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Rapid liquid chromatographic analysis of mitoxantrone in plasma with C_{18} sample purification

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Abstract: A useful and rapid method for the analysis of mitoxantrone in human plasma is described. We purified the sample with a C_{18} Sep Pak^{TM} cartridge pre-treated with methanol, water and 0.05 M ammonium phosphate (pH = 2.75). The drug and internal standard (Methylene Blue) were eluted from the cartridge with 1 M acetic acid in methanol and were separated on a 10 μm particle size CN Resolve^{TM} cartridge in conjunction with a radial compression liquid chromatograph. The mobile phase consisted of a mixture of 0.05 M ammonium phosphate, acetonitrile and methanol (60:35:5, by vol), and the detection was performed spectrophotometrically at 660 nm. The peak height ratio (drug/internal standard) varied linearly ($r > 0.993$) with concentration over the range examined 0.01–3 μg ml^{-1}, and the inter- and intra-run precision at high, medium and low concentrations were good; CV ranged from 2.52 to 7.2%. There was no interference from other anticancer drugs or analgesics. We applied the described method to investigate the pharmacokinetics of mitoxantrone using a rabbit as an *in vivo* model.

Keywords: *Liquid chromatography; mitoxantrone; C_{18} sample clean-up; pharmacokinetics.*

Introduction

Mitoxantrone, 1,4-dihydroxy-5,8-bis{2-[(2-hydroxyethyl)amino]ethyl}-9,10-anthracenedione dihydrochloride is an analogue of doxorubicin which has exhibited antitumor activities towards varieties of experimental and human malignancies. Because of much reduced cardiotoxicity, in comparison to the structurally related anthracyclines, this drug has found a place in the treatment of lymphoma [1, 2], leukaemia [3, 4], and advanced breast cancer [5, 6]. However, myelosuppression, leukopenia and adverse gastrointestinal effects are dose-limiting toxic aspects which require careful monitoring.

Mitoxantrone has been analysed in biological samples mainly by C_{18} reversed-phase high-performance liquid chromatography (HPLC) [7–15]. While some of these methods

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utilize ion-pair systems [7, 10, 14], others employ organic solvent–buffer mixtures with relatively long retention times (i.e. >16 min) [12, 14, 15] or low retention capacity factors (i.e. k' <1.8) [7, 11, 13]. Additionally, several of these methods lack an internal standard [8, 12–14], and some use non-specific detection at 254 nm [10, 12, 14] making the assay less sensitive and prone to interferences from endogenous compounds. The use of radial compression liquid chromatography and CN reversed-phase packing for mitoxantrone has not been previously reported. While maintaining a high k', this approach in combination with C_{18} sample clean-up yields a high chromatographic efficiency (i.e. sharper peaks), enhanced expediency and a shorter analysis time. The described method is also accurate and sensitive which makes it suitable for pharmacokinetic studies of this drug.

Experimental

Chemicals

The mitoxantrone (Lederle Laboratories, Cyanamid of Great Britain, Hampshire, UK) and Methylene Blue (internal standard; Fisher Scientific Co., Fair Lawn, NJ, USA) samples used were pharmaceutical and analytical grade, respectively. Methanol, acetonitrile, ammonium phosphate and acetic acid were all HPLC grade (Fisher Scientific Co., Fair Lawn, NJ, USA). The water for HPLC was prepared by passing deionized water through a Norganic™ cartridge to remove trace amounts of organic compounds and a 0.45-μm (pore-size) membrane filter from Millipore Co. (Milford, MA, USA).

Chromatography

We used a liquid chromatograph (Waters Associates, Milford, MA, USA) comprising an autosampler (WISP), a pump (Model 6000 A), a system controller (Model 720), a radial compression separation module (Z-Module) equipped with a 10-μm-particle-packed, 8 mm 10 cm CN-Resolve Radial Pak™ cartridge and a Guard-Pak™ pre-column module with a C_{18} insert, a variable-wavelength UV detector (Model 480) set at a wavelength of 660 nm, and a data module (Model 730).

To prepare the mobile phase, we mixed 600 ml of 0.05 M of ammonium phosphate, 350 ml of acetonitrile and 50 ml of methanol. After adjusting the pH to 3, the solution was filtered through a filter of 0.45-μm pore size and degassed before use. The flow rate was 3 ml min^{-1} at a pressure of 6.19 MPa (900 psi).

Standard curves

The concentration of mitoxantrone in the "unknown" samples was calculated by use of standard curves constructed by supplementing 1-ml portions of blank plasma with 0.01, 0.025, 0.05, 0.25, 0.5, 0.75, 1.0, 1.5 or 3 μg of mitoxantrone, 0.110 μg of the internal standard and 100 μl of 5% ascorbic acid for stabilization [13, 14]. After the pH was adjusted to 4 with phosphoric acid, the sample was diluted to 1.5 ml with HPLC water and subjected to the purification procedure described below.

Extraction of plasma samples

Mitoxantrone and Methylene Blue (internal standard) were extracted from plasma samples by use of Sep Pak™ cartridges (Waters Associates, Milford, MA, USA). The cartridge was pre-treated with 10-ml fractions each of methanol, water, 0.05 M

ammonium phosphate (pH = 2.75) followed by 10 ml of water prior to vacuum drying for 2 min. The diluted sample was then slowly injected into the cartridge which was then washed with 20 ml of water and vacuum-dried for 2 min. The compounds were eluted from the cartridge with 3.6 ml of 1 M acetic acid in methanol. The eluate was dried under a gentle stream of nitrogen gas and the residue redissolved in 200 μl of mobile phase by placing the tube in an ultrasound water bath for 5 min at ambient temperature (i.e. 23°). The solution was then transferred into an autosampler microvial and 100–200 μl was injected.

Pharmacokinetic study

We used a mature New Zealand albino rabbit weighing 5.29 kg to examine the applicability of the described method to study the pharmacokinetics of mitoxantrone. We catheterized the marginal vein of the left ear [16], using the catheter to infuse the drug and later, after thoroughly flushing with sterile normal saline solution to collect blood samples. The dose (i.e. 1.038 mg kg^{-1}) was dissolved in normal saline solution and delivered at a constant rate over 30 min using a variable-speed syringe type infusion pump (Sage Instruments, Cambridge, MA, USA). Water was offered to the rabbit *ad libitum* for the duration of the experiment. We collected blood samples (2.1–2.2 ml, at different intervals over 24 h and harvested the plasma by centrifugation at 1746 ***g***. The drug was stabilized in the plasma by adding 100 μl of 5% ascorbic acid to each sample and storing immediately at −20° until analysis.

The samples were subjected to the above purification procedure after adding the appropriate amount of internal standard to each tube, adjusting the pH to 4 and bringing the volume to 1.5 ml. We calculated the concentration of mitoxantrone in each sample by use of standard curves prepared from blank rabbit plasma as described above.

Results and Discussion

Representative chromatograms for intact human plasma and human plasma supplemented with mitoxantrone and Methylene Blue are shown in Fig. 1. As can be seen in this figure, the retention times of the above compounds are 5.5 min (k' = 4.9) and 8.99 min (k' = 8.3), respectively, and the peaks are sharp and symmetrical signifying a high chromatographic efficiency. The use of a CN-Resolve Radial Pak™ cartridge for this assay was the result of elaborate work where C_{18} and phenyl reversed-phase cartridges were employed; while C_{18} packing produced broad, tailing peaks, phenyl packing did not retain the compounds adequately, even at much lower concentrations of acetonitrile and methanol in the mobile phase.

Figure 1 also indicates that the sample purification procedure with C_{18} Sep Pak cartridges as described in this report yields clean chromatograms with no interfering peaks from the plasma. We measured the efficiency of extracting mitoxantrone from plasma by dividing the peak heights obtained for purified plasma samples supplemented with 0.05, 0.25, 1 or 3 μg of mitoxantrone by those obtained for the same amounts of the drug injected directly into the cartridge. The extraction efficiency was >72% at the above concentrations. The peak with a retention time of 7.8 min which is fully resolved from the other peaks appears to be due to an impurity originating from the internal standard. We examined the specificity of the assay by measuring the retention times of drugs commonly used in combination with mitoxantrone (Table 1). As demonstrated in this table, no interference from any of these drugs was noted.

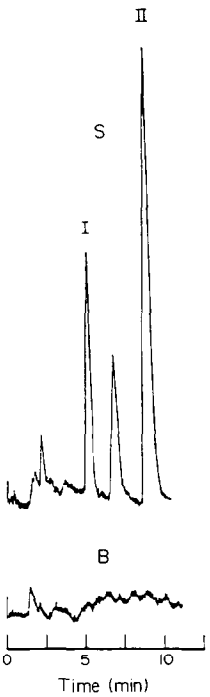

Figure 1
Representative chromatograms of (B) an intact human plasma sample, and (S) human plasma sample to which 0.05 μg ml^{-1} of mitoxantrone (I) and 0.11 μg ml^{-1} Methylene Blue (II, internal standard) were added. Half of the 200-μl of extract was injected.

Table 1
Retention times of drugs which may be used in combination with mitoxantrone

Drug	Retention time (min)
5-Fluorouracil	1.79
Etoposide	1.76
Cyclophosphamide	ND
Carmustine	3.4
Busulfan	1.93
Methotraxate	ND
Lomustine	ND
Procarbazine	2.48
Mytomycin-C	ND
Ifosphamide	ND
Melphelan	ND
Tenoposide	ND
Chlorambucil	ND
Aspirin	ND
Acetaminophen	2.30
Hydrocortisone	ND

ND: Non-detectable.

The linearity of the assay was investigated by preparing on different days calibration curves in the range 0.01–3 μg ml^{-1}. The peak height ratio (drug/internal standard) varied highly linearly with concentration: correlation coefficients (r) for mitoxantrone ranged from 0.9929 to 0.99995 (mean = 0.9972; SD = 0.026; n = 10).

We determined the intra-run precision and analytical recovery of mitoxantrone by analysing eight samples at concentrations of 3, 1, 0.25, or 0.05 μg ml^{-1}. We calculated the analytical recovery as 100 times the amount found/amount added. As shown in Table 2, the CV values for the above concentrations were 2.52, 3.98, 5.39 and 3.65%, respectively, indicating good intra-run precision. The analytical recovery was also good, with values of 98.3, 100, 96.5 and 96.5% at the above concentrations, respectively. The between day precision was determined by analysing on four different days 1-ml plasma samples supplemented with 0.05, 0.5 or 1.5 μg ml^{-1} of mitoxantrone and 0.110 μg of the internal standard. The CV values obtained at the above concentrations were 7.2, 2.9 and 6.7%, respectively, signifying an equally good between day precision.

We examined the applicability of this assay to study the pharmacokinetics of mitoxantrone by infusing 1.038 mg kg^{-1} of the drug over 30 min into the marginal ear vein of a rabbit and analysing the plasma samples collected at different intervals as described above. Figure 2 shows a representative chromatogram of a sample collected

Table 2
Inter-run precision and analytical recovery of mitoxantrone

Amount added (μg)	Amount found (μg)	Coefficient of variation (%)	Analytical* recovery (and SD) (%)
3.0	2.947	2.52	98.3 (2.64)
1.0	0.9997	3.98	100.0 (3.98)
0.25	0.241	5.39	96.5 (5.39)
0.05	0.0483	3.65	96.5 (3.52)

*Mean of eight experiments.

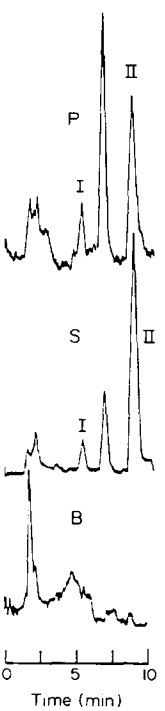

Figure 2
Representative chromatograms of (B) an intact rabbit plasma; (S) rabbit plasma sample to which 0.05 μg ml^{-1} of mitoxantrone (I) and 0.11 μg ml^{-1} Methylene Blue (II, internal standard) were added; and (P) rabbit plasma sample collected 1.5 h after the termination of the infusion to which 0.11 μg ml^{-1} of II was added. The volumes injected were 100, 100 and 110 μl of the reconstituted extract residue (200 μl) of B, S and P, respectively.

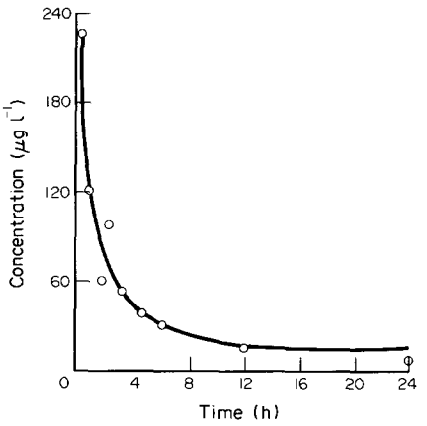

Figure 3
A non-linear regression curve of the concentration of mitoxantrone in plasma versus time data obtained after the termination of 30-min infusion of 1.038 mg kg^{-1} of the drug into the marginal ear vein of a rabbit, and plotted according to the three-compartment model.

1.5 h after the termination of the infusion. We fitted the concentration–time data to the three-compartment model [17] using a non-linear least-squares analysis (Fig. 3). The half-lives for the alpha-, beta- and gamma-phases were 7.1 min, 4.39 h, and 69.87 h, respectively. The area-under-the-curve from time = 0 to time = 24 h was 647.61 $\mu g \cdot h \cdot l^{-1}$ and the total body clearance was 0.601 $l \cdot h^{-1} \cdot kg^{-1}$.

In conclusion, we describe in this report a useful and accurate method for the analysis of mitoxantrone in plasma by use of radial compression liquid chromatography and C_{18} sample clean-up. The assay is rapid and sensitive which makes it suitable for investigating the pharmacokinetics of this drug.

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