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LIQUID CHROMATOGRAPHIC DETERMINATION OF DAUNORUBICIN AND DAUNORUBICINOL IN PLASMA FROM LEUKEMIC PATIENTS

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SUMMARY

A method is given for the determination of daunorubicin and its main metabolite, daunorubicinol, in plasma from leukemic patients after administration of daunorubicin as the free drug or as a complex with DNA. Daunorubicin and daunorubicinol are extracted from 2 ml of plasma (pH 8.1) using a mixture of chloroform and 1-heptanol (9:1). After re-extraction into phosphoric acid (0.1 *M*), 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 a photometric detector, was better than 2% within the range 20 ng/ml to 200 ng/ml. Some determinations of plasma levels of daunorubicin and daunorubicinol are presented.

INTRODUCTION

Daunorubicin (Fig. 1) is an antraquinone glycoside widely used for the treatment of acute leukemia[1]. It is extensively metabolized to daunorubicinol (Fig. 1) with

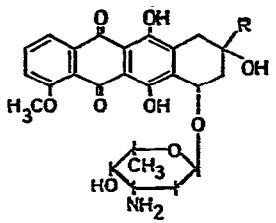

Fig. 1. Structural formulae. R = $-COCH_3$ (daunorubicin); $-CH(OH)CH_3$ (daunorubicinol).

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similar cytotoxic activity[2]. Cardiac irregularities and myocardiopathy are the most serious side effects from treatment with daunorubicin[3,4]. The cardiac toxicity has, in animal models, been found to decrease significantly by administration of daunorubicin as a complex with DNA[5].

A sensitive and selective method for the determination of daunorubicin and daunorubicinol in plasma from leukemic patients after administration of daunorubicin as free drug, or as complex with DNA, would be of a great value for comparative pharmacokinetic studies. Plasma levels of daunorubicin and its metabolites have previously been determined by measurement of total fluorescence after extraction with acidified ethanol[6] or after separation by thin-layer chromatography (TLC)[7]. High-performance liquid chromatography was used for the separation and quantitative determination of daunorubicin and daunorubicinol after extraction from alkalized rabbit plasma[8]. Recently a comparision of plasma daunorubicin levels by fluorescence and radioimmunoassay has been presented[9].

This paper gives a method for the determination of plasma levels of daunorubicin and daunorubicinol comprising extraction with chloroform and 1-heptanol and separation by reversed-phase liquid chromatography with a photometric detector.

EXPERIMENTAL

Apparatus

This consisted of an Aminco-Bowman 4-8202 B spectrofluorimeter and 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, was silanized before use, by treatment with dichlorodimethylsilane (5% by volume) in toluene, followed by washing with dry methanol.

Chromatographic system

The two detectors used were the LDC Spectromonitor I (500 nm) and the Altex Model 153 (253.7 nm). Both were equipped with cells of 10 mm path length and volume 8 μl.

The pump was of the LDC 711 Solvent Delivery System type, and the columns were of stainless steel (length 150 mm, I.D. 4 mm, O.D. 1/4 in.). The column end fittings were modified Swagelok connectors. A Rheodyne (Model 70-10) injection valve with a sample loop of 300 μl was used.

The LiChrosorb RP-2 support (E. Merck, Darmstadt, G.F.R.) had a mean diameter of 5 μm, and the chromatographic system was thermostated to 25.0 $\pm$ 0.1°.

Chemicals

Daunorubicin and daunorubicinol were obtained from Pharma Rhodia (Stockholm, Sweden). Desipramine was supplied by AB 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 were used without further purification.

Chromatographic technique

The columns were packed by the balanced density slurry technique[10] with tetrachloroethylene as the 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. Usually less than 50 ml were required.

Drug administration

Administration of daunorubicin as free drug. Daunorubicin (0.70–1.5 mg/kg body weight in 200 ml 0.9% NaCl solution) was given as an intravenous infusion for 45 min.

Administration of daunorubicin as complex with DNA. Daunorubicin (100 mg) was added to 500 ml of 0.9% NaCl solution containing 2.34 mg herring sperm DNA/ml. The mixture was given as an intravenous infusion (100 ml/h) with a total dose of 0.70–1.5 mg daunorubicin/kg body weight.

Plasma samples from leukemic 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 start of drug administration. The samples were immediately cooled in an ice bath and centrifuged at 4080 *g* for 10 min at 0°. The plasma fraction was carefully aspirated and frozen at −20° until assay.

Spiking of plasma samples

The accuracy and precision of the method was studied as follows: (i) Daunorubicin and daunorubicinol dissolved in 0.1 *M* phosphoric acid (50 μl/ml plasma) were added to drug free plasma. After neutralization with 0.1 *M* sodium hydroxide solution of the spiked plasma samples were treated as described under Analytical method. (ii) Daunorubicin, daunorubicinol and herring sperm DNA (1:1:24, w/w/w) dissolved in distilled water (50 μl/ml plasma) were added to drug free plasma. The spiked plasma samples were treated as described under Analytical method.

Spiking plasma samples with daunorubicin and daunorubicinol (25–200 ng of each per ml plasma) dissolved in distilled water (no DNA present) gave recoveries within the range 30–80%, the recovery being increased with increasing concentration. These results are probably due to adsorption to glass surfaces. No similar effect was observed on addition of daunorubicin and daunorubicinol as DNA complexes dissolved in distilled water.

ANALYTICAL METHOD

Extraction procedure

A 2.00-ml sample of plasma is mixed with 0.2 ml phosphate buffer pH 8.1, $\mu = 1.0$ and extracted with 10.00 ml chloroform–1-heptanol (9:1) for 10 min. After centrifugation, 7.00 ml of the organic phase is extracted with 0.400 ml phosphoric acid 0.1 *M* containing 5 μg/ml of desipramine for 10 min. The aqueous (upper) phase from the extraction procedure is transferred into a centrifuge tube with a tapered base

(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; mobile phase: acetonitrile–water–0.1 *M* phosphoric acid, (25:65:10); mobile phase speed: 0.8–1.0 ml/min). The absorbance of the eluate is measured at 253.7 or 500 nm. Quantitation is based on peak area measurement and molar absorptivity of the migrating compounds.

RESULTS AND DISCUSSION

Metabolic activity in whole blood samples

Daunorubicin has been reported to convert into daunorubicinol under the influence of human hematological components, while no conversion was detected in cell-free plasma[11]. However, no detectable amount of daunorubicinol (<3 ng/ml) was found after incubation of heparinized blood with daunorubicinol (200 ng/ml) at 4° for 30 min.

Extraction procedure

The extraction of daunorubicin and daunorubicinol using chloroform and 1-pentanol as the organic phase has been extensively studied, and is reported elsewhere[12]. The degree of extraction is strongly dependent upon the pH of the aqueous phase as illustrated in Fig. 2. Optimum extraction is obtained at pH 8.0 for daunorubicin and 8.1 for daunorubicinol with an extraction degree of 97 and 91%, respectively (equal phase volumes). Quantitative extraction (>98%) of daunorubicin and daunorubicinol is obtained by using a volume ratio organic phase:aqueous phase of 5.

To avoid an interfering peak in the chromatogram caused by 1-pentanol, chloroform with addition of 1-heptanol was used for the quantitative extraction of daunorubicin and daunorubicinol. It is not likely that this modification affects the extraction profile presented in Fig. 2 (*cf.* ref. 13). No influence on the extraction degree

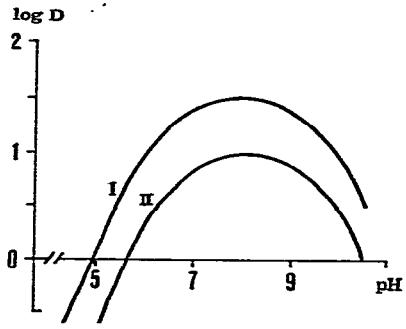

Fig. 2. Distribution ratio and pH of the aqueous phase. Organic phase: chloroform–1-pentanol (9:1); aqueous phase: buffer solution (ionic strength 0.1). I = Daunorubicin, II = daunorubicinol. *D* = distribution ratio = (total concentration in the organic phase)/(total concentration in the aqueous phase) (equal phase volumes). The curves are calculated from constants given in ref. 12.

due to formation of DNA complexes was observed by the extraction of daunorubicin and daunorubicinol (200 ng/ml) in buffer solution pH 8.1 containing 1.0–5.0 μg/ml of herring sperm DNA using chloroform–1-heptanol (9:1; equal phase volumes) as the organic phase. Daunorubicin and daunorubicinol are separated from aglycones, formed as metabolites[14], by re-extraction into a small acidic aqueous phase.

Conditions for quantitative re-extraction can be calculated from determined constants[12]. A phase volume ratio, organic phase:aqueous phase of 20 makes it necessary to use an aqueous phase of pH <1.7 for quantitative transference of daunoribicin and daunorubicinol from the organic into the aqueous phase. In practice, however, quantitative re-extraction was not obtained under the calculated conditions, probably as a result of adsorption phenomena. This assumption is supported by the fact that the yield from plasma samples, spiked with daunorubicin and daunorubicinol, was considerably higher than from spiked buffer samples, *i.e.* adsorption was to a great extent prevented by masking of adsorption sites by coextracted plasma components. The effect of the adsorption phenomena could completely be eliminated by the addition of a secondary amine, desipramine (5 μg/ml), to the small acidic aqueous phase[15]. Transference of liquid phases was to a significant extent simplified by silanization of glass equipment. Both daunorubicin and daunorubicinol were stable at pH 1.7 (25°) for at least 7 days as confirmed by liquid chromatography.

Chromatographic isolation

Reversed-phase liquid chromatography of daunorubicin and daunorubicinol has been studied with respect to selectivity and retention[16]. 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 daunorubicin and daunorubicinol is strongly dependent upon the concentration of acetonitrile, Fig. 3[16]. In our method a mobile phase containing 25% acetonitrile is used, giving a complete separation of daunorubicin and daunorubicinol within 10 min. Minor deviations from this concentration may some-

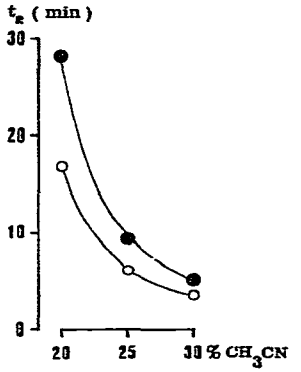

Fig. 3. Retention time and concentration of acetonitrile in mobile phase. Support: LiChrosorb RP–2 (5 μm); sample: daunorubicin (●) and daunorubicinol (○), 2.5 nmoles of each in 100 μl of mobile phase; mobile phase: phosphoric acid (10^{-2} *M*) in water–acetonitrile; mobile phase speed: 0.90 ml/min.

times be necessary when monotoring the eluate at 253.7 nm to increase the resolution between daunorubicin and daunorubicinol and other coextracted UV-absorbing drugs and metabolites.

Detection selectivity and sensitivity

The chromatographic system used showed a high selectivity in the separation of anthraquinone glycosides with minor differences in substitution of the side chain[16]. A chromatogram of drug free plasma samples showed no interfering peaks when measuring the eluate at 253.7 nm. An increase in the detection selectivity may sometimes be necessary, and can be obtained by measuring the eluate at 500 nm. This results, however, in a 3–5 times lower detection sensitivity caused by a combination of lower molar absorptivity (see below) and higher noise level of the detector.

Quantitative determination

Quantitation is based on peak area measurement, eqn. 1.

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

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[17]. The molar absorptivity of daunorubicin and daunorubicinol were found to be identical (7.89×10^3 at 500 nm, and 1.92×10^4 at 253.7 nm). From eqn. 1, it follows that the peak area is independent of chromatographic parameters such as column efficiency and capacity factors of the solutes, as well as of length and diameter of the used chromatographic column. Hence, once the molar absorptivity of the solute has been determined no calibration graph has to be constructed.

Recovery and precision

The recovery and precision of our method at various drug levels are presented in Table I. It shows that daunorubicin and daunorubicinol can be determined with a precision better than 2% at plasma levels >20 ng/ml.

TABLE I

RECOVERY AND PRECISION

Measuring wave length: 500 nm.

Plasma level (ng/ml)	*Recovery (%)*		*Added as*
	Daunorubicin	*Daunorubicinol*	
25	99.3 ± 1.1*	98.6 ± 2.1	Free drug**
100	99.6 ± 1.6	99.7 ± 0.9	Free drug
200	100.0 ± 0.3	103.5 ± 1.8	Free drug
20	99.3 ± 1.7	99.7 ± 1.8	DNA complex**
70	99.9 ± 1.5	99.0 ± 1.2	DNA complex
200	99.5 ± 1.2	101.0 ± 0.8	DNA complex

* Relative standard deviation (n = 8).

** See text for details.

Plasma samples from leukemic patients

A typical chromatogram of a plasma sample from a leukemic patient treated with daunorubicin is shown in Fig. 4. Examples of plasma levels of daunorubicin and daunorubicinol after administration of daunorubicin as the free drug and as a DNA complex to leukemic patients are presented in Fig. 5. Further pharmacokinetic studies are in progress.

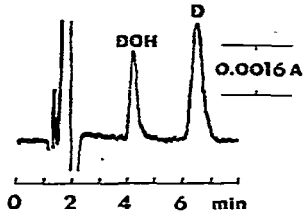

Fig. 4. Chromatogram from leukemic patient plasma containing daunorubicin and daunorubicinol. Chromatographic conditions as given in the text (measuring wave length: 500 nm); plasma sample taken after infusion of 1.4 mg daunorubicin/kg for 45 min; plasma concentrations found: daunorubicin 370 ng/ml: daunorubicinol 90 ng/ml.

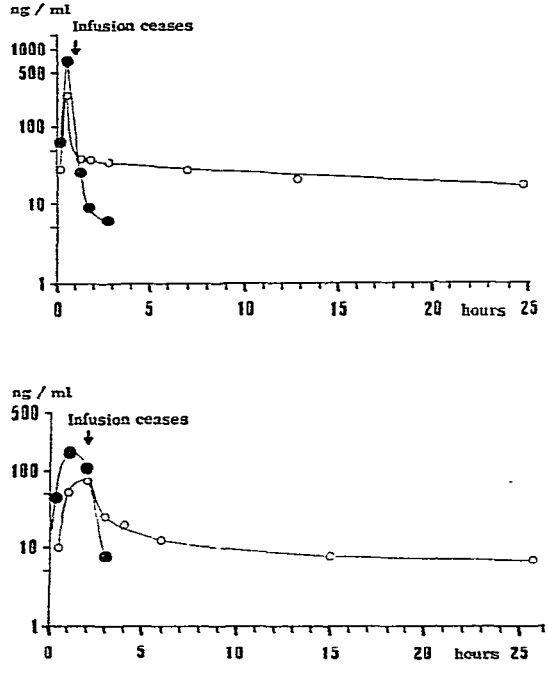

Fig. 5. Plasma levels of daunorubicin and daunorubicinol after intravenous infusion of daunorubicin: ● = daunorubicin; ○ = daunorubicinol. Administered dose of daunorubicin: 1.30 mg/kg as free drug (a); 0.81 mg/kg as DNA complex (b).

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