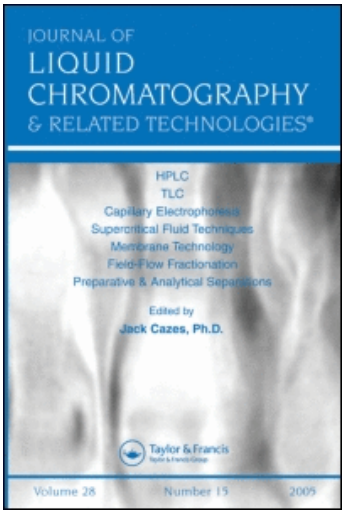

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EXTRACTION OF ANTHRACYCLINES FROM BIOLOGICAL FLUIDS FOR HPLC EVALUATION

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ABSTRACT

A new technique for the extraction of anthracyclines and their metabolites from plasma or serum is described, which gives suitable extracts for HPLC analysis of these drugs in patients. This technique consists in a very rapid chromatographic step on the bonded silica contained in small open columns (C-18 Sep-Paks, Waters Associates) . This extraction gives quantitative recoveries of all the anthracyclines and metabolites that have been tested ; 0.2 to 3 ml of plasma can be processed, with concentrations up to 5,000 ng/ml. The advantages of this technique over classical techniques using an organic extraction with a partition between two phases are : speed, simplicity, efficacy, reproducibility and similarity of recoveries for various anthracyclines.

INTRODUCTION

Anthracyclines are a very powerful group of antitumor antibiotics. Adriamycin and daunorubicin are extensively used for treatment of a wide variety of solid tumors and leukemias. Moreover, several analogues have been synthesized and are studied, at the present time, in phase I and phase II clinical trials. Pharmacokinetic studies of these drugs can provide a much better knowledge of their metabolization in the body and, therefore, of their bioavailability to the various tissues, normal or tumoral. Such studies require

techniques for the measurement of parent drugs and their metabolites in biological fluids. These techniques mustbe highly sensitive (in the nanomolar range) , highly specific and reproducible.

Several techniques have been published recently for the HPLC analysis of anthracyclines in plasma or other biological fluids (1-5). All of them present similar sensitivities and are able to detect less than 10 ng of drug in absolute amount. Among them, the technique proposed by Israel et al. (1) appears as the most suitable and reproducible. Various techniques have also been published for the extraction of these compounds from biological fluids (1-7) . Those techniques are all based upon the physical properties of anthracyclines, which can be extracted by organic solvents. The anthracyclines partition between an organic and an aqueous phase depending on the pH. All these techniques present several drawbacks :

- the extraction is not quantitative and the recovery often ranges between 50 and 70 %, even if claimed higher ;

- when the recovery reaches 90 %, either a huge quantity of organic solvent is required for the extraction of the drug from 1 ml plasma, or only a very little quantity of plasma can be used (0.1 ml), which is not enough, in most cases, for clinical evaluation of low levels of drug ;

- the recovery is not similar for all anthracyclines and for the parent drugs vs. their metabolites. Recovery depends upon the partition coefficient between the organic and the aqueous phases at the pH of the extraction ;

- Recovery is dependent upon the amount of drug present in the sample, and this recovery may be reduced when more than 1μg of drug is present in 1 ml of plasma (personal observations) ;

- most techniques are time-consuming and require usually large quantities of various chemicals ; since there is a partition between an organic and an aqueous phase, several phase separations must be performed with careful and sometimes difficult removal of the organic layers.

All the techniques already proposed in the literature do not present all the drawbacks listed here, but all of them present at least one of them. We report here on an original extraction technique of anthracyclines and their metabolites which tentatively avoids the drawbacks listed above. This technique is based on the HPLC behavior of these compounds. On a bonded silica, it has been shown that anthracyclines are eluted with solvents of increasing polarity (1). Therefore, the use of pre-columns filled with a bonded silica may replace the organic extraction. Such pre-columns are commercially available and their chromatographic behavior is roughly similar to that of the HPLC columns containing the same solid phase.

MATERIALS AND METHODS

C-18 Sep-Paks were obtained from Waters Associates, Milford, MA, U.S.A. (Ref. N° 51910) . They consist of a polyethylene cartridge filled with C-18 bonded silica, and are used as minichromatographic open columns. Solvents or fluids can be pushed through the column with a glass syringe (Fig. 1) . For the extraction of anthracyclines from up to 3 ml plasma or serum, we have used them as follows. We have run through the Sep-Pak :

I. 3 ml pure methanol
II. 3 ml methanol/water 1/1 (v/v)
III. 10 ml Na_2HPO_4 0.05 M
IV. the plasma sample (0.2 to 3 ml)
V. 3 ml Na_2HPO_4 0.05 M (eluate discarded)
VI. 4 ml chloroform/methanol 2/1 (v/v)

This last organic eluate is evaporated to dryness in a warm water-bath under a stream of nitrogen ; it is then reconstituted with a small volume of the solvent used for the HPLC separation (see

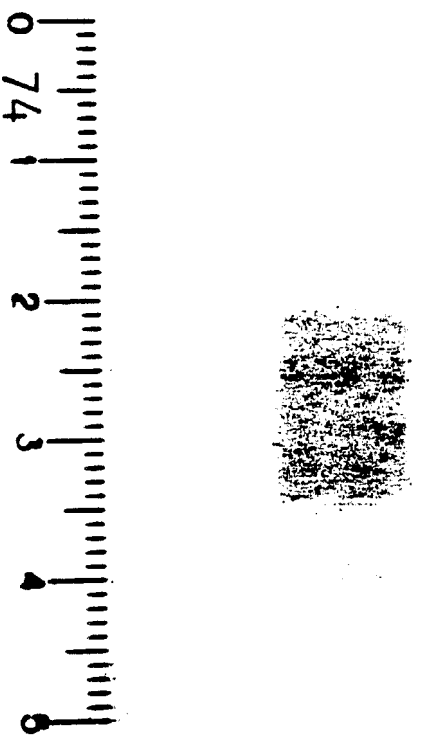

FIGURE 1

A C-18 Sep-Pak (Waters Associates)
The reference scale is in centimeters.

below) . This volume was 100-2500 μl according to the quantity of anthracyclines expected to be present in the sample. A centrifugation in small conical tubes (5 min, 5,000 rpm) eliminates, as a precipitate, the lipid material extracted ; an aliquot of the clear supernatant (5 to 50 μl) is then injected into the liquid chromatograph.

The HPLC conditions were very similar to those described by Israel et al. (1) with minor modifications. The HPLC was achieved with a Waters Associates liquid chromatograph on a column of microbondapak-phenyl (30 x 0.4 cm) . The solvent was a mixture of acetonitrile and formate buffer (32/68, v/v). The formate buffer was prepared with 0.1 % ammonia adjusted to pH 4.00 with pure formic acid. This solvent was used isocratically at a flow rate of 3 ml/min. Detection of the peaks was accomplished with a Schoeffel fluorometer (model SF-970) with an excitation wavelength of 254 nm and an emission cut-off filter at 580 nm. All the chromatograms presented here were obtained with the minimal sensitivity of the fluorometer (range 1 μA) and the minimal time-constant (0.5 sec.) . The fluorescence signal was recorded with an Omniscribe recorder and integration of the peaks was obtained with an ICAP 10 calculator.

RESULTS AND DISCUSSION

The use of C-18 sep-paks gave a quantitative recovery of the anthracyclines present in serum or plasma. The manipulation of the anthracyclines (evaporation, centrifugation, changes of tubing, ...) gave a loss of about 5-10 % of the drugs (Fig. 2). If the same amount of anthracyclines was added to 1 ml of an untreated subject and extracted with Sep-Paks as described in the Materials and Methods section, a recovery of 98-102 % was obtained when compared to the manipulation of the drugs without extraction. We can thus assume that the yield of the recovery of the anthracyclines from the C-18 sep-paks is 100 %. Addition of an internal

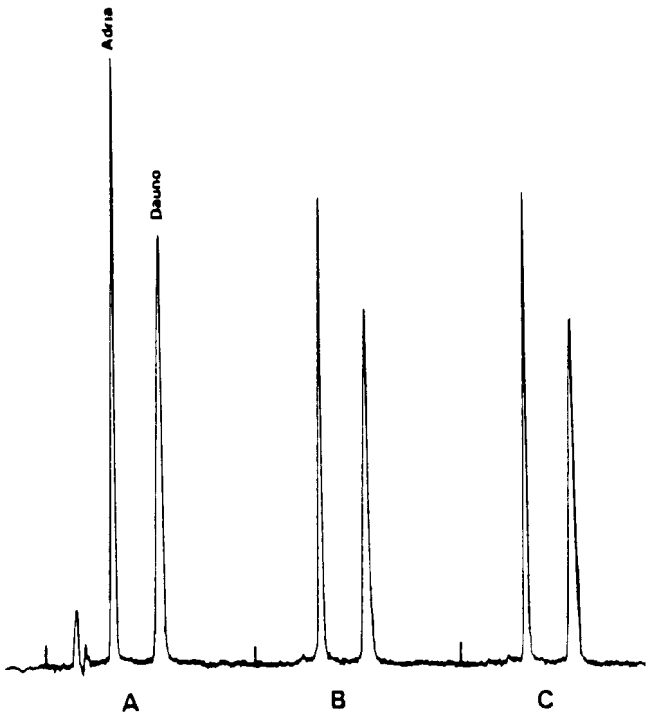

FIGURE 2

Recovery after anthracyclines extraction on Sep-Paks
The chromatographic conditions are described in the text. Chromatogram A : pure adriamycin and daunorubicin standards (20 ng each) were injected ; Chromatogram B : 1000 ng of each standard were added to 1 ml methanol; this was transferred to another tube, evaporated to dryness, redissolved in 1 ml of formate buffer/acetonitrile mixture and transferred to a small conical tube ; 20 μl were injected ; Chromatogram C : 1000 ng of each standard were added to 1 ml blank plasma, a Sep-Pak extraction was performed as described in the text, 20 μl from 1 ml were injected.

standard to the serum prior to extraction allows quantitative measurements.

The 0.05 M Na_2HPO_4 solution has a pH of approximatively 9.0 It was used to ensure that all the molecules of anthracyclines were under the same ionized form. Replacement of this solution by pure water gave a loss of 10-20 % in the recovery of the Sep-Pak extraction. An acidic buffer can also be used instead of the basic solution of Na_2HPO_4. We have used the formate buffer used for the preparation of the HPLC solvent. Similar results were obtained with a 100 % recovery of the drugs from the Sep-Paks. This buffer can be useful for detorubicin extraction since this anthracycline is very unstable at alkaline pH.

We have evaluated the recoveries from the Sep-Paks of a variety of anthracyclines and derivatives (Fig. 3). We have obtained similar quantitative recoveries for adriamycinol, adriamycinone, daunorubicin, daunorubicinol, 4'-epi adriamycin, rubidazone and detorubicin using for the last compound a pH 4.0 buffer for washing the Sep-Paks and a mixture of formate buffer/acetonitrile 60/40 for the HPLC.

We have measured the linearity of the recovery of the drugs in plasma over a wide range of concentrations. Results obtained for adriamycin are shown in Fig. 4. 10 to 5,000 ng were added to 1 ml serum portions of an untreated subject and extracted as described ; aliquots containing 5 to 20 ng of adriamycin were then injected into the chromatograph and the peak areas obtained were multiplied by the dilution factor. A straight line was obtained between 10 and 5,000 ng ; this represents, together, the linearity of the Sep-Pak extraction recovery and the repetability of the injection. This linearity was obtained for concentrations of drugs less than 10 ng/ml, down to 2 ng/ml, which is the limit of sensitivity of the fluorescence detection. A precaution must be taken when such experiments are performed : the drug added to the plasma must be solubilized in water to avoid the need to add methanol to the plasma : this would result in loss of

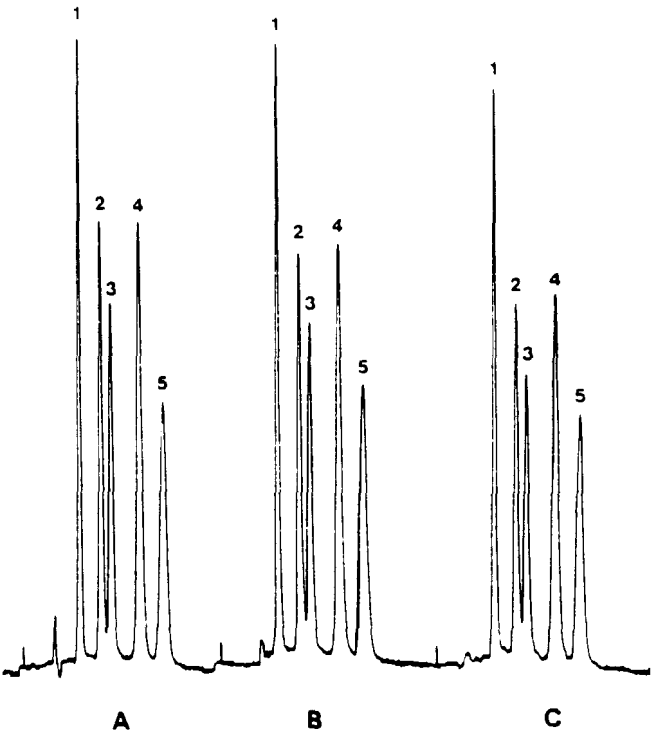

FIGURE 3

Similarity of the recoveries of various anthracyclines after Sep-Pak extraction

The chromatographic conditions are described in the text. Chromatograms A and B : various anthracyclines were injected as standards dissolved in methanol (A) or in formate buffer/acetonitrile 68/32 (B) ; Chromatogram C : the same anthracycline mixture was injected after it has been added to 1 ml serum and extracted with a Sep-Pak as described in the text. No difference can be seen in the relative proportions of the various anthracyclines. Identification of the peaks : (1) adriamycinol ; (2) adriamycin ; (3) 4' epi-adriamycin ; (4) adriamycinone ; (5) daunorubicin.

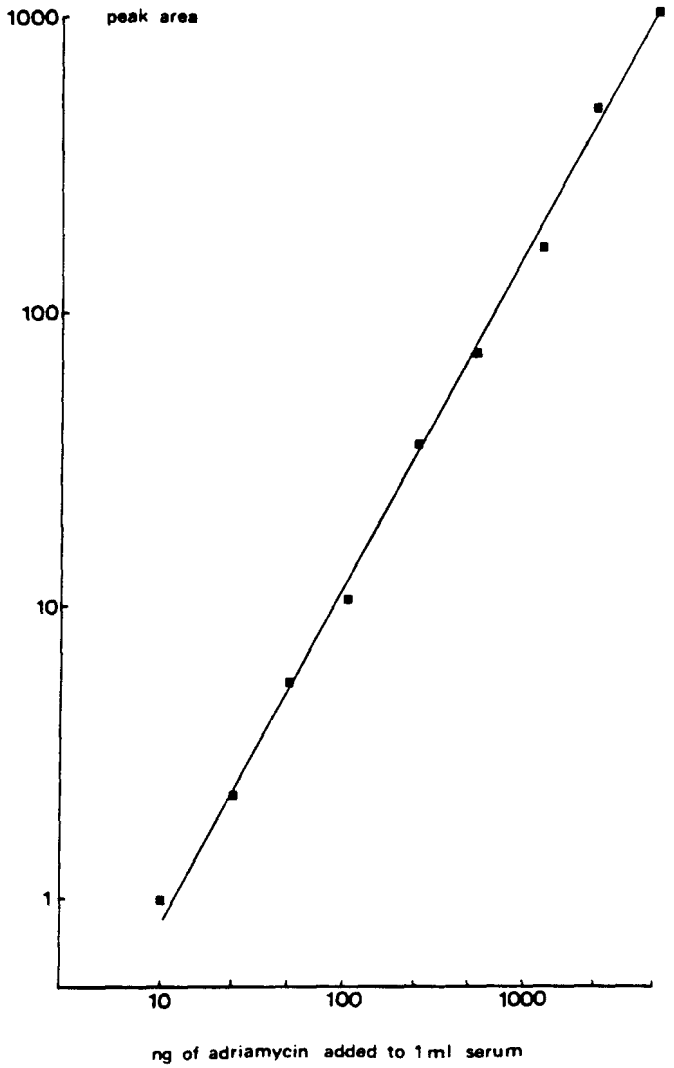

FIGURE 4

Linearity of the Sep-Pak extraction efficacy

10 to 5000 ng of adriamycin were added to 1 ml blank serum portions ; a Sep-Pak extraction was carried out as described in the text ; the volume of reconstitution ranged between 100 μl and 5000 μl ; the volume of injection ranged between 20 μl and 50 μl ; peak area was multiplied by the dilution factor and plotted as a function of concentration of adriamycin in the serum (double logarithmic plotting) . The linearity obtained can be used to evaluate simultaneously : (i) the constancy of extraction yield ; (ii) the repetability of the injection technique ; (iii) the calibration curve of the HPLC technique.

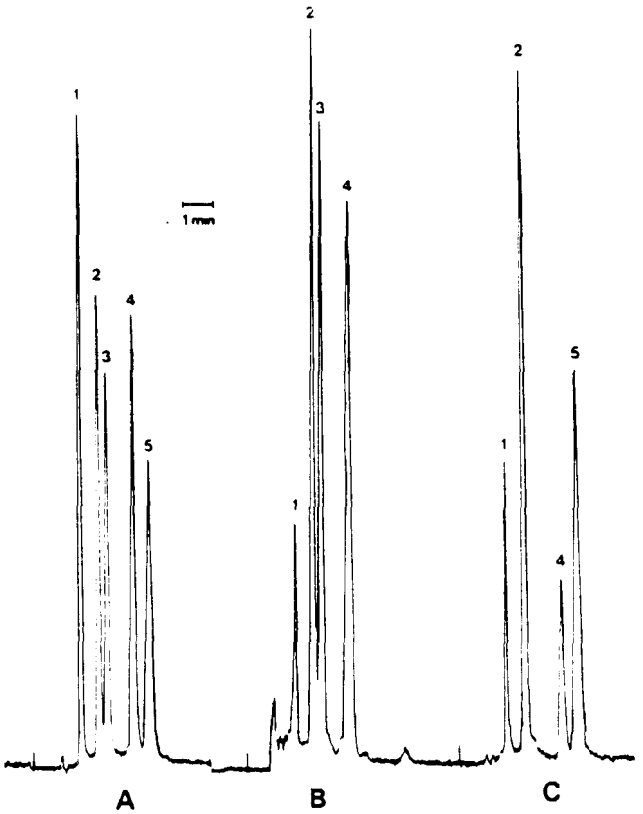

FIGURE 5

Examples of chromatograms obtained from extracts of patients plasmas

Chromatogram A : mixture of pure standards in methanol (20 ng each) ; Chromatograms B and C : anthracyclines extracted from the plasma of patients treated with adriamycin ; 4'-epiadriamycin was added to the serum as an internal standard (B) ; daunorubicin was added to the serum as an internal standard (C). Identification of the peaks as in Figure 3.

the drug when extracting it. Another possibility is to add the plasma to the drug after having dried it in a tube by evaporation of the methanol in which the drug is usually dissolved.

The technique we propose here is a very rapid one which requires about 5 min. manipulation, 20 min. evaporation and 10 min. centrifugation. Since the HPLC technique we have chosen (1) is very rapid (5 min.), up to 25 samples of plasma can be entirely processed within a day. An example of the chromatograms obtained from patients treated with adriamycin is shown in Fig. 5.

ACKNOWLEDGEMENTS

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