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A rapid chromatographic procedure for the determination of adriamycin, daunomycin and their 13-OH metabolites adriamycinol and daunomycinol*

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Summary. A rapid chromatographic procedure for the quantitative determination of the anthracycline antibiotics adriamycin and daunorubicin and their chief metabolites adriamycinol and daunorubicinol in plasma and urine is described. The extraction is performed using SEP-PAK silica cartridges. After filtration the eluate is chromatographed on a reversed-phase column.

Introduction

The anthracyclines adriamycin and daunorubicin are useful in the treatment of neoplasms [2, 3]. The main metabolites found in plasma and urine are their 13-OH derivatives adriamycinol and daunorubicinol. They can be well separated from the parent drugs by HPLC [1]. A liquid-liquid extraction from biological fluids is mostly performed [1]. This time-consuming technique requires relatively large quantities of solvents, and the recovery is not sufficient in all cases. Reversed-phase cartridges are used by Robert [8]. As they must be pretreated before sample application, different solvents are necessary for this procedure. Our aim was to optimize the extraction and determination of adriamycin, daunomycin, and their chief metabolites for pharmacokinetic studies.

Material and methods

Chemicals. Adriamycin (ADR), daunomycin (DRB), adriamycinol (AOL), daunorubicinol (DOL) and 4'-epidoxorubicin (Epi-DX) were gifts from Farmitalia, Milan, Italy. Acetonitrile for chromatography and *o*-phosphoric acid were purchased from Merck, Darmstadt, and SEP-PAK silica cartridges from Waters, Koenigstein. Water was double distilled.

Standards. Lyophilized plasma (Intersero, Wiesbaden) was dissolved in water (1 mg ml^{-1}). The pooled urine was that of five healthy adults. Aliquots of the aqueous anthracycline stock solutions ($1.7-1.9 \times 10^{-4}$ *M*) were added to the plasma and urine. ADR and DRB were used as each other's internal standard [6, 7] and Epi-DX was used for the determination of ADR/AOL, dissolved in methanol (6.9 or 7.1×10^{-9} *M*, respectively). The standard solutions for HPLC consisted of 67 ng ml^{-1} of each compound ($1.15-1.27 \times 10^{-7}$ *M*) in the mobile phase (diluted 4:1 with water, cf. *Results*). All solutions were stored at −20 °C.

HPLC-System. The chromatographic system consisted of a pump 740B with pump control and pressure monitor (Spectra Physics, Darmstadt), an RF 530 fluorescence detector (Shimadzu, Düsseldorf), an ASI 120 autosampler with 500-µl loop (ABC, Munich) and an integrator SP 4100 (Spectra Physics). Hyperchrome columns with 2-cm precolumns (Bischoff, Leonberg) were used.

Analytical conditions (modified from [7]): Column 250×4.6 mm (µBondapak C_{18}) or 120×4.6 mm (Sperisorb ODS 5 µm), precolumn filled with the latter. Mobile phase: Acetonitrile-0.32 *M* *o*-phosphoric acid, 27:73 (ADR/AOL) and 29:71 (DRB/DOL). Flow rate 1 ml min^{-1}; temperature ambient; excitation 480 nm; emission 560 nm. The mobile phase was degassed and filtered through a 0.5-µm filter (HVLP 04700, Millipore). The peaks were automatically identified according to their retention time related to the internal standard (window ±3%) and quantified on the basis of their peak height.

Sample preparation. Prior to and at different times after drug administration [5] 5 ml blood were taken into tubes containing EDTA and immediately centrifuged at 5000 rpm for 10 min. The 24-h urines were collected in a refrigerator (8 °C). The samples were stored at −20 °C.

Plasma or urine (1 ml) containing 200 ng of the appropriate internal standard was applied to the silica cartridge. After flushing with 5 ml double distilled water, the anthracyclines were eluted with the same mobile phase as was used for HPLC (see above). The first milliliter of the eluate was discharged, after which 3 ml was collected and passed through a 0.5-µm membrane filter (Millex SR, Millipore); 500 µl was injected onto the column.

Results

Extraction procedure

The average percentage recoveries (±SD) from plasma over the whole range between 1.8×10^{-8} and 3.6×10^{-6} *M*

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were as follows (calculated from the means of 5 different concentrations, each level measured 3 to 5 times): 89.7±3.3 (ADR), 92.1±4.3 (AOL), 87.5±1 (DRB), and 88.5±5.3 (DOL). The recovery of Epi-DX was 90.1%±4.5% (3.5×10^{-7} *M*). The recoveries from pooled urine were similar.

A loss during the filtration step was not measurable even at low concentrations. The variation coefficients within the different levels were ≦5, at the detection limit about 10. This limit (signal-to-noise ratio of 3) was about 1.7×10^{-8} *M* for the parent drugs and 9.2×10^{-9} *M* in plasma for the metabolites. Linearity between concentration and recovery was found over the whole range ($r \leqq 0.999$).

As shown in Fig. 1, most fluorescent compounds naturally occurring in urine are eliminated during the extraction.

The anthracyclines were very stable in the eluate (pH about 2): stored at room temperature the loss was less then 2% after 24 h, which is consistent with observations published by Eksborg et al. [4].

HPLC

The above chromatographic conditions allow baseline separation of the parent drugs, their chief metabolites, and the appropriate internal standard. The use of Epi-DX as internal standard instead of DRB shortens the analysis time for determination of ADR/AOL (Fig. 2 and 3).

During elution of the anthracyclines from the cartridge the mobile phase is diluted about 4:1 with the water remaining after the wash step. Therefore, the anthracyclines show sharper peaks on the reversed-phase column than in the solution in the eluent alone. To achieve an identical chromatographic behavior the standards for HPLC were also dissolved in the appropriate diluted solvent.

Up to now no interfering substances have been found either in plasma or in urine from patients receiving the following drugs before and during anthracycline treatment: actinomycin D, arumil, chlorpromazine, colistine, cyclophosphamide, dacarbazine, gentamicin, levopromazine, methoclopramide, methotrexate, methylprednisolone, mezlocillin, pethidine, 6-thioguanine, vincristine.

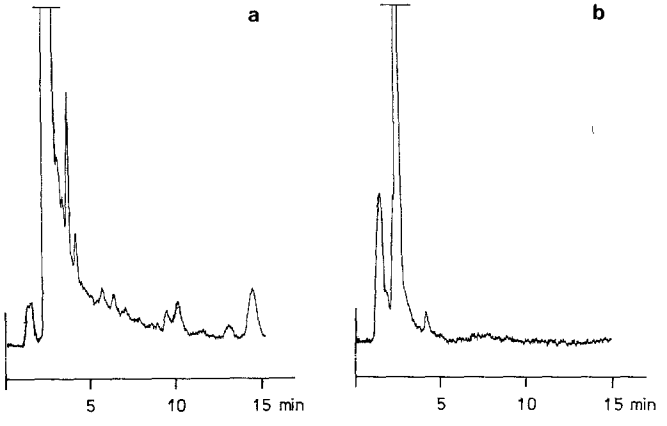

Fig. 1a, b. Chromatographic separation of pooled urine. **a** Urine diluted with water (3:1), 500 µl injected without extraction procedure. **b** Urine extracted as described in the text using silica cartridges. *Chromatographic conditons:* Column (120×4.6 mm) and precolumn (20×4.6 mm), Spherisorb ODS 5 µm; mobile phase: acetonitrile – 0.3 *M* *o*-phosphoric acid (27:73); flow rate 1 ml min^{-1}; temperature ambient; fluorescence detection, excitation 480 nm, emission 560 nm

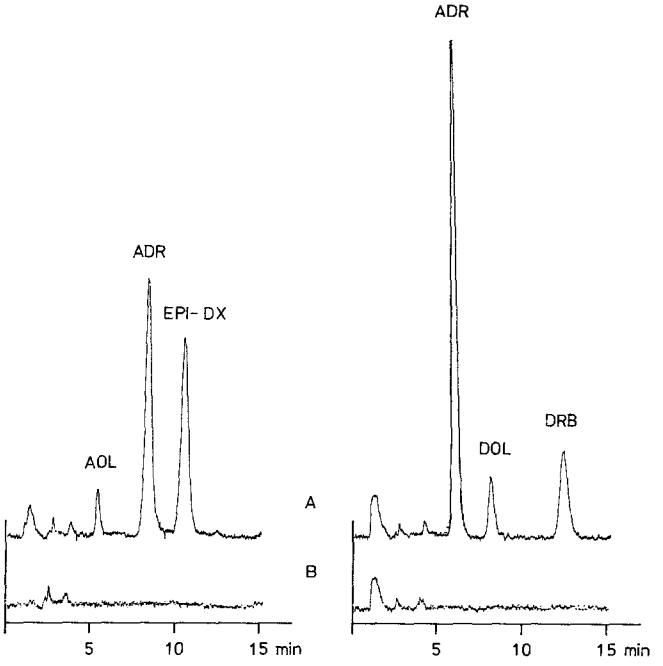

Fig. 2a, b. Plasma extracts of a patient 20 min after receiving 45 mg m^{-2} body surface ADR as bolus injection. Chromatographic conditions as in Fig. 1. **a** Blank plasma; **b** 20 min after injection. *Measured values: AOL (5.3'): 20.0 ng ml^{-1}* plasma, ADR (8.2'): 210.8 ng ml^{-1}, EPI-DX (internal standard, 10.4'): 200 ng ml^{-1}. The retention time of DRB under these conditions was 20.7'

Fig. 3. Plasma extracts of a patient 20 min after receiving 36 mg m^{-2} body surface DRB as bolus injection. Chromatographic conditions as in Fig. 1, except that mobile phase was acetonitrile – 0.3 *M* *o*-phosphoric acid (29:71). **a** Blank plasma; **b** 20 min after injection. *Measured values:* ADR (internal standard, 6.0'): 200 ng ml^{-1} plasma, DOL (8.1'): 21.1 ng ml^{-1}, DRB (12.4'): 52.1 ng ml^{-1}

Discussion

The procedure described allows very rapid and reproducible extraction of the anthracyclines adriamycin and daunomycin and their 13-OH metabolites from biological fluids. It avoids the laborious liquid-liquid extraction, and no concentration step is necessary prior to the high-performance liquid chromatographic determination. The sensitivity is comparable to that of other methods [1]. In contrast to RP cartridges, as used by Robert [8], silica cartridges must not be pretreated prior to the application of aqueous samples and are therefore more useful for the extraction of the anthracyclines.

Other fluorescent compounds, which could be additional metabolites, were found only in some plasma samples and were not identified or determined.

We have tested two different batches of DOL. The first one, received in 1982, was chemically reduced from DRB. We found two peaks (eluent containing 25% acetonitrile), which have recently been recognized as a 1:1 mixture of stereoisomers at C-13 (G. Casinelli, 1984, personal communication). The human metabolite is identical with DOL

obtained by stereoselective microbial reduction and corresponds with the less polar isomer (G. Cassinelli, 1984, personal communication). For our studies we used only this microbially reduced compound. Some of the previously published results should perhaps be considered with reservations if the chemically reduced form was used.

The results of pharmacokinetic studies will be published elsewhere [5].

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