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Short Communication

Determination of the new morpholino anthracycline MX2·HCl and its metabolites in biological samples by high-performance liquid chromatography

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ABSTRACT

Methods for determining concentrations of a new morpholino anthracycline MX2·HCl and its metabolites in biological samples using reversed-phase high-performance liquid chromatography and fluorescence detection are described. The limits of detection were less than 1 ng/ml for all compounds after extraction from 0.5 ml of plasma using C_{18} Sep-Pak cartridges and consecutive solvent extraction. The recoveries from rat plasma ranged from 72.0 to 89.3%. The peak-height ratio of the fluorescence intensities of these compounds *versus* internal standard showed a linear correlation for concentrations up to at least 500 ng/ml in the plasma (correlation coefficient $r > 0.999$). The within-day and between-day precisions of this assay were in the range 0.8–8.7% ($n = 5$) and 2.0–3.5% ($n = 5$), respectively. The concentrations of these compounds in the blood and urine can be also determined by a slight modification of the extraction procedure.

INTRODUCTION

Adriamycin (ADM) is an anthracycline antibiotic used in the treatment of a variety of human neoplastic diseases [1]. However, the use of the agent is restricted by dose-limiting cardiotoxicity [2] and myelosuppression [3]. Furthermore, the frequent emergence of tumour cells resistant to treatment with antitumour drugs is one of the major problems resulting in failure of cancer chemotherapy.

MX2 · HCl (3′-deamino-3′-morpholino-13-deoxo-10-hydroxycarminomycin

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hydrochloride, I) is a new morpholino anthracycline [4], which shows an interesting pharmacological and toxicological profile. This compound shows similar or superior chemotherapeutic effects to ADM against several experimental murine tumours, and is almost equipotent following oral or intravenous administration [4–6]. Moreover, this compound shows antitumour activity against certain drug-resistant tumours [6]. The subacute cardiocytotoxicity was found to be much weaker than that of ADM in a rabbit model (unpublished data). The major dose-limiting factor of I is myelosuppression, not cardiotoxicity, and it may be used for a longer duration than ADM. This new anthracycline is now under investigation in clinical phase II studies and, consequently, its pharmacokinetics are of much interest, with a precise analytical method for this anthracycline in biological samples being desirable.

The concentrations of anthracyclines in biological fluids have been determined by a number of methods, among which reversed-phase high-performance liquid chromatography (HPLC) is the most important [7]. This paper describes a sensitive, accurate and reproducible analytical procedure for I and its metabolites in biological samples, using reversed-phase HPLC and fluorescence detection.

EXPERIMENTAL

Drugs and reagents

Compound I and its metabolites (II, N-(2″-hydroxyethyl)-13-deoxo-10-hydroxycarminomycin; III, 13-deoxo-10-hydroxycarminomycin or oxaunomycin [8]; IV, γ-rhodomycinone [9]; V, β-rhodomycinone [10]) were prepared in our laboratory and are shown in Fig. 1. Adriamycinone (AON) and daunomycinone (DON) were used as internal standards for the determination of I, II and III as their glycosides and IV and V as their aglycones, respectively, and were prepared from adriamycin and daunomycin in our laboratory. Standard methanolic solu-

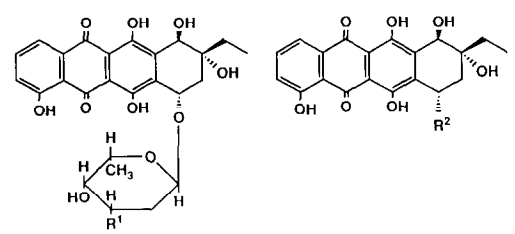

Fig. 1. Structures of I and its metabolites.

tions of these compounds, prepared at concentrations of 0.1 mg/ml, were protected from light and stored at −20°C. Chromatographic solvents were of HPLC grade. All other reagents were of analytical grade and were used without further purification. Deionized and distilled water was used for preparation of the mobile phase solutions and buffers.

High-performance liquid chromatography

The chromatographic system consisted of a Jasco (Tokyo, Japan) 880-PU pump, a Jasco 855-AS intelligent sampler equipped with a 100-μl sample loop, a Jasco 860-CO column oven, a Hitachi (Tokyo, Japan) F1000 fluorescence detector and a Jasco 805-GI graphic integrator. A 5-μm C_{18} column (150 mm × 6 mm I.D.; YMC A312 ODS, YMC, Kyoto, Japan) was used. The excitation and emission wavelengths used for fluorescence detection were 485 and 550 nm, respectively.

The mobile phase was tetrahydrofuran–1% triethylamine, pH adjusted to 2.0 with H_3PO_4 (20:80, v/v) for the determination of I, II and III (system I). For IV and V the mobile phase was acetonitrile–methanol–1% triethylamine, pH adjusted to 2.0 with H_3PO_4 (35:15:50, v/v) (system II). The flow-rates were 1.0 ml/min (system I) or 1.2 ml/min (system II), and the injection volume was 100 μl in both cases. Analyses were performed at 40°C.

Quantitation was based on the ratio of the peak heights of compounds *versus* the peak height of the internal standard.

Extraction procedure

Plasma. Rat plasma was diluted 1:1 in 1% H_3PO_4, and the mixture (1 ml) was filtered through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA) previously washed with methanol (5 ml) and distilled water (10 ml). The cartridge was washed with 2 ml of 0.1% H_3PO_4 and 10 ml of distilled water, then eluted with 2.5 ml of methanol. This eluate was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 0.5 ml of 0.2 *M* citrate buffer (pH 2.2) and 2.5 ml of diethyl ether. Then the solution was stirred vigorously for 30 s with a vortex mixer. After centrifugation at 1100 *g* for 5 min, the diethyl ether layer was evaporated. The residue was dissolved in 0.2 ml of system II mobile phase containing 50 ng/ml DON. To the aqueous layer, 0.25 ml of 1 *M* NaOH, 1 ml of 0.2 *M* phosphate buffer (pH 8.0) and 5 ml of chloroform–methanol (4:1) were added, and mixed for 30 s with a vortex mixer. After centrifugation at 1100 *g* for 5 min, the organic layer was collected and evaporated. The residue was dissolved in 0.2 ml of system I mobile phase containing 100 ng/ml AON.

Blood. Rat blood was diluted 1:1 in 20 m*M* KH_2PO_4–H_3PO_4 (pH 2.0) and haemolysed by vigorous mixing. The mixture was diluted 1:1 in 80% acetonitrile containing 10 m*M* $MgCl_2$, and was then mixed for 30 s with a vortex mixer. After centrifugation at 10 000 *g* for 5 min, a 1-ml sample of supernatant was collected. To the supernatant 1 ml of 0.2 *M* phosphate buffer (pH 8.0) and 8 ml of chloro-

form–methanol (4:1) were added. The mixture was shaken for 10 min and centrifuged at 1100 *g* for 10 min. The organic layer was evaporated, and the residue was dissolved in 0.5 ml of 0.2 *M* citrate buffer (pH 2.2) and 2.5 ml of diethyl ether. The mixture was subjected to the same treatment as that for the plasma sample.

Urine. Rat urine was diluted 1:1 in 80% acetonitrile containing 10 m*M* $MgCl_2$ and was mixed for 30 s with a vortex mixer. The mixture was subjected to the same treatment as the blood sample.

RESULTS AND DISCUSSION

High-performance liquid chromatography

The large differences in the polarities of the metabolites made it necessary to use two mobile phases (systems I and II) to achieve rapid separation.

Fig. 2a and d show typical chromatograms of I and its metabolites from rat plasma spiked with these compounds. AON and DON were used as internal standards for system I and system II, respectively. These anthracyclines were well separated from each other. Chromatograms obtained from similarly treated blank plasma are shown in Fig. 2b and e. Fig. 2c and f show the chromatograms for plasma samples obtained 4 h after intravenous administration of I (2 mg/kg) to a male rat.

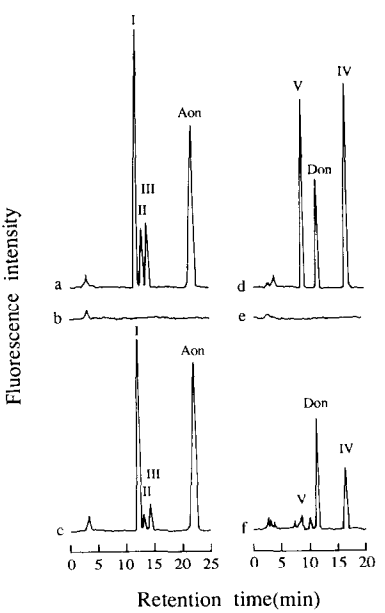

Fig. 2. HPLC of I and its metabolites: (a, b and c) glycoside fraction (HPLC system I); (d, e and f) aglycone fraction (HPLC system II); (a and d) plasma spiked with I (20 ng/ml), II (5 ng/ml), III (5 ng/ml), AON (100 ng/ml), IV (10 ng/ml), V (10 ng/ml) and DON (50 ng/ml); (b and e) blank plasma; (c and f) typical chromatograms of plasma from a rat 4 h after intravenous administraiton of 2 mg/kg I: I (14.20 ng/ml), II (1.39 ng/ml), III (2.05 ng/ml), AON (100 ng/ml), IV (3.09 ng/ml), V (0.82 ng/ml) and DON (50 ng/ml).

TABLE I

DETECTION LIMITS OF I AND ITS METABOLITES

Compound	Detection limit (ng/ml)		
	Plasma	Whole blood	Urine
I	0.45	0.70	0.41
II	0.61	0.92	0.52
III	0.53	0.84	0.59
IV	0.21	0.29	0.19
V	0.22	0.36	0.23

After extraction from the 0.5-ml plasma sample, the detection limits at a signal-to-noise ratio of 3 were in the range 0.21–0.61 ng/ml (Table I). The limits for whole blood (extracted from a 0.25-ml sample) and for urine (extracted from a 0.5-ml sample) are also shown in Table I.

The calibration lines for I and its metabolites in biological samples were obtained by using various amounts of these compounds and fixing the concentration of the internal standards. The relationship between the peak-height ratio and the concentration of I and its metabolites was linear in the range 1–500 ng/ml of plasma, whole blood and urine ($r > 0.999$).

Extraction procedure

The recoveries of the anthracyclines from the spiked biological samples were calculated by comparing the peak heights of the chromatograms of extracted samples with those of standards of identical concentrations in the mobile phases. The recoveries of I and its metabolites from rat plasma are shown in Table II. Coefficients of variation (C.V.) were all below 8.7% for these compounds. The

TABLE II

RECOVERY AND REPRODUCIBILITY OF THE DETERMINATION OF I AND ITS METABOLITES FROM RAT PLASMA

Concentration (ng/ml)	Recovery (mean ± C.V., $n = 5$) (%)				
	I	II	III	IV	V
1.0	86.7 ± 8.7	82.3 ± 3.8	77.6 ± 2.4	79.6 ± 4.1	83.8 ± 2.9
2.5	81.9 ± 2.7	79.1 ± 1.4	75.1 ± 2.8	80.4 ± 3.7	83.6 ± 2.6
10.0	83.9 ± 1.2	87.3 ± 3.2	75.7 ± 0.8	80.6 ± 2.4	84.6 ± 1.8
50.0	83.6 ± 3.0	79.7 ± 3.8	73.6 ± 4.2	82.8 ± 2.5	87.1 ± 1.7
200.0	83.7 ± 2.0	79.9 ± 3.4	72.0 ± 3.4	84.6 ± 1.8	89.3 ± 2.1

TABLE III

BETWEEN-DAY ACCURACY OF THE RECOVERY FROM RAT PLASMA, WHOLE BLOOD AND URINE (50 ng/ml)

Compound	Recovery (mean ± C.V., n = 5) (%)		
	Plasma	Whole blood	Urine
I	84.5 ± 2.1	90.8 ± 5.3	92.0 ± 6.7
II	80.2 ± 2.4	88.8 ± 5.2	84.9 ± 8.4
III	76.6 ± 3.4	81.4 ± 5.0	72.6 ± 7.2
IV	85.7 ± 2.0	91.7 ± 2.3	98.3 ± 3.1
V	92.3 ± 3.5	92.1 ± 2.3	97.0 ± 1.8

average percentage recoveries (± S.D.) from plasma over the range 1.0–200 ng/ml were as follows (calculated from the means of five different concentrations): 84.0 ± 1.7 (I), 81.7 ± 3.4 (II) 74.8 ± 2.1 (III), 81.6 ± 2.1 (IV) and 85.7 ± 2.5 (V).

The between-day accuracy of the assay, as indicated by the C.V., was calculated from the daily mean of analysis (n = 5) of the biological samples spiked with I and its metabolites at a concentration of 50 ng/ml analysed over five days (Table III). The C.V. were in the ranges 2.0–3.5% (plasma), 2.3–5.3% (whole blood) and 1.8–8.4% (urine).

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