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REVERSED-PHASE LIQUID CHROMATOGRAPHIC DETERMINATION OF PLASMA LEVELS OF ADRIAMYCIN AND ADRIAMYCINOL

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SUMMARY

A method is given for the determination of adriamycin and its main metabolite, adriamycinol in plasma from cancer patients after administration of adriamycin as the free drug or as a complex with DNA.

Adriamycin and adriamycinol are extracted in a column from 1 ml of plasma (pH 8.6) using a mixture of chloroform—1-heptanol (8:2). After re-extraction into phosphate buffer pH 2.2, the separation is performed as reversed-phase liquid chromatography on a LiChrosorb RP-2 (5 μm) column with a mobile phase of acetonitrile—water, acidified with phosphoric acid.

The precision by quantitation with photometric detection was better than 5% within the range 50—300 ng/ml. Plasma levels of adriamycin and adriamycinol in a cancer patient are presented in this paper.

INTRODUCTION

Adriamycin (Fig. 1) is one of the most promising new antineoplastic drugs [1,2]. The therapy with adriamycin, like with other anthraquinone glycosides, is restricted by drug-induced myocardial infarction [3,4]. The cumulative dose of adriamycin should not exceed 550 mg/m^2 [3]. Administration of adriamycin as a complex with a macromolecular carrier, herring sperm DNA, has recently been used to overcome the serious heart toxicity of the drug [5—7].

Pharmacokinetic studies of adriamycin in man after administration as free drug or as DNA complex require a reliable method for determination of plasma levels. Such a method should permit simultaneous determination of adriamycinol (Fig. 1), a metabolite of adriamycin, with similar cytotoxic activity as the intact drug [8,9]. The pharmacokinetics of adriamycin has previously been

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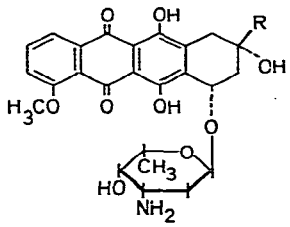


Fig. 1. Structural formulae. Adriamycin, R = $COCH_2OH$; adriamycinol, R = $CH(OH)CH_2OH$.

studied with unselective methods such as the radioimmunoassay (RIA) technique and measurements of total fluorescence of plasma extracts [10—12]. Analytical methods including a chromatographic step offer a higher selectivity, and have been used recently [13—16].

The present paper gives an analytical method for simultaneous determination of adriamycin and adriamycinol in plasma after administration of adriamycin as the free drug or as the DNA complex. The proposed analytical method comprises of column extraction from the buffered plasma sample into an organic phase, re-extraction into a small acidic aqueous phase and separation by reversed-phase liquid chromatography. A high detection sensitivity as well as a high detection selectivity of the method is obtained by the use of a photometric or a fluorimetric chromatographic detector. The construction of the method has been based upon previously reported studies of the extraction properties and reversed-phase liquid chromatographic separation of antraquinone glycosides [17,18], cf. ref. 19.

EXPERIMENTAL

Apparatus

The spectrofluorimeter used was an Aminco-Bowman 4-8202 B, and the pH meter an Orion Research Model 701/digital pH meter equipped with an Ingold combined electrode type 401.

Glass equipment. All glass equipment, with the exception of micro-pipettes, were silanized before use by treatment with dichlorodimethylsilane (5% by volume) in toluene, followed by washing with dry methanol.

Chromatographic system. The detectors were a LDC Spectromonitor II (500 nm, 10-mm path length, 8-μl volume) and a Schoeffel Instrument FS 970 Fluorimetric Detector (435/550 nm; 5-μl cell volume). A LDC 711 Solvent Delivery System pump was used. The columns were stainless steel (length 150 mm, 4 mm I.D., 1/4 in. O.D. Column end fittings were modified Swagelok connectors. A Rheodyne (Model 70-10) injection valve with a sample loop of 300 μl was used. The support employed was LiChrosorb RP-2 (Merck, Darmstadt, G.F.R.) with a mean particle diameter of 5 μm. The chromatographic system was thermostated to 25.0 ± 0.1°.

Chemicals

Adriamycin and adriamycinol were kindly supplied by Farmitalia (Milan, Italy), and desipramine chloride by A.B. Hässle (Mölndal, Sweden). The mobile

phases were prepared from acetonitrile (Merck, Uvasol), phosphoric acid (Merck, p.a.) and distilled water. All other chemicals were of analytical grade and used without further purification.

Chromatographic technique

The columns were packed by the balanced density slurry technique previously described [20] with tetrachloroethylene as suspending medium. The columns were washed with *n*-hexane and acetone (100 ml of each) before use. The mobile phase was passed through the chromatographic system until constant retention of the solutes was obtained. Less than 50 ml was usually required.

Incubation of whole blood samples

To 4-ml whole blood samples (heparinized) were added 100 μl of 10^{-2} *M* phosphoric acid containing adriamycin (40 μg/ml). After addition of 100 μl of 10^{-2} *M* NaOH the samples were incubated at 25° for between 5 min and 22 h. At the end of the incubation time the samples were centrifuged at 4080 *g* for 10 min. The plasma fractions were analysed for adriamycin and adriamycinol according to the proposed analytical method.

Plasma samples from cancer patients

Blood samples (5—7 ml) were collected in 10-ml glass test-tubes (Vacutainer) containing 250 I.U. heparin (freeze dried) immediately before and at appropriate times after the commencement of drug administration. The samples were immediately centrifuged at 4080 *g* for 10 min. The plasma fraction was carefully aspirated and frozen at —20° until assay.

Spiking of plasma samples

The precision and accuracy of the proposed analytical method were tested by analysis of blank plasma samples, spiked with adriamycin and adriamycinol [19].

Analytical method

Extraction procedure. The plasma sample (1.0 ml) is carefully mixed with 0.10 ml phosphate buffer pH 8.6, μ = 1.0 and 1.5 g of diatomaceous earth (acid washed Celite 545), and quantitatively transferred into an empty extraction column (6 mm I.D.). The column is eluted with chloroform—1-heptanol (8:2, v/v), the first 7 ml being collected in a centrifuge tube. The organic phase is extracted for 10 min with 0.300 ml phosphate buffer, pH 2.2, μ = 0.1, containing 10 μg/ml of desipramine. The aqueous (upper) phase from the extraction procedure is transferred into a centrifuge tube with tapered bottom (0.2 ml) containing 2 ml of hexane and centrifuged. (This step is included to facilitate the transfer of the aqueous phase into the chromatographic column without contamination with organic phase.)

Liquid chromatographic isolation and quantitation. Part of the aqueous (lower) phase (0.050—0.300 ml) is injected into the chromatographic column (support: LiChrosorb RP-2, 5 μm mean particle diameter; mobile phase: acetonitrile—water—0.1 *M* phosphoric acid (20:70:10); mobile phase flow-rate

0.8—1.0 ml/min). The concentration of the solutes in the eluate is measured by photometric (500 nm) or fluorimetric (435/550 nm) detection. Quantitation is based on peak area measurements.

RESULTS AND DISCUSSION

Metabolic activity in whole blood samples

Adriamycin has been reported not to be convertable into adriamycinol by human blood cells [21]. No traces of adriamycinol were found in plasma after incubation of whole blood samples with adriamycin for 0—2 h (Table I). Low amounts of adriamycinol were, however, found after incubation for 4—22 h, but the conversion of adriamycin to adriamycinol was found to be considerably slower than the corresponding formation of daunorubicinol from daunorubicin, a cytostatic drug with a structure similar to adriamycin [19]. No formation of adriamycinol, when handling the blood samples according to the procedure described above, is likely to occur.

TABLE I

FORMATION OF ADRIAMYCINOL FROM ADRIAMYCIN IN WHOLE BLOOD SAMPLES

Whole blood samples from a healthy volunteer incubated with 1 μg of adriamycin per ml at 25°.

Time (h)	Relative amounts adriamycinol/adriamycin
0	<0.01
1	<0.01
2	<0.01
4	0.04
6	0.08
22	0.24

Extraction procedure

Optimum degree of extraction is obtained at pH 8.4 for adriamycin and 8.6 for adriamycinol [17]. Under these conditions 94% and 62% of the drug and its metabolite, respectively, are extracted into the organic phase using equal phase volumes. Quantitative extraction can be obtained by an increased phase volume ratio. For the quantitative extraction (>99%) of adriamycinol from 1 ml of an aqueous phase, the volume of the organic phase must exceed 60 ml. Such a large volume is very inconvenient in the re-extraction step preceeding the injection of the sample into the liquid chromatograph.

In the present method, column extraction is used for quantitative transfer of adriamycin and adriamycinol from buffered plasma (pH 8.6) into an organic phase [chloroform—1-heptanol (8:2, v/v)]. The drug and the metabolite are completely eluted within the first 7 ml of the organic extractant passing through the column.

The extraction degree of adriamycin and adriamycinol from a buffer solution (pH 8.6, μ = 0.1) were found to be strongly influenced by the presence of herring sperm DNA, most likely as a result of complex formation [19]. A

25-fold excess (by weight) of DNA reduced the extraction degree of adriamycin and adriamycinol (200 ng/ml) by 20 and 35%, respectively, in batch experiments. When using the column extraction technique as in the proposed method, the influence of DNA on the extraction yield is negligible within therapeutic plasma levels of adriamycin and adriamycinol.

Adriamycin and adriamycinol are separated from aglycones, formed as metabolites [8], by re-extraction into a small volume of acidic phase. Conditions for quantitative re-extraction can be calculated from constants given in ref. 17. A phase volume ratio organic phase:aqueous phase of 25 makes it necessary to use an aqueous phase of pH < 2.6 for quantitative transfer of adriamycin and adriamycinol from the organic into the aqueous phase.

To avoid the effect of adsorption phenomena by the extraction, a secondary amine, desipramine (10 μg/ml) was added to the acidic aqueous phase [19], used in the re-extraction step.

The stability of adriamycin and adriamycinol at 25° was studied by liquid chromatography. At pH 2.2 no degradation was observed within 24 h. Storage for 2 h at pH 8.6 gave recoveries of 94 and 97% of adriamycin and adriamycinol, respectively.

Chromatographic isolation

Separation of anthraquinone glycosides by reversed-phase liquid chromatography has previously been studied in respect to selectivity and retention [18]. The highest performance of the chromatographic system was obtained using LiChrosorb RP-2 as the support with a mobile phase containing 20—30% (v/v) of acetonitrile in water, acidified with phosphoric acid. The retention of adriamycin and adriamycinol was strongly affected by the concentration of acetonitrile, Fig. 2 [18]. In the method 20% of acetonitrile is used giving a complete separation of the drug and the metabolite within 10 min.

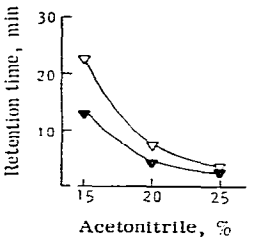


Fig. 2. Retention time and concentration of acetonitrile in mobile phase. Support: LiChrosorb RP-2 (5-μm mean particle diameter). Sample: adriamycin (▽) and adriamycinol (▼), 2.5 nmoles of each in 100 μl of mobile phase. Mobile phase, phosphoric acid (10^{-2} M) in acetonitrile—water; mobile phase flow-rate, 1.0 ml/min.

Detection selectivity and sensitivity

Liquid chromatographic determination of adriamycin and adriamycinol is possible with a very high detection selectivity. A high detector response is obtained by photometric detection at 500 nm as well as by fluorimetric detection at 435/550 nm, i.e. under conditions where almost no endogenous compounds

and other drugs may interfere. Highest detection sensitivity was given by the fluorimetric detector. A sample containing 2 ng of each adriamycin and adriamycinol gave a signal-to-noise ratio of 3 under the chromatographic conditions used in the analytical method. Photometric detection at 500 nm gave about 3—5 times lower sensitivity. Besides the high detection selectivity and sensitivity a further advantage of the fluorimetric detector is given by the fact that, compared to photometric detectors considerably larger sample volumes can be injected. Only minor disturbances of the baseline are caused by the non-retarded solvent peak. Band broadening of the solute peaks is minimized by a proper choice of sample solvent [22]. In this method, phosphate buffer pH 2.2 is used by which the solutes are almost completely retarded.

Quantitative determination

Fluorimetric detection (435/550 nm). Evaluation of the amount of adriamycin and adriamycinol in an unknown sample is based on peak area measurement and the use of a calibration graph, obtained by running known amounts of adriamycin and adriamycinol through the chromatographic system. Identical standard curves [Y (mm^2) = 8.58 + 6.51 X (ng); r = 0.9999, n = 20] were obtained for the two compounds within the range 10—300 ng. By increasing the sensitivity setting of the detector it was possible to quantitate as little as 2 ng of each compound.

Photometric detection (500 ml). Quantitation is based on peak area measurement. The amount of sample in the chromatographic peak can be calculated by means of eqn. 1:

$$M = Y \times u \times b \times \epsilon^{-1} \quad (1)$$

where M = amount of sample in mmoles, Y = peak area in mm^2, u = ml/mm chart paper, b = absorbance/mm chart paper and ϵ = molar absorptivity of the migrating compound [22]. The molar absorptivity of adriamycin and adriamycinol were found to be identical (7.89×10^3).

TABLE II

RECOVERY AND PRECISION

Photometric detection at 500 nm.

Plasma level (ng/ml)	Recovery (%)		Added as**
	Adriamycin	Adriamycinol	
50	85.9 ± 2.24*	93.3 ± 1.64	Free drug
100	89.7 ± 4.66	90.4 ± 3.89	Free drug
280	90.5 ± 3.43	93.2 ± 2.04	Free drug
50	95.4 ± 3.83	95.7 ± 4.11	DNA complex
100	95.1 ± 3.00	96.3 ± 3.19	DNA complex
225	99.7 ± 1.07	96.4 ± 2.40	DNA complex

*Relative standard deviation (n=9).

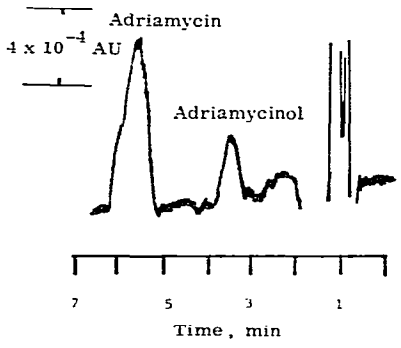
**For details see ref. 19.

Recovery and precision

The recovery and precision of the method at various drug levels are presented in Table II. Adriamycin and adriamycinol can be determined with a precision better than 5% at plasma levels above 50 ng/ml (photometric detection).

Plasma samples from patients

A chromatogram of a plasma sample from a cancer patient treated with adriamycin is shown in Fig. 3. Plasma levels of adriamycin and adriamycinol in a cancer patient treated with adriamycin are shown in Fig. 4. Further pharmacokinetic studies are in progress.



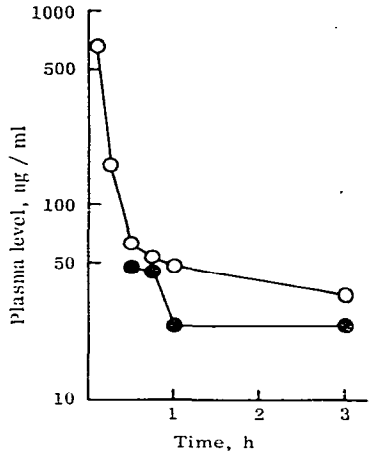


Fig. 3. Chromatogram from cancer patient plasma containing adriamycin and adriamycinol. Chromatographic conditions as given by the analytical method (photometric detection). Plasma concentrations: adriamycin, 44 ng/ml; adriamycinol, 8 ng/ml.

Fig. 4. Plasma levels of adriamycin and adriamycinol after intravenous infusion of adriamycin. ○ = Adriamycin, ● = adriamycinol. Administered dose of adriamycin, 1.0 mg/kg.

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SPECTROFLUORIMETRIC DETERMINATION OF DIPYRIDAMOLE IN SERUM — A COMPARISON OF TWO METHODS

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SUMMARY

Two spectrofluorimetric methods for the determination of dipyridamole in plasma are described. The thin-layer chromatographic—fluoridensitometric method utilizes 1 ml of plasma which is extracted at pH 10 with diethyl ether—dichloromethane (80:20). The organic phase is evaporated to dryness, reconstituted in 250 μl dichloromethane and 5 μl are spotted on a silica gel 60 plate. The plate is developed in ethyl acetate—methanol—ammonia (85:10:5), dried, dipped in a paraffin wax solution, dried, and scanned using 380 nm as excitation wavelength, a 430 nm cut-off filter, and collecting all emitted light on the photomultiplier. Quantitation was done by the external standard method, peak heights being measured and a calibration graph constructed. For the spectrofluorimetric method 1 ml of plasma is extracted at pH 10 with 8 ml of hexane—isoamyl alcohol (95:5) and the organic phase used directly for the measurement of the fluorescence intensity (excitation 405 nm, emission 495 nm). Quantitation was done by measuring the fluorescence of standards that were treated as above and constructing a calibration graph of concentration versus fluorescence intensity. Concentrations of unknowns were found by interpolation from this graph. The two methods were found to exhibit good correlation but the spectrofluorimetric method proved to be more amenable to the analysis of a large number of samples.

INTRODUCTION

When asked to perform a bioavailability study on tablets containing dipyridamole (Fig. 1) a review of the literature revealed that only a few analytical procedures for this drug had been published [1—4]. None of these methods suited our particular needs and an analytical method for the determination of dipyridamole in plasma had to be developed. Due to the highly fluorescent nature of this drug, it was decided to utilize this property as a means of determination.

This paper describes two fluorimetric procedures for the determination of dipyridamole in plasma; namely, a thin-layer chromatographic (TLC)—fluoridensitometric method, and a spectrofluorimetric method where the fluores-