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Distribution of 4-Hydroxy-*N*-desmethyltamoxifen and Other Tamoxifen Metabolites in Human Biological Fluids during Tamoxifen Treatment[1]

Ernst A. Lien,[2] Einar Solheim, Oscar A. Lea, Steinar Lundgren, Stener Kvinnsland, and Per M. Ueland

Clinical Pharmacology Unit, Department of Pharmacology and Toxicology [E. A. L., P. M. U.], Department of Pharmacology and Toxicology [E. S.], Oncology Research Laboratory [O. L.], Department of Oncology [S. L.], University of Bergen, N-5021, Bergen, Norway, and Department of Oncology [S. K.], University of Trondheim, 7000 Trondheim, Norway

ABSTRACT

Several metabolites of tamoxifen, including 4-hydroxy-*N*-desmethyltamoxifen (metabolite BX), 4-hydroxytamoxifen (metabolite B), *N*-desmethyltamoxifen (metabolite X), the primary alcohol (metabolite Y), and *N*-desdimethyltamoxifen (metabolite Z) were identified and their concentrations determined in fluids and feces from patients receiving chronic tamoxifen treatment. The biological samples investigated were serum, pleural, pericardial and peritoneal effusions, cerebrospinal fluid, saliva, bile, feces, and urine.

In serum, tamoxifen itself, and the metabolites X and Z were the prevailing species, but significant amounts of the metabolites Y, B, and BX were also detected. About 3 h after drug intake tamoxifen as well as Y, B, BX, X, and Z showed a peak in serum. This may be explained by efficient metabolism of the metabolite precursor before being distributed to peripheral compartments. Upon drug withdrawal all metabolites showed first-order elimination curves which paralleled that of tamoxifen suggesting that their rate of elimination exceeded that of tamoxifen and that the serum levels are production rate limited. The protein binding of tamoxifen and its major serum metabolites (Y, X, Z) was determined and found to be higher than 98%. Albumin was the predominant carrier for tamoxifen in human plasma.

The concentrations of tamoxifen and its metabolites in pleural, pericardial, and peritoneal effusions equalled those detected in serum, corresponding to an effusion/serum ratio between 0.2 and 1. Only trace amounts of tamoxifen and metabolite X were detected in cerebrospinal fluid (CSF/serum ratio <0.02). In saliva, concentrations of tamoxifen and X exceeded the amounts of free drug in serum, suggesting active transport or trapping of these compounds in the salivary gland. Bile and urine were rich in the hydroxylated, conjugated metabolites (Y, B, and BX), whereas in feces unconjugated metabolite B and tamoxifen were the predominating species.

INTRODUCTION

The nonsteroid antiestrogen tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene] has since 1973 been widely used for the palliative treatment of breast cancer (1). Tamoxifen has also been evaluated in the management of several other conditions, including pancreatic (2), ovarian and endometrial cancer, anovulatory infertility (3), cyclic mastalgia (4), and trials on prophylactic intervention in women at high risk of developing breast cancer are planned (5, 6).

Tamoxifen seems to be extensively metabolized and knowledge of its biotransformation is important since this is a major determinant of elimination and some metabolites may be active.

Most information on the distribution of tamoxifen and its metabolites are based on animal studies. Borgna and Rochefort found polar metabolites of tamoxifen in plasma, uterus, liver, and oviduct of immature female rats and chickens injected with [^{3}H]tamoxifen. 4-Hydroxytamoxifen and another hydroxylated metabolite, "M2," were the major tritiated metabolites in the cytosol and KCl-extracted nuclear fraction from rat uterus (7). This metabolite may be 4-hydroxy-*N*-desmethyltamoxifen, the formation of which has been demonstrated after incubation with rat liver microsomes (8) and in isolated rat hepatocytes in suspension (9). In the female rat, mouse, monkey, and dog most [^{14}C]tamoxifen was excreted via bile into the feces, but a significant fraction of the biliary radioactivity was reabsorbed and underwent enterohepatic circulation (10). In the rat most tamoxifen metabolites in bile and feces were present as glucuronides and other conjugates (10).

Despite the wide use of tamoxifen as a drug, knowledge on its fate in humans is sparse. Several metabolites of tamoxifen have been identified in human plasma, *i.e.*, the primary alcohol (metabolite Y[3]), metabolite B, metabolite X, metabolite Z (3). Fromson gave a single dose of [^{14}C]tamoxifen to four patients and found that the peak plasma concentration occurred after 4–7 h and the terminal half-life was longer than 7 days (11). Other pharmacokinetic studies confirmed these findings (12). Also in humans, most tamoxifen is excreted as metabolites into feces. Less than 20% are eliminated in the urine (11).

Among the serum metabolites the hydroxylated metabolite B has received particular attention since it has higher *in vitro* affinity for the estrogen receptor than the parent drug (8, 13, 14). Another hydroxylated metabolite, 4-hydroxy-*N*-desmethyltamoxifen (metabolite BX), has similar properties (8).

We recently demonstrated high concentrations of metabolite BX in bile from a patient receiving chronic tamoxifen treatment (15). We here report on the investigation of the presence of metabolite BX and other tamoxifen metabolites in various biological samples from patients treated with tamoxifen. These samples include serum, pleural, pericardial and peritoneal effusions, cerebrospinal fluid, saliva, urine, and bile, and an extract from feces.

MATERIALS AND METHODS

Chemicals. Tamoxifen, metabolite B, and metabolite X were obtained from Pharmachemie B.V., Haarlem, Holland. Metabolite Y, metabolite Z, and metabolite BX were gifts from Imperial Chemical Industries PLC, Pharmaceuticals Div., Macclesfield, Cheshire, UK. The latter reference compound was obtained as a mixture of about 85% *cis* isomer and 15% *trans* isomer. Attempts to enrich the *trans* isomer by boiling in ethanol, were not successful. [*N-methyl*-^{3}H]tamoxifen (specific activity, 84 Ci/mmol) and [^{14}C]tamoxifen citrate S.A. 25 mCi/mmol were obtained from Amersham International, UK. Purity was checked by thin-layer chromatography on silica gel in ethyl ether:triethylamine (99:1). *N*-Methyl-tritiated tamoxifen was highly unstable and was not used in quantitative analysis.

β-Glucuronidase was obtained from Sigma Chemical Company, St. Louis, MO. This β-glucuronidase, which is a preparation of the intestinal juice of the snail, *Helix pomatia*, also contains sulfatase activity

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[2] To whom requests for reprints should be addressed, at Clinical Pharmacology Unit, Central Laboratory, University of Bergen, N-5016 Haukeland Hospital, Norway.

[3] The abbreviations used are: metabolite Y, *trans*-1(4-β-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene; metabolite B, 4-hydroxytamoxifen; metabolite BX, 4-hydroxy-*N*-desmethyltamoxifen; metabolite X, *N*-desmethyltamoxifen; metabolite Z, *N*-desdimethyltamoxifen; ODS, octadecylsilane; AcN, acetonitrile; LC/MS, liquid chromatography/mass spectrometry; MPA, medroxyprogesteroneacetate; SIM, selected-ion monitoring; CSF, cerebrospinal fluid.

(β-glucuronidase 126,000 units/ml, sulfatase 4000 units/ml).

Monospecific antiserum used for albumin determinations (16) was purchased from Dakopatts, Copenhagen, Denmark.

Patients. 36 patients, who received tamoxifen as a palliative treatment of breast cancer, were included, and they all gave their informed consent to participate in the study. Their age, duration of treatment, dosing, and biological samples obtained, are listed in Table 1.

Sample Collection. Pericardial, pleural and peritoneal effusion, and cerebrospinal fluid were obtained during diagnostic or therapeutic procedures, collected, and stored in glass tubes until analysis. Blood samples were drawn on the same occasions. Bile was collected into polyethylene bags from a biliary T-drain from Patient E. B. with breast cancer and total bile duct occlusion due to metastases.

Blood samples were drawn from three patients (A. B., E. M. C., and G. T.) after tamoxifen was discontinued to obtain the decay curves for tamoxifen and its metabolites. Blood samples were collected at intervals of 4 to 14 days for 35 to 41 days after drug withdrawal. Feces were collected from three patients (A. H., K. R., and A. L. H.) for 3 days and urine from 13 patients for 24 h. Urine was collected into plastic cans. Saliva was obtained from 11 patients while chewing waxed sheet (Parafilm) to stimulate salivary flow.

Blood samples were obtained in these patients at the time of collecting feces, urine, or saliva.

All samples were stored in darkness at −20°C until analysis.

Sample Processing. The bile and urine were thawed, and treated with β-glucuronidase from *Helix pomatia*, essentially as described by Bakke and Scheline (17). To increase the yield of unconjugated tamoxifen and metabolites from urine, the treatment with β-glucuronidase was extended to 24 h, and the amount of β-glucuronidase used was increased from 2520 to 5040 units/ml urine. The yield was also increased by resuspending the urine precipitate by vigorous shaking before the β-glucuronidase-treatment. After incubation with the enzyme, the pH was adjusted to 7 by adding 1 N NaOH. The neutralized samples were mixed with an equal volume of AcN, and after centrifugation the supernatants were transferred to sample vials, capped, and analyzed.

In a preliminary study aliquots of urine (5 ml) were refluxed for 2.5 h with water (5 ml) and titrated to pH 1 with 18 N sulfuric acid. The samples were then neutralized and handled as described above.

Feces were mechanically homogenized, and about 3 g of feces were then mixed with 20 ml of water or 50% AcN, vigorously shaken and treated with ultrasound for 5 min. The extract was then centrifuged for 15 min at 420 × g_{av}. The supernatant obtained from extraction with water was treated with β-glucuronidase as described in the previous paragraph for urine. The solution obtained by direct AcN extraction was properly diluted with mobile phase, transferred to sample vials, capped, and analyzed.

The samples of pleural, pericardial and peritoneal effusions, cerebrospinal liquor, and saliva were treated with AcN, as previously described for serum (18).

Determination of Protein Binding. Protein binding of tamoxifen was determined both for drugs in patient serum by ultracentrifugation and for radiolabeled drug added to drug free serum by column chromatography and equilibrium dialysis.

Serum (5 ml) from Patient B. L. who had been treated with 40 mg tamoxifen b.i.d. for 78 days, was transferred to Ultra-Clear tubes from Beckman and centrifuged at 215,000 × g_{av} for 24 h in an ultracentrifuge model Beckman L8-60M. The temperature was kept at 4°C. The centrifugation tubes were harvested by careful aspiration of fractions of 400 μl from the top of the generated gradient. The concentrations of tamoxifen and its metabolites and albumin were determined in the separate fractions and in the same serum sample before centrifugation.

[^{3}H]Tamoxifen (3 μCi) was added to serum diluted 1:1 with isotonic phosphate buffer (pH 7.4), and incubated for 24 h at 4°C. A sample of 1 ml was subjected to affinity chromatography on concanavalin A-Sepharose 4B from Pharmacia, as described previously (19). Another sample was analyzed by gel filtration on Sephadex G-100 column (2.5 x 70 cm). Radioactivity was determined in 0.5-ml aliquots of eluent fractions. Absorbance was determined at 280 nm.

Equilibrium saturation analysis was performed by dialysis of five different concentrations of [^{14}C]tamoxifen citrate against a 60-μM solution of human serum albumin (Koch-Light Chemicals, UK) in isotonic phosphate buffer, pH 7.4 (20).

High-Performance Liquid Chromatography. We used a liquid chromatography system which was developed for the determination of tamoxifen and metabolites in serum (18). The assay was modified to improve the separation and isolation of the early eluting, hydrophilic metabolites. The method and the modification are briefly described below.

Large samples of 250 μl were injected into a small precolumn with an internal diameter of 0.21 cm, packed with 5 μm ODS material. The length of this column was increased from 2 to 3 cm (15). The samples were on-column concentrated by equilibrating the precolumn with 50% AcN in water, containing 3 mM acetic acid and 2 mM diethylamine. The analytes were then directed into an analytical ODS-Hypersil column (0.21 x 10 cm) by changing the mobile phase followed by column switching. The composition of the mobile phase was 91% AcN containing 1 mM acetic acid and 0.67 mM diethylamine, and the flow rate was 0.3 ml/min. Small adjustments in the AcN concentration of the mobile phase were made to compensate for different composition of the extracts.

Tamoxifen and its metabolites were eluted in the following order: metabolite Y, metabolite B, metabolite BX, tamoxifen, metabolite Z, and metabolite X. These compounds were postcolumn converted to fluorophors by UV illumination while passing through a quartz tube, and then monitored by a fluorescence detector.

Table 1 *Patient characteristics*

Patient	Age	Treatment: Duration (days)	Treatment: Dose	Biological sample[a]
UA	71	77	30 mg daily	Pleural effusion
		203	30 mg daily	Saliva
AB	65	56	30 mg daily	Multiple serum samples[b]
EB	57	71	30 mg daily	Bile[c]
SB	70	184	30 mg daily	Saliva
EMC	61	118	30 mg daily	Multiple serum samples[b]
MC	70	23	30 mg daily	Pericardial effusion
		112	30 mg daily	Pericardial effusion
DE	64	720	30 mg daily	Urine
IE	76	73	30 mg daily	Multiple serum samples,[d] urine
		134	30 mg daily	Multiple serum samples,[d] urine
SE	73	161	30 mg daily	Saliva
AF	67	20	30 mg daily	Pleural effusion
EF	65	21	30 mg daily	Peritoneal effusion
AH	62	57	30 mg daily	Feces
ALH	58	13	30 mg daily	Feces
RH	73	11	30 mg daily	Pleural effusion
		66	30 mg daily	Pleural effusion
		93	30 mg daily	Pleural effusion
		178	30 mg daily	Saliva
ITH	58	175	30 mg daily	Urine
EJ	51	14	30 mg daily	Pleural effusion
DK	51	22	30 mg daily	Pleura
BL	50	835	30 mg daily	Cerebrospinal fluid
		78[e]	40 mg b.i.d.	
OL	83	716[e]	40 mg b.i.d.	Urine
BO	70	120	30 mg daily	Urine
JGO	64	172	30 mg daily	Saliva
EN	67	28	30 mg daily	Saliva
SP	72	651	30 mg daily	Saliva
AR	42	10	30 mg daily	Pleural effusion
ALR	68	135	30 mg daily	Urine
KR	59	47	30 mg daily	Feces, Urine
		54	30 mg daily	Urine
NS	68	62[e]	40 mg b.i.d.	Pleural effusion
OS	79	296	30 mg daily	Urine
SBS	62	296	30 mg daily	Urine
RS	79	132	30 mg daily	Saliva
AT	76	68	30 mg daily	Urine
		129	30 mg daily	Multiple serum samples,[d] urine
GT	74	990	30 mg daily	Multiple serum samples[b]
ST	72	54	30 mg daily	Saliva
KZ	81	133	30 mg daily	Saliva
LMØ	41	480	30 mg daily	Saliva
MØ	44	48	30 mg daily	Urine

[a] Refers to biological sample other than a single serum sample, which was obtained from all patients.
[b] After drug withdrawal.
[c] External biliary catheter.
[d] During one dosing interval.
[e] Days after dose escalation from steady state on 30 mg daily.

The instruments and the construction of the postcolumn converter have been described (18).

Liquid Chromatography/Mass Spectrometry. The analytical column was connected to a LC/MS, thermospray system (Model 201; Vestec, Houston, TX). Before entering the thermospray, the effluent from the column was mixed with 0.1 M ammonium acetate reagent, delivered at a rate of 0.7 ml/min via a zero dead volume T-connector.

Determination of Albumin. Albumin was quantitated in individual fractions by "rocket" immunoelectrophoresis (16). Column dimensions were 2.5 × 70 cm and flow rate 52 ml/h. Temperature, 4°C.

RESULTS

Identification of Tamoxifen and Its Metabolites in Biological Fluids and Extracts. Samples of serum, pericardial, pleural and peritoneal effusions were deproteinized with AcN and analyzed by microdispersion LC and fluorescence detection, as described in a previous publication (18). Tamoxifen, metabolites Y, X, and Z, could be demonstrated in the chromatogram in substantial amounts whereas metabolites BX and B were present only in low concentrations (Fig. 1). The metabolites seem to be unconjugated in these fluids, since β-glucuronidase treatment did not increase peak height (data not shown).

The amounts of metabolite BX in serum were below the detection limit of our LC/MS system. To verify the identity of this metabolite it was concentrated by extraction of 10 ml serum with hexane-2% butanol, evaporated and redissolved in 500 μl 50% AcN. A compound with molecular ion $(M + 1)^+$ of 374 *m/z*, corresponding to that of authentic metabolite BX, cochromatographed with the fluorescence peak of metabolite BX (data not shown).

The amount of tamoxifen and metabolites in cerebrospinal fluid was below the detection limit of our routine assay based on fluorescence detection (Fig. 1).

In urine and bile the existence of tamoxifen, metabolite BX

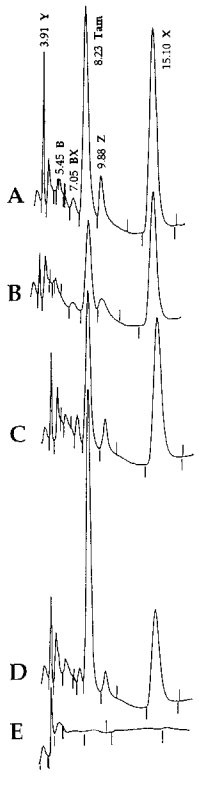

Fig. 1. Chromatograms of serum and various biological fluids. Samples from *A*, serum; *B*, pleural effusion; *C*, pericardial effusion; *D*, peritoneal effusion; and *E*, cerebrospinal fluid were subjected to reversed-phase chromatography, as described in the text. The samples are from different patients.

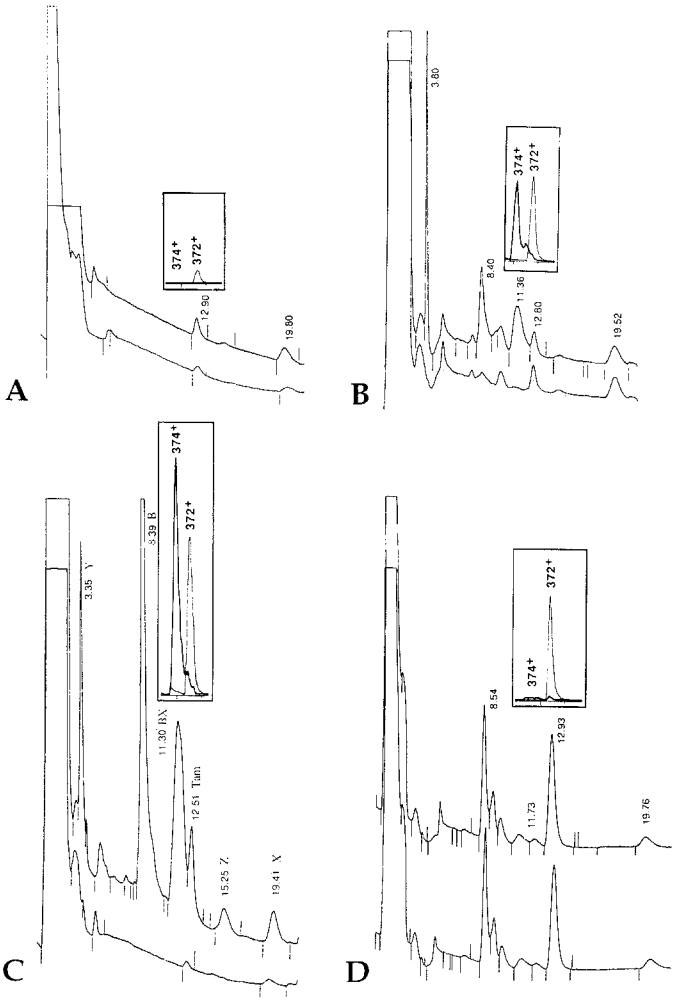

Fig. 2. Chromatography of extracts from *A*, saliva; *B*, urine; *C*, bile; and *D*, feces; and identification of compounds with LC/MS. The samples were divided into two portions, one of which was treated with β-glucuronidase. Samples were subjected to reversed-phase chromatography as described in the text. The fluorescence profiles were obtained after postcolumn photoactivation (18). *Low traces*, untreated samples; *upper traces*, samples treated with β-glucuronidase. *Insets*, LC-SIM traces for the M^+ ion for tamoxifen (374 *m/z*) and metabolite BX (372 *m/z*). In these separate runs the postcolumn reactor was by-passed.

as well as other hydroxylated metabolites like metabolites Y and B could be verified with LC/MS (SIM). This is shown for metabolite BX in Fig. 2. In feces the amount of metabolite BX was small and the abundance of the 374^+ ion gave a response only slightly but significantly above the baseline (Fig. 2). Metabolite B and tamoxifen could be detected and verified in feces by this method.

Metabolite Concentrations and Kinetics during Chronic Dosing. Fig. 3 shows the variations in serum concentrations of tamoxifen and its metabolites, including metabolite BX, throughout 24 h in two patients (A. T., I. E.) during steady state (Table 1). About 2 h after drug ingestion, serum tamoxifen showed a distinct peak, which was followed by a less pronounced serum peak. The concentration then slightly declined. Similar serum profiles were observed for metabolites Y, B, BX, X, and Z (Fig. 3).

The serum concentration curves were determined for tamoxifen and its metabolites in three patients after drug withdrawal. The elimination curves were monophasic and corresponded to serum half-lives between 7 and 11 days for tamoxifen and 10 and 11 for metabolite X. Notably, the curves for metabolites Y, B, BX, X, and Z were parallel to that of the parent compound, tamoxifen (Fig. 4).

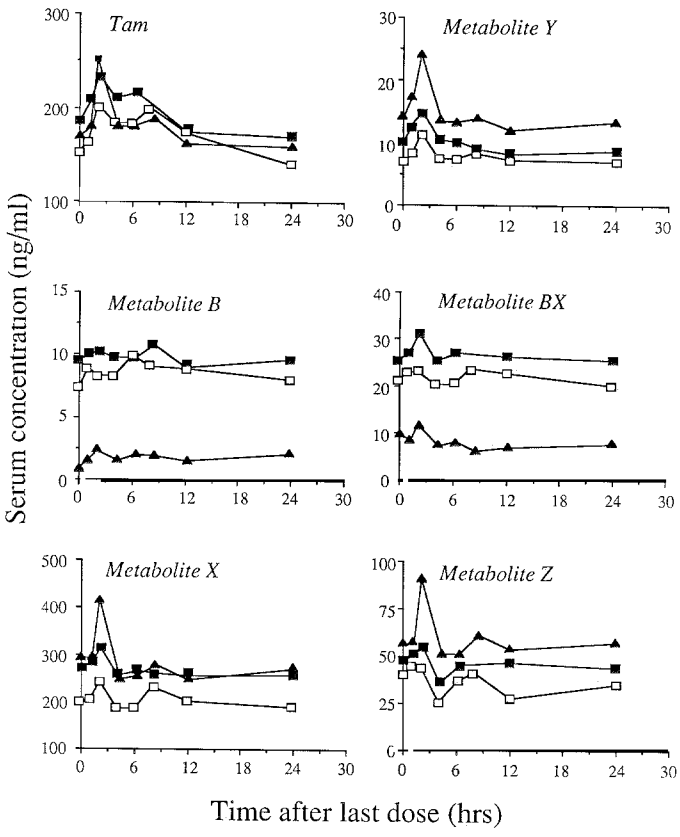

Fig. 3. Fluctuations in serum levels of tamoxifen and metabolites during one dosing interval. 30 mg tamoxifen was given at time 0. ▲, Patient AT; □, Patient IE (8/8/87); ■, Patient IE (10/9/87).

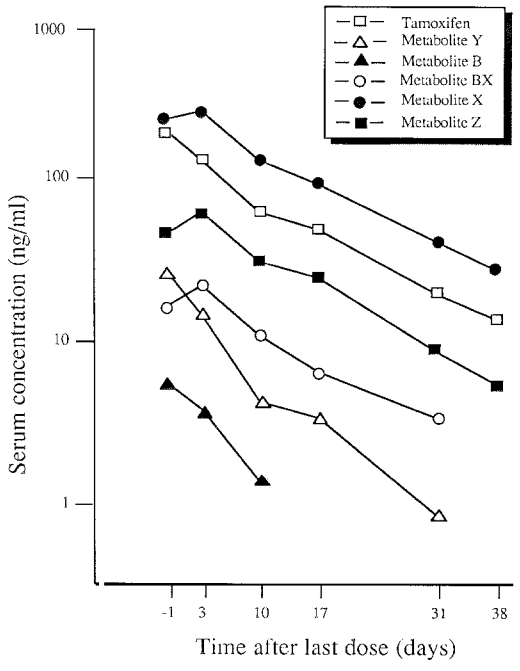

Fig. 4. Elimination curves for tamoxifen and metabolites in serum after tamoxifen withdrawal. Patient (*A. B.*) was treated with 30 mg tamoxifen daily for 60 days. At time 0 days tamoxifen was replaced with MPA.

The apparent terminal distribution volume (V_z) for tamoxifen during steady state could be calculated from the equation (21):

$$V_z = \frac{D \cdot \tau}{C \cdot k_{el}}$$

where τ is the dosing interval, C the mean concentration during steady state, and k_{el} the elimination constant.

V_z values were 52 liter/kg (A. B.), 61 liter/kg (G. T.) and 53 liter/kg (E. M. C.). The Patient A. B. changed directly over from tamoxifen to MPA after tamoxifen treatment for 8 weeks. Patient E. M. C. used acetaminophen, allopurinol, codeine, digoxin, furosemide, nifedipine, and warfarin, whereas G. T. used no other drugs.

Protein Binding. Serum from a patient (B. L.) receiving 40 mg tamoxifen b.i.d. was subjected to ultracentrifugation. A gradient of albumin was created, and amount of albumin in the top fraction was 0.5% of that in whole serum. The concentration of tamoxifen, metabolites Y, X, and Z closely followed the albumin concentration (data not shown). Based on the residual amount of albumin in the top fraction and the detection limit of the method (about 1 ng/ml) the protein binding of these three compounds were calculated to be higher than 98%. Because of the low level of metabolites B and BX in serum, the protein binding of these compounds could not be assessed.

The protein binding was further studied by characterizing the interaction of labelled drug with serum proteins. Most [^{3}H] tamoxifen added to serum comigrated with albumin when fractionated by gel filtration on Sephadex G 100. A peak of similar magnitude and elution volume was observed when serum was replaced by pure human serum albumin at a concentration equivalent to that present in serum (Fig. 5). Furthermore, Scatchard analysis (22) (not shown) of the binding data obtained from equilibrium dialysis of [^{14}C]tamoxifen *versus* human serum albumin revealed an association constant of 1.3×10^5/M and approximately 1 binding site per molecule.

From these data one can calculate that albumin alone could bind 98.8% of tamoxifen in serum. Other binding proteins do not seem to be involved to any great extent. Fractionation of serum incubated with labeled tamoxifen on concanavalin A-Sepharose columns (19) showed no label to be associated with the adsorbed glycoprotein fraction (Fig. 5). This excludes significant binding to α_1-acid glycoprotein and the steroid-binding proteins cortisol binding globulin and sex hormone binding globulin.

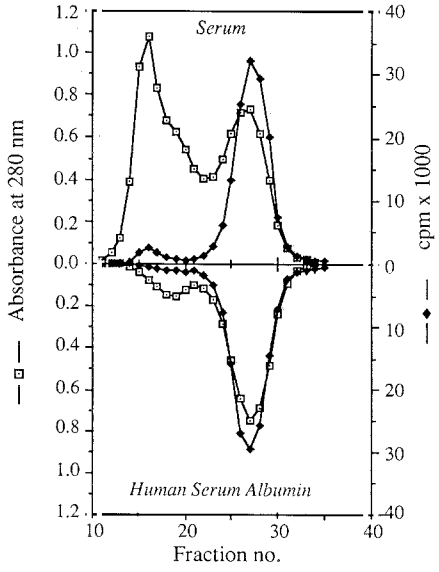

Fig. 5. Gel filtration of human serum and human serum albumin incubated with [^{3}H]tamoxifen. *Top*, gel filtration on Sephadex G-100 (2.5 x 70 cm) of 1 ml serum diluted 1:1 with phosphate buffer (pH 7.4) incubated with 3 μCi [^{3}H] tamoxifen. Flow rate was 52 ml/h, temperature 4°C. Radioactivity was determined in 0.5-ml aliquots of eluent fractions. Absorbance was measured at 280 nm. Albumin was quantitated in each fraction by "rocket" immunoelectrophoresis. *Bottom*, gel filtration as in the top, but this time 2 ml 2% human serum albumin was used instead of human serum.

A small fraction of labeled tamoxifen was associated with large protein(s) eluted in the void volume of Sephadex G-100. This binding could be due to the presence of a high affinity, low capacity binding protein analogous to the antiestrogen binding site on rat serum low density lipoprotein (23).

Distribution into Pleural, Pericardial and Peritoneal Effusions and Cerebrospinal Fluid. The concentrations of tamoxifen and its serum metabolites were determined in pleural, pericardial and peritoneal effusions from nine patients receiving chronic tamoxifen dosing. A serum sample was drawn and analyzed on the same occasions (Table 2).

There was only a marginal increase (<5%) in concentrations of tamoxifen and its metabolites in these effusions as well as serum after incubation with β-glucuronidase (data not shown), suggesting that they exist mainly as unconjugated species in these fluids.

The distribution ratios between these fluids and serum varied between 0.3 and 1 in most cases (Table 2). These values were compared with the distribution of albumin (most ratios between 0.5–0.8) but no obvious correlation could be demonstrated. The distribution ratios for the different compounds were similar. Tamoxifen and its metabolites therefore seem to be distributed into these fluids to about the same extent. Notably, the distribution of tamoxifen and its metabolites showed a marked inter patient variation (Table 2). For example, the ratio was between 0.2 and 0.6 for Patient R. H. on three different occasions (on Days 11, 66, and 93 after initiation of treatment) and between 0.7 and 1.2 for Patient M. C. on two occasions (on Days 23 and 112).

The amounts of tamoxifen and its metabolites in cerebrospinal liquor from Patient B. L. were below the detection limit (about 1 ng/ml) of our routine LC method. We improved the sensitivity of the method by concentrating these compounds from 1.5 ml of CSF on top of the guard column. On-column concentration was improved by equilibrating the system with water, and then eluting it as described in "Materials and Methods." Tamoxifen and metabolite X was then detected in significant, but low, concentrations. The CSF/serum ratio was of the same magnitude for tamoxifen and metabolite X as for albumin (Table 2).

Tamoxifen and Metabolite X in Saliva. Analysis of saliva from 11 patients showed that the compounds most abundant in serum, tamoxifen, and metabolite X were present in substantial amounts, whereas the other tamoxifen metabolites were essen-

Table 2 *Distribution of tamoxifen and its metabolites between serum and pleural, pericardial and peritoneal effusions and between serum and CSF*

Patient	Days of treatment	Sample	TAM[a] (ng/ml)	Y[a] (ng/ml)	B[a] (ng/ml)	BX[a] (ng/ml)	X[a] (ng/ml)	Z[a] (ng/ml)	Albumin (g/liter)
UA	71	Pleural effusion	87.1	34.8	2.0	7.2	176.2	50.6	28
		Serum	173.0	67.1	4.3	8.0	278.8	66.6	40
		Ratio	0.50	0.52	0.47	0.90	0.63	0.76	0.70
AF	20	Pleural effusion	148.1	3.1	3.0	2.8	114.9	9.6	15
		Serum	321.7	8.5	7.1	7.8	200.9	20.8	25
		Ratio	0.46	0.37	0.42	0.36	0.57	0.46	0.60
RH	11	Pleural effusion	63.7	3.7	0.8	1.3	56.9	8.3	30
		Serum	145.2	13.3	3.3	4.0	121.0	18.3	38
		Ratio	0.44	0.28	0.24	0.33	0.47	0.45	0.79
RH	66	Pleural effusion	72.0	8.0	1.5	5.5	121.3	29.3	30
		Serum	151.5	28.3	3.5	8.6	203.6	47.5	34
		Ratio	0.48	0.28	0.43	0.64	0.60	0.62	0.88
RH	93	Pleural effusion	73.7	9.1	0.8	3.4	110.3	26.3	25
		Serum	198.0	33.9	4.4	13.4	281.0	65.2	38
		Ratio	0.37	0.27	0.18	0.25	0.39	0.40	0.66
EJ	14	Pleural effusion	66.4	9.2	2.0	9.2	85.4	23.1	34
		Serum	95.5	13.1	5.5	10.0	103.2	25.8	47
		Ratio	0.70	0.70	0.36	0.92	0.83	0.90	0.72
DK	22	Pleural effusion	50.5	4.7	0.3	2.2	113.0	15.8	26
		Serum	138.7	13.2	0.7	3.6	187.3	48.0	40
		Ratio	0.36	0.36	0.43	0.61	0.60	0.33	0.65
AR	10	Pleural effusion	67.5	1.7	1.2	0	38.9	0	16
		Serum	159.7	5.2	4.5	3.0	75.7	3	25
		Ratio	0.42	0.33	0.27		0.51		0.64
NS	62[b]	Pleural effusion	200.0	43.1	3.5	3.5	395.7	86.8	30
		Serum	240.2	57.7	3.8	3.9	410.1	89.8	40
		Ratio	0.83	0.75	0.92	0.90	0.97	0.97	0.75
MC	23	Pericardial effusion	96.0	15.9	3.0	13.4	152.1	25.7	24
		Serum	89.6	13.5	3.1	15.4	128.0	26.2	25
		Ratio	1.07	1.18	0.97	0.87	1.19	0.98	0.96
MC	112	Pericardial effusion	71.4	19.2	2.5	10.5	149.0	39.3	24
		Serum	79.0	22.5	3.5	10.0	163.5	42.1	28
		Ratio	0.90	0.85	0.71	1.05	0.91	0.93	0.86
EF	21	Peritoneal effusion	179.9	15.2	4.0	9.6	101.1	17.9	13
		Serum	227.9	23.7	7.0	28.0	238.6	54.9	25
		Ratio	0.79	0.64	0.57	0.34	0.42	0.33	0.69
BL	835	CSF	1.1				1.2		0.2
		Serum	103.2	15.8	1.6	6.7	171.5	33.7	31.8
		Ratio	0.011				0.007		0.006

[a] TAM, tamoxifen; Y, metabolite Y; B, metabolite B; BX, metabolite BX; X, metabolite X; Z, metabolite Z.
[b] 62 days after dose escalation from 30 mg daily to 40 mg twice daily.

tially absent. Furthermore, we found no strict correlation between serum level and the concentration in saliva (saliva/serum ratio between 0.003 and 0.081 for tamoxifen and 0.002 and 0.040 for metabolite X), but saliva from patients with high serum levels often contained relatively high amounts of tamoxifen and metabolite X (Table 3). Notably, there was a correlation between the amount of tamoxifen and metabolite X in saliva (Fig. 6), suggesting that salivary tamoxifen is not derived from tablet residues.

Table 3 *Distribution of tamoxifen and metabolite X into saliva*

Patient	Sample	Tamoxifen (ng/ml)	Metabolite X (ng/ml)
UA	Saliva	0.6	0.8
	Serum	110.6	202.8
	Ratio	0.005	0.004
SB	Saliva	1.2	1.3
	Serum	146.0	197.3
	Ratio	0.008	0.007
SE	Saliva	7.8	5.8
	Serum	283.5	215.0
	Ratio	0.028	0.027
RH	Saliva	2.2	3.0
	Serum	124.6	186.8
	Ratio	0.018	0.016
EN	Saliva	0.6	0.7
	Serum	104.4	105.9
	Ratio	0.006	0.007
JGO	Saliva	2.1	1.2
	Serum	188.5	206.8
	Ratio	0.011	0.006
SP	Saliva	1.7	1.1
	Serum	90.3	178.3
	Ratio	0.019	0.006
RS	Saliva	0.3	1.1
	Serum	23.4	69.9
	Ratio	0.013	0.016
TS	Saliva	0.6	0.8
	Serum	192.0	368.8
	Ratio	0.003	0.002
KZ	Saliva	5.0	4.8
	Serum	252.4	425.8
	Ratio	0.020	0.011
LMØ	Saliva	14.6	11.1
	Serum	179.0	279.6
	Ratio	0.081	0.040

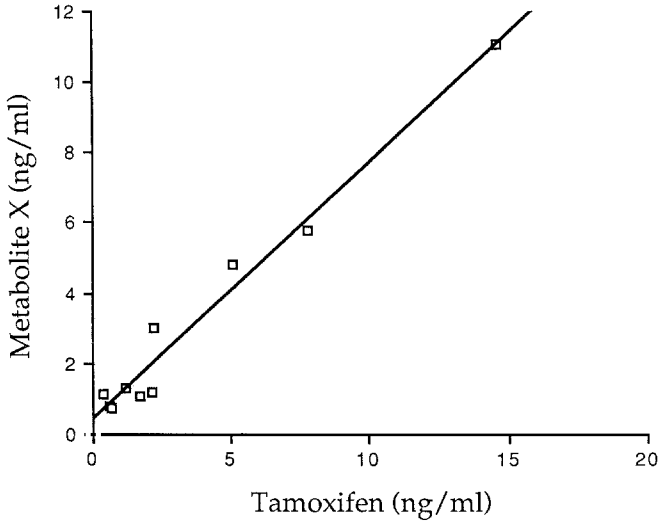

Fig. 6. Correlation between tamoxifen and metabolite X in saliva.

Urine, Bile, and Feces. Fig. 2 shows that the hydroxylated metabolites Y, B, and BX, are the predominating species in urine as well as in bile. Metabolite B and the new metabolite, BX, are about equally abundant in urine, whereas the amount of metabolite BX exceeds that of metabolite B in bile. These metabolites are probably conjugated in urine and bile since their chromatographic peaks appear after β-glucuronidase treatment (Fig. 2, *B* and *C*).

The bile serum ratio for Patient E. B. after 71 days of tamoxifen treatment and 21 days external T-tube was for tamoxifen and metabolites Y, B, BX, X, and Z 0.3, 1.3, 69.0, 47.0, 0.01, and 0.2, respectively.

In feces the metabolite profile is quite different (Fig. 2*D*). Large amounts of tamoxifen and metabolite B were found, whereas smaller amounts of metabolite BX and X were recovered. The recovery of these compounds was about twofold higher when feces was extracted with AcN compared with water (data not shown). Treatment of the fecal extract from one patient with β-glucuronidase increased concentration of tamoxifen and metabolites by less than 27%, whereas the chromatographic profile was unchanged after β-glucuronidase treatment in the two other samples investigated. This suggests that tamoxifen and metabolites are mainly unconjugated in feces.

The amounts of tamoxifen and its metabolites found in urine, bile and feces after β-glucuronidase treatment are summarized in Table 4.

In a preliminary investigation we have found that the amounts recovered from urine after boiling with sulfuric acid increased more than 10-fold. This indicates that other conjugates than glucuronides may be present. However acid hydrolyses may alter the aglycone. These results are therefore difficult to interpret.

DISCUSSION

The present paper describes the distribution of tamoxifen and its metabolites into effusions, cerebrospinal fluid, saliva, urine, bile and feces of patients receiving chronic dosing with tamoxifen. The metabolites investigated include metabolite BX which was recently discovered in our laboratory to be present in human bile (15), but evaluation of its role will depend on knowledge of its concentration in serum and other fluids. Notably, this compound exists in serum and effusions in small amounts which exceed that of metabolite B (Fig. 1, Table 2). Thus, these hydroxylated metabolites may reach the target cells. This may be important since they have a higher affinity than tamoxifen towards the estrogen receptor and may therefore possess significant biological activity (8). However, the largest amounts of metabolite BX are found in conjugated form in excretory fluids like bile and urine (Fig. 2, Table 4), suggesting that metabolism along this pathway contributes to the metabolic clearance of tamoxifen.

Table 4 *Tamoxifen and metabolites extracted from 24-h samples of urine, bile and feces*

All samples were treated with β-glucuronidase, and the values are given in μg/24 h.

		Metabolite				
Material	TAM	Y	B	BX	X	Z
Urine[a]	3.5 ± 2.2	5.6 ± 4.3	8.9 ± 5.1	11.4 ± 5.2	4.7 ± 3.0	0
Bile[b]	12	5	68	98	1	2
Feces[c]	609	24	314	141	92	2
	230–1092	13–41	123–579	92–189	61–121	0–7

[a] Values are given as mean ± SD, $n = 14$.
[b] Sample from one single Patient (E. B.) with liver metastases and external biliary drainage.
[c] Values are given as mean and range, $n = 3$.

We monitored serum tamoxifen, metabolite BX and other metabolites during one dosing interval in two patients (Fig. 3). The concentrations are within the range reported by others (24). Tamoxifen in serum had a peak concentration which subsided within 5 h after drug intake (Fig. 3). This transient increase is probably related to drug absorption (25). A second moderate increase in serum concentration could be explained by enterohepatic circulation which has been reported for tamoxifen in the rat and dog (10).

Most metabolites, especially Y, X, and Z, showed a serum peak concurrent with the absorption peak for tamoxifen (Fig. 3). This could be explained by efficient formation of these metabolites from tamoxifen before the parent drug is distributed to peripheral compartments.

The elimination curve for tamoxifen after drug withdrawal was monoexponential and consistent with a half-life of 7–11 days (Fig. 4) which is in accordance with data reported by others (12). In some patients (A. B., E. M. C., and G. T.) the curves of metabolite X and Z paralleled the tamoxifen decay curve. The same relation to tamoxifen seems to exist for metabolite Y, B, and BX, but determination of the terminal data points was hampered by low concentrations approaching the detection limit of the assay. Our data suggest that the serum levels of metabolites X and Z and probably of Y, B, and BX are production rate limited, and that the elimination rates for these metabolites equal or exceed that of tamoxifen. This conclusion is in variance with the results from single dose experiments showing that metabolite X has a longer half-life than tamoxifen (12, 26). Our data should be interpreted with caution because our patients have various manifestations of breast cancer. Furthermore, one of them changed over from tamoxifen to MPA, a drug which may influence tamoxifen pharmacokinetics (26). Another used a combination of eight drugs including warfarin. The possibility that tamoxifen (and its metabolites) may affect its own metabolism through influence on microsomal enzymes (27) should also be considered.

To the best of our knowledge the distribution volume of tamoxifen has not been previously determined. We calculated the terminal distribution volume (V_z) for tamoxifen to be 50–60 liter/kg. This estimation is based on the assumption that the bioavailability (F) of tamoxifen is equal to one, as reported in animals (10). F has not been directly determined in humans for this drug because no intravenous formulation of tamoxifen is available. However, Adam *et al.* (12) demonstrated equal availability of different oral formulations of tamoxifen, suggesting efficient absorption.

The protein binding of tamoxifen and its metabolites in plasma may affect distribution as well as elimination, but data on protein binding of tamoxifen are sparse (23, 28, 29) and have not been published for tamoxifen metabolites. We investigated the protein binding by ultracentrifugation of patient serum and found that tamoxifen and metabolites Y, X, and Z cosedimented with serum albumin. Our data were consistent with protein binding higher than 98% for these compounds during chronic tamoxifen administration.

The gel filtration experiment with labeled tamoxifen (Fig. 5) suggests that albumin is the main but not the sole carrier of tamoxifen in human serum. A small fraction of labeled tamoxifen was associated with large protein(s) eluting in the void volume of the Sephadex G-100 column (Fig. 5). This binding could be due to the presence of a high affinity, low capacity binding protein analogous to the tamoxifen binding site on low density lipoprotein described in rat serum (23). In view of the high concentration of tamoxifen, *i.e.*, micromolar range, circulating in the blood of treated patients, the importance of such a binding protein seems to be negligible compared to albumin, even at the very low (nM) concentration of tamoxifen investigated (Fig. 5).

The equilibrium dialysis studies revealed high affinity binding of tamoxifen to serum albumin, and there is about one site per albumin molecule. From these parameters and the albumin concentration in human serum one can calculate that albumin alone may bind 98.8% of the tamoxifen in serum. However, the binding parameters obtained for tamoxifen should be viewed with some caution. Only 60% of radiolabeled tamoxifen was recovered in our dialysis experiments (data not shown). We have observed that free tamoxifen adheres to glass surfaces and membranes during dialysis as well as ultracentrifugation. This may lead to underestimation of the free tamoxifen fraction.

Fractionation of serum incubated with labeled tamoxifen on a concanavalin A-Sepharose affinity column showed no radioactivity to be associated with the absorbed glycoprotein fraction (data not shown). This excludes tamoxifen binding to steroid-binding proteins like cortisol binding globulin and sex hormone binding globulin, as well as to α_1-acid-glycoprotein (19). This finding is important because α_1-acid-glycoprotein is a highly fluctuating "acute phase protein" which increases during malignant diseases (30). α_1-Acid-glycoprotein accounts for significant protein binding of many lipophilic basic drugs (30) and has also been shown to bind to steroids (31).

The high degree of binding of tamoxifen to human serum albumin is a factor which tends to retain the drug within the circulation. This is in contrast to the relatively large volume of distribution for tamoxifen (V_z about 50–60 liter/kg), which is in accordance with recovery of high concentration of radioactive tamoxifen in several tissues following i.v. injection into mice (32) and rats (33). Due to the limited binding capacity of the estrogen receptor this may only contribute to the binding of a small fraction of tamoxifen in tissue. However, there are high affinity, high capacity binding sites for tamoxifen, so-called "antiestrogen binding sites," in most tissues (34–36). Such peripheral sites may contribute to the extensive distribution and long half life of tamoxifen. In addition, tamoxifen is a lipophilic drug which may accumulate in brain (32) and fat tissue.

Tamoxifen and all serum metabolites are readily distributed into peritoneal, pericardial, and pleural effusions (Table 2) where these compounds may affect cancer cells. Others have reported on the attainment of a rapid equilibrium of drugs between serum and such effusions (37) which are regarded as shallow compartments. The differences in concentrations between serum and these effusions may be related to fluctuation in serum levels during a dosing interval (Fig. 3) and the turnover of these fluids.

Only trace amounts of tamoxifen and metabolite X (about 0.5% of serum level) were found in cerebrospinal fluid from one patient receiving 30 mg tamoxifen daily (Fig. 1, Table 2). To detect these small concentrations we had to add on-column concentration of extract of 1.5 ml cerebrospinal fluid to our routine assay. Others have reported no detectable tamoxifen in cerebrospinal fluid from a patient receiving 150 mg tamoxifen twice daily (38).

Our results show that tamoxifen and metabolite X cross the blood-brain barrier. There are several possible reasons why only small amounts were detected. Free tamoxifen and metabolites may be avidly adsorbed from a solution like CSF with low protein content to surfaces during sample collection. Assuming that protein-binding of tamoxifen and metabolites approaches 100% in CSF, the protein-bound fraction accounts for most of the drug also in this fluid. Thus, low albumin content explains

the low drug concentration in CSF. The observation that the CSF/serum distribution ratios for tamoxifen, metabolite X, and albumin are nearly equal (Table 2) supports this possibility.

The presence of protein-bound tamoxifen in CSF implies that at least trace amounts of free, active drug exist. Free drug may become distributed into tumor and brain tissue. Notably, Wilking (32) found eight times higher concentrations of radioactivity in brain tissue then in blood of mice injected with [^{14}C] tamoxifen. Small amounts of drug may become available to brain metastases. Such cancer cells are often estrogen receptor negative (39). This may explain the lack of effect of tamoxifen on brain metastases (39).

Compared with cerebrospinal fluid, saliva was rich in the two compounds most abundant in serum, tamoxifen, and metabolite X. Trace amounts of metabolite Z were also found in some samples (Fig. 1). No strict correlation with serum levels was found (Table 3). This could be related to partial drug and metabolite adsorption, which could be demonstrated to occur to waxed sheet (Parafilm) chewed by patients to stimulate salivary flow.

Comparing the protein-binding of tamoxifen and metabolite X (higher than 98%) with the distribution of these compounds into saliva (distribution ratio of 0.004–0.081, Table 3) it is suggested that the level of drug in saliva exceeds the concentration of free drug in serum. High concentrations in saliva lends support to the conclusion that lipid soluble drugs readily cross the gland epithelium (40). For some antiepileptic drugs levels in saliva have been regarded to reflect the free fraction (41). At least two explanations could be offered for the disparity between the unbound fraction and the salivary levels. Firstly, tamoxifen and its predominating serum metabolites may be actively transported into the saliva or trapped in the saliva through interaction with salivary proteins including albumin (42), or because of pH-dependent change in drug ionization (43). Secondly, the free fraction of tamoxifen and tamoxifen metabolites in serum is in fact underestimated due to adsorption, as discussed above.

High concentrations of tamoxifen and tamoxifen metabolites in the salivary glands point to the possibility that these compounds may be accumulated in other exocrine glands as well. In mice injected with [^{14}C]tamoxifen, the drug was enriched in pancreas and lung (32). Tamoxifen is presently under evaluation as a drug in the treatment of pancreas cancer (2).

Conjugated, hydroxylated tamoxifen metabolites (Y, B, and BX) seem to prevail in bile and urine (Fig. 2, Table 4) suggesting that biotransformation to such species are important processes in tamoxifen elimination. In addition, conjugated tamoxifen could also be recovered in significant amounts from bile (Fig. 2, Table 4). However, our results with bile should be interpreted with caution since they are obtained from a single patient with liver metastases and bile drainage; both are factors which may modify bile composition (44). The high bile/blood concentration ratio of metabolites B and BX suggests an active secretory process responsible for their transfer from blood to bile (45). From bile these compounds reach feces which seems to be a major excretory route for tamoxifen in humans (11).

The metabolite profile in feces differs markedly from that of bile and urine. Tamoxifen and metabolites exist in feces mainly as unconjugated species. This may be explained by deconjugation catalyzed by enzymes derived from intestinal microorganisms (46).

Tamoxifen itself and metabolite B are the most abundant compounds in feces. Significant amounts of metabolite BX are also detected (Fig. 2, Table 4). Thus, these hydroxylated metabolites may represent quantitatively important excretory products of tamoxifen, as has been suggested for metabolite B in laboratory animals and humans by others (10, 11). It is possible that tamoxifen in feces is produced through the action of bacterial enzymes on tamoxifen metabolite(s). This is a prerequisite for the proposed (10) enterohepatic circulation of tamoxifen.

Metabolite Y was recovered from feces in only trace amounts (Fig. 2, Table 4) but this metabolite is abundant in bile (Fig. 2) and present in significant amounts in serum from patients receiving high-dose tamoxifen (38). These findings are consistent with reabsorption of metabolite Y from the intestinal contents.

In conclusion, tamoxifen is extensively metabolized. Small amounts of hydroxylated metabolites are detected in serum, where tamoxifen and the most abundant metabolites are found to be highly protein bound. Both tamoxifen and its serum metabolites are readily distributed into pericardial, pleural and peritoneal effusions, whereas only trace amounts seem to cross the blood-brain barrier. Moderate quantities exceeding the free fractions were detected in saliva. Conjugated, hydroxylated metabolites are the prevailing species in excretory fluids like urine and bile, and formation of these compounds may be important for tamoxifen elimination.

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