Journal of Chromatography, 131 (1977) 375–381

CHROM. 9466

DETERMINATION OF DOXORUBICIN HYDROCHLORIDE IN PHARMACEUTICAL PREPARATIONS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

HOWARD G. BARTH** and ALBERT Z. CONNER
*Hercules Research Center***, Wilmington, Del. 19899 (U.S.A.)*
(First received April 2nd, 1976; revised manuscript received June 18th, 1976)

SUMMARY

An improved high-pressure liquid chromatographic method for the determination of doxorubicin hydrochloride (Adriamycin™) in pharmaceutical preparations has been developed. A liquid–solid system using microporous silica (Zorbax) as the stationary phase and 3.8% 0.5 *M* sodium acetate buffer (pH 4.5) in isopropanol as the mobile phase gave optimum separation of doxorubicin hydrochloride from the excipient (lactose) and minor impurities. The doxorubicin hydrochloride content of a 10-mg vial can be determined in 25–30 min with a relative standard deviation of 3.2%. Results compare favorably with those obtained by established thin-layer chromatography and spectrophotometric procedures. The sensitivity of the method is sufficiently high (nanogram range) for possible use in metabolic or pharmacological studies.

INTRODUCTION

Doxorubicin hydrochloride (Fig. 1) is a cancer chemotherapeutic agent that was originally isolated from *Streptomyces peucetius* var. *caesius*. This anthracycline compound consists of an aglycone, adriamycinone, combined with an amino sugar, daunosamine. The finished dosage form which contains lactose as an excipient, is manufactured by Farmitalia (Milan, Italy) and sold under the trademark of Adriamycin.

At present, both thin-layer chromatographic (TLC) and spectrophotometric methods are used for quality control of Adriamycin preparations. The TLC procedure is time-consuming and precise quantitation is difficult. The spectrophotometric procedure is non-specific since it measures all compounds absorbing at 495 nm.

Several paper chromatographic and TLC procedures have been developed for

* Presented at the 123rd American Pharmaceutical Association Annual Meeting, April 3–8, 1976, New Orleans, La., U.S.A.

** To whom correspondence should be addressed.

*** Hercules Research Center Contribution Number 1682.

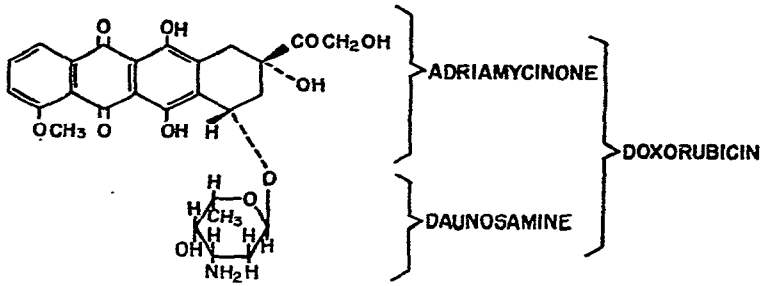

Fig. 1. Structure of doxorubicin.

the separation of doxorubicin[1,2] from related anthracycline compounds. Majors[3] has applied high pressure liquid–solid chromatography to the separation of a structurally similar anthracycline antibiotic, daunomycin, from its aglycone, daunomycinone. In addition, Carpenter[4] has proposed a similar method for the assay of doxorubicin. Recently, a reversed-phase high-pressure liquid chromatographic (HPLC) method has been developed for the determination of traces of doxorubicin and its metabolites in urine with subsequent quantitation by radioimmunoassay[5].

The use of HPLC for the quality control of doxorubicin was investigated, since this technique is capable of giving high resolution, short analysis times, and high precision. This paper describes an improved HPLC method for the determination of doxorubicin and associated impurities in Adriamycin pharmaceutical preparations.

EXPERIMENTAL

Apparatus

A Varian Model 8500 liquid chromatograph and a DuPont 254 nm photometer (Model 842) were employed. Samples were injected using a 10-μl external loop valve (Catalog No. ACV-6-uHPa-C-20, Valco Instruments Company, Houston, Texas, U.S.A.). The valve was pneumatically actuated with about 80 p.s.i. of nitrogen controlled by a four-port manual valve (Model No. RP 3/8, S.P. Manufacturing Corporation, Solon, Ohio, U.S.A.). The loop was filled with sample solution from a 5-ml gastight syringe (Model No. 1005, Hamilton Company, Reno, Nev., U.S.A.).

Columns

The following packing materials were evaluated: Phenyl/Corasil (Waters Assoc., Milford, Mass., U.S.A.), Pellidon (Reeve Angel, Clifton, N.J., U.S.A.), Vydac (Applied Science, State College Park, Penn., U.S.A.), Micropak SI-10 and SI-5 (Varian, Palo Alto, Calif., U.S.A.), HCP/Zipax, Zipax, Zorbax, and ODS/Zorbax (DuPont, Wilmington, Del., U.S.A.). Of all the supports examined, Zorbax ($\approx 5\,\mu$m; purchased prepacked in 2.1 mm $\times$ 25-cm column) was found the most useful in terms of efficiency and selectivity. The column was not thermostatted.

Mobile phase

The mobile phase was 3.8% 0.5 *M* sodium acetate (adjusted to pH 4.5 with acetic acid) in isopropanol. Together with the Zorbax column, it was found to give optimum separation of doxorubicin from the excipient (lactose) and minor impurities.

Isopropanol was "distilled in glass" (Burdick & Jackson, Labs., Muskegon, Mich., U.S.A.) and all chemicals were of reagent grade.

Reference standards and samples

Doxorubicin hydrochloride and adriamycinone were supplied by Farmitalia. Adriamycin samples were obtained from Adria Labs. (Wilmington, Del., U.S.A.) in 10-mg vials with 50 mg of lactose.

Assay procedure

The contents of a 10-mg vial of Adriamycin were transferred quantitatively with 5.0 ml of distilled water into a 50-ml volumetric flask and then diluted to volume with isopropanol. A 10-μl aliquot of the sample was injected in duplicate at a mobile phase flow-rate of 10 ml/h (approx. 3200 p.s.i.). The attenuation was set to 0.04 absorbance units full scale (a.u.f.s.) to detect adriamycinone and associated impurities and 0.32 for doxorubicin. The recorder chart speed was 0.25 in./min.

Calibration

A 10-mg quantity of doxorubicin hydrochloride working standard was accurately weighed to ± 0.01 mg and transferred to a 50-ml volumetric flask. The sample was dissolved in 5.0 ml of distilled water and diluted to volume with isopropanol. At the beginning and end of each day, 10-μl injections were made in duplicate.

Calculations

The weight of doxorubicin hydrochloride used to prepare the standard solution was corrected for moisture and amount of impurities by use of the following equation:

$$W_c = t\left[W - W\left(\frac{I_{std}}{I_{std} + A_{std}}\right)\right]$$

where W_c is the corrected weight of doxorubicin hydrochloride, t is the fraction of dry material based on moisture content (Karl Fischer titration), W is the weight in mg of doxorubicin used in preparing the standard solution, A_{std} is the area of the standard doxorubicin peak at an attenuation of 0.32 a.u.f.s., I_{std} is the area of the impurity peaks in the standard normalized to 0.32 a.u.f.s.

The mg amount of doxorubicin per vial is:

$$W_v = A_a \frac{W_c}{A_{std}}$$

where A_a is the area of the sample doxorubicin peak at an attenuation of 0.32 a.u.f.s.

RESULTS AND DISCUSSION

Evaluation of the chromatographic system

A typical chromatogram of an Adriamycin preparation is shown in Fig. 2. Excellent separation of doxorubicin from associated impurities was achieved. Peak 1 was caused by the solvent front. Peak 2 has been identified as adriamycinone by retention time comparison with standards, and peaks 3 and 4 have been identified as related

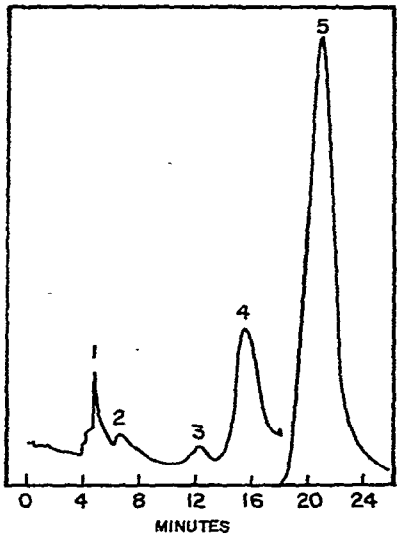

Fig. 2. High-pressure liquid chromatogram of doxorubicin. Column, Zorbax, 2.1 mm × 25 cm. Mobile phase, 3.8% sodium acetate buffer (pH 4.5) in isopropanol at a flow-rate of 10 ml/h (3200 p.s.i.). Detector, 254 nm DuPont photometer. Sample, 10 μl of 9.91 mg of doxorubicin hydrochloride working standard in 50 ml of a 10% aqueous solution of isopropanol. 1 = solvent front; 2 = adriamycinone; 3 and 4 = anthracycline-related compounds; 5 = doxorubicin. Peaks 1, 2, 3 and 4 were measured at an attenuation of 0.04 a.u.f.s., peak 5 at ×0.32 a.u.f.s.

anthracycline compounds[6]. The total analysis time was about 26 min which was sufficiently rapid for use as a quality control procedure.

Although satisfactory resolution was obtained with this chromatographic system, plate heights (HETP) calculated from the doxorubicin peak (<0.5 mm) were higher than expected for the microporous silica column. This discrepancy was probably caused by the high viscosity of the mobile phase (2.3 cP) which decreased the rate of mass transfer and increased the pressure drop across the column. An attempt was made to reduce mobile phase viscosity by substituting either methanol or ethanol for isopropanol. However, doing so led to increased tailing of the doxorubicin peak. In addition, the column was heated to as high as 55° with no improvement in efficiency. In fact, the efficiency decreased at these temperatures. This decrease might have been caused by exposure of more active silanol sites at elevated temperatures.

As the concentration of sodium acetate buffer in isopropanol was increased from 3 to 10%, there was a corresponding decrease in the capacity factor, k', of doxorubicin from 5.7 to 0.7 and an increase in theoretical plate number from 600 to 1000. Therefore, a buffer concentration somewhat greater than 3% was required to give rapid analysis time with adequate resolution. In addition, as the molarity of sodium acetate buffer was decreased from 0.5 to 0.05 *M*, keeping the volume ratio of buffer to isopropanol constant, severe tailing occurred, with a corresponding increase in k'. Thus, the buffer molarity selected was 0.5 *M*. Because of the lability of doxorubicin at extreme pH ranges, no attempt was made to evaluate other buffer systems.

As shown in Table I there was no significant change in the capacity factors of doxorubicin over a period of six consecutive days. Because the column was not

TABLE I

COLUMN STABILITY

Conditions: 2.1 mm × 25 cm Zorbax column with 3.8% 0.5 *M* sodium acetate buffer (pH 4.5) in isopropanol as the mobile phase; flow-rate, 10 ml/h.

Day		*k' of doxorubicin*
1		3.38
2		3.28
3		3.22
4		3.41
5		3.50
6		3.30
	Average	3.35
	Standard deviation	0.10

thermostatted, slight variations were probably caused by ambient temperature fluctuations.

Samples were prepared in 10% aqueous solutions of isopropanol to prevent the precipitation of lactose. Although lactose was introduced during each injection (10 μg), column performance was not affected by this small amount. It was demonstrated that the lactose could be removed from the column by eluting with water (approx. 10 ml) and the column regenerated to its initial activity with 20 ml of water–isopropanol (1:1) followed by elution overnight with 100–120 ml isopropanol, and finally with 25 ml of 3.8% acetate buffer in isopropanol.

Calibration and detection limit

Because a sample loop valve was used, no internal standard was necessary. Figs. 3 and 4 show peak area and height response *versus* amount of doxorubicin injected, respectively. Peak areas exhibited a linear relationship with amount of doxorubicin injected (up to approx. 2.5 μg) and, therefore, can be used for quantitation. For peak height measurements a non-linear response was evident in this range. This was caused by peak broadening which increased with the amount of doxorubicin injected.

This method was also capable of determining nanogram quantities of doxorubicin. Both peak areas and heights showed linear behavior with amount of doxorubicin injected up to approx. 40 ng. The limit of detection, as determined by the peak height at twice the noise level, was about 0.8 ng. The sensitivity of this method was sufficiently high for possible use in metabolic or pharmacological studies.

Quantitation of doxorubicin

The doxorubicin content of the finished dosage form was determined by comparing the major peak area, measured by triangulation, with that of a standard. Because the same 10-μl loop was used with both samples and standards, it was not necessary to calibrate the loop. The anthracycline related impurities were determined by area normalization. This was done by assuming that the ultraviolet (UV) absorptivities of these compounds were essentially the same as of doxorubicin.

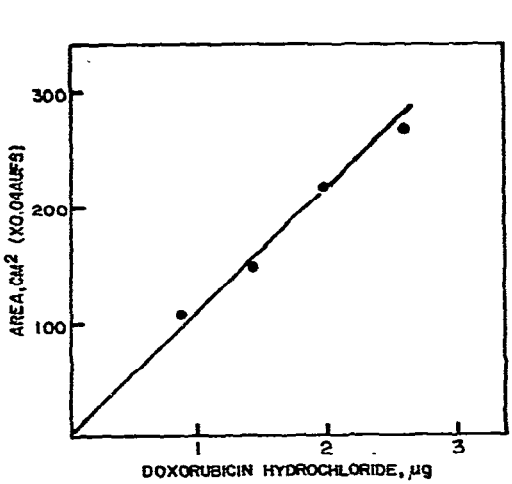

Fig. 3. Peak area as a function of amount of doxorubicin hydrochloride injected. Conditions as in Fig. 2. Chart speed, 0.25 in/min; areas measured at an attenuation of 0.04 a.u.f.s.

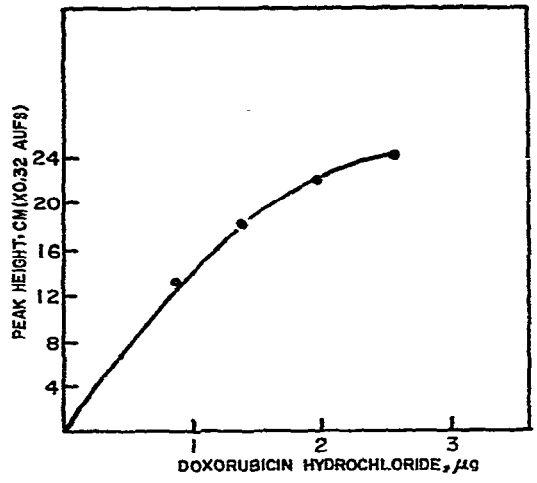

Fig. 4. Peak heights as a function of amount of doxorubicin injected. Conditions as in Fig. 2. Heights were measured at an attenuation of 0.32 a.u.f.s.

Five vials from each of five batches were analyzed for doxorubicin. The contents of each vial were analyzed in duplicate and the results are shown in Table II. The relative standard deviation within a batch ranged from 0.8 to 5.8%. Reproducibility of the method as determined by pooling the relative standard deviations of thirty sets of measurements ($n = 67$) was 3.2%. However, better precision has since been obtained by means of an electronic integrator which was not available at the time of this work.

The TLC procedure gave 9.7 mg of doxorubicin per vial for each of the five batches analyzed. The TLC system which was used consisted of polyamide–cellulose (1:1) as the sorbent and a complex mobile phase which was obtained from the upper layer of a mixture of *n*-butanol, isopropyl alcohol, isopropyl ether, acetic acid, and

TABLE II

HPLC ASSAY OF PHARMACEUTICAL PREPARATIONS

Conditions: same as in Table I. Each vial was analyzed in duplicate.

Vial	*Amount of doxorubicin per vial (mg)*				
	Batch 1	*Batch 2*	*Batch 3*	*Batch 4*	*Batch 5*
1	9.34	10.25	9.98	10.04	9.92
2	9.26	9.53	9.82	9.78	10.3
3	9.30	9.16	9.95	9.35	10.6
4	9.29	8.97	9.77	9.20	9.27
5	9.45	9.30	9.95	9.31	
Average	9.33	9.44	9.89	9.54	10.0
Standard deviation within a batch	0.07	0.49	0.09	0.36	0.58

water (12:2:2:3:15). Quantitation was accomplished by extracting the doxorubicin spot with acidified methanol and measuring its absorbance at 495 nm. The TLC procedure could not distinguish small variations of the doxorubicin content among different vials, as compared with the HPLC procedure, because it lacks the high resolving power found in HPLC.

Table III compares the average doxorubicin and anthracycline related impurities per vial in each of the batches assayed with the HPLC and spectrophotometric procedures. These results show excellent agreement between the two methods. In addition, the TLC procedure gave a consistent value of 10.0 mg/vial for the batches tested.

TABLE III

COMPARISON OF HPLC AND SPECTROPHOTOMETRIC ANALYSIS

Conditions: Same as in Table I. n is the number of vials analyzed within a batch.

Method	*Average amount (mg) of doxorubicin and related anthracycline compounds per vial*				
	Batch 1	*Batch 2*	*Batch 3*	*Batch 4*	*Batch 5*
HPLC ($n = 5$)	9.81	9.95	10.4	10.1	10.5
Spectrophotometry ($n = 10$)	10.1	9.85	10.3	10.1	10.4

ACKNOWLEDGEMENTS

This work was supported by Adria Laboratories, Incorporated, Wilmington, Delaware. The authors gratefully acknowledge the technical assistance given by Judy DeFranco and Allen Orr.

REFERENCES

1 F. Arcamone, G. Cassinelli, G. Fantini, A. Grein, P. Orezzi, C. Pol and C. Spalla, *Biotechnol. Bioeng.*, 11 (1969) 1101.
2 K. K. Chan and P. A. Harris, *Res. Commun. Chem. Path. Pharmacol.*, 6 (1973) 447.
3 R. E. Majors, *Liquid Chromatography at Work*, No. 7, Varian, Palo Alto, Calif., 1973.
4 R. H. Carpenter, Food and Drug Administration, personal communication, Febr. 1974.
5 J. J. Langore, H. V. Vunakis and N. R. Bachur, *Biochem. Med.*, 12 (1975) 283.
6 Farmitalia, Milan, personal communication.