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ION-PAIRED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MITOXANTRONE IN PHYSIOLOGICAL FLUIDS

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SUMMARY

Mitoxantrone is one of the newer anthracenedione derivatives which has already been studied in phase I and II trials, where it has shown significant antitumour activity against a variety of human tumours. To determine the prolonged terminal half-life of mitoxantrone, we developed a sensitive high-performance liquid chromatographic method, providing a detection limit of 1 ng/ml of extracted serum. This system uses a C_{18} reversed-phase column. The mobile phase consists of a mixture of acetonitrile (30%, v/v) and an ammonium formate buffer (70%, v/v) with a pH of 2.7. Hexane sulphonic acid is added as an ion-pair former. Detection at a wavelength of 658 nm provides a highly selective system, showing no interfering peaks. Ametantrone, another anthracenedione derivative, is used as an internal standard. The extraction procedure for serum also uses hexane sulphonic acid in an ion-paired system. Because of the highly selective detection wavelength, urine samples can be injected without a sample clean-up procedure.

This very sensitive method, combined with high selectivity and a fast and inexpensive serum clean-up procedure, has allowed us to document the prolonged terminal plasma half-life of mitoxantrone (levels of 2—5 ng/ml of plasma can still be detected six days after an

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intravenous infusion of 15 mg/m^2 over 30 min). In urine an as yet unidentified metabolite was discovered.

INTRODUCTION

Mitoxantrone (Fig. 1), 1,4-dihydroxy-5,8-bis{[2-[(2-hydroxyethyl)amino]-ethyl]amino}-9,10-anthracenedione dihydrochloride, or DHAD (NSC-301739), is an anthracenedione derivative which has shown significant antitumour activity in animal models [1—4]. It has also been studied in phase I and II studies and proved to have equal activity in breast cancer to that of doxo-rubicin, but with lower cardiotoxicity [5—11]. Previous reports dealing with the determination of mitoxantrone in plasma have two major disadvantages: either they have poor sensitivity [12—15] or else they lack an internal standard [16].
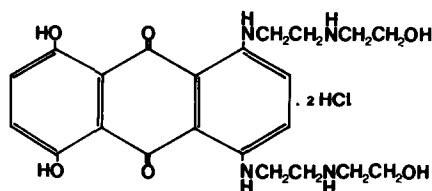

Fig. 1. Structural formula of mitoxantrone.

For those reasons and in order to evaluate the prolonged terminal half-life of this drug we tried to develop a new high-performance liquid chromatographic (HPLC) method with a sensitivity of at least 1 ng/ml of serum and using an internal standard.

MATERIALS AND METHODS

Materials

Mitoxantrone was kindly provided by American Cyanamid (Louvain-la-Neuve, Belgium) and ametantrone by the Drug Synthesis and Chemistry Branch of the National Cancer Institute, Bethesda, MD, U.S.A. (Dr. J.P. Davignon).

All glasswork was siliconized using Glass-treat (5% solution in hexane) from Chrompack (Middelburg, The Netherlands). Formic acid, 25% ammonia and sodium tetraborate · 10H$_2$O were purchased from E. Merck (Amsterdam, The Netherlands) and were analytical grade. Hexane sulphonic acid was obtained from Kodak (Oostdijk, The Netherlands), and *l*-ascorbic acid from Brocacef B.A. Holland (Maarssen, The Netherlands); both were analytical grade. Aceto-nitrile and dichloromethane were purchased from Pharmachemie (Haarlem, The Netherlands) and were HPLC grade.

Human plasma containing citrate—phosphate—dextrose as anticoagulant was kindly provided by the National Blood Bank of The Netherlands (Amster-dam, The Netherlands).

All aqueous solutions were filtered through a 0.22-μm filter (Sartorius SM

11107) and the organic solutions through a 0.22-μm filter (Sartorius SM 11607).

Sample clean-up procedure

Blood samples were collected at time 0, 15, 30, 40, 45, 60, 90 and 120 min after the beginning of the infusion, and afterwards at 4, 8, 12, 24, 36, 48 h up to six days after time 0.

After centrifugation at 1500 *g*, and separation, the plasma was immediately frozen and stored at -20°C.

Plasma samples were warmed up quickly just prior to extraction and mixed on a vortex for 10 sec. One millilitre of a solution containing hexane sulphonic acid (0.01 mg/ml), ascorbic acid (0.5 mg/ml) and ametantrone (1.2 mg/ml) as internal standard was added to 1 ml of plasma. After vortexing for 30 sec, 1 ml of a 0.1 *M* borax buffer (pH 9.5) was added and again vortexed for 30 sec. Extraction was performed with 5 ml of dichloromethane; after centrifugation at 1500 *g*, and separation, the organic layer was dried at 40°C under nitrogen. The dry residue was kept at -20°C until HPLC analysis was performed.

Urine was prepared as follows: 1 ml of frozen urine was brought to room temperature, mixed with 1 ml of an aqueous solution containing the internal standard and 100 μl were directly injected.

HPLC analysis

Plasma or urine was assayed for mitoxantrone by reversed-phase HPLC using an ion-pair system.

The HPLC system employed a Spectra Physics chromatography pump Model 740B or a Waters Assoc. solvent delivery system Model 6000A, and a Waters Assoc. injector Model U6K.

Separation of mitoxantrone, possible metabolites and the internal standard was carried out using a 30 × 0.39 cm μBondapak C_{18} column (Waters Assoc.) with a particle size of 10 μm. A guard column (7 × 0.21 cm) packed with the same material was used to protect the chromatographic column. The packing was changed every two weeks or when necessary. The mobile phase consisted of a mixture of acetonitrile (30%) and 0.16 *M* ammonium formate buffer (70%) pH 2.7.

Hexane sulphonic acid was added in a concentration of 0.025 *M*. The flow-rate was 1.6 ml/min.

Detection at 658 nm was performed either on a Pye Unicam LC-UV type variable-wavelength spectrophotometer or a Waters Assoc. fixed-wavelength detector Model 441. The peaks were recorded on a Hewlett-Packard integrator recorder Model 3380 A.

Quantitation, recovery and precision

Quantitation was done by the internal standard method, using the peak heights as well as the integrated areas under the peak.

Plasma standard curves were established for mitoxantrone in aqueous solution, ametantrone in aqueous solution, mitoxantrone extracted from aqueous solution and from plasma, in absolute values and after plotting against the internal standard level.

Recovery was calculated by comparing the measured levels of spiked serum to those of standard aqueous solutions.

Five samples, spiked with 100, 250, 500 or 1000 ng of mitoxantrone per ml of serum, were assayed.

Three additional samples with concentrations of 5, 10 or 20 ng of mitoxantrone per ml of serum were used to calculate the recovery data in the range close to the detection limit.

Precision and variation were determined by assaying five to ten samples of spiked serum.

All experiments were done in triplicate and repeated on different occasions.

RESULTS

Fig. 2 shows the chromatogram of an aqueous solution containing 250 ng of ametantrone and 300 ng of mitoxantrone, when determined at 254 nm; or 3000 ng of ametantrone and 300 ng of mitoxantrone when assayed at 658 nm. Here there is a ten-fold decrease in peak height for ametantrone when the wavelength is changed from 254 to 658 nm.

A representative chromatogram at 0.005 a.u.f.s. of both drugs extracted from plasma is shown in Fig. 3. This sample of serum contained 250 ng of ametantrone and 5 ng of mitoxantrone per ml. This demonstrates that a sensitivity of 1 ng/ml of extracted serum can easily be reached, knowing that only half of the reconstituted extract is injected.

A blank serum showed no peaks at the position of the internal standard or mitoxantrone. The coefficient of variation, estimated on five samples, varied
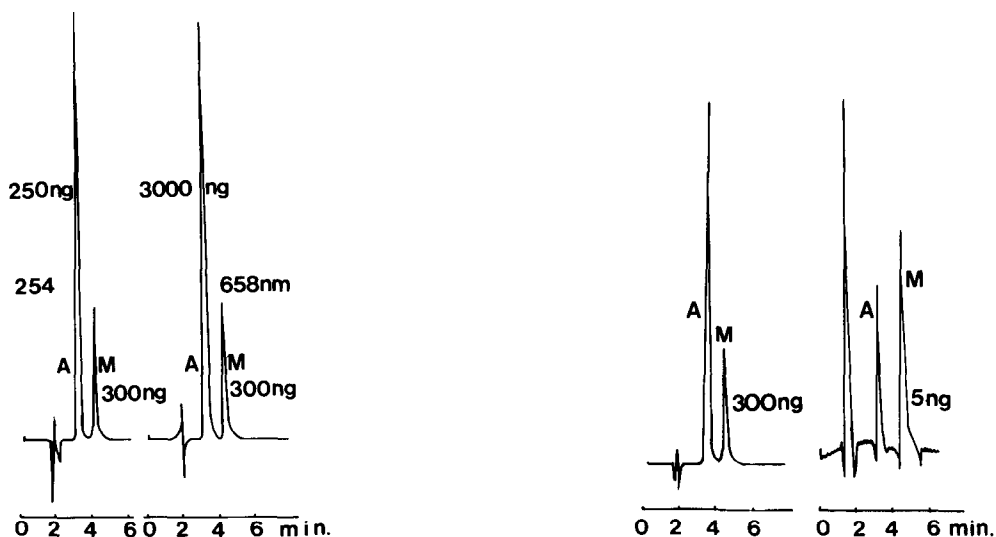

Fig. 2. Chromatograms of mitoxantrone (300 ng) and ametantrone (250 ng) in aqueous solution at 254 nm, and of mitoxantrone (300 ng) and ametantrone (3000 ng) in aqueous solution at 658 nm. Peaks: A = ametantrone; M = mitoxantrone.

Fig. 3. Chromatograms of mitoxantrone and ametantrone at 658 nm, after extraction from serum; serum was spiked with 300 ng or 5 ng of mitoxantrone. Peaks: A = ametantrone; M = mitoxantrone.

TABLE I

COEFFICIENT OF VARIATION FOR EXTRACTED PLASMA SAMPLES

Concentration (ng/ml)	n	C.V. (%)
5	10	11.9
10	10	14.6
20	10	8.5
200	5	2.7
400	5	2.3
1000	5	2.7
2000	5	2.24

from 2.24% for a concentration of 2000 ng of mitoxantrone per ml of extracted serum to 23% for 10 ng mitoxantrone per ml. When five additional samples were assayed for the low concentrations (5, 10 and 20 ng) the respective coefficients of variation were 11.9%, 14.6% and 8.5% (Table I).

The recovery of the sample clean-up was 62 ± 2% at concentrations of 100, 250, 500 or 1000 ng/ml, and 58 ± 7.6% at concentrations of 5, 10 or 20 ng/ml.

Fig. 4 shows the result of the determination of a urine sample. Note the absence of other peaks, except for the one before the internal standard which could represent a still unidentified metabolite. This chromatogram can only be measured at 658 nm; at 254 nm there are a lot of disturbing peaks.

Fig. 5 shows the various standard curves. All correlation coefficients were above 0.9995. Note the difference in slope between curve 4 (absolute values of mitoxantrone after extraction from plasma) and curve 5 (internal standard — corrected values); this is due to the recovery of the sample clean-up of 62%.
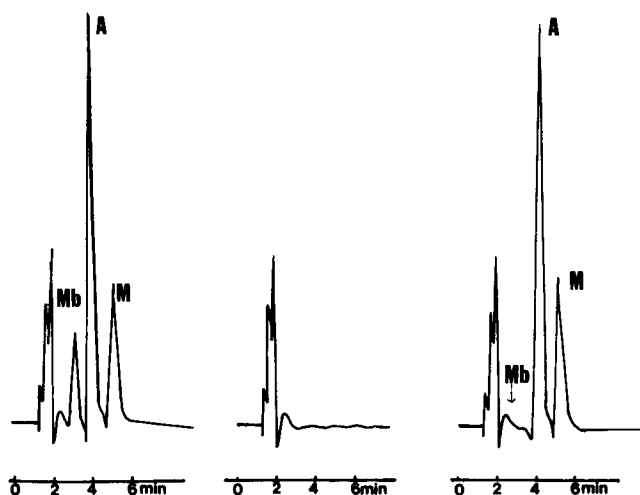

Fig. 4. Chromatograms of urine samples of a patient treated with mitoxantrone (15 mg/m^2 intravenous infusion over 30 min). The left chromatogram is of a sample of urine containing a metabolite of mitoxantrone (Mb), ametantrone (A) as internal standard and unchanged mitoxantrone (M). The centre chromatogram is representative of blank urine, and the right is of spiked urine containing ametantrone and mitoxantrone.
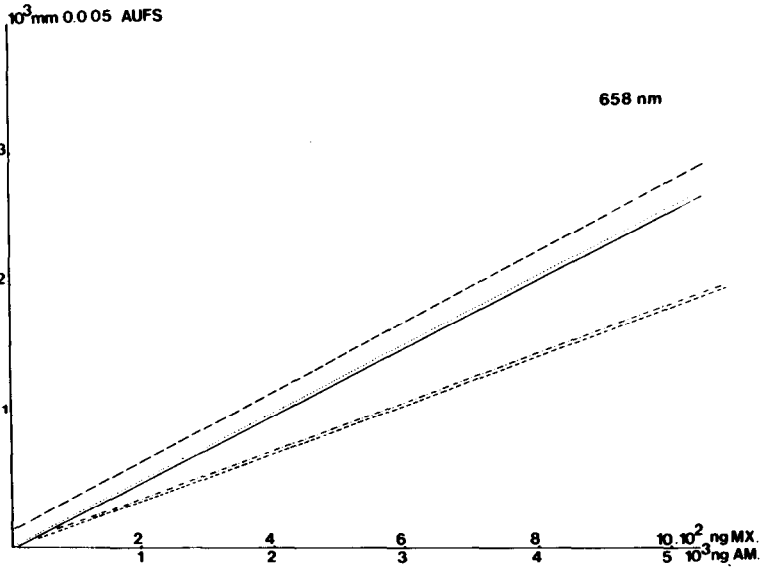
Fig. 5. Standard curves of mitoxantrone (MX) in aqueous solution (———), ametantrone (AM) in aqueous solution (· · · · ·), mitoxantrone extracted from aqueous solution (. . . . .) in absolute values and after plotting against the internal standard, mitoxantrone extracted from plasma in absolute values (— · — · — · —) and after plotting against the internal standard (— — — —).
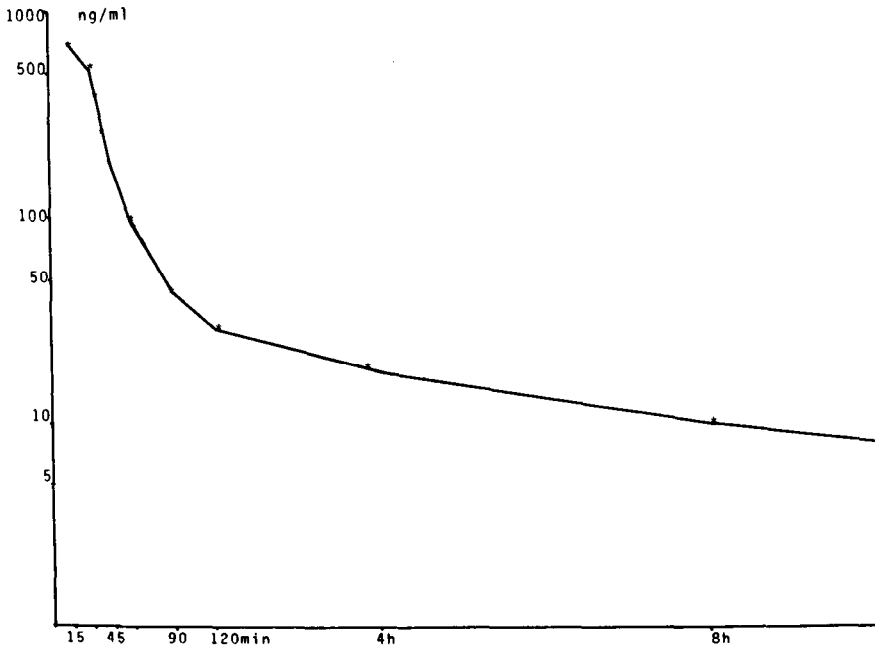

Fig. 6. Representative plasma elimination curve from twenty patients (mean values), treated with 15 mg/m² mitoxantrone, intravenously, over 30 min. Levels of 3—5 ng/ml plasma are still found 48 h to six days after the infusion.

After plotting against the internal standard, the slope of the curve is the same as that representing mitoxantrone in aqueous solution. Fig. 6 represents a typical plasma elimination curve, which shows the mean values of twenty patients treated with an infusion of 15 mg/m^2 mitoxantrone over 30 min. Mitoxantrone concentrations of 2—5 ng/ml of serum can still be detected after six days (not shown in curve).

DISCUSSION

We used ametantrone, another antracenedione derivative, as the internal standard. This method leads to more accuracy than the use of cresyl violet [14], because ametantrone is structurally related to mitoxantrone and the total elution time is less than 5 min. Ostroy and Gams [14] used cresyl violet as internal standard but the elution time is more than 50 min after mitoxantrone. Hulhoven and Desager [15] also used ametantrone as internal standard but their sensitivity level was not below 15 ng/ml. We could determine 1 ng of mitoxantrone per ml of plasma, even without using available electronic methods to magnify the recorded peaks.

On the other hand, Peng et al. [16] reached the same sensitivity level of 1 ng/ml but they used the external standard method which adds considerably to the cost [16]. Their system is based on the same constituents for the mobile phase: acetonitrile and an ammonium buffer. But when using an ammonium formate buffer with a pH of 2.7 instead of an ammonium acetate buffer with a pH of 4.0 we had better results. Reduction of pH from 4.0 to 2.7 gave better peaks, but adding hexane sulphonic acid to the mixture led to a spectacular increase in peak sharpness, and an increase in recovery of 15—20%.

We chose the wavelength of 658 nm to determine mitoxantrone for two major reasons. First, it is a very specific wavelength since commonly taken drugs do not absorb at 658 nm. Secondly, we could use a fixed-wavelength detector since there is a mercury-lamp filter available for the 658 nm wavelength. Other absorption peaks of the mitoxantrone spectrum are 254, 546 and 611 nm. The two cited wavelengths of 546 and 611 nm are somewhat less sensitive and a variable-wavelength detector is necessary to reach them.

The sensitivity at 254 nm and 658 nm is almost equal for mitoxantrone but differs by a factor of ten for ametantrone which has better absorption peaks at 254, 589 and 618 nm. Ascorbic acid was used by Peng et al. [16] to protect against degradation of mitoxantrone in solutions. We had an increase of about 10% in recovery when adding ascorbic acid to the sample before the clean-up procedure. On the other hand, adding it to the fresh sample, before freezing, made no difference, compared to samples directly frozen at —20°C. The whole sample clean-up procedure takes about 30 min.

There were no interfering peaks in the urine chromatogram, so no clean-up was used. The peak preceding the ametantrone peak (Fig. 4) can only be seen in urine from patients treated with mitoxantrone; it was absent in urine spiked with the internal standard or mitoxantrone and in blank urine. For these reasons we think it is a metabolite which we have not yet been able to identify. It must be a substance structurally related to mitoxantrone and ametantrone, since it also absorbs at 658 nm and it is localized near the peaks of these two anthracenedione derivatives.

Possible reasons why other authors until now could not visualize such a metabolite are the lack of a very sensitive HPLC method to separate the substances and possible loss or alteration during sample clean-up procedures.

The cumulative urinary excretion of unchanged mitoxantrone was about 6—12% after 48 h. The possible metabolite was seen only during the first 24 h. Along with the remaining plasma levels this suggests that mitoxantrone is probably stored in a third space from where it is afterwards slowly released. Some data suggest that it could be stored partly in red blood cells [17].

Assay of samples of haemolysed blood showed peak levels six to eight times higher than non-haemolysed samples (unpublished data).

In conclusion, we have developed an HPLC method and sample clean-up procedure based on the property of ion-pair formation by hexane sulphonic acid. It has allowed us to document the prolonged terminal half-life of mitoxantrone in plasma and we could also demonstrate a possible metabolite in urine. The sample clean-up method is inexpensive, fast and reliable. Another advantage is that the same technique can be used to determine the pharmaco-kinetics of ametantrone, using mitoxantrone as internal standard.

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