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DETECTION AND SEPARATION OF MITOXANTRONE* AND ITS METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

GERHARD EHNINGER*, BARBARA PROKSCH and ERDMUTE SCHILLER

Medizinische Universitätsklinik, Otfried-Müller-Strasse 10, D-7400 Tübingen (F.R.G.)

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SUMMARY

A high-performance liquid chromatographic method using ion-pair chromatography on reversed-phase C_{18} material was developed. After sample clean-up on XAD columns, mitoxantrone at concentrations below 1 ng/ml in serum and 0.2 ng/ml in urine were measurable with a coefficient of variation of < 9.3% at a wavelength of 658 nm. Four metabolites were separated in urine. The two major metabolites co-chromatographed with the synthesized mono- and dicarboxylic acid derivatives of mitoxantrone. The method allowed the measurement of mitoxantrone and its metabolites in serum up to more than one week and in urine up to four weeks after administration of the drug

INTRODUCTION

Mitoxantrone, an anthracenedione derivative, has shown cytostatic effects in both animal and in vitro test systems [1, 2]. In clinical studies, antitumour activity has been demonstrated in patients with advanced breast cancer, lymphoma and leukaemia [3—7].

Various sample clean-up procedures and high-performance liquid chromatographic (HPLC) conditions have been used. These methods have limited sensitivity [8, 9], could only measure concentrations shortly after administration of mitoxantrone and could not demonstrate the terminal elimination phase. Our group has previously reported on the extensive metabolism in the isolated perfused rat liver [10, 11] and described metabolites in man [12]. No published HPLC method could detect these metabolites in serum and urine.

*Trade-name · Novantrone.

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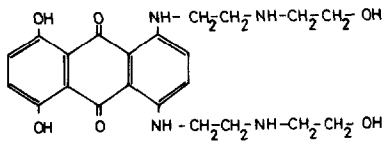
The method described here enables serum levels and urinary excretion of mitoxantrone and its proposed metabolites to be monitored at low concentrations. This may yield information that could elucidate the pharmacokinetics where controversial data with an elimination half-life between 56 min and 42.6 h have been reported [13—17] and could elucidate the metabolism of this first anthracenedione derivative.

EXPERIMENTAL

Mitoxantrone and its mono- and dicarboxylic acid analogues were supplied by Lederle Labs. (Pearl River, NY, U.S.A.). Their structures are shown in Fig. 1.

Water for HPLC use was prepared by filtering doubly distilled water through Norganic cartridges (Millipore, Eschborn, F.R.G.). Acetonitrile, LiChrosorb, L-ascorbic acid, ammonium dihydrogen phosphate, citric acid hydrate, 2-propanol (Uvasol) and methanol (Uvasol) were obtained from Merck (Darmstadt, F.R.G.). 1-Pentanesulphonic acid was obtained from Waters Assoc. (Eschborn, F.R.G.). Disposable Pasteur capillary pipettes (short size, 150 mm) were obtained from WU (Mainz, F.R.G.), quartz-wool (5—30 μm) from Roth (Karlsruhe, F.R.G.) and XAD-2 absorbent, research grade, particle size 0.05—0.1 mm, from Serva (Heidelberg, F.R.G.).

I



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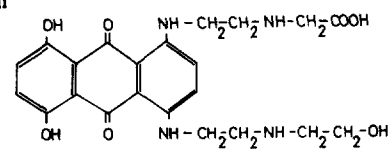

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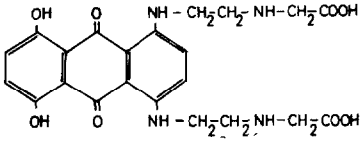

Fig. 1. Structures of mitoxantrone (I) and its mono- (II) and dicarboxylic acid analogues (III).

HPLC apparatus

The chromatographic system consisted of a Waters Assoc. 6000A and an M-45 solvent delivery system, a Model 710A intelligent sample processor

(WISP), a Lambda-Max Model 481 LC spectrophotometer, a Data Module M730 recorder and a Model 720 system controller for mobile phase optimization. Separation was obtained with a Waters Assoc. μBondapak C_{18} column (30 cm × 3.9 mm I.D.; 10 μm particle size). The guard column was packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) and refilled whenever a back-pressure was recorded.

Blood samples were taken before treatment and at 10, 20, 30, 45 and 60 min and 2, 4, 6, 24 and 48 h following the infusion and on subsequent days if the patient was still in hospital. Urine was voided spontaneously. A 250-ml volume of doubly distilled water was added to prevent precipitation.

Chromatographic conditions

The optimum isocratic system for the resolution of mitoxantrone and its metabolites was found to be acetonitrile—water (25:75) containing 5 m*M* 1-pentanesulphonic acid at ambient temperature. The flow-rate was maintained at 1 ml/min. Mitoxantrone and its metabolites were detected at 658 nm at a sensitivity of 0.01 a.u.f.s.

Sample stabilization and clean-up procedure

Disposable columns were prepared according to the method of Reynolds et al. [9]. As a modification we used 150 mg of XAD-2 beads suspended in 2 ml of methanol, which were packed in Pasteur capillary pipettes, containing quartz-wool in the tip. After introducing a second quartz-wool plug at the top, the columns were washed with water and 0.05 *M* phosphate buffer (pH 7.4) and stored in this buffer. Serum was stabilized by the immediate addition of 5% (w/v) of ascorbic acid in 0.1 *M* citrate buffer (pH 3) (9 parts of serum + 1 part of stabilizer) as described by Reynolds et al. [9].

Volumes of 1—10 ml of serum were applied directly to the XAD-2 columns and washed with two 2-ml portions of 0.05 *M* ammonium dihydrogen phosphate solution (pH 2.7). Then the column was washed with 0.2 ml of 2-propanol—0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) (30:70, v/v) and mitoxantrone and its metabolites were eluted with a further 0.4 ml of this solution into glass microvials (Waters No. 9298). A 0.1-ml volume was injected into the HPLC system. Volumes of 10—30 ml of urine were applied directly to the XAD-2 columns within a few hours after voiding, washed with two 2-ml portions of 0.05 *M* ammonium dihydrogen phosphate solution (pH 2.7) and extracted with 1 ml of 2-propanol—0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) (30:70, v/v) into glass microvials and volumes of 0.1 ml were injected into the HPLC system.

Quantitative analysis

Quantitation was effected by the external standard method. Calibration graphs for plasma and urine were obtained by plotting the peak areas (for more than 10 ng) or peak heights (for less than 10 ng) against the known concentration of standards. The reproducibility of the external standard procedure was determined by measurement of different concentrations of mitoxantrone added to the mobile phase ten times. Results for metabolites were expressed in mitoxantrone equivalents.

Recovery, precision and detection limit

The recovery was calculated by comparing the peak height or peak area of the mitoxantrone added to serum and urine with that of the standard added to the mobile phase. Precision and accuracy were determined by assaying duplicate serum samples on twelve different days. The detection limit of the assay was set at a response equal to twice the average noise level.

RESULTS

The calibration graphs were linear with respect to peak height over the range 0.5—50 ng ($r > 0.999$) and with respect to peak area over the range 50—2000 ng ($r > 0.999$). The reproducibility (coefficient of variation, C.V.) of the external standard procedure measured by ten analyses was 0.3% at 100 ng, 1.0% at 20 ng and 1.5% at 10 ng for peak area and 0.8% at 10 ng and 0.9% at 2 ng for peak height.

TABLE I

RECOVERY OF MITOXANTRONE FROM DIFFERENT VOLUMES OF SERUM AND URINE

All experiments were run ten times.

Sample concentration (ng/ml)	Sample volume (ml)	Sample origin	Recovery (%)	C.V. (%)
10	1	Serum	96.7 ± 2.6	2.7
10	10	Serum	96.7 ± 8.3	8.6
200	10	Urine	97.2 ± 1.2	1.2
5	10	Urine	97.5 ± 4.1	4.2
2	10	Urine	96.9 ± 6.2	6.4
0.2	10	Urine	96.3 ± 9.0	9.3

The precision and recovery data are summarized in Table I. The C.V. of the reproducibility at a serum concentration of 200 ng/ml measured by duplicate independent analyses on twelve different days was 5.4%. The limit of sensitivity of the assay was about 1 ng. Volumes of 10 ml or less of serum and urine could be extracted with C.V. values less than 9.3%, as shown in Table I. Therefore, concentrations of 0.2 ng/ml in urine and below 1 ng/ml in serum could be measured. The sensitivity was also increased by injecting 0.39 ml of the eluate from the XAD column. However, this volume of 2-propanol—ammonium dihydrogen phosphate buffer was observed occasionally to disturb the separation characteristics of the reversed-phase C_{18} columns. The recovery of metabolites was checked by comparing the peaks after direct injection of urine containing these metabolites into the HPLC system with those after the usual sample clean-up procedure. No loss of metabolites was observed.

Serum and urine from patients before treatment contained no peaks that occurred at the same positions as those of the compounds of interest. All natural constituents that had an absorption at 658 nm were eluted between

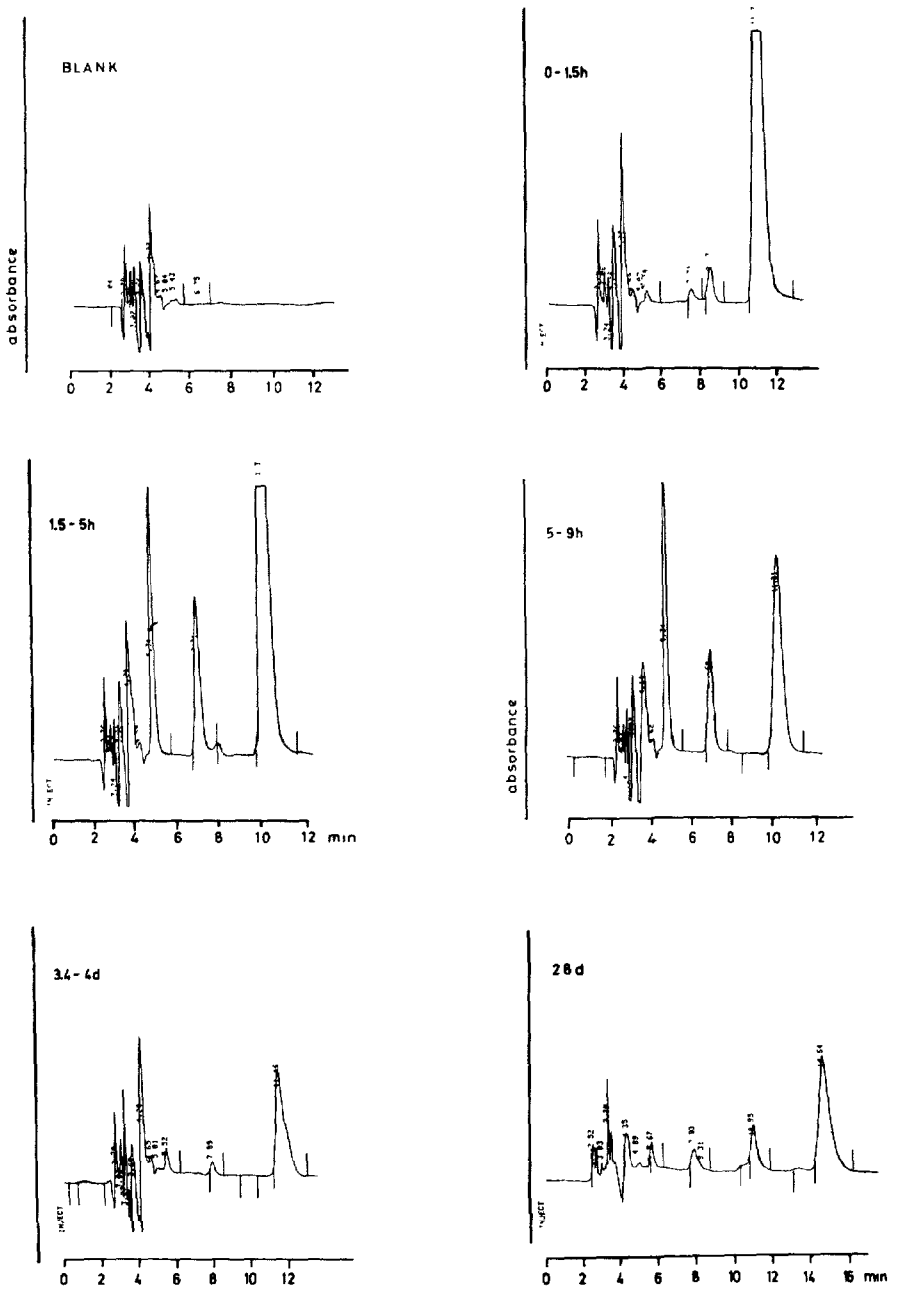

Fig. 2. HPLC profiles of urine samples collected after administration of 14 mg/m^2 mitoxantrone to patient 1, cycle 3. Top, left shows the blank before treatment. The sample 0—1.5 h after treatment shows mitoxantrone as the dominant peak at a retention time (t_R) of 11.31 min. Metabolite 1 (t_R 5.34 min) and metabolite 2 (t_R 7.71 min) were detected in small amounts. Metabolite 3 (t_R 9.73 min) is predominant. The samples from the periods 1.5—5 and 5—9 h after treatment show metabolites 1 and 2 in greater amounts. In the samples 3.4—4 and 28 days after administration of the drug, mitoxantrone and metabolite 1 and 2 are still present but a new peak occurred at t_R 14.64 min.

Fig. 2 shows the chromatographic profile of urine collected at different time intervals and Fig. 3 the profile of serum withdrawn before and after administration of mitoxantrone. The mono- and dicarboxylic acid analogues of mitoxantrone co-chromatographed in spiked urine together with metabolites 2 and 1, respectively. The metabolites were purified by preparative HPLC. All metabolites were blue, except the material that eluted with a retention time of 14.64 min, which was reddish brown (Table II). A computer-fitted curve of serum concentrations and urinary excretion is shown in Fig. 4.

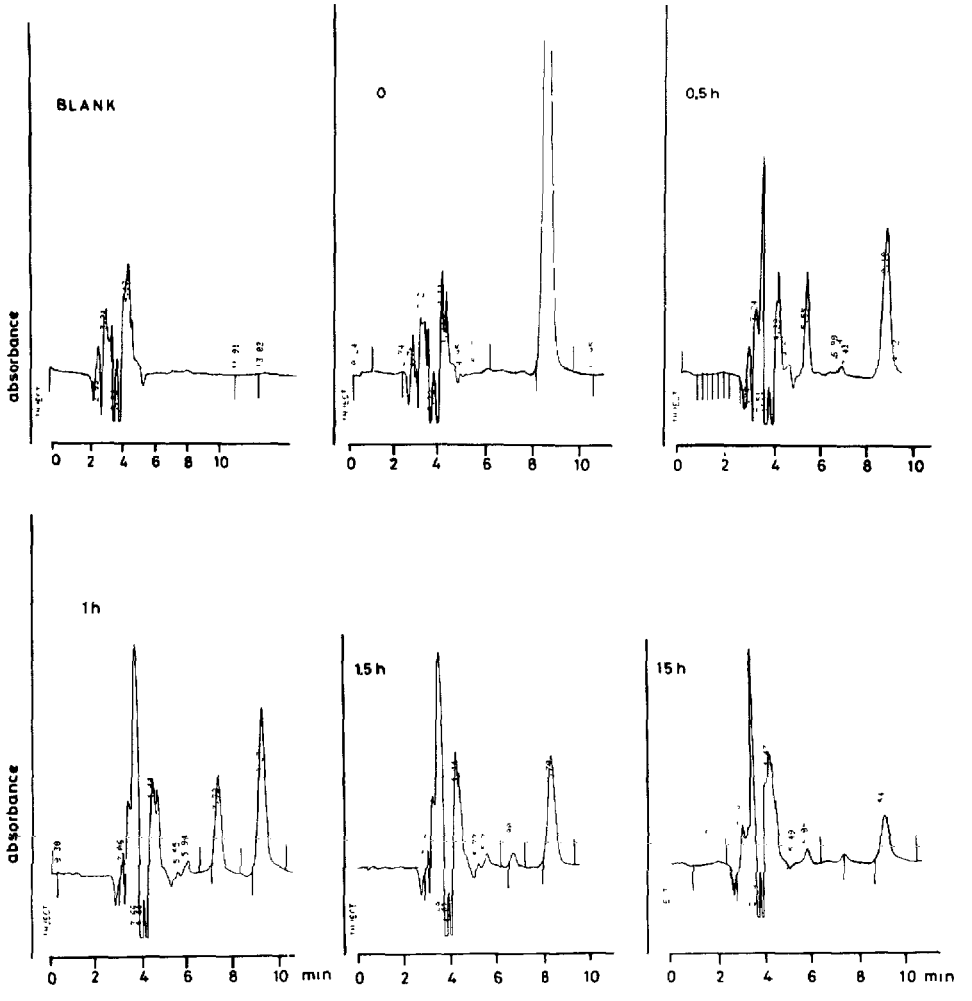

Fig. 3. HPLC profiles of serum samples (cycle 2) at different times after mitoxantrone infusion. Top left shows the blank before treatment. The sample at 0 h shows mitoxantrone at t_R 8.85 min, that at 0.5 h two metabolites at t_R 5.55 and 7.41 min, and mitoxantrone at 9.18 min. For the sample 1 h after treatment mitoxantrone at t_R 9.27 min and metabolite 2 at t_R 7.32 min are predominant, with metabolite 1 at t_R 5.94 min. In the samples 1.5 and 15 h after treatment mitoxantrone and two other peaks are still present.

TABLE II

RETENTION TIMES (t_R) WITH THE DESCRIBED HPLC SYSTEM AND COLOURS OF MITOXANTRONE, THE DICARBOXYLIC ACID ANALOGUE (METABOLITE 1), METABOLITE 2, METABOLITE 3 AND SUBSTANCE 4

Substance	Colour	t_R (min)
Mitoxantrone	Blue	11.3
Dicarboxylic acid analogue (= metabolite 1?)	Blue	5.4
Monocarboxylic acid analogue (= metabolite 2?)	Blue	7.4
Metabolite 3	Blue	8.9
Substance 4	Reddish brown	14.6

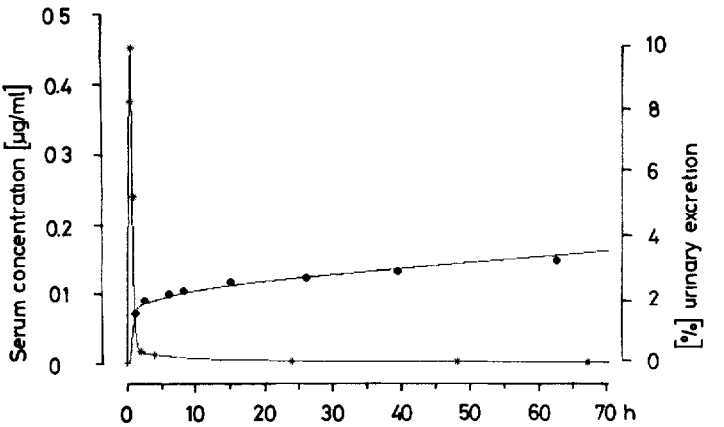

Fig. 4. Computer-fitted curves for mitoxantrone serum (*) and urinary (•) concentrations (percentage dose versus time) for patient 1, cycle 2.

DISCUSSION

The above methods allowed us to measure mitoxantrone and its metabolites in serum up to more than one week and in urine up to four weeks after administration of the drug. A concentration step involving pre-elution of the XAD columns, the ability to extract up to 30 ml of sample and changing the wavelength to 658 nm allowed us to measure these low drug concentrations.

A high peak resolution was achieved by ion-pair chromatography with 1-pentanesulphonic acid. In the isocratic system described by Peng et al. [18], the retention time of mitoxantrone was too short and the metabolites were not separated from impurities owing to their different polarities. Double liquid extraction with chloroform as reported by Ostroy and Gams [8] also resulted in a loss of metabolites.

Two of the four separated peaks have retention times identical with those of the mono- (metabolite 2) and dicarboxylic (metabolite 1) acid derivatives of mitoxantrone. These materials have been described by Chiccarelli and co-workers [19, 20] as metabolites isolated from the urine of leukaemia patients and dogs and the bile of rats. These structural analyses carried out by negative-ion chemical ionization mass spectrometry should still be verified by nuclear magnetic resonance (NMR) examination. Until NMR spectra are available, one

should consider these substances to be proposed metabolites. The additional two metabolites (3 and 4) have not previously been reported in man. Their chemical structural analysis is in progress.

By use of the proposed method, serum concentration curves and urinary excretion after administration of mitoxantrone were measured in several patients and fitted by use of TOPFIT [21, 22]. The estimated half-life was 214.8 h, which corresponds well with data from animal studies where the terminal half-life of ^{14}C-labelled mitoxantrone was estimated to be 8.5 ± 3.7 days [23]. Previously reported [13—17] terminal half-life values have been much shorter (56 min to 42.6 h), and probably reflect the limitations of the assay sensitivity rather than the "true" terminal half-life.

Alberts et al. [13] reported that ^{14}C-labelled mitoxantrone-related material was present in relatively high concentrations in several tissues taken at autopsy 35 days after administration of the drug. These results suggest a "deep" tissue compartment with slow release, which is consistent with a long terminal half-life.

The described method offers a practical approach for an investigation of the pharmacokinetics and the metabolic pattern of mitoxantrone whilst being effective in separating mitoxantrone and its metabolites and being very sensitive.

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