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DIRECT DETERMINATION OF MITOXANTRONE AND ITS MONO- AND DICARBOXYLIC METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The simultaneous isolation and determination of mitoxantrone (Novantrone®) and its two known metabolites (the mono- and dicarboxylic metabolites) were carried out using a high-performance liquid chromatographic (HPLC) system equipped with an automatic pre-column-switching system that permits drug analysis by direct injection of biological samples. Plasma or urine samples were injected directly on to an enrichment pre-column flushed with methanol–water (5:95, v/v) as the mobile phase. The maximum amount of endogenous water-soluble components was removed from biological samples within 9 min. Drugs specifically adsorbed on the pre-column were back-flushed on to an analytical column (Nucleosil C_{18}, 250×4.6 mm I.D.) with 1.6 *M* ammonium formate buffer (pH 4.0) (2.5% formic acid) containing 20% acetonitrile. Detection was effected at 655 nm. Chromatographic analysis was performed within 12 min. The detection limit of the method was about 4 ng/ml for urine and 10 ng/ml for plasma samples. The precision ranged from 3 to 11% depending on the amount of compound studied. This technique was applied to the monitoring of mitoxantrone in plasma and to the quantification of the unchanged compound and its two metabolites in urine from patients receiving 14 mg/m^2 of mitoxantrone by intravenous infusion for 10 min.

INTRODUCTION

Mitoxantrone, 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]-amino]-9,10-anthracenedione dichloride (Novantrone®), is an analogue of the anthracycline synthesized by Murdock et al. [1] in the 1970s. It is a potent inhibitor of DNA and RNA synthesis [2–6]. Mitoxantrone shows better antitumour activity than the parent compounds (daunorubicin, doxorubicin) against a wide range of murine tumours [7] without presenting induced cardiotoxic side-effects in treated patients [8, 9].

Phase II studies indicated that mitoxantrone also shows activity particularly against breast cancer and leukaemia in humans [10, 11]. Its clinical use is lim-

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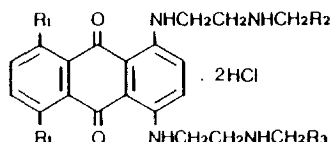

	R_1	R_2	R_3
MITOXANTRONE	OH	CH_2OH	CH_2OH
CL 285-049	OH	CH_2OH	COOH
CL 283-981	OH	COOH	COOH

Fig. 1. Structures of mitoxantrone and its mono- (CL 285 049 X) and dicarboxylic acid (CL 283 981) derivatives.

ited, however, by haematological disorders [8, 12–14]. Useful information about the elimination patterns of the drug (metabolism, excretion) could be obtained by monitoring the levels of the unchanged compound and its mono- and dicarboxylic metabolites (Fig. 1) in biological fluids.

Several techniques have been proposed for the measurement of mitoxantrone in biological fluids. High-performance liquid chromatographic (HPLC) methods together with various sample clean-up procedures have been reported for the determination of mitoxantrone in urine and plasma [15–23] but few of these HPLC methods characterize the different mitoxantrone metabolites reported in human plasma [19] and urine [19–23]. Also, these methods yield controversial data on the elimination half-lives, with values ranging between 0.5 and 210 h for the unchanged compound [24–29]. This may be due to the varying sensitivities of the methods, ranging from 0.2 to 75 ng/ml [15–22].

In this paper an HPLC method is described that permits drug measurements in biological fluids by direct injection of urine and plasma without sample pretreatment. The automatic pre-column-switching system described by Roth et al. [30] is used as a sample clean-up procedure. This technique offers the advantage of rapidly obtaining individual plasma kinetic information for a new patient, permitting his or her drug schedule adjustment by a Bayesian estimator. The method has been applied to kinetic and metabolic studies of mitoxantrone in man. Mitoxantrone and its two reported metabolites [the mono- (M1) and dicarboxylic (M2) derivatives] were monitored in plasma and urine during 24 and 48 h, respectively, after the end of a 10-min intravenous (i.v.) infusion of 14 mg/m^2 Novantrone.

EXPERIMENTAL

Reagents and standard solutions

Mitoxantrone and the monocarboxylic (CL 285 049X) and dicarboxylic (CL 283 981) metabolites were kindly donated by Lederle Labs. (Oullins, France). These compounds were used without further purification.

Stock solutions (1 mg/ml) of mitoxantrone and the metabolites were prepared in distilled water containing 10% of 0.1 *M* citrate buffer (pH 3.0) (5% ascorbic acid) and stored at $-20°C$ without apparent degradation. Serial dilutions of

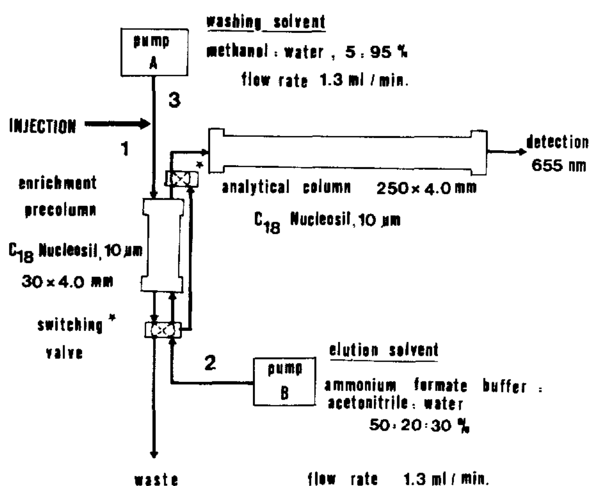

Fig. 2. Principle of the method for one of the two alternating enrichment pre-columns. (1) Biological samples were injected into the enrichment pre-column, and flushed with the washing solvent stream (pump A) for 10 min. (2) At the end of the purge procedure, the time relay triggered the switching valve and the compounds were back-flushed (pump B) on to the analytical column by the elution solvent stream, while the opposite working pre-column was being conditioned with the washing solvent stream before the next injection (3).

standards were made within the working range 12.5–2000 ng/ml in distilled water and normal drug-free urine and plasma.

Ammonium formate (98% purum) was purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical-reagent grade.

HPLC analysis

Blood samples were centrifuged at 2500 *g* for 5 min at 4°C, and plasma containing 10% of 0.1 *M* citrate buffer (pH 3.0) (5% ascorbic acid) was immediately stored in a polypropylene tube at −20°C until HPLC analysis. Urine was collected over a 48-h period after the end of Novantrone administration. Diuresis was noted and an aliquot of each fraction kept at −20°C until analysis. Just before HPLC assay, the urine and plasma samples were centrifuged at 2500 *g* for 5 min and 10 000 *g* for 3 min, respectively, to eliminate solid particles.

The HPLC apparatus [30] consisted of a purge phase linked to an analytical phase by a column-switching module (Gynkotek, Munich, F.R.G.) bearing the injection system and three pneumatically driven valves. The first and second valves connected two pre-columns, PC1 and PC2 (Nucleosil C_{18}, 30×4.0 mm I.D., 10-µm particles) (Knauer, West Berlin, F.R.G.), with the injection system and pump A (Model 6000 A, Waters Assoc., Milford, MA, USA), while the third valve, in the back-flush mode, connected the pre-column (PC1 or PC2) with an analytical column (Nucleosil C_{18}, 250 mm×4.0 mm I.D., 10-µm particles) (Knauer) and pump B. A programmable time relay limited the washing period. Detection was carried out at 655 nm on a Lambda Max Model 481 LC spectrophotometer (Waters Assoc.). The signal (height of absorbance peak) was calculated by means of an HP 3390 A integrator (Hewlett-Packard, Les Ulis, France).

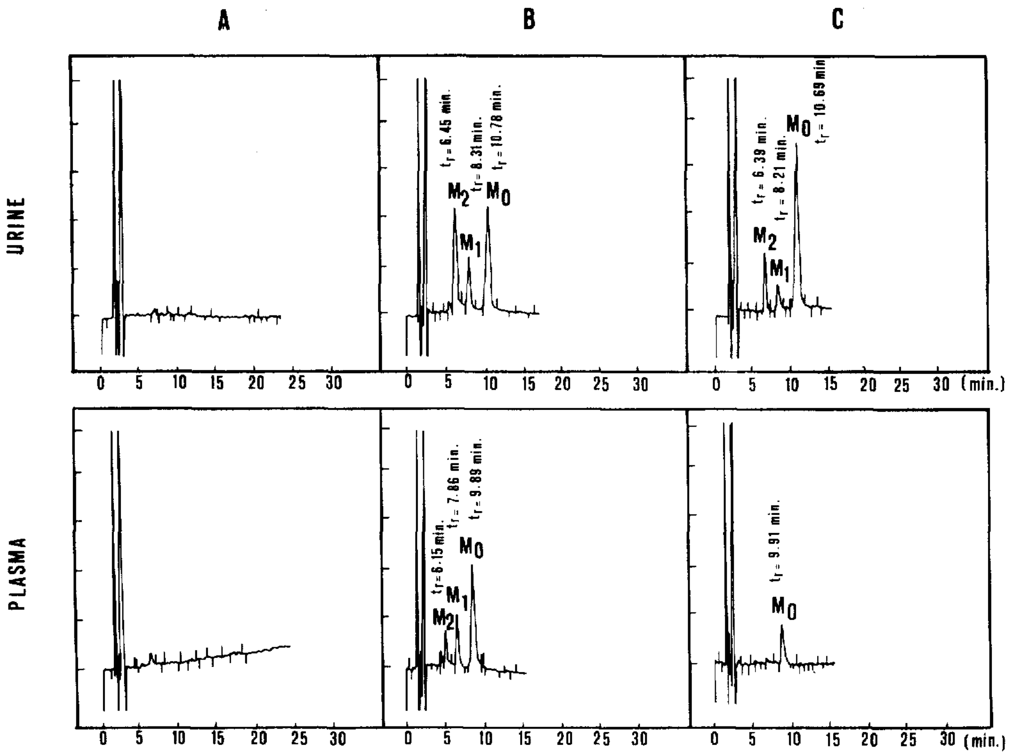

Fig. 3. Typical chromatograms of urine (top) and plasma (bottom) samples. (A) Control urine (500 μl) and plasma (300 μl). (B) Control urine and plasma spiked with known amounts of mitoxantrone, M1 and M2. (C) Patient's urine and plasma removed 42 h and 2 h, respectively, after a 16.8-mg i.v. infusion of mitoxantrone for 10 min. Peaks: M_0 = mitoxantrone; M_1 = monocarboxylic derivative; M_2 = dicarboxylic derivative.

The principle of this back-flush mode is illustrated in Fig. 2 for one of the working pre-columns.

Biological samples were injected directly into the enrichment pre-column (PC1 and PC2) through injection loops of 50, 200 or 500 μl. The mobile phase for the washing–enrichment procedure was methanol–water (5:95, v/v) delivered by pump A at a flow-rate of 1.3 ml/min. The compounds concerned were selectively retained on the pre-column and water-soluble endogenous compounds were eliminated with the waste phase. At the end of a 10-min purge procedure, the time relay triggered the two driven valves and the compounds were back-flushed on to the analytical column with a 1.6 *M* ammonium formate buffer (pH 4.0) (2.5% formic acid)–acetonitrile–water (50:20:30, v/v/v) mobile phase, delivered by pump B at a flow-rate of 1.3 ml/min.

The overall analytical procedure required 20 min because while PC1 was in the analytical phase PC2 was in the conditioning phase, thus permitting a new injection before the end of the PC1 analytical phase.

TABLE I

WITHIN-DAY LINEARITY

Compound	Concentration added* (ng/ml)	Injection volume (μl)	Equation of the linear regression curve**	Correlation coefficient
Plasma				
Mitoxantrone	25–500	200	$x=3.14\cdot 1^{-3}y+0.90$	0.9992
	100–2000	50	$x=4.54\cdot 10^{-3}y+1.67$	0.9998
M1	25–500	200	$x=6.00\cdot 10^{-3}y+0.80$	0.9998
	100–2000	50	$x=8.42\cdot 10^{-3}y+1.28$	0.9996
M2	25–500	200	$x=5.60\cdot 10^{-3}y+2.03$	0.9997
	100–2000	50	$x=5.44\cdot 10^{-3}y+1.38$	0.9994
Urine				
Mitoxantrone	10–200	500	$x=3.96\cdot 10^{-3}y+0.46$	0.9996
	25–500	200	$x=4.25\cdot 10^{-3}y-0.57$	0.9998
	100–2000	50	$x=3.51\cdot 10^{-3}y+0.99$	0.9996
M1	10–200	500	$x=6.17\cdot 10^{-3}y+1.30$	0.9997
	25–500	200	$x=7.83\cdot 10^{-3}y-1.65$	0.9996
	100–2000	50	$x=6.83\cdot 10^{-3}y+0.76$	0.9999
M2	10–200	500	$x=3.83\cdot 10^{-3}y+1.07$	0.9985
	25–500	200	$x=3.96\cdot 10^{-3}y-2.07$	0.9995
	100–2000	50	$x=3.90\cdot 10^{-3}y-0.92$	0.9995

*Linearity of the response was studied between 5 and 100 ng injected in 50 and 200 μl of plasma and 50, 200 and 500 μl of urine, with amounts of 5, 10, 25, 50, 75 and 100 ng.
**y=peak height of the signal as reported in the Hewlett-Packard Model 3390 A integrator.

RESULTS

Different percentages of methanol in the washing solvent were tried. No loss of the compounds was observed below 50% of methanol in water after washing for 1 h. A concentration of 5% methanol in water was chosen because it represented the lowest risk of protein precipitation. Moreover, the maximum amount of endogenous water-soluble components was removed from urine and plasma within 9 min even when large volumes of biological samples were injected.

Fig. 3 shows typical chromatograms obtained under analytical conditions. The compounds were eluted within 12 min. The retention times of mitoxantrone and its mono- and dicarboxylic metabolites (M1 and M2) were 11, 8 and 6 min, respectively. The three compounds were baseline-separated; no endogenous peaks appeared.

Calibration graphs were obtained by plotting directly the peak height against the amount of compound injected. The parameters for mitoxantrone, M1 and M2 in plasma and urine are shown in Table I. The within-day linearity proved satisfactory for plasma concentrations ranging from 25 to 2000 ng/ml and for urine concentrations ranging from 10 to 2000 ng/ml.

The accuracy ranged from −6.0 to 7.0% for 5 ng injected, from −3.2 to 5.0% for 25 ng injected and from −1.6 to 6.8% for 125 ng injected. The precision of the method was assessed for mitoxantrone, M1 and M2 in urine and plasma by injec-

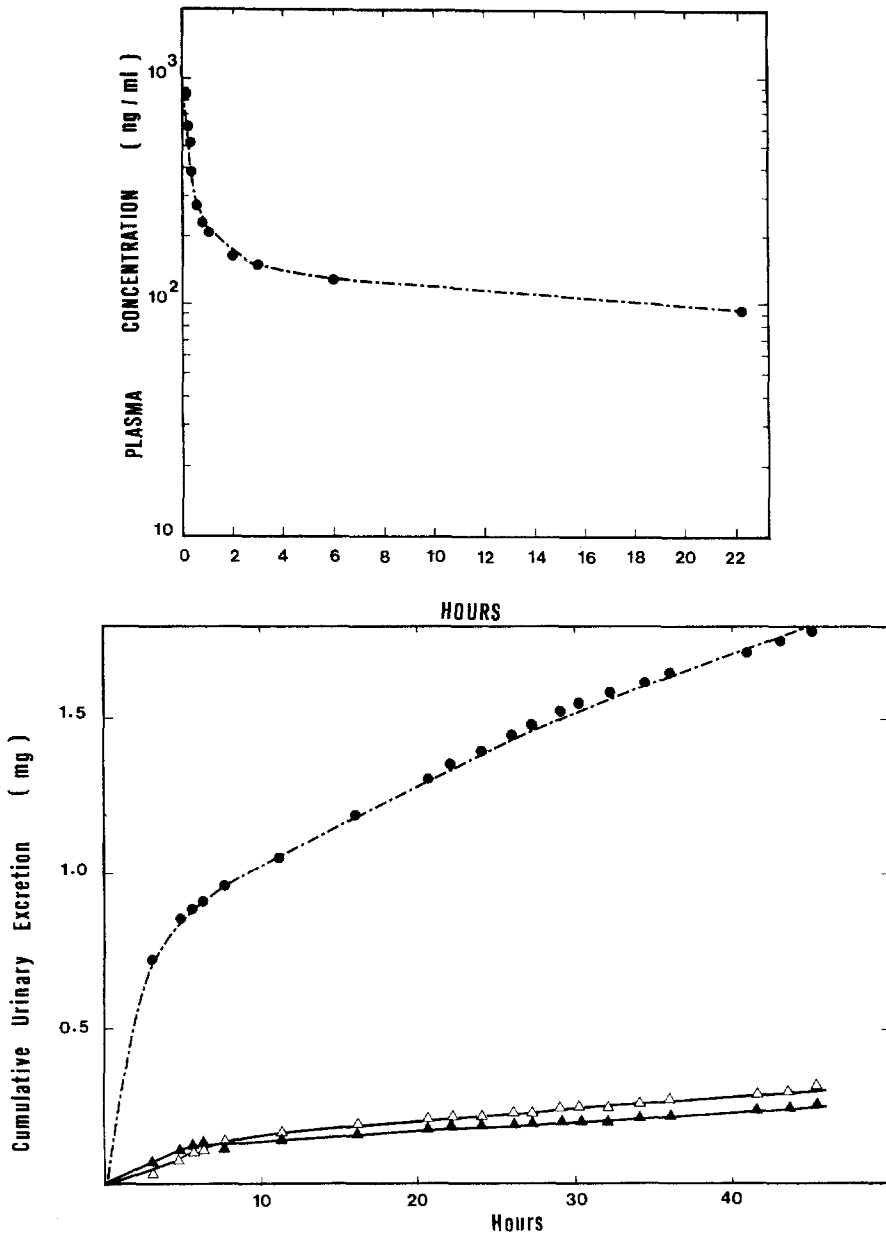

Fig. 4. Top: plasma time-concentration curve for mitoxantrone in a patient receiving a 10-min i.v. infusion of Novantrone (14 mg/m^2). Bottom: cumulative urinary excretion of (●) mitoxantrone, (▲) mono- and (△) dicarboxylic metabolites in the same patient.

tion of the same sample seven times. It varied from 3.0 to 11.0% for concentrations ranging from 5 to 125 ng/ml.

The limit of quantification, defined by a signal-to-noise ratio higher than 3:1, was 4 ng/ml in urine and 10 ng/ml in plasma for the three compounds. The selectivity of the method was not altered by the presence of co-administered me-

dicaments such as daunorubicin, doxorubicin, *cis*-platinum, vincristine, vinblastine, cytosine arabinoside and methotrexate.

DISCUSSION

Detection was carried out at 655 nm; Ostroy and Gams [15] had reported that mitoxantrone showed several absorption maxima, the major bands being located at 662, 611 and 246 nm. The maximum absorbance values for mitoxantrone and its two metabolites in the buffer used as the analytical mobile phase were 655, 610 and 240 nm. The wavelength of maximum absorbance of 240 nm was disadvantageous in HPLC analysis because of the interference of plasma endogenous peaks. A detection wavelengths of 655 nm was therefore used.

The method proved linear between 5 and 100 ng of mitoxantrone, M1 and M2 injected on to the pre-column. Using different injection loops, the concentration ranges for a linear response were 25–2000 ng/ml in plasma and 10–2000 ng/ml in urine. For the same range of linearity, the slopes of the calibration graphs (peak height versus amount injected) were similar whatever the volume injected. Owing to the enrichment procedure in the back-flush system, the peak width is independent of the volume injected and peak tailing is negligible even with large injection volumes (200 μl). The injection volume did not interfere with the HPLC determination of mitoxantrone, M1 and M2.

The sensitivity of the method was calculated for an amount of 2.0 ng injected. Pre-column life-time allowed the direct injection of a maximum of 200 μl of plasma and 500 μl of urine. Consequently, the minimal concentrations quantifiable were 4 ng/ml for urine and 10 ng/ml for plasma samples. These values are in agreement with the sensitivity values reported in the literature, i.e., 0.2–75 ng/ml [15–22, 24].

In conclusion, the advantages of the method are that no sample pre-treatment is required, direct injection of body fluids is possible, it is inexpensive and the analysis time is short (20 min), so that pharmacokinetic information about a new patient can be readily obtained. A Bayesian estimation of a patient's kinetic parameters can be obtained from data for previous patients, permitting the adjustment of the drug schedule for this new patient before the end of the designed infusion period.

APPLICATIONS

The method was applied to a pharmacokinetic and metabolic study of mitoxantrone in man. The plasma levels of mitoxantrone, M1 and M2 were determined at the following times: 3, 10, 20, 30 and 40 min, then 1, 2, 4, 5, 12 and 24 h after a 10-min infusion of 14 mg/m^2 Novantrone. The plasma concentration–time curve (Fig. 4) was biphasic in a patient receiving 16.8 mg of Novantrone by short i.v. infusion. No circulating metabolites were detectable in plasma. The plasma kinetic parameters for mitoxantrone were calculated according to a model-independent approach. The plasma clearance of the unchanged compound was 98 ml/min/m^2 and the half-life was 24 h.

The urinary cumulative excretions of mitoxantrone and its two carboxylic metabolites are illustrated in Fig. 4 for the same patient; 42 h after the end of the infusion of Novantrone, 10% of the administered dose remained unchanged and only 3% of the dose was excreted as metabolites (1.7% for the dicarboxylic metabolite, 1.3% for the monocarboxylic metabolite). These results agree with previous studies in man [19–25].

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