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Tamoxifen Metabolites in Patients on Long-term Adjuvant Therapy for Breast Cancer

Susan M. Langan-Fahey, Douglass C. Tormey and V. Craig Jordan

Serum concentrations of tamoxifen, 4-OH-tamoxifen, N-desmethyltamoxifen, and metabolites E and Y were assayed to assess the variation of tamoxifen-metabolism during short-term and long-term endocrine treatment for breast cancer. Once steady-state was achieved, serum levels of tamoxifen and its metabolites in individual patients were stable in the short (10 weeks) and long term (over 7 years) (coefficient of variation [CV], 10–15%), but the variation between individuals (CV 50–70%) was high. Serum tamoxifen and N-desmethyltamoxifen levels were not correlated with indices of obesity. Thus this does not explain the large variation between individuals. In addition to the metabolites that were measured, 4-hydroxy-N-desmethyltamoxifen was tentatively identified in patients' serum. Overall, this study demonstrated that the metabolites of tamoxifen are stable (i.e. no metabolic tolerance) for up to 10 years of drug administration.
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INTRODUCTION

SEVERAL METHODS have been described to measure tamoxifen and its metabolites [1–8]. However, their use has often been confined to the analysis of a few patient samples. Sometimes no internal extraction standards have been reported and none was reported to be validated to the good laboratory practice standards required by the Food and Drug Administration (FDA) for reporting clinical trials.

There are several reasons for our study. Tamoxifen is being used ubiquitously for breast cancer therapy and for long periods. Many clinical trials use the drug as an adjuvant for more than 5 years, so it is important to have automated methods to monitor compliance and the correlation of responses to drug levels. In addition, it is important to analyse metabolites during different treatment schedules to ensure that some patients do not demonstrate toxicities or treatment failures based upon their metabolic handling of the drug.

We report validation of an automated high-performance liquid chromatography (HPLC) method based upon our earlier study that described post-column ultraviolet (UV) irradiation to form fluorescent phenanthrene derivatives [3]. We varied the mobile phase composition to measure tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen (4-OH-tamoxifen) and metabolites Y and E in patients' serum. Metabolite Y is the deaminated glycol derivative of tamoxifen whereas metabolite E is the oestrogenic metabolite of tamoxifen that is cleaved at the ether link.

PATIENTS AND METHODS

Solvents and reagents

Solvents were HPLC grade from EM Science (Cherryhill, New Jersey). Glacial acetic acid HPLC grade was from Baker Chemicals (Phillipsburg, New Jersey) and diethylamine was from Aldrich (Milwaukee, Wisconsin). Tamoxifen and its metabolites were gifts from ICI (Macclesfield, UK). Tamoxifen N-oxide was synthesized from tamoxifen freebase by the method of Foster *et al.* [9]. Enclomiphene was a gift from Merrel Dow (Cincinnati, Ohio). Standard stock solution of 1 mg/ml in ethanol were stored in amber vials at −20°C. Working standards were prepared in either ethanol or mobile phase. The working standards in ethanol were used to spike serum standards for extraction.

HPLC

All chromatography was done isocratically on a Gilson 4000 chromatograph with a Gilson 121 filter fluorometer (excitation 254 nm and re-emission filter 330 nm) or a Shimadzu 'rf 535' dual monochromator fluorometer and a Gilson 231 autosampler. Metabolites were separated on a silica, 5 μm particle-size column, 100 × 4 mm (Scientific Glass Engineering, Austin, Texas), with one of two mobile phase systems. After separation, the compounds were converted on-line to their corresponding phenanthrene by UV irradiation. The conversion takes place in a quartz tube approximately 70 cm × 0.3 mm bent into a zigzag pattern and positioned between two mercury (ozone producing) lamps 6.5 cm apart.

System I mobile phase consisted of iso-octane/ethanol/isopropanol/diethyl-amine/acetic acid (75/22/3/0.05/0.05) and used enclomiphene 100 ng in 10 μl ethanol added to each sample and standard as internal standard for assay of tamoxifen, 4-OH-tamoxifen and N-desmethyltamoxifen. The retention times were: enclomiphene 2.25 min, tamoxifen 3.2 min, 4-OH tamoxifen 5.5 min, N-desmethyltamoxifen 8.6 min and 4-OH-N-desmethyltamoxifen 14.7 min at 1 ml/min. N-oxide eluted at approximately 21.8 min as a broad peak.

System II used a mobile phase of hexane with 1% isopropanol and metabolite E as internal standard (20 ng was added in 10 μl ethanol for samples being measured for metabolite Y). Metabolite E had a retention time of 4.1 min and metabolite Y had a retention time of 7.0 min at 1 ml/min. Tamoxifen had a broad peak that was not quantifiable. Eight samples were injected for 10 min runs, then 10% isopropanol in hexane was pumped through the column for 30 min to elute tamoxifen and the 4-hydroxy and N-desmethyl metabolites. The column was equilibrated back to 1% isopropanol in hexane before injecting another set of samples. Samples that were examined for metabolite E were not spiked with this metabolite as internal standard.

Samples were thawed and mixed on the day of the assay.

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Spiked serum standards were prepared by spiking 0.5 ml serum with 20 μl appropriate standard in ethanol. All standards and samples were run in duplicate.

Standard curve levels for tamoxifen were 5 to 100 ng per 500 μl serum; for 4-OH-tamoxifen, 2 to 20 ng per 500 μl; and for N-desmethyltamoxifen, 5 to 200 ng per 500 μl. The standard curve levels for metabolite Y were 1 to 20 ng per 500 μl extract. 5 ml hexane with 2% amyl alcohol was added to each sample and standard. The samples were mixed for 5 min and then centrifuged for 5 min to separate the layers. The upper hexane layer was transferred to a conical tube and dried under N_2 at 45°C. The aqueous layer was extracted with 2 ml hexane/amyl alcohol as before. The second extract was added to the first and dried completely. Finally, the sample was redissolved in 100 μl mobile phase and injected for HPLC.

Quality control samples were run with each set of standards and samples. Batches of quality control samples were prepared ahead of time by spiking control serum with tamoxifen and the 4-hydroxy and N-desmethyl metabolites. These samples were stored frozen until needed.

Stability studies

We assessed the effects of freezing and thawing, dry storage of the extract and haemolysis on the assay for tamoxifen, 4-OH-tamoxifen and N-desmethyltamoxifen. Spiked samples were thawed and refrozen for three cycles and then analyzed. Haemolyzed serum was spiked and then assayed. We also examined the stability of the HPLC assay system for 10 h sample runs. 5 patients' samples of 6 ml serum each were extracted and portions equivalent to 335 μl serum were injected repeatedly overnight. The remaining extracts were left for 24 and 48 h before injection.

Patients

All patients attended the clinic at the University of Wisconsin Clinical Cancer Center (UWCCC). Patients' serum was drawn routinely, logged and stored at −70°C in the UWCCC serum bank. We assayed samples from 35 breast cancer patients who received adjuvant tamoxifen (10 mg twice a day) for at least 2 years at the time of sampling to measure the variation of serum tamoxifen concentrations at steady-state. We assayed 5 patients' samples who had their bloods drawn once per week for up to 10 weeks to assess the within-individual variation. All 5 patients had received tamoxifen (10 mg twice a day) for at least 2 years. We also measured serial serum samples (10–22 samples per patient) from 10 patients who had taken the drug for at least 7 years to assess the long-term variation of levels of tamoxifen and metabolites.

Calculations

The Gilson 714 software prints out peak height on a Tandy 3000 computer connected to the HPLC equipment. Each metabolite peak height was divided by the appropriate internal standard peak height to give a ratio that was plotted against its corresponding standard level. Quality control patients' sample concentrations were calculated from the linear regression equation obtained with the spiked serum curve data. Recoveries were calculated by (peak height serum extract injected ÷ peak height metabolite injected in mobile phase) ÷ fraction of extract injected.

RESULTS

Validation

A 3 day validation study showed that extracted serum curves for tamoxifen, 4-OH-tamoxifen and N-desmethyltamoxifen were linear and reproducible within FDA guidelines for HPLC-based assays. Tamoxifen averaged over all levels for 3 days (2 repeats per day at each of 6 levels) had a coefficient of variation (CV) of 5.4%. We found CVs of 5.4% and 12.4% for 4-OH-tamoxifen and N-desmethyltamoxifen, respectively. The absolute recoveries when averaged for 3 days over all levels were

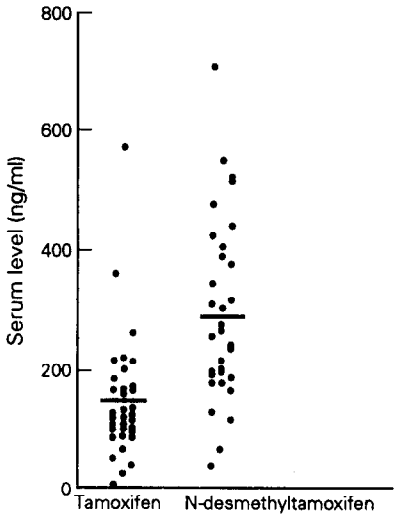

Fig. 1. Serum tamoxifen and N-desmethyltamoxifen levels measured in 35 breast cancer patients who had received 20 mg daily for at least 2 years. Solid bar = mean.

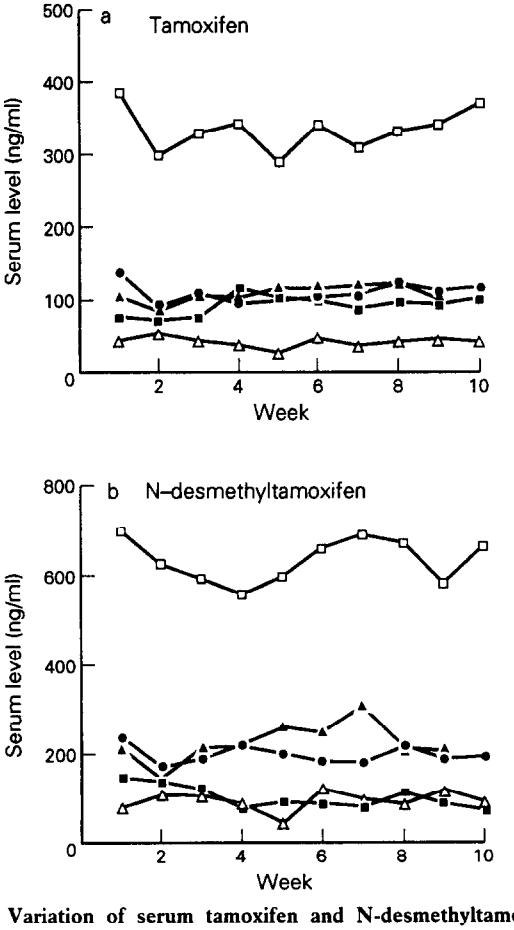

Fig. 2. Variation of serum tamoxifen and N-desmethyltamoxifen levels within individual patients who were measured once per week for 10 weeks. □ = patient 11, • = 12, ▲ = 13, ■ = 14 and △ = 15.

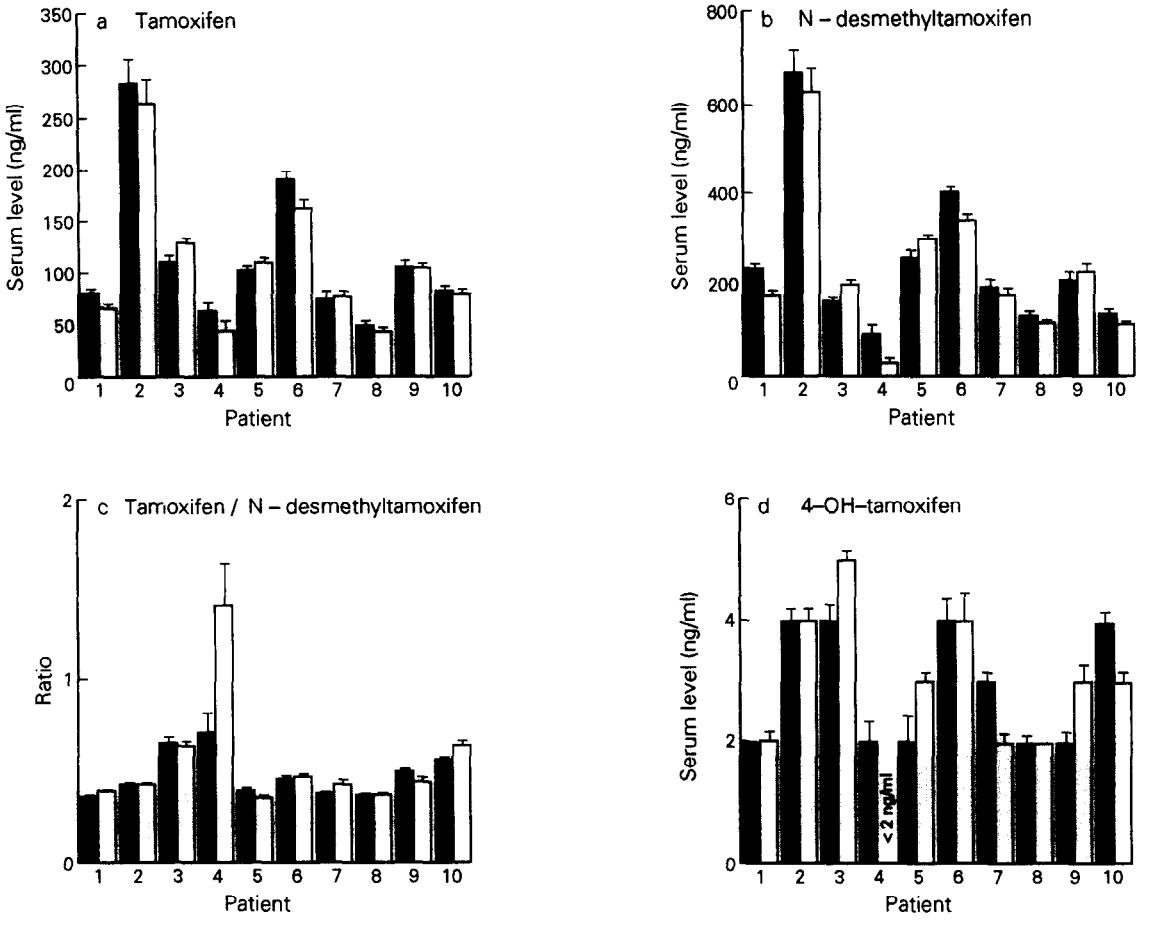

Fig. 3. 10 patients treated for 7–11 years with tamoxifen. For each patient the values were grouped and then averaged post chemotherapy for years 0–4 (solid bar) and > 4 (open bar). Error bars are S.E.

75% for tamoxifen, 86% for 4-OH-tamoxifen and 71% for N-desmethyltamoxifen.

We also measured the absolute recoveries for extracting once with 2.5 ml of hexane/amyl alcohol and found them to be similar to the twice-extracted data (tamoxifen 87%, 4-OH-tamoxifen 86% and N-desmethyltamoxifen 59%). Enclomiphene extracted with an efficiency of 71%. Under these conditions, tamoxifen and its N-desmethyl metabolite can be accurately measured at 10 ng/ml serum and the 4-hydroxymetabolite at 2 ng/ml with the Gilson 121 fluorometer.

Quality control samples, run on each of the 3 days, averaged 96%, 96% and 87% of the expected values for tamoxifen (10 and 50 ng), 4-OH-tamoxifen (5 and 20 ng) and N-desmethyltamoxifen (10 and 125 ng) over the 3 day period. We found no interference from haemolyzed blood or three cycles of freeze/thaw on the extraction of spiked quality control samples.

Our 10 h stability study showed that the system gave reproducible results. The CVs for tamoxifen and the N-desmethyl metabolite were 10% and 11%, respectively, when averaged for the 5 samples (each patient's sample was injected 10 times). There was no apparent trend to higher or lower values at the end of 10 h for either compound. The 4-hydroxy metabolite was not detected.

Metabolite Y gave a linear response in the concentration range measured that was reproducible over the 3 day period. The recovery of metabolite Y when averaged over all levels was 92%. The quality control samples measured to within 10% of the expected values. We found the limit for accurate measurement of metabolite Y to be 1 ng/ml serum. The detection limit for metabolite E was about 0.3 ng/ml.

The sensitivity of the assay can be increased by extracting larger volumes of serum (1–2 ml), spiking smaller amounts of standards for the standard curves and using the Shimadzu fluorometer. The maximum sensitivity with appropriate standard quantities would be approximately 0.6 ng/ml for tamoxifen and the 4-hydroxy and N-desmethyl metabolites and for metabolites Y and E.

Serum levels of tamoxifen and metabolites

In serum samples from 35 patients who had received tamoxifen for at least 2 years, tamoxifen levels ranged from 8 to 574 ng/ml (mean 148, S.E. 16.9) and N-desmethyltamoxifen ranged from

Table 1. *Quality control data for HPLC analysis of tamoxifen, 4-OH-tamoxifen and N-desmethyltamoxifen*

	Tamoxifen	4-OH-tamoxifen	N-desmethyltamoxifen
Low			
Measured	9 (0.34)	5 (0.17)	10 (0.45)
Expected	10	5	10
High			
Measured	43 (1.02)	20 (0.54)	116 (3.72)
Expected	50	20	125

Each value is mean (ng/ml) of nine daily means (S.E.).

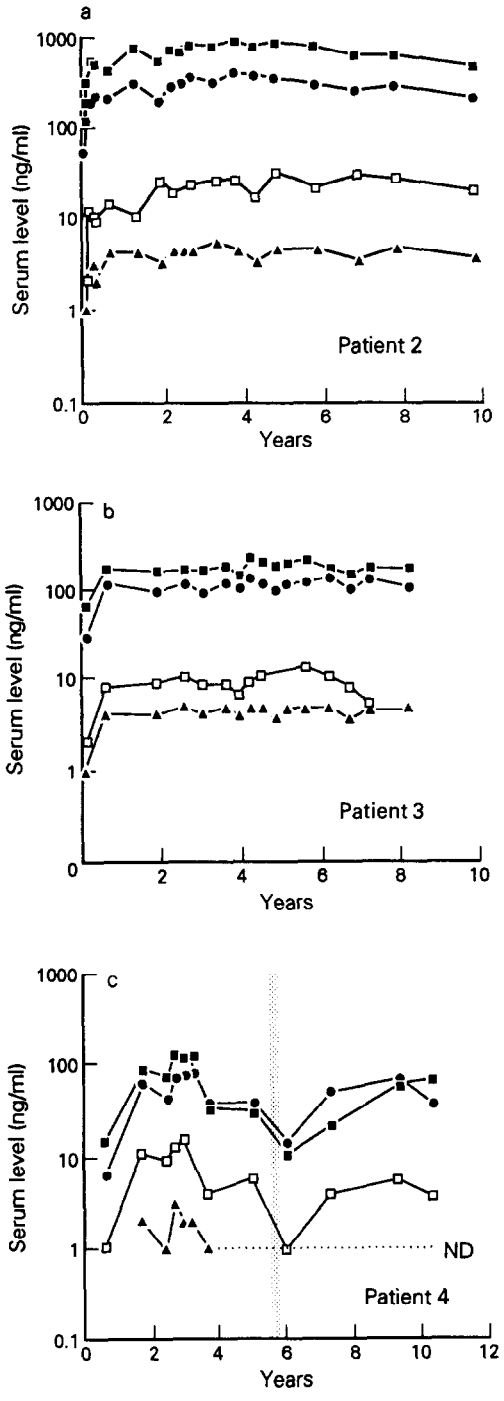

Fig. 4. Long-term serum levels of tamoxifen and three metabolites in individual patients: patient 2 = highest serum tamoxifen level, patient 3 = average level, and patient 4 = most variable level (shaded area represents 98 days of withdrawal from tamoxifen treatment). • = tamoxifen, ■ = N-desmethyltamoxifen, ▲ = 4-OH-tamoxifen and □ = metabolite Y. ND = not detected, < 1.0 ng/ml.

35 to 709 ng/ml (290, 24.5) (Fig. 1). Both compounds had high CVs: 68.9% and 50.7%, respectively. The ratio of tamoxifen to the N-desmethylmetabolite was constant over the range of serum tamoxifen levels except at the highest and lowest values. The mean value was 0.49 with a CV of 30%. Serum tamoxifen or N-desmethyltamoxifen levels were not correlated with weight, height, weight ÷ (height)^2 or body surface area.

The week-to-week variation in patients sampled once per week for up to 10 weeks was less than that between individuals (Fig. 2). Samples were measured by the Gilson fluorometer. Overall, the 5 patients gave a CV of 12.3% for tamoxifen and 15.4% for N-desmethyltamoxifen. The ratio had a CV of 13.4% and averaged 0.65 for all 5 patients. 1 patient had a ratio of 1.21 and was clearly different from the other patients. Metabolite Y is a minor metabolite of tamoxifen and was present at low concentrations ranging from 10 to 16 ng/ml serum.

Patients on long-term tamoxifen

Because of limited serum available from these patients, we extracted 250 μl serum and spiked half the usual amounts of standards for the standard curve. We also used the Shimadzu fluorometer. All metabolite data yielded a straight line when plotted. Quality control values for each metabolite were consistent at the high and low range over the 9 day sample analysis (Table 1).

We identified 10 patients who had taken tamoxifen for 7–11 years. 9 patients took the drug continuously; patient 4 was withdrawn from tamoxifen for 98 days during year 6 and then resumed treatment. All patients were disease-free. We found the average serum tamoxifen level varied from 48 to 283 ng/ml. Despite the wide range of values in these patients, those taking the drug continuously did not show a trend toward higher or lower values when the serum levels were grouped and averaged for the first 4 years of treatment and then compared with the average serum level after 4 years of treatment (Fig. 3).

To further illustrate the stability of the serum metabolite levels during long-term treatment, data from a patient with high levels (patient 2) and average TAM levels (patient 3) are shown in Fig. 4. In both cases, the serum levels of tamoxifen and three metabolites were constant for 10 years. Data from patient 4, who had the most variable levels, are also included in Fig. 4.

We were unable to detect metabolite E (an oestrogenic compound) in system II in the 3 patients shown in Fig. 4. However, we noted an additional peak using system I. We have tentatively identified the metabolite as 4-OH-N-desmethyltamoxifen because it co-chromatographed with the authentic compound. The chromatographs of 9 patients on long-term treatment exhibited a peak with a retention time similar to authentic 4-OH-N-desmethyltamoxifen.

DISCUSSION

The long-term use of tamoxifen [10–13] has made it important to monitor compliance and establish baseline levels of the drug for a particular patient. It is clear that patients exhibit a broad range of serum levels of tamoxifen [14–16]. We found the minor metabolite, 4-OH-N-desmethyltamoxifen, in patients' serum during long-term tamoxifen therapy. This metabolite has been found in bile [17] and serum [18] from patients receiving tamoxifen and in athymic mouse serum during long-term therapy [19]. The metabolite would be predicted to be a potent antioestrogen because of the 4-hydroxyl group. The variations observed between individuals were not usually seen in serial samples from the same patients, as evidenced by the lower CVs found in the serial samples compared with the CVs of individual samples.

This finding raises important considerations. Firstly, without the continous monitoring of patients, it is virtually impossible to establish whether a woman is non-compliant (as in the case we previously reported [20]) or has a low steady-state level of the drug. This cannot be judged from a single measurement. Also, adjuvant studies often assume that patients take the drug or not (depending in which arm of the study a patient finds herself), and use a random sample to monitor compliance. Again

it is unclear how the overall compliance is evaluated for the trial or whether the actual dose of drug is routinely taken. Indeed, much attention had focused upon the response to adjuvant chemotherapy and the dose actually given [21] but no studies are being considered in clinical trials with tamoxifen; however, this is now possible with an automated system to monitor serum levels.

Unlike chemotherapy, tamoxifen is given as a daily dose rather than a dose per metre squared. To determine whether this was a factor in the widely different values we found in patients, we related the serum level of tamoxifen and the N-desmethylmetabolite to indices of obesity, but saw no association.

Could patients who have higher circulating levels of tamoxifen have a better response to the drug? This does not seem to be true for unselected patients with advanced disease [22]; however, adjuvant therapy may be somewhat different, especially if groups are preselected by hormone receptor level. The hypothesis would be that patients with low circulating levels of tamoxifen would fail therapy before those with high levels. Low concentrations of anti-oestrogens can stimulate breast cancer cell replication *in vitro* [23–25], which has been suggested [26] as a possible mechanism of the tumour flare sometimes seen when patients are first given tamoxifen [27, 28]. An initially low level of the drug may stimulate the tumour cells to grow, but as serum levels rise to steady-state, blockade of the cell cycle may occur. This hypothesis, however, has never been tested clinically and there are no data to correlate what is occurring in the tumour with circulating levels. A patient who has rapid clearance of the drug and perhaps low levels in the plasma may be at greater risk for stimulating breast cancer cells to replicate than a patient with higher levels of the drug. Compliance may also significantly affect the patient with initially low serum levels of tamoxifen. If the drug is stopped, tumoristatic serum levels will be cleared rapidly so that drug fluctuations caused by the sporadic taking of the drug may cause tamoxifen-stimulated growth. These questions can only be answered by correlating blood levels of parent compound and metabolites with disease-free survival of patients in adjuvant trials.

It is increasingly important to monitor tamoxifen and its metabolites in clinical trials to determine whether certain features of drug handling are associated with toxicities or therapeutic failures. The proposed use of tamoxifen as a chemosuppresive agent in women at high risk for the disease [29] underscores the need to evaluate the clinical pharmacology of the drug carefully before treating patients without breast cancer. Although many drugs produce metabolic tolerance after months of therapy, tamoxifen has steady serum levels and consistent ratios of metabolites for up to a decade of treatment

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Cellular Retinol Binding Protein and Breast Carcinoma

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Cellular retinol binding protein (C-RBP) levels were measured in 87 malignant and 18 non-malignant breast cancer tissues. C-RBP, sedimenting in the '2S' region on 5–20% sucrose density gradients, was detectable in 70% of malignant tissues examined. None of the non-malignant tissues contained detectable C-RBP. No significant association between tumour steroid receptors status, patients' obesity or menopausal status and C-RBP contents was observed. However, patients with stage IV disease had higher C-RBP levels than patients at stages II and III ($P < 0.0001$), which suggested altered intracellular mobilization of retinol in the tumour, probably as an indirect consequence of inadequate nutrient intake.
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INTRODUCTION

VITAMIN A and retinoids are important in the growth and differentiation of epithelial cells [1]. Under experimental conditions, cells deprived of vitamin A are prone to malignant transformation [2, 3]. Dietary vitamin A deficiency increases the frequency of spontaneous and virus or carcinogen induced epithelial malignant tumours in laboratory animals [4–8]. A synthetic analogue of vitamin A (4-hydroxy-phenylretinamide [4HPR]) is effective against carcinogen N-nitromethylsourea (NMU) induced tumours in rats [9, 10]. Also, synergistic or additive inhibitory action of 4HPR and ovariectomy or anti-oestrogen has been reported [10]. However, reports on the chemopreventive action of vitamin A in humans are conflicting. Prospective and retrospective studies in humans suggest a relation between vitamin A deficiency and malignancies of epithelial origin [2, 11]. Epidemiological studies on a specific geographic population also suggested an inverse association between blood levels of vitamin A and risk of developing cancer [12–16]. In contrast, Willett *et al.* [17] failed to observe such a relation between risk of developing cancer and blood vitamin A levels. Prospective studies on blood levels of vitamin A, β-carotene or retinol binding protein (RBP) failed to correlate vitamin A levels and development of cancer [17].

We have studied plasma RBP in premenopausal breast carcinoma patients receiving adjuvant chemotherapy. Women with lower plasma RBP during the course of chemotherapy had tumour recurrence earlier than those who had higher RBP levels [18]. No such correlation between plasma vitamin A or β-carotene levels and time to tumour recurrence was observed. In the present study, we measured cellular RBP (C-RBP) levels in malignant and non-malignant breast tissues. We investigated correlations between C-RBP status in the tumour and the patient's menopausal, steroid receptor and disease status.

MATERIALS AND METHODS

Clinical materials

Clinical material was available through the cooperation of surgeons and pathologists associated with the Division of Surgical Oncology, University of Illinois Hospital, and Cook County Hospital. Malignant ($n = 87$) and non-malignant ($n = 18$) breast tissues were obtained from women with confirmed diagnosis of cancer undergoing biopsy or mastectomy. Following surgical excision, tissues were immediately transported on ice to our laboratory, freed from connective tissues and blood vessels, and stored at −80°C until assayed for receptors or C-RBP. Information about the patients' age, body weight, height, menopausal status and clinical staging of disease were maintained in the divisional computer system.

Chemicals

[^{3}H]-oestradiol (2,4,6,7,^{3}H-N-oestradiol, specific activity 362 × 10^{10} Bq/mmol), [^{3}H]progesterone (17-N-methyl-^{3}H, 322 × 10^{10} Bq/mmol) and ^{3}H-retinol (74 × 10^{10} Bq/mmol) were obtained from New England Nuclear. All assays were performed with 'Tris'–EDTA buffer (10 mmol/l Tris–HCl, 1.5 mmol/l

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