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Improved LC assay for the determination of mitozantrone in plasma: analytical considerations

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Abstract: Preliminary method development studies on mitozantrone (MTZ) revealed a number of characteristics which were found to be important in the analysis of patient samples for pharmacokinetic studies. MTZ rapidly bound to glass, particularly at low concentrations (<10 ng ml^{-1}), necessitating the use of silanized glassware or polypropylene tubes for the handling of all solutions containing MTZ. MTZ was also found to react with two commonly-used antioxidants; sodium metabisulphite and EDTA. However, solutions containing MTZ were found to be stabilized by the addition of ascorbic acid (0.5% w/v). In the absence of ascorbic acid, MTZ underwent rapid, biphasic degradation in plasma at 24 and 37°C, with terminal half-lives of approximately 70 h. Ascorbic acid (0.5% w/v) was found to stabilize plasma samples containing MTZ throughout work-up procedures and during frozen storage. The addition of ascorbic acid to the sample collection vial was also necessary to prevent MTZ degradation in the eluting solvent of the solid-phase extraction system. Another important consideration was the requirement for an equilibration period of >5 min after the addition of ametantrone (AM) internal standard to plasma samples. This was essential, since the slope of the calibration plot obtained using non-equilibrated plasma was approximately 30% of that obtained for calibration plots using equilibrated plasma, and would result in erroneous determination of MTZ plasma concentrations. The fully developed assay was rapid, precise and sensitive (relative errors at 1 ng ml^{-1} = 2.3%). MTZ concentrations determined using the LC method described in this report correlated well with an independently developed ELISA technique (r = 0.995, n = 20).

Keywords: *Mitozantrone; rapid LC assay; plasma concentration; analytical considerations; systematic errors.*

Introduction

Mitozantrone (1,4-dihydroxy-5,8-bis-([2-(2-hydroxyethyl)amino]ethylamino-9,10-anthracenedione dihydrochloride) is an antitumour drug of the antitumour antibiotic classification. Phase II clinical and pharmacokinetic studies on mitozantrone (MTZ) in low-dose continuous infusion regimens at this institution required the development of a sensitive assay system capable of determining MTZ at plasma concentrations some 1000× lower than the peak concentrations obtained with conventional bolus injections.

Previous studies [1–16] on the determination of MTZ in plasma describe liquid chromatography (LC) systems based on a C_{18} stationary phase both with and without the inclusion of anionic ion-pairing reagents in the mobile phase. Despite the requirement for extensive sample pre-treatment (see later) only a few published methods detail the use of an internal standard [1, 3, 5–8, 16].

Various sample 'clean-up' methods to extract MTZ from plasma have been reported. These include adsorption of MTZ onto glass wool [9] or XAD-2 resin [4, 10, 15], liquid–liquid extraction [3, 5, 6, 11, 12, 16] and solid-phase extraction using disposable mini-columns [1, 8, 9, 13, 14]. In some cases the reported limit of detection (LOD) for MTZ was 10–75 ng ml^{-1}, which would not have been adequate for continuous infusion studies where it was envisaged that an LOD of <1 ng ml^{-1} would be required. Greater sensitivity (LOD 1–2 ng ml^{-1}) was achieved using either solid-phase extraction, direct injection [7] or enrichment pre-columns [17] in combination with a more selective detection wavelength (658 nm), or by using electrochemical detection [3] or fluorescence detection [18].

Difficulties in the bioanalysis of MTZ have been associated with drug instability, non-specific drug binding and the reactivity of MTZ with other reagents. The adsorption of MTZ onto laboratory glassware was first reported by

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Ostroy and Gams [6]. This observation was confirmed by Lin *et al.* [8] who demonstrated that 95% of the drug in an MTZ solution (400 ng ml^{-1}) was absorbed onto a glass LC injection syringe. The bound MTZ could be eluted under acidic conditions or alternatively adsorption of the drug could be prevented if glassware was silanized. The significance of adsorption at low MTZ concentrations (e.g. 1–10 ng ml^{-1}) and the protection offered by silanization have not hitherto been investigated.

Reynolds *et al.* [15] noted the instability of MTZ in plasma and proposed that all analytical techniques must include an antioxidant to be valid. This and other reports [14, 19] on the stability of MTZ in plasma (at 1 μg ml^{-1}) have given half-lives of 6 days at 4°C, 36 days at −17°C and 24 h at 24°C, and have also recommended the use of an antioxidant. Ice-cold blood collection tubes containing EDTA have been used to minimize MTZ loss during blood centrifugation [10], and conflicting reports on the use of sodium metabisulphite have also appeared in the literature; metabisulphite at a concentration of 0.001% w/v appeared to stabilize MTZ in plasma [3], but at higher concentrations (2.6% w/v) it has been reported to react rapidly with MTZ [15]. Ascorbic acid (either in the presence or absence of a citrate buffer) was the most frequently used antioxidant in previously reported pharmacokinetic studies on MTZ [1, 4, 5, 7, 8, 10, 13, 15–17]. It would, therefore, seem appropriate that the selection of antioxidant should be validated under the work-up and analytical conditions employed unless these follow exactly the conditions of a previously published method.

The aim of this study was to further develop and refine an LC assay for MTZ in plasma to achieve the sensitivity necessary for pharmacokinetic studies on patients receiving low-dose continuous infusions which, in turn, required the identification of sources of drug loss and systematic error during sample preparation and assay. Since it was envisaged that sample clean-up prior to assay would be relatively complex, it was considered necessary to include an internal standard in the LC assay. A structural analogue of MTZ, ametantrone (AM; 5,8-bis([2-(2-hydroxyethyl)amino] ethylamino)9,10-anthracenedione), which exhibited comparable retention characteristics and a similar absorption spectrum to MTZ, was considered a potential candidate for internal standard. Both MTZ and AM bind extensively to plasma proteins [16] which potentially introduces a further source of error into the assay. Studies were, therefore, undertaken to establish whether the binding of AM and MTZ to plasma proteins was competitive and time-dependent so that the necessary equilibration time could be determined.

The binding of MTZ to untreated and to silanized glassware was investigated together with MTZ binding to ultrafiltration centrifuge tubes (used to determine bound and unbound drug fraction). Similarly, the reactivity of MTZ towards the anticoagulant heparin in blood collection tubes was examined.

Stability studies were undertaken to determine the degradation kinetics of MTZ in plasma under storage conditions with and without the addition of antioxidants. The reactivity of MTZ with antioxidants was also studied. Further stability studies were carried out on MTZ in the solid-phase extraction eluting solvent to evaluate the effect of antioxidants on drug degradation during sample clean-up.

By characterizing the reactivity of MTZ through each critical stage of the procedure (sample collection and storage, sample clean-up, LC assay) sources of error could be eliminated and the sensitivity and precision of the assay improved.

The final part of this study was concerned with the comparison between assay values obtained for MTZ in plasma using the LC method and those obtained by an ELISA technique [20].

Experimental

Analytical standard and reagents

Mitozantrone dihydrochloride (1,4-dihydroxy-5,8-bis([2-(2-hydroxyethyl)amino]-ethylamino)-9,10-anthracenedione dihydrochloride; mitoxantrone; novantrone) was a gift from Lederle (Pearl River, USA). The internal standard ametantrone 5,8-bis([2-(2-hydroxyethyl)amino]ethylamino)9,10-anthracenedione) was a gift from the National Cancer Institute (Bethesda, MD, USA). Methanol and acetonitrile were of LC grade (BDH Ltd, Poole, UK). The silanizing agent was 'Glass-Treet' (Phase Separations Ltd, Clywd, UK). All other reagents were of analytical reagent grade from either BDH Ltd (Poole, UK),

The Sigma Chemical Co. (Poole, UK) or Aldrich Chemical Co. Ltd (Gillingham, UK).

Apparatus

Liquid chromatography (LC) System: ConstaMetric 3000 Solvent Delivery System, SpectroMonitor 3100 UV/Visible detector, CI-10B Integrator/Printer-Plotter and Rheodyne 7125 Loop-Valve Injector (LDC Analytical Stone, UK). Solid-phase extraction system: 'Baker'-10 (HPLC Technology Ltd, Macclesfield, UK). Solid-phase extraction columns: 100 mg C18 'TechElut' minicolumns (HPLC Technology Ltd, Macclesfield, UK). Ultrafiltration tubes: Centrisart 1, molecular weight cut-off 10,000 Da (Sartorius, Belmont, UK).

LC assay procedure

An LC system was developed for MTZ using AM as the internal standard, based on previously described methods of Ostroy and Gams [6] and Van Belle *et al.* [16] which used a reversed-phase C18 column with a mobile phase containing formate buffer in place of acetate buffer, in order to reduce peak tailing. Of the five UV-visible absorption maxima for MTZ (242, 279, 525, 620 and 658 nm), the wavelength selected for detection was 658 nm (the wavelength with the second highest extinction co-efficient) on the basis that interference by endogenous plasma compounds was less likely at this wavelength. The LC conditions are summarized below: LC column: Techopak 10 C18 (15 cm × 3.8 mm i.d., 10 μm particle size); mobile phase: ammonium formate (0.5 M, pH 3.0: acetonitrile, (73.27, v/v) at a flow rate of 1.1 ml min^{-1}; chromatography temperature: 37°C (column bath) and mobile phase warming coil (length 1 m); injection volume: 100 μl loop; detection: visible detection at 658 nm, sensitivity 0.001 AUFS.

Sample preparation

A method was developed for the extraction of MTZ and the internal standard ametantrone (AM) from aqueous and biological samples using solid-phase C18 mini-columns. The method used was based on previously described methods [1, 13] with the added modifications of 10% acetonitrile in the eluting solvent which in preliminary studies was found to optimize the co-elution of both MTZ and AM, and the addition of ascorbic acid (10% w/v) to the collecting vials, to prevent the oxidative degradation of MTZ and AM in the strongly acidic eluting solvent (pH 1.0).

Venous blood samples were collected into 10-ml lithium–heparin 'Exetainer' blood collection tubes and immediately transferred into pre-cooled (4°C) polypropylene tubes for centrifugation (1620*g*, 15 min). The resulting plasma was immediately aspirated using polypropylene pipettes and transferred to pre-cooled polypropylene tubes containing ascorbic acid powder (50 mg). Samples were frozen at −20°C for a maximum of 7 days before analysis.

Frozen samples were thawed at room temperature and 10 μl ascorbic acid solution (50% w/v) and 10 μl ametantrone solution (10 μg ml^{-1}) were added per ml sample. The mixtures were then vortexed (10 s), allowed to equilibrate for 10 min at 4°C followed by 5 min at room temperature, and then 1–5 ml portions (depending on the volume of sample available) applied to pre-conditioned solid phase extraction (SPE) columns for clean up and concentration.

The SPE columns (C18 100 mg 'TechElut' columns) were conditioned by aspirating 2 ml methanol, under vacuum, followed by 2 ml distilled water, ensuring that the column packing did not dry out at any stage. Unfiltered samples of 1–5 ml volume (as above) were applied to previously conditioned minicolumns and aspirated under vacuum. The columns were then washed with 2 ml distilled water, and the analytes of interest eluted with 2 × 200 μl of methanolic HCl (0.5 M)–acetonitrile (90:10, v/v) followed by 200 μl of ascorbic acid solution (0.5% w/v) which served to 'wash out' any remaining solvent and act as an antioxidant. Eluate was collected into silanized 1 ml volumetric flasks containing 50 μl ascorbic acid solution (10% w/v). The eluate was then adjusted to volume with ascorbic acid solution (0.5% w/v) for analysis by LC.

Stability-indication of assay

The stability-indicating ability of the LC assay system was determined using forced degradation studies. To four 10 ml volumetric flasks (a)–(d) was added 1 ml of the following solutions: to flask (a), 1 M HCl; to flask (b), 1 M NaOH; to flask (c), 6 vol hydrogen peroxide solution; and to flask (d), water for injection. Flasks (a)–(c) were heated in a water bath at 70°C for 30 min. Flask (d), the control, was

protected from light and stored at 4°C. Each flask was then equilibrated to room temperature, adjusted to volume with distilled water, and the resulting solutions subjected to assay.

Binding of MTZ to glassware

In order to investigate the binding of MTZ to glassware, 20 ml volumes of solutions of MTZ [10 ng ml^{-1} in sodium acetate buffer (0.6 mM, pH 4.0)] were distributed among glass volumetric flasks (20 ml volume) and previously silanized glass volumetric flasks (20 ml volume). The flasks were stored in the dark at ambient temperature. At various intervals over 1 h, flasks of each type (untreated and silanized) were removed for assay.

Binding of MTZ and AM to centrisart tubes

Solutions of MTZ (2 ng ml^{-1}), AM (100 ng ml^{-1}) and a solution of MTZ plus AM (2 ng ml^{-1} and 100 ng ml^{-1}, respectively) in ascorbic acid (0.5% w/v) were centrifuged (400*g*, 5 min) in Centrisart tubes (cut off 10,000 Daltons). To determine the extent of loss of MTZ and AM onto the container walls or onto the filtration membrane, solutions were assayed before and after ultrafiltration.

Reaction of MTZ with heparin

Solutions of MTZ (50, 10 and 2 ng ml^{-1} in distilled water) were incubated in unsilanized glass lithium–heparin 'Exetainer' blood sampling tubes for time periods of up to 30 min, and were then subjected to assay. In a separate experiment, solutions of MTZ (5 ng ml^{-1}) were incubated in polypropylene lithium–heparin tubes under identical conditions and assayed as above.

Reaction of MTZ with antioxidants

A solution of MTZ (10 ng ml^{-1}) was divided into four portions, and each portion incubated with either ascorbic acid (to 0.5% w/v), EDTA (to 0.001 M), sodium metabisulphite (to 0.001% w/v) or no addition (the control). The mixtures were incubated at 25°C for 15 min, after which time all solutions were subjected to assay.

Effect of ascorbic acid on stability of MTZ in water and in methanolic HCl (0.5 M)–acetonitrile eluting solvent

To investigate whether ascorbic acid (0.5% w/v) was effective in preventing MTZ degradation in the solid-phase extraction eluent, solutions of MTZ (500 ng ml^{-1}) in 2 ml of either water or 0.5 M methanolic HCl–acetonitrile (90:10, v/v) in previously silanized volumetric flasks (10 ml volume) were subjected to accelerated degradation by incubation for 20 min at 60°C in the presence and absence of ascorbic acid (0.5% w/v). The solutions were then cooled to ambient temperature and adjusted to volume with ascorbic acid solution (0.5% w/v) for assay. In a further experiment both aqueous and organic solutions containing ascorbic acid (0.5% w/v) were incubated at 25°C for 20 min (sufficient time for four LC analyses) and then subjected to assay as previously described.

Stability of MTZ in plasma

The stability of MTZ in plasma was investigated at three temperatures (24, 37 and −20°C) both in the presence and absence of the antioxidant ascorbic acid. Blank plasma in a sterile polypropylene tube was spiked with MTZ (to 10 ng ml^{-1} for 24 and 37°C studies, and 5 ng ml^{-1} for −20°C study) using 10 or 5 μl of a solution of 1 μg ml^{-1} per ml plasma, respectively. In duplicate experiments, the 10 ng ml^{-1} spiked plasma was divided into 20 aliquots of 1 ml of which 10 were incubated at 24°C and 10 were incubated at 37°C. For the frozen (−20°C) study, 5 ng ml^{-1} spiked plasma was divided into two portions, and solid ascorbic acid (to 0.5% w/v) was added to one portion. Both fractions were then divided into 10 1 ml aliquots and frozen at −20°C.

The spiked plasma incubated at 24 and 37°C was assayed in duplicate after 0, 2, 4, 6, 8, 24, 48, 72 and 96 h incubation. Duplicate samples of the frozen (−20°C) spiked plasma (with and without ascorbic acid) were thawed at ambient temperature and assayed at 0, 1, 2, 4 and 12 weeks.

Effect of equilibration time on binding of MTZ and AM to plasma proteins

Blood was sampled from healthy volunteers who had not received any medication for at least 7 days. Blank plasma was prepared from these samples and pooled. Blank plasma (10 ml) was spiked with MTZ (to 4 ng ml^{-1}), AM (to 100 ng ml^{-1}) and ascorbic acid to (0.5% w/v) in a polypropylene tube. The mixture was vortexed for 10 s, refrigerated at 4°C and samples (0.5 ml) taken for assay at intervals of 0, 5, 10, 15, 20 and 30 min. The LC

peak height ratio MTZ/AM was recorded for each sample.

Comparison of LC assay for MTZ with ELISA method

Plasma samples from patients receiving MTZ by bolus injection (14 mg m^{-2}) were divided into two portions. One portion was subjected to the LC assay for MTZ while the second portion was frozen and submitted to a second laboratory (Department of Haematology, Southampton General Hospital, Southampton, UK) for immediate assay by an ELISA method. For each patient sample, the MTZ concentration obtained by the method was plotted against the concentration obtained by the ELISA technique.

Results and Discussion

Validation of LC assay

A typical example of a LC chromatogram of MTZ using the method described with AM internal standard is presented in Fig. 1.

The relationship (obtained on two separate days) between MTZ/AM peak height ratio (R) and MTZ concentration (C) obtained for equilibrated spiked plasma was linear (unweighted linear regression) over the concentration range 1–100 ng ml^{-1}, satisfying equation [1];

$$[R = 0.1108\ C - 0.0908\ (r = 0.999,\ n = 8)] \quad (1)$$

Table 1 shows the calibration data together

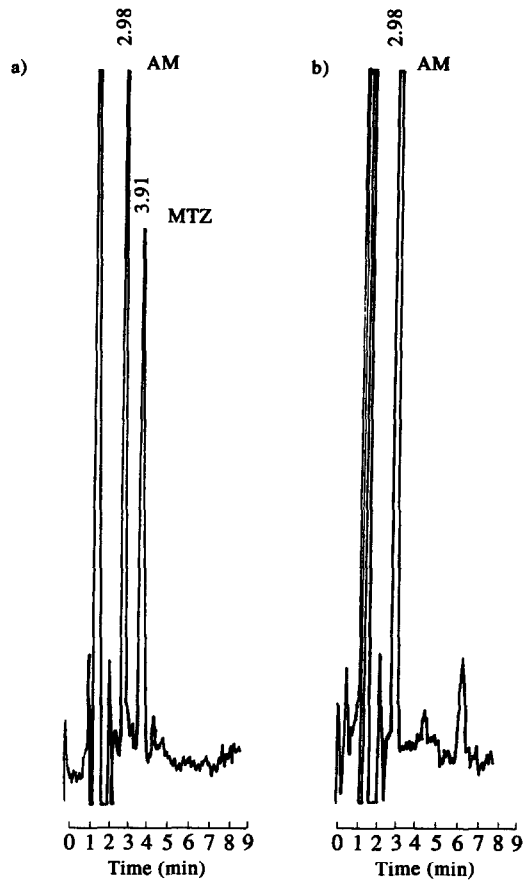

Figure 1
LC chromatogram obtained for a solid-phase extract of (a) mitozantrone (10 ng ml^{-1}) (MTZ) and the internal standard ametantrone (100 ng ml^{-1}) (AM) in spiked plasma, and (b) a patient blank sample.

Table 1
Accuracy and precision of the method

Cs* (ng ml^{-1})	Volume of plasma extracted (ml)	MTZ/AM pk ht ratio
Calibration standards		
1	3	0.1198
2	3	0.1572
5	1	0.4744
10	1	0.9502
15	1	1.4720
20	1	2.0669
40	1	4.4346
100	1	10.9864

Cs* (ng ml^{-1})	Volume of plasma extracted (ml)	Mean MTZ/AM pk ht ratio	Cc† (mg ml^{-1})	MTZ assay (% of theoretical)	RSD (%)	RE‡ (%)
Calibration quality controls						
1	3	0.0225	1.02	102.3	7.57, $n = 5$	2.3
5	1	0.5052	5.38	107.6	4.67, $n = 6$	7.6
10	1	0.9810	9.67	96.7	4.55, $n = 5$	3.3

*Theoretical concentrations of standard solutions of MTZ in plasma.
†Mean concentration of MTZ calculated for standard solutions in plasma from equation (1). $y = 0.1108\ x - 0.0908$.
‡Relative error.

with the quality control data: the theoretical concentrations of MTZ in spiked plasma, the corresponding observed concentrations determined from the calibration plot, the corresponding RSDs for the observed concentrations and the relative errors (RE) are given. The limit of detection of MTZ in plasma was calculated using an extrapolation of the point $2S_{y/x}$ to the x-axis of the calibration plot, where $S_{y/x}$ is given by:

$$S_{y/x} = \sqrt{\frac{\sum(yi' - yi)^2}{n - 2}} \quad (2)$$

The limit of detection was calculated at 2 ng ml^{-1}, however the up to five-fold sample concentration effect of solid-phase extraction increased the achievable limit of detection of the method (RE at 1 ng ml^{-1} = 2.3%, see Table 1).

The mean absolute recovery and inter-day precision for the extraction and LC assay of MTZ [5 ng ml^{-1} in ascorbic acid (0.5% w/v)] on 12 separate days was:

$$\bar{x} = 90.6\%, \text{ RSD} = 3.3\%, n = 12.$$

The absolute recovery of MTZ from spiked plasma, as determined by the difference in slopes between the calibration plots prepared in ascorbic acid (0.5% w/v) and spiked plasma, was 86.6%.

Stability indication

Stress under acidic conditions at 70°C for 30 min resulted in a reduction of the MTZ peak height by 63.7%. Under basic and oxidative conditions the MTZ peak was completely abolished. The assay was therefore stability-indicating for non-specific drug degradation.

The improved LC system combined with optimized solid-phase extraction conditions and the incorporation of an internal standard provided adequate sensitivity for pharmacokinetic studies on MTZ by low-dose infusional regimens. The elimination of systematic errors from the storage and handling of plasma samples prior to assay is considered below.

Binding of MTZ to glassware and ultrafiltration tubes

The data presented in Table 2 shows the time-dependent binding of MTZ to untreated glassware with drug loss from a 10 ng ml^{-1} solution reaching almost 50% over 1 h. There was no significant loss over the same period when the MTZ solution was stored in silanized glassware. On the basis of this study, all glassware used in the preparation and dilution of MTZ solutions was silianized prior to use. Two-piece polypropylene syringes were used to introduce samples into the LC loop-valve in place of conventional glass syringes.

Table 2
Binding of MTZ (10 ng ml^{-1} solution) to untreated and silanized glassware

Incubation time (min)	% MTZ recovered untreated glassware	% MTZ recovered silanized glassware
0	100	100
10	92.8	ND*
20	81.6	ND
30	71.7	ND
60	54.2	99.5

*Not done.

Significant binding of MTZ and the internal standard AM to the Centrisart ultrafiltration membrane or tube precluded ultrafiltration as a sample 'clean-up' procedure. Of a solution of MTZ, 50.4% (2 ng ml^{-1}) and 21.2% of a solution of AM (100 ng ml^{-1}) were lost on centrifugation in Centrisart tubes. The reduced binding for both MTZ and AM (32.7% and 9.7%, respectively) when both drugs were present simultaneously in the test solution was indicative of competitive binding by the two agents. This observation initiated the studies on plasma protein binding of MTZ and AM described in this report.

The product information leaflet supplied by the manufacturers of MTZ [21] indicates that precipitates are formed when MTZ is mixed with heparin in the same infusion. Since most blood collection tubes contain heparin as an anticoagulant this was a potential source of error in pharmacokinetic studies. The data presented in Table 3 confirm that MTZ recovery from solutions incubated in glass lithium–heparin blood collection tubes decreases with time. MTZ loss is particularly significant with dilute solution (2–10 ng ml^{-1}). However, the loss of MTZ for a 5 ng ml^{-1} solution incubated in polypropylene lithium–heparin collection tubes was only 4% after 30 min incubation. This finding suggests that although a small fraction of the analyte could react with heparin, the loss of MTZ from solution in glass lithium–heparin blood collection tubes was mainly attributable to adsorption of MTZ onto glass. Since the loss of

Table 3
Recovery of MTZ after incubation in lithium–heparin blood collection tubes

Incubation time (min)	% MTZ recovery			
	MTZ, 50 ng ml^{-1} (g)*	MTZ, 10 ng ml^{-1} (g)	MTZ, 2 ng ml^{-1} (g)	MTZ, 5 ng ml^{-1} (p)†
0	101.8	100.5	91.5	100.0
10	100.2	94.9	66.5	103.1
20	95.4	82.4	61.5	98.0
30	96.0	65.9	59.9	96.0

*Glass tubes.
†Polypropylene tubes.

MTZ in the polypropylene lithium–heparin tubes was minimal over 20 min, this type of blood collection tube should be used in pharmacokinetic studies on MTZ.

Reaction of MTZ with antioxidants

The incubation of MTZ (10 ng ml^{-1} in distilled water) with various antioxidants for 15 min at 25°C showed that ascorbic acid (0.5% w/v) alone stabilized this solution. A loss of 12.5% MTZ was recorded after 15 min incubation with EDTA (0.001 M), and sodium metabisulphite (0.001% w/v) gave 100% loss of MTZ over the incubation period. The UV/visible absorption spectrum of the MTZ solution was unchanged after treatment with sodium metabisulphite and although the peak corresponding to MTZ was abolished in the LC chromatogram, an additional peak eluting with a relative retention time to MTZ of 1.39 was detected. These findings suggested that metabisulphite did not react with substituents of the anthracenedione ring system but with the hydroxyethylaminoethyl side-chains. Further studies are required to identify the MTZ-metabisulphite reaction product, although the findings of this study are consistent with the formation of a sulphated adduct from the reaction of SO_3^{2-} (from the reaction of metabisulphite with water) with the terminal hydroxyethyl group of the MTZ side-chains by nucleophilic substitution, in analogy with the reaction of adrenaline and metabisulphite [22]. There was no loss of MTZ after incubation with ascorbic acid and this study lends credence to the selection of ascorbic acid as an antioxidant in previous reports on the determination of MTZ in plasma [2, 5, 7, 8, 10, 13, 16].

Stability of MTZ in solid-phase extraction solvents

In preliminary studies the eluting solvent giving optimal recovery of MTZ and AM from the solid-phase extraction columns was 0.5 M methanolic HCl–acetonitrile (90:10, v/v). However, MTZ peak heights rapidly decreased (by 40%) between the first and second LC injections of MTZ in this solution. Under accelerated degradation conditions (60°C), MTZ rapidly degraded in both the solid-phase washing solvent (water) (63.3% remaining at 20 min) and the methanolic HCl–acetonitrile eluting solvent (0% remaining at 20 min). The addition of ascorbic acid (to 0.5% w/v) abolished MTZ degradation in water at elevated temperature (99.3% remaining at 20 min) and significantly reduced the rate of degradation in the organic eluting solvent (69.2% remaining at 20 min). At ambient temperature (25°C), the addition of ascorbic acid prevented analyte decomposition in both solvents (99.8% remaining at 20 min, sufficient time to perform four LC runs). It was concluded that ascorbic acid should be added to the washing and eluting solvents used in the solid-phase extraction procedure. Previous studies [1, 13] employing methanolic HCl (0.5 M) as the eluting solvent in solid-phase extraction conditions did not consider the stability of MTZ during extraction and consequently did not include an antioxidant. In one of these studies [13], methanolic HCl extracts were stored at −20°C prior to assay, thus increasing the potential for error in MTZ determination.

Stability of MTZ in plasma

In studies on the *in vitro* stability of MTZ in plasma at 24 and 37°C, the loss of MTZ (10 ng ml^{-1}) was found to be apparently biphasic

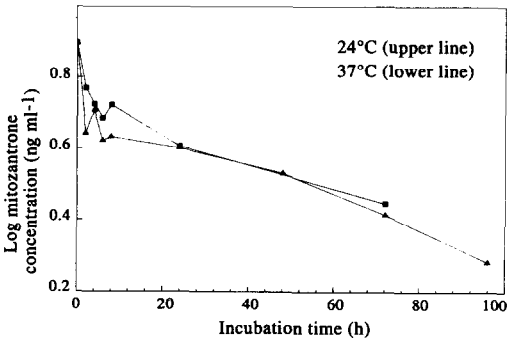

Figure 2
Semilogarithmic plot of the loss of mitozantrone after incubation in spiked plasma at 24 and 37°C.

(Fig. 2) with terminal half-lives of approximately 70 h. MTZ loss was more rapid at 37°C than 24°C. Previous studies on the loss of MTZ in plasma have reported differing half-lives. Batra *et al.* [19] reported a half-life of 10.5 days for MTZ (50 ng ml^{-1}) in plasma at 24°C, whereas under the same conditions Reynolds *et al.* [15] reported a half-life of 24 h. Neither group reported a biphasic degradation process although this may reflect the infrequent sampling times in these studies. MTZ was found to degrade in frozen plasma with a half-life of 34 days, which increased to 53 days on the addition of ascorbic acid (t_{90} = 10.5 days) (as calculated by the regression equations of $y = -0.272\ x + 4.314$, $r = 0.91$ and $y = -0.333\ x + 5.135$, $r = 0.94$, respectively). A previous report [16] on the degradation of MTZ in frozen plasma recorded no changes in the rate of MTZ loss on the addition of ascorbic acid, however, the antioxidant concentration (one-tenth of that used in this study) may explain the apparent absence of antioxidant activity. However, Reynolds *et al.* [15] have shown that in plasma adjusted to pH 5.3, the addition of 0.5% (w/v) ascorbate resulted in <4% loss of MTZ at 4°C over 7 days, the additional decrease in stability being attributed to the increased stabilization of ascorbate at pH 5.3.

On the basis of our data we recommend that blood samples are transferred to pre-chilled (4°C) polypropylene tubes containing solid ascorbic acid (to 0.5% w/v) immediately after collection. Whole blood should be centrifuged under refrigerated conditions and the aspirated plasma stored at −20°C for no more than 7 days prior to assay (storage time could perhaps be increased by reducing sample pH to 5.3, however this was not evaluated in our study). Spiked plasma samples prepared in this manner were stable throughout the work-up procedures, and gave coefficients of variation of 3.62%, $n = 7$ (10 ng ml^{-1} MTZ) and 6.86%, $n = 5$ (1 ng ml^{-1} MTZ), with percentage recoveries of 97 and 102%, respectively.

Effect of equilibration time on MTZ and AM protein binding

Samples of plasma spiked with MTZ (in polypropylene tubes) subjected to extraction and assay immediately after addition of AM and vortex mixing produced low MTZ:AM peak height ratios resulting in low estimation of MTZ (Table 4). In samples taken after 5 min the MTZ:AM peak ratio had stabilized and the MTZ concentrations determined in these samples were close to the expected values (see Table 4). In addition, calibration curves prepared using equilibrated and non-equilibrated plasma gave significant differences in slope. These observations are difficult to explain, but perhaps may be attributed to the displacement of MTZ from protein binding sites by AM, although this would indicate non-disruption of protein binding by solid-phase extraction (SPE). A previous study

Table 4
Effect of equilibration time on MTZ: AM peak height ratio

Equilibration time (min)	MTZ/AM peak height ratio	MTZ assay (% of spiked plasma)
0	0.14	52.1
5	0.35	99.5
10	0.36	101.7
15	0.37	104.2
20	0.36	101.7
30	0.34	97.2

[15] has reported significant disruption of protein binding of MTZ on hydrophobic XAD-2 beads, and therefore, it was questionable whether protein binding effects would be observed by SPE. However, calibration curves prepared using equilibrated plasma ultrafiltrate (prepared using Centrisart tubes 10,000 Da cut-off) either directly injected onto the LC system, or extracted by SPE gave regression equations which compare with equilibrated and non-equilibrated plasma calibration curves as follows: Equilibrated plasma: $R = 0.1108\ C - 0.0908$, $r = 0.999$, $n = 8$; Non-equilibrated plasma: $R = 0.031\ C + 0.076$, $r = 0.99$, $n = 7$; Equilibrated ultrafiltrate, direct injection: $R = 0.057\ C - 0.004$, $r = 0.99$, $n = 6$; Equilibrated ultrafiltrate, extracted: $R = 0.047\ C + 0.014$, $r = 0.99$, $n = 6$, where C represents MTZ concentration (ng ml^{-1}) and R is the peak height ratio (MTZ/AM). The difference in the slopes of the extracted and non-extracted ultrafiltrate slopes is accounted for by absolute recovery (82%). The differences in slopes between extracted plasma ultrafiltrate and whole plasma support the hypothesis that MTZ and AM exhibit different binding affinities to plasma proteins. Table 4 clearly demonstrates the difference in MTZ/AM peak height ratio between samples assayed immediately after spiking and samples equilibrated for 5–30 min after spiking, and this difference is confirmed (by comparison of regression equations) over the concentration range of the calibration plots of equilibrated and non-equilibrated plasma, which were linear over MTZ concentrations of 1–100 ng ml^{-1}. Failure to allow sufficient time for equilibration therefore results in a large systematic error: plasma samples must be allowed to equilibrate for at least 5 min after addition of the internal standard. To our knowledge no other report on the LC determination of MTZ in which an internal standard is used has considered this significant source of error.

Comparison of LC assay with ELISA method

MTZ concentrations determined in patient samples by the LC assay were linearly related to MTZ concentrations determined from the same samples using an ELISA method, as described by the linear regression equation: $y = 1.63\,x + 1.59$ ($r = 0.995$, $n = 20$) (Fig. 3). The slope of the regression line was >1 and reflects the fact that MTZ concentrations

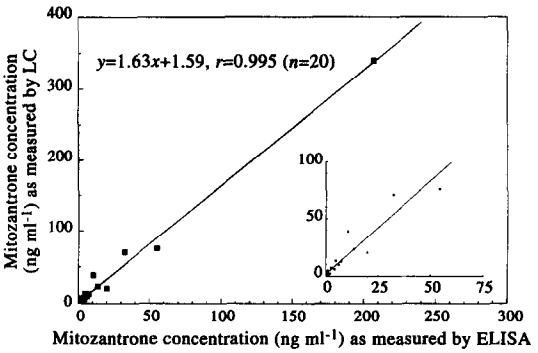

Figure 3
Correlation between LC-measured and ELISA-measured plasma concentrations of MTZ.

determined by the ELISA method were consistently higher than the corresponding LC assay values. This finding is not uncommon with ELISA and RIA techniques because these methods are unable to discriminate between the parent drug and degradation products or metabolites. Accordingly, similar observations have been reported for other drug assays, for example cyclosporin [23].

The degradation products and metabolites of many antitumour drugs are highly toxic and the specific determination of the parent compound and metabolites/degradation products is usually required. Unless a panel of highly specific monoclonal antibodies are available for this purpose, the use of immunoassay techniques may not be appropriate for pharmacokinetic studies on this class of drug.

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