Journal of Chromatography, 377 (1986) 415—422
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3028

Note

Rapid quantitative determination of four anthracyclines and their main metabolites in human nucleated haematopoietic cells

P.A.J. SPETH*, P.C.M. LINSSEN, J.B.M. BOEZEMAN, J.M.C. WESSELS and C. HAANEN

Department of Haematology, St. Radboud University Hospital, P.O. Box 9101, Nijmegen (The Netherlands)

(First received August 27th, 1985; revised manuscript received November 22nd, 1985)

Anthracyclines belong to the most potent cytostatic drugs in the treatment of cancer [1]. Their concentrations in body fluids have been determined using a number of methods, among which high-performance liquid chromatography (HPLC) has proved to be the most valuable [2–5].

This paper describes factors that determine the extraction recovery of anthracyclines from human haematopoietic cells. A modification of the method of Baurain et al. [3] permits rapid determination of the cellular concentration of four anthracycline compounds and their cytostatic active 13-hydroxy metabolites in the target cell. The method has been applied in studies of in vivo cellular pharmacokinetics in leukaemic cells, of which examples are given.

EXPERIMENTAL

Drugs and chemicals

Adriamycin (ADM), daunomycin (DNM), 4′-epi-adriamycin (E-ADM) and 4-demethoxydaunomycin (D-DNM) were kindly supplied by Farmitalia Carlo Erba (Milan, Italy). The 13-hydroxy metabolites adriamycinol (ADMol), 4′-epi-adriamycinol (E-ADMol), daunomycinol (DNMol) and 4-demethoxydaunomycinol (D-DNMol) were kindly supplied by Professor F. Arcamone (Farmitalia Carlo Erba). Desipramine was a gift from Ciba Geigy (Arnhem, The Netherlands). All other chemicals were purchased from Merck (Darmstadt, F.R.G.) and were of analytical grade.

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Preparation of cell suspension

Blood samples from patients were obtained in heparinized polypropylene tubes on ice. After immediate centrifugation, plasma was removed for analysis of the anthracycline concentration. To obtain a pure white blood cell suspension, the erythrocytes in the pellet were lysed with at least four volumes of ammonium chloride (8.29 g of ammonium chloride, 1 g of potassium bicarbonate and 37 mg of K_2EDTA per l) on ice [6]. After centrifugation at 0°C, the white cells were resuspended in 500 μl of cold phosphate buffered saline (pH 7.4). Cell numbers were counted. The recovery of the white cells after the lysis step amounted to 80—90%. Plasma and cell suspensions were stored at −20°C until analysis by HPLC.

Extraction

A 250-μl volume of the thawed cell suspension containing $< 10^7$ cells was sonicated at 75 W for 15 s and extracted twice with 3 ml of chloroform—methanol (9:1) supplemented with 100 μl of 1 *M* Tris buffer (pH 8.8) containing the internal standard (DNM for ADM, and ADMol for E-ADM, DNM and D-DNM). The two chloroform phases were collected and evaporated in one step at 35°C in air. The dry residue was dissolved in 750 μl of chloroform—methanol (9:1). After addition of 50 μl of Tris buffer, mixing and 5 min centrifugation at 1000 *g*, 500 μl of the organic phase were injected into the chromatographic system. Samples of 500 μl of plasma were extracted in a similar way.

High-performance liquid chromatography

The chromatographic system consisted of a double-head pump (LC3-XP, Pye Unicam, Cambridge, U.K.), a sampling valve (Valco, Houston, TX, U.S.A.) and a sampling loop of 500 μl. The analytical column (10 cm × 3.0 mm I.D.) was packed with spherical silica gel SI-60, particle size 7 μm (Chrompack, Middelburg, The Netherlands). A fluorescence detector (FS 970 L.S. Fluorometer, Schoeffel, Ramsey, NJ, U.S.A.) was used with an excitation wavelength of 488 nm. Emission was detected above 550 nm. The column was eluted with chloroform—methanol—glacial acetic acid—water (720:210:40:30) supplemented with 0.3 n*M* magnesium chloride and 10 μg/ml desipramine. The flow-rate was set at 0.8 ml/min. Quantification of the cellular drug concentrations was achieved by measuring the peak-height ratio of the drug and the internal standard, and it was calculated from a calibration curve of peak height versus concentration. The detection limit of the drugs investigated was 1 ng at a signal-to-noise ratio of 3:1. The results are expressed in ng/ml for plasma and cells, assuming that 10^9 cells equalize 1 ml.

Calibration curve

Samples of 250 μl of cell suspension were spiked with the appropriate drugs. A calibration curve in the anticipated range of 1—500 ng/ml was made each time before analysis. The correlation coefficients of the calibration curves were always > 0.96.

Day-to-day variation and recovery

At the start of this study for each of the investigated drugs, a suspension of nucleated blood cells was prepared, divided into numerous samples and stored at −20°C. These samples were used as so-called "reference samples" each time cells were analysed for their anthracycline concentration. Thus, the day-to-day variation over the whole procedure of extraction and analysis could be calculated over a long period of time. This variation was expressed as the standard deviation, divided by the mean and multiplied by 100%. The recoveries of the parent drug and the 13-hydroxy metabolites after extraction were measured over a concentration range of 5—200 ng/ml and were compared with the direct assay of the standard in chloroform–methanol (9:1).

Cellular pharmacokinetics

With the method described, the cellular and plasma pharmacokinetics of ADM, E-ADM, DNM and D-DNM were studied in patients treated for acute myelogenous leukaemia and solid tumours. The plasma and cell concentration data were fitted according to a two-compartment open model, and the pharmacokinetic parameters were calculated according to conventional procedures [7].

RESULTS AND DISCUSSION

Extraction efficiency

A number of factors determine the recovery of the extraction of anthracyclines from cell suspensions. Best results were obtained with the procedure that consisted of sonication of a 250-µl cell suspension containing $< 40 \cdot 10^6$ cells/ml, followed by two extraction steps with 3 ml of chloroform–methanol

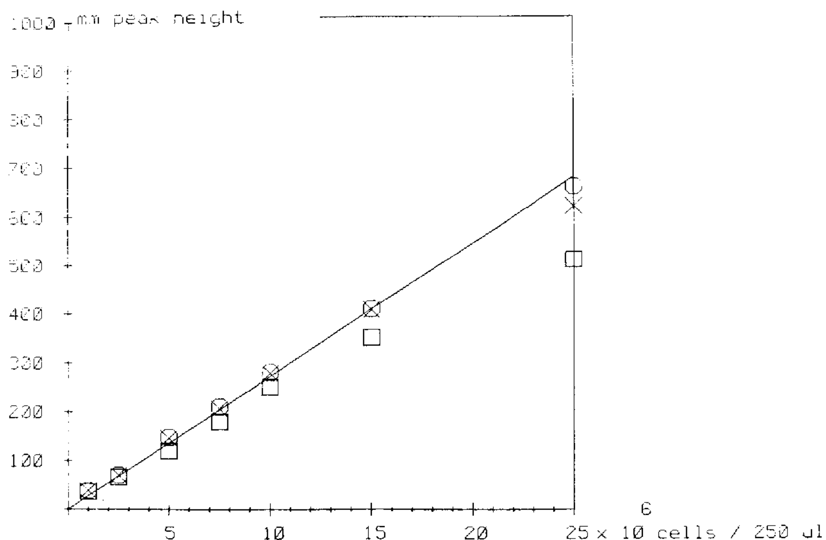

Fig. 1. Influence of absolute cell number on the recovery of the ADM extraction. Concentration of ADM per 10^6 cells after one (□), two (×), and three (○) extractions with 3 ml of chloroform—methanol (9:1) of a 250-µl cell suspension (data of one out of three experiments).

(9:1) under the addition of 100 μl of 1 M Tris buffer (pH 8.8), and evaporation of the collected organic phases in one step at 35°C in air. The data thus obtained were set at 100%. Variables in this method are discussed below and are related to these data.

Sonication of the cell suspension at 75 W for 15 s improved the extraction of anthracyclines from the non-sonicated cell suspension from 64 ± 18% to 100 ± 8% in the case of sonication (n = 9). The effects of cell concentration, as well as the number of extraction steps, on the recovery of ADM from cells is illustrated in Fig. 1. After three extractions with 3 ml of chloroform–methanol (9:1), the curve is linear up to 25 · 10^6 cells per 250 μl (r = 0.9993). After two extractions, linearity is observed to 15 · 10^6 cells per 250 μl (r = 0.9993). Two extractions of 25 · 10^6 cells per 250 μl, or one extraction of > 5 · 10^6 cells per 250 μl, revealed data which were outside the 95% confidence interval compared with the three extractions. Extraction with chloroform–methanol (4:1) resulted in recoveries of 77% (ADM), 68% (DNM), 74% (E-ADM) and 68% (D-DNM) compared to the extraction with chloroform–methanol (9:1) (n = 5).

With an increase in the volume of the cell suspension from 100 to 500 μl, a gradual decrease in recovery from 100 to 76% was observed after one extraction. A second extraction improved the recovery.

With the method described for ADM, E-ADM, DNM and D-DNM, linearity of extraction efficiency was observed up to 60 · 10^6 cells/ml (n = 16), with r values of 0.98 (ADM), 0.96 (E-ADM), 0.97 (DNM) and 0.96 (D-DNM) (Fig. 2).

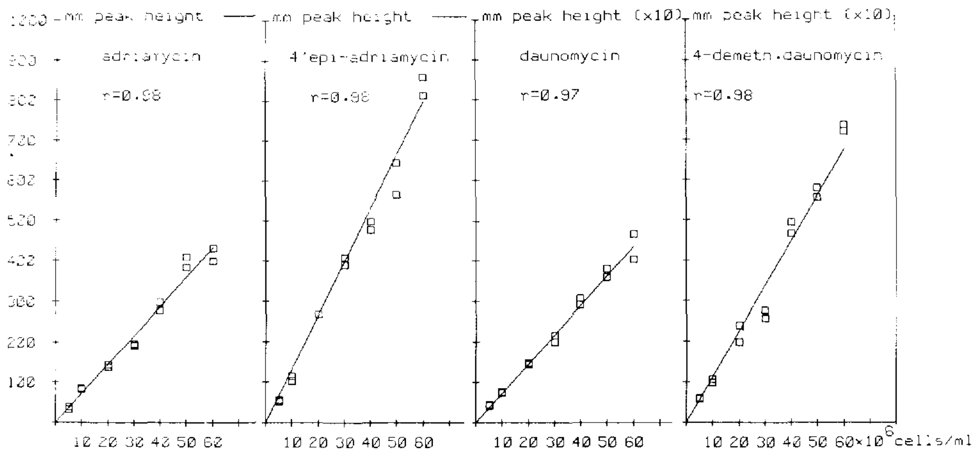

Fig. 2. Extraction efficiency of ADM, E-ADM, DNM and D-DNM from suspensions with increasing cell concentrations per ml. With the method described in the text, linearity is observed to > 40 · 10^6 cells/ml.

Stability, day-to-day variation and recovery

Each time the cellular anthracycline concentrations were measured, two reference samples were extracted as well. The storage period of the reference samples varied from six to nine months. The cellular concentration of the different anthracyclines did not change during this storage period (Table I). The

TABLE I

CELLULAR ANTHRACYCLINE CONCENTRATION OVER A STORAGE PERIOD OF UP TO NINE MONTHS AT − 20° C

n = 4.

	Concentration (ng per 10^6 cells)		
	One month	Nine months	Decrease
Adriamycin	2100 ± 200	2100 ± 200	N.S.*
4'-Epi-adriamycin	2500 ± 100	2400 ± 200	N.S.
Daunomycin	4600 ± 200	4500 ± 200	N.S.
4-Demethoxydaunomycin	3800 ± 300	3700 ± 300	N.S.

*N.S. = Not significantly different.

day-to-day variation over the whole procedure of extraction and chromatographic determination of the reference samples was 6.9% for ADM (n = 20), 9.1% for DNM (n = 18), 9.0% for E-ADM (n = 21) and 10.1% for D-DNM (n = 23%). The recoveries after the extraction procedure of the four anthracyclines were 92.4 ± 12.8% (ADM), 91.9 ± 8.8% (DNM), 83.9 ± 13.8% (E-ADM) and 94.3 ± 8.1% (D-DNM).

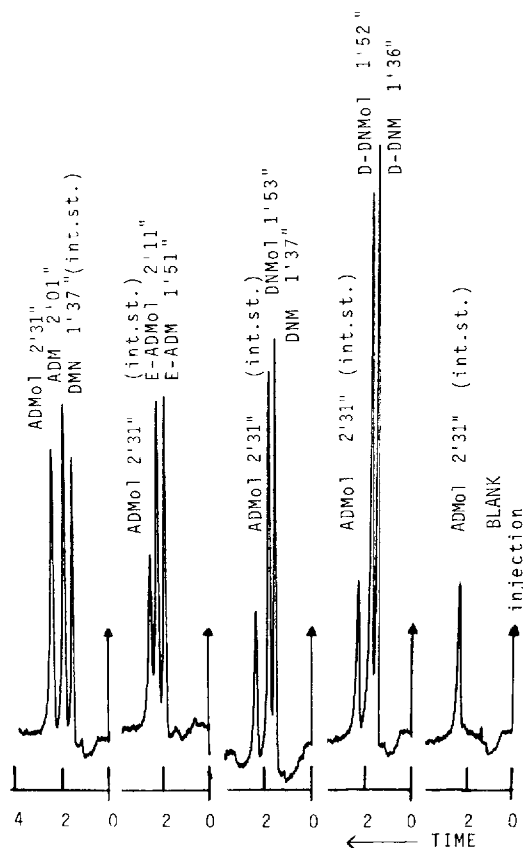

Fig. 3. Chromatograms of blank and spiked cellular samples. Samples were spiked with 25 ng/ml D-DNM, DNM, E-ADM, ADM and their 13-hydroxy metabolites.

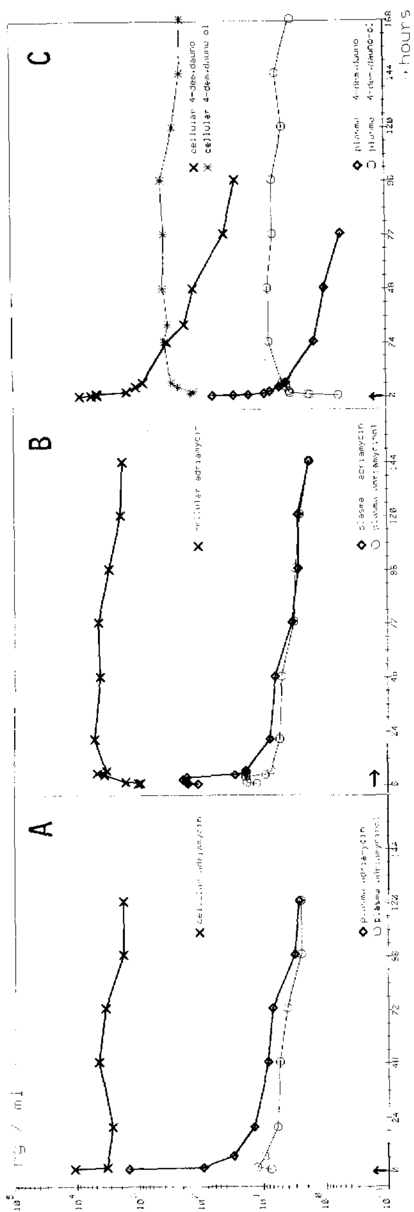

Fig. 4. The cellular and plasma concentration–time profiles of ADM and ADMol measured over a period of six days after 75 mg of ADM administered as a bolus injection (A) and during a 4-h infusion (B) or 18 mg of D-DNM as a bolus injection (C) in one patient.

Chromatography

Chromatograms of cell samples spiked with a 25 ng/ml cell suspension of ADM, E-ADM, DNM or D-DNM and their 13-hydroxy metabolites are shown in Fig. 3. Blank cells showed only the injection peak. Retention times were, at most, 2 min 31 s.

Cellular pharmacokinetics

Fig. 4 and Table II illustrate the importance of cellular pharmacokinetics, which differ considerably from plasma pharmacokinetics. ADM (75 mg) was administered either as a bolus injection or as a 4-h continuous infusion. D-DNM (18 mg) was administered as a bolus injection only. The most remarkable facts are the rapid cellular uptake during and the considerable loss a few minutes after the end of the bolus injection, the continuous cellular uptake in the case of prolonged infusion (up to a cellular/plasma ratio of > 800) and the long cellular terminal half-life. While no cellular ADMol was observed, D-DNMol very soon was the main intracellular drug.

Although the maximum plasma level was reduced one decade compared to a bolus injection in the case of prolonged ADM infusion, the cellular concentrations at the end of the infusion and thereafter were of the same order of magnitude as observed in the case of the bolus injection.

TABLE II

CELLULAR AND PLASMA PHARMACOKINETICS OF 75 mg OF ADRIAMYCIN GIVEN AS A BOLUS INJECTION AND AS A 4-h INFUSION IN ONE PATIENT, AND OF 18 mg OF 4-DEMETHOXYDAUNOMYCIN ADMINISTERED AS A BOLUS INJECTION

$t_{1/2\alpha}$ = first half-life time; $t_{1/2\beta}$ = terminal half-life time; A and B are calculated concentration constants; AUC = area under the curve.

Drug	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	A (ng/ml)	B (ng/ml)	AUC (μg h/l)
Plasma					
Adriamycin bolus	0.3	34.3	818	25.5	1601
Adriamycin infusion	0.05	42.8	900	14	1552
4-Demethoxydaunomycin	0.4	47.2	37.6	2.4	188
Cellular					
Adriamycin bolus	0.1	103	11900	4237	637700
Adriamycin infusion	0.1	117	9500	4511	765880
4-Demethoxydaunomycin	0.6	15	6635	1035	28457

CONCLUSION

In pharmacokinetic studies of anthracycline antibiotics, great effort is generally given to evaluating the disappearance rate of plasma concentrations [2, 8—10]. However, cellular concentration--time profiles are much more relevant in vivo, since cells are the target of the drug. In studies on cellular pharmacokinetics so far [11—14], no attention has been paid to the specific factors that affect the extraction recovery of anthracyclines from human haematopoietic cells. Extraction of suspensions containing $> 40 \cdot 10^6$ cells/ml

is likely to underestimate the actual cellular drug concentration, unless attention is paid to the absolute cell number and the volume that is extracted, to prior sonication, and to the number of extraction steps.

Eksborg et al. [15] described a decrease in ADM plasma concentration after a prolonged storage time. This was not confirmed by Oosterbaan et al. [9]. In our study, no decline in cellular concentration was observed over a storage period of nine months at −20°C in cell suspensions containing ADM, DNM, E-ADM and D-DNM.

Preliminary data from our laboratory suggest good correlations between cellular drug concentrations and cytotoxicity. Studies on drug concentrations in the target cells in vivo should be extended to improve our knowledge of the working mechanism of cytostatic drugs. Since only mean cellular drug concentrations are obtained with this method, these observations also have to be extended to flow cytometric studies of anthracycline concentrations in individual cells [16]. However, chromatography will remain necessary and will be complementary to this technique.

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