women given ^{14}C-labelled tamoxifen, it was found [5] that slow excretion of radioactivity in the faeces occurred, that unchanged drug and conjugated hydroxylated metabolites accounted for less than 30 per cent of faecal radioactivity, and that the 4-hydroxy derivative **2** was a major serum metabolite.

More recently, Adam *et al.* [6] have reported that the major serum metabolite of tamoxifen in humans is the *N*-desmethyl derivative compound **3** and not the 4-hydroxy derivative **2**.

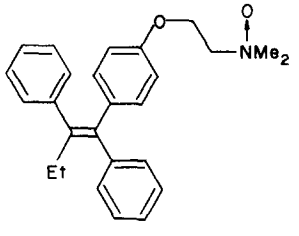
The metabolites **2** and **3** could be formed by cytochrome P-450-mediated oxidation of tamoxifen and in seeking to ascertain the balance between aromatic hydroxylation and *N*-demethylation, the metabolism of tamoxifen using initially rat liver microsomes, was investigated.

Metabolism was carried out in the conventional manner [7] using microsomes prepared from the livers of male Wistar rats pretreated with sodium phenobarbital. Tamoxifen and its metabolites were extracted with ether from the basified incubate and subjected to reverse-phase high pressure liquid chromatography (h.p.l.c.). The major metabolite (20 per cent) had the same retention time and mass spectrum as the synthetic [6] *N*-desmethyl derivative **3**. A minor metabolite (1.5 per cent) with the retention time of the 4-hydroxy derivative **2** was also present, but its structure was not confirmed. In addition to **2** and **3** a third metabolite (6 per cent) was detected and subsequently identified as tamoxifen *N*-oxide (compound **4**).

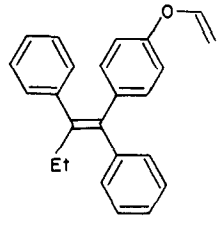
The electron impact (EI) mass spectrum of the small amount of metabolite **4** isolated contained no peak for the molecular ion (*m/z* 387) but a prominent signal at *m/z* 326 corresponding to the olefin compound **5** was present which would be formed [8] by thermal elimination of the dimethylamine oxide moiety from **4**. Otherwise, the EI mass spectrum of **4** resembled that of tamoxifen, as would be predicted from the readiness with which *N*-oxides lose an oxygen atom under EI conditions [9]. Structural investigation of the metabolite was restricted by the small amounts available, but this limitation was removed by the finding that the treatment of tamoxifen with aqueous 30% hydrogen peroxide in methanol (conditions used to convert *N,N*-dimethylcyclohexylmethylamine into its *N*-oxide [10]) gave a product with chromatographic and mass spectral properties identical to those of the new metabolite. The ^{1}H-n.m.r. spectrum (60 MHz) of the synthetic product, which indicated that it was a single substance, was similar to that [1, 2] of tamoxifen, except that the signals for the NMe_2 and $—CH_2CH_2N$ groups were shifted downfield, consistent with deshielding consequent on *N*-oxidation.



1. R′ = H, R″ = Me (Tamoxifen)
2. R′ = OH, R″ = Me (4-Hydroxytamoxifen)
3. R′ = R″ = H (*N*-Desmethyltamoxifen)



4. (Tamoxifen *N*-oxide)



5.

Moreover, the only signal in the high mass range (i.e. $m/z>400$) of the field desorption (FD) spectrum of the synthetic product occurred at m/z 775, which is the cluster ion $[2M + H]^+$ for tamoxifen *N*-oxide (**4**). Such cluster ions are typical [11] of FD spectra and when formed are diagnostic of molecular weight. Thus, the synthetic product is tamoxifen *N*-oxide (**4**) and it follows that the new metabolite has the same structure.

The finding that *N*-demethylation greatly preponderates over aromatic hydroxylation during metabolism of tamoxifen with rat liver microsomes does not necessarily mean that it occurs *in vivo* and in this context [12] the metabolism of tamoxifen using freshly isolated hepatocytes is being investigated. It is possible that the *N*-oxide **4** is an intermediate [13] in the *N*-dealkylation leading to **3**. It is noteworthy that **4** is immobile in the t.l.c. system [Kieselgel GF_{254} (Merck), benzene-triethylamine, 9:1] used by Fromson *et al.* [4, 5] for analysis of the ^{14}C-labelled components present in human serum following the administration of a single dose of [^{14}C]-tamoxifen. The material remaining at the origin in their study contained 28–47 per cent of the total serum radioactivity, some of which might be tamoxifen *N*-oxide. With the solvent system chloroform–methanol (9:1), the *N*-oxide **4** had R_f 0.13 (cf. 0.50 for tamoxifen). The antiestrogenic properties and toxicity of tamoxifen *N*-oxide are being studied.

Metabolism. The preparation of microsomes from male Wistar albino rats was as previously described [7].

Incubations were carried out in 25-ml conical flasks which were shaken gently at 37° for 50 min after gassing with oxygen. Each flask contained 1.5 ml of a microsomal suspension (equivalent to 375 mg of liver), 1.43 μmoles of $NADP^+$, 30.6 μmoles of D-glucose 6-phosphate, 24.6 μmoles of $MgCl_2.6H_2O$ and 2.5 μl of a solution of glucose-6-phosphate dehydrogenase (5 mg/ml, 140 u/mg) in a total volume of 10 ml buffered at pH 7.4 with 0.1M Tris–HCl. Cofactors and microsomes were each added in three portions after 0, 15 and 30 min. Tamoxifen citrate (500 μg) was added as a solution in *N,N*-dimethylformamide (25 μl). Since the substrate was not completely soluble in the aqueous solution, its final concentration in the incubate was <100 μg/ml. Controls involved adding tamoxifen citrate after the incubation period.

After incubation, the pH of each incubate was adjusted to 9 with 0.13 M NaOH (2.8 ml) which was then extracted with ether [14] (3 × 32 ml). The combined extracts were dried (Na_2SO_4) and concentrated. A solution of the residue in methanol (5.8 ml) was applied to a C_{18} Sep-Pak cartridge which was eluted with methanol (8 ml). The eluate was passed through a 0.5 μm Fluoropore membrane filter (Millipore Corp.), concentrated, and a solution of the residue in methanol (300 μl, h.p.l.c. grade) was subjected to reverse-phase h.p.l.c. using a Waters Model ALC/GPC 204 liquid chromatograph equipped with a Model 6000A solvent delivery system, a U6K injector, a Model 440 dual channel absorbance detector operated at 254 nm, and a μ Bondapak C_{18} column (30 cm × 3.9 mm i.d.). The column was eluted with methanol–water–diethylamine (90:10:0.1) at 1 ml/min. H.p.l.c.–grade methanol was purchased from Rathburn Chemicals (Walkerburn) Ltd., water was doubly distilled in all glass apparatus, and diethylamine was redistilled commercial material (b.p. 55.0–55.5°).

Retention times in minutes of (a) standards were: 4-hydroxytamoxifen, 4.66; tamoxifen, 8.02; *N*-desmethyltamoxifen, 9.84; and of (b) products extracted from incubate were 4-hydroxytamoxifen(1.5%, determined from peak area), 4.7; tamoxifen *N*-oxide (6%), 6.2; tamoxifen (72%), 8.2; (*N*-desmethyltamoxifen (20.5%), 9.84. Under these conditions the peak for 4-hydroxytamoxifen was poorly resolved. Better resolution was obtained with a more polar solvent system (methanol–water–diethylamine 80:20:0.1).

In subsequent separations the peaks (other than for 4-hydroxytamoxifen) were collected and the contents were subjected to mass spectrometry (AEI MS-12 spectrometer, 70eV, trap current 100 μA, ion-source temperature 130–150°, direct insertion technique). The mass spectra of tamoxifen are noted below; *N*-desmethyltamoxifen (synthetic and metabolic) gave *inter alia* peaks at m/z 357 (M^+ of **3**, 77%), 300 ($[\mathbf{3}\text{-}CH_2CH_2NHCH_2]^+$, 100%) and 58 ($[CH_2CH_2NHMe]^+$, 59.3%).

Tamoxifen-N-oxide (compound **4**). A solution of tamoxifen (50 mg, 0.135 mmole) in methanol (3 ml) and aqueous 30% hydrogen peroxide (1 ml) was stored for 2 days at room temperature. The mixture was then stirred with platinum oxide (20 mg) until evolution of oxygen ceased (~4 hr), filtered, and concentrated below 40° under reduced pressure. Benzene was distilled from the residue to remove residual water. Crystallization from benzene–cyclohexane then gave **4** (52 mg) as small colourless needles, m.p. 134–136°, repeated elemental analyses of which were consistent and indicated $H_2O + H_2O_2$ of crystallization. The tendency of *N*-oxides for form hydrates and H_2O_2 adducts is well documented [13]. (Found: C, 70.85; H, 7.23; N, 3.21. $C_{26}H_{29}NO_2.H_2O.H_2O_2$ requires C, 71.07 per cent; H, 7.52 per cent; N, 3.19 per cent). On heating **4** at ~80° and 12 mmHg for 2 days the loss in weight corresponded to volatilization of $H_2O+H_2O_2$. The EI mass spectrum of **4** contained a prominent peak at m/z 34 corresponding to $H_2O_2^{+\cdot}$.

The product **4** was homogeneous by t.l.c. (chloroform-methanol, 9:1; R_f 0.13; *cf.* 0.50 for tamoxifen) and h.p.l.c. (see below). The EI mass spectrum contained, *inter alia*, peaks at m/z 387 ($M^{+\cdot}$, 1.7%), 385 ($[M - 2H]^+$, 0.6%), 371 ($[M - O]^+$, 12%), 357 ($M^{+\cdot}$ of **3**, 4.4%), 326 ($M^{+\cdot}$ of **5**, 29.9%), 311 ($[\mathbf{1}\text{-}CH_2NMe_2]^+$, 3.4%), 300 ($[\mathbf{1}\text{-}CH_2CH_2N(Me)CH_2]^+$, 11.2%), 72 ($[CH_2CH_2NMe_2]^+$, 28.9%), 58 ($[CH_2{=}NMe_2]^+$, 100%); the EI mass spectrum of tamoxifen contained peaks at m/z 371 ($M^{+\cdot}$, 14.8%), 300 (3.4%), 72 (23.7%) and 58 (100%). FD spectrum (Varian MAT 731 spectrometer, emitter heating current 0–25 mA) of **4** (signals m/z <775 >10% of base peak): m/z 775 ($[2M + H]^+$, 1.34%), 387 ($M^{+\cdot}$, 27.4%), 385 ($[M - 2H]^+$, 27.3%), 372 ($[M - O + H]^+$, 32.5%), 371 ($[M - O]^+$, 100%), 357 (14.1%), 327 (11.6%), 326 (52.5%); the FD spectrum of tamoxifen (no emitter heating) contained only peaks at m/z 371 ($M^{+\cdot}$, 100%), 372 (30.8%) and 373 (10%).

N.m.r. data (60 MHz, $CDCl_3$, internal Me_4Si): δ 0.91 (t, 3 H, *J* 7 Hz, $C\underline{H}_3CH_2$), 2.45 (q, 2 H, $CH_3C\underline{H}_2$), 3.25 (s, 6 H, NMe_2), 3.60 (t, 2 H, *J* 5 Hz, $NC\underline{H}_2CH_2$), 4.40 (t, 2 H, $OC\underline{H}_2CH_2$), 6.49 and 6.75 (2 d, each 2 H, *J* 8.5 Hz, aromatic AB system), 7.09 and 7.24 (2 s, each 5 H, 2 Ph). The corresponding signals in the n.m.r. spectrum of tamoxifen (same coupling constants unless otherwise stated) were at δ 0.88, 2.44, 2.24, 2.60 (*J* 6 Hz), 3.88, 6.50, 6.76, 7.09 and 7.24.

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