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Note

Quantitative determination of low concentrations of adriamycin in plasma and cell cultures, using a volatile extraction buffer

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Since its introduction, adriamycin (doxorubicin) has become one of the most intensively studied cytostatics. Our interest focuses primarily on low-dose and long-term (days) pharmacokinetics, both in vivo and in vitro (cell culture). Determination of the drug in such experiments requires an extraction—chromatographic system, adapted to low levels of drug (10 ng/ml).

Chemical structures (Fig. 1) show that alkaline extraction conditions (pH ca. 8.5) are needed to simultaneously extract the parent drug and its metabolites. Adriamycin, however, is known to be unstable at pH values above 6.5 [1, 2]. Indeed, we repeatedly observed low recoveries and chromatographically detectable degradation products when phosphate, borate buffers or sodium hydroxide were used. This occurred especially when, after evaporation of the organic solvent, the concentrated extract was delayed in further analysis. To circumvent these problems, we propose to substitute a volatile buffer (ammonium formate) for the classical non-volatile phosphate buffer.

One-step extraction procedures, using chloroform—isopropanol mixtures, are convenient for matrices containing little protein, such as cell culture medium (10% foetal calf serum) or cultured cells suspended in buffer or medium. Such a technique is less suitable, however, in the case of plasma or serum, since after extraction and centrifugation a thick protein interface is formed, making precise separation of the phases virtually impossible. We devised a two-step modification, in which isopropanol is separately used as a

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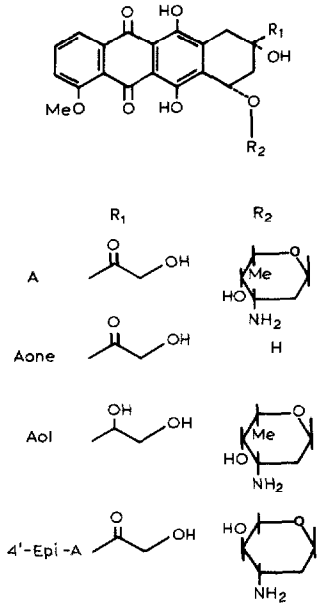

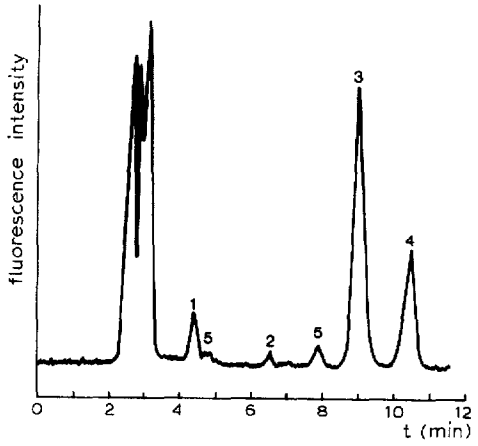

Fig. 1. Chemical structure of adriamycin (A), adriamycinone (Aone), adriamycinol (Aol) and 4'-*epi*-adriamycin (4'-Epi-A).

Fig. 2. Chromatogram of a liver cell culture extract. Cells were cultured for 24 h in the presence of 100 ng/ml adriamycin. Chromatographic conditions are given in the text. Peaks: 1 = Adriamycinone; 2 = adriamycinol; 3 = adriamycin; 4 = 4'-*epi*-adriamycin (internal standard); 5 = unknown metabolites.

protein denaturing agent, followed by the addition of chloroform. As internal standard, the structurally similar analogue 4'-*epi*-adriamycin (Fig. 1), which elutes very close to adriamycin in the chromatographic system (Fig. 2), is used.

EXPERIMENTAL

Chemicals

Adriamycin, adriamycinol, adriamycinone and 4'-*epi*-adriamycin were kindly donated by F. Arcamone (Farmitalia, Milan, Italy). Cell culture medium ("medium") was Eagle's Minimal Essential Medium, supplemented with 10% foetal bovine serum (Gibco, Paisley, U.K.). Siliconizing fluid was a ready-made solution (Siliconlösung Serva), obtained from Serva (Heidelberg, F.R.G.). All other chemicals were of standard analytical quality and were purchased from Merck (Darmstadt, F.R.G.).

Chromatography

The chromatographic system consisted of a DuPont Series 8800 chromatograph (DuPont, Wilmington, DE, U.S.A.,), coupled with a Varian filter-type fluorescence detector set at excitation wavelength of 480 nm and emission wavelength of 580 nm (Varian, Walnut Creek, CA, U.S.A.). The Spherisorb ODS-5 column (25 × 0.46 cm I.D.; 5 μm particle size) was obtained from Phase Separations (Queensferry, U.K.). As described previously [3],

the mobile phase was a water—acetonitrile mixture (42:48) containing 20 m*M* phosphoric acid and 10 m*M* sodium dodecyl sulphate, but the solvent flow-rate was 1.5 ml/min instead of 1 ml/min in the original description.

Extraction of adriamycin added to cell culture medium

Adriamycin was added to the test tubes as 100-μl aliquots of stock solution in methanol. The methanol was evaporated under a stream of nitrogen and the adriamycin redissolved in 1 ml of medium. Final concentrations of the drug were 0, 20, 40, 60, 80 and 100 ng/ml. To the spiked medium, 100 μl of buffer solution were added (1 *M* sodium phosphate or 1 *M* ammonium formate, pH 8.5, prepared immediately before use), together with 5 ml of an ice-cold chloroform—isopropanol mixture (4:1) and — if needed — 100 μl of the internal standard stock solution (4′-*epi*-adriamycin, 1 μg/ml in methanol). Extraction was carried out by gentle swirling for 10 min, after which the phases were separated by centrifugation for 5 min at 1000 *g*. The water layer and protein interface were discarded and the lower organic layer was evaporated to dryness. The residue was redissolved in 100 μl of methanol and 50 μl were injected into the chromatograph. For recovery studies, no internal standard was added, but the residue was dissolved in 100 μl of 4′-*epi*-adriamycin stock solution. As extraction vessels, 16 × 100 mm test tubes in ordinary glass, siliconized glass or polypropylene were used. Siliconization was carried out by leaving the siliconizing fluid in the tubes for 5 min and, after emptying, curing the film for 1 h at 100°C.

Extraction of adriamycin added to human plasma

Plasma was supplemented with adriamycin as described above. To 1 ml of spiked plasma, 2.5 ml of ice-cold isopropanol and 100 μl of buffer solution were added. After 10 min of swirling, proteins were precipitated by centrifuging at 1000 *g* for 20 min. A 5-ml volume of ice-cold chloroform was added to the resulting water—isopropanol mixture and extraction continued as above.

Extraction from cultured cells

Cells ($5 \cdot 10^6$—$10 \cdot 10^6$) were dispersed in 1 ml of culture medium, to which 100 μl of 1 *M* ammonium formate (pH 8.5), 100 μl of internal standard solution and 5 ml of chloroform—isopropanol mixture were added. The procedure was continued as described above. Cells were not lysed prior to extraction, as the cell walls readily dissolved in the organic mixture. Quantitation was done by comparing peak-height ratios (adriamycin/internal standard) of unknowns with those of standard solutions of the drug and internal standard, dissolved in medium.

RESULTS

Volatile versus non-volatile buffers

Recovery, using sodium phosphate, was 80.3 ± 4.2% (n = 5, 100 ng/ml adriamycin). Using ammonium formate, 89.9 ± 2.2% recovery was obtained.

Using the latter buffer substance, redissolved concentrates could be stored without any deterioration for up to 24 h (4°C, in the dark). This was, however, not the case when a phosphate buffer was used: after 2 h extra peaks, probably representing deglycosylated degradation compounds, were seen.

Choice of vessel material

When nanogram amounts of drug are to be handled, losses due to adsorption on glass walls can be very important. When non-treated glass vessels were used, recovery was 23.0 ± 30.2% (n = 5, 100 ng/ml adriamycin); siliconization improved this figure up to 76.0 ± 3.8%. However, these results are still inferior to those obtained with polypropylene tubes, where a recovery of 86.9 ± 1.8% was obtained.

Recovery, precision and lowest detectable amount

Using the extraction scheme described above, the analytical reliability criteria for the entire procedure were established, both for the parent molecule and for the two main metabolites. Recovery and relative standard deviation (R.S.D.) for adriamycin were 89.9 ± 2.2% (n = 10, 100 ng/ml adriamycin) in medium and 84.7 ± 1.6% in plasma. Between-run R.S.D. was 3.6% (n = 10) and 2.9%, respectively. The lowest detectable amount was approximately the same for both matrices, i.e. 1 ng.

Recoveries of the two metabolites were comparable. For the glycosylated metabolite it was 86.0 ± 2.2% from plasma and 83.9 ± 2.9% from medium. For the less polar deglycosylated adriamycin, the corresponding values were 104.9 ± 4.7% and 74.3 ± 4.0%, respectively.

Linearity studies

Figs. 3 and 4 show the relationship between peak-height ratios and concentrations for adriamycin and its metabolites. In all cases, virtually perfect linearity was obtained ($r \geqslant 0.9990$). This was also the case when 4′-*epi*-adriamycin was added to plasma or medium and adriamycin was used as an internal standard (not shown).

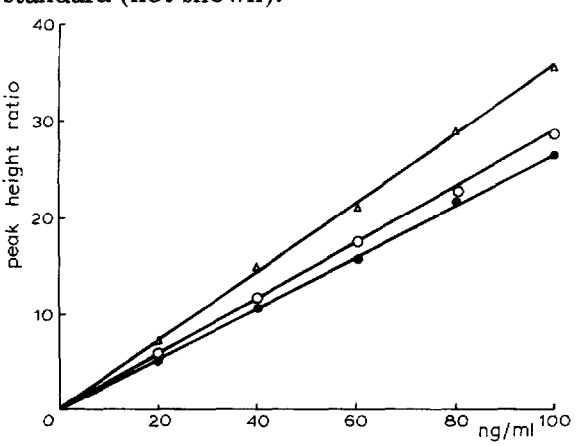

Fig. 3. Relationship between peak-height ratios and concentration of adriamycin (○, r = 0.9998), adriamycinol (●, r = 0.9999) and adriamycinone (△, r = 0.9997) after extraction of supplemented cell culture medium.

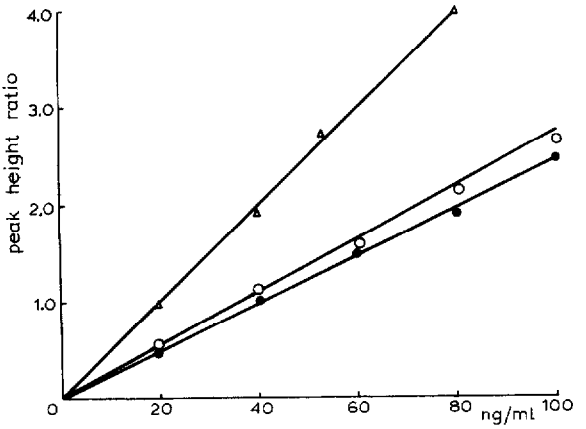

Fig. 4. Relationship between peak-height ratios and concentration of adriamycin (○, r = 0.9991), adriamycinol (●, r = 0.9992) and adriamycinone (△, r = 0.9990) after extraction of supplemented human plasma.

Application to cell cultures

Fig. 2 shows a typical chromatogram, obtained after 24 h of incubation of liver cells in medium containing 100 ng/ml adriamycin. Fig. 5 shows an example of drug uptake and efflux versus time.

Determination of plasma levels in man

In Fig. 6, the kinetics of intravenously injected adriamycin (40 mg/m^2) and its metabolites are shown. After the initial rapid decline of plasma levels, the drug persists for up to four days in plasma. Adriamycinol and adriamycinone, the main metabolites, appear in the first few minutes, disappearing thereafter along with the parent drug.

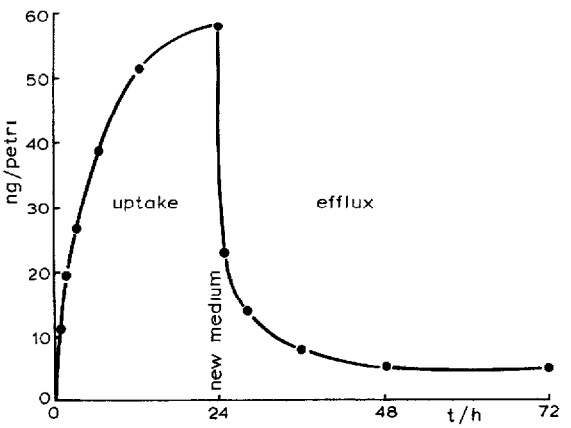

Fig. 5. Uptake and efflux of adriamycin in a liver cell culture. To replica petri dishes containing 5 · 10^6 of HeLa cells and 5 ml of medium, adriamycin stock solution was added so that the concentration in the medium was 100 ng/ml. Uptake was followed over 24 h by harvesting all the cells of a dish at regular intervals and carrying them through the entire procedure as described. Efflux was studied by renewing the medium with adriamycin-free medium and harvesting dishes at the intervals showed.

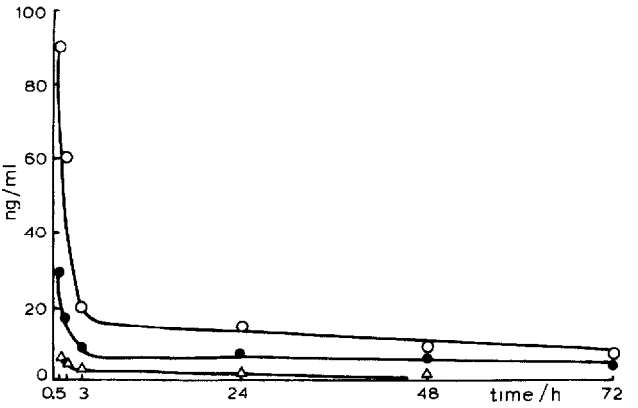

Fig. 6. Time course of plasma levels of adriamycin (○), adriamycinol (●) and adriamycinone (△) after intravenous bolus injection of 40 mg/m² adriamycin.

DISCUSSION

Recovery and precision of our extraction—chromatographic method compare well with a previously described system for small amounts of drug, but aimed only at glycosylated compounds [4]. In our assay, all known metabolites can be determined in the same run. Performance is, however, not as good as for those schemes suitable for microgram amounts of drug [5, 6].

The superiority of the volatile buffer over the phosphate buffer is hard to rationalize, as the pH of the extraction mixtures was identical in both cases. There is probably some co-extraction of certain buffer constituents, which are then concentrated along with the drug. Thus, very high concentrations of the alkaline buffer can be present in the residue, and will account for the destruction of adriamycin. In fact, a very high pH (up to 10) was noted with phosphate buffer extraction when residues were redissolved in a small amount (200 μl) of water. As shown, this was not the case when ammonium formate was substituted for the phosphates.

Although intended for determination of adriamycin, the whole system can easily be used for the determination of 4′-*epi*-adriamycin, a drug which is now being investigated in preliminary clinical trials.

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