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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MITOXANTRONE IN PLASMA UTILIZING NON-BONDED SILICA GEL FOR SOLID-PHASE ISOLATION TO REDUCE ADSORPTIVE LOSSES ON GLASS DURING SAMPLE PREPARATION

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SUMMARY

Mitoxantrone, a highly active antineoplastic agent, was found to bind strongly to non-bonded silica gel and glassware. When a Hamilton syringe was used to load and inject a mitoxantrone solution (0.4 μg/ml in water) on to a high-performance liquid chromatographic (HPLC) system, about 95% of the loaded compound was found to bind to the glass surface of the syringe barrel and could not be removed by rinsing with water. It could, however, be removed slowly with an acidic solution and thus a small peak of mitoxantrone was present on the chromatogram whenever a blank acidic solution was injected with the syringe. The bound mitoxantrone could be removed effectively from the syringe surface with a solution of tetramethylammonium chloride, citric acid, methanol and water (elution solvent). This binding introduces a large error in assay results and might be one of the major factors responsible for contradictory pharmacokinetic data that have been reported. A new plasma preparation scheme and an HPLC method for mitoxantrone were developed to address this binding problem. Mitoxantrone was extracted directly from plasma samples with a plastic mini-column packed with non-bonded silica gel and eluted with the above elution solvent. The eluent was analysed by HPLC on an ODS column with an absorbance detector at 658 nm. The mobile phase was 0.1 *M* triethylamine phosphate (pH 3.0) in water–tetrahydrofuran–methanol (69:1:30) containing 0.02 *M* tetramethylammonium chloride. Methylene blue was added as an internal standard. Preliminary results showed that mitoxantrone levels in human plasma followed a triphasic decay curve after an intravenous bolus injection. The terminal elimination half-lives measured in three patients (mean $t_{\frac{1}{2}\gamma} = 25$ min) were all shorter than the published values which ranged from 56 min to 9 days.

INTRODUCTION

Mitoxantrone [1,4-dihydroxy-5,8-bis({2[(2-hydroxyethyl)amino]ethyl}amino)-9,10-anthracenedione dihydrochloride; CAS 70476-82-3; NSC 301739; Novantrone; Fig. 1] is an affective chemotherapeutic agent currently used against a wide variety of neoplastic diseases in humans[1–5]. Although many pharmacokinetic studies

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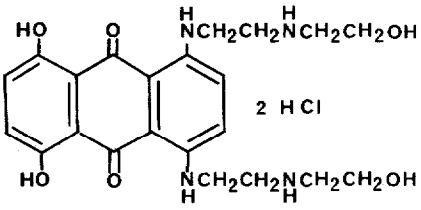
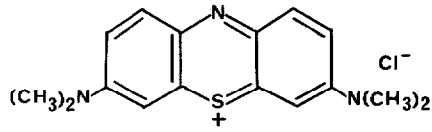


MITOXANTRONE



METHYLENE BLUE

Fig. 1. Structures of mitoxantrone and methylene blue.

have been carried out, the reported data are controversial with the elimination half-life varying widely from 56 min to 9 days[6–10]. Most of the data were obtained by high-performance liquid chromatographic (HPLC) analysis with some differences in the experimental conditions, such as mobile phase or absorption wavelength for detection[10–15]. The differences in the HPLC systems do not appear to be sufficient to account for such large discrepancies in the reported data. The largest differences among these assay methods lies in the sample preparation procedures which include extraction by liquid–liquid partition[11,12], by solid-phase retention with a hydrophobic resin[10,13] or with reversed-phase silica gel[14], or by a column-switching technique[15,16]. We initially tried to find a possible explanation for the discrepancies by comparing the various sample clean-up procedures. In the process, we found that mitoxantrone bound strongly to silica gel and to laboratory glassware. This property appears to have escaped the attention of previous workers and may be responsible to a great extent for the differences reported.

In this paper we present evidence to show that the binding of mitoxantrone to the laboratory glassware can result in erroneous assay data. We also describe a new plasma sample preparation scheme and an HPLC assay system for mitoxantrone that addresses the binding problem. The proposed system involves the use of methylene blue (Fig. 1) as an internal standard and a solid-phase extraction technique for sample preparation.

EXPERIMENTAL

Materials

Mitoxantrone was obtained form Cyanamid Canada (Toronto, Canada) with a purity of 101.1% as its dihydrochloride salt (free base content, 86.9%). Methylene blue was purchased from Mallinckrodt (St.Louis, MO, U.S.A.), tetramethylammonium chloride and triethylamine from Fluka (Ronkonkoma, NY, U.S.A.), methanol, acetonitrile and monobasic ammonium phosphate (all of HPLC grade) from Fisher Scientific (Fair Lawn, NJ, U.S.A.), tetrahydrofuran (distilled-in-glass grade) from Caledon Laboratories (Georgetown, Canada), orthophosphoric acid (85.9%, analytical reagent grade), *l*-ascorbic acid (biochemical-reagent grade) and citric acid monohydrate (analytical grade) from J. T. Baker (Phillipsburg, NJ, U.S.A.) and citrated normal human plasma from American Hospital Supply (Miami, FL, U.S.A.).

Plastic mini-columns (65 × 6 mm I.D., Bond-Elut), aspiration apparatus (Vac-Elut) and non-bonded silica gel (Sepralyte, 40 μm) were obtained from Analytichem International (Harbor City, CA, U.S.A.).

A stock solution of triethylamine phosphate (1 *M*) was prepared by mixing 67.8 ml of orthophosphoric acid with 800 ml of deionized water, titrating to pH 3.0 with triethylamine and then diluting to 1000 ml with deionized water. It was filtered through a 0.45-μm type HA filter (Millipore, Bedford, MA, U.S.A.) and stored at 4°C. The deionized water used throughout the experiments was obtained by connecting the distilled water system of the hospital to a Barnstead three-module Nanopure II system (Bybron/Barnstead, Boston, MA, U.S.A.).

HPLC system

The apparatus consisted of an LKB (Bromma, Sweden) Model 2150 pump, a Waters Assoc. Model 441 absorbance detector at 658 nm (Millipore, Bedford, MA, U.S.A.), a Hitachi (Tokyo, Japan) Model 561 chart recorder, a Rheodyne (Cotati, CA, U.S.A.) injection valve with a 50μl sample loop and a Waters Nova-pak ODS 4-μm column (75 × 3.9 mm I.D.). The mobile phase was prepared by mixing 10 parts (v/v) of triethylamine phosphate (1 *M*, pH 3.0), 1 part of tetrahydrofuran, 30 parts of methanol, 2 parts of tetramethylammoniumchloride (1 *M*) and 57 parts of deionized water. It was filtered through a 0.5-μm Type FH filter (Millipore) under maximum suction from a hydraulic pump and used without further degassing. HPLC was carried out at room temperature (22–23°C) with a flow-rate of 1.0 ml/min.

Binding on to syringes

Before injection of any solution the Hamilton syringe (Type A; size 100 μl) was rinsed three times with the elution solvent (see *Isolation procedures*) and each time the solvent was allowed to fill up to the full capacity of the syringe. The syringe was then rinsed three times with deionized water, five times with 0.1 *M* Cl in 50% aqueous methanol and again rinsed ten times with deionized water. The syringe was then used to load and inject 100 μl of the elution solvent on to the HPLC system to verify that no interfering peak was detected on the chromatogram. The syringe was again rinsed ten times with deionized water. It was then used to load 100 μl of test solution, the solution was allowed to remain in the syringe for 1 min at room temperature and then all 100 μl were injected on to the HPLC system (which was equipped with a 50-μl sample loop). The syringe was rinsed ten times with deionized water and used to inject 100 μl of the solution as described or subjected to the cleaning procedures as described above before injection of a new test solution. A plastic syringe (plastic tuberculin syringe, capacity of 1 ml in divisions of 0.01 ml; Becton-Dickinson, Rutherford, NJ, U.S.A.) fitted with a Hamilton removable needle for the Rheodyne injector was also used in the experiment. Great care was taken to fill up the solution to the 0.1-ml mark and all other conditions were followed precisely as those for the Hamilton syringe described above.

Collection of plasma samples

Venous blood (2.5 ml) was withdrawn with a polypropylene syringe and transferred into a polypropylene test-tube (100 × 17 mm I.D.) which contained 0.06 ml of 15% ascorbic acid (prepared in 0.6 *M* citrate buffer, pH 3.0) and chilled in ice–water

immediately. The mixture was centrifuged at 2000 *g* for 5 min at 4°C (with a Model NH-SII centrifuge; IEC, Needham Heights, MA, U.S.A.) and the plasma was stored at −20°C in a 1.5-ml Eppendorf polypropylene centrifuge tube equipped with a cap until analyzed. All plasma samples were frozen at least overnight before assay.

Isolation procedures

Plastic mini-columns, each containing 100 ml of non-bonded silica gel, were inserted into Luer fittings of the aspiration apparatus (Vac-Elut). Each column was then washed with 1 ml of 1 *M* hydrochloric acid, followed by 3 ml of water and finally with 0.5 ml of 2 *M* monobasic ammonium phosphate (pH unadjusted). The frozen plasma sample was thawed at 4°C and an aliquot (1.0 ml) was pipeted into a 1.5-ml Eppendorf polypropylene centrifuge tube. A 5-μl volume of methylene blue solution (5 μg/ml) was added to each sample as an internal standard. After centrifugation at 12 000 *g* for 1 min at 4°C (with a Model 5412 Eppendorf centrifuge; Brinkmann, Westbury, NY, U.S.A.), the supernatant was transferred into the above-mentioned mini-column and allowed to pass through at a flow-rate of about 1 ml/min by adjusting the suction of the pump. The precipitate remaining in the centrifuge tube was resuspended in 0.5 ml of 0.3% ascorbic acid solution (1:50 dilution of the aforementioned 15% ascorbic acid in 0.6 *M* citrate buffer, pH 3.0) by vortexing and subsequently transferred into the same mini-column. This two-step transfer of plasma sample was found to be essential to ensure a good flow of sample through the mini-column, which was frequently blocked by the small clots that invariably formed after the plasma sample was stored. The column was then washed with 0.5 ml of water followed by 0.5 ml of acetonitrile saturated with tetramethylammonium chloride and suction was applied for a further 5 min. It was subsequently removed from the aspiration unit, suspended above a plastic test-tube (100 × 7.5 mm I.D.) and eluted with 0.3 ml of elution solvent by centrifugation at 2000 *g* for 5 min at 4°C with an IEC Model NH-SII centrifuge. The elution solvent was prepared by mixing 10 parts (v/v) of tetramethylammonium chloride solution (1 *M*), 32 parts of methanol, 20 parts of citric acid solution (1 *M*) and 38 parts of deionized water.

Quantitation

The minimum quantifiable concentration for mitoxantrone by the detector was 1 ng/ml at a peak height-to-noise ratio of 5. The baseline on the chart recorder was very stable (fluctuation 0.3 mm; full-scale = 25 cm) with the recorder at its maximum sensitivity ($R = 0.005$) and the mobile phase running at a flow-rate of 1.0 ml/min. Citrated normal human plasma blank together with those spiked with mitoxantrone at concentrations of 2.5, 5, 10, 25, 100 and 500 ng/ml were each added to 20 μl of 15% ascorbic acid (in 0.3 *M* citrate buffer, pH 3.0) and then prepared as described under *Isolation procedures*. After HPLC analysis, a calibration graph of the peak-height ratio of mitoxantrone to methylene blue *versus* mitoxantrone concentration was constructed. The graph was linear over the range 2.5–500 ng/ml with $Y = 0.056x + 0.22$, and a correlation coefficient (r) = 1.001. The mitoxantrone concentrations in clinical samples were obtained from the calibration graph by reference to the peak-height ratio of mitoxantrone to methylene blue determined by HPLC.

Pharmacokinetic study

An intravenous bolus of mitoxantrone (10 mg/m^2 body surface area) was given at 0 min and the blood samples were taken at 1, 2, 5, 10, 15, 20, 30, 45, 60, 120, 180 and 480 min. A blank sample was also taken immediately before drug administration. For those at 1, 2 and 5 min, the diluted samples (1:10 in 0.3% ascorbic acid) and also the undiluted samples were analyzed. For all other samples, only the undiluted plasma were analyzed. As the timing gap between the first three samples was narrow, sampling times were varied appropriately from the schedule owing to slow blood flow.

RESULTS AND DISCUSSION

It is well known that protonated amines can be strongly retained by the surface of silica gel and the mechanism for the retention is thought to involve interaction with the silanol groups of the gel[17,18]. With four amino groups present on its molecule (Fig. 1), it is therefore not surprising that mitoxantrone binds very strongly to silica gel and glassware.

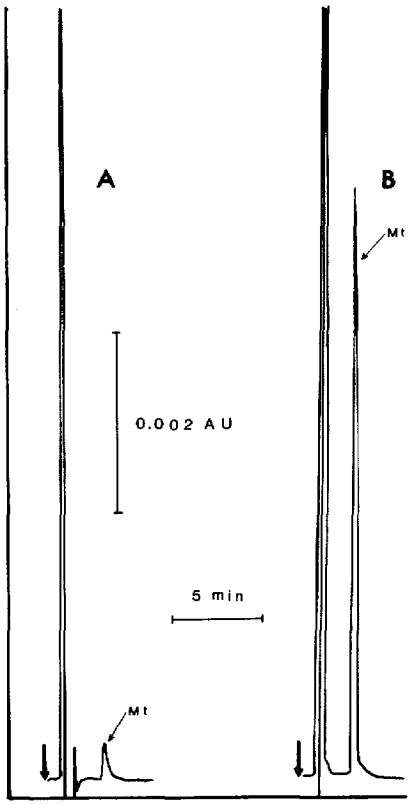


Fig. 2. Chromatographic profiles of (A) mitoxantrone solution (0.4 μg/ml in water) and (B) blank elution solvent injected into the HPLC system with a Hamilton syringe.

One of the most commonly used items of glassware in HPLC is the Hamilton syringe. Fig. 2A shows a chromatogram of mitoxantrone solution (0.4 μg/ml) prepared in deionized water and injected into the HPLC system with a Hamilton syringe. After rinsing ten times with deionized water the syringe was used to inject a blank elution solvent (see *Isolation procedures*) and the chromatogram shown in Fig. 2B was obtained. The peak height of mitoxantrone in Fig. 2B is about 16.4 times that in Fig. 2A, indicating that mitoxantrone solution (prepared in deionized water) binds to the glass surface of the syringe barrel and can subsequently be removed by rinsing with a solution containing a competing amine, but not by rinsing extensively with deionized water.

Fig. 3A shows a chromatogram of mitoxantrone solution (0.4 μg/ml) prepared in the elution solvent and injected into the HPLC system with a Hamilton syringe. After the injection the syringe was rinsed ten times with deionized water and a blank elution solvent was then injected. Only a very minor mitoxantrone peak (about 1% of the peak height in Fig. 3A) was detected on injection of the blank elution solvent (Fig. 3B). Another injection of blank elution solvent gave no mitoxantrone peak (not shown). The experiments demonstrated that, if a competing amine is present in the injection solution (such as the elution solvent), very little mitoxantrone is adsorbed on

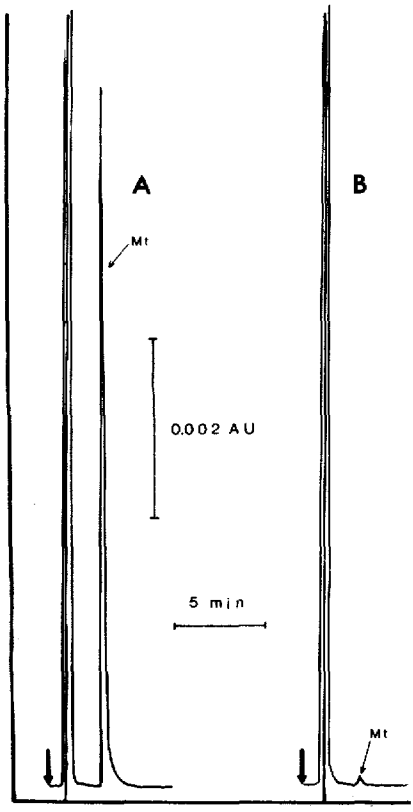


Fig. 3. Chromatographic profile of (A) mitoxantrone solution (0.4 μg/ml in the elution solvent) and (B) blank elution solvent injected into the HPLC system with a Hamilton syringe.

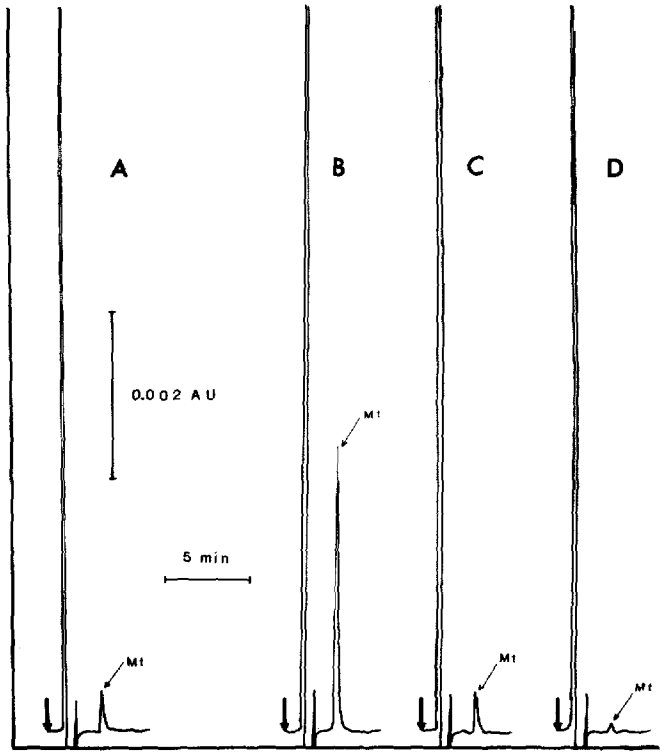


Fig. 4. Chromatographic profiles of (A) mitoxantrone solution (0.4 μg/ml in water) and successive injections of 0.1 *M* hydrochloric acid (B, C and D) with a Hamilton syringe. (B) First injection; (C) second injection; (D) fifth injection.

the syringe wall and if the Hamilton syringe is rinsed with the elution solvent between injections, almost no carry-over contamination of the Hamilton syringe by mitoxantrone will occur. The peak height of mitoxantrone in Fig. 2A is only 5% of that in Fig. 3A, suggesting that about 95% of loaded mitoxantrone in a solution prepared only with deionized water binds to the glass wall of the Hamilton syringe.

Fig. 4 shows the results obtained by injection of mitoxantrone solution (0.4 μg/ml in water) (A) with a Hamilton syringe, followed by a succession of aliquots of 0.1 *M* hydrochloric acid injected with the same Hamilton syringe (B, C and D). The syringe was rinsed ten times with deionized water between each injection. The experiment demonstrates that mitoxantrone adsorbed on the glass surface of the barrel of a Hamilton syringe can be removed slowly and inefficiently with an acidic solution. Thus, if a contaminated Hamilton syringe is used by an unsuspecting analyst to inject an acidic extract, a small mitoxantrone peak will be produced even though no mitoxantrone is present in the extract. One of the consequences of this error will be an artificially long elimination half-life for mitoxantrone in human plasma.

Fig. 5A shows the chromatographic profiles of a mitoxantrone solution (0.4 μg/ml in water) injected with a polypropylene syringe. The syringe was then rinsed with deionized water and the elution solvent was injected (Fig. 5B). There appeared to be some adsorption of mitoxantrone on the syringe, but the extent of the binding was

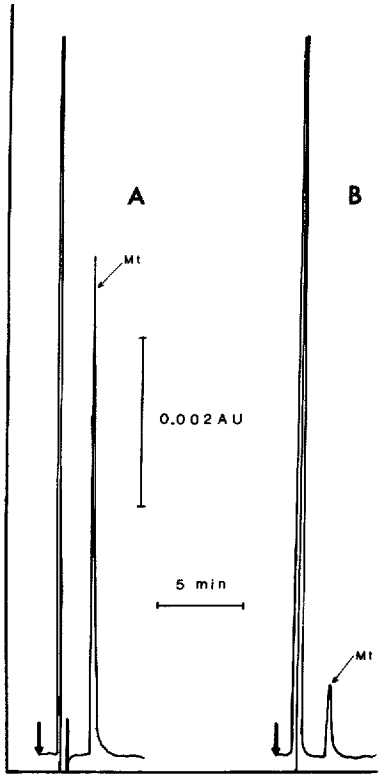


Fig. 5. Chromatographic profiles of (A) mitoxantrone solution and (B) blank elution solvent injected into the HPLC system with a polypropylene syringe.

far less than that observed with a Hamilton syringe. It must be noted that the plastic syringe was equipped with a rubber-tipped plunger, and therefore the source of this minor binding has not been located. The experiment nevertheless suggests that laboratory equipment or hardware made of polypropylene rather than glass should be used in mitoxantrone assays. We also avoid using rubber stoppers for vessels for the storage of plasma samples.

Taking advantage of this property, we isolated mitoxantrone directly from plasma samples with a mini-column packed with non-bonded silica gel and then eluted mitoxantrone and methylene blue (internal standard) from the column with an elution solvent that consisted of tetramethylammonium chloride, citric acid, methanol and deionized water. The composition of the elution solvent was formulated on the basis of three factors, a competing amine, an acid and an organic solvent. The selection of each compound could be carried out visually as both mitoxantrone and methylene blue show a deep blue color. When 1 ml of mitoxantrone or methylene blue solution (1 μg/ml in water) was allowed to pass through the plastic mini-column packed with non-bonded silica gel it formed a sharp blue band on top of the gel. A series of test solutions at different concentrations were added 100 μl at a time to the silica gel to evaluate their efficiency in washing down the blue band from top of the gel. Tetramethylammonium chloride is known to block silanol groups on silica gel[19],

but alone it was found to be not as effective in eluting down the bound mitoxantrone or methylene blue. Addition of citric acid to the tetramethylammonium chloride solution made both blue compounds move down more sharply and faster through the silica gel. Many other acids tested, including hydrochloric, phosphoric, formic and acetic acid, were not as effective. The effectiveness of the elution was also enhanced by the addition of methanol to the solvent, but its concentration could not be too far above that in the HPLC mobile phase otherwise the chromatographic system would be destabilized[20].

Methylene blue serves well as an internal standard. It is a polyamine with an absorption spectrum very similar to that of mitoxantrone and allowed the detection procedure to be carried out at 658 nm. Methylene blue also binds to Hamilton syringes and similar results to those for mitoxantrone in Fig. 2–4 were observed. It binds to silica gel even more strongly than mitoxantrone and required a larger volume of solvent for elution. In order to elute both compounds in a small volume of solvent, the sample-loaded column was washed once with acetonitrile that had been saturated with tetramethylammonium chloride. This washing pushed the methylene blue band so that it moved forward without much effect on mitoxantrone and thus allowed the two compounds to be eluted from the mini-column at about the same time with the elution solvent formulated. It should be noted that when methylene blue was added to the sample as an internal standard it was decolorized immediately by ascorbic acid (added as a stabilizer for mitoxantrone) in the plasma. The reduced form of methylene blue, however, was rapidly reoxidized once it had been injected into the HPLC system, because any residual amount of ascorbic acid that might be present in the injection solution would be separated quickly by the ODS column and the remaining methylene blue was continuously interacting with a mobile phase that contained air (N.B., the mobile phase was only filtered using the hydraulic pump, without further degassing). We verified this fact by injecting into the HPLC system methylene blue solution (100 ng/ml in the elution solvent) with and without 0.3% ascorbic acid and found that identical peaks (same retention time and peak shape and height) were produced.

Fig. 6 shows a representative chromatographic profile from the HPLC analysis of a plasma sample from a leukemic patient who had received an intravenous bolus injection of mitoxantrone. The first plasma sample was taken 2.5 min after administration of the drug. Subsequent plasma samples obtained 5 and 20 min after the injection of the drug showed a rapid decline of the mitoxantrone peaks, whereas the peaks for the internal standard (methylene blue) remained relatively unchanged. There was no interfering peak in blank plasma (Fig. 6, -5 min).

The results obtained in studies on three patients are summarized in Table I. The plasma concentration at 0.5 – 1 min after administration of the drug was 1.2 – 3.4 μg/ml; it then followed a triphasic decay curve with mean half-lives of 2.2 min ($t_{\frac{1}{2}}\alpha$), 8.7 min ($t_{\frac{1}{2}}\beta$) and 25 min ($t_{\frac{1}{2}}\gamma$)[21]. The terminal elimination half-life ($t_{\frac{1}{2}}\gamma$) obtained by us is the shortest that has ever been reported to date. The data, however, do not conflict with the observation that [^{14}C]mitoxantrone "equivalents" could be identified in autopsy tissues that were obtained more than 1 month after drug administration[22], as the workers concerned measured only the recovery of radioactivity without identifying the nature of the compounds (the site of the label on mitoxantrone was not identified).

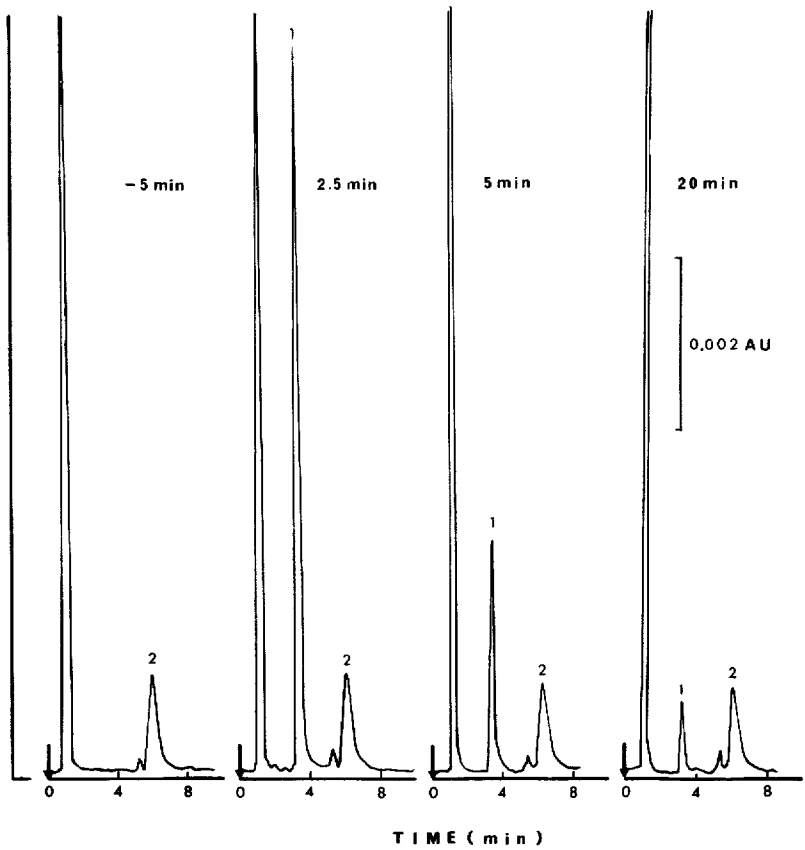


Fig. 6. Representative chromatographic profile for the analysis of plasma samples from a leukemic patient who had received an intravenous bolus of mitoxantrone (10 mg/m^2 body surface area) at 0 min. Plasma samples were taken at 1, 2.5, 5, 10, 15, 20 min continuing up to 4 h. A blank sample (−5 min) was also obtained immediately before administration of the drug. Only the results of −5, 2.5, 5 and 20 min are shown. Peaks: 1 = mitoxantrone; 2 = methylene blue. The minor peak between these two peaks is an impurity carry-over from the methylene blue solution.

Many sample preparation techniques for mitoxantrone, including solid-phase extraction, have been described[10–16]. The advantages for using solid-phase extraction over liquid–liquid extraction are well recognized[23]. Our solid-phase extraction method is superior to other methods in several respects. First, we include in our system an internal standard, in contrast to all other solid-phase extraction methods published so far[10,13,14]. Second, our extraction mechanism is based on adsorption and desorption of an amine with silanol groups, whereas extraction of mitoxantrone using an XAD-2 column [10,13] or a reversed-phase silica gel column[14] was based on hydrophobic interaction or a mixed mechanism. Obviously our method is more selective. Third, our elution solvent for the mini-column has a similar composition to the HPLC mobile phase, and therefore the HPLC system can withstand repeated injections of samples without deterioration. Other methods use a strong organic solvent, such as 30% propanol[10,13] or 0.5 *M* methanolic hydrochloric acid[14], for the elution. Is is well

TABLE I

HPLC ANALYSIS OF PLASMA SAMPLES FROM PATIENTS WHO HAD RECEIVED AN INTRAVENOUS BOLUS INJECTION OF MITOXANTRONE

Patient	*Age (yr)*	*Weight (kg)*	*Peak concentration*[a] *(μg/ml)*	*Half-lives (min)*		
				$t_{\frac{1}{2}}\alpha$	$t_{\frac{1}{2}}\beta$	$t_{\frac{1}{2}}\gamma$
F.D.	16	76	3.4 (0.5 min)	2	9	25
S.B.	16	47	2.1 (1.0 min)	1.5	8	20
I.F.J.	15	70	1.2 (1.0 min)	3	9	30

[a]The peak concentration represents the mitoxantrone concentration in the first plasma sample obtained after administration of the drug. The time in parentheses indicates the time when the sample was taken.

known that injecting a sample in a stronger organic solvent than the mobile phase can result in peak broadening and distortion[20]. Fourth, our method addressed the problem of binding of mitoxantrone to the laboratory glassware and thus avoided potential errors. None of the published methods for the determination of mitoxantrone has addressed this binding problem.

In conclusion, we found that mitoxantrone bound strongly to silica gel and glassware. We have developed a reliable and sensitive HPLC method for monitoring plasma mitoxantrone that addressed the binding problem. We recommend that all future studies on mitoxantrone, including drug monitoring and *in vitro* incubation, should avoid using any glassware to prevent loss of the compound. Polypropylene vessels should be used in a place of glass vessels.

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