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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY: THIN-FILM FLUORESCENCE SCANNING ANALYSIS OF ADRIAMYCIN AND METABOLITES IN TISSUE

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SUMMARY

A rapid, non-hydrolytic thin-film fluorescence scanning method is described for the quantitation of adriamycin and metabolites in tissues. Adriamycin, with daunomycin added as the internal standard, was extracted from tissue homogenate which contained 500 μg of oxalic acid, with ice-cold 0.5 *N* hydrochloric acid–85% isopropanol, separated by thin-layer chromatography, and quantitated *in situ* via a fluorescence scanning technique. This method has a sensitivity to 0.05 μg per gram of tissue.

INTRODUCTION

Investigation of tissue uptake of adriamycin (Ad), a widely used anticancer drug, and its metabolites has been a subject of considerable interest owing to the dose-related cardiac toxicity. Previously published methods of the analysis of Ad and its metabolites from tissues have been either hydrolytic[1–3], which obscured the analysis of adriamycinone and other heat-labile metabolites, or were applicable only to non-tissue-bound material. In those procedures utilizing non-hydrolytic conditions for the analysis of tissue-bound Ad, no recovery data were reported[2,3]. In this paper we describe a low-temperature acid–alcohol extraction procedure coupled with a recently published thin-film fluorescence procedure for the analysis of Ad and its fluorescent metabolites in tissues. In addition, this method can be applied to the quantitative study of *in vitro* metabolism of adriamycin.

EXPERIMENTAL

Materials and methods

All solvents were of reagent grade. Analytical glassware was silanized with dimethyldichlorosilane (Pierce, Rockford, Ill., U.S.A.). Silica gel plates (silica gel 60

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pre-coated plate, E. Merck, Elmsford, N.Y., U.S.A.) were scored into 16 1-cm wide lanes and pre-washed with methanol before use. Tissues were homogenized with a high speed motor-driven homogenizer (Brinkman Polyton, Model PT 10/35; Brinkman, New York, N.Y., U.S.A.). Fluorescence scanning was performed on a spectrophotofluorimeter (Aminco-Bowman, Silver Spring, Md., U.S.A.), equipped with a thin-film scanner and an electronic noise filter (Spectrum Scientific, Newark, Del., U.S.A.) in a manner previously described[4]. The output was recorded on a strip-chart recorder (Perkin-Elmer-Coleman 165; Perkin-Elmer, Beaconsfield, Great Britain).

Procedure

Thawed or fresh tissue (1 g) was homogenized in 4.0 ml of ice-cold normal saline containing 500 μg of oxalic acid. To the mixture was added 5 μg of daunomycin (Da) as an internal standard. The mixture was agitated and allowed to equilibrate in an ice-bath for 20 min. Then 10 ml of ice-cold 0.5 *N* hydrochloric acid in 85% isopropanol were added. The mixture was vortexed briefly and then allowed to stand in an ice-bath for 20 min. After centrifugation at 3000 *g* at 5° for 15 min, a 4.0-ml aliquot of the supernatant was removed into an ice-cold test-tube which contained 2.0 ml of cold chloroform. The mixture was vortexed for 20 sec, followed by centrifugation at 3000 *g* at 5° for 5 min. The upper aqueous layer was removed by aspiration and discarded and a volume of cold saturated sodium hydrogen carbonate solution equal to the remaining organic layer was added to the tube. The mixture was agitated for 5 min and then centrifuged in the cold at 3000 *g* for 5 min. The aqueous layer was removed by aspiration and the chloroform layer was pipeted into a silanized test-tube. The organic solution was evaporated using a stream of nitrogen. The side of the tube was rinsed with 20 μl of methanol–chloroform (1:1) and was again evaporated to dryness. The residue was dissolved by adding about 3–4 μl of methanol–chloroform (1:1) and the entire sample was spotted on to silica gel plates using a 2-μl Lang Levy pipet. On each plate were spotted 11 unknown samples, one of which was a blank, and five of which were Ad-spiked tissue (rabbit liver) standards. The plate was developed and then scanned as previously described[4]. Essentially, the plate was first developed ascendingly to 12 cm from the origin in a filter-paper-lined tank containing solvent I (chloroform–methanol–acetic acid, 93:5:2). The plate was then removed, dried briefly for 1 min with an air gun and placed in solvent II (chloroform–methanol–acetic acid, 76:20:4). The development height was again 12 cm from the origin. The plate was again dried briefly and allowed to stand in the hood for 10 min before scanning.

The excitation and emission wavelengths for the fluorescence scanning were set at 475 and 580 nm, respectively. At each lane, the internal standard spot was centered by manipulation of the *x–y* axis on the thin-layer chromatographic (TLC) scanner until the maximal fluorescence intensity was obtained. The plate was then returned to the origin of the spot and the scanning was carried out in the direction of the chromatographic development at a speed of approximately 2.2 cm/min.

A standard graph of Ad:Da fluorescence peak intensity ratio *versus* concentration of Ad in each tissue was constructed for each plate. The tissue levels of Ad (μg/g) were obtained from this standard graph. When the amount of certain blank tissue was limited, either blank skeletal muscle or liver was used for the construction of the standard graph. In each instance no significant difference was found.

RESULTS AND DISCUSSION

The recovery of Ad and Da extractions from rabbit liver homogenate was approximately 40% when compared with the peak height of the same amount of each applied directly on the same plate. Similar recovery data were obtained with rabbit kidney homogenate.

The use of acid–alcohol extraction appeared to have an advantage over the usual organic solvent extraction for these drugs as both Ad and Da are amines and have a high affinity for nuclear components which may not be entirely released by extraction with common organic solvents. Facile hydrolysis at the glycosidic linkage in the presence of strong acid has previously hampered the use of acid–alcohol mixtures for the analysis of intact Ad and Da. Using rapid extraction with 0.3 *N* hydrochloric acid in 85% isopropanol at low temperature, we have found no significant degradation (<5%) of Ad and Da. The choice of isopropanol rather than ethanol has enabled us to reconstitute the desired solvent composition, chloroform–isopropanol (1:1), previously found to be effective for the extraction of Ad and Da from plasma[4], by direct addition of an estimated amount of chloroform to the mixture. In addition, the use of Da as an internal standard has provided procedural advantages as discussed[4].

Typical thin-layer chromatograms of Ad- and Da-spiked tissue extract and of the extracts from nine organ tissues of a rabbit given 3 mg/kg of Ad, together with the corresponding tissue blanks, are shown on Fig. 1a–c. The standard graphs plotted as the relative fluorescence ratios of Ad to Da *versus* concentration of Ad and obtained from spiked kidney and liver homogenates are shown in Fig. 2a and b. Typical tissue levels of Ad and fluorescent metabolites of a rabbit given 3 mg/kg of the drug are shown in Table I. Comparison of this method with two other literature methods[1,2] for the analyses of the same tissue samples is shown in Table II.

As shown in Fig. 4, essentially no interference peaks with Ad, Da and major Ad metabolites were observed in nine tissue blanks measured (liver, kidney, heart, small intestine, bone marrow, lung, fat, muscle and spleen). As previously found in the plasma extract, Ad, Da and major Ad metabolites were well resolved from each other in the solvent system used. Similar results were found with tissue extracts (Fig. 1a and c). As shown in Fig. 2a and b, measurements were linear in the range 0.05–5.0 μg. The background noise levels were generally low, although they varied with the tissue being examined. However, most of the tissue levels of Ad in the treated animals are generally much higher than those in the plasma. The sensitivity level is more than adequate for the analysis of Ad in tissue samples after normal dosing. Therefore, no attempt was made to improve the sensitivity below 0.05 μg/g.

The reproducibility of this method was reflected by the excellent duplicate results between determinations of spiked tissue (Table I) and tissue samples from adriamycin-treated rabbits.

One complication in the construction of a standard graph for tissue was the metabolic enzymic activity of the tissue homogenate, even at low temperatures, especially the reductases. Generation of undesirable daunomycinol was seen when spiked with Da. The addition of 500 μg of oxalic acid to the tissue homogenate was found to alleviate this problem.

As shown in Fig. 1 and Table I, 3 h after administration of 3 mg/kg of Ad in a

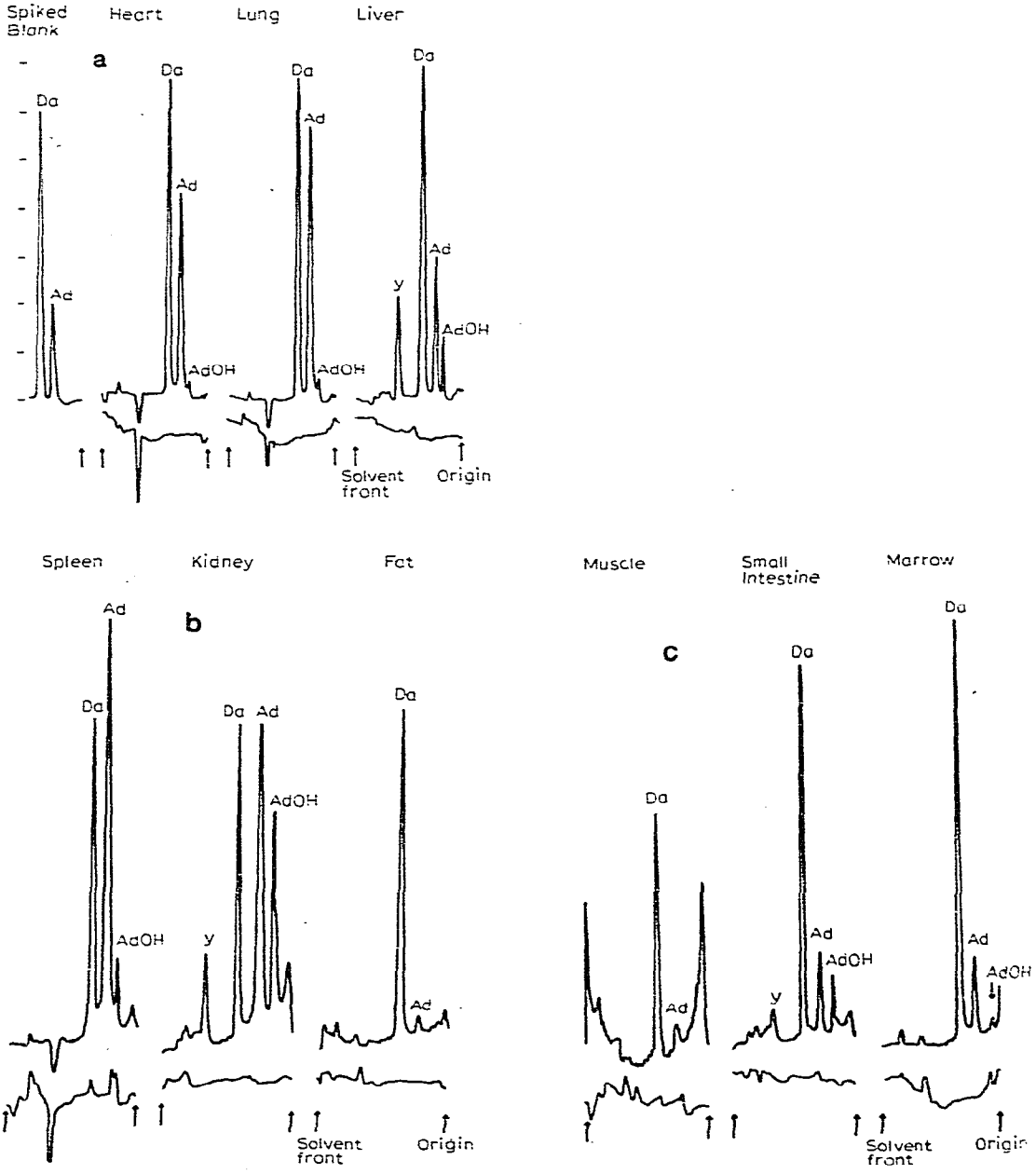

Fig. 1. TLC fluorescence scannograms of extracts from rabbit tissues. The upper curves represent tissue extracts with drugs. Spiked tissue is liver. Other organ tissues are indicated as labeled and are from a rabbit treated with 3 mg/kg of adriamycin. Setting: photomultiplier at 3, recorder at 100 mV, gain at 5, cut-off frequency at 0.1 kHz. The lower curves are blank tissue extracts. Setting: photomultiplier at 10, recorder at 20 mV, gain at 5, cut-off frequency at 0.05 kHz. These were performed on a different plate and the settings are approximately one third more sensitive than those used on the upper curves.

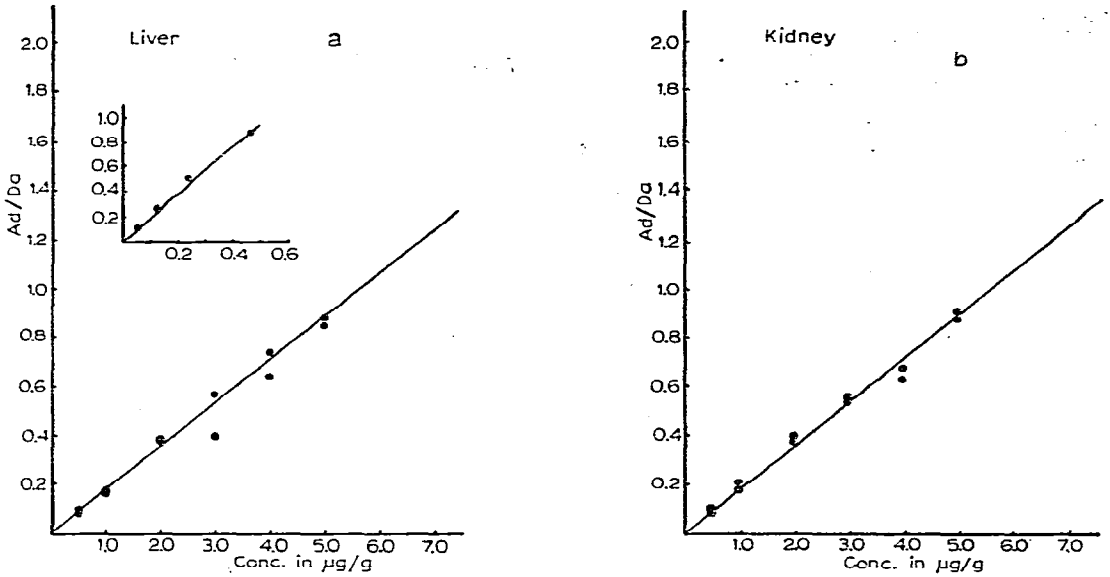

Fig. 2. Tissue standard graphs of Ad. Horizontal axis, Ad concentration; vertical axis, fluorescence peak-height ratio between Ad and Da. (a) Liver; (b) kidney. Upper curve on (a) shows the linearity at low concentrations on a different plate.

rabbit, two major metabolites, namely adriamycinol (AdOH) and an unknown aglycone metabolite Y, were detected in liver, kidney and spleen, but very little in heart, fat and skeletal muscle. This observation was consistent with the reported results[3]. The Ad level was found to be highest in the spleen, followed by kidney, and this order is consistent with that reported. As there were neither recovery data nor standard graphs for the metabolites, their levels in tissue were expressed in Ad equivalents only.

TABLE I

ADRIAMYCIN AND METABOLITES LEVELS IN RABBIT TISSUES 3 h AFTER ADMINISTRATION OF 3 mg/kg OF ADRIAMYCIN

Results are averages of at least duplicate determinations.

Tissue	*Ad (μg/g)*	*AdOH* (μg/g)*	*Metabolite Y**
Spleen	22.29 ± 1.24	3.03	N.D.**
Kidney	14.67 ± 2.59	11.10 ± 2.99	3.73 ± 0.25
Lung	13.73 ± 0.31	0.774 ± 0	N.D.
Liver	6.72 ± 0.09	3.18 ± 0.29	4.95
Heart	9.61 ± 0.52	0.48 ± 0.12	N.D.
Small intestine	2.60 ± 0.17	1.80 ± 0.04	0 84 ± 0.01
Fat	0.63 ± 0.01	N.D.	N.D.
Muscle	1.23 ± 0.18	N.D.	N.D.
Marrow	5.92 ± 0.19	N.D.	3.74

* Expressed as Ad equivalents.
** N.D. = not determined.

TABLE II

TISSUE AD CONCENTRATIONS (AVERAGE μg/ml ± S.D.)* IN RABBITS AS DETERMINED BY THREE METHODS

Results are averages of three determinations. Total dose: 60 mg.

Tissue	*Described method*	*Hydrolytic instant TLC method*[1]	*Non-hydrolytic TLC Method*[2]
Skeletal muscle	4.24 ± 0.36	4.37 ± 1.30	—*
Heart	13.93 ± 0.55	23.29 ± 1.95	14.89 ± 7.6
Liver	9.84 ± 0.31	3.57 ± 0.23	13.59
Lung	28.71 ± 5.18	48.86 ± 2.96	30.64 ± 6.97
Kidney	31.75 ± 4.40	62.88 ± 3.30	167.37 ± 46.68
Spleen	55.19 ± 2.40	79.52 ± 4.60	25.91 ± 5.6
Marrow	20.47 ± 0.65	13.89	—*
Small intestine	8.24 ± 0.11	24.52 ± 1.81	—*

* Samples not analyzed.

Comparison with the Literature

Tissue samples from a rabbit given 60 mg of Ad were analyzed by two other literature methods[1,2]. The results (Table II) indicated that the values from this method were in general agreement with those by the non-hydrolytic method[2] except for the kidney, where the value from the latter method was considerably higher. The values from the hydrolytic method were in general higher than those obtained by this method. The possible explanation of this discrepancy could be the lower resolution of the latter method and the inability to distinguish adriamycinone from that generated metabolically.

Method evaluation

Quantitative TLC analyses for drugs are usually hampered by various problems, such as non-uniform plate thickness, difference in coating consistency, low sensitivity in detection and poor reproducibility, although recent advances in methodology have greatly improved these deficiencies[5–8]. In the method described, a standard graph is prepared for each plate run together with the samples. This procedure corrects the problems of variability in replicate determinations. The use of daunomycin, a structurally similar compound to adriamycin, as the internal standard also eliminates the need for standardization of the instrument at different wavelengths and provides procedural corrections for extraction and difference in spot size, as both Da and Ad posses identical chromophores and similar extraction characteristics. In addition, the relatively high wavelengths of excitation and emission alleviate interferences due to common endogenous fluorescent materials. Therefore, this method, similar to that reported in plasma, is a reliable and sensitive quantitative TLC procedure.

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