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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR MITOXANTRONE IN PLASMA USING ELECTROCHEMICAL DETECTION

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SUMMARY

A sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the quantitation of mitoxantrone in plasma using electrochemical detection. Bisantrene was chosen as the internal standard. A reversed-phase, 10-μm μBondapak C_{18} analytical column (30 cm × 3.9 mm) with an isocratic mobile phase of 28% acetonitrile in 80 m*M* sodium formate buffer (pH 3.0) was used. The eluent was monitored by both electrochemical detection at an applied potential of +0.75 V vs. Ag/AgCl and visible absorbance at 660 nm. Only electrochemical detection was able to quantitate the internal standard and provided ten times higher sensitivity than visible absorbance for mitoxantrone with a detection limit as low as 0.1 ng/ml. Calibration curves in the range 0.1–1000 ng/ml showed good linearity ($r = 0.998$) and precision (coefficient of variation < 10%). This HPLC method utilized a reproducible and inexpensive liquid–liquid extraction procedure. Using methylene chloride, the extraction efficacy of mitoxantrone from plasma was 85.3% with a coefficient of variation less than 2.1%. This new assay was then applied to measure mitoxantrone concentrations in plasma obtained from two leukemic patients receiving 12 mg/m^2 mitoxantrone as a 1-h infusion.

INTRODUCTION

Mitoxantrone (NSC 301739 or Novantrone™) and bisantrene (NSC 337766 or CL 216942) are new anthracene derivatives which have shown significant antitumor activity in preclinical studies [1–3] and clinical trials [4–7]. Pre-clinical and clinical phase I–II testing of mitoxantrone has shown a spectrum of antitumor activity similar to doxorubicin and a more favorable toxicity spectrum. Early studies with bisantrene showed clinical effectiveness but problems with intravenous drug precipitation have compromised further study of this compound [8].

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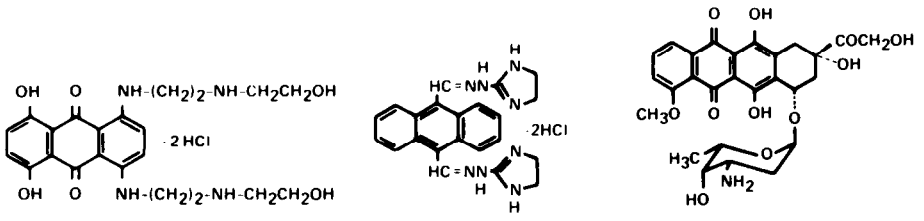

Fig. 1. Chemical structures of mitoxantrone, bisantrene (internal standard) and doxorubicin.

Mitoxantrone, like doxorubicin, inhibits the synthesis of DNA, RNA and protein but is believed to kill cells primarily by intercalation with DNA [4,9]. Structural comparisons of mitoxantrone, bisantrene and doxorubicin are illustrated in Fig. 1.

A prerequisite for the performance of pharmacologic studies with new, investigational anticancer drugs is the development of very sensitive, specific and precise methods for the quantitation of the drug and its metabolites in biological fluids. In previous reports pertaining to the quantitation of mitoxantrone in preclinical and clinical pharmacokinetic studies, the detection limit of the published methods has varied from 1 to 75 ng/ml using reversed-phase high-performance liquid chromatography (HPLC) with either UV–visible or electrochemical detection [10–14]. Only a few of the published methods have utilized an internal standard method which is more precise when liquid–liquid extraction of the drug from biological matrices is involved. We have chosen bisantrene as the internal standard because it possesses similar electrochemical behavior to mitoxantrone [15]. In this paper, we report on the development of an HPLC assay method with electrochemical detection (ED) for the determination of mitoxantrone concentrations in plasma and pharmaceutical solutions.

EXPERIMENTAL

Materials and reagents

Mitoxantrone as the dihydrochloride salt and bisantrene were gifts from Lederle Labs., American Cyanamid (Pearl River, NY, U.S.A.). Reagents and HPLC-grade solvents were purchased from Sigma (St. Louis, MO, U.S.A.). All water used for HPLC was deionized, distilled and filtered with a Milli-Q™ water purification system (Milford, MA, U.S.A.).

Extraction of biological fluids

Exactly 1.0 ml of plasma was spiked with 50 μl of a 100 μg/ml stock solution of bisantrene. Samples were then alkalinized to pH 11 with 50 μl of 1 *M* sodium hydroxide solution, and the drugs extracted into 5.0 ml of methylene chloride in 100×16 mm borosilicate glass tubes. The extract was evaporated to dryness under a stream of nitrogen at room temperature (22–23°C). The residue was reconstituted with 250 μl of mobile phase, centrifuged at 15 600 *g* for 1 min in a Model

5414 Eppendorf microcentrifuge prior to transfer to autosampler vials and HPLC injection (injection volume, 150 μl).

Chromatographic conditions

The chromatographic system was composed of a Waters Model 510 pump, a Model 710B WISP sample processor, a Model 481 variable-wavelength detector set at 660 nm (one of the λ_{max} of mitoxantrone) connected in series with a BAS LC-4B electrochemical detector with a Model TL-5 glassy-carbon electrode at an applied potential of +0.75 V vs. Ag/AgCl reference electrode. Waters Expert™ HPLC software, System Interface Module (SIM), a Digital Professional 350 computer and a Model LA-50 printer were used for methods development, data reduction, data storage and hard copy print-outs.

An isocratic mobile phase of 28% (v/v) acetonitrile in 80 m*M* sodium formate buffer solution (pH 3.0) was used. The flow-rate was maintained at 1.0 ml/min through a 10-μm μBondapak™ reversed-phase C_{18} analytical column (30 cm × 3.9 mm) and guard column packed with C_{18}/Corasil 37–50 μm (from Millipore/Waters, Milford, MA, U.S.A.).

The concentration of mitoxantrone was computed by comparing the mean mitoxantrone/bisantrene peak-area ratio of duplicate or triplicate injections (injection volume, 150 μl) to a calibration curve equation calculated by linear least-squares regression. Calibration curves were analyzed in the range 0.1–1000 ng/ml. Only ED was able to provide a bisantrene detector response because bisantrene does not absorb light at 660 nm; therefore, the external standard method was used when determining concentrations with the variable-wavelength UV detector.

Extraction efficiency

The extraction efficiency was calculated by comparing the mitoxantrone area and mitoxantrone/bisantrene area ratio in plasma to equivalent amounts injected directly onto the HPLC system. The efficiency at four different plasma concentrations (1.0, 10, 100 and 1000 ng/ml mitoxantrone) was assessed.

Assay validation

Ten extractions and injections of the 50 ng/ml calibrator were made in a single day for the determination of intra-assay precision. To assess the inter-assay precision, duplicate injections of two calibrators (500 and 5 ng/ml) were performed daily for five consecutive days. On two separate days, calibrators of 1000 and 0.1 ng/ml were analyzed in triplicate for assessment of high/low precision. Accuracy was determined by triplicate analysis of five different operator-blinded concentrations (0.1, 5, 50, 500 and 1000 ng/ml).

Stability studies in plasma and buffered solutions

Because of the reported instability of the drug in biological fluids [10], the stability of mitoxantrone in plasma with or without the addition of an antioxidant (0.001% sodium metabisulfite) was assessed at various times (0, 12, 24 and 36 h) at ambient room temperature (22–23°C). The concentrations were 10, 100

and 1000 ng/ml. The stability of the drug in 100 m*M* sodium phosphate buffer at pH 4.0, 5.5, 7.0, 8.5 and 10.0 was also evaluated using a scanning spectrophotometer (from 220 to 700 nm) and by HPLC. Each solution containing 100 μg/ml mitoxantrone was stored at room temperature and 4°C over 21 days. The sampling times were 0, 1, 2, 3, 4, 7, 9, 11, 16 and 21 days after preparation.

Pharmacokinetic studies

Two adult cancer patients with refractory acute non-lymphocytic leukemia received mitoxantrone at a dose of 12 mg/m^2 intravenously over 1 h. Serial blood samples were drawn 5 min before the infusion, immediately after the infusion (peak level) and at 5, 10, 20, 30 and 60 min and 2, 3, 6, 12, 24, 36 and 48 h after the 1-h infusion. Blood samples were collected in heparinized tubes containing 0.001% sodium metabisulfite, immediately centrifuged at 4°C and stored at −70°C until HPLC analysis.

RESULTS

Based on the cyclic voltammograms of mitoxantrone and bisantrene provided by BAS (West Lafayette, IN, U.S.A.), we chose +0.75V for optimal sensitivity for both compounds. Typical HPLC profiles of mitoxantrone and bisantrene are shown in Fig. 2. Visible detector response at 660 nm is compared to the electrochemical detector response. The variable-wavelength UV detector was at maximal detector sensitivity (0.001 a.u.f.s.) while the electrochemical detector could have been adjusted 50-fold more sensitive for mitoxantrone. Using ED, the detection limit determined based on a signal-to-noise ratio of 3 was 0.1 ng/ml, which is approximately ten-fold more sensitive than visible detection.

The extraction efficiency of mitoxantrone from plasma was 85.3% with a coefficient of variation (C.V.) less than 2.1% in the concentration range studied. The liquid–liquid extraction procedure after alkalinization appeared to be quite reproducible, inexpensive and comparable to solid-phase extraction methods reported in the literature [10,11]. The retention times for these compounds were quite precise with a day-to-day C.V. of less than 2%. As shown in Table I, precision (C.V.) for high and low mitoxantrone concentrations was 5.3 and 7.6%, respectively, intra-assay precision was 4.9% and the day-to-day precision was 6.5%. With ED, the CV at 0.1 and 5.0 ng/ml was in the 6–8% range, and better precision was obtained at the higher concentrations. With visible detection, the C.V. was less than 10% in the concentration range studied. Linear least-squares regression analysis of mitoxantrone peak areas, peak-area ratios relative to the internal standard and peak-height ratios to internal standard versus known plasma concentrations were linear from 0.1 to 1000 ng/ml using ED. The linear correlation coefficients of the best-fit lines were greater than 0.998 for all of the different methods. Accuracy of the analytical method, determined by the triplicate analysis of five pre-spiked, operator-blinded unknowns, was within ±5% of the true values.

The stability of mitoxantrone in plasma with or without the addition of an antioxidant was assessed, and drug recoveries after a 36-h incubation with plasma containing sodium metabisulfite were 92.2, 89.4 and 86.7% at concentrations of

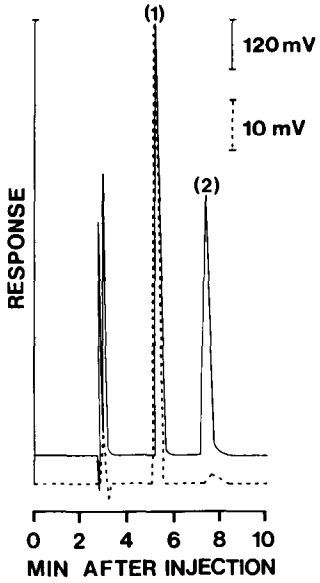

Fig. 2. Comparison of chromatograms obtained by electrochemical (——) and visible (- - - - - -) detection at an applied potential of +0.75 V and wavelength of 660 nm, respectively. This plasma sample contained approximately 500 ng/ml mitoxantrone (1) and 2000 ng/ml bisantrene (2). 10 mV equal 0.02 a.u.f.s. (for visible absorbance) and 120 mV equal 30 nA (for ED).

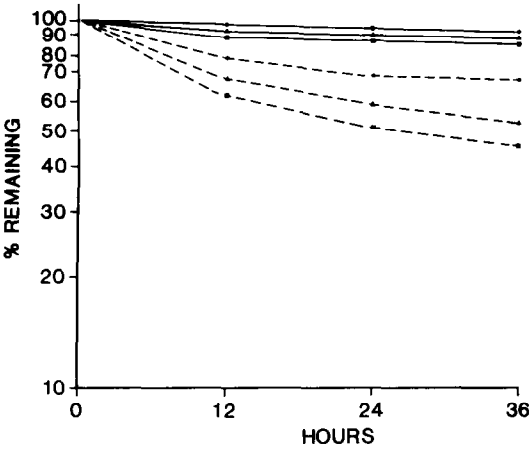

Fig. 3. The stability of mitoxantrone in plasma with (——) or without (- - -) the addition of sodium metabisulfite (0.001%) at ambient room temperature. Three concentrations were analyzed: ●, 10 ng/ml; ▲, 100 mg/ml and ■, 1000 ng/ml.

10, 100 and 1000 ng/ml, respectively, as shown in Fig. 3. Fig.-3 also shows the stability of mitoxantrone at three different concentrations with no antioxidant over 36 h. Mitoxantrone was less stable in plasma containing no antioxidant, suggesting that sodium metabisulfite effectively stabilizes the drug. The effect of pH on the stability of mitoxantrone in aqueous solutions was investigated at both 4 °C and room temperature over 21 days, and our results show that the drug

TABLE I

SUMMARY OF PRECISION EVALUATION OF THE NEW HPLC METHOD FOR MITOXANTRONE IN PLASMA

Plasma concentration (ng/ml)	*n*	Coefficient of variation* (%)	
		Electrochemical detection	Visible detection
0.1	6	7.6	9.5
5	10	6.5	7.7
50	10	4.9	8.5
500	10	6.4	6.7
1000	6	5.3	7.0

*See the *Assay validation* section for the assessment of precision.

decomposed up to 15% at room temperature at pH 8.5 and 10.0 while the drug remained intact at pH lower than 7.0 over the study period (Table II).

We then applied this new method for the quantitation of mitoxantrone in patient samples. A typical chromatogram of a patient's pre-infusion sample and a peak plasma sample are shown in Fig. 4. Fig. 5 shows concentration–time plots of two patients who received 12 mg/m^2 mitoxantrone as a 60-min infusion. Peak concentrations of mitoxantrone were 510 and 567 ng/ml. Plasma mitoxantrone concentrations at 48 h after the end of the infusion were 1.4 and 1.6 ng/ml.

DISCUSSION

Both mitoxantrone and bisantrene are undergoing clinical investigation for the treatment of a variety of pediatric and adult malignancies. For this reason, a sensitive, specific and simple analytical method for the determination of the clinical pharmacokinetics is necessary. We have developed a relatively inexpensive

TABLE II

PERCENTAGE OF MITOXANTRONE (100 μg/ml) REMAINING AS A FUNCTION OF TIME IN 100 m*M* SODIUM PHOSPHATE BUFFER AT VARIOUS pH AT ROOM TEMPERATURE AND 4°C OVER 21 DAYS

Data on days 1, 2, 4, 9 and 16 are not listed. RT = room temperature (22–23°C). All samples were analyzed in triplicate.

Time (days)	Percentage mitoxantrone remaining									
	pH 4.0		pH 5.5		pH 7.0		pH 8.5		pH 10.0	
	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C
0	100	100	100	100	100	100	100	100	100	100
3	98.1	99.8	99.6	100.6	101.1	100.7	98.6	98.7	93.6	98.1
7	98.6	98.6	98.8	98.5	99.6	100.0	95.5	98.5	88.5	97.1
11	98.8	98.9	101.5	101.8	102.0	100.6	91.2	97.5	85.8	96.1
21	98.9	99.5	100.6	100.6	100.4	100.4	87.5	97.7	85.0	95.8

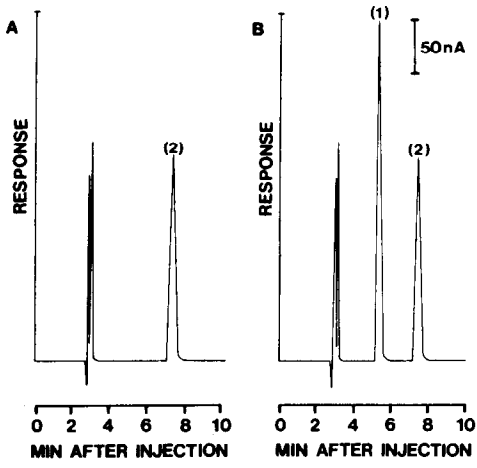

Fig. 4. HPLC-ED profiles of (A) pre- and (B) post-dose plasma samples obtained from a patient receiving 12 mg/m^2 mitoxantrone (1). Both samples (1.0 ml of plasma) were spiked with 50 μl of 100 μg/ml stock solution of bisantrene (2), internal standard.

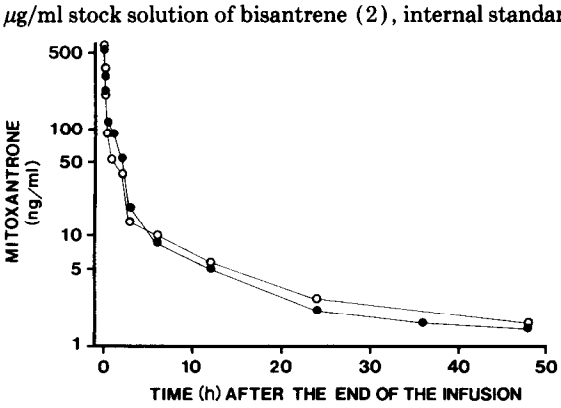

Fig. 5. Mitoxantrone plasma concentration–time curves of two patients receiving the drug at a dose of 12 mg/m^2.

and rapid analytical method for the simultaneous determination of these compounds in pharmaceutical solutions and biological fluids. Both detection methods are linear and have adequate precision and sensitivity.

Other published analytical methods have attained a sensitivity or detection limit ranging from 1 to 75 ng/ml [10–13]. In addition, a few published methods employ a non-specific quantitation method with a detection limit of about 10 ng/ml [5]. In 1983, Houpt and Baldwin [14] reported an HPLC assay for mitoxantrone in urine. They also employed electrochemical monitoring of the drug which provided a detection limit of 2 ng. Compared to our HPLC method, there are several specific differences in sample sources (urine versus plasma), sample treatment procedures (60% recovery with solid-phase extraction versus 85% with methylene chloride extraction), quantitation methods (external versus internal standard method) and sensitivity (2 ng versus 0.04 ng or 0.1 ng/ml). Our HPLC method in combination with the use of ED has allowed at least ten-fold increase

in sensitivity relative to other published procedures. Since it also yields better precision and accuracy in measuring drug concentrations lower than 5 ng/ml, quantitation of plasma mitoxantrone levels in the terminal phase is more reliable. To our knowledge, there is only one HPLC method that has the ability to detect mitoxantrone at concentrations below 1 ng/ml in serum with a C.V. less than 9.3% at a visible wavelength of 658 nm [16]. This method is able to measure metabolites, mono- and dicarboxylic acid analogues of mitoxantrone. We had no opportunity to evaluate the ability of our HPLC method to separate those metabolites from the parent drug primarily because the standard references were not available to us. Instead, we determined the peak purity using an LKB photodiode array detector and confirmed that no co-eluting peaks underneath the mitoxantrone peak appeared.

In summary, a specific and very sensitive HPLC assay using ED has been developed for the determination of mitoxantrone concentrations in plasma. If mitoxantrone is administered by continuous infusion for longer than 24 h at low dosages, the steady-state plasma concentrations should be detectable with this method.

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