Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 4, pp. 483–490, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00
Pergamon Journals Ltd.

Reversed-phase ion-pair high-performance liquid chromatography of adriamycin and adriamycinol in rat serum and tissues

J. M. GALLO†, C. T. HUNG and D. G. PERRIER*

Department of Pharmacy, University of Otago, Dunedin, New Zealand

Abstract: An ion-pair reversed-phase high-performance liquid chromatographic method is presented for the determination of adriamycin and adriamycinol concentrations in rat serum and tissues. The scheme for developing the chromatographic conditions is discussed. A good separation of adriamycinol and adriamycin was obtained by using a high sodium lauryl sulphate concentration in the mobile phase. The retention times of adriamycinol, adriamycin and daunomycin (internal standard) were less than 3 min with no interfering peaks from endogenous constituents. A protein precipitation method was used to prepare the serum and tissue samples for injection.

Keywords: *Ion-pair HPLC; adriamycin and adriamycinol.*

Introduction

Adriamycin (doxorubicin) was introduced as an anthracycline anticancer agent in 1969 [1, 2]. Since then both non-specific and specific analytical methods have been used for the quantitation of adriamycin and its metabolites in biological samples. Non-specific fluorometric and radioimmunoassay procedures show adequate sensitivity but lack specificity, making these methods unattractive [3, 4]. Specific analyses initially began with the separation of adriamycin and adriamycinol by thin-layer chromatography and more recently by high-performance liquid chromatography (HPLC) [5–10]. HPLC methods are preferred due to their relative simplicity plus specificity and sensitivity.

Adriamycin is a basic compound with the pKa of the amino group reported to be about 8 [11, 12], and therefore, will ionize in the acidic and neutral mobile phases usually employed in HPLC. The technique of increasing solute retention by suppressing solute ionization is not suitable for weak bases because the high mobile phase pH required would dissolve the silica column. Recently, an interest in the application of ion-pair chromatography for the analysis of anthracyclines in biological samples has been noted [13]. Haneke *et al.* [14] used 5 mM heptane sulphonic acid as the pairing-ion to quantitate daunorubicin, doxorubicin and their aglycones in pharmaceutical preparations. Van Lancker *et al.* [15] employed sodium lauryl sulphate as an ion-pair reagent to separate a number of anthracyclines, although the system was not tested with biological samples.

*To whom correspondence should be addressed, at: Faculty of Pharmacy, University of Toronto, Toronto, Ontario M5S 1A1, Canada.
†Present address: College of Pharmacy, University of Georgia, Athens, GA 30602, U.S.A.

Therefore, this method was developed for the quantitation of adriamycin and adriamycinol in biological samples using ion-pair chromatography. The method uses a high concentration of ion-pair reagent and provides adequate separation of adriamycin, adriamycinol and daunomycin (internal standard) with very short times for analyses. The strategies involved in developing such an assay for these compounds by an empirical optimization technique are discussed.

Experimental

Chemicals

Adriamycin, adriamycinol, adriamycinone and daunomycin were kindly donated by Farmitalia Carlo Erba (Milan, Italy). HPLC grade acetonitrile and analytical grade isopropanol were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Water was double distilled and MilliQ® filtered. Analytical grade potassium dihydrogen phosphate, phosphoric acid and formic acid were purchased from BDH Chemical Co. (Poole, UK). Silanizing agent, Aquasil, was obtained from Pierce Chemical Co. (Rockford, IL) and sodium lauryl sulphate (99% pure) from Sigma Chemical Co. (St. Louis, MO).

Apparatus

The chromatographic system consisted of a Waters Associates 6000A pump, a Rheodyne 7125 injector with a 100 μl sample loop, a Schoeffel FS970 fluorometer and a Linseis L650 recorder. The chromatograpic column was stainless steel, 100 × 4.6 mm i.d., packed with ODS-Hypersil (Shandon Southern Products Ltd, Cheshire, UK). The tissue homogenizer was a Silverson® (Machines Ltd, London, UK).

Serum extraction

The extraction of adriamycin and adriamycinol from biological samples has included single and multiple organic extractions, back extractions, solid–liquid extractions and protein precipitation methods [8, 10, 16–19]. In the present analysis a protein precipitation method was used for the preparation of both rat serum and tissues for injection.

Fifty microlitre aliquots of either a 2 μg ml^{-1} or 10 μg ml^{-1} daunomycin HCl stock solution in methanol–water (30:70, v/v) were added to 1 ml of rat serum in 15 ml silanized glass centrifuge tubes and vortexed briefly. Three millilitres of acetonitrile was added to the tubes while vortexing for a total of 30 s. The tubes were then centrifuged at 4°C for 20 min at 3000 rpm. Resulting supernatants were transferred to 15 ml silanized glass tubes and 50 μl aliquots of formic acid–acetonitrile (10:90, v/v) were added to each tube and vortexed briefly. The solvents were evaporated to dryness under nitrogen at a temperature of 37 ± 3°C. Dried residues were reconstituted in 120 μl of acetonitrile–1 mM phosphoric acid, pH = 2 (25:75, v/v). The tubes were centrifuged at 4°C for 10 min at 3000 rpm to separate any particulate matter.

Tissue extraction

Rat tissues were collected over ice. The animals were killed by exsanguination after anesthetizing with ether. Tissues were weighed and homogenized for about 2 min at maximum speed in a 1:4 (weight:volume) ratio with ice-cold saline. A minimum of 3 ml of normal saline was used.

The internal standard, daunomycin HCl, solution was added to 1 ml of tissue

homogenate as described for serum and vortexed briefly. Three millilitres of isopropanol–acetonitrile (10:90, v/v) was added to each tube while vortexing for 30 s. The tubes were centrifuged at 4°C for 20 min at 3000 rpm. The supernatant was transferred to silanized glass tubes and 25 μl aliquots of formic acid–acetonitrile (10:90, v/v) were added and the tubes were vortexed briefly. The samples were then treated in the same manner described for serum.

Chromatography

A mobile phase of acetonitrile–aqueous 80 mM sodium lauryl sulphate and 30 mM KH_2PO_4 (1:1, v/v) was used. The final pH was adjusted to 2.0 with phosphoric acid. A flow rate of 2 ml min^{-1} was employed and the excitation and emission wavelengths on the fluorometer were set at 470 and 580 nm respectively. Injection volumes were between 20 and 100 μl.

To obtain optimum separation the pairing-ion (sodium lauryl sulphate) concentration, organic modifier (acetonitrile) concentration, pH and ionic strength of the mobile phase were evaluated.

Results and Discussion

Chromatographic separation

One of the most important requirements in a routine analytical method, besides the required sensitivity and specificity, is the reproducibility of the results. In order to determine optimal and reproducible chromatographic conditions for the separation of adriamycin, adriamycinol, adriamycinone and daunomycin, a number of preliminary studies were performed. In ion-pair chromatography the retention time of an ionic solute depends on the choice of the pairing-ion and its concentration, organic modifier concentration, pH and ionic strength of the mobile phase.

Influence of organic modifier. The effect of the acetonitrile concentration in the mobile phase on the capacity factor (k') of the solutes was similar to that observed by Van Lancker [15]. An increase in the acetonitrile concentration decreased k' and reduced selectivity. Below 50% (v/v) acetonitrile slight variations in acetonitrile concentrations cause marked changes in the capacity factors and peak tailing became apparent. At higher concentrations of acetonitrile selectivity became minimal. Therefore, a 50% (v/v) acetonitrile/aqueous mobile phase was used.

Influence of pH. The pH of the mobile phase, acetonitrile–aqueous 20 mM KH_2PO_4 (1:1, v/v) was varied over a range of 2–7 using phosphoric acid to adjust pH. It was observed that the k' values for adriamycin, adriamycinol and daunomycin increased as the pH of the eluent increased. Such an effect for basic compounds has been demonstrated by other investigators [20, 21]. However, the k' values of the three basic solutes changed greatly in the region from pH 3 to 7 with observable peak tailing. At pH values below 3 the k' for these compounds was relatively constant with no peak tailing. A pH value of 2 was therefore chosen based on the insensitivity of k' to small changes in pH, and the fact that the peaks were the sharpest.

Influence of buffer salt concentration. Insufficient buffer capacity of the eluent in ion-pair chromatography will cause poor column efficiency and may result in peak splitting

[22]. On the other hand, the addition of a buffer salt into the mobile phase will alter its ionic strength. This has been demonstrated to have a significant influence on solute retention [23]. Over a total mobile phase concentration range of 10–50 mM KH_2PO_4 in 50% (v/v) acetonitrile, measured pH = 2, a general decrease in k' occurred for adriamycin, adriamycinol, adriamycinone and daunomycin as the KH_2PO_4 concentration increased. The decrease in retention of protonated basic substances has been explained by the added salt competing for the free silanol groups on the C_{18} surface [24]. This general decrease in solute retention was insignificant at and above 30 mM KH_2PO_4. Thus, a 30 mM KH_2PO_4 concentration in the mobile phase was chosen for the relative constancy of k' and the absence of peak tailing.

Influence of ion-pair reagent. Sodium lauryl sulphate (SLS) was employed as the anionic pairing-ion because of its hydrophobicity and for its adsorption characteristics onto the C_{18} stationary phase. It has been demonstrated that SLS is appreciably adsorbed onto the C_{18} surface even at high organic modifier concentrations [25]. Figure 1

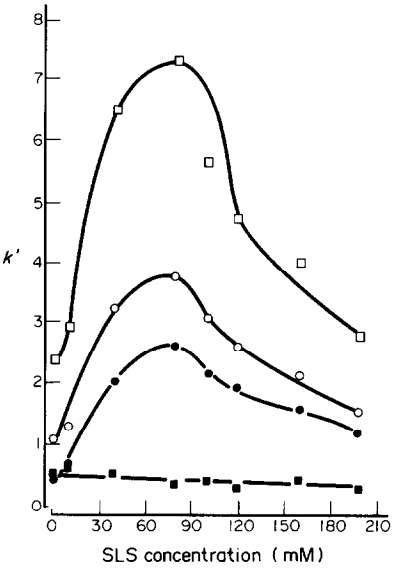

Figure 1
Capacity factor, k', vs sodium lauryl sulphate (SLS) mobile phase concentration. □, Daunomycin; ○, adriamycin; ●, adriamycinol; ■, adriamycinone. Mobile phases consisted of various SLS concentrations in acetonitrile-aqueous 30 mM KH_2PO_4 (1:1, v/v) the final pH was adjusted to 2 with phosphoric acid.

shows a plot of k' versus the SLS concentration in the mobile phase which is similar to that shown by Van Lancker *et al.* [15]. It can be seen that the k' values for the ionic solutes pass through a maximum as predicted by the ion-exchange desolvation mechanism [25, 26]. This mechanism considers that the decrease in k' of an ionic solute after the maximum is due to the decrease in the surface area of the C_{18} support. This is a result of adsorption of the hydrophobic portion of the pairing ion. For the non-ionic solute, adriamycinone, the k' gradually decreased as the concentration of SLS increased according to the ion-exchange desolvation mechanism. The maximum retention and the optimal separation was achieved at a SLS mobile phase concentration of 80 mM. In addition, at this SLS concentration the retention of the ionic solutes was insensitive to small changes in mobile phase SLS concentration. Therefore, an 80 mM SLS concentration was utilized in this investigation.

Extraction method

A protein precipitation method was used for the extraction of adriamycin, adriamycinol and daunomycin from rat serum and tissues. This method was chosen rather than organic extraction methods because it was simple and more reproducible. Acetonitrile gave higher recoveries than acetone and methanol. It was found that isopropanol–acetonitrile (10:90, v/v) significantly increased the recoveries of the compounds from the tissue but not from serum. The apparent pH of both the serum and tissue supernatants were adjusted to approximately 5 with formic acid–acetonitrile (10:90, v/v). The evaporation of the supernatants without pH adjustment and at a pH less than 4 caused significant drug degradation. Tables 1 and 2 give the percentage recovery of adriamycin, adriamycinol and daunomycin from rat serum and tissues. The percentage recovery was determined by comparing the peak heights resulting from the injection of samples relative to those of standards.

Serum and tissue determinations

Figure 2 shows a chromatogram of blank rat serum and rat serum to which known amounts of adriamycin, adriamycinol and daunomycin were added. The retention times are 1.4 min for adriamycinol, 1.8 min for adriamycin and 2.95 min for daunomycin. Figure 3 shows chromatograms from a blank kidney homogenate and from an *in vivo* sample. Blanks from other tissues are similar to the one presented here. Table 3 gives the

Table 1
Percentage recovery from rat serum

Compound	Added concentration (ng ml^{-1})	Percentage recovery*
Adriamycinol	10	74.3 (4.8)
	200	73.4 (11.3)
Adriamycin	9.4	68.9 (12.0)
	188	74.9 (11.3)
	1880	82.2 (2.7)
Daunomycin	500	81.3 (4.0)

*Each mean (relative standard deviation) is based on a minimum of nine samples.

Table 2
Percentage recovery from rat tissue homogenates

	Percentage recovery*		
Tissue	Adriamycinol	Adriamycin	Daunomycin
Brain	48.7 (3.8)	39.3 (9.4)	42.5 (4.95)
Heart	52.2 (2.8)	40.8 (7.1)	42.8 (5.80)
Kidney	49.7 (11.5)	43.2 (3.3)	42.5 (2.45)
Liver	47.4 (11.5)	39.2 (2.1)	38.0 (3.04)
Lung	45.8 (6.4)	25.9 (3.3)	39.5 (5.94)
Small intestine	43.4 (13.7)	46.5 (12.2)	35.7 (8.54)
Spleen	40.1 (4.3)	33.2 (9.7)	43.2 (3.82)

*Each mean (relative standard deviation) is based on three samples. For all tissues the concentrations added were 50, 100 and 100 ng ml^{-1} of adriamycinol, adriamycin and daunomycin, respectively.

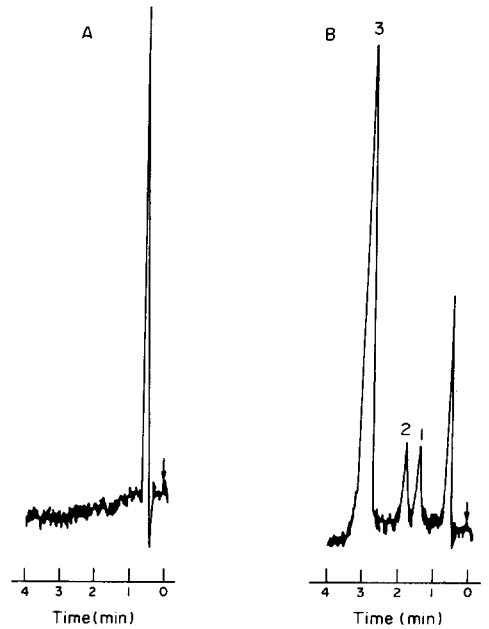

Figure 2
Chromatograms from rat serum. (A) Blank, injection volume = 100 μl; (B) 1, adriamycinol (10 ng ml^{-1}); 2, adriamycin HCl (10 ng ml^{-1}); 3, daunomycin (100 ng ml^{-1}); injection volume = 100 μl. Chromatographic conditions, see text.

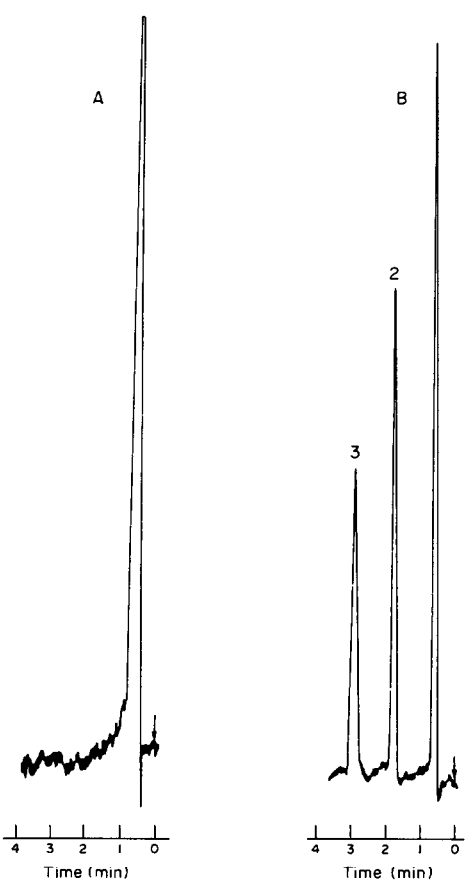

Figure 3
Chromatograms from rat kidney. (A) Blank, injection volume = 100 μl; (B) rat given 2 mg kg^{-1} of adriamycin HCl i.v., sample taken at 4 h post-dose, adriamycin concentration = 3.14 μg g^{-1}. Peak identification as in Fig. 3, injection volume = 50 μl. Chromatographic conditions, see text.

Table 3
Adriamycin and adriamycinol concentrations at 4 h after a 2 mg kg^{-1} dose of adriamycin HCl i.v. in the rat

Sample	Adriamycin	Adriamycinol (ng g^{-1})
Serum	12.8 ng ml^{-1} (32.6)*	†
Brain	29.3 ng g^{-1} (13.3)	†
Heart	3.03 μg g^{-1} (6.3)	72.0 (5.2)
Kidney	4.32 μg g^{-1} (13.7)	58.8 (14.9)
Liver	2.02 μg g^{-1} (25.9)	56.3 (11.5)
Lung	4.40 μg g^{-1} (4.4)	37.9 (6.2)
Small intestine	1.77 μg g^{-1} (9.2)	56.0‡
Spleen	4.04 μg g^{-1} (15.6)	32.4‡

*Each mean (relative standard deviation) is based on three rats.
†Below assay sensitivity.
‡Insufficient sample to perform analysis in all rats.

actual serum and tissue concentrations of rats which had received a 2 mg kg^{-1} i.v. dose of adriamycin HCl. Although adriamycinone was separated from adriamycin and adriamycinol it could not be quantitated as it eluted with the endogenous constituents in serum and tissue samples. However, adriamycinone is an inactive metabolite, and therefore, quantitation is not necessary for most kinetic studies.

Linearity and sensitivity

Standard curves for adriamycinol (10–200 ng ml^{-1}) and adriamycin (9.4–188 ng ml^{-1}, and 188–1880 ng ml^{-1}) were prepared in rat serum and tissue homogenates. All standard curves were linear with r values greater than 0.98.

Based on a signal to noise ratio of 3, the lowest detectable concentration of adriamycin and adriamycinol in rat serum was 5 ng ml^{-1} (on-column weight approximately 3 ng). In rat tissues, the lowest detectable amount was between 25 and 50 ng g^{-1} (on-column weight range, approximately 5–18 ng) for both adriamycin and adriamycinol depending on the tissue.

Table 4
Accuracy and precision in rat serum and tissues

Sample		Concentration (ng ml^{-1})*		
		Known	Measured	RSD
Serum	Adriamycinol	10.0	9.9	9.17
	Adriamycin	9.4	9.3	10.4
	Adriamycin	188.0	193.8	6.55
	Adriamycin	1880.0	1857.5	4.76
Heart	Adriamycin	970.0	987.9	8.57
Kidney	Adriamycin	582.0	573.5	6.28
Liver	Adriamycin	194.0	209.5	7.80
Lung	Adriamycin	582.0	589.3	4.48
Small intestine	Adriamycin	582.0	589.5	10.86
Spleen	Adriamycin	970.0	971.5	8.33

*Each mean and relative standard deviation (RSD) is based on 12 samples for serum and 6 samples for all tissues. The measured concentrations were determined from a standard curve prepared by adding known amounts of drug to the serum and tissue samples.

Accuracy and precision

Table 4 gives the accuracy and precision of this assay in rat serum and tissue homogenates to which known amounts of adriamycin, adriamycinol and daunomycin were added.

Conclusion

The analytical procedure described here is simple and reproducible. The chromatographic conditions produced an adequate separation of adriamycin and adriamycinol in rat serum and rat tissues within an analytical time of 4 min.

Acknowledgements: This investigation was supported in part by the New Zealand MRC project grant 84/93. The facilities provided by the Department of Clinical Biochemistry are gratefully acknowledged.

References

[1] A. Dei Marco, M. Gaetani and B. Scarpinato, *Cancer Chemother. Rep.* (part 1) **53**, 33–37 (1969).
[2] R. H. Blum and S. K. Cater, *Ann. Intern. Med.* **80**, 249–259 (1974).
[3] N. R. Bachur, C. E. Riggs, Jr, M. R. Green, J. J. Langone, H. Van Vunakis and L. Levine, *Clin. Pharmacol. Ther.* **21**, 70–77 (1976).
[4] R. S. Benjamin, C. E. Riggs, Jr and N. R. Bachur, *Clin. Pharmacol. Ther.* **14**, 592–599 (1973).
[5] K. K. Chan and P. A. Harris, *Res. Commun. Chem. Pathol. Pharmacol.* **6**, 447–463 (1973).
[6] E. Watson and K. K. Chan, *Cancer Treat. Rep.* **60**, 1611–1618 (1976).
[7] K. K. Chan and C. D. Wong, *J. Chromatogr.* **172**, 343–349 (1979).
[8] M. Israel, W. G. Pegg, P. M. Wilkinson and M. B. Garnick, *J. Liquid Chromatogr.* **1**, 795–809 (1978).
[9] S. Eksborg, H. Ehrsson and I. Andersson, *J. Chromatogr.* **164**, 479–486 (1979).
[10] R. N. Pierce and P. I. Jatlow, *J. Chromatogr.* **164**, 471–478 (1979).
[11] S. Eksborg, in *Clinical Pharmacology of Antineoplastic Drugs* (H. M. Pinedo, Ed.), p. 193. Elsevier/North Holland Biomedical Press, Amsterdam (1978).
[12] P. M. May, G. K. Williams and D. R. Williams, *Inorg. Chim. Acta* **46**, 221–228 (1980).
[13] C. E. Riggs, Jr and N. R. Bachur, in *Pharmacokinetics of Anticancer Agents in Humans* (M. M. Ames, G. Powis and J. S. Kovach, Eds), p. 241. Elsevier, Amsterdam (1983).
[14] A. C. Haneke, J. Crawford and A. Aszalos, *J. Pharm. Sci.* **70**, 1112–1115 (1981).
[15] M. A. Van Lancker, H. J. C. F. Nelis and A. P. DeLeenheer, *J. Chromatogr.* **254**, 45–52 (1983).
[16] A. J. Quattrone and D. F. Ranney, *J. Anal. Toxicol.* **4**, 12–15 (1980).
[17] W. Bolanowska, T. Gessner and H. Preisler, *Cancer Chemother. Pharmacol.* **10**, 187–191 (1983).
[18] A. M. B. Bots, W. J. Van Oort, J. Noordhoek, A. Van Dijk, S. W. Klein and Q. G. C. M. Van Hoesel, *J. Chromatogr.* **272**, 421–427 (1983).
[19] J. Robert, *J. Liquid. Chromatogr.* **3**, 1561–1572 (1980).
[20] H. F. Proeless, H. J. Lohman and D. G. Miles, *Clin. Chem.* **24**, 1948–1953 (1978).
[21] P. P. Pashankov, P. S. Zikolov and O. B. Budwsky, *J. Chromatogr.* **209**, 149–160 (1981).
[22] W. R. Melander, J. Stoveken and C. Horvath, *J. Chromatogr.* **185**, 111–127 (1979).
[23] W. E. Rudzinski, D. Bennett, V. Garcia and M. Seymour, *J. Chromatogr. Sci.* **21**, 57–61 (1983).
[24] E. Papp and G. Y. Vigh, *J. Chromatogr.* **259**, 49–58 (1983).
[25] C. T. Hung and R. B. Taylor, *J. Chromatogr.* **209**, 175–190 (1981).
[26] C. T. Hung and R. B. Taylor, *J. Chromatogr.* **202**, 333–345 (1980).

[*First received 18 December 1984; in final form 9 August 1985*]