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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE NEW ANTINEOPLASTIC AGENTS BISANTRENE AND MITOXANTRONE

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SUMMARY

Bisantrene and mitoxantrone are two new anthracene derivatives which have shown significant antitumor activity against a wide variety of animal tumors and in human phase I and II clinical trials. We have developed a rapid, simple and sensitive sample cleanup procedure and high-performance liquid chromatographic (HPLC) assay for both drugs. This method uses a commercially available mini-cartridge with C_{18} reversed-phase packing to isolate the drugs from the biological matrix prior to HPLC. For both drugs the average recovery of the assay was 98 ± 6% with a coefficient of variation (C.V.) of less than 7%. Using this new method our assay sensitivity has improved to less than 10 ng/ml for bisantrene and 1 ng/ml for mitoxantrone, allowing us to document a prolonged terminal phase plasma half-life for both bisantrene and mitoxantrone. Equilibrium dialysis studies showed that both drugs are highly protein bound. Mitoxantrone appears less stable in human plasma than bisantrene. Recoveries from plasma after a 24-h incubation at 25 and 37°C were 40 and 20% for mitoxantrone and 90 and 85% for bisantrene, respectively. Addition of ascorbic acid prior to incubation of mitoxantrone in human plasma at 37°C resulted in less than a 10% decrease in the latter's concentration over a 24-h period. To maintain sample integrity, all plasma samples should be fortified with ascorbic acid and kept frozen prior to analyses.

INTRODUCTION

Bisantrene {9,10-antracenedicarboxaldehyde-bis-[(4,5-dihydro-1H-imidazol-2-yl)hydrazone] dihydrochloride, NSC 337766} and mitoxantrone {1,4-

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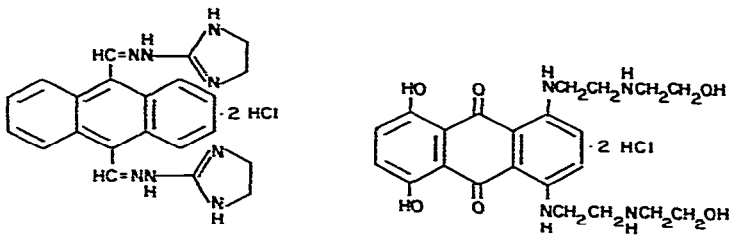
dihydroxy-5,8-bis-[2-(2-hydroxyethyl)amino ethylamino]-9,10-anthracenedione dihydrochloride, NSC 301739} (Fig. 1) are two new anthracene derivatives which have shown significant antitumor activity against a wide variety of animal tumors [1, 2] and in phase I and II clinical trials [3—6]. Our preliminary pharmacokinetic studies [7, 8] showed that bisantrene has a relatively long terminal half-life. The reported pharmacokinetic data of mitoxantrone are, however, controversial [9—11].



BISANTRENE (NSC 337766)

MITOXANTRONE (NSC 301739)

Fig. 1. Chemical structure of bisantrene and mitoxantrone.

A simple, precise and sensitive analytical method is essential for pharmacokinetic studies of both drugs. We have recently reported a high-performance liquid chromatography (HPLC) method for the measurement of bisantrene in biological samples [12]. In that report, we used a more extensive sample cleanup procedure, including perchloric acid precipitation of proteins and ethyl-acetate partitioning of bisantrene. Other published HPLC methods for mitoxantrone also require lengthy sample cleanup procedures, including double liquid extraction with chloroform [13] or XAD-2 column isolation [14]. Additionally, these methods have limited sensitivity (i.e., sensitivity ca. 75 ng/ml) [14] and fail to identify a terminal phase of mitoxantrone plasma elimination after the administration of a standard dose (12 mg/m^2).

In the present study, we have developed a new sample cleanup procedure and HPLC assay for both bisantrene and mitoxantrone. This method uses a commercially available mini-cartridge with C_{18} reversed-phase packing to isolate the drugs from biological matrix prior to HPLC. This system allows accurate determination of bisantrene and mitoxantrone with an analysis time of less than 10 min. Using this improved method we have studied the stability and the protein binding of the drugs and have measured the plasma concentrations of both bisantrene and mitoxantrone in patients.

EXPERIMENTAL

Materials

Bisantrene and mitoxantrone reference standards, obtained from Lederle Labs., American Cyanamid Company (Pearl River, NY, U.S.A.), were dissolved in methanol and stored at —80°C. Organic solvents were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and filtered through a 0.45-μm fluoropore filter (Millipore, Bedford, MA, U.S.A.) prior to use. Aque-

ous solvents for HPLC use were filtered through a 0.45-μm cellulose acetate filter prior to use. Ammonium acetate (HPLC grade) was obtained from Fisher Scientific, Fair Lawn, NY, U.S.A., L-ascorbic acid was obtained from Gibco, Grand Island, NY, U.S.A., human albumin and γ-globulin were obtained from Sigma, St. Louis, MO, U.S.A. and 3 *M* methanolic HCl was obtained from Supelco, Bellefonte, PA, U.S.A.

Sample cleanup procedure

A Vac-Elut™ system equipped with Bond-Elut™ 1-ml C_{18} cartridges (Analytichem International, Harbor City, CA, U.S.A.) was used for sample cleanup. The Vac-Elut's components included a stainless-steel vacuum basin. Vacuum was applied through a 1/8-in. NTP hose fitting located on one end of the basin. A 10-place molded cover with a foam polyethylene gasket precisely fitted the top of the basin. Bond-Elut cartridges were inserted into luer fittings which were an integral part of the cover. Sample eluates were collected in test tubes which were held under the cartridges in a stainless-steel removable rack.

Plasma samples of 1—2 ml were passed through a cartridge, which was sequentially preconditioned by washing with 10 ml of methanol and 5 ml of water. After the plasma had passed through the cartridge, it was washed with 5 ml of water and 300 μl of 0.5 *M* methanolic HCl was used to elute the drug. The eluate was collected, vortexed and kept at —20°C for HPLC analysis.

HPLC apparatus

HPLC was performed with an apparatus consisting of a Model 660 solvent programmer, two Model 6000A solvent delivery systems, a Model U6K injector, a Model 440 detector (Waters Assoc., Milford, MA, U.S.A.), and a Model A-25 recorder (Varian, Palo Alto, CA, U.S.A.). A Waters Assoc. μBondapak C_{18} (30 cm × 3.9 mm I.D.; particle size 10 μm) reversed-phase column preceded by a 7 cm × 2.1 mm I.D. guard column packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was used for all analyses. The guard column packing was changed every two weeks or whenever there was significant back pressure build up.

Chromatographic conditions

Bisantrene was eluted isocratically at ambient temperature with acetonitrile—0.2 *M* ammonium acetate pH 4.0 (27:73) as solvent at a flow-rate of 2.0 ml/min. Bisantrene was detected at 436 nm using a Waters Assoc. Model 440 fixed-wavelength detector.

Mitoxantrone was eluted isocratically at ambient temperature with acetonitrile—0.2 *M* ammonium acetate pH 4.0 (25:75) as solvent at a flow-rate of 1.5 ml/min. Mitoxantrone was detected at 658 nm using a Waters Assoc. Model 440 fixed-wavelength detector.

Quantitation

Quantitation of both drugs was done by the external standard method of analysis. Plasma standard curves were obtained by plotting the resulting peak heights against the known concentration of standards added to the plasma samples.

Recovery and precision

Various amounts of standard bisantrene or mitoxantrone were added to 1 ml of human plasma at room temperature. The plasma was immediately prepared for HPLC analysis. Recovery was calculated by comparing the peak heights of the spiked samples to that of the standards. All experiments were run in triplicate.

Precision and accuracy were determined by assaying patient's plasma in triplicate on different days.

Mass spectral identification

Confirmation of bisantrene and mitoxantrone in the plasma samples was accomplished as previously described, using a Finnigan Model 3300 mass spectrometer coupled to an Incos Model 2061 data system (Finnigan Instruments, Sunnyvale, CA, U.S.A.). Both drugs were analyzed for complete spectra and then plasma samples were analyzed by selected ion monitoring [12, 15].

Stability studies

The stability of bisantrene and mitoxantrone in human plasma and in the presence of human plasma proteins (5% human albumin + 3% γ-globulin) was studied at 37 and 25°C. Samples were assayed for bisantrene or mitoxantrone concentration at various time intervals up to 24 h.

The stability of mitoxantrone in human plasma containing ascorbic acid was also studied. A volume of 100 μl of 5% L-ascorbic acid in citrate buffer (0.1 *M*, pH 3.0) was added to 1 ml of human plasma (maintained at 37°C) at the following intervals: (a) prior to the addition of mitoxantrone, or (b) 1—2 h after the addition of mitoxantrone.

Equilibrium dialysis study

The dialysis apparatus (Chemical Rubber Co., Cleveland, OH, U.S.A.) had a 2-ml capacity. A cellulose acetate membrane separated the system into two symmetrical 1-ml chambers. Two sets of apparatus were run simultaneously, one containing 1 ml bisantrene or mitoxantrone (20 μg/ml) in normal saline dialyzed against 1 ml human plasma, the other containing 1 ml bisantrene or mitoxantrone in normal saline dialyzed against 1 ml normal saline. The dialysis apparatus was kept at room temperature with gentle shaking. Aliquots were removed from both chambers at time intervals up to 24 h for the measurement of bisantrene or mitoxantrone.

RESULTS

Figs. 2 and 3 show the chromatographic profiles of standard bisantrene and mitoxantrone extracted from normal plasma and a typical 0 time patient's plasma. There were no co-eluting peaks at the chromatographic positions of bisantrene and mitoxantrone, demonstrating the clean background associated with our sample cleanup procedure for both drugs.

Excellent linearity ($r > 0.996$) was observed for the standard curves over a 10—2000 ng/ml range for bisantrene and a 1—2000 ng/ml range for mitoxantrone. The precision and recovery data for the assay are shown in Tables I

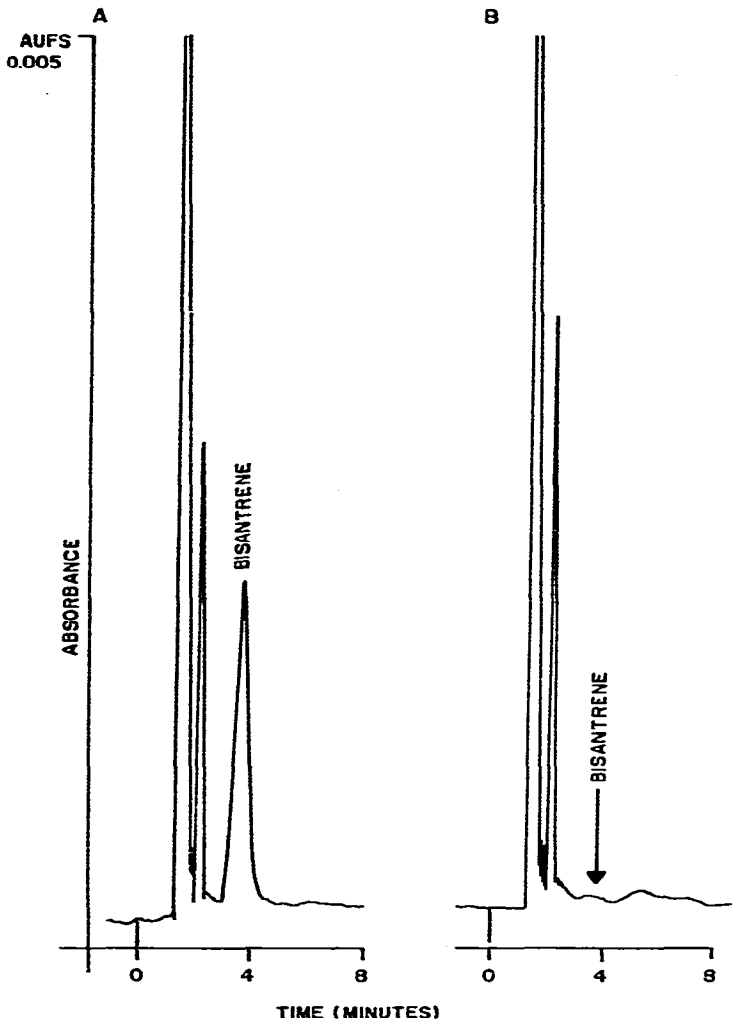


Fig. 2. Chromatographic profiles of (A) standard bisantrene (200 ng/ml) extracted from normal plasma and (B) a typical 0 time patient's plasma. Equivalent of 0.5 ml plasma was injected at 0.005 a.u.f.s.

and II. The average recovery was 98 ± 6% with a coefficient of variation (C.V.) of less than 7%. The sensitivity of the assay was 10 ng/ml for bisantrene and 1 ng/ml for mitoxantrone.

The full mass spectra of bisantrene and mitoxantrone are shown in Figs. 4 and 5. Note the prominant molecular ion at *m/e* 398. This ion and three other prominant ions (313, 228, 215) were used in the selected ion monitoring analysis of patient samples.

Fig. 6 shows the plasma disappearance curves for bisantrene and mitoxantrone following 60- and 30-min infusions, respectively, in two patients. Note that both drugs had prolonged terminal plasma half-lifes in these two patients.

Fig. 7 shows the stability of mitoxantrone and bisantrene in human plasma.

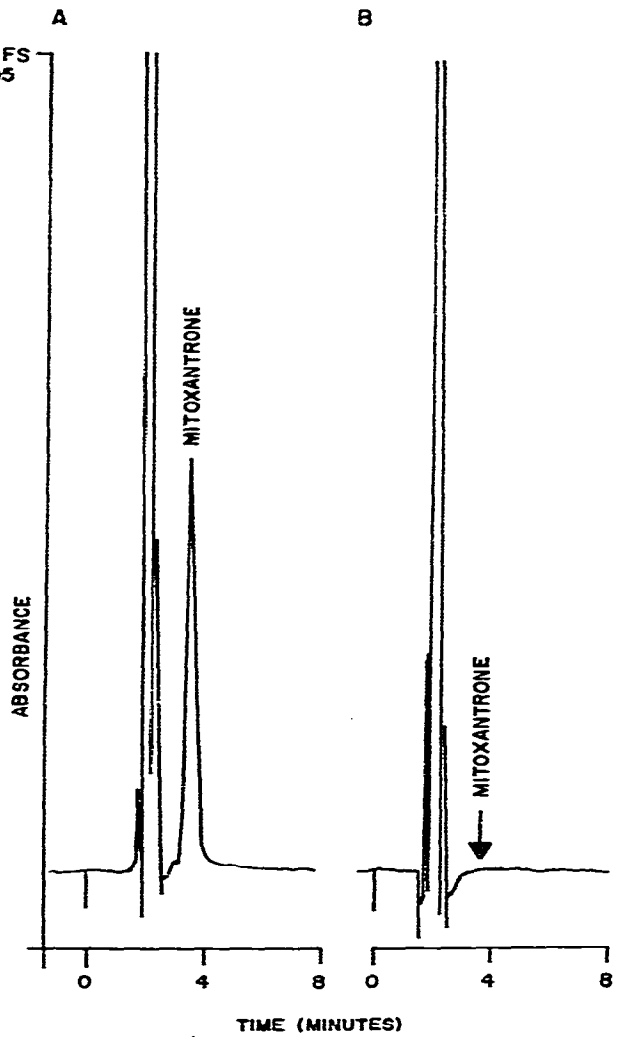


Fig. 3. Chromatographic profiles of (A) standard mitoxantrone (50 ng/ml) extracted from normal plasma and (B) a typical 0 time patient's plasma. Equivalent of 0.5 ml plasma was injected at 0.005 a.u.f.s.

Recoveries after a 24-h incubation at 25 and 37°C were 40 and 20% for mitoxantrone and 90 and 85% for bisantrene, respectively. Incubation of mitoxantrone with 5% human albumin + 3% human γ-globulin for 24 h resulted in recoveries of 80% at 25°C and 60% at 37°C.

To determine whether ascorbic acid stabilizes mitoxantrone in plasma as has been reported by Reynolds et al. [14] we incubated mitoxantrone with human plasma containing ascorbic acid. Mitoxantrone incubation for 24 h at 37°C resulted in drug recovery of greater than 90% (Fig. 8). Ascorbic acid was unable to regenerate but did prevent further loss of mitoxantrone when it was added to plasma following a 30% decrease in mitoxantrone concentration (Fig. 8).

TABLE I

RECOVERY OF BISANTRENE AND MITOXANTRONE FROM PLASMA

All experiments were run in triplicate on different days.

Amount added (ng/ml)	Recovery* (%) ($\bar{x}$ ± S.D.)	C.V. (%)
Bisantrene		
2000	105 ± 2.5	2.4
500	101 ± 5.1	5.0
100	93 ± 2.3	2.5
Mitoxantrone		
1000	105 ± 5.1	4.8
100	94 ± 2.0	2.1
10	94 ± 2.3	2.4
Average**	98 ± 5.6	5.7

*The immediate recovery of bisantrene and mitoxantrone at 25°C using sample extraction method as described in text.
**This is the average of all concentrations tested.

TABLE II

PRECISION OF ASSAY

Samples A, B, C and D are patient's plasma samples obtained after an intravenous infusion of bisantrene or mitoxantrone.

Experiment	Concentration* (ng/ml) ($\bar{x}$ ± S.D.)	C.V. (%)
Mitoxantrone		
Sample A	185 ± 10	5.4
Sample B	1.75 ± 0.12	6.8
Bisantrene		
Sample C	1560 ± 20	1.3
Sample D	114 ± 7.2	6.3

*Each patient sample concentration represents the average of three independent analyses on different days.

The results of dialysis experiments are shown in Fig. 9. Because of the instability of mitoxantrone in plasma, we placed the drug in normal saline for dialysis against plasma. After 24 h only 5% of the initial bisantrene and mitoxantrone concentrations remained in the dialysis chamber containing saline, suggesting that both drugs are highly protein bound. In contrast when both dialysis chambers contained saline, after 24 h 50% of the initial drug concentrations were detected in both chambers, suggesting that there was no significant adsorption of either drug onto the dialysis membrane.

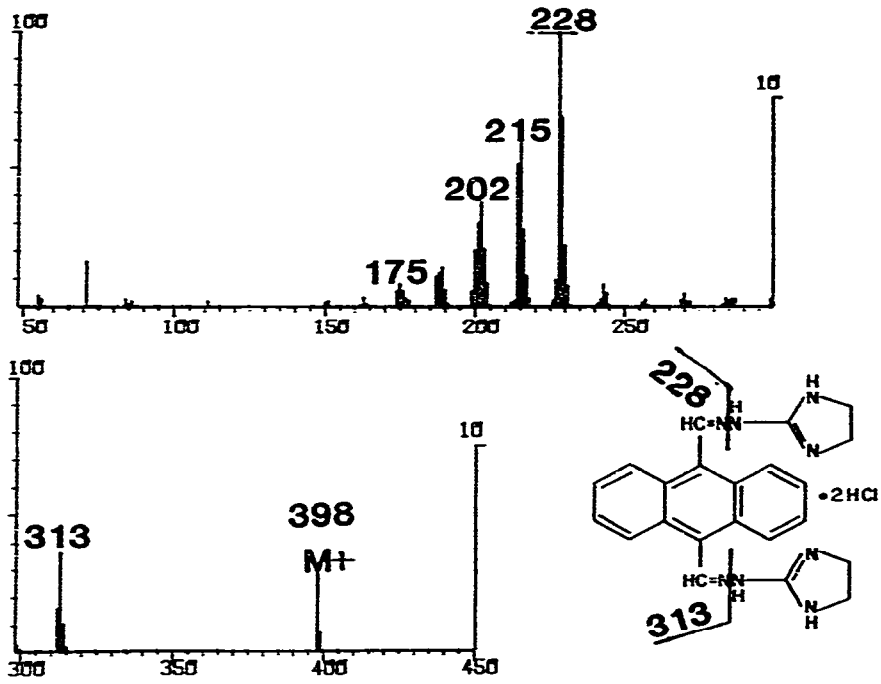


Fig. 4. Complete mass spectra of bisantrene from pure standard.

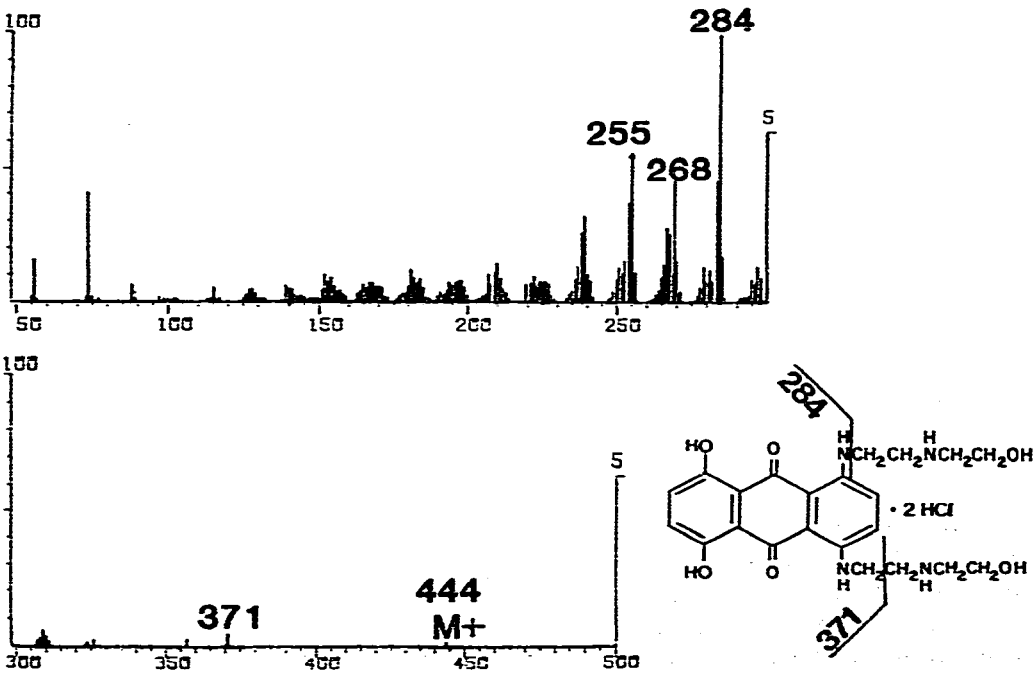


Fig. 5. Complete mass spectra of mitoxantrone from pure standard.

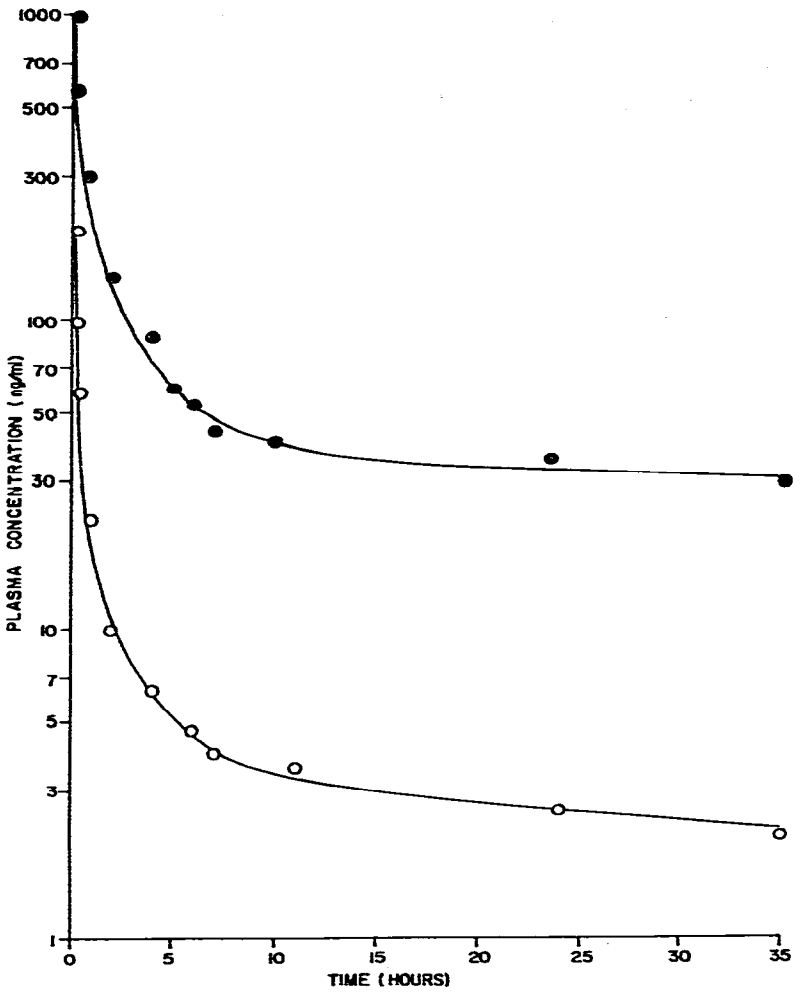


Fig. 6. Plasma disappearance curves of bisantrene (260 mg/m²) (•) and mitoxantrone (12 mg/m²) (○) after an intravenous infusion.

DISCUSSION

In a previous report [12], we used ethyl acetate for the extraction of bisantrene from biological fluids. Liquid extraction often resulted in a large volume of solvent, which had to be evaporated to dryness and reconstituted into a small volume for subsequent HPLC analysis. We found this process to be time consuming. Other published HPLC methods for mitoxantrone also require lengthy sample cleanup procedures, such as the double liquid extraction with chloroform described by Ostroy and Gams [13] or the XAD-2 column isolation described by Reynolds et al. [14].

Using commercially available and relatively inexpensive C_{18} mini-cartridges, we developed a simple, precise and quantitative extraction method for both bisantrene and mitoxantrone. The drug was separated from plasma constituents

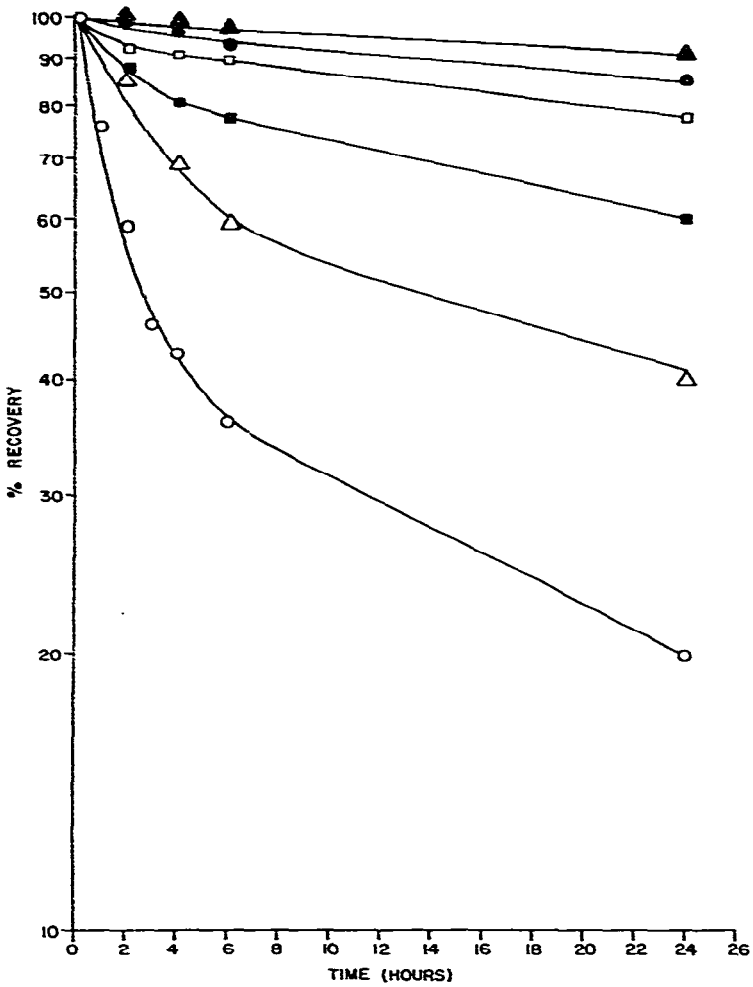


Fig. 7. Recovery of bisantrene (10 μg/ml) and mitoxantrone (10 μg/ml) in human plasma and in the presence of human plasma proteins (5% albumin + 3% γ-globulin) at 25 and 37°C. (▲) Bisantrene in plasma at 25°C, (●) bisantrene in plasma at 37°C, (△) mitoxantrone in plasma at 25°C, (○) mitoxantrone in plasma at 37°C, (□) mitoxantrone in plasma protein at 25°C, (■) mitoxantrone in plasma protein at 37°C.

by its retention on the C_{18} mini-cartridge. One advantage of the mini-cartridge is that it can be loaded with large quantities of plasma. This allows the assay of very low bisantrene and mitoxantrone drug concentrations.

Several solvents were tested for the washing and elution process in an effort to obtain optimal analytical conditions. Washing with distilled water alone resulted in a relatively clean chromatographic background (Figs. 2 and 3). Subsequent washing with acetonitrile, methylene chloride, or ethyl acetate did not improve the chromatography. Washing with methanol or chloroform resulted in poor drug recoveries (i.e., <50%). The 0.5 *M* methanolic HCl is a

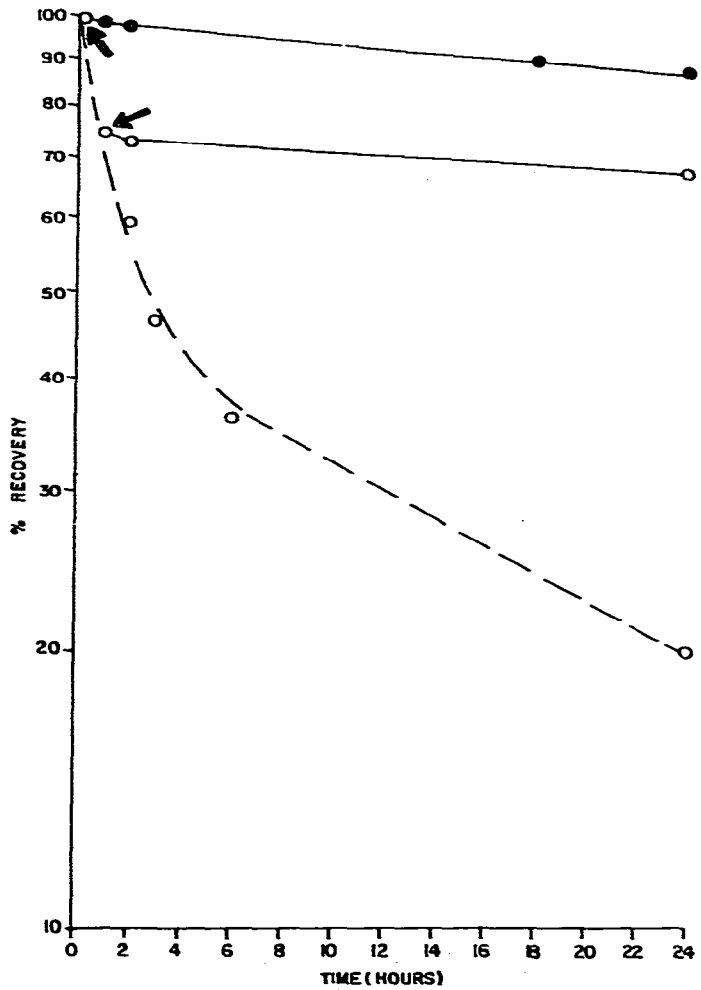


Fig. 8. Effect of ascorbic acid on the recovery of mitoxantrone in plasma at 37°C. To 1 ml of plasma containing mitoxantrone (10 μg/ml), 100 μl of 5% ascorbic acid in citrate buffer (0.1 *M*, pH 3.0) was added at the time as indicated by the arrow point. (o — — — o) Mitoxantrone in plasma without ascorbic acid, (•—•) ascorbic acid was added at time 0, (o—o) ascorbic acid was added at 1 h after incubation.

unique solvent chosen to elute the drug because we were able to accomplish a 98% recovery (Table I) by eluting with only 300 μl methanolic HCl. This is a valuable saving of time and organic solvents as compared to other procedures [12—14] and results in a much better recovery, precision and sensitivity.

Reynolds et al. [14] have described a similar method which utilizes a XAD-2 column for the extraction of mitoxantrone. However, packing the XAD-2 column is tedious, time consuming, and may not be uniform which can lead to variable recoveries and imprecision. Furthermore, the 254-nm detection wavelength used for their assay may not be optimal. Detection in the UV range

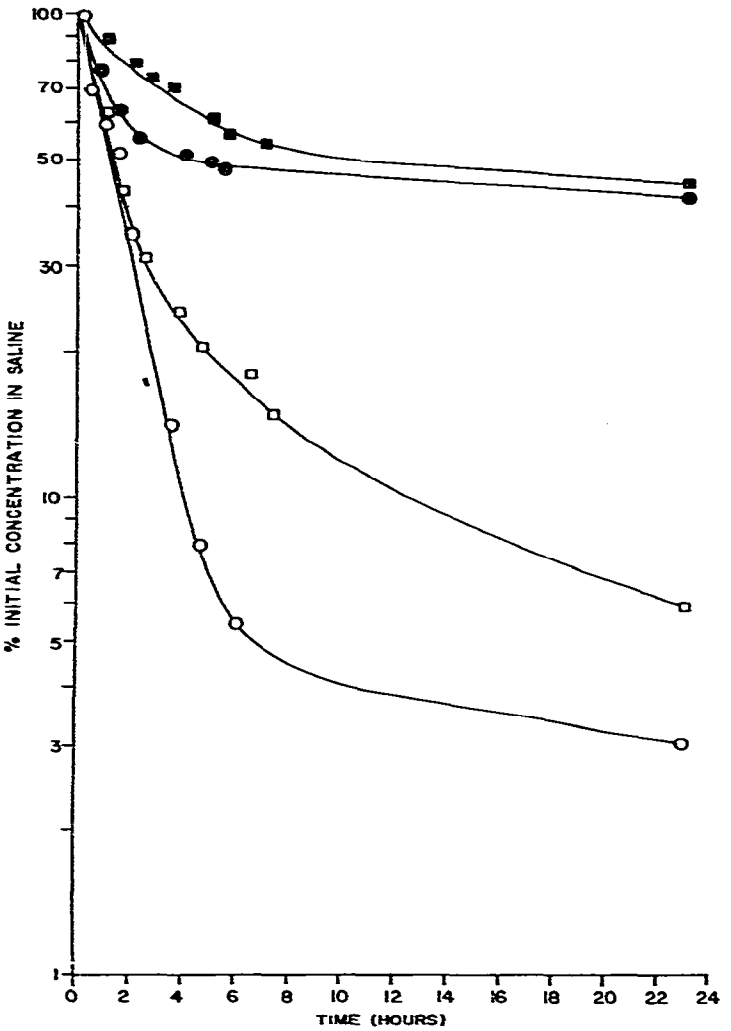


Fig. 9. Equilibrium dialysis of bisantrene and mitoxantrone at room temperature. Bisantrene or mitoxantrone (20 μg/ml) was added to 1 ml of normal saline to dialyze against 1 ml of normal saline or human plasma. Samples from both chambers were taken simultaneously at time intervals and assayed for bisantrene and mitoxantrone. (●) Bisantrene, saline vs. saline; (○) bisantrene, saline vs. plasma; (■) mitoxantrone, saline vs. saline; (□) mitoxantrone, saline vs. plasma.

will usually encounter a great deal of interfering peaks from biological samples. This will hinder the sensitivity of the assay and could lead to misidentification if mass spectrometry is not utilized for peak confirmation. Their reported sensitivity was 75 ng/ml which is not adequate to describe the plasma pharmacokinetics of mitoxantrone.

Mitoxantrone can also be detected at 546 nm as described by Ostroy and Gams [13], but detection at this wavelength is considerably less sensitive than the known λ_{max} of 658 nm. We detected mitoxantrone at 658 nm which has improved detection sensitivity markedly with no interfering peaks when we used the present rapid and precise sample cleanup procedure. The sensitivity

of our assay is below 1 ng/ml which has allowed us to document a prolonged terminal phase plasma half-life for this drug.

Mitoxantrone appears less stable in human plasma than bisantrene. Instability may result from chemical degradation or irreversible interaction of mitoxantrone with plasma proteins. The results of our stability studies of mitoxantrone in human plasma proteins (Fig. 7) suggest that its instability is related to more than just its interaction with plasma proteins. Reynolds et al. [14] have reported a similar instability of mitoxantrone in plasma. They suggested that this instability may result from an oxidative process. We have confirmed that ascorbic acid can stabilize, but not reverse prior loss of mitoxantrone from plasma samples (Fig. 8). Therefore, to maintain sample integrity, all plasma samples should be fortified with ascorbic acid and kept frozen prior to HPLC analyses of mitoxantrone.

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