Determination of Adriamycin and Its Fluorescent Metabolites in Biological Fluids of Inpatients with Lung Cancer by High Performance Liquid Chromatography

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A normal-phase high performance liquid chromatographic determination of adriamycin and its metabolites, adriamycinol and adriamycinone, in human body fluids (plasma, urine and pleural fluids) is described. Samples were collected from lung cancer patients who had received adriamycin by peripheral vein infusion, bronchial arterial infusion and intrapleural infusion. Adriamycin and its metabolites were easily and efficiently extracted from each biological fluid by the use of an Extrelut® column. High performance liquid chromatographic separation was carried out in the presence of Mg^{2+} and the limits of determination of adriamycin, adriamycinone and adriamycinol were found to be 0.52, 0.72 and 0.48 ng/ml, respectively. Chromatographic analyses were completed within 8 min.

The present method was also applied to pharmacokinetic studies of adriamycin, and it was found that the metabolic fate of adriamycin can be well explained by a two-compartment model.

Keywords——adriamycin; adriamycinol; adriamycinone; fluorescence detector; high performance liquid chromatographic determination

Adriamycin (doxorubicin hydrochloride, ADM) is an anthracycline antibiotic used in the treatment of acute leukemia, malignant lymphoma, lung cancer and many other malignant tumors. Doses and the method of administration of ADM should be controlled carefully to avoid its toxic side effects, such as cardiac insufficiency and digestive disturbance. Therefore, it is necessary to monitor the levels of ADM in body fluids to get maximum therapeutic effects and to minimize toxic side effects.

Many methods including high performance liquid chromatography (HPLC) have been developed for the determination of ADM and its metabolites.[1−3] In the present investigation, we developed facile and rapid methods for determination and pretreatment of clinical samples by taking the stability and adsorptive properties of the drugs into consideration. Clinical samples were obtained from lung cancer patients who had received ADM via three routes,[4,5] peripheral vein infusion (PVI), bronchial arterial infusion (BAI) and intrapleural infusion (IPI). Some pharmacokinetic parameters were calculated on the basis of typical plasma concentration–time curves obtained by the present methods.

Experimental

Chemicals and Materials——ADM, adriamycinone (AON), adriamycinol (AOL) were obtained from Farmitalia (Milan, Italy) and daunomycin (DAU) was kindly supplied by Meiji Seika Co. (Tokyo, Japan). Standard solutions of these compounds were prepared in methanol at 5 μg/ml concentration; they were protected from light and stored at −20 °C. ADM injections (Adriacin inj.) were purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). Chromatographic grade methylene chloride and methanol were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and chloroform, glacial acetic acid, magnesium chloride, sodium phosphate mono-hydride and potassium phosphate dihydride were of analytical grade (Koso Chemical Co., Tokyo, Japan). Control plasma and

TABLE I.   Patients' Characteristics

Patient	Sex	Age	Histologic type of carcinoma	Method of administration[a]
No. 1	Male	65	Squamous cell carcinoma	PVI
No. 2	Male	44	Small cell carcinoma	PVI
No. 3	Male	59	Small cell carcinoma	PVI
No. 4	Male	59	Malignant lymphoma	PVI
No. 5	Male	69	Squamous cell carcinoma	BAI
No. 6	Female	57	Adenocarcinoma	BAI
No. 7	Male	51	Adenocarcinoma	BAI
No. 8	Male	51	Adenocarcinoma	IPI
No. 9	Male	83	Adenocarcinoma	IPI
No. 10	Male	40	Adenocarcinoma	IPI
No. 11	Female	59	Small cell carcinoma	IPI
No. 12	Female	44	Adenocarcinoma	IPI

Amount of ADM administered was 50 mg for all cases, except for No. 4 (80 mg).   a) PVI, peripheral vein infusion; BAI, bronchial arterial infusion; IPI, intrapleural infusion.

urine were supplied by several healthy volunteers and pleural fluids were collected from patients before drug administration.

Apparatus——A Shimadzu LC-3A high performance liquid chromatograph equipped with a Rheodyne model 7125 syringe loading sample injector with a 50 μl loop was used throughout this investigation. The chromatographic detector used was a Shimadzu RF-530 fluorescence monitor (flow-cell volume 12 μl), operating at 480 and 590 nm (excitation and emission, respectively). Peak areas were computed on a Shimadzu C-R1B Chromatopac integrator. The stationary phase was silica gel (Zorbax® BP-SIL, Du PONT, 5 μm) packed into a 4.6 i.d. × 250 mm stainless-steel column. The mobile phase was a mixture of methylene chloride, methanol, glacial acetic acid and 0.01 M magnesium chloride solution (200 : 50 : 7 : 5, v/v) and was used at a flow rate of 1.5 ml/min at room temperature.

Routes of Administration——The subjects were twelve lung cancer patients admitted to Tokyo National Chest Hospital; the clinical data are summarized in Table I. ADM was administered to these patients at a dosage of 50 or 80 mg via one of the following three routes.

PVI: A 50 ml isotonic sodium chloride solution containing ADM was administered via the antecubital vein on one arm over 20 min. Blood samples were collected from the vein of the other side.

BAI: The same ADM solution as above was administered over 20 min via an infusion catheter placed into the bronchial artery through the femoral artery by Seldinger's technique.

IPI: This method of administration was applied to the patients who had accumulated pleural effusion in the thorax. ADM solution (100 ml) prepared in an isotonic sodium chloride or 10% Tween 80 solution was injected to the pleural cavity via a draining tube. An attempt was made to distribute the administered drug equally in the pleural cavity by means of several postural changes.

Methods of Sampling——In the case of IPI, pleural fluids were collected at 3, 7, 15 and 27 h after injection through a draining tube.

Blood samples were collected at 5, 15, 30 min, or 1, 2, 12 and 24 h after administration for PVI or BAI. In the case of IPI, they were collected at 1, 2, 4 and 8 h after administration. Five ml of blood was collected in a test-tube containing heparin at the indicated time after the end of infusion. The sample was centrifuged at 2500 g for 10 min, and the plasma fraction was separated and kept frozen at −20 °C until assay.

Urine samples were collected every 6 h during the first 24 h and every 24 h thereafter up to 72 h. In some cases, however, samples could not be collected due to the condition of the patients. Urine samples were also stored in the dark and kept frozen at −20 °C.

Extraction Procedure——A mixture of biological fluid (50—500 μl), 0.1 M phosphate buffer solution (pH 7.8, 40—100 μl) and DAU (5 μg/ml, 20 μl) as an internal standard was poured onto the top of an Extrelut® column. The column was eluted with a mixture of chloroform–methanol (10 : 1, v/v; 20—30 ml). The eluate was evaporated to dryness under a vacuum below 35 °C, and the residue was dissolved in a chromatographic elution mixture (100—200 μl). Twenty μl of this solution was injected into the HPLC system.

This extraction method was applicable to all the biological fluids (plasma, urine and pleural fluids).

Results and Discussion

Quantitative Analysis

For quantitation of ADM and its metabolites by HPLC, both normal-phase and

reversed-phase chromatographic systems have been proposed elsewhere.[6−9] In the present investigation, the normal-phase system was chosen to minimize adsorption of ADM and its metabolites in the chromatographic system. Reduction of the adsorption was also achieved by adding Mg^{2+} ion[10,11] to the chromatographic elution mixture. The addition of Mg^{2+} also improved the separation, giving sharper peaks and better reproducibility.

Calibration lines for ADM and its metabolites in control plasma obtained by the peak area method using DAU as an internal standard were found to be linear up to 1.5 ng/ml for each compound, and the correlation coefficients (r) for ADM, AON and AOL were 0.998, 0.991 and 0.994, respectively. The coefficients of variation were 2.0% at lower concentrations (<0.5 μg/ml) and 1.3% at higher concentrations (>1.0 μg/ml). The limits of determination of this assay (at three times the noise level) were about 0.52, 0.72 and 0.48 ng/ml for ADM, AON and AOL, respectively. Elutions were completed within about 8 min.

Recoveries of ADM, AON and AOL by this technique were more than 98% (Table II) as examined by using a control plasma spiked with these compounds. Figure 1 shows typical chromatograms for biological samples obtained under the conditions mentioned above. The capacity factors (k') for AON, DAU, ADM and AOL were 0.55, 1.50, 2.35 and 3.84,

TABLE II.   Recoveries and Reproducibilities of Determination of Adriamycin, Adriamycinone and Adriamycinol in Plasma[a]

Standard drugs added (μg/ml)	Observed concentration mean ± S.D. μg/ml (recovery %, C.V. %)[b]		
	Adriamycin	Adriamycinone	Adriamycinol
0.25	0.251 ± 0.004 (100.3, 1.60)	0.254 ± 0.006 (101.6, 2.36)	0.252 ± 0.003 (100.8, 1.19)
0.50	0.503 ± 0.004 (100.6, 0.79)	0.533 ± 0.018 (106.6, 3.38)	0.497 ± 0.011 (99.4, 2.21)
1.00	0.997 ± 0.004 (99.7, 0.40)	1.037 ± 0.022 (103.7, 2.12)	0.982 ± 0.008 (98.2, 0.81)

a) Standard samples were added to 0.2 ml of standard plasma to obtain concentrations of 0.25, 0.50, 1.00 μg/ml of each compound and 20 μl of 5 μg/ml daunomycin (internal standard) and 0.1 M phosphate buffer (pH 7.8). These preparations were treated with Extrelut® and subjected to HPLC. *b*) S.D.: standard deviation; C.V.: coefficient of variation.
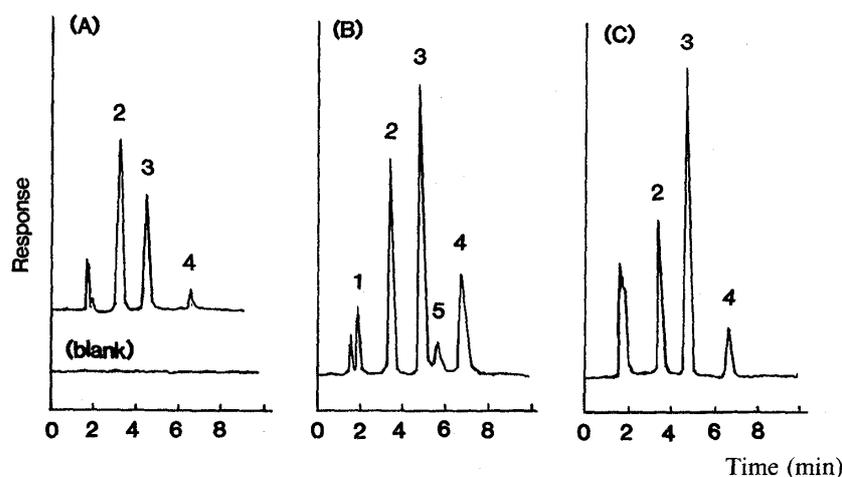

Time (min)

Fig. 1.   Typical Chromatograms of Samples Collected from Lung Cancer Patients Being Treated with Adriamycin

(A), plasma; (B), urine; (C), pleural fluid. 1, adriamycinone; 2, daunomycin (internal standard, 10 ng); 3, adriamycin; 4, adriamycinol; 5, unknown (occasionally seen in the case of the IPI method).

respectively.

As described by Benvenuto *et al.*,[12] determination of ADM was difficult because of its labile nature as well as its adsorption on glassware. In the present investigation, the stability of ADM was studied both in control plasma and in an isotonic sodium chloride solution at 37 °C and at -20 °C. ADM disappeared rapidly in both isotonic sodium chloride solution and plasma at 37 °C with half-lives of 26.2 and 2.30 h, respectively. On the other hand, almost no recognizable change was observed for at least 8 d when kept frozen at -20 °C. Thus, it was decided that samples containing ADM should be frozen at -20 °C immediately after sampling.

Pharmacokinetic Results

Time courses of plasma concentration of ADM after administration by PVI, BAI and IPI are shown in Fig. 2.

In the cases of PVI and BAI, ADM concentration decreased biphasically, and its biological half-lives were 6—8 min for α-$T_{1/2}$ and 3—10 h for β-$T_{1/2}$. Since α-$T_{1/2}$ was very short, it was assumed that the distribution of ADM to tissues was very rapid, while β-$T_{1/2}$, which reflects an excretion process, was long, which indicated that the loss from tissues was very slow.

As for IPI, the transition during the first 1 h after injection was not determined since samples were not available. However, the subsequent course with a half-life of 2—5 h was
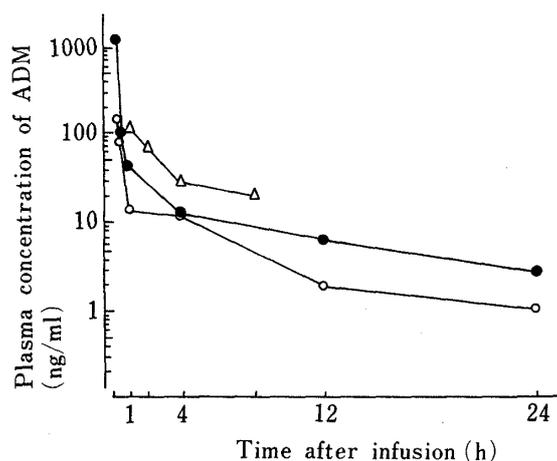

Fig. 2.   Time Course of Plasma Concentration of Adriamycin Plotted on a Semilogarithmic Scale

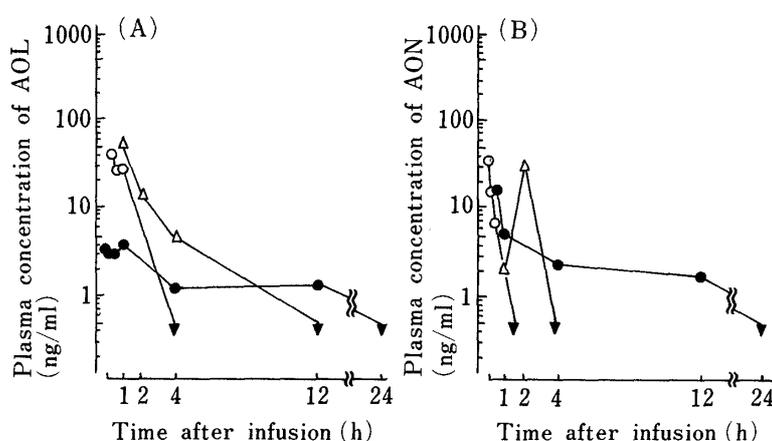Methods of administration were: (○), PVI; (●), BAI; (△), IPI.

Fig. 3.   Time Courses of Plasma Concentration of Adriamycin Metabolites Plotted on a Semilogarithmic Scale

(A), adriamycinone; (B), adriamycinol. Methods of administration were: (○), PVI; (●), BAI; (△), IPI. The symbol (▼) indicates less than 0.5 ng/ml plasma concentration (the limit of determination of this procedure).

similar to that of the β-phase of PVI and BAI.

Plasma concentrations of ADM metabolites are shown in Fig. 3A, B. It was generally observed that the concentration of metabolites in plasma was not as high as that of ADM itself, but after BAI, they were detectable even during 12—24 h after infusion.

Excretion in Urine

Cumulative urinary excretion of ADM is shown in Fig. 4. Total recoveries of residual ADM in urine were 1.4—10.5% of the amount of ADM administered for PVI, 8.5—15.0% for BAI and 3.4—10.0% for IPI. In each case, more than 80—90% of total ADM excreted in the urine was excreted within 24 h after treatment, and in some cases ADM was detected 72 h after administration.

It should be noted that urinary excretions varied among individuals, as they were directly related to the volume of urine. For example, a patient with pleuritis carcinomatosa did not excrete much urine due to accumulation of a large quantity of pleural fluid. As a consequence, urinary excretion of metabolites was decreased significantly in the case of IPI.

Cumulative amounts of metabolites excreted in urine are shown in Fig. 5A, B as percentages of ADM administered. A small quantity of AON was detected in the cases of BAI (0.3%) and IPI (0.4%). AOL was found to be slightly larger in quantity than AON, 0.4—1.4% with PVI, 0.7—2.4% with BAI and 0.3—2.7% with IPI. The amount of ADM excreted decreased gradually (Fig. 4), while AOL was excreted at a constant rate (*ca.* 4—20 μg/h AOL) up to 72 h after administration.

As shown in Fig. 6, 5—16% of ADM administered in pleural fluid was recovered unchanged and 0.3—1.0% of it was recovered as AOL.

There were some unknown fluorescent compounds which were separated and detected on HPLC, especially when ADM was injected into the pleural cavity by the IPI method. The amount of these unknowns was larger in urine or pleural fluid than in plasma (Fig. 1). It remains to be determined whether these unknown ADM metabolites have either negative or positive effects on chemotherapy.

Pharmacokinetic parameters calculated on the basis of plasma concentration versus time curves (Fig. 2) are summarized in Table III, though data for IPI are excluded due to lack of adequate sample numbers. The parameters were computed by using a nonlinear iterative least-squares method, and were found to be best fitted by a two-compartment model. The results were not averaged because of relatively large variances, which could be attributed to differences of sex, age and condition of patients. However, each set of parameters obtained with individual patients indicates that ADM has a tendency to be adsorbed in tissues. This view is based on the fact that the biological half-lives β-$T_{1/2}$ were much larger than α-$T_{1/2}$.
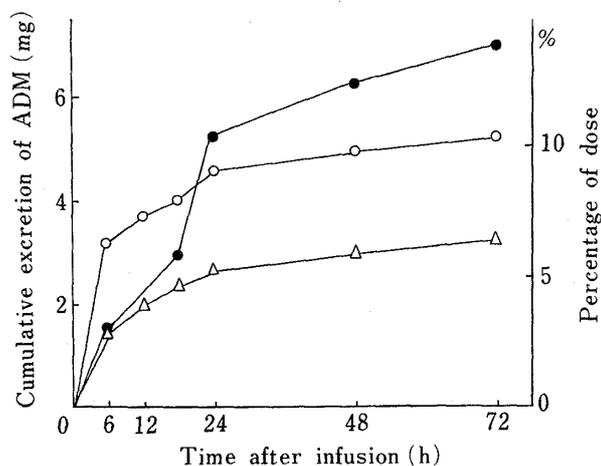

Fig. 4. Cumulative Urinary Excretion of Adriamycin

Methods of administration were: (O), PVI; (●), BAI; (△), IPI. The results are expressed as the total quantity (mg) and as percentage of the dose.
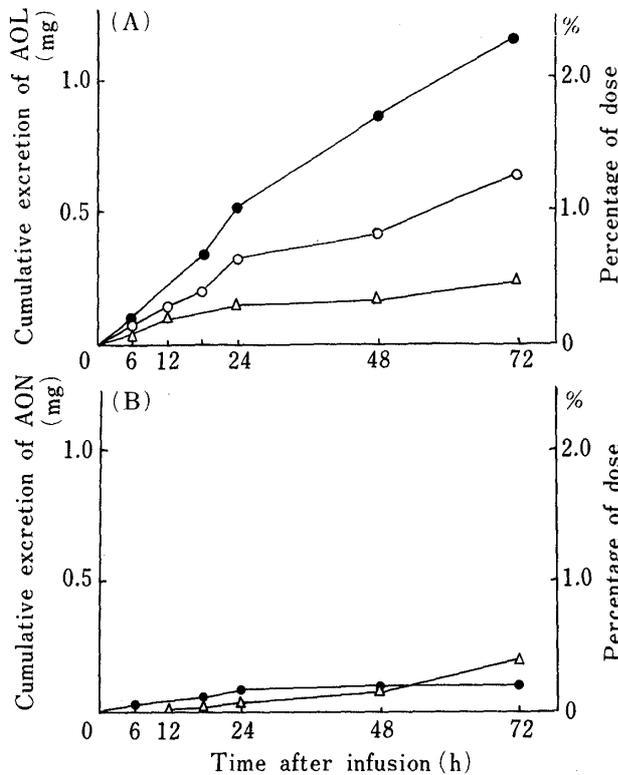
Fig. 5. Cumulative Urinary Excretion of Adriamycin Metabolites

The results are expressed as total adriamycin equivalents (mg) and as percentage of the dose, (A), adriamycinol; (B), adriamycinone. Methods of administration were: (O), PVI; (●), BAI; (△), IPI.
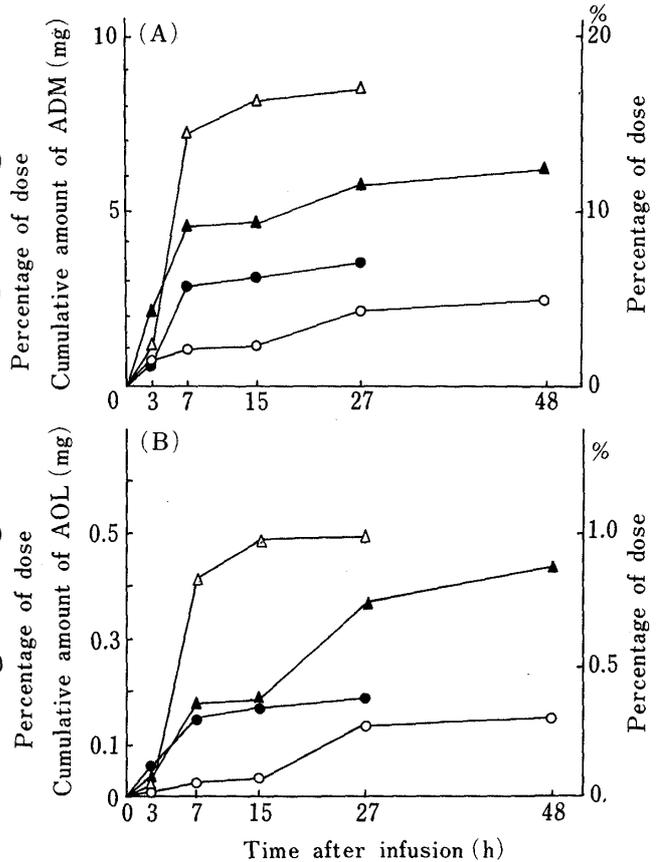
Fig. 6. Cumulative Amounts of (A) Adriamycin and (B) Adriamycinol in Pleural Fluid after IPI Administration

Each line represents an individual patient: (O), No. 8; (●), No. 9; (△), No. 10; (▲), No. 11 (Table I). The results are expressed as total quantity (mg) and as percentage of the dose.

TABLE III. Pharmacokinetic Parameters for Adriamycin Obtained from the Plasma Concentration *versus* Time Curves[a]

| Patient No. | $C = A \times \exp(-\alpha \times t) + B \times \exp(-\beta \times t)$ | | | | $\alpha - T_{1/2}$ (min) | (Eq. 1) $\beta - T_{1/2}$ (h) |
	A	α	B	β		
1	1054.2	5.20	8.82	0.09	8.00	7.72
2	1486.0	4.30	11.08	0.04	9.67	19.35
3	520.5	8.66	45.25	0.18	4.80	3.95
4	749.1	16.88	36.41	0.09	2.47	7.75
5	441.2	4.42	22.96	0.07	7.67	9.27
6	1336.8	5.42	18.22	0.07	9.42	9.68
7	578.3	5.47	87.80	0.06	7.62	10.98

a) Calculated parameters were fitted to a two-compartment model (Eq. 1). b) Refer to Table I for the patient numbers. $\alpha - T_{1/2}$ or $\beta - T_{1/2}$ are biological half-lives for the α- or β-phase.

In conclusion, it was shown that the method of determination described in this paper is practically useful, since it can provide accurate data after a simple pretreatment of samples, the volume of which is small enough to be readily tolerable by the patients. This claim was also supported by pharmacokinetic studies which revealed a reasonable metabolic fate of

ADM in terms of a two-compartment model.

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