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Short Communication

Quantification of mitoxantrone in bone marrow by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

An analytical method for the determination of mitoxantrone in bone marrow was developed using high-performance liquid chromatography with electrochemical detection. The extraction procedure was optimized by investigating several factors which potentially could influence the recovery of mitoxantrone from bone marrow cells. The mean recovery of mitoxantrone from rat bone marrow was found to be 81.7% with a coefficient of variation 3.8%. High-performance liquid chromatography was carried out to quantitate mitoxantrone using ametantrone as internal standard. The detection limit of our analytical method amounts to 100 pg on-column, corresponding to 1 ng/ml of cell suspension containing $2 \cdot 10^7$ cells and a day-to-day variation of maximally 8%. Storage of bone marrow samples, containing mitoxantrone, for one to fourteen days resulted in a mean recovery of 94%, as compared to freshly analysed samples. Subsequently we studied the pharmacokinetics of mitoxantrone in rat bone marrow. It appeared that after an intravenous bolus injection of mitoxantrone (2.5 mg/kg) in rats, the drug accumulated in the femoral bone marrow for about four days, and thereafter gradually declined.

INTRODUCTION

Mitoxantrone (Novantrone®; 1,4-dihydroxy-5,8-bis({2-[(2-hydroxyethyl)-amino]ethyl}amino)-9,10-anthracenedione dihydrochloride) is an anti-cancer drug with activity against breast cancer [1] and acute non-lymphocytic leukemia [2]. The pharmacokinetics of mitoxantrone in bone marrow compartments has not been studied so far, in spite of the facts that the bone marrow is the target organ in cases of hematological malignancy and that the major dose-limiting toxicity of mitoxantrone is reported to be hematological [1,3].

Several high-performance liquid chromatographic (HPLC) methods have been described for the determination of mitoxantrone in biological fluids and tissues [4–8], but no method is available yet for the determination of mitoxan-

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trone in bone marrow. This paper describes an HPLC method for the quantitative determination of mitoxantrone in bone marrow. The method was used to study the *in vivo* pharmacokinetics of mitoxantrone in rat bone marrow.

EXPERIMENTAL

Chemicals

Mitoxantrone (Novantrone) and ametantrone were obtained from Lederle Nederland (Etten-Leur, The Netherlands). Formic acid, dichloromethane, ammonia (25%) and sodium chloride, all of analytical grade, were purchased from Merck (Amsterdam, The Netherlands). Ethylenediaminetetraacetic acid (disodium salt) was obtained from BDH (Poole, U.K.). 1-Hexanesulphonic acid (sodium salt) was obtained from Kodak (Oostdijk, The Netherlands), and acetonitrile from Baker (Deventer, The Netherlands); both were of HPLC quality. Water was made of HPLC quality by a Milli-Q™ (Millipore, Etten-Leur, The Netherlands) water purification system.

Animals

Twelve-week-old barrier-derived male Brown Norway (BN/BiRij) rats, weighting *ca.* 250 g, were used in the experiments.

HPLC system

The HPLC system consisted of a Waters Model 6000A pump (Waters Assoc., Etten-Leur, The Netherlands) and a Rheodyne 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) with a 100-μl sample loop. Separation of mitoxantrone and the internal standard (ametantrone) was performed using a Waters μBondapak C_{18} (30 cm × 0.39 cm I.D.; particle size 10 μm) analytical column. Detection was carried out with an electrochemical detector (AMOR/Spark, Emmen, The Netherlands) at an applied potential of +0.75 V between the glassy carbon electrode and the Ag/AgCl electrode. Peak recording and integration was performed using a Hewlett Packard 3390A integrator.

The mobile phase was a modification of the one described by Van Belle *et al.* [4], and contained 0.16 *M* ammonium formate buffer (pH 2.7)–acetonitrile (70:30) supplemented with 25 m*M* hexanesulphonic acid, 2 m*M* sodium chloride, and 1.34 m*M* ethylenediaminetetraacetic acid. The flow-rate was 1.6 ml/min. Sodium chloride was added to give the Ag/AgCl (reference) electrode a constant potential.

Quantification of mitoxantrone in rat bone marrow cells was achieved using the internal standard method.

Preparation of rat bone marrow suspensions

To obtain a bone marrow suspension, a rat femur was cut into two parts and each part was flushed with 1 ml of ice-cold physiological saline in a plastic (poly-

styrene) container. The resulting suspension was filtered through a Nylon sieve, and the cells were counted.

Extraction procedure for mitoxantrone from bone marrow

The final extraction procedure after optimization (see *Recovery*) was as follows. A 1-ml volume of a bone marrow suspension containing $2 \cdot 10^7$ nucleated cells was spiked with 50 ng of internal standard (ametantrone). After addition of 1 ml of 0.1 *M* borate buffer (pH 10.0), extraction was performed with 5 ml of dichloromethane in a borosilicate glass tube. To separate the organic and the aqueous phase the samples were centrifugated for 15 min at 1000 *g*. The total dichloromethane phase was collected by carefully pipetting this phase from below the aqueous phase, and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dry residue was reconstituted in 1 ml of mobile phase, and 100 μl of this solution were injected into the HPLC system.

Recovery

An optimal extraction procedure was obtained by investigating the influence on the recovery of the pH, the number of cells during extraction and the volume of the cell suspension.

The recovery was calculated by comparing peak heights of mitoxantrone or ametantrone extracted from bone marrow with peak heights of equivalent mitoxantrone or ametantrone concentrations injected directly into the HPLC system.

To study the influence of the pH on the recovery, 1 ml of bone marrow ($2 \cdot 10^7$ cells) spiked with 50 ng of mitoxantrone and 50 ng of ametantrone was extracted at pH 8.5, 9.0, 9.5, 10.0, 10.5, 11.0 and 12.0. The recovery was also examined when the extraction was performed at pH 10.0 with different amounts of cells (0.5, 1.0, 2.0, 3.0, and $4.0 \cdot 10^7$ cells) and with different volumes of cell suspensions (0.5, 1.0, 2.0, 3.0 ml) in the presence of 50 ng of mitoxantrone and 50 ng of ametantrone.

Using the final extraction procedure established after optimization, the recovery was subsequently examined by the extraction of different mitoxantrone concentrations (1000, 100, 10 and 1 ng) from $2 \cdot 10^7$ bone marrow cells.

Stability

The stability of mitoxantrone in bone marrow suspensions was studied. Bone marrow suspensions (1 ml, $2 \cdot 10^7$ cells) spiked with 50 ng of mitoxantrone were stored at -20°C for one to fourteen days. After a certain storage period the samples were thawed, and the mitoxantrone concentration was assayed and compared with directly assayed samples.

RESULTS AND DISCUSSION

Electrochemical detection

The optimal potential between the glassy carbon and the Ag/AgCl electrodes for the detection of mitoxantrone and ametantrone (with the electrochemical detector) was determined by making voltammograms. Fig. 1 shows the voltammograms for mitoxantrone, ametantrone (both 0.5 μg/ml) and the background current from mobile phase constituents. The optimal potential for the detection of mitoxantrone and ametantrone was found to be + 0.75 V.

Using the electrochemical detector we found no evidence for a decrease in time of the activity of the glassy carbon working electrode, owing to adsorption of oxidation products. Probably the clean samples and regular cleaning of the glassy carbon electrode prevented a gradual decrease in response.

We found electrochemical detection to be ten times more sensitive than UV–VIS detection at 658 nm (data not shown). This is in agreement with the results that Choi *et al.* [5] reported for the detection of mitoxantrone in plasma. Another advantage of electrochemical detection is the almost equal responses of mitoxantrone and ametantrone. With UV–VIS detection at 658 nm, their different responses necessitate high ametantrone concentrations [4].

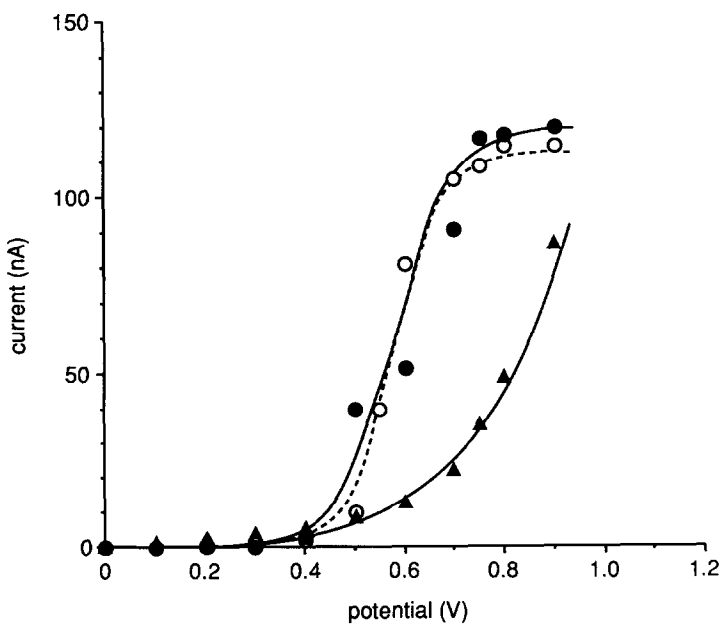

Fig. 1. Voltammograms of mitoxantrone (●), ametantrone (○) and the background current from mobile phase constituents (▲).

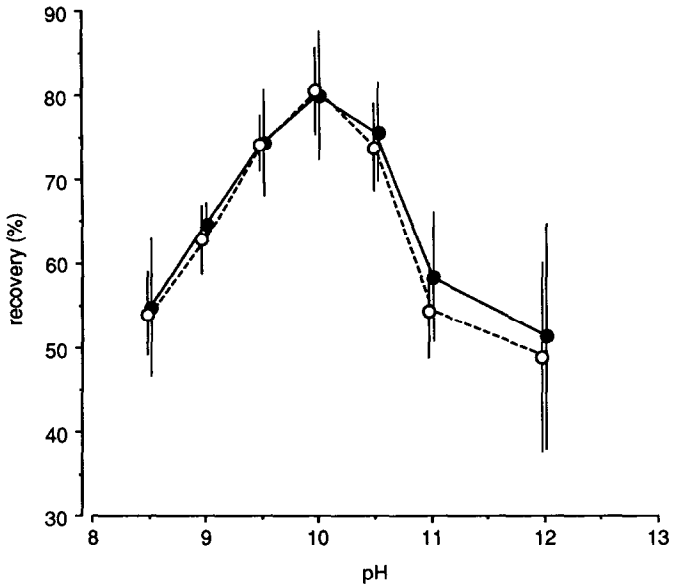

Fig. 2. Influence of the pH of the aqueous phase during extraction on the recovery of mitoxantrone (●) and ametantrone (○). Means ± S.D. of three experiments are plotted.

Recovery and precision

Several factors that can potentially influence the recovery were studied to optimize the extraction procedure for quantification of mitoxantrone in rat bone marrow. Fig. 2 shows the influence of the pH of the aqueous phase during extraction on the recovery of mitoxantrone and ametantrone. It was found that mitoxantrone and ametantrone were maximally extracted at pH 10.0. The influence of the number of cells to be extracted and the volume of the cell suspension on the recovery were also studied. The results are shown in Fig. 3, and the data indicate a minimal effect of both factors on the recovery. To eliminate these minor differ-

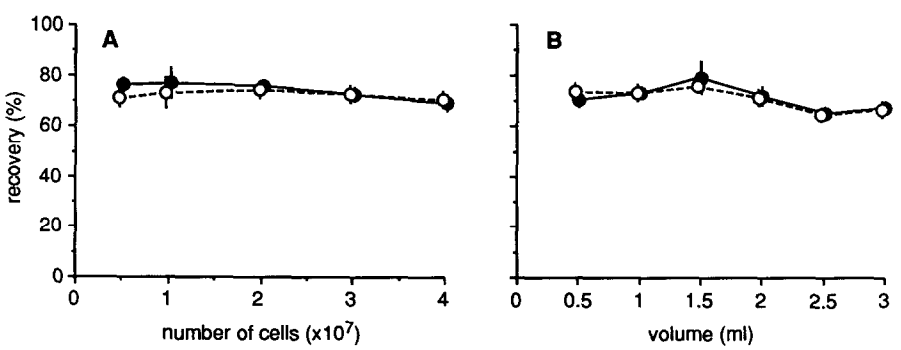

Fig. 3. Influence of the number of cells (A) and of the volume of the cell suspension (B) during extraction on the recovery of mitoxantrone (●) and ametantrone (○). Means ± S.D. of three experiments are plotted.

ences in recovery a fixed number of cells and a fixed volume of cell suspension were used ($2 \cdot 10^7$ cells and 1.0 ml, respectively) in further experiments. Fig. 3B also shows that the recovery is independent of the phase ratio. This can be explained by an almost complete transfer of mitoxantrone and ametantrone into the organic phase at all phase ratios, in combination with a non-specific loss of mitoxantrone and ametantrone of *ca.* 20% during the sample clean-up procedure.

The recovery of different concentrations of mitoxantrone extracted from rat bone marrow cells was assessed. The recoveries were 82.9, 80.7, 82.8 and 80.4% at concentrations of 1000, 100, 10 and 1 ng of mitoxantrone per $2 \cdot 10^7$ rat bone marrow cells, respectively. The mean recovery in the concentration range studied was 81.7%, with a coefficient of variation of 3.8%.

The day-to-day variation of the analytical method was assayed by five-fold analysis of bone marrow samples spiked with 100, 75, 50, 25, 12.5 and 6.25 ng of mitoxantrone on five different days, and was found to be 0.6, 0.5, 2.0, 1.8, 2.1 and 8.1%, respectively.

In conclusion, extraction at pH 10 of a fixed number of cells and a fixed volume ($2 \cdot 10^7$ cells and 1 ml, respectively) resulted in a precise and quantitative extraction procedure, with a detection limit of 1 ng/ml cell suspension containing $2 \cdot 10^7$ cells and a maximum day-to-day variation of *ca.* 8%.

Chromatograms

Fig. 4 shows typical chromatograms from blank rat bone marrow (Fig. 4A) and bone marrow of a rat after intravenous treatment with 2.5 mg/kg mitoxantrone (Fig. 4B). The retention times for ametantrone and mitoxantrone are 3.64 and 4.99 min, respectively.

Quantification

Quantification of mitoxantrone in unknown rat bone marrow samples was done by the internal standard method. The peak-height ratios of mitoxantrone and ametantrone (internal standard) were determined, and the concentration of mitoxantrone was calculated from a calibration curve. The curve was made by plotting the peak-height ratios of mitoxantrone and ametantrone against known concentrations of mitoxantrone (range 6.25–100 ng/ml of cell suspension) in the calibration samples. The correlation coefficients of these curves following least-squares regression were always better than 0.998.

Stability

To investigate whether or not it is allowed to store bone marrow samples at −20°C for analysis at a later time, stability studies were performed. Concerning the stability of mitoxantrone in bone marrow samples, we found recoveries of 94.2, 92.8, 95.5 and 94.2% after storage at −20°C for one, two, seven and fourteen days, respectively. The mean recovery of mitoxantrone over the whole storage period was 94.2%.

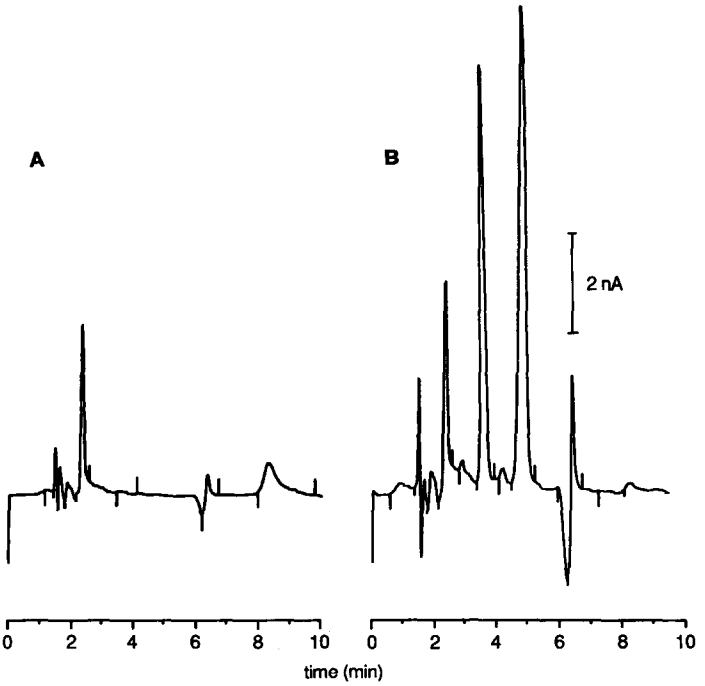

Fig. 4. Chromatograms of a blank rat bone marrow sample (A) and a spiked (ametantrone) bone marrow sample obtained from a rat treated with mitoxantrone (25 mg/kg, intravenously). Ametantrone is eluted at 3.64 min and mitoxantrone at 4.99 min (B). The mitoxantrone peak represents 5 ng absolutely.

Thus, bone marrow samples can be stored for at least fourteen days at −20°C without significant loss of mitoxantrone.

Animal experimentation

Rats were treated with an intravenous bolus injection of 2.5 mg/kg mitoxantrone. The rats were killed at various times after the injection, and the concentration of mitoxantrone in bone marrow was assayed using the method described. Fig. 5 shows a concentration–time curve of mitoxantrone in rat bone marrow: the concentration is expressed as μg per 10^9 nucleated bone marrow cells, because drug concentrations in tissues are generally expressed as μg per gram wet weight tissue and 10^9 nucleated bone marrow cells are equal to *ca.* 1 g of wet weight tissue. It was found that mitoxantrone accumulates in the bone marrow for *ca.* four days after which there is a decline, although mitoxantrone is still detectable in the bone marrow eighteen days after the injection. It is our experience that in the bone marrow such a long drug exposure period is not observed for comparable intercalating drugs, such as anthracycline antibiotics [9]. For example, 48 h after an intravenous bolus dose of daunorubicin (7.5 mg/kg) in rats, no drug is detectable in the femoral bone marrow.

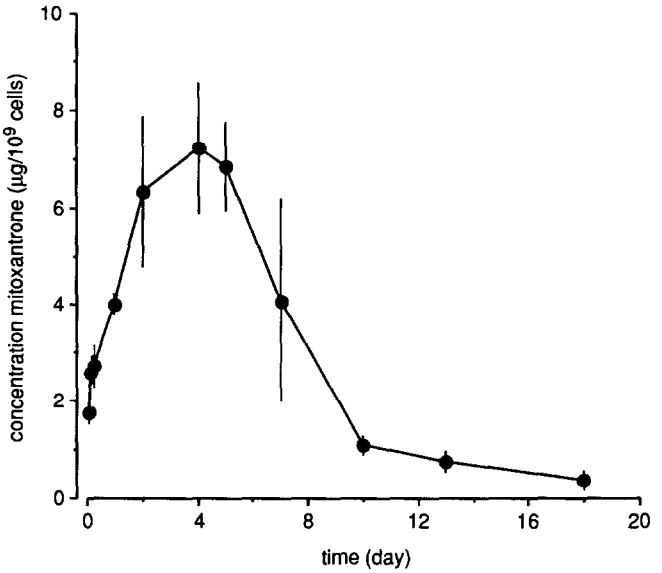

Fig. 5. Bone marrow concentration–time curve of mitoxantrone in rats after an intravenous injection of 2.5 mg/kg mitoxantrone. Means ± S.D. of four rats are plotted.

It is a clinical observation that mitoxantrone is far more myelosuppressive than, for example, daunorubicin or doxorubicin, and the observed persistence of mitoxantrone in the femoral bone marrow might contribute to the observed difference in myelotoxicity.

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