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IDENTIFICATION AND BIOLOGICAL ACTIVITY OF TAMOXIFEN METABOLITES IN HUMAN SERUM

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Abstract—During the examination of serum samples from patients on chronic 'Nolvadex' therapy three major metabolites (**X**, **Y** and **Z**) were detected in addition to the parent drug. Two of these metabolites have been positively identified as *N*-desmethyltamoxifen (**X**) and a side-chain primary alcohol (**Y**). The third metabolite (**Z**) has been tentatively identified as *N*-desdimethyltamoxifen. Quantitative analysis of these metabolites in sera from patients undergoing chronic Nolvadex therapy (20 mg ~ b.d.) has shown that the mean *N*-desmethyltamoxifen concentration was 481 ng/ml, the mean metabolite **Y** concentration was 49 ng/ml and that desdimethyltamoxifen concentrations were in the range 20–40 ng/ml. The corresponding mean unchanged drug level in these patients was 310 ng/ml. 4-Hydroxytamoxifen could not be detected in these samples. Measurements of the relative binding affinities of tamoxifen and its metabolites for rat uterus oestrogen receptors have shown that 4-hydroxytamoxifen had a relative binding affinity similar to oestradiol while tamoxifen and its side-chain metabolites had lower affinities. It has been shown that all the metabolites examined are antioestrogenic, as demonstrated by their ability to prevent implantation in pregnant rats and inhibit oestradiol-induced uterine weight gain. It is therefore possible that the metabolites of tamoxifen collectively contribute to the therapeutic activity of the drug.

The antioestrogen tamoxifen (Nolvadex)§ has been used since the early 1970s in the treatment of advanced breast cancer [1–3] and infertility [4]. The pharmacokinetics and metabolism of this agent were originally examined using [^{14}C] tamoxifen and the main metabolites were isolated from bile and faeces [5, 6] rather than serum. The major metabolic pathway was identified as aromatic hydroxylation to form phenolic metabolites, including 4-hydroxytamoxifen (4-OH). Since this metabolite is a potent antioestrogen it has been suggested that it contributes to the antioestrogen effect observed in patients treated with tamoxifen [7].

Recently the pharmacokinetics and metabolism of tamoxifen have been re-examined using specifc procedures for the measurement of the parent compound and its metabolites [8–10]. The thin-layer densitometric method [9] has the advantage of detecting and quantifying any unconjugated metabolite with the triphenylethylene structure. This paper reports the analysis of human serum samples using this method, the elucidation of the structures of new metabolites found and the biological activity of these new metabolites.

A preliminary communication on some of the results presented here has already appeared [11].

MATERIALS AND METHODS

Reagents. Amyl alcohol, toluene [AR grade, BDH, Poole U.K.], hexane, methanol [HPLC grade (Rathburn Chemicals, Wakeburn, U.K.)], cyclohexane (Spectrosol grade, Hopkin & Williams, Chadwell Heath, U.K.) liquid paraffin [reagent grade (Hopkin & Williams), acetyl chloride [SynchemicA grade (Hopkin & Williams)] and *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Pierce & Warriner UK Ltd, Chester U.K.) were used without further purification. Dansyl chloride (BDH) was used in the form of a 0.1% solution in redistilled acetone. Diazomethane was freshly prepared before use from *N*,*N*-dimethylterephthalamide [E.I.Dupont De Nemours and Co. (Inc.), DE]. Triethylamine [SynchemicA grade (Hopkin & Williams)] was redistilled from phthallic anhydride (BDH) before use and the 89–90° fraction collected. *n*-Butanol, chloroform and ethyl acetate [AnalaR grade (Hopkin & Williams)] were redistilled before use. 0.1 M, pH 7 phosphate buffer was prepared using equimolar disodium hydrogen and potassium dihydrogen orthophosphates [AnalaR grade (Hopkin & Williams)] dissolved in double distilled water. Buffers for the pH profiles and differential extractions were prepared by the method of Britton and Robinson [12].

Authentic samples of tamoxifen, its metabolites and their isomers were synthesised in the laboratories of Imperial Chemical Industries plc (Macclesfield, U.K.).

Instruments. The densitometer used was a Vitatron Model TLD 100 (Fisons Ltd, Loughborough, U.K.). This equipment was used with a mercury lamp source filtered by a UVB liquid filter, with a maximum wavelength of 366 nm, and emission was measured at 410 nm using an interference filter. The u.v. light source for cyclisation of tamoxifen and its metab-

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§ Nolvadex is a trade mark, propery of Imperial Chemical Industries plc.

olites to phenanthrenes was the mercury lamp (λ_{max} 254 nm) of a thin-layer plate viewing cabinet [Model C5 Chromato-Vue cabinet (Ultraviolet Products Inc., San Gabriel, CA)].

The gas chromatography–mass spectrometry was carried out using an LKB 9000 gas chromatograph–mass spectrometer linked to an LKB 2130 data system (LKB Products Ltd, Bromma, Sweden). The compounds were chromatographed on glass columns (1.5 m long, 4 mm i.d.) of OV-1 and OV-17 [1% (Phase Separations Ltd, Queensferry, U.K.)] on 80–100-mesh Gas Chrom Q (Field Instruments, Richmond, U.K.), coated by filtration of 1% solutions of OV-1 and OV-17 in chloroform/toluene. The columns, column temperatures and retention times for individual compounds are shown in Table 1. The injection port temperature was 250° and the carrier gas was helium (30 ml/min). The mass spectrometer was operated in the repeat mode with an ion source temperature of 270°, a trap current of 50 μA and an accelerating voltage of 3.5 kV.

Samples. Serum samples were obtained from patients undergoing Nolvadex therapy as previously described [13, 14]. These were stored frozen at −20° until required.

TLC. All separations were carried out using precoated silica gel 60 plates without fluorescent indicator (E. Merck, Darmstadt, F.R.G.).

The following solvent mixtures were used for separations: (1) toluene, triethylamine, absolute alcohol (8:1:1); (2) toluene, methanol (9:1); (3) toluene, triethylamine, *n*-butanol (80:15:5); (4) chloroform, cyclohexane, triethylamine (5:4:1) + 0.2 ml absolute alcohol/50 ml; (5) chloroform, cyclohexane, triethylamine, absolute alcohol (45:35:10:10).

Ten millilitres of liquid paraffin per 100 ml of solvent was added to each for the reasons already stated [9].

Quantitative evaulation. The parent drug, and its metabolites were extracted from serum at pH 7 using an organic solvent, separated by TLC and converted to fluorescent phenanthrene derivatives prior to *in situ* densitometric evaluation as previously described [9]. The procedure was modified as necessary by alteration of the pH and the use of alternative chromatographic solvent mixtures as outlined earlier.

A procedure for the exclusion of phenolic metabolites from the final extract was developed. This involved protein precipitation of 200 μl of serum with 400 μl of methanol, centrifugation, adjustment of the pH to 14 by the addition of 10 N sodium hydroxide to the supernatant and extraction into hexane, prior to TLC.

Differential extraction of metabolite ***X****.* To 1 ml of serum was added 1 ml of pH 2.45 buffer; this was then extracted 3 times using 5 ml of 1.5% amyl alcohol in *n*-hexane. The organic layers were discarded and the aqueous layer basified with 1 ml of 1 N sodium hydroxide before reextracting with 5 ml of 1.5% amyl alcohol in *n*-hexane. The organic layer was finally evaporated to dryness using oxygen-free nitrogen.

A parallel experiment was carried out using 1 ml of control serum to which the same concentration of authentic tamoxifen and *N*-desmethyltamoxifen [15] had been added.

Two-dimensional chromatography of metabolite **Z**. A 1-ml aliquot of serum was extracted by the standard analytical procedure and the resultant residue was transferred as a single spot to the corner of a TLC plate. This was then chromatographed in solvent system (1). The area of plate corresponding to metabolite **Z** was overspotted with either acetyl chloride or dansyl chloride and the plate was rechromatographed at 90° to the original direction. Authentic *N*-desdimethyltamoxifen was spotted onto the plate and derivatised with either acetyl or dansyl chloride and chromatographed at the same time as metabolite **Z**. Paraffin was omitted from the first run as this caused poor chromatography in the second run.

pH profiles (Table 2). Extractions under various conditions of pH were carried out by substituting the pH 7 buffer used in the routine analytical procedure with 1 ml of the appropriate buffer solution.

Derivatisations. Methylation was carried out overnight, by addition of 500 μl of ethereal diazomethane to the dry residue of a serum extract redissolved in 100 μl of ethyl acetate.

Acetylation was carried out *in situ* by overspotting with 50 μl of acetyl chloride and heating at 110° for 10 min.

Dansylation was carried out *in situ* by overspotting

Table 1. Major ions for gas chromatography–mass spectrometry peaks for tamoxifen, its metabolites and isomers

Compound	Parent peak	Base peak	Other significant peaks	GC conditions and retention time
Tamoxifen	371	58	72	OV-1 241° TR2
Metabolite **X**	357	44	58, 91, 285, 300	OV-17 258° TR1
N-Desmethyltamoxifen	357	44	58, 91, 285, 300	OV-17 258° TR1
TMS metabolite **Y**	416	73	57, 91, 117, 129, 132, 191, 299, 300, 357, 401	OV-1 241° TR3
TMS primary alcohol	416	73	57, 91, 117, 129, 132, 191, 299, 300, 357, 401	OV-1 241° TR3
Primary alcohol	344	344	91, 165, 191, 207, 227, 284, 299, 300, 329	OV-1 246° TR2
G	344	326	91, 207, 221, 235, 249, 281, 297, 311	OV-1 246° TR1
H	344	313	91, 107, 191, 207, 235	OV-1 246° TR2
F*	360	360	208, 299, 300, 301, 315, 345	

* Fromson *et al.* [5, 6].

Table 2. % extraction efficiencies of tamoxifen and its metabolites, into 1.5% amyl alcohol in hexane, under varying conditions of pH (pH 7 taken nominally as 100%)

Compound	1 N NaOH	pH 7	pH 3	pH 2.45	pH 2.2	0.1 NH_2SO_4
Tamoxifen	83	100	94	60	45	6
X	80	100	44	9	7	4
Y	76	100	93	88	102	99
Z	82	100	23	3	10	1
E*	71	100	—	—	90	102

* Fromson *et al.* [5, 6].

with the solution of dansyl chloride in redistilled acetone. The derivatives were detected using the densitometer with a filter to cut out light with a wavelength < 525 nm in place of the 410-nm interference filter.

Silyl derivatives for gas chromatography–mass spectrometry were prepared using BSTFA containing 1% TMCS, 10 μl being added to the dry residue of a serum extract redissolved in 10 μl of ethyl acetate and allowing 15 min reaction time.

Competition of tamoxifen and its metabolites for rat uterus oestrogen receptor. Cystosol prepared from mature rat uterus was incubated with [^{3}H]oestradiol in the absence or presence of increasing concentrations of oestradiol or antioestrogens and relative binding affinities were calculated as previously described [16].

Antioestrogenic activity of tamoxifen and its metabolites. Antioestrogenic activity was determined: (a) by measurement of the inhibition of implantation in pregnant rats given a single oral dose of test compound at 4 p.m. on day 4 of pregnancy [17], and (b) by measurement of the inhibition of oestradiol-induced uterine weight gain in immature rats given three daily oral doses of test compound and concurrent subcutaneous administration of 0.5 μg/rat of oestradiol benzoate [18].

Chemical synthesis. The synthesis of metabolites **X** and **Y** has previously been described [15, 19]. Compounds described in this paper as metabolite **Z** and compounds **G** or **H** were synthesised in an analogous manner.

RESULTS

When a serum sample obtained from a patient who had received 100 mg Nolvadex b.d for at least 12 months [14] was examined by the thin-layer densitometric procedure [9] three components, in addition to the parent drug, were present in sufficient quantity to be measured. The fluorescent trace of the thin-layer chromatogram is shown in Fig. 1 and the new compounds were labelled **X**, **Y** and **Z**. The relationship of components **X**, **Y** and **Z** to the parent drug was demonstrated by the lack of fluorescent product at the relevant R_f values prior to irradiation with u.v. light.

Metabolite X

Metabolite **X** was distinguished from 4-OH by a slight difference in R_f value (0.36 and 0.32 for metabolite **X** and 4-OH, respectively, in solvent 1). Clearer separation of the two metabolites was subsequently obtained using solvents 3–5. In view of the work of Fromson *et al.* [5, 6] it was considered that **X** could be a phenolic metabolite. Methylation of a serum extract resulted in no change of R_f for metabolite **X** although it was confirmed separately that the phenolic metabolites **B**, **D** and **E** [5, 6] underwent almost complete reaction. Further confirmation that metabolite **X** did not contain a phenolic group was obtained by its extractability under strongly basic conditions, whereas **B**, **D** and **E** were not extractable. The extraction efficiency of metabolite **X** under various conditions of pH (Table 2) showed it to contain a basic function and its reaction with acetyl and dansyl chlorides showed that it possessed a derivatisable group. In view of the similarity in side chain between imipramine and tamoxifen it was postulated that tamoxifen could follow the same metabolic route, i.e. *N*-desmethylation [20]. *N*-desmethyltamoxifen was synthesised [15] and found to have an identical R_f value to metabolite **X** in solvent systems

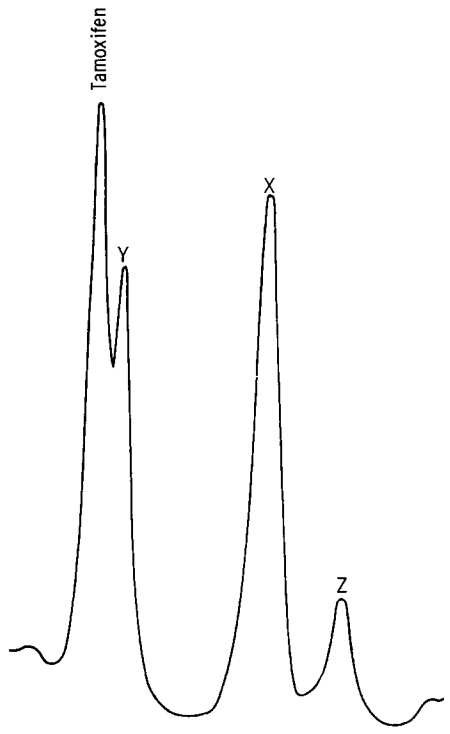


Fig. 1. Thin-layer densitometric trace for a serum extract from a patient treated with 100 mg tamoxifen b.d.

1, 3 and 4, a similar pH extraction profile and not to react with diazomethane.

Final proof of the identity of metabolite **X** was obtained from gas chromatography–mass spectrometry. Initial attempts to identify metabolite **X**, using a pH 7 serum extract, failed because the GLC retention times of tamoxifen and the metabolite were identical. However by careful adjustment of the pH a differential extraction based on this (see Materials and Methods) allowed a 20-fold concentration of *N*-desmethyltamoxifen relative to tamoxifen in a control sample. The unknown was treated in an identical manner and the extract thus obtained was subjected to gas chromatography–mass spectrometric analysis. The resultant retention times and mass spectrometry data (Table 1) were identical for metabolite **X** and authentic *N*-desmethyltamoxifen. It was necessary to use an OV-17 column for this gas chromatography as on-column losses were encountered when using an OV-1 column for metabolite **X**.

It has been shown that the method of Adam *et al.* [9] can be used in the determination of serum levels of *N*-desmethyltamoxifen [21] and the concentration in the serum extract from the patient who had received 100 mg b.d for at least 12 months (Fig. 1) was 2.4 μg/ml. *N*-Desmethyltamoxifen concentrations have also been measured in serum samples from a clinical study in which patients were treated with tamoxifen at a dose of 20 mg b.d [13]. These are shown in Table 3. The extraction efficiency determined for *N*-desmethyltamoxifen using this procedure was 84%.

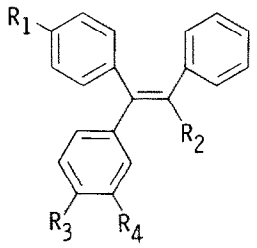
Metabolite Y

Metabolite **Y** had properties similar to Fromson's metabolite **E** (Fig. 2) in that their R_f values in solvent systems 1 and 2 were identical and both compounds had the same pH extraction characteristics. Metabolite **Y** did not react with diazomethane and was extractable from base treated serum, thus demonstrating that metabolite **Y** was not phenolic. It was subsequently shown that **E** and **Y** could be resolved using solvent systems 3–5. It was found that metabolite **Y** reacted with dansyl and acetyl chlorides and it was postulated that metabolite **Y** had lost the basic nitrogen but contained an alkyl hydroxyl group. A sample of the primary alcohol was synthesised [19] and when compared with metabolite **Y** they were found to be identical in all solvent systems and also in their reactions and pH profile. Initial attempts to obtain a positive identification of metabolite **Y**, by extraction from acidified serum and analysis of the residue by gas chromatography–mass spectrometry, proved problematic due to high endogenous background interference.

However, the absence of a basic nitrogen allowed complete separation of metabolite **Y** from the other compounds using the non-basic solvent system [2] for a pH 7 serum extract. **Y** was located by running the primary alcohol alongside it and preventing the conversion of **Y** to its phenanthrene by covering it with black paper during the irradiation with u.v. light. The required area of silica was then scraped from the plate and transferred to a small column prepared from a pasteur pipette fitted with a methanol-washed cotton-wool plug. **Y** was eluted using 5 ml of methanol. The eluent was then evaporated to dryness using oxygen-free nitrogen. All the solvents were distilled and paraffin was omitted from the solvent mixture used for this isolation procedure to ensure that the extract was as clean as possible. A parallel experiment was carried out using

Table 3. Mean steady-state serum levels of tamoxifen and two of its major serum metabolites, after 20 mg b.d. dosing, for serum samples from a clinical study [13].

Patient No.	Tamoxifen (ng/ml)	*N*-Desmethyl-tamoxifen (**X**) (ng/ml)	Primary alcohol (**Y**) (ng/ml)
6	385	548	33
7	255	425	39
12	302	575	46
15	377	463	40
16	447	486	55
19	391	733	68
22	350	851	136
23	196	348	37
31	306	300	22
32	164	311	39
36	180	337	26
37	216	395	64
41	494	532	66
42	258	307	44
43	326	606	21
Mean	309.8	481.1	49.1
S.E.	25.5	42.2	7.3



Identity	R_1	R_2	R_3	R_4
Tamoxifen	CH_3 \ NCH_2CH_2O / CH_3	C_2H_5	H	H
N-Desmethyl Tamoxifen (Metabolite X)	CH_3 \ NCH_2CH_2O / H	C_2H_5	H	H
Primary Alcohol (Metabolite Y)	OH \ CH_2CH_2O	C_2H_5	H	H
N-desdimethyl (Metabolite Z)	$H_2NCH_2CH_2O$	C_2H_5	H	H
B*	CH_3 \ NCH_2CH_2O / CH_3	C_2H_5	OH	H
C*	CH_3 \ NCH_2CH_2O / CH_3	C_2H_5	OH	OCH_3
E*	HO	C_2H_5	H	H
F*†	CH_3CH_2O	$CHCH_3$ \ OH	OH	H
G	CH_3CH_2O	$CHCH_3$ \ OH	H	H
H	CH_3CH_2O	CH_2CH_2OH	H	H

* Identified by Fromson *et al.* [5, 6]
† Tentative assignment.

Fig. 2. The structures of tamoxifen and its metabolites.

1 ml of control serum spiked with the primary alcohol, tamoxifen and *N*-desmethyltamoxifen to confirm recoveries.

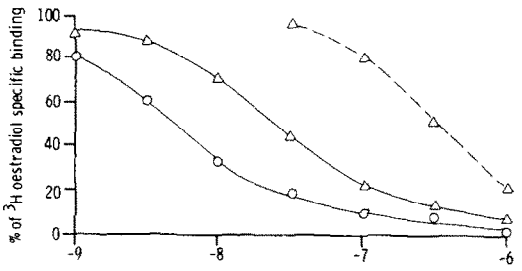
Both extracts were derivatised using BSTFA containing 1% TMCS and analysed by gas chromatography–mass spectrometry. The gas chromatography retention times and mass spectrometry fragmentation patterns of the silylated derivatives were identical with those of metabolite **Y** and the primary alcohol (Table 1). Because of the possible positional isomers for metabolite **Y**, the alcohols **G** and **H** (Fig. 2) were synthesised (see Materials and Methods) and compared with the primary alcohol. They were found to have different R_f values in solvents 1 and 2, different gas chromatography retention times and different mass spectrometric fragmentation patterns from the primary alcohol (Table 1).

Metabolite **Y** can be quantified in serum samples using the non-basic chromatography solvent system 2. Its extraction efficiency was 91% and the concentration in the serum extract from the patient who had received 100 mg b.d. for at least 12 months (Fig. 1) was 0.8 μg/ml. The concentrations of metabolite **Y** in serum samples from a clinical study in which patients were treated with tamoxifen at a dose of 20 mg b.d. [13] are shown in Table 3.

Metabolite Z

Metabolite **Z** was more polar than the other metabolites and its pH extraction profile showed that it retained the base function. Its lack of reaction with

Figure 3 Competition of Tamoxifen for Rat Uterus Oestrogen Receptor



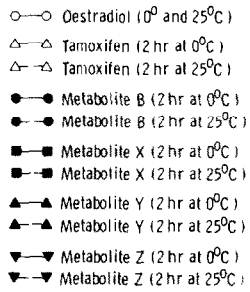


Figure 4 Competition of Metabolite B for Rat Uterus Oestrogen Receptor

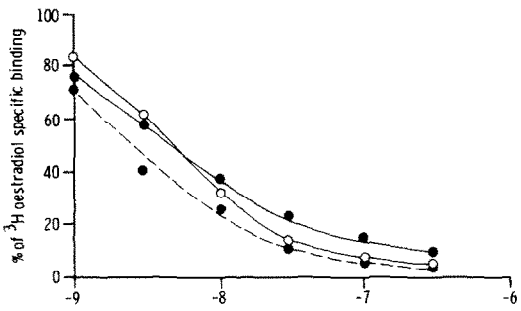


Figure 5 Competition of Metabolite X for Rat Uterus Oestrogen Receptor

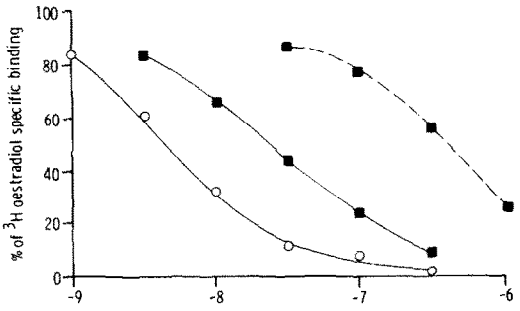


Figure 6 Competition of Metabolite Y for Rat Uterus Oestrogen Receptor

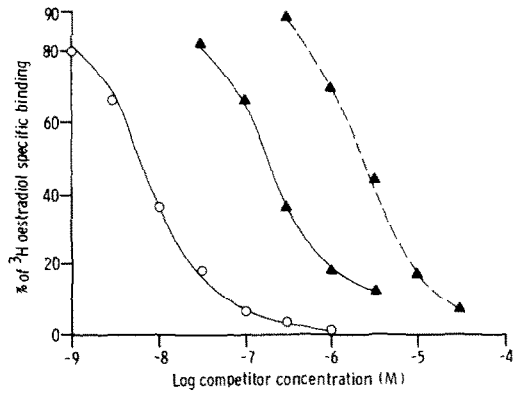
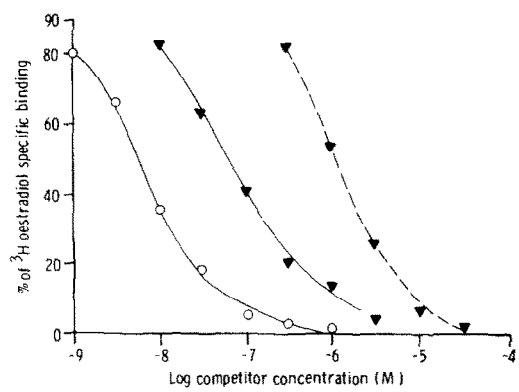


Figure 7 Competition of Metabolite Z for Rat Uterus Oestrogen Receptor



Figs 3–7. The competition of tamoxifen and its metabolites for rat uterus oestrogen receptor.

Table 4. Relative binding affinities (RBA) of tamoxifen and its metabolites

	2 hr at 0°	2 hr at 25°	Ratio
Tamoxifen	38.2	1.8	21.2
Metabolite **X**	21.0	1.1	19.1
Metabolite **Y**	3.84	0.25	15.7
Metabolite **Z**	9.34	0.51	18.4
Metabolite **B***	109.8	187.8	0.58

* Fromson *et al.* [5, 6].

diazomethane and its extractability from base-treated serum indicated that it did not contain a phenolic group although it reacted with both dansyl and acetyl chlorides. In view of this it was postulated that metabolite **Z** could be the *N*-desdimethyl metabolite, a route which is also found with imipramine [22]. A sample of *N*-desdimethyltamoxifen was synthesised (see Materials and Methods) and compared with metabolite **Z**. It was found that metabolite **Z** had the same R_f value as *N*-desdimethyltamoxifen in solvent systems 1, 3, 4 and 5. It was also found that the dansyl and acetyl derivatives of *N*-desdimethyltamoxifen and metabolite **Z** had the same R_f values in system 1. Final proof of the postulated structure of metabolite **Z** by gas chromatography–mass spectrometry has been hindered by the low levels found in the serum.

N-Desdimethyltamoxifen has a low fluorescent yield, approximately half that of tamoxifen. This low yield and a poor extraction efficiency prevents determination of serum concentrations below 7.5 ng/ml. However, assuming that metabolite **Z** is *N*-desdimethyltamoxifen the steady-state concentrations reached after 20 mg b.d. dosing could be up to 40 ng/ml.

*Metabolite **B** (4-OH [5, 6])*

Although the solvent system used in the previously reported assay procedure for tamoxifen (solvent 1 [9]) resolved metabolites **B** and **X** the resolution was insufficient to allow measurement of concentrations of metabolite **B**, i.e. once the ratio **X**/**B** exceeded 10:1. A wider separation of metabolites **B** and **X** could be achieved by the use of solvent systems 3 or 5. However the relative R_f values of metabolites **B** and **Z** then prevented a baseline separation. Use of solvent system 4 resolved metabolite **B** from both metabolites **X** and **Z** but this resulted in a low R_f value for metabolite **B** and interference from endogenous background material.

It is clear from these studies that metabolite **B** could have been present in human serum but at very low concentrations compared with the side-chain metabolites and low concentrations of metabolite **B** (< 1–11.4 ng/ml) have been reported in patients during chronic Nolvadex therapy when samples were analysed by gas chromatography–mass spectrometry [8, 23].

Receptor binding of tamoxifen and its metabolites

The competition of tamoxifen and its metabolites with [^{3}H]oestradiol for ctyoplasmic oestrogen receptors is shown in Figs 3–7 and the receptor binding affinities are shown in Table 4. In all cases non-radioactive oestradiol competed with [^{3}H]oestradiol to give a classic competition curve which was identical at 0 and 25° suggesting that equilibrium was rapidly established. At both 0 and 25° metabolite **B** behaved very much like oestradiol, while tamoxifen and its side-chain metabolites demonstrated lower affinities for the receptor. When the temperature was raised to 25° the apparent affinity of metabolite **B** increased slightly while the curves of tamoxifen and its side-chain metabolites shifted to apparent affinities much less than those observed at 0°.

Antioestrogenic activity of tamoxifen and its metabolites

The antioestrogenic activity of tamoxifen and its metabolites was compared by measuring their efficacy in terminating early pregnancy in the rat. When administered on the evening of day 4 of pregnancy antioestrogens prevent implantation by antagonizing the pre-implantation surge of oestradiol [17]. The data in Table 5 shows that tamoxifen and metabolites **B** and **X** have a very similar antioestrogenic potency in this test; the minimal fully effective dose was in the range 0.05–0.1 mg/kg for all three compounds. Metabolites **Y** and **Z** were also clearly antioestrogenic, although of somewhat lower potency (0.2 and 0.5 mg/kg respectively).

The antiuterotrophic test (see Fig. 8) again showed that all compounds [except **Z** (not tested)] were clearly antioestrogenic. Metabolite **B** was equipotent with tamoxifen, but metabolites **X** and **Y** were somewhat less potent than tamoxifen.

Table 5. Prevention of implantation in rats by tamoxifen and its metabolites (single oral dose at 4 p.m. of day 4 of pregnancy)

Dose (mg/kg)	% egg loss				
	Tamoxifen	Metabolite **X**	Metabolite **Y**	Metabolite **Z**	Metabolite **B***
1.0	—	—	100	—	—
0.5	—	—	100	—	—
0.2	100	100	68	100	100
0.1	100	98	51	98	100
0.05	100	98	38	63	96
0.025	30	31	—	—	9
0.01	—	—	—	10	—

* Fromson *et al.* [5, 6].

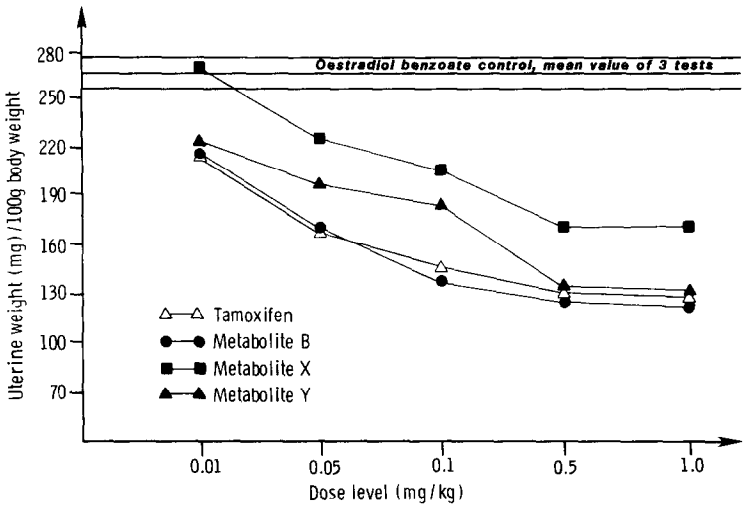


Fig. 8. The inhibition of oestradiol-induced uterine weight gain by tamoxifen and its metabolites.

DISCUSSION

The metabolites identified by Fromson *et al.* [5, 6] were mainly obtained after hydrolysis of bile and faecal extracts. All contained hydroxyl groups and it is suggested that these metabolites are rapidly conjugated and eliminated via the bile. We have been unable to detect these metabolites in human serum. Daniel *et al.* [8, 23] have reported low levels of 4-OH in human serum after chronic tamoxifen administration.

The present work has shown that metabolism of the side chain is also important and a theoretical metabolic route is shown in Fig. 9. The fact phenolic metabolites were not detected in human serum using our procedure suggests that they are rapidly eliminated, whereas the parent drug and metabolites **X**, **Y** and **Z**, which were found in serum, cannot be readily conjugated and probably need to be further metabolised prior to excretion. Tamoxifen *N*-oxide has been found in rat liver microsome preparations [24] and it may be that this is an intermediate in the formation of the *N*-desmethyl metabolite, although hydroxylation at the α-carbon is probably the most likely first step to *N*-dealkylation (e.g. Ref. 25).

The present work has cast doubt on the structure tentatively assigned by Fromson to metabolite **F** [5, 6]. In discussion on this structure he argued that the mass spectral fragmentation pattern of **F** was not compatible with that expected from a primary alcohol formed by oxidation of the ethyl group of the tamoxifen molecule. We have confirmed this argument by examining the mass spectrum of such a compound [**H** (Fig. 2)]. As predicted by Fromson, the major fragmentation pattern was loss of 31 mass units to yield the base peak at *m/z* 313. This loss was not observed for metabolite **F**. However the additional argument to assign the secondary alcohol at R_2 (Fig. 2), i.e. oxidation of the ethyl group, has not been confirmed. Fromson argued that, if a primary alcohol had been formed by cleavage of the dimethylamino side chain, then losses of 30, 31 and 44 mass units would have been expected in its mass spectrum. This has been shown not to be the case for metabolite **Y**, which contains the side-chain primary alcohol function. Although a small peak at

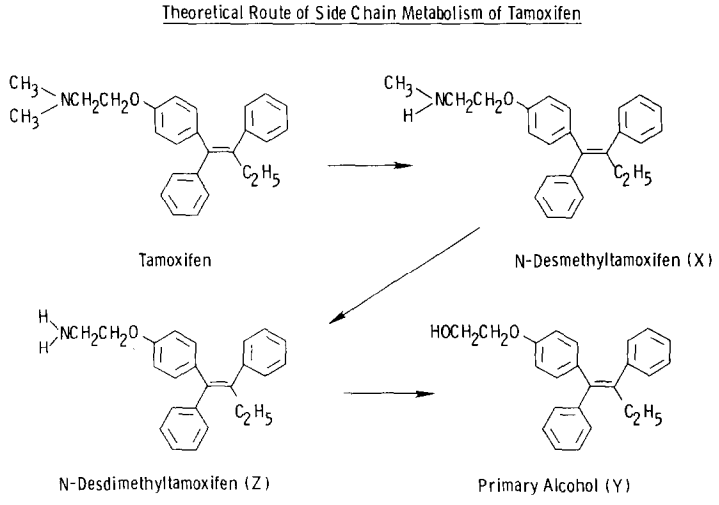


Fig. 9. The theoretical route for the side-chain metabolism of tamoxifen.

M/z 357 [$M^+ - 44$] was observed, no losses of 30 or 31 mass units were seen. Finally compound **G**, which contains the same secondary alcohol function suggested to be present in metabolite **F**, has a mass spectrum dominated by loss of water to yield the base peak at m/z 326. No such loss was recorded for metabolite **F**.

No authentic metabolite **F** was available in the present work. Thus complete elucidation of the problem was not possible. However, in the light of present knowledge, it is suggested that the metabolite labelled as **F** by Fromson is a hydroxylated derivative of our metabolite **Y**. No authentic sample of this compound was available.

Metabolite **X** can be analysed routinely in human serum at the same time as tamoxifen using the procedure of Adam *et al.* [9], and **Y** can be analysed by a similar procedure using a non-polar solvent and determination of concentration by peak height. Concentrations of metabolite **Z** have not yet been determined in serum but assuming that it is *N*-desdimethyltamoxifen the levels reached in normal clinical therapy could be up to 40 ng/ml. Table 3 shows steady-state concentrations of tamoxifen and metabolites **X** and **Y** in serum from patients undergoing tamoxifen therapy using a dose of 20 mg b.d. The serum concentrations shown here are in good agreement with those determined by Daniel *et al.* [23] who used an alternative analytical method [8].

The biological studies reported have shown that tamoxifen and its metabolites are potent antioestrogens. Although the oestrogen receptor binding sites showed major differences in binding characteristics between tamoxifen, its side-chain metabolites and metabolite **B**, the difference in receptor binding kinetics were not reflected by major differences in their antioestrogenic potencies.

In female patients treated with tamoxifen, serum concentrations of metabolite **X** may equal or exceed those of tamoxifen while concentrations of metabolites **Y** and **Z** are somewhat lower. It is possible that these metabolites contribute to the regression of oestrogen-dependent mammary carcinoma. However, because of the low serum concentrations of metabolite **B**, it is less likely to play a significant role in inducing tumor regression in patients treated with tamoxifen.

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