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High-performance liquid chromatographic analysis of tamoxifen, toremifene and their major human metabolites

François Berthou* and Yvonne Dréano

Laboratoire de Biochimie, Faculté de Médecine, B.P. 815, 29285 Brest Cedex (France)

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ABSTRACT

The chromatographic behaviour of tamoxifen, toremifene and their major metabolites was investigated by reversed-phase high-performance liquid chromatography on four stationary phases. Two packings were the usual octadecylsilane type and the other two were octylsilane and octadecylsilane of the type specific for basic compounds. The results provide new insight into variations in selectivity with column type for drugs whose basic properties, owing to the presence of an ionizable nitrogen atom, make their chromatography difficult. The results allow an improvement of the separation of metabolites of tamoxifen and toremifene, two triphenylethylene drugs widely used for the treatment of breast cancer. A method is described for the identification and determination of metabolites formed by incubating the parent drugs with human liver microsomal preparations. The assay has been optimized for the identification and quantification of three major metabolites formed by N-oxidative demethylation of the side-chain, 4-hydroxylation of the aromatic ring and a side-chain deamination followed by hydroxylation. These catalytic activities involve cytochrome P450 enzymes.

INTRODUCTION

Recent literature has provided new insights into the molecular pharmacology of non-steroidal antiestrogens widely used in the treatment of estrogen receptor-positive breast cancer [1]. Tamoxifen, *Z*-1-[4-[2,2-(N,N-dimethylamino)ethoxy]-phenyl]-1,2-diphenylbut-1-ene (ICI 46,474), has been used as a first-line drug since 1960 in the treatment of breast cancer, while a new drug of the same triphenylethylene class, namely toremifene, 4-chloro-1,2-diphenyl-1-[4-[2,2-(N,N-dimethylamino)ethoxy]phenyl]but-1-ene (Farmos Fc1157 a), has been developed since 1980. Thus, there is now considerable interest in their pharmacokinetics and metabolism because many metabolites are biologically active and may have higher affinity for the estrogen receptor than the parent drug [2,3].

Tamoxifen and toremifene[a] have similar chemical structures with the exception of substitution of a chlorine atom for a hydrogen atom on the ethylene alkyl chain of TAM[b] (Fig. 1). Both anticancer drugs are extensively metabolized and several metabolites have been detected in human serum [4,5]. The main metabolic pathway consists of N-oxidative demethylation leading either to N-demethyl-TAM (compound X) or to N-demethyl-TOR (compound I). Other more hydrophilic metabolites, especially the deaminohydroxy (TAM Y or TOR III) and 4-hydroxy (TAM B or TOR II) metabolites, have been con-

[a] Toremifene and tamoxifen are both geometric isomers of *Z* type and not *trans* as mentioned by many authors.

[b] Abbreviations used: see Fig. 1. Trivial nomenclature was used for metabolites: letters X, Z, Y, B, BX and F for tamoxifen and roman numbers for toremifene (I, II, III, IV, VI and X).

* Corresponding author.

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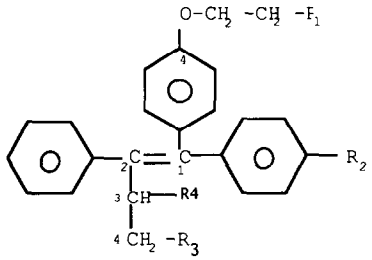

Number	Compound R_3 = Cl	R_1	R_2
1	TOR	$N-(CH_3)_2$	H
2	TOR I or DMTOR	$NH-CH_3$	H
3	TOR X or di-DMTOR	NH_2	H
4	TOR III or deaminohydroxy-TOR	OH	H
5	TOR II or 4 - OH - TOR	$N-(CH_3)_2$	OH
6	TOR IV or 4 - OH - DMTOR	$NH-CH_3$	OH
7	TOR VI or 4 - OH - deamino - hydroxy-TOR	OH	OH

Number	Compound R_3 = H	R_1	R_2
1	TAM	$N-(CH_3)_2$	H
2	TAM X or DMTAM	$NH-CH_3$	H
3	TAM Z or di-DMTAM	NH_2	H
4	TAM Y or deaminohydroxy-TAM	OH	H
5	TAM B or 4 - OH - TAM	$N-(CH_3)_2$	OH
6	TAM BX or 4 - OH - DMTAM	$NH-CH_3$	OH
7	TAM F or 4 - OH - deamino - hydroxy-TAM	OH	OH
8	TAM No or TAM - N - oxide	$-N^{+}(O^{-})-(CH_3)_2$	H
9	α - OH - TNO or α- hydroxy - TAM N-oxide	$-N^{+}(O^{-})-(CH_3)_2$	R4 = OH

Fig. 1. Chemical structure of toremifene (R_3 = Cl) and tamoxifen (R_3 = H) and their metabolites.

firmed in serum from women treated with either tamoxifen [6,7] or toremifene [8,9]. Many other metabolites have been tentatively identified in plasma and human tissues [10] and in the rat [11]. There has been little use of established techniques to study *in vitro* the metabolism of such drugs. It almost seems obvious that the use of human liver microsomes could provide invaluable information about the metabolic pathways of these two antiestrogens in man. Furthermore, until now, the nature of enzymes involved in these metabolic pathways and their localization have not been studied. For this study, a single high-performance liquid chromatographic (HPLC) assay was needed in order to separate a broad range of metabolites. To this end, we carried out a systematic study of their chromatographic behaviour. The improvement in the separation parameters allowed the identification of three main metabolites of the two triphenylethylene drugs produced in human liver microsomes by cytochrome CYP 3A4/3A5 enzymes [12,13].

EXPERIMENTAL

Chemicals

Toremifene citrate and all its metabolites were kindly provided by Farmos (Oulu, Finland). Tamoxifen and 4-hydroxytamoxifen were obtained from Sigma (St. Louis, MO, USA); the other tamoxifen metabolites were kindly provided by Dr. McCague (Institute of Cancer Research, Sutton, UK). HPLC-grade acetonitrile, methanol, ammonium acetate, acetic acid and triethylamine were obtained from Merck (Darmstadt, Germany).

Apparatus and chromatography

The anlayses were performed on Spectra-Physics (Santa Clara, CA, USA) 8750 or 8810 pump systems with one solvent-delivery system, a Rheodyne 7125 manual injector, an LDC-UV 3100 detector (Riviera Beach, FL, USA) and an SP 4290 integrator. The four reversed-phase columns were either the usual octadecylsilane (ODS) phases such as Nucleosil C_{18} or Nova-Pak C_{18} with 7 and 13% carbon loading, respectively (Table I), or octyl- and octadecylsilane phases specially treated for the analysis of basic compounds, namely LiChrosorb RP-Select-B and Nucleosil C_{18} A/B with 12 and 25% carbon loading, respectively. The mobile phase consisted of acetonitrile–100 m*M* ammonium acetate–triethylamine (46:54:0.05, v/v/v) adjusted to pH 6.4 with acetic acid for the classical ODS phases; the flow-rate was 1 ml/min. For the two specific

TABLE I

PHYSICOCHEMICAL CHARACTERISTICS OF FOUR STATIONARY PHASES USED

ND = Information not available from suppliers.

HPLC column	Alkyl chain	Particle diameter (μm)	End-capped	Pore diameter (nm)	Carbon loading (% C)	Specific area (m^2/g)	Surface coverage (μmol/m^2)	Length × I.D. (mm)	Supplier
RP-Select-B	C_8	5	Yes	6	12	360	3.2	250 × 4	Merck
Nucleosil C_{18} A/B	C_{18}	5	Yes	10	25	300	ND	250 × 4	Macherey-Nagel
Nucleosil C_{18}	C_{18}	5	Yes	10	13	300	2.1	250 × 4.6	Macherey-Nagel
Nova-Pak C_{18}	C_{18}	4	Yes	6	7	120	3.2	150 × 3.9	Waters-Millipore

phases, the mobile phase consisted of methanol–water–triethylamine (80:20:0.01, v/v/v) with a flow-rate of 0.8 ml/min. Eluates were monitored by UV detection at either 277 or 238 nm at 0.01 a.u.f.s. The capacity factor (k') was calculated from the solute retention time (t_R) and dead time (t_0) according to the equation: $k' = (t_R - t_0)/t_0$. Dead time was determined by means of injection of methanol detected at 220 nm.

In order to identify tamoxifen and toremifene metabolites in some biological samples, detection was carried out by spectrofluorimetry after on-line photocyclization of HPLC eluates. The on-line UV photoreactor was from Knauer (Strasbourg, France) and the spectrofluorometer was an SFM 25 (Kontron, Zurich, Switzerland) whose the excitation and emission wavelengths were 258 and 318 nm, respectively, with a 10-nm slit width.

Biological applications

Incubations of tamoxifen or toremifene with microsomal preparations from human livers were carried out as previously described [12,13]. In outline, the standard incubation mixture contained 0.1 *M* potassium phosphate buffer pH 7.4, 1 m*M* EDTA, 3 m*M* magnesium chloride, 0.5 m*M* tamoxifen or toremifene and 1 mg microsomal protein. The reaction was started at 37°C by addition of 1.2 m*M* NADPH. After 60 min shaking at 37°C, the reaction was stopped by addition of 5 ml of chilled chloroform. After vortex-mixing, the aqueous phase was re-extracted at pH 9.0 with 5 ml of chloroform. The organic extracts were pooled and dried at 40°C under nitrogen stream. To the dried residue was added 0.2 ml of a methanol–water (85:15, v/v) mixture for HPLC analysis. Control incubations were run as described above except that NADPH was omitted or the incubation mixture was bubbled with carbon monoxide before NADPH addition.

For the identification of metabolites of toremifene, ^{3}H-labelled toremifene (specific activity 6 mCi/mmol, from Farmos) was incubated at 0.5 *M* with 2 mg of microsomal proteins from Br032, Br039 and Br047 samples (prepared as previously described in ref. 14) for 100 min under the conditions described above. The chloroformic extracts were analysed by HPLC on either Nova-Pak C_{18} or LiChrosorb RP-Select-B as described above. HPLC eluates were collected and their radioactivity counted by liquid scintillation spectrometry. Metabolites were identified on the basis of their retention times, UV spectral characteristics and ^{3}H label.

RESULTS AND DISCUSSION

Chromatographic behaviour

Table II shows the capacity factor (k') of seven

TABLE II

CAPACITY FACTORS (k') OF TOREMIFENE (TOR) AND TAMOXIFEN (TAM) AND THEIR METABOLITES ANALYSED ON FOUR STATIONARY PHASES

HPLC conditions are described in the Experimental section. For nomenclature of compounds, see Fig. 1. ND = Not determined.

Compound	LiChrosorb RP-Select-B		Nucleosil C_{18} A/B		Nucleosil C_{18}		Nova-Pak C_{18}	
	TOR	TAM	TOR	TAM	TOR	TAM	TOR	TAM
1	16.2	43.2	42.1	66.5	17.4	27.8	25.7	34.1
2	25.5	67.0	35.8	57.1	11.2	17.5	17.0	22.4
3	14.8	38.5	27.5	42.6	7.8	11.7	12.2	15.7
4	4.8	12.6	16.6	25.3	14.8	22.3	32.5	40.9
5	7.4	19.1	12.9	17.7	5.5	7.2	6.3	6.7
6	11.4	28.9	11.3	16.0	4.2	5.4	6.1	5.0
7	2.7	6.9	6.4	8.9	5.9	7.5	8.8	9.2
8	–	21.5	–	18.3	–	ND	–	52.2
9	–	8.1	–	5.0	–	8.9	–	4.1

and nine metabolites of toremifene and tamoxifen, respectively, on the four reversed phases studied. The capacity factors of TAM and its metabolites were higher than those of TOR and its metabolites, except for TAM BX on the Nova-Pak C_{18} column. The reduced retention of TOR *versus* TAM was due to the polar chlorine atom of the ethylene alkyl chain.

The highest capacity factors were observed on the Nucleosil C_{18} A/B for the most apolar compounds, namely TOR, TAM and their mono-demethylated derivatives DMTOR and DMTAM (compounds **2**). This chromatographic behaviour could be explained by the strong hydrophobic interaction between apolar solutes and the most apolar stationary phase as a result at its higher carbon loading.

A salient feature in the retentions of TAM and TOR and their mono-methylated derivatives, namely DMTAM and DMTOR (compounds **2**), was that their retentions were reversed on LiChrosorb RP-Select-B compared with the other stationary phases studied. (Fig. 2). This allowed us to take advantage of such chromatographic behaviour because the major metabolites of TAM and TOR, *i.e.* DMTAM and DMTOR, could easily be separated from the parent drug and other metabolites (Fig. 3). The same order of elution of compounds with a triphenylethylene ring bearing either a mono-methylamino or a dimethylamino function on the ethoxy group of aromatic ring was also observed on LiChrosorb RP-Select-B: 4-OH-TOR (compound **5**) was eluted before 4-OH-DMTOR (compound **6**) like 4-OH-TAM (compound **5**) *versus* 4-OH-DMTAM (compound **6**), while this order of elution was reversed on all the three other phases studied. Such a chromatographic behaviour of N-desmethyltamoxifen was previously reported on Hypersil ODS [15] and ODS-2 [16] stationary phases (carbon loading of 9.5 and 10.5% and surface coverage of 2.6 and 2.2 μmol/m^2, respectively) with very different mobile phases.

The basic derivatives (TOR X or TAM Z; compounds **3**) were always eluted after the phenolic (TOR II, TOR IV, TAM B and TAM BX; compounds **5** and **6**, respectively) derivatives. Another unexpected retention was observed for derivatives with a triphenylethylene ring bearing a hydroxy-ethoxy group, namely TOR III and TAM Y (compounds **4**). These two compounds had the most apolar behaviour only on Nova-Pak C_{18}, as previously reported [9]. This allowed a clear identification of these metabolites of

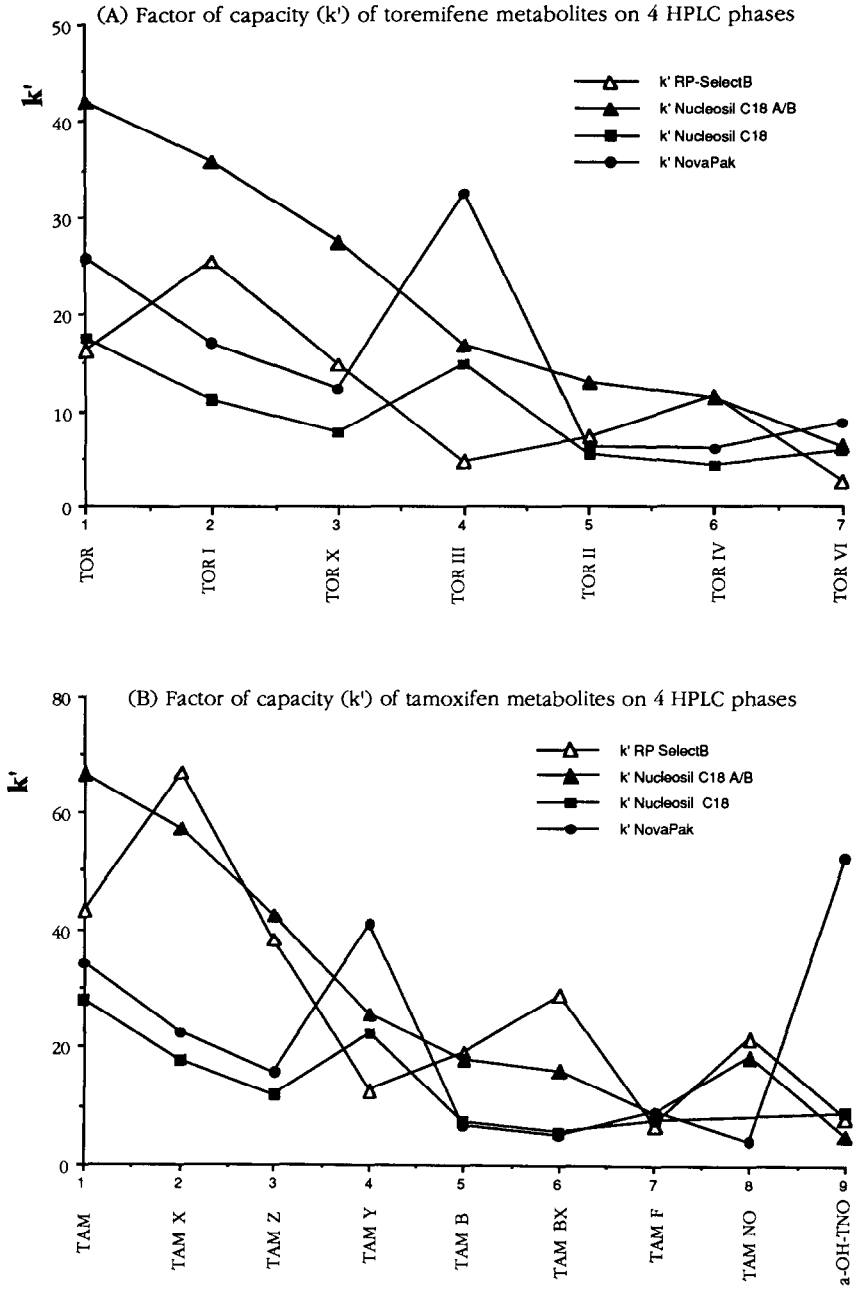

Fig. 2. Variation in capacity factor (k') of (A) toremifene and its metabolites and (B) tamoxifen and its metabolites according to their chemical structures on four HPLC stationary phases. For identification of metabolites, see Fig. 1.

TAM or TOR incubated with human liver microsomes (Figs. 3 and 4). Their formation needed two successive N-demethylations of the parent drug followed by a hydroxylation step [3]. One serious drawback was the stability of packings, especially Nova-Pak C_{18}, under the conditions described above, resulting in great variations in the retention times of TOR III *versus* TOR. Ta-

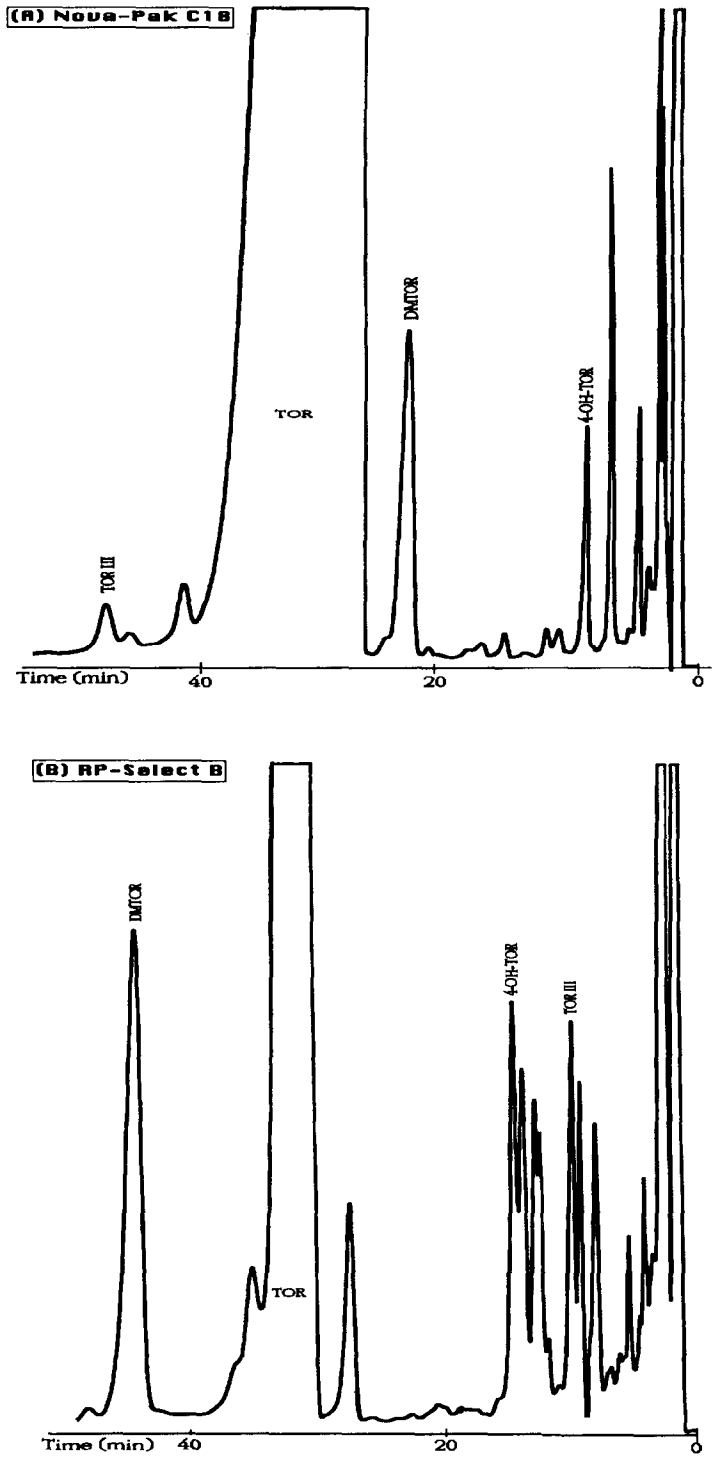

Fig. 3. HPLC profiles of toremifene (TOR) metabolism by the same human liver microsomal preparation (Br032) on two stationary phases, Nova-Pak C_{18} (A) and LiChrosorb RP-Select-B (B). For experimental conditions: see Experimental section. UV detection: (A) 277 nm; (B) 238 nm.

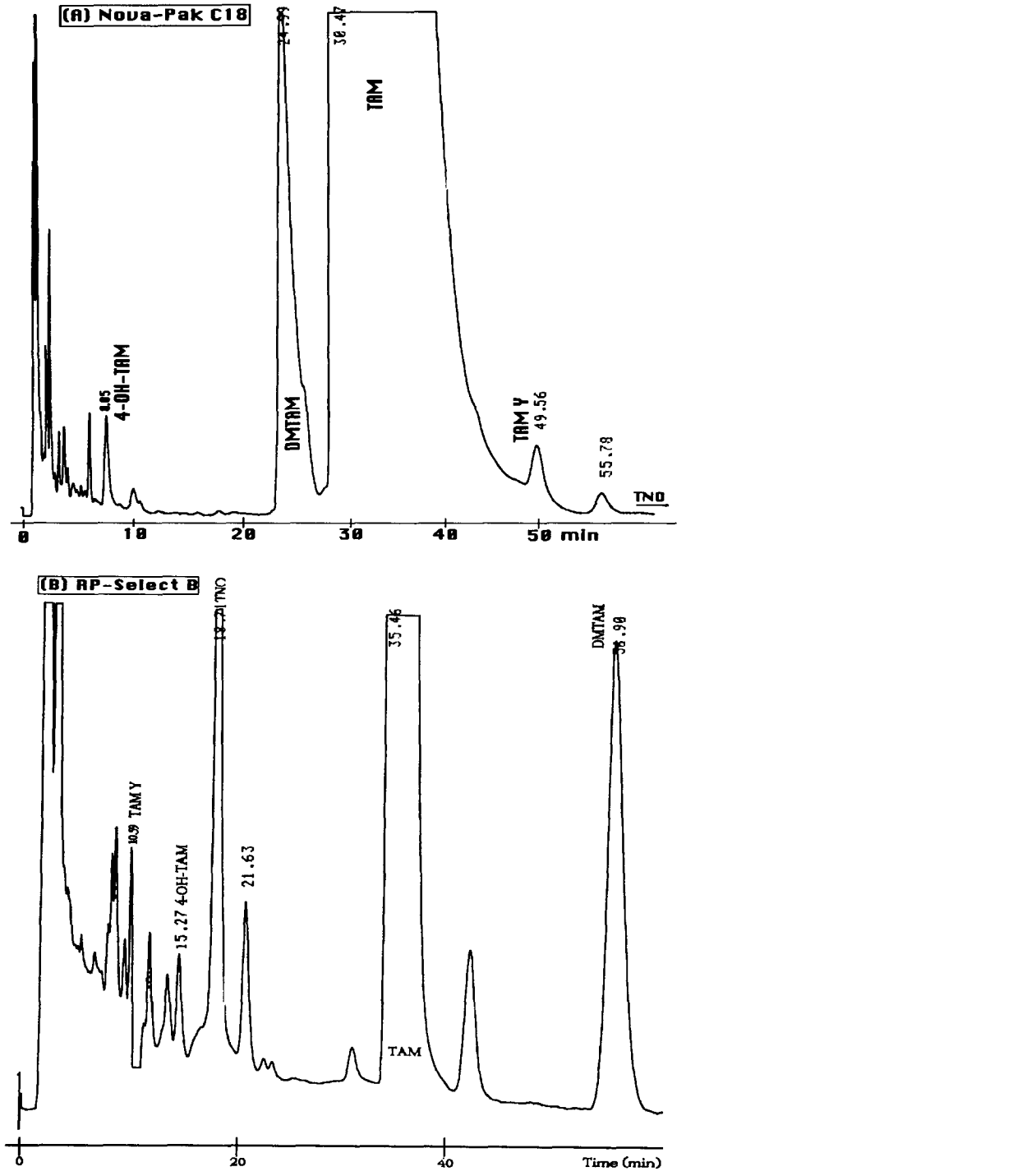

Fig. 4. HPLC profiles of tamoxifen (TAM) metabolism by the same human liver microsomal preparation (Br035) on two stationary phases, Nova-Pak C_{18} (A) and LiChrosorb RP-Select-B (B). For experimental conditions: see Experimental section. UV detection: (A) 277 nm; (B) 238 nm.

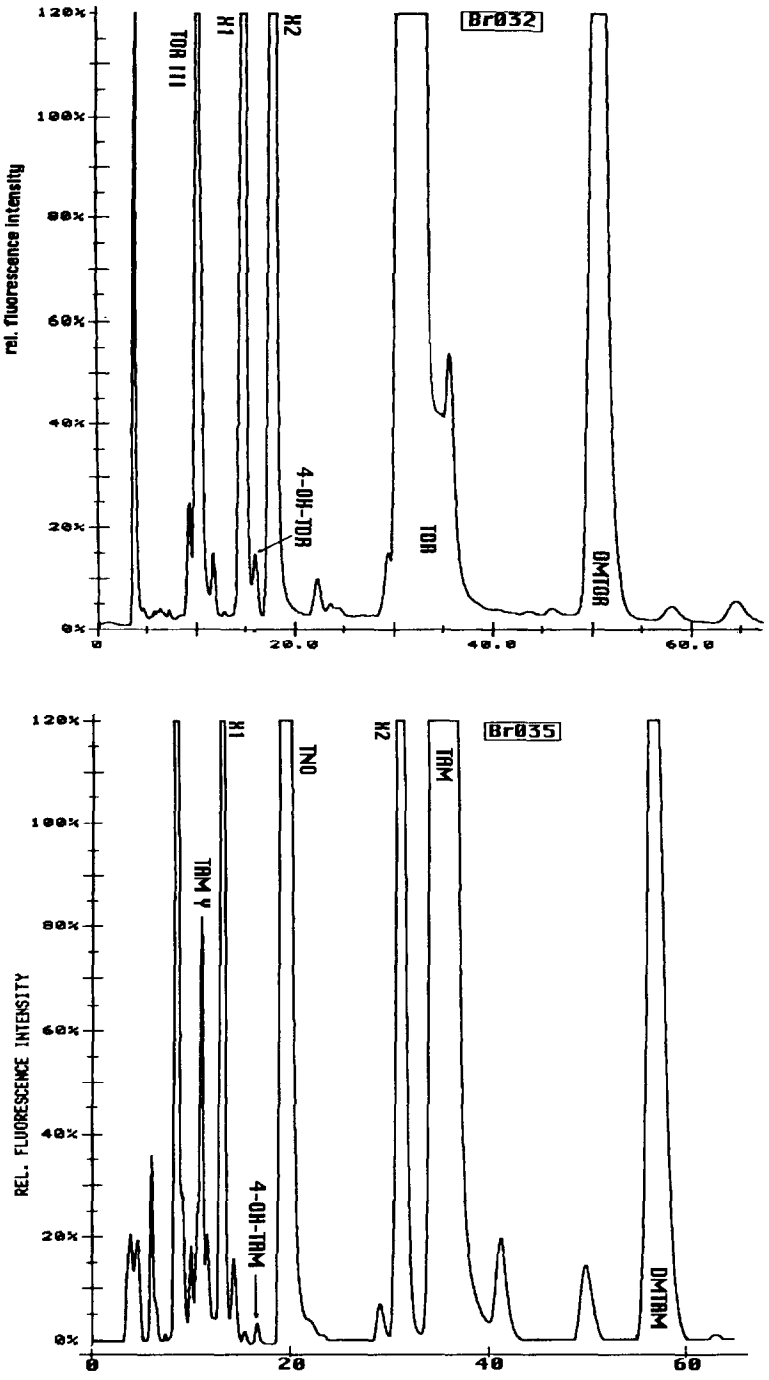

Fig. 5. HPLC profiles of toremifene (TOR) and tamoxifen (TAM) metabolism by two human liver microsomes: Br032 and Br035. Detection by fluorescence: excitation at 258 nm and emission at 318 nm after photocyclization of HPLC eluates. HPLC conditions: LiChrosorb RP-Select-B; mobile phase, methanol–water–triethylamine (80:20:0.1, v/v/v); X1 and X2 metabolites are not yet identified.

moxifen N-oxide (TNO, compound **9**) was so hardly retained on Nova-Pak C_{18} that it was eluted as a non-Gaussian peak.

Detection

The UV absorption maxima of tamoxifen and toremifene are 238 and 277 nm with a relative ratio of absorbance of 1.7. The UV characteristics are quite similar for the different metabolites but with different relative ratios, namely 0.9 for DMTOR and 2.7 for tamoxifen N-oxide. Thus, UV detection could be carried out at either 238 or 277 nm, depending on the sensitivity required, the background of detection and the presence of interfering compounds in biological samples, as previously described [17]. Figs. 3 and 4 show that the shoulder on the solvent front decreased dramatically when detection was set at 277 nm (Figs. 3A and 4A).

The sensitivity of detection can be improved by on-line post-column photochemical activation of HPLC eluates followed by fluorescence detection [16–19]. This UV irradiation technique allows the photocyclization of triphenylethylene derivatives into phenanthrene derivatives [19], which are easily detectable by fluorescence. Fig. 5 shows the HPLC profiles of toremifene and tamoxifen metabolized by two human liver microsomes, Br032 and Br035, with on-line photocyclization followed by spectrofluorimetric detection. When compared with Figs. 3 and 4, it is obvious that the detection was more specific, eliminating from biological samples the interfering peaks not originating from triphenylene substrates. Furthermore, if the limit of detection of minor metabolites was dramatically increased, it is worthwhile noticing that the yield of photocyclization of the 4-hydroxylated derivative was less than that of other compounds.

Recovery from biological samples

The mean recovery of tamoxifen was estimated by comparing the peak-height ratios of spiked plasma before extraction according to the procedure described above with a standard curve made up in methanol (10–40 ng injected: see Fig. 6). Mean recovery was 96 ± 25% (n = 6) for tamoxifen and 104 ± 8% (n = 6) for N-desmethyltamoxifen in the range 25–250 ng/ml. When ^{3}H-radiolabelled toremifene was incubated with human liver microsomal preparations, the identified compounds, *i.e.* TOR, 4-OH-TOR, DMTOR and TOR III, represented 68.6 ± 8.4% (n = 10) of added radioactivity [13], while the total radio-

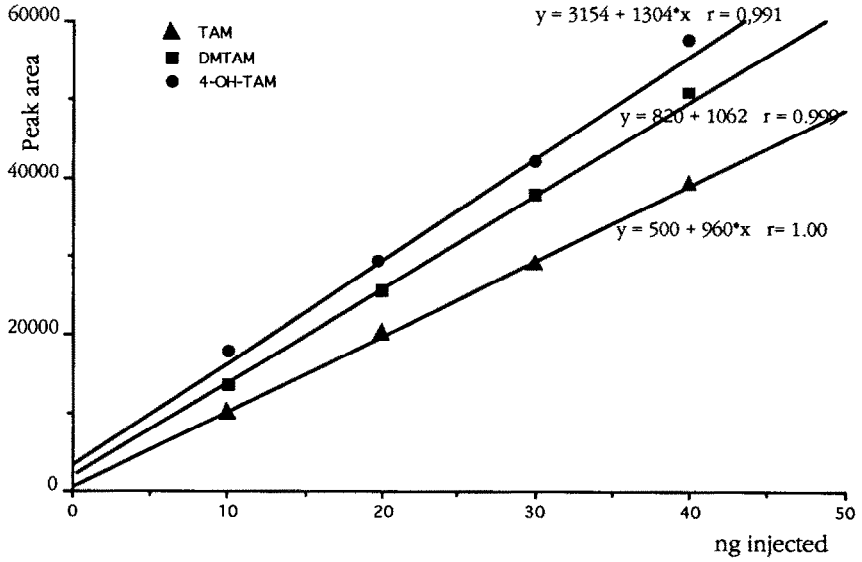

Fig. 6. Calibration curves of tamoxifen (▲), N-desmethyltamoxifen (■, DMTAM) and 4-hydroxytamoxifen (●, 4-OH-TAM): peak area *versus* amount injected (ng) on LiChrosorb RP-Select-B with UV detection at 238 nm.

TABLE III

METABOLIC RATES OF [^{3}H]TOREMIFENE (0.5 m*M*) BY THREE HUMAN MICROSOMAL PREPARATIONS ON TWO HPLC COLUMNS

A = LiChrosorb RP-Select-B; B = Nova-Pak C_{18}. [^{3}H]Toremifene (500 μM) was incubated with 2 mg of protein. For analytical conditions, see Experimental section.

Compound	Metabolic rate (pmol/min/mg protein)					
	Br032		Br039		Br047	
	A	B	A	B	A	B
DMTOR	200	208	108	104	128	123
4-OH-TOR	28	34	28	23	27	22
TOR III	21	15	14	15	15	18

activity extracted from incubation medium represented 82.3 ± 8.45% of added radioactivity.

Identification of metabolites in human liver microsomes

The assay with tamoxifen or toremifene was developed using human liver microsomal preparations in order to determine the major metabolic pathways catalysed by cytochrome P450 enzymes and the nature of P450 isozymes involved.

Figs. 3 and 4 show the HPLC profiles of toremifene and tamoxifen metabolites formed by two human liver microsomes. The same metabolic pathways were observed. The major route was N-oxidative demethylation leading to either N-DMTOR or N-DMTAM. These primary metabolites were successively N-demethylated then hydroxylated to either TOR III or TAM Y. These two secondary metabolites were not easily quantified by HPLC on LiChrosorb RP-Select-B because of the presence of interfering compounds in the solvent front. This problem was overcome by analysis on the Nova-Pak C_{18} packing, which allowed the elution of TOR III or TAM Y after the parent drug. The third metabolite resulted from the hydroxylation of the aromatic carbon number 4, giving 4-OH-TOR or 4-OH-TAM. Until now, it has been difficult to detect and quantify the minor metabolite 4-OH-TAM [12]. By combining optimized HPLC conditions on LiChrosorb RP-Select-B and Nova-Pak C_{18}, this metabolite could be measured. Such a metabolite represented about one fifth of that of DMTOR, whereas 4-hydroxylation was a very minor pathway in TAM metabolism [12,13,20] (see Figs. 3 and 4). Finally, the HPLC peaks of these three metabolites of toremifene, namely DMTOR, 4-OH-TOR and TOR III, were shown to be pure. Indeed their metabolic rates, as determined by separating the radiolabelled metabolites of [^{3}H]TOR on LiChrosorb RP-Select-B and Nova-Pak C_{18} were quite similar (Table III).

The identity of these three metabolites of the two triphenylene class drugs, namely DMTOR or DMTAM, 4-OH-TOR or 4-OH-TAM and TOR III or TAM Y, was confirmed by spectrofluorimetric detection after on-line photocyclization into phenanthrene derivative (Fig. 5). Finally, these metabolic pathways were catalysed by cytochrome P450 enzymes as they were NADPH-dependent and were inhibited by carbon monoxide (Table IV).

The method of analysis of metabolites of toremifene and tamoxifen described above, by combining both HPLC columns and mode of detection, is reliable and suitable. It allowed successful quantification of metabolites in human liver microsomes and therefore identification of cytochrome P450 isoenzymes involved [12,13].

TABLE IV

PERCENTAGE INHIBITION OF TOREMIFENE (TOR) AND TAMOXIFEN (TAM) METABOLISM BY HUMAN LIVER MICROSOMES AFTER BUBBLING SAMPLES WITH CARBON MONOXIDE

ND = Not determined.

Compound	Inhibition (%)	Compound	Inhibition (%)
DMTOR	79 ± 5	DMTAM	71 ± 4
4-OH-TOR	72 ± 3	4-OH-TAM	75 ± 2
TOR III	79 ± 5	TAM Y	ND

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