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Direct liquid chromatographic micro-measurement of tamoxifen in plasma of cancer patients

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Abstract

We describe in this report a sensitive and direct method for the analysis of tamoxifen (TAM) in microsamples of plasma. The drug and internal standard (quinine bisulfate, I.S.) were separated on a 10-μm particle, 10 cm×8 mm CN cartridge in conjunction with a radial compression system. The mobile phase was a mixture of 0.1 *M* sodium acetate in 0.001 *M* tetrabutylammonium phosphate solution (pH 6) and methanol (30:70, v/v) at a flow-rate of 4 ml/min. After addition of I.S. and *o*-phosphoric acid in acetonitrile (0.6 *M*) to the plasma (30 μl), the mixture was placed in an ultraviolet shortwave transluminator for 2 min prior to injection into the chromatograph. The compounds were detected in the effluent fluorometrically at excitation and emission wavelengths of 258 and 378 nm, respectively. Under these conditions, no interference in the assay from any endogenous substance or other concurrently used drugs was observed and the retention times of I.S. and TAM were 4.4 and 10.15 min, respectively. The concentration of TAM in plasma was linearly (r>0.9983) related to the peak height ratio (TAM/I.S.) in the range 0.01–2.0 μg ml^{-1} and C.V. at 0.075, 0.4 and 1.2 μg ml^{-1} was ≤4.96%. We are currently using this assay for monitoring TAM in plasma and investigating its pharmacokinetics in cancer patients receiving cytotoxic drugs in addition to TAM as a multi-drug resistance modifier.

Keywords: Tamoxifen

1. Introduction

Tamoxifen (TAM), a triphenylethylene derivative, is a non-steroidal anti-estrogen commonly employed in the treatment of breast carcinoma. The mechanism of antitumor activity is thought to be related to competitive inhibition of the binding of estradiol to estrogen receptor by TAM. Renewed interest has recently been displayed in this drug as a potential modifier of multi-drug resistance (MDR) of tumor cells [1]. However, this action is achieved at high doses requiring a careful monitoring of its concentrations in plasma.

TAM has been analyzed in plasma by high performance liquid chromatography [2–8]. With most of these methods, TAM is converted by UV irradiation into a fluorescent compound which is measured fluorometrically after chromatographic separation. However, in spite of this manipulation these methods require plasma volume >0.2 ml which can only be obtained by venipuncture, an undesirable burden to most patients. Also, the majority of these methods [2,3,7,8] do not include an internal standard. Further, some of these assays

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[3,5,6] involve elaborate liquid–liquid or solid–liquid extraction prior to chromatography and others require home-built on-line photocyclisation reactor [4–6,8] or on-column switching device [4,7,8] which may not be available.

In this report, we describe a direct and accurate method for the analysis of TAM in 30 μl of plasma. The sensitivity, accuracy and small sample size used which may be obtained by fingertip puncture make this assay highly suitable for monitoring and investigating the pharmacokinetics of TAM in cancer patients.

2. Experimental

2.1. Materials

Analytical samples of tamoxifen and 4-hydroxytamoxifen were purchased from Sigma (St. Louis, MO, USA) and Research Biochemical International (Natick, MA, USA), respectively. A small sample of N-desmethyltamoxifen was kindly provided by Dr. B.M. Vose, Zeneca (Macclesfield, Cheshire, UK). Methanol, *o*-phosphoric acid (both from Fisher Scientific, Fair Lawn, NJ, USA), quinine bisulfate (internal standard, I.S.) and tetrabutylammonium phosphate (Kodak, Rochester, NY, USA) and sodium acetate (Fluka, Buchs, Switzerland) were all of HPLC grade. Water for HPLC was prepared by passing deionized water through a 0.45-μm (pore-size) membrane filter (Millipore, Milford, MA, USA). Stock solutions of TAM and I.S. in methanol were prepared every week and stored in multiple light-protected flasks at −18°C. On the day of analysis, one of these flasks is taken out of the freezer and the solution is allowed to thaw at room temperature and used. Under these conditions, no degradation (i.e., no change in peak height for the same amount injected) of either of these compounds was observed.

2.2. Instrument

We used for this analysis a liquid chromatograph (Waters, Milford, MA, USA) consisting of a data module (Model 730), system controller (Model 720), an automatic sample injection module (Model 710B), a solvent delivery pump (Model 501) and a radial compression separation module (RCM-module) equipped with a 10-μm-particle-packed, 10 cm×8 mm CN Radial Pak cartridge and a Guard-PAK precolumn module with a CN Resolve insert. The insert was regularly changed every 12–15 injections. After placing the samples to which the internal standard was added in an ultraviolet short-wave transluminator (UVP, San Gabriel, CA, USA), with 0.25 J min^{-1} energy, for 2 min to photoactivate TAM, the compounds were detected in the effluent fluorometrically (Model 470, Waters) at excitation and emission wavelengths of 258 and 378 nm, respectively.

2.3. Mobile phase

The mobile phase was prepared by mixing 700 ml methanol with 300 ml of 0.1 *M* sodium acetate in 0.001 *M* tetrabutylammonium phosphate solution adjusted to pH 6 with *o*-phosphoric acid. The mixture was filtered through 0.45-μm (pore size) membrane and degassed before use. A flow-rate of 4 ml min^{-1} at a pressure of 7.56 MPa or 1100 p.s.i. was used.

2.4. Standard curves and linearity

The linearity of the assay was established by preparing on different days concentration versus peak height ratio (TAM/I.S.) standard curves in the range 0.01–2.0 μg/ml. The curve which consisted of 8 concentrations was constructed by adding to 30 μl fractions of intact plasma, appropriate amounts of TAM in methanol, 12 μl of I.S. in methanol (15 μg/ml) and an adequate aliquot of 0.6 *M* *o*-phosphoric acid in acetonitrile to make the final volume 90 μl. The sample was then placed in a shortwave UV transluminator for 2 min and 50 μl was injected into the chromatograph.

2.5. Precision and accuracy

We examined the intra-day and inter-day precision and accuracy of the assay by analyzing replicate plasma samples supplemented with 0.075, 0.4 or 1.2 μg ml^{-1} which represent low, medium and high concentrations of TAM in plasma on the same day

and on 10 different days according the aforementioned procedure. The intra-day and inter-day precision was determined from the C.V. values for the concentrations analyzed, whereas the accuracy calculated as the percent deviation from actual concentration was estimated as 100 (concentration found − concentration analyzed)/concentration analyzed.

2.6. Analysis of patients' samples

The applicability of the assay to measure the concentrations of TAM in plasma was examined by analyzing samples collected at mid-dosing interval from patients treated for non-Hodgkin's lymphoma with cytotoxic drugs (i.e., doxorubicin, cyclophosphamide, vincristine, prednisone) in addition to TAM as a multi-drug resistance modifier. The tamoxifen dose administered was 480 mg daily for 4 days prior to chemotherapy.

To 30 μl of the patient sample, the internal standard was added and the analysis was performed according to the described procedure.

3. Results and discussion

Representative chromatograms for blank plasma, plasma supplemented with TAM and quinine bisulfate and a plasma sample collected from a patient receiving 480 mg of TAM daily are depicted in Fig. 1. As demonstrated in this figure, the compounds were eluted rapidly (<12 min) with large retention capacity factors (k') for both TAM and quinine bisulfate (k'=12.5 and 4.8, respectively), indicating a high chromatographic efficiency.

To optimize the chromatographic conditions for the assay, the suitability of different reversed-phase packings and various mobile phases at different solvent compositions was thoroughly investigated. While C_{18} (10 μm) packing yielded a poor peak for TAM, C_{18} (4 μm) produced an early, sharp peak with a poor resolution from that of the plasma. The use of a phosphate buffer, methanol and acetonitrile mixture at different proportions as a mobile phase gave a very early peak for TAM which was unresolved from that of the plasma. Changing the inorganic portion to sodium acetate (0.1 *M*) in 0.001 *M*

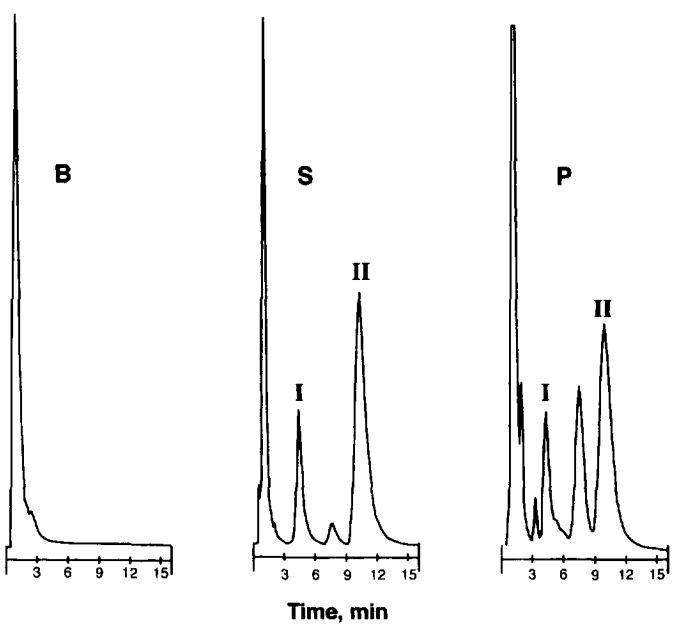

Fig. 1. Representative chromatograms of (B) an intact plasma; (S) a plasma to which 0.9 μg ml^{-1} of TAM (II) and 12 μg ml^{-1} of internal standard (I) were added; and (P) a plasma sample collected at mid-dosing interval from a patient treated with 480 mg of TAM daily (concentration measured=0.87 μg/ml) and to which 12 μg ml^{-1} of internal standard was added.

tetrabutyl ammonium solution (pH 5) and using methanol in lieu of methanol–acetonitrile mixture produced a poor resolution between TAM and I.S. However, when the pH of the inorganic solution was changed to 6, a full separation between peaks was observed at organic–inorganic solvent ratio of 30:70. These conditions were therefore employed for the analysis.

The impact of duration of UV irradiation on the sensitivity of the assay for TAM was also investigated (Fig. 2). As demonstrated in this figure, there was a linear (r=0.988), positive relationship between the duration of irradiation and the peak height for TAM. However, to ensure the shortest analysis time possible while achieving the desired sensitivity for the small sample size employed (i.e., 30 μl), 2 min irradiation was selected. It is noteworthy that the minimum analyzable limit of the assay was 0.01 μg/ml.

As shown in Fig. 1, a direct injection of plasma after dilution with 0.6 M solution of o-phosphoric acid in acetonitrile yielded clean chromatograms with no interference from plasma endogenous substances. Also, the recovery of TAM from plasma at concentration equivalent to 0.4 and 1.2 μg ml^{-1} as determined from the ratio of the peak height of 30 μl of spiked plasma to that of the same volume and concentration of TAM in methanol solution was almost complete (i.e., 96.4 and 99.2%, respectively). Further, none of the drugs that may concomitantly be prescribed with TAM, and none of the metabolites of TAM that we were able to obtain commercially or otherwise (i.e., 4-hydroxytamoxifen and N-desmethyltamoxifen) interfered in the assay (Table 1). It is noteworthy that the peak between peaks I and II is that of 4-hydroxytamoxifen.

The linearity of the assay was equally good with correlation coefficient for the peak height ratio (drug/internal standard) vs. concentration standard curves of >0.9983 (mean r=0.9991, n=10). It is noteworthy that the day–day variability of the peak-height ratio over the concentration range was reasonably small with coefficient of variation (C.V.) of 7.3, 5.8, 6.2, 6.4, 5.1, 6.3, 6.9 and 5.5% at 0.01, 0.05, 0.1, 0.2, 0.3, 0.5, 1.2 and 2 μg/ml, respectively. The accuracy of the assay was equally good where the deviation from the nominal values at the concentrations shown in Table 2 was <4.6% (intra-day) and 7.3% (inter-day). It should be noted that the accuracy, as indicated earlier, was determined by

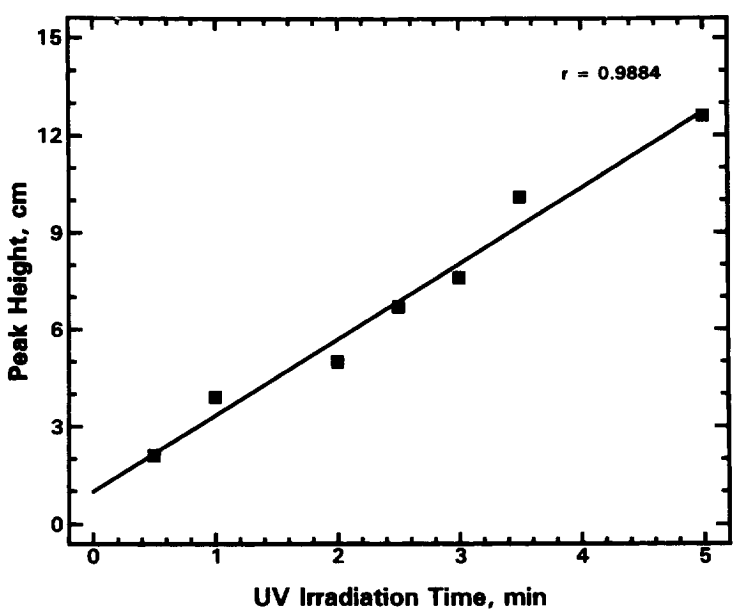

Fig. 2. Effect of duration of UV irradiation on the peak height of tamoxifen under the described chromatographic conditions.

Table 1
Retention times of drugs that may concomitantly be prescribed with tamoxifen

Drug	Retention time (min)
Aspirin	1.29
Azathioprine	0.95
Carmustine	2.87
Chlorambucil	0.83
Cyclophosphamide	ND
Cytarabine	0.79
Dacarbazine	0.79
Doxorubicin	ND
Etoposide	1.12
Ifosfamide	ND
Indomethacin	1.20
Lomustine	3.05
Methotrexate	2.98
Mitomycin C	ND
Prednisone	ND
Procarbazine	1.16
Quinine bisulfate	4.36
Sodium salicylate	0.85
Tamoxifen	10.13
4-Hydroxytamoxifen	7.76
N-Desmethyltamoxifen	6.30
Taxol	ND
Tenoposide	1.35
Thioguanine	1.05
Vincristine	ND

ND=non-detected during the run under the described conditions.

analyzing spiked plasma samples whose concentrations of TAM were calculated from the equation of a standard curve constructed on the same day.

Table 2 also presents data on the intra-day and inter-day precision of the described assay. As shown in this table, the C.V. values of 0.075, 0.4 and 1.2 $\mu g\ ml^{-1}$ which represent low, medium and high concentrations of TAM in plasma was <4.96% (intra-day, $n=10$) and ≤7.59% (inter-day, $n=10$). These values undoubtedly signify a good intra-day and inter-day precision. The change of the pre-column insert every 12–15 injections did not appear to influence the precision of the assay.

This assay was employed to measure TAM in plasma of patients with non-Hodgkin's lymphoma treated with cytotoxic drugs in addition to daily dose of 480 mg of TAM as a MDR modifier. The concentrations measured at mid-dosing interval on the 4th day of TAM administration in ten of these patients ranged from 0.503 to 1.434 μg/ml.

In contrast to previously published methods for TAM which all require plasma volume >0.2 ml and either do not include an internal standard [2,3,7,8] or involve elaborate liquid–liquid or solid–liquid extraction [3,5,6,9,10] or derivatization [10] step prior to chromatography, the described assay is direct, expedient and does not suffer from any of these deficiencies. Also, it requires no special instrumentation such as home-built on-line photocyclisation reactor [4–8], on-column switching device [4,7,8], or mass spectrometer [10] as is the case with other techniques. It is of interest to note that the described assay enjoys these advantages while maintaining an excellent specificity (full separation of the metabolites and no interference from any drug or endogenous substance), linearity ($r>0.9983$), accuracy (deviation from nominal concentration <7.3%) and

Table 2
Accuracy and precision of the described assay for tamoxifen in plasma

	Concentration analyzed (μg/ml)	Concentration found[a] (μg/ml)	Precision C.V. (%)	Accuracy[b] (%)
Intra-day	0.075	0.0756	4.96	0.8
	0.400	0.406	3.44	1.5
	1.200	1.145	2.20	−4.6
Inter-day	0.075	0.0717	6.67	−4.4
	0.400	0.429	5.16	7.3
	1.200	1.228	7.59	2.3

[a] Mean of 10 determinations.
[b] Deviation from nominal value; calculated as 100 (concentration found−concentration analyzed)/concentration analyzed

precision (C.V. <7.59%) that are comparable to those reported for previous assays of TAM.

In summary, the assay described in this report is an accurate and reproducible assay for tamoxifen in microsamples of plasma by direct injection into a radial compression liquid chromatographic system. The small sample size, ease and speed of the analysis (i.e. total analysis time <20 min) make this method highly suitable for monitoring and pharmacokinetic investigation of this drug in cancer patients.

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