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Novel assay method for mitoxantrone in plasma, and its application in cancer patients

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ABSTRACT

Mitoxantrone is an anthracene derivative that acts as a cytostatic in a variety of cancers. A quantitative analytical method has been established for the determination of mitoxantrone in plasma The method employed C_{18} reversed-phase ion-pair chromatography with an isocratic mobile phase of 50.0% methanol in 10 m*M* phosphate buffer (pH 3.0) plus 0.09% 1-pentanesulphonic acid and ultraviolet detection. Sample preparation consisted of two extraction steps using same organic solvent system at different pH to remove plasma impurities efficiently Potential adsorption of mitoxantrone onto glassware was considered. Silanization of all glassware with 5% dichlorodimethylsilane in chloroform increased the extraction recovery in plasma from 50 to 85% with high reproducibility. Mitoxantrone was unstable in human plasma. To maintain plasma sample integrity, each millilitre of sample should be fortified with 0 1 ml of 5% vitamin C (in citrate buffer) and kept frozen until analysis. Using this new method, the calibration curve of mitoxantrone in plasma in the range of interest (1–500 ng/ml) showed good linearity (r = 0.996) and precision (both between-day and within-day coefficients of variation less than 10%) The lower detection limit of this assay method was 1 ng The application of this method allowed us to study the stability of mitoxantrone in plasma, and the pharmacokinetics of mitoxantrone in nasopharyngeal carcinoma patients receiving 12 mg/m^2. The study revealed a prolonged terminal phase half-life for mitoxantrone

INTRODUCTION

Mitoxantrone, 1,4-dihydroxy-5,8-bis{{2-[(2-hydroxyethyl)amino]ethyl}amino}-9,10-anthracenedione dihydrochloride (Fig. 1), is an anthracene derivative with significant cytotoxic effects in both pre-clinical studies [1,2] and clinical studies

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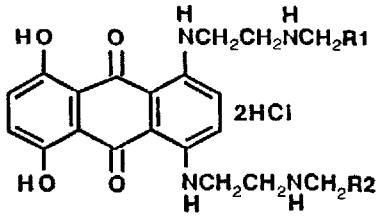

R1	R2	MW	COMPOUND
CH_2OH	CH_2OH	444	MITOXANTRONE
COOH	COOH	472	METAB A
COOH	CH_2OH	458	METAB B

Fig 1. Molecular structures of mitoxantrone and two metabolites.

[3–10]. It was approved by the FDA in 1987 for treatment of leukemia. Phase II studies have been carried out by a number of oncology groups and institutions in many other tumour types. Clinical studies demonstrated that mitoxantrone was generally well tolerated, causing a very low incidence of nausea, vomiting and alopecia, and virtually no phlebitis or soft tissue damage in instances of extravasation.

A simple, precise, sensitive and reproducible analytical method for biological samples is necessary for pharmacokinetic studies. Radioimmunoassay of mitoxantrone in serum has been developed [11]. Although the antibody appears to be specific for the parent compound, clinical pharmacokinetic studies using this assay are awaiting publication. Several high-performance liquid chromatographic (HPLC) methods for the determination of mitoxantrone in human plasma have been reported [12–18]. The first published HPLC method [12] used a gradient elution procedure for separating nine aminoanthraquinone analogues. Although temperature changes were used to enhance the resolution, the authors did not address the possible interference from biological impurities and the method may not be particularly suitable for routine clinical use. Some published HPLC methods need a very time-consuming sample clean-up procedure, leading to a relatively inefficient recovery of mitoxantrone and imprecise determination of the terminal elimination phase. Only a few of the published reports utilized an internal standard method, which is more precise when liquid–liquid extraction is involved [18].

The stability of mitoxantrone in human plasma has been discussed [14]. The technique of introducing vitamin C as an antioxidant, and also decreasing the plasma pH to further stabilize mitoxantrone in plasma samples, was applied. However, whether or not the amount of antioxidant needed to stabilize the drug is dependent on the concentration of mitoxantrone is still awaiting investigation. Besides the problem of stability, a significant amount of mitoxantrone adsorbed on the surface of glassware during sample preparation is another obstacle to be solved.

In the present study, we have developed a novel method, with a low detection limit of 1 ng, for the determination of mitoxantrone in biological fluids. We used this method to study the stability of mitoxantrone in plasma and the pharmacokinetics of the drug in cancer patients. Also, the effects on the recovery of the

method of plasma pH values and adsorption on glassware during the sample preparation were studied.

EXPERIMENTAL

Materials and reagents

Mitoxantrone dihydrochloride was purchased from Lederle Labs. Division (Cynamid of Great Britain, Gosport, U.K.). Haloperidol reference standard was kindly supplied by Janssen Pharmaceutica (Beerse, Belgium). The sodium salts of *n*-pentanesulphonic acid, l-ascorbic acid, citric acid and potassium phosphate disodium salt were purchased from Merck (Darmstadt, F.R.G.) and were of analytical grade. Chloroform and carbon tetrachloride (Merck) were used for extraction. HPLC-grade methanol was used throughout the study after filtration through a 0.22-μm Millipore filter followed by degassing. Milli-Q water was prepared through a Milli-RO 60 water purification system (Millipore, Bedford, MA, U.S.A.).

Chromatography

HPLC was performed on a Kratos liquid chromatographic system with a Spectroflow Model 400 solvent-delivery pump, a Model 783 variable-wavelength UV detector (Kratos, Ramsey, NJ, U.S.A.) set at 242 nm (λ_{max} of mitoxantrone) at a sensitivity of 0.005 a.u.f.s., a Model 710B WISP sample processor (Waters Assoc., Milford, MA, U.S.A.) and a column oven (Waters) thermostatted at 40°C. A Waters Assoc. μBondapak C_{18} (10 μm particle size, 25 cm × 4 mm I.D.) column preceded by a guard column was used for all analyses. The packing material of the guard column was replaced every one or two weeks or when there was significant build-up back-pressure.

Mitoxantrone and haloperidol were eluted isocratically with methanol–0.01 *M* KH_2PO_4 buffer (pH 3.0) (50:50, v/v), containing 0.09% pentanesulphonic acid sodium salt as the solvent system at a flow-rate of 1.5 ml/min. Peak areas were integrated and recorded by a SIC-12 integrator (System Instruments, Tokyo, Japan).

Glassware adsorption studies

The adsorption of mitoxantrone on the surface of glassware in stock solution and in plasma was studied. All glassware used in the extraction process, including tubes with PTFE screw-caps (Pyrex) and disposable culture tubes (Corning), were pretreated using two different methods to compare the efficiencies of adsorption prevention. Silicon coating of the glassware was done by immersing the glass tubes in 2% silicon solution for 10 min. An alternative silanization process was performed by immersing the tube with 5% dichlorodimethylsilane (DCDM-Si) in chloroform for 24 h, then incubating at 100°C overnight to complete the silanization. In addition to the tube-coating, 3% 2-propanol was added to the extraction solvent to enhance the prevention effect.

Standard solutions of mitoxantrone (100 ng/ml) were prepared in coated or non-coated tubes containing organic solvent, dried by filtered gas and reconstituted with mobile phase. The mitoxantrone concentration was determined directly by HPLC. Mitoxantrone in plasma samples (200 ng in 2 ml of plasma) was first stabilized by adding 200 μl of vitamin C in citrate buffer solution, then extracted using organic solvent either with or without 2-propanol.

Stability studies in plasma

The stability of mitoxantrone in plasma with or without the addition of ascorbic acid as an antioxidant was assessed at 25, 4, −20 and −60°C. A volume of 200 μl of 5% L-ascorbic acid in citrate buffer (0.1 *M*, pH 3.0) was added to 2 ml of human plasma prior to the addition of mitoxantrone for the antioxidant-added group. The plasma samples stored at 25°C were assayed for the mitoxantrone immediately after preparation and once an hour for 4 h, other samples were assayed at various intervals up to six months. In each case, $n = 3$. Data were expressed as percentages of the initial concentration. The half-life, T_{50}, is the time taken for the plasma mitoxantrone concentration [D] to become $[D]_0/2$, *i.e.* one-half of the original concentration. The shelf-life, T_{90}, of plasma mitoxantrone is taken to be the time for [D] to reach $0.90[D]_0$, that is, 10% decomposition.

According to Garrett and Carper's technique [20], the rate constant (k) for the decomposition of mitoxantrone in plasma at various temperatures and the activation energy (E_a) were obtained from an Arrhenius plot using the following equation:

$$\text{slope} = \frac{-E_a}{2.303\ R}$$

where R is the gas constant.

To establish whether the amount of antioxidant vitamin C required is dependent on the mitoxantrone concentration, two groups of plasma samples with mitoxantrone concentrations of 50 and 1000 ng/ml were prepared and assessed at 4°C. Three times the amount of vitamin C (300 μl/ml of plasma) was added to another 100 ng/ml plasma sample. Samples were assayed, and the percentages of the initial mitoxantrone concentration remaining at various times up to 70 days were assessed.

Extraction procedures

Several solvent systems containing different ratios of chloroform and carbon tetrachloride were tested for the clean-up and extraction procedures. Plasma samples (2 ml) containing 100 ng/ml mitoxantrone or less were stabilized by the immediate addition of 200 μl of 5% ascorbic acid solution (prepared in 0.1 *M* citrate buffer, pH 3.0). A 150-μl haloperidol stock solution (25 μg/ml) was added as internal standard. Addition of 1 *M* hydrochloric acid removed the impurities from 7.0-ml portions of several selected solvent systems by mechanical rotation

for 15 min. After centrifugation, the upper aqueous layer was aspirated into another screw-capped tube. Then, mitoxantrone was extracted into the same organic solvent selected after alkalization by adding 1 *M* sodium hydroxide to the aqueous layer and rotating the tube for 1 h. Following the phase separation, the organic solvent was evaporated by filtered dry air and the residue reconstituted in 200 μl of mobile phase. A portion of 100 μl of the reconstituted solution was injected directly into the HPLC apparatus.

The overall extraction recovery of mitoxantrone from plasma was studied at various plasma pH values in the first clean-up step (pH 4.3, 4.9, 4.9, 4 9 and 5.1) and in the second extraction procedure (pH 9.0, 11, 12 and 13) by using various volumes of 1 *M* hydrochloric acid and 1 *M* sodium hydroxide solutions. The extraction recovery (ER) was calculated from the following equation:

$$ER = \frac{PA_p}{PA_s} 100\%$$

where PA_p is the peak area of mitoxantrone in plasma, and PA_s is the peak area of the equivalent amount of mitoxantrone in stock solution without extraction.

Assay precision and validation

Precision and accuracy were determined by constructing the within-day and between-day standard curves in triplicate and determining various standard mitoxantrone solutions (1–500 ng/ml) using these curves. To assess the precision of the within-day assay, triplicate extractions of plasma samples (2 ml) in the concentration range of 1–500 ng/ml were performed on a single day. For the between-day assay, the determination was carried out for three consecutive days. The mean peak-area ratio used throughout this study was calculated by dividing the mitoxantrone peak area by the haloperidol peak area in plasma.

Pharmacokinetic analysis

Two adult patients with metastatic nasopharyngeal carcinoma (NPC) receiving a mitoxantrone dose of 12 mg/m^2 in 250 ml of normal saline intravenously over 30 min were studied. Serial blood samples were drawn 10 min before drug administration, immediately after the infusion and at 15, 30, 60 min as well as 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 60 and 72 h from a forearm vein after infusion. Blood samples without anticoagulant were collected. Plasma was separated immediately by centrifugation, and a 10% volume of ascorbic acid was added as antioxidant. All plasma samples were frozen at −60°C until analysis. The method developed in the present study was applied to the determination of mitoxantrone concentration in plasma. Pharmacokinetic parameters were estimated by two computer programs: CSTRIP [21] and PCNONLIN [22].

RESULTS AND DISCUSSION

Extraction procedure and recovery

Initial attempts to extract mitoxantrone from plasma by direct extraction achieved only limited success. The extraction efficiency was better with a proton-donating solvent, such as chloroform, rather than proton-accepting solvents, and was improved by the addition of carbon tetrachloride. The extraction recovery of mitoxantrone from plasma was markedly improved by increasing the fraction of chloroform from 60% (v/v) to 90% (v/v). The extraction recoveries achieved using different ratios of extraction solvent are listed in Table I. After this preliminary study, chloroform–carbon tetrachloride (9:1) was used as the extraction solvent throughout the study.

In order to obtain optimum absolute recovery with an acceptable lower detection limit and increase the sensitivity of the analysis in plasma, various pH values in the first clean-up and second extraction step were tried. Table II shows the overall mitoxantrone recoveries at various plasma pH values in the first clean-up procedure. Although the extraction recovery seems best under the last value in Table II, the clean-up procedure could not be completed if the emulsion was reserved to the next extraction step. Therefore, 80 μl of 1 *M* hydrochloric acid were added to each 2-ml plasma sample in the first step, and only the upper aqueous layer without the emulsion was transferred to the next extraction step. After alkalization of the aqueous layer to pH 12 in the second extraction step, the recovery of mitoxantrone from plasma increased to 60% (Table III).

Owing to the unsatisfactory recovery after the pH adjustment, a further study of the extraction recovery was focused on the adsorption of mitoxantrone on the surface of glassware. From such studies with mitoxantrone in standard solution, the recovery of mitoxantrone was greatly improved, from 60% (60.9 ± 9.33%) to 87% (87.5 ± 8.71%) after silanization of all glass tubes. The method of silicon

TABLE I

EXTRACTION RECOVERY OF MITOXANTRONE FROM PLASMA USING DIFFERENT RATIOS OF EXTRACTION SOLVENTS

The initial concentration of the drug was 5 μg/ml.

$CHCl_3/CCl_4$ ratio	Extraction recovery (%)
6·4	4 64
7:3	55.1
8 2	47.6
9 1	60.9
10:0	58.6

TABLE II

OVERALL MITOXANTRONE RECOVERY AT DIFFERENT pH VALUES IN THE FIRST CLEAN-UP PROCEDURE

The first clean-up procedure was acidification of plasma samples and the second was alkalinization of samples. The intitial concentration of the drug was 100 ng/ml

Plasma pH	1 *M* HCl (ml)	Absolute recovery (mean ± S.D) (%)
5.1	–	27.3 ± 1.32
4.3	0.25	29.8 ± 0.26
4.9	0.08	39.0 ± 2.54
4.9 (extraction twice)[a]	0.08	40.7 ± 6.69
4.9 (emulsion was reserved)	0.08	50.9 ± 13 5

[a] Whether one or two extractions were made, the total volume of extraction solvent was 7 ml.

coating brought about only limited improvement, to 73.6 ± 4.69%. Use of 2-propanol did not enhance the recovery at all (56.6 ± 1.77%). Similar results were obtained with mitoxantrone in plasma (Table IV). The absolute recovery was raised from 63 to 87% by pre-silanizing all the glass tubes. The advantage of glassware silanization is based on the strongly covalent bonding of oxygen atoms in the silica material of glassware to silicon atoms in DCDM-Si. Also, there is no active group available for drug adsorption.

For accurate quantitative assay and definition of pharmacokinetic parameters, such as elimination half-life, a low detection limit is vitally important. Therefore, the significant adsorption of mitoxantrone on glassware should be considered in analysis. In previous reports pertaining to the quantitation of the drug in preclinical and clinical pharmacokinetic studies, the authors failed to take this problem into account [13–19]. Since the present studies have unequivocally demonstrated

TABLE III

OVERALL MITOXANTRONE RECOVERY AT DIFFERENT pH VALUES IN THE SECOND EXTRACTION PROCEDURE

The initial concentration of the drug was 100 ng/ml.

Plasma pH	Absolute recovery (mean ± S D.) (%)
9.00	40.4 ± 1.90
11.0	43 6 ± 3.15
12.0	59 0 ± 5.71
13.0	58.9 ± 2.86

TABLE IV

ABSOLUTE RECOVERY OF MITOXANTRONE FROM PLASMA USING SILANIZED TUBES AND/OR ADDED 2-PROPANOL

Initial concentration of drug, 100 ng/ml.

Treatment[a]	Absolute recovery (mean ± S.D.) (%)
A	63 1 ± 4.51
B	68.0 ± 2.25
C	87.1 ± 8.68
D	75 4 ± 6.37

[a] A: Non-coated tube, no 2-propanol in extraction solvent. B Non-coated tube, 2-propanol in extraction solvent. C. Silanized tube, no 2-propanol in extraction solvent D. Silanized tube, 2-propanol in extraction solvent.

that mitoxantrone is adsorbed on the surface of glassware, the validity of these other methods and the consequent results showing a relatively short elimination half-life for mitoxantrone are in question.

Many published papers use absorbance detection at 658–660 nm to detect mitoxantrone [11,13,15,16], because detection at *ca.* 254 nm usually produced a large number of interfering peaks from biological fluids. In the present study, sample preparation consisted of extraction using the same organic solvent at different pH values to remove plasma impurities efficiently. The clean chromatogram of blank plasma shown in Fig. 2 demonstrates that the optimum detection wavelength at 242 nm is applicable, and has better sensitivity.

Following correction of the problems of pH-dependent extraction and glassware adsorption, the overall extraction recovery from plasma was improved to 87%. The assay was proven to be reproducible with a coefficient of variation within 10% (Table IV). This allowed us to monitor extremely low mitoxantrone concentrations, which is essential for the study of pharmacokinetics in clinical research.

Validation of assay

The highly sensitive sample extraction procedures described in this paper give a clear separation of the drug in blank and spiked samples (Fig. 2). A plasma concentration of 1 ng/ml mitoxantrone (1 ml) was proven as the lower limit of quantitation. The accuracy was confirmed by determining standard mitoxantrone solutions (1–500 ng/ml) using the calibration curve: the maximum error was 4.8%. The standard curves of both between-day and within-day assays, ranging from this low level to 500 ng/ml mitoxantrone, showed good linearity. The correlation coefficient was higher than 0.997, and the linearity of response was determined by least-squares analysis of data points. The coefficients of variation (C.V.)

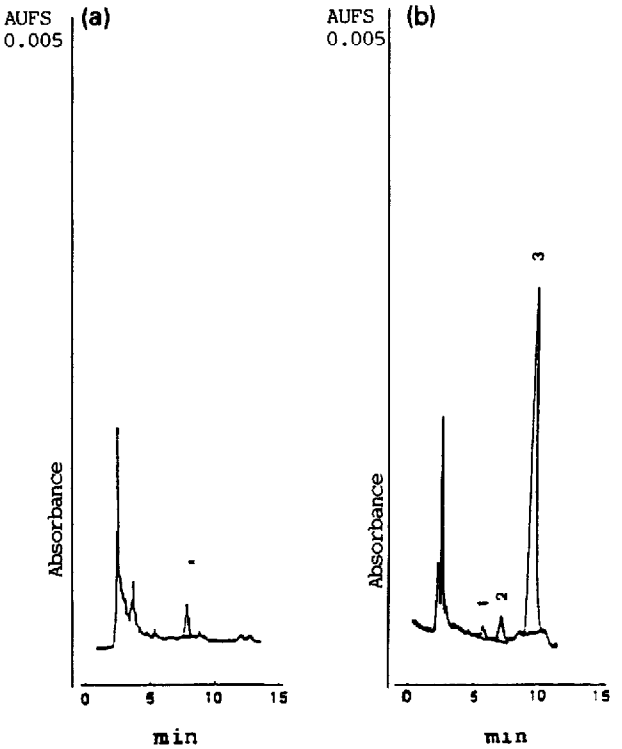

Fig 2. (a) Chromatogram of blank plasma from a volunteer without mitoxantrone and internal standard, haloperidol. Peak 1 = plasma impurity (b) Chromatogram of plasma spiked with mitoxantrone and internal standard, haloperidol Peaks. 1 = mitoxantrone (corresponds to 1 ng), 2 = plasma impurity; 3 = internal standard (corresponds to 3.75 μg)

of the within-day assay was 6.1% for 1 ng/ml and 2.1% for 500 ng/ml mitoxantrone. The C.V. of the between-day assay was within 9%.

Stability studies

The degradation of mitoxantrone in plasma at 4°C followed an apparently pseudo-first-order process without added vitamin C, with T_{50} equal to 13.6 days. More than 10% of the initial concentration was degraded within the first day of storage. This loss could be reduced by storing samples at lower temperatures. When plasma samples were frozen at −20 and −60°C, T_{50} was 31.0 and 62.0 days, respectively, with T_{90} 2.8 and 5.0 days (Table V). The degradation is also pseudo first order. (Fig. 3).

In plasma samples adjusted to pH 5.3, the addition of 5% ascorbate stabilized the system and resulted in less than 10% loss of mitoxantrone in 4 h at room temperature. At refrigerated temperature (4°C), *ca.* 10% loss was observed within fifteen days. However, lowering the storage temperature to −20 and −60°C will prolong T_{90} to 112 and 175 days, respectively (Table V). Table V also shows the

TABLE V

ESTIMATION OF T_{90} AND T_{50} VALUES FOR MITOXANTRONE IN PLASMA WITH AND WITHOUT ADDED VITAMIN C

Temperature (°C)	Without vitamin C			With vitamin C		
	T_{90} (days)		T_{50} (days)	T_{90} (days)		T_{50} (days)
4	0.10		13.6	15.5		104
−20	2.80	(2.10–3.60)[a]	31.0	112	(92–132)[a]	681
−60	5.00	(3.70–6.30)[a]	62.0	175	(131–220)[a]	1120
Activation energy (cal/mol)	2430			3870		

[a] 95% confidence interval.

95% confidence interval of T_{90} at −20 and −60°C. Fig. 3 shows the stability of mitoxantrone in plasma with 5% vitamin C at −20 and 60°C. There is no statistical difference between the two storage temperatures ($p > 0.05$) within six months.

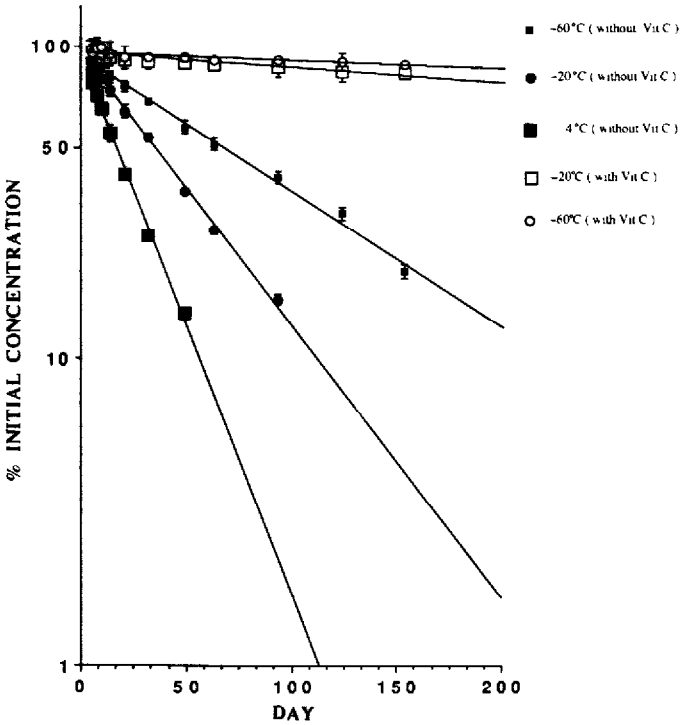

Fig. 3. Stability of mitoxantrone (mean ± S.D., $n = 3$) in plasma (100 ng/ml) with or without vitamin C at various temperatures. A 100-μl volume of 5% vitamin C in citrate buffer was added to each ml of plasma in the vitamin C-added group.

The reason for the much shorter T_{90} at 4°C may possibly be the gradual oxidation of vitamin C in plasma, leading to increased degradation of mitoxantrone in plasma. This proposal is based partly on the observation of a brown colour change of plasma samples after a month of storage and is further strongly supported by the following study.

To determine whether the addition of ascorbate to plasma is mitoxantrone concentration-dependent, and would thereby introduce potential negative deviations into the analysis, two groups of plasma samples with mitoxantrone concentrations of 50 and 1000 ng/ml were prepared and studied at 4°C. The percentage of the initial concentration remaining was almost the same at each sampling point for the three groups. Also the slopes of the degradation lines were apparently parallel (Fig. 4). Another study was performed using three times the amount (300 μl per ml of plasma) of vitamin C to prevent plasma mitoxantrone degradation. Within the study period of 70 days, the mitoxantrone was still stable, and neither significant loss of drug (Table VI) nor brown colour formation in plasma samples was observed. The information from Fig. 4 and Table VI indicates that the plasma mitoxantrone concentration is not a major factor in determining the rate of degradation. Also, increasing the amount of vitamin C could prevent the degradation that resulted from a decrease in the potency of the anti-oxidant. Since we

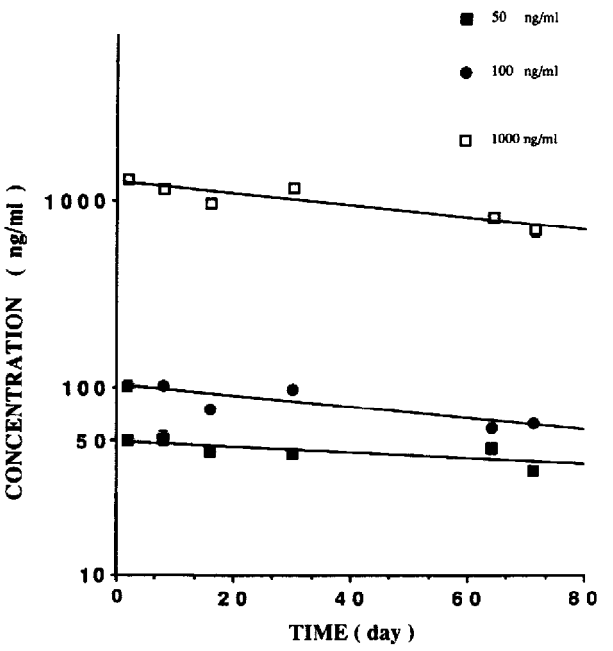

Fig. 4. Stability of various concentrations of mitoxantrone (mean ± S.D., n = 3) in plasma with vitamin C at 4°C. A 100-μl amount of 5% vitamin C in citrate buffer was added to each ml of plasma.

TABLE VI

STABILITY OF MITOXANTRONE IN PLASMA AT 4°C

Initial concentration of mitoxantrone, 100 ng/ml, 300 μl of 5% vitamin C were added per ml of plasma, $n = 3$

Sampling day	Peak-area ratio[a] (mean ± S.D.)
1	0.118 ± 0.014
7	0.092 ± 0.005
15	0.100 ± 0.011
29	0.104 ± 0.011
63	0.079 ± 0.003
70	0.124 ± 0.010

[a] Ratio of the peak area of mitoxantrone to the peak area of haloperidol.

normally used 200 μl of vitamin C in 2 ml of plasma, at a storage temperature at 4°C, vitamin C undergoes self-oxidation, so the T_{50} and T_{90} values listed in Table VI are much shorter than those applying at lower temperatures. Once all the vitamin C was oxidized, the degradation will proceed at the same rate as at 4°C with no added vitamin C.

The rather low activation energy, listed in Table V, indicates that only a simple oxidation process was involved in the degradation of plasma mitoxantrone. In clinical pharmacokinetic studies, it is very rare to analyse the freshly obtained plasma samples immediately. So the proper storage conditions are imperative to prevent drug loss from samples prior to analysis.

Clinical application

We applied this novel method to the quantitation of plasma levels of mitoxantrone-treated patients. Fig. 5 shows concentration–time plots of two NPC patients receiving 12 mg/m^2 mitoxantrone as a 30-min infusion. The peak concentrations of mitoxantrone were 570.8 and 418.8 ng/ml, respectively. The plasma mitoxantrone concentrations 72 h after the end of infusion were 3.8 and 2.3 ng/ml, respectively. The corresponding pharmacokinetic parameters of these two patients are listed in Table VII. Rather prolonged terminal phase half-lives were observed.

CONCLUSION

The rapid, accurate and precise sample clean-up and analysis of the anticancer agent, mitoxantrone, in plasma is described. The study revealed that all plasma samples should be fortified with ascorbic acid to maintain the sample integrity and kept frozen prior to analysis. The problem of adsorption of mitoxantrone on

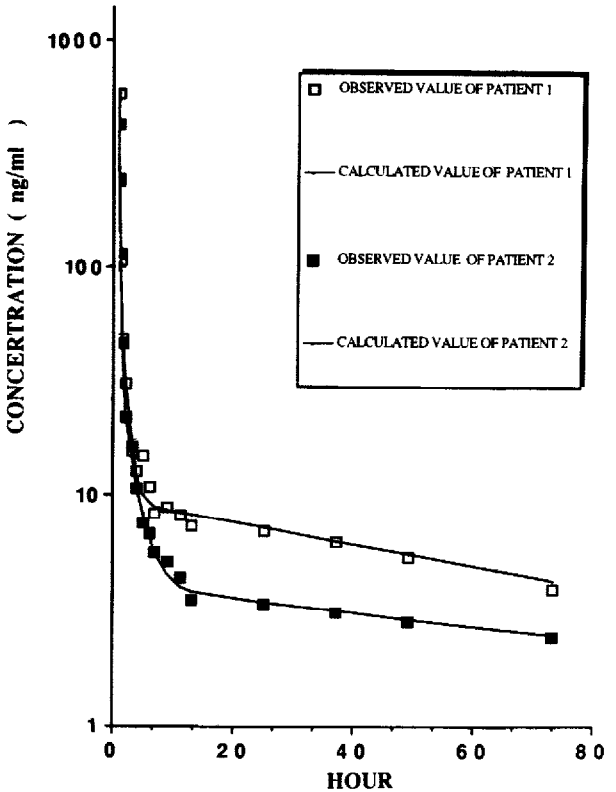

Fig. 5. Plasma concentration-time curve of mitoxantrone in two patients with nasopharyngeal carcinoma after rapid infusion of 12 mg/m^2 mitoxantrone intravenously.

TABLE VII

PHARMACOKINETIC PARAMETERS OF MITOXANTRONE

Parameters were determined in samples from two NPC patients who each received a 12 mg/m^2 infusion of the drug.

Parameter	NPC 1	NPC 2
Body weight (kg)	48 0	43.5
Dose (mg)	16 0	17
α (1/h)	9.94	5.98
β (1/h)	1 01	0.46
γ (1/h)	0 011	0 007
$T_{\frac{1}{2}\alpha}$ (min)	4.20	6 90
$T_{\frac{1}{2}\beta}$ (h)	0.690	1.51
$T_{\frac{1}{2}\gamma}$ (h)	63.0	103
V_c (l)	6.80	14.2
V_d(l)	1540	3632
AUC (ng h/ml)	944	698
Cl (ml/min)	232	336

glassware, which will result in significant variability in quantitative analysis, must not be overlooked. The lower detection limit of this assay method was 1 ng, which allowed us to perform clinical pharmacokinetic studies.

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