Optimised analysis of tamoxifen and its main metabolites in the plasma and cytosol of mammary tumours

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Summary Recent biochemical and pharmacological findings concerning tamoxifen (TMX) have proven that both the unchanged drug and the main metabolites, N-desmethyltamoxifen (NDT) and 4-hydroxytamoxifen (4OHT) are biologically active. An HPLC method based on on-line post-column UV irradiation with fluorescence detection is described. Optimized conditions allowed complete and rapid separation of TMX 4OHT, NDT and two other recently reported metabolites, Y and Z. This method was applied to plasma and cytosol drug and metabolite analyses. In plasma, from the moment of initial drug administration until the steady state (after 1 month or more of continuous oral TMX treatment), the values of NDT to TMX ratios were completely reversed: 22 to 215 in mean %, P < 0.01. The presence of metabolites Y and Z is significant. 4OHT, hardly detectable at the first dose, was measured at the steady state with high interpatient variability. It is hypothesized that metabolite evolution with time may be due to auto-induction of drug metabolism. In cytosols, which were all obtained during continuous TMX treatment, the ratios between TMX and metabolites were comparable to those observed in plasma, but with greater interpatient variability. Metabolite Y was not detectable in cytosols. This variability was not linked to the levels of cytosolic oestradiol receptors before initiation of treatment.

Recent literature has provided new insights into the molecular pharmacology of tamoxifen (TMX), its main metabolites, N-desmethyltamoxifen (NDT), 4-hydroxytamoxifen (4OHT), and its more recently reported metabolites Y (Jordan et al., 1983) and Z (Kemp et al., 1983). Although their biochemical identity has not yet been established, antioestrogen binding sites, which differ from oestriadiol receptors (ER), are the subject of current investigation (Sutherland & Murphy, 1980; Sudo et al., 1983; Fernö & Borg, 1983). The binding affinity of E2, TMX, NDT, and 4OHT relative to ER warrants increased study (Borgna & Rochefort, 1980; Fabian et al., 1981; Reddel et al., 1983; Miller et al., 1984). These investigations have shown that 4OHT, due to its preponderant affinity for ER, may play a critical role in the overall antioestrogenic action of the triphenylethylen derivative. These studies have emphasized the need for an analytical method allowing simultaneous measurement of TMX and its main metabolites not only in plasma, but also in tumour tissue in order to examine the possible relationship between distribution of TMX and/or metabolites and tumour response. Among recently developed methods, HPLC seems the most adequate since it is both sensitive, selective and suitable for routine analysis (Brown et al., 1983; Camaggi et al., 1983). Improvement of analytic parameters has resulted in original conditions compatible with sensitive and selective determination of TMX, NDT, 4OHT, Y and Z. Application to individual pharmacokinetics and cytosol drug assays is presented for breast cancer patients under TMX treatment.

Materials and Methods

Reagents
Tamoxifen (ICI 46 474), N-desmethyltamoxifen (ICI 55 548), 4-hydroxytamoxifen (ICI 79 280), and metabolites Y (ICI 142 269) and Z (ICI 142 268) were provided by ICI (Pharmaceuticals Division, Macclesfield, UK). The internal standard, clomifene (CMF), was provided by Merrel Laboratories (Paris, France). Stock solutions of drugs (100 μg ml⁻¹) were prepared in absolute ethanol and stored in polyethylene flasks at −20°C. Working standards were prepared by appropriate dilutions of these stock solutions in polyethylene tubes. Acetonitrile for HPLC, and Butanol-IPA were obtained from MERCK (Darmstadt, FRG). Methanol, diethyl ether, and hexane (all Normapur) were purchased from Prolabo (Paris, France). Phosphoric acid 85% was from Carlo Erba (Milan, Italy); KH₂PO₄ Rectapur was from Prolabo (Paris, France). Silicone solution was from Serva (Heidelberg, FRG).

Apparatus
Figure 1 details the system used for chromatographic separation, on-line photocyclisation, spectrofluorimetric detection, and recording. A 6000A pump, U6K injecter, and RCM 100 column compression module were supplied by Waters Associates (Milford, MA, USA). A spectrofluorimeter SFM25 (Kontron, Zurich, Switzerland) was equipped with 10 and 15 mm slits; voltage was set at 600 V and the gain between 0.2 and 0.5. A Hewlett-Packard 3390A integrator (Arondale, PA, USA) was used for chromatographic recording. The UV on-line photocyclisation system was derived from a previously described system (Camaggi et al., 1983): at the outlet of the column, a 6.5m long Teflon capillary tube (0.35 mm ID, 1.5 mm OD) was arranged in 7 superposed circulations (20 cm diameter), with a Philips HPK 125 watt high pressure mercury lamp located in the centre. This irradiation system was enclosed in a wooden box with forced ventilation (20 x 30 x 40 cm).

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Chromatographic conditions

Mobile phase: KH₂PO₄ 10⁻² M/20%; H₃PO₄ 0.3 M/10%; H₂O/28%; CH₃CN/42%.

Columns: The performance of a conventional stainless steel column (Zorbax CN 4.6 mm × 25 cm, DuPont Wilmington, DE, USA), flow rate 2.8 ml min⁻¹, was compared to that of a radially compressed column (Rad Pak Cartridge CN 10 μm 100-8, Waters Associates, Milford, MA, USA), flow rate 2.5 ml min⁻¹. Absorption and emission spectra were recorded for MTX, 4-OHT, NDT, Y and Z (1 μg ml⁻¹ by stop-flow after on-line UV irradiation). Optimal routine conditions were: ex = 258 nm and em = 378 nm.

Sample preparation

One ml blood samples were obtained in EDTA tubes. All glass tubes used for extraction were siliconated before use. The different types of plasma extractions described in the literature were compared:

**Organic extraction** Diethyl ether (Golander & Sternson, 1980); hexane-butanol 2% (Brown et al., 1983). Plasma (0.5 ml), spiked with 2 μg clomifene, was extracted twice with 4 vol of organic solvent each time. The organic phases were combined after centrifugation (2000 rpm, 4°C, 10 min) and dried under N₂, at 37°C. The dried residue was reconstituted in 250 μl of methanol, centrifuged (2000 rpm, 4°C, 10 min), and 10–100 μl were injected.

**Sep pak C₁₈ extraction** (Waters Associates, Milford, MA, USA) (Camaggi et al., 1983) One ml plasma, spiked with 4 μg clomifene, was treated with 2 ml water/methanol (1:1). After vortexing and centrifugation (2000 rpm, 4°C, 10 min), the supernatant was filtered through the SEP PAK cartridge previously activated by the passage of 3 ml of methanol followed by 3 ml of H₂O. Successive elutions were performed with 5 ml H₂O, 1 ml H₂O, CH₃CN 1:1, and 0.5 ml CH₃CN. All eluates were discarded. The eluate from the last elution (5 ml 0.3 M phosphoric acid in CH₃CN) was concentrated under vacuum (Büchi Rotavapor R). The dried residue was reconstituted with 250 μl of methanol and 50 μl were injected.

Results

**Choice of the column system**

Figure 2 shows the HPLC profiles of TMX and its main metabolites separated by the conventional stainless steel column which gave the best performances. The values of the capacity factor K* were as follows: 4-OHT, 2.79; Z, 4.07; NDT, 4.93; TMX, 5.71; Y, 7.36; CMF, 7.50.

**Choice of the extraction process**

Recoveries were low (close to 30%) with cartridge extraction. Organic extraction was better; higher recuperation was obtained with 2% hexane-butanol, but the cleanest blank plasma resulted from diethyl ether extraction. This last extraction process was retained, and gave the following recoveries (spiked plasma at 100 ng ml⁻¹): 4-OHT, 68%; Z, 62%; NDT, 92%; TMX, 67%; Y, 95%; CMF, 85%.

**Linearity, sensitivity, reproducibility**

When the mean peak height (Y, mm) is considered as a function of spiked plasma extracted in triplicate by diethyl ether (x = 5, 10, 20, 50, 100 ng ml⁻¹), regression lines (y = a + bx) were obtained with r² at 0.99. The limit of sensitivity 2.5 times the baseline height for 500 μl of diethyl ether extracted plasma (100 μl injected) was 2 ng ml⁻¹ (0.5 ng injected) for 4-OHT, Z and TMX, and 1.5 ng ml⁻¹ (0.3 ng injected) for NDT and Y. Intra- and inter-assay reproducibility (spiked plasma at 100 ng ml⁻¹, 6 points) was respectively (coefficient of variation, %): 4-OHT, 10 and 11; Z, 7 and 11; NDT, 2 and 10; TMX, 3 and 14; Y, 3 and 7.

**Plasma levels in treated patients**

Figure 3a gives the profile of a blank plasma and Figure 3b the plasma profile of a patient under TMX treatment. Quantitatively, NDT appears the major metabolite; 4-OHT, Y and Z were formed to lesser degrees.

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**Figure 2** HPLC profile of pure compounds 4-OHT (1), Z (2), NDT (3), TMX (4), and T (5). Internal Standard (6). 25 μl of a 200 ng ml⁻¹ solution of each compound injected HV = 600 V gain = 0.5.

**Figure 3** Plasmatic profiles HV = 600 V gain = 0.5. (a): blank plasma; (b) extracted plasma of a patient under treatment. For details see Materials and methods. Peaks 1–6 as described in Figure 2.
Table I presents the main pharmacokinetics data for a single dose of TMX and at the steady state for 6 patients. 4OHT was hardly detectable at the first dose. By contrast, 4OHT was present at the steady state (1 month or more). Metabolites Y and Z followed the same mode of evolution, and represented significant metabolites during chronic treatment. High inter-patient variability was seen in both TMX and metabolite plasma levels, particularly at the steady state. It is striking to note that the NDT to TMX ratios were completely reversed from the first dose (mean=22%) to the steady state (mean=215%), \( P<0.01 \). There was a similar but not significant trend for metabolite Z. The proportion of metabolite Y did not vary, on the average, between the first dose and the steady state.

**Drug levels in tumour cytosol**

Table II lists the cytosol in values of TMX and its main metabolites for 5 patients during continuous treatment. Globally, the cytosol distribution of TMX and metabolites reflected the general profile in serum (excess of NDT), although metabolite Z represented a higher percentage than in serum. Metabolite Y was never detectable. There was no apparent link between the ER level and the intra-cytosol concentration of TMX and metabolites.

**Discussion**

The increasing clinical use of the antioestrogen compound TMX has stimulated experimental efforts to elucidate its complex mechanism of action (cf. review of Furr & Jordan, 1984). Sutherland and Murphy (1980) investigated the comparative binding of \(^{3}H\) E\(_{2}\) and \(^{3}H\) TMX in ER positive and negative cytosols of human breast cancer carcinomas; their results support the evidence for the presence of anti-oestrogen binding sites which E\(_{2}\) was unable to saturate in binding. Certain recent investigations have confirmed the existence of such specific triphenylethylene binding sites (Miller et al., 1984) whereas others do not (Raam et al., 1983; Fernö & Borg, 1985). The relative affinity of E\(_{2}\), TMX, NDT, 4OHT and Y for ER has been extensively explored, and general consensus is observed in the literature (Borgna & Rochefort, 1980; Fabian et al., 1981; Jordan et al., 1983; Reddel et al., 1983; Miller et al., 1984; Tate et al., 1984). Thus, 4OHT appears to be equally or more potent than E\(_{2}\) for binding, whereas TMX, NDT and Y are bound with a lesser affinity. The observation that the drug concentration may condition the mode of action of triphenylethylene derivatives is an interesting acquisition (Reddel et al., 1985). In brief, on a submicromolar level, TMX and its metabolites can inhibit cellular proliferation (Reddel et al., 1983; Brand & Lykkefeldt, 1984; Taylor et al., 1984); for other cellular systems, TMX and its metabolites can stimulate cell proliferation (Reddel & Sutherland, 1984). Above micromolar level, the antioestrogens are cytotoxic, the order of potency being NDT > 4OHT > TMX (Reddel et al., 1983). All of these concepts require clinical confirmation and this necessitates a sensitive and specific method for analysis of TMX and related metabolites. In the present method, we adopted the principle of on-line post-HPLC

**Table I** Pharmacokinetics parameters at the first dose and steady state for breast cancer patients treated by adjuvant hormone therapy with TMX only

| Patients | TMX (ng ml\(^{-1}\)) | 4OHT (ng ml\(^{-1}\)) | NDT (ng ml\(^{-1}\)) | Y (ng ml\(^{-1}\)) | Z (ng ml\(^{-1}\)) | \(\frac{NDT}{TMX}\%\) | TMX (ng ml\(^{-1}\)) | 4OHT (ng ml\(^{-1}\)) | NDT (ng ml\(^{-1}\)) | Y (ng ml\(^{-1}\)) | Z (ng ml\(^{-1}\)) | \(\frac{NDT}{TMX}\%\) |
|----------|----------------------|----------------------|----------------------|-------------------|------------------|----------------------|----------------------|----------------------|----------------------|-------------------|------------------|----------------------|
| LIS      | 49.1                 | ND                   | 10.8                 | 1.5               | ND               | 22                   | 74.0                 | 3.4                  | 193.1               | 20.7              | 24.5             | 261                |
| TOU      | 46.0                 | ND                   | 11.5                 | 1.8               | 3.3              | 25                   | 152.6                | 13.5                 | 352.0               | 19.9              | 39.5             | 231                |
| SPA      | 73.9                 | ND                   | 11.9                 | 7.6               | 1.9              | 16                   | 120.0                | 6.5                  | 258.0               | 40.0              | 50.0             | 215                |
| RAM      | 90.0                 | 1.5                  | 10.8                 | 11.8              | 6.3              | 12                   | 77.5                 | 2.9                  | 96.2                | 14.7              | 7.4              | 124                |
| PIS      | 66.0                 | ND                   | 17.8                 | ND                | 2.2              | 27                   | 147.8                | 6.6                  | 214.8               | 28.4              | 33.3             | 145                |
| RAY      | 74.8                 | ND                   | 21.7                 | 11.1              | ND               | 29                   | 298.9                | 5.9                  | 941.8               | 83.9              | 176.2            | 315                |
| Mean     | 66.5                 | (14.1)               | (21.7)               | (11.1)            | (5.6)            | (2.2)                | (145.1)              | (6.5)                | (342.6)             | (34.6)            | (55.1)           | 215                |

\(\text{Significantly different from the first dose} P<0.01 \text{ (t test of paired samples). For the first dose, blood samples were collected at time (h): 1, 2, 4, 6, 12, 24. At steady state (one month or more), blood samples were obtained at 8 am, before TMX intake. ND: Not detectable (below limits of sensitivity, see text for values).}\)

**Table II** Cytosol TMX and metabolite levels

| Patient (material) | Receptor status | TMX and metabolite concentrations (ng mg\(^{-1}\) DNA) |
|--------------------|-----------------|---------------------------------------------------|
| FAG (breast tumour) | 5 25            | 16.8 22.4 95.3 ND 53.2 |
| MAU (breast tumour) | 35 0            | ND ND ND ND |
| UNT (breast tumour) | 5 0             | 28.1 ND 131.8 ND 97.7 |
| AUG (ascitic cells) | 0 0             | 6.8 8.6 ND ND ND |
| (Breast = primary)   |                 |                                                   |
| PAP (breast tumour) | 0 0             | 95.6 ND 227.8 ND 85.2 |

\(\text{Steroid receptors measured before initiation of treatment as previously described (Milano et al., 1983). Biopsies for drug measurement were obtained 8 days after initiation of TMX treatment (30 mg day}^{-1} \text{ orally). Cytosol was extracted, like plasma, by diethyl ether after spiking with internal standard. ND = Not detectable (below limits of sensitivity, see text for values).}\)
column UV photocyclisation and fluorescence detection (Brown et al., 1983; Camaggi et al., 1983). The optimal analytical conditions described herein allowed complete separation and quantification of TMX from all of the metabolites that have been reported until now in humans (Furr & Jordan, 1984). The resulting high sensitivity made possible a limit of detection in plasma in the range of 2 ng ml⁻¹ for a small extracted volume (500 μl), thereby allowing acceptable blood sampling for repeated pharmacokinetic studies. In addition, cytosol measurements of TMX and metabolites were possible for breast cancer patients under treatment. Other workers, using a sophisticated gas chromatography mass spectrometric method, failed to detect 4-OHT in cytosol (Daniel et al., 1981). Application of the present method to blood monitoring and cytosol measurement of TMX and metabolites warrants several comments. (a) The relative proportions of NDT and TMX systematically varied between the administration of the first drug dose and the pseudo-steady state (1 month or more of continuous oral treatment). This fact was reported previously (Fabian et al., 1980; Wilkinson et al., 1980; Kemp et al., 1981). However, this has not been quantified so far for individual patients. This observation may be due to the longer elimination half-life of the metabolite (Adam et al., 1980). Enzymatic induction of N-demethylation is another possible explanation; this hypothesis is supported by the fact that continuous oral administration of the drug predisposes to a hepatic first pass effect. Supporting this view, 4-OHT, which was scarcely detectable at the first dose, was present at the steady state with high intersubject variability. Owing to the inherent difficulties in separating 4-OHT and NDT by classical thin layer chromatography. 4-OHT was not evaluable in previous pharmacokinetic studies (Wilkinson et al., 1980). The presence of metabolite Z has also been previously signaled (Kemp et al., 1983), but not quantified in a series of patients. Present data show that metabolite Z represents a significant part of the circulating drug profile. Experimental studies to evaluate its pharmacology and contribution to the activity of the parent drug appear justified, as has been done for metabolite Y (Jordan et al., 1983). (b) The distribution between the unchanged drug and its metabolites in cytosol is virtually the same as in plasma, although greater dispersion occurs in tumours. Metabolite Y was never detectable. Variability was not linked to the pretreatment ER content of the tumour cytosol. This observation confirms the need for more thorough investigations on the so-called antiestrogen binding sites and their possible role in drug-related actions. Although a correlation has recently been reported in animals between the tumour TMX content and tumour regression (Daniel et al., 1984), clinical extrapolation of the present results is beyond the scope of this paper.

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