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Note

Rapid high-performance liquid chromatographic assay for the anthracyclines daunorubicin and 7-con-O-methylnogarol in plasma

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The anthracyclines doxorubicin and daunorubicin** (Fig. 1) are important antitumour drugs but show a dose-limiting cardiotoxicity [1, 2]; hence, less toxic anthracyclines are being sought [3]. Rapid reliable assays are needed for in vivo studies on such compounds: here we consider daunorubicin and 7-con-O-methylnogarol. The former is a "type" compound for the group, and an assay for it in the blood of infected animals was needed for investigation of its trypanocidal effects [4]; the latter compound is the most promising semi-synthetic derivative of the anthracycline nogalamycin [5].

Fluorescence assay will not distinguish between anthracyclines and their fluorescent metabolites [6], and thin-layer chromatography is also inappropriate since hydrolysis of drug may occur [1]. High-performance liquid chromatography (HPLC) is the method of choice. Normal-phase HPLC has been reported [7—11], but the drug must be extracted into organic phase so there may be problems of recovery. Reversed-phase HPLC has also been used [7, 12—17]; in all cases an extraction (solvent extraction or adsorption on to a pre-column) was performed, yielding an organic phase which was either evaporated or back-extracted, so again there are problems of recovery. The procedure described here maximises recovery of drug and metabolites (irrespective of partition properties), and minimises transferences, by precipitation of protein from the plasma followed by direct injection of supernatant on to the HPLC column. Whilst this work was in progress, a similar method was reported for doxorubicin by Quattrone and Ranney [18].

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**Daunorubicin is the International Non-Proprietary Name for the compound given the trivial name daunomycin.

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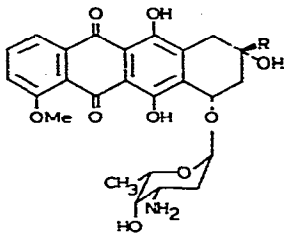
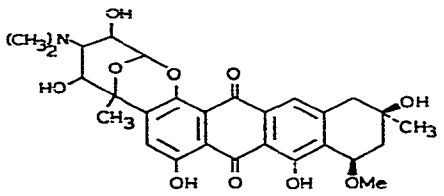


Doxorubicin R=$COCH_2OH$
Daunorubicin R=$COCH_3$
Daunorubicinol R=$CH(OH)CH_3$



7-con-o-methylnogarol

Fig. 1. Structural formulae of doxorubicin, daunorubicin, daunorubicinol and 7-con-O-methylnogarol (the stereochemistry of the latter compound is not yet fully verified but is most probably that shown here [5]).

MATERIALS AND METHODS

Materials

Daunorubicin hydrochloride and daunomycinone were gifts from Dr. F. Arcamone (Farmitalia, Milan, Italy); 7-con-O-methylnogarol was a gift from Dr. P.F. Wiley (The Upjohn Company, Kalamazoo, MI, U.S.A.). Acetonitrile was HPLC grade (Fisons, Loughborough, Great Britain) and phosphoric acid was analytical grade (BDH, Poole, Great Britain). Water was double-distilled before use. All glassware was silanised to prevent adsorption of drug. All drug solutions were protected from light and were stored refrigerated.

Method

General extraction method. Acetonitrile—0.1 *M* H_3PO_4 (4 : 1) (1 ml) was added to human plasma (1 ml) in a stoppered centrifuge tube; the mixture was vortex-mixed for 10 sec then centrifuged at 2200 *g* for 10 min to pellet the precipitated protein. The supernatant (20 μl or 100 μl) was chromatographed and the peak height and peak area were compared with those from standards prepared by the addition of drug to a centrifuged plasma—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1) mixture.

High-performance liquid chromatography. A Pye LC-XPD pump was used with a six-port rotary injection valve (Rheodyne 7125) and either a 20-μl or 100-μl loop, and a 250 × 4.6 mm I.D. LiChrosorb RP-2 (5 μm) column. The mobile phase was 35% acetonitrile in 0.01 *M* H_3PO_4 at a flow-rate of 1 ml min^{-1} (ambient temperature) and detection was with a 25-μl mirrored flow cell in a Perkin-Elmer 3000 fluorescence spectrometer (Figs. 2 and 3).

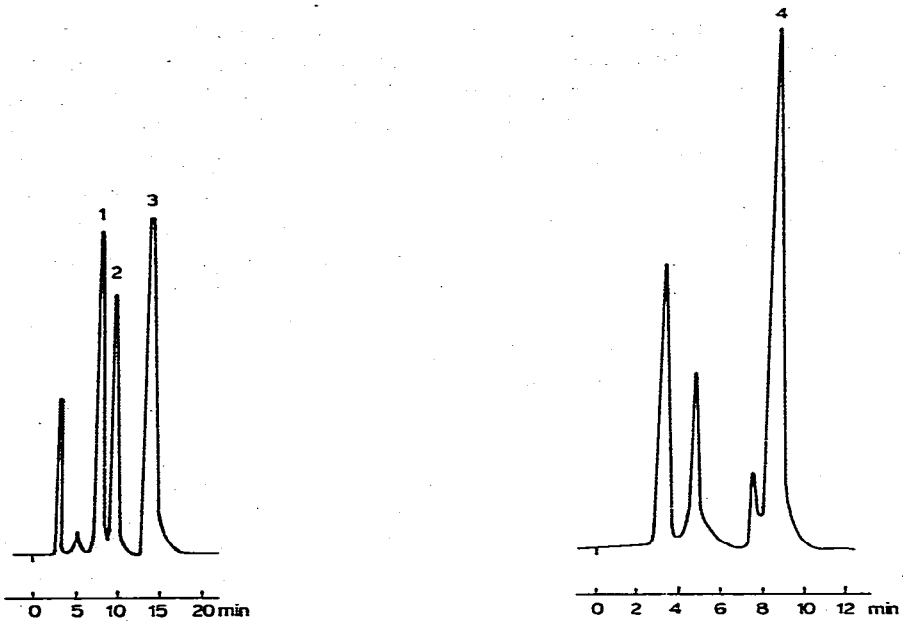


Fig. 2. Separation of daunomycinol (1, retention time 7.5 min) daunorubicin (2, 9.5 min) and daunomycinone (3, 14.0 min) in supernatant from precipitated plasma. Stationary phase, LiChrosorb RP-2; mobile phase, 35% acetonitrile in 0.01 *M* phosphoric acid; pressure, 120 bar; flow-rate, 1.0 ml min^{-1}; fluorescence detection, excitation 475 nm (15 nm slit), emission 557 nm (20-nm slit).

Fig. 3. Separation of 7-con-O-methylnogarol (4, retention time 8.3 min) in supernatant from precipitated plasma. Conditions as for Fig. 2, except excitation 471 nm (15-nm slit), emission 550 nm (20-nm slit).

Evaluation of fluorescence enhancement. Solutions containing daunorubicin (500 ng ml^{-1}) were prepared in mixtures of plasma and acetonitrile—0.01 *M* H_3PO_4 (4 : 1) in ratios from 25 : 75 to 75 : 25. This was repeated using water in place of plasma. All solutions were vortex-mixed for 10 sec then centrifuged at 100,000 *g* for 2 h. The fluorescence of each solution was determined at 30°C, with the excitation monochromator at 475 nm (5-nm slit) and the emission monochromator at 557 nm (10-nm slit).

RESULTS

The method developed here for the assay of daunorubicin in plasma uses precipitation of proteins from the plasma by addition of an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1), so avoiding losses during solvent extraction and during transferences. An aliquot of the supernatant is then chromatographed. Since fluorescence detection was used, it was necessary to verify that there is no change in the fluorescence properties of the drug when it is applied to the column in this form compared to a solution in water. Preliminary experiments showed that the fluorescence of drug detected on elution from the column is enhanced in the treated plasma sample compared to the water standards. This change is presumably due to co-eluting material.

This enhancement of daunorubicin fluorescence was further investigated by determination of the fluorescence of daunorubicin (500 ng ml^{-1}) in centrifuged mixtures of plasma and acetonitrile—0.1 *M* H_3PO_4 (4 : 1) and in centrifuged mixtures of distilled water and acetonitrile—0.1 *M* H_3PO_4 (4 : 1). The fluorescence of daunorubicin in those samples containing water was independent of the ratio of water to acetonitrile—0.1 *M* H_3PO_4. In samples containing plasma, the fluorescence of daunorubicin increased as the ratio of plasma to acetonitrile—0.1 *M* H_3PO_4 (4 : 1) increased, and the fluorescence was enhanced compared to the samples containing water (for example, at a 35 : 65 ratio of plasma to acetonitrile—0.1 *M* H_3PO_4 the fluorescence was 104% of that in samples containing water, whereas at a 65 : 35 ratio it was 122% of that in the water-containing samples). The relevance of this fluorescence enhancement to the HPLC assay was next evaluated by comparison of the peaks from standards of daunorubicin in distilled water to which an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1) was added, with those from solutions prepared by addition of daunorubicin to treated plasma (that is, plasma to which an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1) had been added, followed by centrifugation). Hence, in the first case, the concentration of daunorubicin in water—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1) was accurately known, and in the second case the concentration of daunorubicin in centrifuged plasma—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1) was accurately known. The values of peak height for the daunorubicin in the "plasma" samples were consistently 10% higher than the corresponding values for the "water" samples. Consequently in all further work, drug standards were always prepared in plasma—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1). Standards typically gave calibration curves with a correlation coefficient of 0.998.

The efficiency of recovery of the assay method was next assessed by triplicate assay of spiked plasma samples containing between 10 ng ml^{-1} and 5 μg ml^{-1} daunorubicin. The plasma was treated by addition of an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1) and centrifugation. The recovery of daunorubicin in the 100 ng ml^{-1} to 5 μg ml^{-1} range was 99.1% (s = 1.24, n = 5) and for the whole range, 10 ng ml^{-1} to 5 μg ml^{-1}, was 92.3% (s = 12.3, n = 8). The procedure was repeated for the aglycone (daunomycinone); for the 100 ng ml^{-1} to 5 μg ml^{-1} range the recovery was 96.6% (s = 5.82, n = 5) and for the whole 10 ng ml^{-1} to 5 μg ml^{-1} range was 95.0% (s = 5.44, n = 8). For 7-con-O-methylnogarol there was excellent recovery over the whole range from 10 ng ml^{-1} to 5 μg ml^{-1} (98.5%, s = 1.12, n = 5).

DISCUSSION

Reversed-phase HPLC was chosen as the method here since it gives the opportunity to develop an assay where manipulation of a biological sample is minimised. LiChrosorb RP-2 was used since it has been shown previously [12] that selectivity of the assay is not affected as the chain length of the bonded group is altered; RP-2 gives shorter retention times than octylsilyl- and octadecylsilyl-bonded phases. Isocratic elution with a solvent similar to that of Eksborg et al. [13] gave good separation of daunorubicin, daunorubicinol and daunomycinone, so allowing assay of parent drug and these metabolites.

An evaluation of the effect of the assay procedure on the fluorescence properties of the drug was necessary since the quantum yield of a drug (and hence fluorescence intensity) is sensitive to changes in factors such as solution composition. It was found that fluorescence was enhanced, so standards should always be prepared in a solution of the same composition as the sample.

The assay method developed here is very rapid and is carried out in a single tube, thus minimising losses. It will extract metabolites as well as parent drug. The recovery for all compounds evaluated (daunorubicin, daunomycinone and 7-con-O-methylnogarol) was 96% or greater for the 100 ng ml^{-1} to 5 μg ml^{-1} range. As concentration of drug was reduced, recovery of daunorubicin and daunomycinone decreased but recovery of 7-con-O-methylnogarol remained at 98.5%. The method is thus a very rapid, reproducible assay for anthracyclines and their metabolites in plasma, and the sensitivity compares well with that of other reported methods. We have also extended the assay to determination of anthracyclines in homogenised cell samples.

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