Journal of Chromatography, 196 (1980) 463–469
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,894

DETERMINATION OF THE CONCENTRATION OF ADRIAMYCIN AND ITS METABOLITES IN THE SERUM AND TISSUES OF EHRLICH CARCINOMA-BEARING MICE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SHINYA SHINOZAWA*, YUUICHI MIMAKI and YASUNORI ARAKI
Department of Hospital Pharmacy, Okayama University Medical School, 2-5-1, Shikata-cho, Okayama 700 (Japan)
and
TAKUZO ODA
Department of Biochemistry Cancer Institute, Okayama University Medical School, 2-5-1, Shikata-cho, Okayama 700 (Japan)
(Received March 12th, 1980)

SUMMARY

The concentration of adriamycin and its metabolites in serum and tissues was determined by high-performance liquid chromatography (HPLC) with a lapse of time after a single intraperitoneal administration to Ehrlich carcinoma-bearing mice. HPLC was carried out by using Zorbax Sil as the stationary phase and 3.8% sodium acetate in isopropanol as the mobile phase, at an excitation wavelength of 470 nm and an emission wavelength of 585 nm. Extraction of adriamycin using chloroform–methanol (4:1) gave recoveries of 98% from serum and 66–96% from tissues. Adriamycin rapidly disappeared from the serum, and the content of adriamycin per gram of tissue decreased in the order liver, duodenum, kidneys, spleen, lung, heart and tumour, and a high tissue retention occurred in the spleen and heart. The main metabolites of adriamycin were adriamycinone and adriamycinol, and other minor metabolites were detected.

INTRODUCTION

Adriamycin is an anthracycline antibiotic used extensively for the treatment of leukaemia and various malignant tumours[1]. Adriamycin in biological tissues has been determined by bioassay[2], fluorescence methods[3–7], isotope methods[2,8–10] and high-performance liquid chromatography (HPLC)[10–14]. The mechanism of action of this antibiotic indicates that it has an especially strong affinity with DNA and tissue proteins[15,16], and there are still many problems to be solved in the methods for its determination in biological materials owing to its inactivation[2] and low recovery rate.

We have examined the method of extraction of adriamycin from biological tissues and determined the concentration of adriamycin and its metabolites in the

0021-9673/80/0000–0000/$02.25 © 1980 Elsevier Scientific Publishing Company

serum and tissues of Ehrlich carcinoma-bearing mice by HPLC with a lapse of time after intraperitoneal injection.

MATERIALS AND METHODS

Ehrlich ascites carcinoma ($5 \cdot 10^6$ cells per animal) was inoculated on the backs of ddN female mice (body weight 20–25 g, 6 weeks old) and the animals were submitted to the treatment after 20 days. The animals had free access to diet and water.

Adriamycin hydrochloride, adriamycinone, adriamycinol and daunomycin were kindly donated by Farmitalia (Milan, Italy). Adriamycin hydrochloride was dissolved in sterilized saline solution and administered intraperitoneally to mice at a dose of 8 mg/kg, and there were three mice in each group. At definite intervals (20 min, 60 min, 5 h and 24 h after the administration), blood was collected from the cervical artery and diluted 10-fold with 10 m*M* phosphate-buffered saline (pH 7.8). Liver, kidneys, spleen, duodenum (with contents), lung, heart and tumour tissues were excised, washed with sterilized saline solution and cut into small pieces in 2 ml of 10 m*M* phosphate-buffered saline (pH 7.8). The pieces of the organs were homogenized with a Polytron homogenizer to make 5–10% homogenate, and the homogenates were stored at −20°C until taken for measurement. The procedure for the extraction of adriamycin from the serum and tissues is shown in Fig. 1.

1 ml of diluted serum and 5–10% tissue homogenate in 10 m*M* phosphate-buffered saline (pH 7.8)
↓ + 5 ml of chloroform–methanol (4:1)
Extract by shaking for 10 min at room temperature
↓
Centrifuge at 3000 rpm for 10 min
↓
Lower organic phase
↓
Filter with Millipore filter (0.45 μm)
↓
Evaporate to dryness under vacuum
↓
Dilute with chromatographic elution mixture (200–500 μl)
↓
Inject 5 μl with microsyringe
↓
HPLC analysis

Fig. 1. Procedure for extraction of adriamycin.

High-performance liquid chromatography

A Hitachi Model 635A high-performance liquid chromatograph was connected to a Hitachi Model 650-10S high-sensitivity fluorescence detector, and the results were recorded on a Hitachi Model 056 recorder. The stationary phase was Zorbax Sil (5 μm) packed in a stainless-steel tube (150 × 4.6 mm I.D.). The mobile phase was 3.8% sodium acetate (pH 4.5) in isopropanol at a flow-rate of 1.0 ml/min. Measurements were made by the method previously described[17] at an excitation wavelength of 470 nm and an emission wavelength of 585 nm, using the ratio of the peak area to that of an internal standard (daunomycin).

Recovery of adriamycin

To 1 ml of mouse serum and 5–10% tissue homogenates were added 50 μl of a solution containing 1, 5 or 50 μg of adriamycin hydrochloride. Adriamycin was extracted from the serum and tissue homogenates as shown in Fig. 1, and determined by HPLC, and the recovery rates of the antibiotic were calculated.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on plates coated with a 250-μm layer of silica gel 60 (Merck, Darmstadt, G.F.R.), using as solvent systems chloroform–methanol–acetic acid–water in the proportions (1) 100:50:14:6 and (2) 100:100:14:14. After chromatography the fluorescent areas were detected under 253.7-nm light.

RESULTS

The determination of adriamycin in biological samples by HPLC with a fluorescence detector, at an excitation wavelength of 470 nm and an emission wavelength of 585 nm, was found to be subject to the least effect of the contaminating blank, and the separation of adriamycin, adriamycinone, adriamycinol and daunomycin was good (Fig. 2).

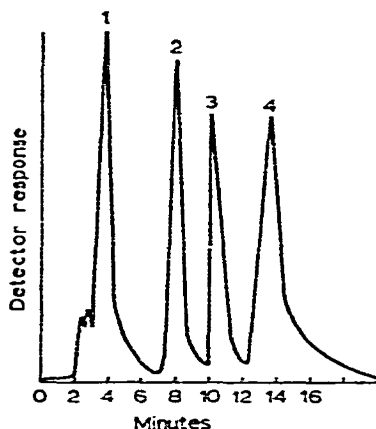


Fig. 2. High-performance liquid chromatogram of a standard sample mixture obtained with a Hitachi Model 635A high-performance liquid chromatograph with a Zorbax Sil column (150 × 4.6 mm I.D.) at room temperature. Mobile phase, 3.8% sodium acetate in isopropanol; flow-rate, 1 ml/min; detector, Hitachi Model 650-10S fluorescence spectrophotometer (excitation, 470 nm; emission, 585 nm); sensitivity, 10; fine, 6; pen range, 5 mV. Peaks: 1 = adriamycinone; 2 = daunomycin; 3 = adriamycin; 4 = adriamycinol.

The recoveries of adriamycin from serum and tissues using the procedure in Fig. 1 were calculated, and were 98.1 ± 2.4% from serum, 90.4 ± 8.8% from the liver, 88.5 ± 5.5% from the kidneys, 75.7 ± 8.3% from the spleen, 66.6 ± 8.0% from the duodenum, 87.5 ± 4.1% from the lung, 96.0 ± 3.0% from the heart and 92.1 ± 4.6% from the tumour tissue (n = 6, mean ± standard deviation).

Tissue distribution

Total adriamycin equivalents (the concentration of adriamycin plus its metabolites in the serum, after its intraperitoneal administration to Ehrlich carcinoma-bearing mice, disappear rapidly and the peak appears 10 min after administration. At that time, the peak level in serum was higher than that in all other tissues examined, but the level declined rapidly thereafter until 120 min. A slight elevation was seen at 5 h but the level was low (0.27 μg/ml) at 48 h (Fig. 3). The distribution of total adriamycin equivalents per gram of tissue was high in the liver, duodenum and kidneys. The antibiotic was present in highest concentration in the liver, the maximum level

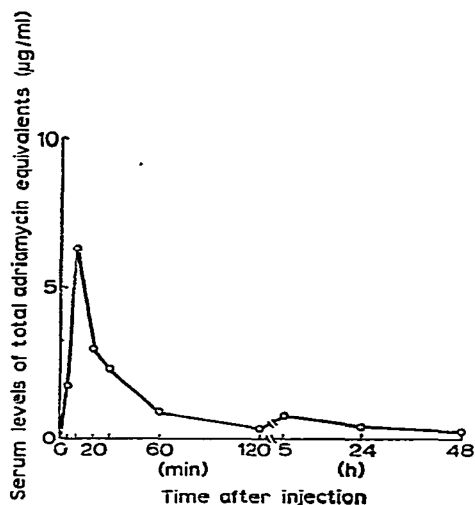


Fig. 3. Serum levels of total adriamycin equivalents in Ehrlich carcinoma-bearing mice after a single intraperitoneal injection, examined by HPLC.

being 31.97 μg/g 20 min after administration, and the high level was maintained until 120 min. A two-peak distribution was seen in the liver duodenum and kidneys, there being high peaks at 20 and 120 min (liver, duodenum) and at 20 and 60 min (kidneys). The highest concentration of adriamycin (2.5 μg/g) in the lung appeared 30 min after administration and the antibiotic disappeared gradually thereafter. The concentration of adriamycin in Ehrlich solid tumour was much lower than in other tissues, the highest value being 0.25 μg/g 20 min after administration. The tissue affinity of adriamycin was high in the heart and spleen, and transitory increases were seen at 48 h (Fig. 4). Although a high concentration of adriamycin was seen 48 h after its administration in the spleen, this is due to diminution of the spleen tissue, and the content per organ as a whole is decreased (Fig. 5).

Metabolites

Typical examples of HPLC and TLC analyses of serum are shown in Figs. 6 and 7. Peaks 1–6 (P1–P6) in Fig. 6 represent adriamycin-related compounds. P3 could be identified as adriamycinone and P6 as adriamycinol, but it was not clear what metabolites P1, P2, P4 and P5 were. P1, when examined by HPLC and TLC,

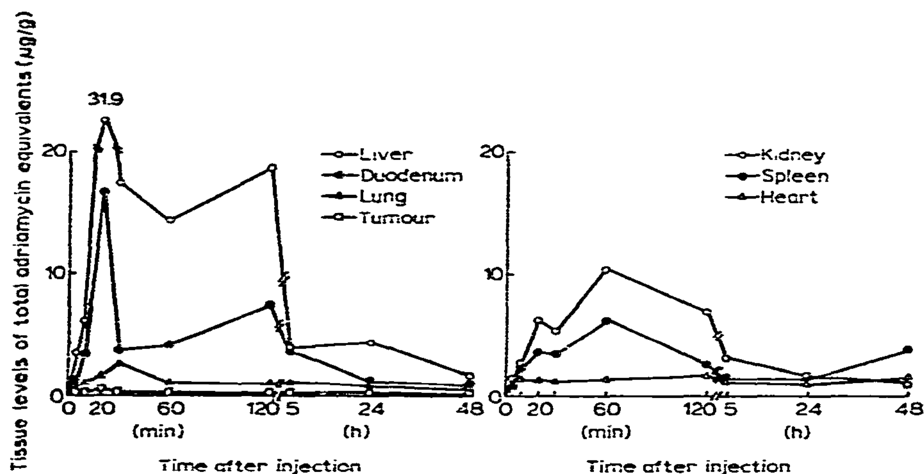


Fig. 4. Tissue levels of total adriamycin equivalents in Ehrlich carcinoma-bearing mice after a single intraperitoneal injection, examined by HPLC.

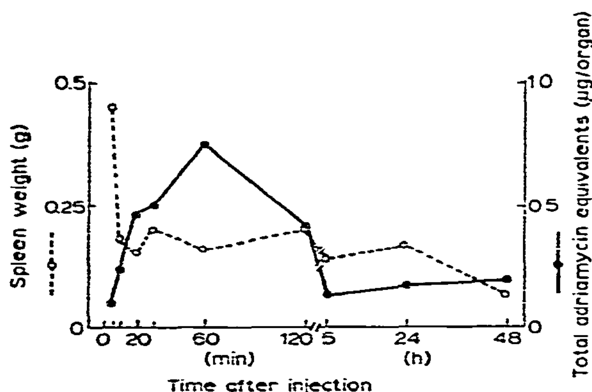
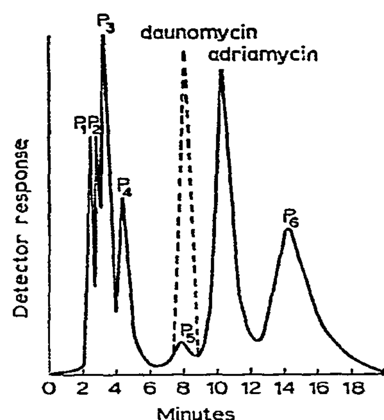


Fig. 5. Changes of spleen weight and total adriamycin equivalents of spleen in Ehrlich carcinoma-bearing mice after a single intraperitoneal injection, examined by HPLC.

showed a fluorescent compound, but it was not clear whether it was due to a tissue blank or an adriamycin metabolite. P5 was detected at the same position as daunomycin when examined by HPLC and TLC, but represented only a trace amount. Therefore, P1 and P5 equivalents were not included in the total adriamycin equivalent values.

The ratios (%) of adriamycin, adriamycinol, AD-NE metabolite (P2 metabolite + adriamycinone) and P4 metabolite to total adriamycin equivalents in serum, liver and tumour tissue are given in Table I. The proportion of AD-NE metabolite was about 50% in serum and more than 50% in the liver and tumour tissue. P4 metabolite was detected in serum in every examination, but in the liver and tumour tissue it was hardly detectable. Adriamycinol was detected in the serum and liver in every examination, but hardly in tumour tissue.



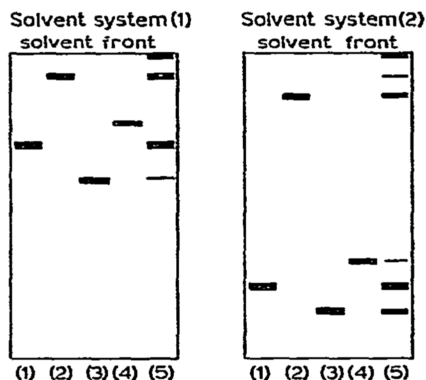


Fig. 6. High-performance liquid chromatogram of adriamycin and its metabolites. Peaks: P3 = adriamycinone; P6 = adriamycinol. HPLC was carried out as in Fig. 1.

Fig. 7. TLC of adriamycin and its metabolites. Solvent system (1): chloroform–methanol–acetic acid–water (100:50:14:6); (1) adriamycin (R_F 0.70), (2) adriamycinone (*ca.* 0.93), (3) adriamycinol (*ca.* 0.59), (4) daunomycin (*ca.* 0.78), (5) serum sample. Solvent system (2): chloroform–methanol–acetic acid–water (100:100:14:14); (1) adriamycin (R_F 0.24), (2) adriamycinone (*ca.* 0.86), (3) adriamycinol (*ca.* 0.16), (4) daunomycin (*ca.* 0.32), (5) serum sample.

TABLE I

RATIO (%) OF ADRIAMYCIN AND ITS METABOLITES TO TOTAL ADRIAMYCIN EQUIVALENTS IN SERUM, LIVER AND TUMOUR TISSUES IN EHRLICH TUMOUR-BEARING MICE AFTER A SINGLE INTRAPERITONEAL INJECTION, EXAMINED BY HPLC

Results are means ± standard deviations ($n = 3$).

Tissue	*Substance*	*Time after administration*			
		20 min	*60 min*	*5 h*	*24 h*
Serum	AD-NE*	46.2 ± 4.5	50.6 ± 15.8	42.0 ± 7.0	57.0 ± 32.9
	P4 metabolite	12.3 ± 2.8	18.4 ± 4.6	20.7 ± 7.7	14.7 ± 1.6
	Adriamycin	36.6 ± 15.9	18.4 ± 5.0	20.7 ± 2.8	16.4 ± 1.6
	Adriamycinol	4.9 ± 3.7	12.6 ± 5.1	16.6 ± 2.1	11.9 ± 4.0
Liver	AD-NE*	76.8 ± 19.1	83.4 ± 26.9	57.9 ± 2.8	67.1 ± 16.1
	P4 metabolite	N.D.**	N.D.**	N.D.**	N.D.**
	Adriamycin	19.3 ± 11.5	11.7 ± 4.9	34.7 ± 3.9	21.3 ± 6.8
	Adriamycinol	3.9 ± 2.6	4.9 ± 1.0	7.5 ± 2.9	11.6 ± 8.4
Tumor	AD-NE*	70.7 ± 34.2	65.0 ± 29.1	83.3 ± 34.7	80.6 ± 28.6
	P4 metabolite	19.5 ± 0.5	Trace	Trace	Trace
	Adriamycin	9.8 ± 2.6	17.5 ± 1.7	16.7 ± 12.2	19.4 ± 15.9
	Adriamycinol	Trace	17.5 ± 1.6	Trace	Trace

* P2 metabolite + adriamycinone.
** Not detected.

DISCUSSION

Adriamycin binds strongly with DNA and tissue proteins[15,16], and its extraction from tissues had been difficult. Since Hulhoven and Desager[11] reported a method

for its extraction from L1210 cells, that was applicable to tissue homogenate, good recoveries were obtained. The maximal serum concentration of total adriamycin equivalents after intraperitoneal administration in mice, as measured by the present method with HPLC, appeared 10 min after administration, but disappeared rapidly thereafter. This agreed with previous reports on adriamycin in rats[6,9], rabbits[2] and humans[5,7]. Kimura *et al.*[2] and Rosso *et al.*[5] observed a rebound in the adriamycin level in serum 8 and 24 h, respectively, after administration. Our experiments also suggested such a rebound in serum 5 h after administration. The total adriamycin equivalent level was high in the liver, duodenum and kidneys from 20 to 120 min after administration.

With respect to tissue retention of adriamycin in the heart, a high retention of adriamycin may be possible because adriamycin is structually similar to quinone compounds present in animal tissues, and can enter coenzyme Q and related enzyme systems[18]. The tissue retention of adriamycin may be related to the diminution of splenic tissue and the appearance of immunosupressive effects of this antibiotic[19].

For the identification of the metabolites of adriamycin, there is no established opinion, and they have been reported as aglycones[6,20], adriamycinol[10] and six metabolites (containing adriamycinol)[21], but in some work no metabolites[5,9] could be detected. In our experiments, the main metabolites in serum and tissues were adriamycinone and adriamycinol, and unidentified minor metabolites were also detected.

REFERENCES

1 G. Bonadonna, S. Monfardini, M. de Lena, F. Fossati-Bellani and G. Beretta, *Proc. 1st Int. Symp. Adriamycin, Milan, Italy, September 9–10th, 1971*, Springer-Verlag, Berlin, Heidelberg, New York, 1972, p. 139.
2 K. Kimura, H. Fujita and Y. Sakai, *Proc. 1st Int. Symp. Adriamycin, Milan, Italy, September 9–10th, 1971*, Springer-Verlag, Berlin, Heidelberg, New York, 1972, p. 124.
3 N. R. Bachur, A. L. Moor, J. G. Bernstein and A. Liu, *Cancer Chemother. Rep.*, 54 (1970) 89.
4 E. Arena, N. d'Alessandro, L. Dusonchet, N. Gebbia, F. Gerbasi, M. Palazzoadriano, A. Raineri, L. Rausa and E. Tubaro, *Arzneim.-Forsch.*, 21 (1971) 1258.
5 R. Rosso, C. Ravazzoni, M. Esposito, R. Sala and L. Santi, *Eur. J. Cancer*, 8 (1972) 455.
6 D. W. Yesair, E. Schwartzbach, D. Shuck, E. P. Denine and M. A. Asbell, *Cancer Res.*, 32 (1972) 1177.
7 R. S. Benjamin, C. E. Riggs and N. R. Bachur, *Clin. Pharmacol. Ther.*, 14 (1973) 592.
8 G. D. Fronzo, L. Lenaz and G. Bonadonna, *Biomedicine*, 19 (1973) 169.
9 T. Negishi and H. Takahira, *Kiso to Rinsho*, 7 (1973) 425.
10 J. J. Langone, H. Van Vunakis and N. R. Bachur, *Biochem. Med.*, 12 (1975) 283.
11 R. Hulhoven and J. P. Desager, *J. Chromatogr.*, 125 (1976) 369.
12 H. G. Barth and A. Z. Conner, *J. Chromatogr.*, 131 (1977) 375.
13 S. Eksborg, *J. Chromatogr.*, 149 (1978) 225.
14 R. Baurain, A. Zenebergh and A. Trouet, *J. Chromatogr.*, 157 (1978) 331.
15 T. Negishi and H. Takahira, *Yakugaku Zasshi*, 93 (1973) 1498.
16 P. Chandra, *Cancer Chemother. Rep., Part 3*, 6 (1975) 115.
17 S. Shinozawa, Y. Mimaki, H. Tomano, Y. Araki and T. Oda, *J. Chromatogr.*, 190 (1980) 489.
18 T. Kishi and K. Folkers, *Cancer Treat. Rep.*, 60 (1976) 223.
19 P. Obrecht, M. Westerhausen and A. Simon, *J. Amer. Med. Ass.*, 216 (1971) 179.
20 E. H. Herman, R. M. Mhatre, I. P. Lee and V. S. Waravdekar, *Proc. Soc. Exp. Biol. Med.*, 140 (1972) 234.
21 R. S. Benjamin, C. E. Riggs and N. R. Bachur, *Cancer Res.*, 37 (1977) 1416.