Biochemical Pharmacology, Vol. 41, No. 12, pp. 1911–1919, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

IDENTIFICATION OF THE CYTOCHROME P450 IIIA FAMILY AS THE ENZYMES INVOLVED IN THE N-DEMETHYLATION OF TAMOXIFEN IN HUMAN LIVER MICROSOMES

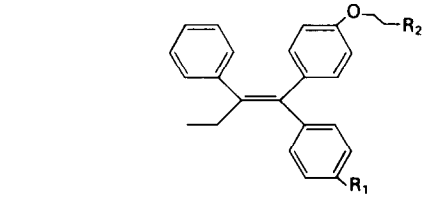
FRANÇOISE JACOLOT,* ISABELLE SIMON,* YVONNE DREANO,* PHILIPPE BEAUNE,† CHRISTIAN RICHE* and FRANÇOIS BERTHOU*‡

* Laboratoire de Biochimie-Pharmacologie, Faculté de Médecine, BP 815-29285 Brest Cedex, France; and † INSERM U-75, CHU Necker, 75230, Paris, France

(*Received* 17 *October* 1990; *accepted* 19 *January* 1991)

Abstract—The antiestrogen tamoxifen (Tam or Nolvadex, ICI)—Z-1-[4-[2-(dimethylamino) ethoxy]phenyl]-1,2-diphenyl-1-butene is widely used in treatment of hormone-dependent breast cancer. The drug is extensively metabolized by cytochrome P450 dependent hepatic mixed function oxidase in man, yielding mainly the *N*-desmethyl metabolite (DMT). This study has been carried out to determine the P450 enzyme involved in the N-oxidative demethylation of Tam in microsomal samples from 25 human livers (23 adults, two children). This metabolic step was inhibited by carbon monoxide up to 75%. Tam was demethylated into DMT with an apparent K_m of 98 ± 10 μM; rates varied between 37 and 446 pmol/min/mg microsomal protein. These metabolic rates were strongly correlated with 6β-hydroxylation of testosterone ($r = 0.83$) and erythromycin *N*-demethylase ($r = 0.75$), both activities known to be associated with P450 IIIA enzyme. To further assess whether or not the Tam demethylation pathway is catalysed by the same P450, the inhibitory effect of TST on this reaction was determined. The competitive inhibition had an apparent K_i of 100 ± 10 μM. Drugs such as erythromycin, cyclosporin, nifedipine and diltiazem were shown to inhibit *in vitro* the metabolism of tamoxifen. Furthermore the P450 IIIA content of liver microsomal samples, measured by Western blot technique using a monoclonal P450NF (nifedipine) antibody, was strongly correlated with DMT formation ($r = 0.87$). Tam *N*-demethylase activity was inhibited by more than 65% with polyclonal anti-human anti-P450NF. All these *in vitro* observations establish that a P450 enzyme of the IIIA sub-family is involved in the oxidative demethylation of tamoxifen in human liver.

Tamoxifen§ [compound **1** (see Fig. 1), Nolvadex from ICI [46, 474] is a non-steroidal anti-estrogen which is currently used for the treatment of human hormono-dependant breast cancer [1]. Metabolism may play an important role in modulating the biological activity of the drug in so far as it is thought to act by competing with cytoplasmic estradiol receptor by means of its metabolites, especially 4-hydroxy-metabolite (compound **2**). The drug is extensively metabolized by hepatic cytochrome P450 dependent mixed function oxidase in man [2] and various other mammalian species [3–9]. Originally the 4-OH-T was the only serum metabolite detected in human serum [2], but later Adam *et al.* [10], showed the major serum metabolite to be the *N*-desmethyl derivative, DMT (compound **3**). Up to now five metabolites have been identified in human serum: DMT, 4-OH-T, 4-OH-DMT, compounds **Z** and **Y** [11–14]. *In vitro* studies with rat [5, 7], rabbit [6] liver microsomes or isolated rat hepatocytes [3, 4]



Compound 1	TAM	$R_2 = N\text{-}(CH_3)_2$	$R_1 = H$
Compound 2	4-OH-TAM	$R_2 = N\text{-}(CH_3)_2$	$R_1 = OH$
Compound 3	DMT	$R_2 = NH\text{-}CH_3$	$R_1 = H$
Compound 4	TNO	$R_2 = N \rightarrow O$ | $(CH_3)_2$	$R_1 = H$

Fig. 1. Chemical structure of tamoxifen and its major metabolites.

identified other metabolites such as TNO (compound **4**) and derivatives. All these metabolites may together with the parent drug contribute to the observed clinical response. Thus, the present study was undertaken to determine the metabolic pathways of Tam in human liver microsomes. In addition previous studies suggest the involvement of the microsomal cytochrome P450 superfamily in Tam metabolism by rodents [6, 9]. So in order to identify the P450 enzyme involved in the metabolism of Tam, a thorough investigation was carried out with human liver microsomes. In this paper evidence is presented

‡ To whom requests for reprints should be addressed.

§ Abbreviations: Tam, tamoxifen, *Z*-1-[4-[2-(dimethylamino) ethoxy]phenyl] - 1,2 - diphenyl - 1 - butene; DMT, *N*-desmethyltamoxifen; TNO, tamoxifen *N*-oxide; 4-OH-T, 4-hydroxy tamoxifen; 4-OH-DMT, 4-hydroxy-*N*-desmethyl tamoxifen; **Z**, *N,N* - didesmethyltamoxifen; **Y**, *Z* - 1[4 - [2 - (hydroxy)ethoxy]phenyl] - 1,2 - diphenyl - 1 - butene; ER, estrogen receptor; PBS, phosphate buffer saline; TST, testosterone.

that human P450 IIIA* enzymes family, previously identified as nifedipine, cyclosporine, erythromycin oxidases [15–17] are the major enzymes involved in the N-demethylation of Tam.

MATERIALS AND METHODS

Chemicals

Tam was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and its metabolites are a gift of Dr McCague (Institute of Cancer Research, Sutton, U.K.). Erythromycin, cyclosporin and diltiazem were supplied by Sigma, Sandoz (Rueil-Malmaison, France) and Synthelabo-LERS (Meudon la Forêt, France) respectively. Testosterone and its metabolites were from Steraloids (Wilton, NH, U.S.A.). [4-^{14}C]Testosterone (sp. act. 57 mCi/mmol) was from Amersham (Amersham, U.K.).

Human liver samples and microsomes preparation

Human liver samples were obtained from 23 adult organ donors immediately after death (19 males, four females, mean age: 37 ± 12 years) and two children: 5 months old (Br023) and 2 years old (Br025). Sampling was made in accordance with French legal considerations. Ethical Committee approval was obtained prior to this study. Pre-death dietary habits and exposure to environmental chemicals were not known. Liver fragments were frozen immediately and stored in liquid nitrogen. Microsomal fractions were prepared as previously described [18] and stored at −80° until use.

Determination of monooxygenase activities

Protein content of microsomal preparations was determined by the method of Lowry *et al.* [19]. Total cytochrome P450 levels were measured according to Omura and Sato [20], with molecular extinction coefficient of 91 mM $^{-1}$ cm $^{-1}$.

Tamoxifen metabolism. Incubations were run in polypropylen test tubes or siliconized glass tubes in the dark. The standard incubation mixture contained, in a final volume of 0.5 mL, 40 mM potassium phosphate buffer pH 7.4, 120 mM potassium chloride, 5 mM magnesium chloride, 0.5 mM tamoxifen and 2.4 mM NADPH. After 2 min of pre-incubation at 37°, the reaction was started by addition of 1 mg microsomal proteins. After 15 min shaking at 37°, the reaction was stopped by addition of 5 mL chloroform. The mixture was vortexed and re-extracted at pH 9.0 with 5 mL chloroform. The organic extracts were pooled and dried at 40° under nitrogen stream. To the drug residue was added 0.5 mL methanol–water (90/10; v/v) mixture for HPLC analysis. Control incubations were run as described except that microsomal proteins or NADPH were omitted. Tam metabolites were analysed by HPLC with a Lichrosorb C-18 RP-Select B column, 250 × 4 mm, from Merck (Darmstadt, F.R.G.) eluted by a mobile phase consisting of methanol, water, triethylamine (90/10/0.1; v/v/v) with a flow rate of 0.8 mL/min. Eluates were detected by UV at 238 nm with a sensitivity of 0.005 AUFS. Peaks were identified by their retention time and spectral characteristics in comparison with standard compounds. Peaks were quantified by an integrator-calculator SP-8100 from Spectra-Physics (Santa Clara, CA, U.S.A.). Overall biotransformation was expressed as the percentage of substrate transformed into known metabolites relative to the untransformed drug.

Testosterone metabolism. The standard incubation mixture contained, in a final volume of 1 mL, 100 mM potassium phosphate buffer pH 7.4, 0.05 mM EDTA, 0.5% glycerol, 25 μM [4-^{14}C]testosterone (sp. act. 5 mCi/mmol) and 0.2 mg microsomal proteins. After pre-incubation at 37°, the reaction was started by addition of 2.4 mM NADPH. After 15 min shaking at 37°, the reaction was stopped by addition of 5 mL methylene chloride. The organic phase was taken to dryness under nitrogen stream at 45°. Testosterone metabolites were analysed either by thin-layer chromatography according to Waxman *et al.* [21] or by HPLC according to Sonderfan *et al.* [22]. In all cases, 6β-hydroxy-testosterone was identified according to its chromatographic behavior and its radioactivity was counted by liquid scintillation spectrometry.

Erythromycin demethylase. Erythromycin demethylation activity was determined at 37° in an incubation mixture containing 1 mM erythromycin, 1 mg of microsomal protein, 2.5 mM hydrochloride semicarbazide in a final volume of 1 mL of 0.1 mM potassium phosphate buffer pH 7.4. The reaction was initiated by the addition of 2 mM NADPH, proceeded for 15 min, and formaldehyde formation was measured according to Werringloer [23].

Inhibition of Tam metabolism by different compounds

Incubations were performed as above described with 0.1 mM tamoxifen, except that testosterone, erythromycin, cyclosporin, nifedipine, estradiol or diltiazem were added at concentrations ranging between 25 and 500 μM. Control experiments were conducted in the same conditions with the same amount of organic solvent, acetone or methanol, needed for solubilization of drugs. Three liver samples were used for these experiments: FH-2A, Br022 and Br024 on the basis of their high tamoxifen-metabolizing activity.

*Inhibition of Tam metabolism by anti-*P450 IIIA *antibody*

Diluted microsomes from FH-2A sample

* P450 nomenclature. The new recommended nomenclature for cytochrome P450 (Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FP, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Philips IR, Sato R and Watermann MR, The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8**: 1–13, 1989) is used throughout this paper. The human P450 IIIA subfamily appears to have at least four distinct genes. As these genes are encoding proteins whose primary sequences are at least 82% homologous, polyconal antibodies against any P450 IIIA form are expected to cross-react with all other proteins of the subfamily. So, it is not possible to decide which form(s) of this subfamily is the one involved in Tam, nifedipine or cyclosporin metabolism. We shall accordingly only use the term P450 IIIA to designate the P450 IIIA involved in these activities.

(0.50 nmole cytochrome P450/mL) in 40 mM potassium phosphate buffer pH 7.4 were incubated at room temperature for 20 min in the absence or in the presence of increasing amounts of polyclonal P450-NF antibody [24] or non-immune rabbit IgG. Tam and NADPH were added and the reaction was allowed to proceed as indicated above.

Immunoblot analysis

Protein samples (20 μg) were separated by electrophoresis on 9% sodium dodecyl sulphate (SDS)–polyacrylamide gel according to Laemmli [25], and transferred electrophoretically to a nitrocellulose sheet [26]. After incubation at 37° for 30 min by 3% bovine serum albumin and 10% newborn calf serum in PBS, the nitrocellulose sheet was sequentially treatd with monoclonal anti-P450-NF human [24] overnight at 4°, washed with PBS then PBS containing rabbit anti-mouse immunoglobulins conjugated to peroxidase (Dako, Versailles, France). Finally the sheet was washed with PBS and the peroxidase activity was detected with 4-chloronaphtol and H_2O_2. The quantification of P450 IIIA was performed by densitometry. The integrated peak area of the various microsomal preparations was expressed as arbitrary units relatively to the amount of proteins.

Data analysis

The values are means ± SD from 24 livers; the sample from the 5-month-old subject Br023 was not included. Correlation coefficients were calculated using an ANOVA table by the least-squares regression analysis from the raw data. As a normal gaussian distribution in the population was observed, correlation coefficients were determined with 25 samples.

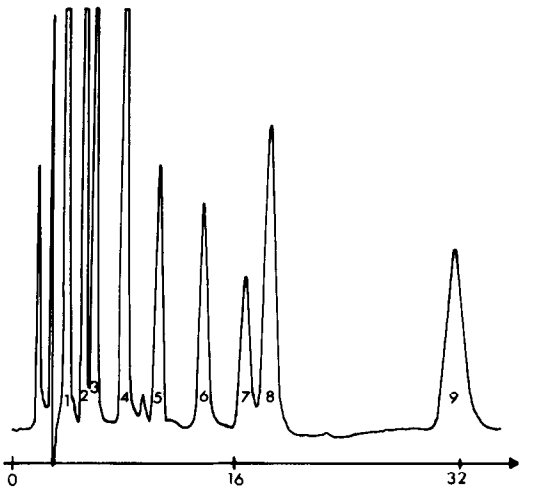


Fig. 2. HPLC chromatogram of Tam metabolites. Analysis was carried out on a Lichrosorb RP-select B 5 μm column (25 × 0.4 cm); mobile phase was methanol/water/triethylamine (80/20/0.1, v/v/v) at 0.8 mL/min. Eluates were detected at 238 nm with 0.005 AUFS sensitivity. For identification of peaks, see Fig. 1. Tam metabolites (100 ng each) are numbered as follows: 1: metabolite F or *Z*-1-[4-(2 - hydroxyethoxy)phenyl] - 1 - [(4 - hydroxy)phenyl]1,2 - diphenyl-1-butene; 2: α-hydroxy-tamoxifen-*N*-oxide; 3: metabolite Y or *Z* - 1 - [4 - (2 - hydroxyethoxy)phenyl] - 1,2 - diphenyl - 1 - butene; 4: 4 - hydroxytamoxifen; 5: tamoxifen - *N* - oxide; 6, 7: *Z* and *E* - 1 - [4 - (2 - methylamino) ethoxy)phenyl] - 1 - [4 - hydroxyphenyl] - 1,2 - diphenyl - 1 - butene; 8: tamoxifen; 9: desmethyl-tamoxifen.

RESULTS

Metabolites of tamoxifen

By using the optimized HPLC conditions, eight metabolites were totally separated within 35 min (Fig. 2). In order to reduce analysis time, the mobile phase was modified as indicated in Materials and Methods. These modified conditions did not allow separation of α-hydroxy-Tam-*N*-oxide from metabolite **Y**. Since these two compounds were not detected in biological samples from human microsomes, these analytical HPLC conditions were used. Figure 3 shows metabolic profiles of Tam incubated with human and rat microsomal samples in comparison with control incubation. DMT, 4-OH-Tam and TNO metabolites were identified by their chromatographic behavior and their UV-spectra. Furthermore, DMT peak was shown to be homogeneous by its constant absorbance ratio 240/260 nm during its elution. Whereas 4-OH-Tam was formed at a very low level (about 1.6 ± 1.3 pmol/min/mg protein) which is close to the minimum amount detectable, TNO was produced in detectable amounts, however very similar in control and human microsomal samples (Fig. 3A and B). It is worth noting that TNO was formed in amounts greater when using rat microsomes than human microsomes. Accordingly TNO was not taken into account in calculating the metabolic rate of Tam in human microsomes.

Cytochrome P450 *dependence of tamoxifen metabolism*

Preliminary experiments suggested the involvement of cytochrome P450 in the Tam metabolism by human liver microsomal samples. This evidence included localization of activity in the microsomal subcellular fraction, heat lability, absolute dependence upon the presence of NADPH for catalytic activity. Furthermore, the binding of Tam to human liver microsomes was determined by differential spectroscopy. Type I difference spectra with minima at 420 nm and maxima at 390 nm were obtained upon addition of increasing amounts of Tam in liver microsomes.

The reciprocal plot of the absorbance change at 390 minus 420 nm against Tam concentration (Fig. 4) allowed determination of an apparent dissociation constant K_s of 9 μM and a maximal absorbance change $\Delta A_{max} = 0.0064$. Moreover, DMT formation from Tam was inhibited up to 75% when the incubation mixture was bubbled with CO before addition of microsomal sample.

Kinetic parameters

Figure 5 shows the linear kinetics of DMT formation from Tam by microsomes from human liver FH-2A. K_m was determined as 98 ± 10 μM and V_{max} as 550 pmol/min/mg microsomal protein.

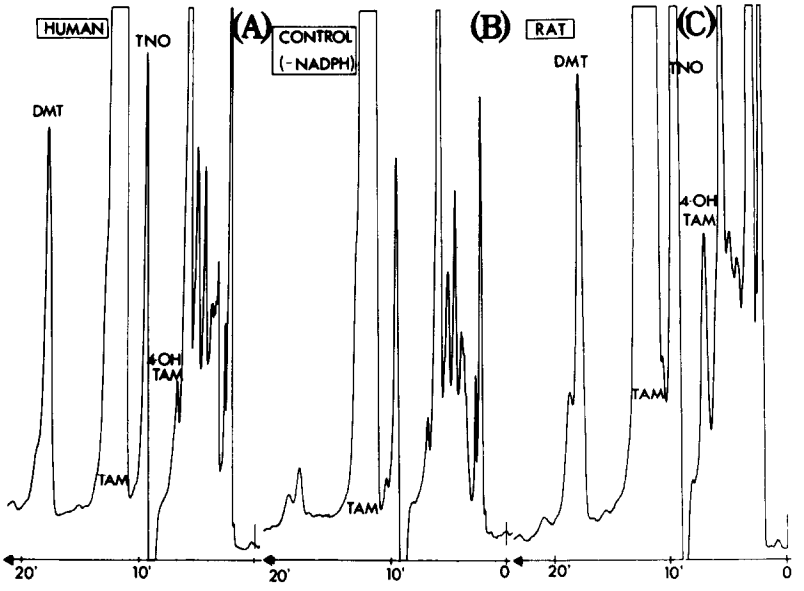


Fig. 3. Typical HPLC chromatograms of incubation medium of 0.5 mM Tam with microsomal samples from FH-2A human liver containing NADPH (A) and not containing NADPH (B = control) and from rat liver (C). For HPLC conditions, see Fig. 2. Mobile phase consisted on methanol/water/triethylamine (90/10/0.1; v/v/v) at 0.8 mL/min.

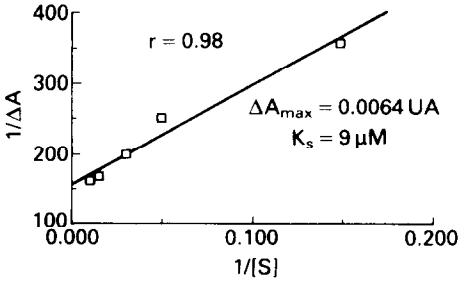


Fig. 4. Reciprocal plot of A ($A_{390} - A_{420}$) against Tam concentration. Microsomal sample from FH-2A liver was diluted at 1.6 mg protein/mL or 0.8 nmol cytochrome P450/mL. Increasing amounts of Tam were added to the sample cuvette and the same amount of ethanol to the reference cuvette. After zero recording, differential spectra were recorded.

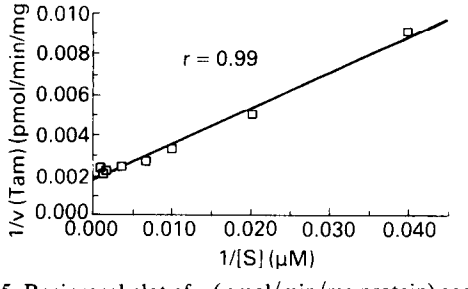


Fig. 5. Reciprocal plot of v (pmol/min/mg protein) against Tam concentration (μM) according to Lineweaver and Burk. Microsomal sample from FH-2A liver was used.

Correlation studies between Tam metabolism and different monooxygenase activities in human liver microsomes

Table 1 shows the rate of biotransformation of Tam and testosterone by 24 adult human and one newborn liver microsomes. The rate of N-oxidative demethylation of Tam ranged from 37 to 446 pmol/min/mg whereas the rate of 6β-hydroxylation of TST varied between 165 to 4075 pmol/min/mg. An excellent correlation between these two activities was observed (Fig. 6B) ($r = 0.83$; N = 25; $P < 0.001$). Similarly, Tam demethylation was significantly correlated with erythromycin *N*-demethylase activity (Fig. 6A) ($r = 0.75$; N = 24; $P < 0.001$).

Immunoblot analysis of microsomal proteins

Immunoblot analysis of isolated human liver microsomes using a monoclonal antibody reactive with P450-NF revealed a band of 52,000 Daltons as the major reactive polypeptide (Fig. 7). In two of the liver samples (Br015 and Br017) shown in the insert of figure, however, a band of 52,500 Daltons was also detected with the monoclonal antibody.

Correlation of monooxygenase activities with immunoquantitated P450 IIIA

N-Oxidative demethylation of tamoxifen as well as 6β-hydroxylation of testosterone in 25 microsomal samples was highly significantly correlated with the amount of P450 IIIA immunodetected ($r = 0.87$, $r = 0.90$ respectively) (Fig. 8).

Inhibition studies

To further assess whether or not the N-demethylation of Tam is catalysed by the P450 enzyme involved in 6β-hydroxylation of TST (i.e.

Table 1. Cytochrome P450-dependent activities in microsomal sample from 25 human livers

Subjects	Sex, age (years)	Total P450 (pmol/mg)	Tam *	TST *	ER †	P450 IIIA Δ
FH1	M,18	269	79	815	1.14	1.21
FH2A	M,41	527	446	4075	2.58	5.61
FH3	M,47	470	151	3420	1.86	3.65
Br015	M,43	414	88	985	0.85	2.81
Br016	M,44	369	123	1850	1.15	2.89
Br017	M,26	593	164	1795	1.42	2.58
Br018	F,45	334	93	950	1.00	1.80
Br019A	M,45	241	37	740	0.83	1.07
Br021	M,49	257	104	1105	1.06	1.23
Br022	M,23	286	225	3650	1.90	3.34
Br024	F,15	505	299	3935	2.12	4.49
Br025	M,2	110	128	1085	1.00	1.20
Br027	M,21	100	54	435	0.76	1.02
Br028	M,27	165	100	845	0.50	1.90
Br029	M,36	77	66	165	0.35	0.71
Br031	M,23	154	51	430	0.17	0.40
Br032	M,36	286	134	2455	1.66	2.95
Br033	M,44	363	92	1626	0.74	1.46
Br034	M,32	286	72	747	0.44	1.26
Br035	M,35	252	172	2120	1.57	2.36
Br036	M,57	153	147	1310	0.64	1.12
Br037	M,56	230	117	279	0.32	0.91
Br038	F,51	120	44	324	0.24	1.00
Br039	F,46	538	328	2450	0.98	3.88
Mean		296	138	1566	1.05	2.12
± SD		150	98	1200	0.63	1.34
Br023	F,5 months	228	97	375	ND	2.04

* pmol/mg/min.
† nmol/mg/min.
Δ, expressed as relative arbitrary relative unit.
Tam, N-oxidative demethylation; TST: 6β-hydroxylation.
ER, erythromycin *N*-demethylase.
ND, not determined.

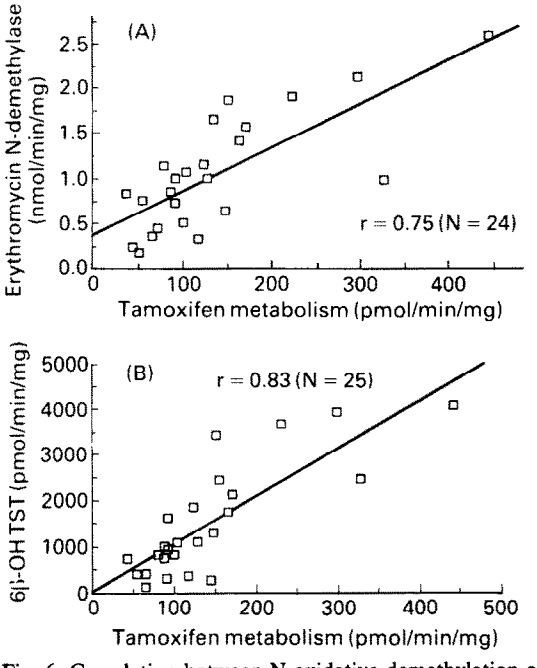


Fig. 6. Correlation between N-oxidative demethylation of Tam and erythromycin *N*-demethylase (A) and 6β-hydroxylation of TST (B). The correlation coefficient (*r*) was determined by the least-squares linear regression analysis.

P450 IIIA), the effect of TST on this reaction was determined. Figure 9 illustrates the competitive inhibition of TST on Tam demethylation with an apparent K_i of 100 ± 10 μM and the same effect of Tam on 6β-hydroxylation of TST with an apparent K_i of 10 μM.

Immunoinhibition of Tam demethylase in human liver microsomes

In order to confirm the role of P450 IIIA as the major enzyme involved in the N-demethylation of Tam, immunoinhibition experiments were carried out on microsomal preparation from FH-2A liver. Results are reported in Fig. 10.

Tam *N*-demethylase activity was inhibited by more than 65% of control activity with 5 mg immune Ig G per nmole P450.

Tamoxifen drug interactions

In order to assess drugs that interfere with the hepatic metabolism of Tam, erythromycin, cyclosporin A, nifedipine, diltiazem and estradiol were tested. Table 2 reports the results.

Clearly erythromycin, cyclosporin, nifedipine and diltiazem competitively inhibited Tam *N*-demethylase activity with apparent K_i of 20, 1, 45 and 30 μM, respectively. However, estradiol did not significantly inhibit Tam metabolism.

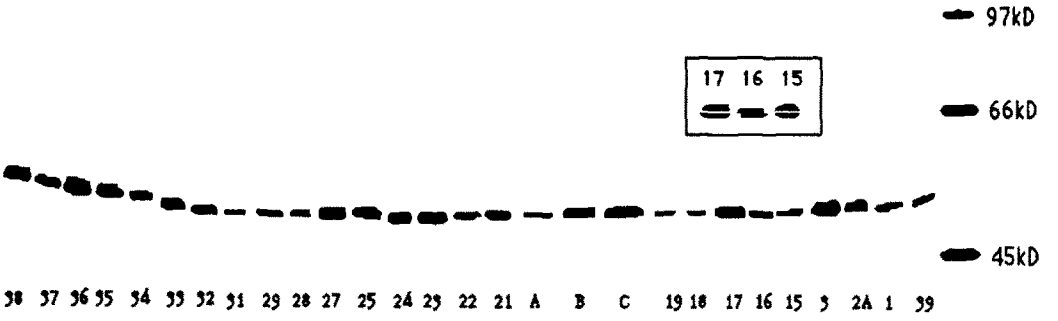


Fig. 7. Immunoblot of P450 IIIA protein. Microsomal proteins (20 μg) were probed with monoclonal anti-human P450 NF. Lanes are numbered according to human samples of Table 1. Lanes A, B, C: purified human cytochrome P450IIIA (1, 2 and 4 pmoles respectively). Right lane: molecular weight markers silver-stained. Insert: lanes 15, 16 and 17 developed on another electrophoretic gel.

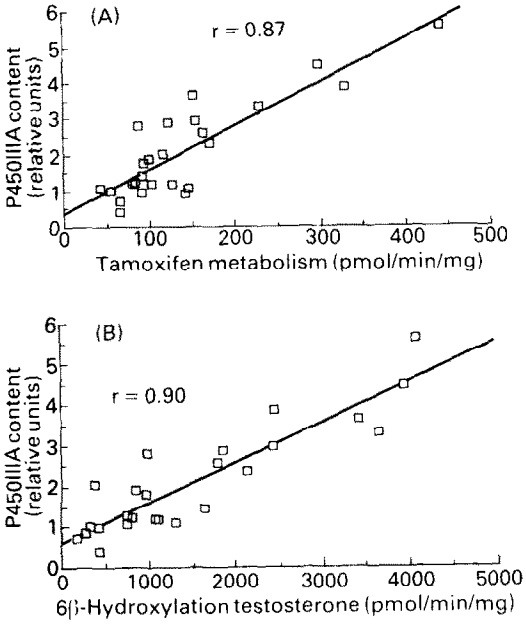


Fig. 8. Correlation between Tam-*N*-demethylase activity (A), 6β-hydroxylation of TST (B) and immunoquantitated P450 IIIA in human liver microsomes. Microsomal preparation from 25 human livers were analysed for Tam biotransformation and 6β-hydroxylation of TST. P450 IIIA content determined by immunoblot analysis of 20 μg of microsomal protein using monoclonal anti-human P450 NF. Intensity units are relative arbitrary units determined by densitometry. Correlation coefficient (*r*) was calculated by the least-squares linear regression method ($P < 0.001$).

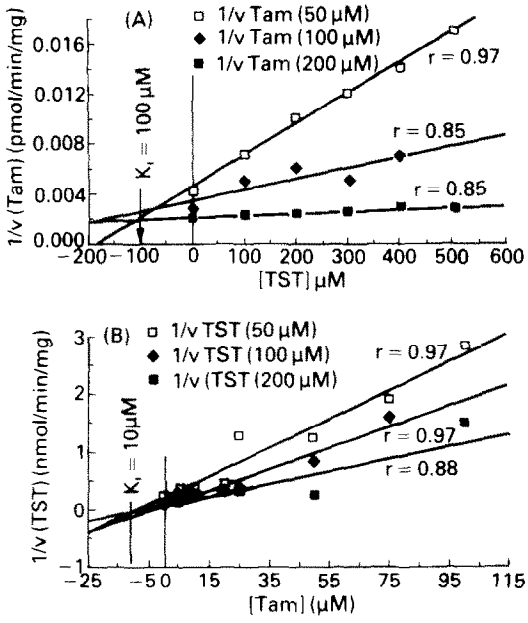


Fig. 9. Competitive inhibition of Tam demethylation by testosterone (A) and of testosterone-6β-hydroxylation by Tam (B). Upper plot: liver microsomal preparation from FH-2A donor was assayed for Tam (50, 100 and 200 μM) demethylase activity in the presence of increasing amounts of TST. Lower plot: liver microsomal sample preparation from Br039 liver was assayed for 6β-hydroxylation TST (50, 100 and 200 μM) activity in the presence of increasing amounts of Tam. The data were analysed according to the Dixon plot.

DISCUSSION

Metabolism of tamoxifen in mammalian species appears to have been subject to controversy. While Fromson *et al.* [2] detected the 4-OH-T as the only Tam metabolite in human serum, Adam *et al.* [10] showed the major serum metabolite to be DMT. Since these earlier works, it has been clearly established that this N-demethylated derivative is the most abundant metabolite detected in plasma [13, 14, 27, 28]. This is consistent with our observation that this metabolite was the major compound produced by human microsomes. N-Demethylation of Tam varied between 37 and 446 with a mean of 138 ± 98 pmol/min/mg protein. No significant difference was observed according to gender or age of liver donors. The 4-OH-Tam was produced at a very low level, 1.6 ± 1.3 pmol/min/mg protein. It represented about 1% of DMT; this is in agreement with the DMT/4-OH-Tam ratio measured in the

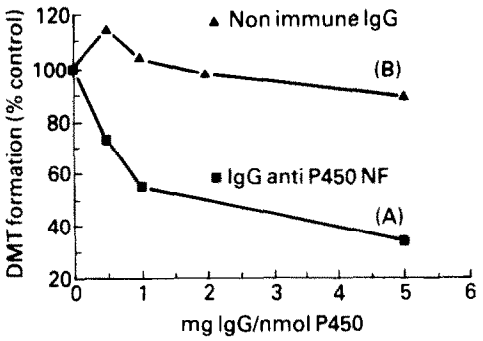


Fig. 10. Inhibition of Tam *N*-demethylase activity in microsomal preparation by anti-P450 IIIA antibody. Liver microsomes (diluted to 0.5 nmole P450/mL) from FH-2A liver was incubated 20 min at room temperature in the presence of increasing amounts of immune Ig G anti-P450 NF (A) or non-immune Ig G (B). Uninhibited activity was 275 pmol/min/mg.

Table 2. Inhibition of Tam *N*-demethylase activity in human liver microsomes by different compounds

Inhibitor (μM) concentration	25	50	100	200	250	500
Erythromycin	—	—	83	—	92	95
Cyclosporin	95	97	100	—	—	—
Nifedipine	—	16	38	66	75	81
Estradiol	0	0	0	—	15	21
Diltiazem	51	60	76	82	—	—

Values are expressed as percentage of inhibition of Tam metabolism by the Br022 hepatic microsomal sample in presence of an associate drug. Control value (0% inhibition) was 292 pmol/min/mg protein. Tam concentration was 100 μM.

serum of patients [13, 14]. The metabolic profile of Tam was quite different in rodents. For example, the 4-OH-Tam was formed at the metabolic rate of 250, 230 and 290 pmol/min/mg in microsomal preparations from rat, mouse and rabbit respectively, i.e. at a level close to that of DMT (results not shown). Such a result is in agreement with a previous study conducted on rabbit and rat liver microsomes [6]. Amounts of TNO were also detected in incubation medium but they did not differ significantly from those found in incubation where NADPH was not added. Thus, TNO may be considered as an artefact of *in vitro* experiments in so far as N-oxidation of tamoxifen was shown to be strongly dependent on oxygen availability in isolated rat hepatocytes [4].

N-Demethylation of tamoxifen was shown to be cytochrome P450 dependent. This enzymatic reaction was inhibited by carbon monooxide and needed NADPH. Spectra binding studies indicated a very high affinity of Tam for the type I binding site of P450 with a K_s of 9 μM. Such binding type was described using rat [9] and rabbit liver microsomes [8]. Furthermore, Tam did not inactivate Ph50 enzyme (results not shown). It should be noted that the Michaelis constant K_m and apparent spectral dissociation constant K_s are in disagreement in one order of magnitude: 100 vs 9 μM. Such a result was not surprising since K_s is obtained from spectral changes which are determined for the ferric protein in the absence of reducing equivalents while K_m is obtained from an actively metabolizing system [29]. However, Tam *N*-demethylase activity was poorly correlated with the total P450 amount ($r = 0.66$; N = 24). These results suggest that the variations observed are due to the content of specific P450 isoform(s). All the results reported in this paper demonstrated clearly that cytochromes P450 from the P450 IIIA subfamily are the major enzymes involved in the N-demethylation of Tam in human liver.

This conclusion was derived from the following observations:

1. Correlation of Tam *N*-demethylase activity with TST 6β-hydroxylation ($r = 0.83$; $P < 0.001$), erythromycin *N*-demethylase ($r = 0.75$; $P < 0.001$) and nifedipine oxidase ($r = 0.75$; $P < 0.001$, results not shown), all these activities known to be supported by P450 IIIA [21, 30, 31];
2. Correlation of Tam *N*-demethylase activity with P450 IIIA level determined by Western blot ($r = 0.87$; $P < 0.001$), but no significant correlation with P450 IA ($r = 0.38$; N = 25) and P450 IIE1 ($r = 0.11$; N = 25) determined by immunoblotting [32];
3. Inhibition of Tam *N*-demethylase activity by anti-P450 NF (IIIA) antibody;
4. Competitive inhibition between Tam and TST, cyclosporin, erythromycin, nifedipine and diltiazem, all compounds known to be metabolized by P450 IIIA enzymes [15–17, 21, 30, 33].

The human P450 IIIA gene subfamily [34] appears to contain at least four members, HLp or IIIA3 [35], P450 NF or IIIA4 [31], hPCN3 or IIIA5 [36] and HFL 33 or IIIA6 [37], the latter being expressed in the fetus. These forms are at least 82% homologous in terms of amino acid sequences. Thus antibodies against any P450 IIIA isoforms are expected to cross-react with all other proteins of the subfamily. So, it is not yet known whether only or several of these isoform(s) is (are) involved in cyclosporin, nifedipine, diltiazem monooxygenase activities. Furthermore, enzymatic analyses of the P450 IIIA purified forms have generally yielded very low catalytic activities. Thus, experiments reported here do not allow to distinguish between closely related P450 III family forms involved in the metabolism of tamoxifen. However, the P450 IIIA4 isoform seems to be the most important member of the family, while the P450 IIIA5 or hPCN3 is a minor form expressed in about 15% of the general population [36], probably in the Br015 and Br017 samples in this study. Concerning the other isoform P450 IIIA3, it constitutes a very minor form in human liver [38]. Accordingly, knowing that 6β-hydroxylation of testosterone is mainly mediated by hPCN1 or P450 IIIA4 [36], our results may suggest that this P450 IIIA isoform is probably involved in the N-demethylation of tamoxifen.

The list of drugs and endogeneous compounds which have been characterized as specific substrates

of human P450 IIIA includes erythromycin [30], nifedipine [15], cyclosporin [16, 17], testosterone [21], diltiazem [33] and cortisol [39]. It can be therefore anticipated that any of these drugs should lead to interaction with tamoxifen when given in association. This hypothesis was confirmed by our study *in vitro*. Thus, erythromycin, cyclosporin, and diltiazem at 100 μM concentration inhibited the biotransformation of Tam to DMT by more than 75%. In opposite, estradiol significantly inhibited N-demethylation of Tam only at concentrations greater than 500 μM. This is consistent with the observation that estradiol was mainly metabolized by a cytochrome P450 IA and to a lesser extent by P450 IIIA4 [40]. In theory, metabolism of tamoxifen should be decreased during concomitant administration of one of these drugs. However, it should be kept in mind that the extent of the effect is likely to be modulated by various factors such as dose, biodisponibility and relative K_m of both drugs. Up to now, a severe interaction of tamoxifen with warfarin was described [41]; the investigation pointed out that inhibition of cytochromes P450 enzymes by tamoxifen may be the basis for the interaction between tamoxifen and warfarin. As warfarin is known to be mainly metabolized by the cytochrome P450IIIA enzymes family (named cytochrome P450-5 in Ref. 42) this drug interaction could be due to a competitive inhibition. In this respect, it has to be emphasized that tamoxifen therapy may be complicated when given in association with drugs known to be inducers or inhibitors of P450 IIIA enzymes.

Acknowledgements—We wish to thank Drs R. MacCague (Institute of Cancer Research, Sutton, U.K.) and J.-P. Thenot (Synthelabo-Lers, Meudon La Forêt, France) for the gifts of tamoxifen and diltiazem metabolites respectively. Technical assistance of Ms C. Guillon is gratefully acknowledged. The authors wish to thank Ms V. Rouxel for careful typing of the manuscript. This work was financially supported by Ministère Education Nationale—Equipe recommandée BI* and Association de Recherche sur le Cancer (ARC Grant Number 6590 to F.B.).

REFERENCES

1. Furr BJA and Jordan VC, The pharmacology and clinical uses of tamoxifen. *Pharmacol Ther* **25**: 127–205, 1984.
2. Fromson JM, Pecuson S and Bramah S, The metabolism of tamoxifen (ICI 46,474) Part I: in laboratory animals; Part II: in female patients. *Xenobiotica* **3**: 693–714, 1973.
3. Bates DJ, Foster AB, Gribbs LJ and Jarman M, Metabolism of tamoxifen by isolated rat hepatocytes: anti-estrogenic activity of tamoxifen *N*-oxide. *Biochem Pharmacol* **31**: 2823–2827, 1982.
4. Parr IB, McCague R, Leclecq G and Stoessel S, 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. *Biochem Pharmacol* **36**: 1513–1519, 1987.
5. Foster AB, Gribbs LJ, Jarman M, Van Maanen JMS and Schulten HR, Metabolism of tamoxifen by rat liver microsomes: formation of the *N*-oxide, a new metabolite. *Biochem Pharmacol* **29**: 1977–1979, 1979.
6. Ruenitz PC, Bagley JR and Pape CW, Some chemical and biochemical aspects of liver microsomal metabolism of tamoxifen. *Drug Metab Dispos* **12**: 478–483, 1984.
7. McCague R and Seago A, Aspects of metabolism of tamoxifen by rat liver microsomes. Identification of a new-metabolite: *E*-1-[4-(2-dimethylaminoethoxy) - phenyl]-1,2-diphenyl-1-buten-3-ol *N*-oxide. *Biochem Pharmacol* **35**: 827–834, 1986.
8. Ruenitz PM and Toledo MM, Inhibition of rabbit liver microsomal oxidative metabolism and substrate binding by tamoxifen and the geometric isomers of clomiphene. *Biochem Pharmacol* **29**: 1583–1587, 1980.
9. Meltzer NM, Stang P and Sternson LA, Influence of tamoxifen and its *N*-desmethyl and 4-hydroxy metabolites on rat liver microsomal enzymes. *Biochem Pharmacol* **33**: 115–123, 1984.
10. Adam UK, Patterson JS and Kemp JV, Studies on the metabolism and pharmacokinetics of tamoxifen in normal volunteers. *Cancer Treat Rep* **64**: 761–764, 1980.
11. Jordan VC, Metabolites of tamoxifen in animals and man: identification, pharmacology and significance. *Breast Cancer Res Treat* **2**: 123–138, 1982.
12. Kemp JV, Adam KH, Wakeling AE and Slater R, Identification and biological activity of tamoxifen and metabolites in human serum. *Biochem Pharmacol* **32**: 2045–2052, 1983.
13. Murphy C, Fotsis T, Pantza RP, Aldercreutz H and Martin F, Analysis of tamoxifen and its metabolites in human plasma by GC-MS using selected ion monitoring (S.I.M.). *J Steroid Biochem* **26**: 547–555, 1987.
14. Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S and Ueland PM, Distribution of 4-hydroxy *N*-desmethyl tamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res* **49**: 2175–2183, 1989.
15. Guengerich FP, Martin MV, Beaune P, Kremers P, Wolff T and Waxman DJ, Characterization of rat and human liver cytochrome P450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J Biol Chem* **261**: 5051–5060, 1986.
16. Combalbert J, Fabre I, Dalet I, Derancourt J, Cano JP and Maurel P, Metabolism of cyclosporin A. Purification and identification of the rifampicin-inducible human liver cytochrome P450 (cyclosporin oxidase) as a product of P450 IIIA gene subfamily. *Drug Metab Dispos* **17**: 197–207, 1989.
17. Kronbach T, Fischer V and Meyer UA, Cyclosporine metabolism in human liver: identification of a cytochrome P450 III gene family as the major cyclosporine-metabolizing enzyme explains interactions of cyclosporine with other drugs. *Clin Pharmacol Ther* **43**: 630–635, 1988.
18. Berthou F, Ratanasavanh D, Riché C, Picart D, Voisin T and Guillouzo A, Comparison of caffeine metabolism by slices, microsomes and hepatocyte cultures from adult human liver. *Xenobiotica* **19**: 401–417, 1989.
19. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
20. Omura T and Sato E, Carbon monoxide binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2379, 1964.
21. Waxman DJ, Attisano C, Guengerich FP and Lapenson D, Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6β-hydroxylase cytochrome P450 enzyme. *Arch Biochem Biophys* **263**: 424–436, 1988.
22. Sonderfan AJ, Arlotto MP, Dutton DR, McMillen SK and Parkinson A, Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P450. *Arch Biochem Biophys* **255**: 27–41, 1987.
23. Werringloer J, Assay of formaldehyde generated during

microsomal oxidation reactions. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. LII, pp. 297–302. Academic Press, New York, 1978.
24. Beaune P, Kremers P, Letawe-Goujon F and Gielen J, Monoclonal antibodies against human liver cytochrome P450. *Biochem Pharmacol* **34**: 3547–3552, 1985.
25. Laemmli UK, Change of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
26. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of protein from polyacrylamide gel to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1970.
27. Stevenson D, Briggs RJ, Chapman DJ and Devos D, Determination of tamoxifen and five metabolites in plasma. *J Pharm Biomed Anal* **6**: 1065–1068, 1988.
28. Etienne MC, Milano G, Fischel JL, Frenay M, François E, Formento JL, Gioanni J and Namer M, Tamoxifen metabolism: pharmacokinetic and *in vitro* study. *Br J Cancer* **60**: 30–35, 1989.
29. Schenkman JB, Sligar SG and Cinti DL, Substrate interaction with cytochrome P450. *Pharmacol Ther* **12**: 43–71, 1981.
30. Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B and Guzelian PS, Identification of the cytochrome P450 induced by macrolide antibiotic in rat liver as the glucocorticoid responsive cytochrome P450p. *Biochemistry* **24**: 2171–2178, 1985.
31. Beaune PH, Umbenhauer DR, Bork RW, Lloyd RS and Guengerich FP, Isolation and sequences determination of a cDNA clone related to human cytochrome P450 nifedipine oxidase. *Proc Natl Acad Sci USA* **83**: 8064–8068, 1986.
32. Berthou F, Flinois JP, Ratanasavanh D, Beaune P, Riché C and Guillouzo A, Evidence for the involvement of several cytochromes P450 in the first steps of caffeine metabolism by human liver microsomes. *Drug Metab Dispos*, in press.
33. Pichard L, Gillet G, Fabre I, Dalet-Beluche I, Bonfils C, Thenot JP and Maurel P, Identification of the rabbit and human cytochromes P450 IIIA as the major enzymes involved in the N-demethylation of diltiazem. *Drug Metab Dispos* **18**: 711–719, 1990.
34. Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FP, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Philips IR, Sato R and Watermann MR, The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8**: 1–13, 1989.
35. Molowa DT, Schnetz EG, Wrighton SA, Watkins PB, Kremers P, Mendez-Picon G, Parker GA and Guzelian PS, Complete cDNA sequence of a cytochrome P450 inducible by glucocorticoids in human liver. *Proc Natl Acad Sci USA* **83**: 5311–5315, 1986.
36. Aoyama T, Yamano S, Waxman DJ, Lapenson DP, Meyer UA, Fischer V, Tyndale R, Inaba T, Kalow W, Gelboin HV and Gonzalez FJ, Cytochrome P450 hPCN3, a novel cytochrome P450 IIIA gene product that is differentially expressed in adult human liver. *J Biol Chem* **264**: 10388–10395, 1989.
37. Komori M, Nishio K, Ohi M, Kitada M and Kamataki T, Molecular cloning and sequence analysis of cDNA containing the entire coding region for human fetal liver cytochrome P450. *J Biochem* **105**: 161–163, 1989.
38. Bork RW, Muto T, Beaune PH, Srivastava PK, Lloyd RS and Guengerich FP, Characterization of mRNA species related to human liver cytochrome P450 nifedipine oxidase and the regulation of catalytic activity. *J Biol Chem* **264**: 910–919, 1989.
39. Ged C, Rouillon JM, Pichard L, Combalbert J, Bressot N, Bories P, Michel H, Beaune P and Maurel P, The increase on urinary excretion of 6β-hydroxycortisol as a marker of human hepatic cytochrome P450 IIIA induction. *Br J Clin Pharmacol* **28**: 373–383, 1989.
40. Aoyama T, Korzekwa K, Nagata K, Gillette J, Gelboin HV and Gonzalez FJ, Estradiol metabolism by complementary desoxyribonucleic acid expressed human cytochrome P450s. *Endocrinology* **126**: 3101–3106, 1990.
41. Tamoxifen and warfarin: a life-threatening drug interaction. *Perspect Clin Pharmac* **8**: 3–5, 1990.
42. Beaune PH, Kremers PG, Kaminsky L, De Graeve J, Albert A and Guengerich FP, Comparison of monooxygenase activities and cytochrome P450 isozyme concentration in human liver microsomes. *Drug Metab Dispos* **14**: 437–442, 1986.