Analysis of Doxorubicin, 4'-Epidoxorubicin, and Their Metabolites by Liquid Chromatography

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Abstract An analytical method based on isocratic HPLC separation and fluorescence detection was developed to allow for sensitive and specific analysis of anthracyclines and their metabolites in plasma and urine. The method is particularly advantageous when comparing the metabolism and/or pharmacokinetics of analogues, such as doxorubicin (85, 105)-10-{3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)-oxy]-8-glycopyrrol-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedicone (1) and 4'-epidoxorubicin (2), since both drugs and their metabolites can be analyzed under identical conditions. The analytical properties of 1, 2, and eight metabolites were studied in plasma, serum, buffer solution, and urine. The detection limit in plasma was 4 x 10^-8 M for the glucuronides, 7 x 10^-8 M for the glycosides, and 1 x 10^-9 M for the aglycones. In plasma, 1, 2, doxorubicin (3), 4'-epidoxorubicin (4), doxorubicinone (5), and doxorubicinol aglycone (6) showed a linear concentration-response relationship from their detection limit up to 5 x 10^-6 M. A linear calibration graph for plasma samples was also obtained for 7-deoxydoxorubicinone (7) and 7-deoxydoxorubicinol (8); however, these compounds had a significantly lower upper limit (5 x 10^-7 M). Urine samples were acidified to pH 2.5 and analyzed by HPLC without further purification. A linear calibration curve was obtained in the clinically relevant range. The detection limit in urine was 5 x 10^-8 M. Plasma and urine of two patients who had received 4'-epidoxorubicin by iv bolus injection were analyzed.

4'-Epidoxorubicin (2) is a new anthracycline, differing from doxorubicin only in the orientation of the C-4' hydroxyl in the amino sugar.1 Compound 2 and doxorubicin (85, 105)-10-{3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)-oxy]-8-glycopyrrol-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedicone (1) are thus C-4' epimers.2 The drugs, however, are significantly different pharmacologically,3-6 and 2 is now considered to have some advantages. The antitumor spectrum of 2 appears to closely resemble that of 1,7 but 2 appears to have a somewhat better therapeutic index. In patients it is less myelosuppressive by weight than 1,8 and less cardiotoxic whether administered at equal doses or at equally myelosuppressive doses.9

The pharmacological differences underlying the improved clinical performance of 4'-epidoxorubicin have been suggested to be related to its clinical pharmacokinetic and metabolic performance5-6 as well as its distribution.10,11 A complete and quantitative study of the analysis of doxorubicin, 4'-epidoxorubicin, and their metabolites has not been reported yet.12-15 Therefore, we have developed and extensively investigated a sensitive and universal method for precise quantitation of 1, 2, and their metabolites. The resulting analytical procedures and data from two patients who received 2 iv are reported.

Experimental Section

Chemicals—The following abbreviations were used: 1, doxorubicin; 2, 4'-epidoxorubicin; 3, doxorubicinol; 4, 4'-epidoxorubicinol; 5, doxorubicinone; 6, doxorubicinol aglycone; 7, 7-deoxydoxorubicinone; 8, 7-deoxydoxorubicinol; 9, daunorubicin; 10, 4'-epidoxorubicinol-glucuronide; and 11, 4'-epidoxorubicinol glucuronide. Compounds 1-8 were kindly supplied by Farmitalia Carlo Erba (Milan, Italy). Daunorubicin (9) was obtained from SPECIA (France). Compounds 10 and 11 were isolated from human urine as described earlier.4 Acetonitrile was of HPLC grade (Rathburn Chemicals, England), and all other chemicals were of analytical grade. Water was deionized and purified by a Milli-Q Reagent Water System (Millipore, Ettten-Leur, The Netherlands).

Sample Preparation—Stock solutions of 1, 2, and metabolites in methanol (10^-3 M) were prepared and stored in the dark at 5-20 °C. Plasma samples were kept frozen (-20 °C) until analysis. Immediately before analysis, the plasma samples were carefully thawed and vortex mixed. Then, a 1-mL aliquot was transferred to a polypropylene tube containing 50 mL of internal standard solution (5 x 10^-8 M daunorubicin in methanol). The contents were mixed, 8 mL of chloroform/isopropyl alcohol (4:1, v/v) was added, and the tubes were again vortex mixed for 40 s. The tubes were then centrifuged for 20 min at 2500 rpm. The organic layer was removed and evaporated under an air stream at 40 °C. After evaporation was completed the walls of the tube were rinsed with 0.25 mL of chloroform, which was subsequently evaporated. The residue was redissolved at 50 °C in 50 mL of methanol/chloroform (4:1, v/v) and centrifuged in closed tubes at 0 °C. The tubes were placed in an ultrasonic bath for 15 min at 0 °C and subsequently cooled for at least 5 min to -20 °C, or stored until analysis. Finally, the tubes were centrifuged for 10 s at 1500 rpm (0 °C) and the samples were injected onto the column (30 μL) using a WISP automatic injector.

Urine samples were carefully thawed and shaken at 37 °C for 30 min, followed by sonication for 15 min. The precipitate usually present in thawed urine generally dissolved by this procedure. The pH of the urine samples was measured and adjusted to pH 2.5 ± 0.2 with 10 M H3PO4 (usually 10-15 μL/mL urine). The internal standard was then added (50 μL of 6 x 10^-5 M or 2 in methanol per mL of urine) and mixed thoroughly before it was injected onto the HPLC column (30 μL).

Liquid Chromatography—The chromatographic system consisted of an Orilta pump (Giessen, G.F.R.), a model 710B WISP automatic injector (Waters Assoc., Milford, MA) and a fluorescence detector (Perkin-Elmer model 3000, Perkin-Elmer, Gouda, The Netherlands) connected to a BD41 recorder (Kipp & Zn, Delft, The Netherlands). A 3-μm CP Microsphere C18 column (Chrompack, Middelburg, The Netherlands), with dimensions of 250 x 4.6 mm, was used. Excitation and emission wavelengths used for fluorescence detection were 470 and 580 nm, respectively. The mobile phase consisted of 0.06 M KH2PO4 buffer (pH 4.0):acetonitrile (70:30, v/v) for plasma samples (solvent system I). The glucuronides 10 and 11 in plasma as

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well as in urine samples were analyzed using 0.02 M KH$_2$PO$_4$ (pH 4.0):acetonitrile (50:20, v/v) as the mobile phase (solvent system II). The flow rate was 0.7 mL/min and the injection volume was 30 µL in all cases. Quantitation was based on the ratio of the peak heights of drug or metabolite versus the peak height of the internal standard. Detection limits were determined as the minimum concentration which produced a peak at least three times as high as the baseline noise.

Recoveries—Recoveries were determined by analysis of six independently prepared citrate plasma samples containing 1 or 2 and their respective metabolites at a concentration of $10^{-6}$ M. The recoveries were calculated by comparison with standard solutions at the same concentration in methanol:chloroform (4:1, v/v). Linearity and Precision—Stock solutions of 1 or 2 and their metabolites were diluted with methanol, and 50 µL of solution was added to blank citrated plasma to obtain a concentration range from $5 \times 10^{-9}$ to $5 \times 10^{-6}$ M. Samples were analyzed in triplicate, and the range of linear response was determined by the maximum correlation coefficient obtained by the linear least-squares method.

The precision of the method was measured by analysis of six independently prepared citrated plasma samples at a concentration of $7 \times 10^{-9}$ M for the glucosides and aglycones and $4 \times 10^{-8}$ M for the glucuronides.

Thin-Layer Chromatography of Aglycones—To investigate self-association of the aglycone metabolites, 10-µL samples of 3 and 8 in methanol ($6 \times 10^{-4}$ M) were spotted on a C8 reversed-phase thin-layer chromatography plate (RP-TLC). The plate was developed ascendingly with a mixture of acetonitrile:KH$_2$PO$_4$ (0.065 M, pH 4.0, 2:1, v/v) in a standard glass developing tank. The experiment was repeated with the following modifications: 5 and 8 dissolved in chloroform:isopropyl alcohol (4:1, v/v) and developed as before.

Patients—Citrate plasma and urine samples were collected from two female patients with progressive advanced breast cancer (age 53 and 65 years) during the first course of treatment which consisted of a dose of 75 mg/m$^2$ of 2 as an iv bolus injection. Prior therapy consisted of radiotherapy in one patient, while no prior therapy was given to the other patient.

Results

An example of a chromatogram of a standard mixture of 1, 2, and their major metabolites (except 10 and 11) is given in Fig. 1. Plasma samples containing 1 and its metabolites were analyzed once with solvent system I, as shown in Fig. 2. However, plasma samples containing 2 and its metabolites were analyzed twice, once with solvent system I, as shown in Figs. 1 and 2, and once with solvent system II, as shown in Fig. 3. The large differences in the polarity of the metabolites of Linearity and Precision—Stock solutions of 1 or 2 and their metabolites were diluted with methanol, and 50 µL of solution was added to blank citrated plasma to obtain a concentration range from $5 \times 10^{-9}$ to $5 \times 10^{-6}$ M. Samples were analyzed in triplicate, and the range of linear response was determined by the maximum correlation coefficient obtained by the linear least-squares method.

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The precision of the method was measured by analysis of six independently prepared citrated plasma samples at a concentration of $7 \times 10^{-9}$ M for the glucosides and aglycones and $4 \times 10^{-8}$ M for the glucuronides.

Thin-Layer Chromatography of Aglycones—To investigate self-association of the aglycone metabolites, 10-µL samples of 3 and 8 in methanol ($6 \times 10^{-4}$ M) were spotted on a C8 reversed-phase thin-layer chromatography plate (RP-TLC). The plate was developed ascendingly with a mixture of acetonitrile:KH$_2$PO$_4$ (0.065 M, pH 4.0, 2:1, v/v) in a standard glass developing tank. The experiment was repeated with the following modifications: 5 and 8 dissolved in chloroform:isopropyl alcohol (4:1, v/v) and developed as before.

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The precision of the method was measured by analysis of six independently prepared citrated plasma samples at a concentration of $7 \times 10^{-9}$ M for the glucosides and aglycones and $4 \times 10^{-8}$ M for the glucuronides. 

± 1% under these circumstances. The detection limit in plasma was $7 \times 10^{-9}$ M for the glucosides, $4 \times 10^{-8}$ M for the glucuronides, and $1 \times 10^{-9}$ M for the aglycones. The analytical methods described gave good linear correlations in the range normally encountered in plasma of patients. The linear correlation coefficients were >0.998 for all compounds studied in the range from the detection limit up to $5 \times 10^{-8}$ M, except for the 7-deoxyaglycones. Compound 7 gave a linear correlation with a correlation coefficient of 0.996 from $10^{-9}$ to $10^{-6}$ M, and both 7-deoxyaglycones gave a linear correlation with linear correlation coefficients >0.998 from $10^{-9}$ to $5 \times 10^{-7}$ M. At higher concentrations of the 7-deoxyaglycones, the fluorescence efficiency sharply decreased. We have discovered that the presence of CHCl$_3$ in the extraction medium and the sample solvent had a beneficial effect on the linearity of the analysis of these compounds, and we have implemented this in our procedures. To investi-
gate the underlying mechanism of this phenomenon, we analyzed 7 and 5 by reversed-phase TLC. When 7 was dissolved in methanol its $R_f$ value was virtually zero; in other words, it did not migrate. However, when 7 was first dissolved in CHCl3/isopropyl alcohol (4:1, v/v), its $R_f$ value was 0.53, almost the same as the $R_f$ value of 5 in both cases. A possible explanation for this is suggested by the observation that anthracyclines tend to undergo self-association in polar solvents at high concentrations.57 Doxorubicin aggregates formed in this way dissociate in nonpolar solvents. The reason why the 7-deoxyxyglycones would undergo self-association at even lower concentration than C-7 oxygenated anthracyclines, as suggested by our observations, is not clear. Solvation at the C-7 oxygen possibly hinders ring stacking and therefore inhibits self-association.

The precision of the method was determined as the standard deviation of six citrated plasma samples at a concentration of $7 \times 10^{-9}$ M and was found to be 8.7% (1), 12.0% (2), 14.7% (3), 12.5% (4), 4% (5), 4% (6), 13.5% (7), and 8.6% (8). For the glucuronides, the precision was determined to be 19% at a concentration of $4 \times 10^{-8}$ M.

Plasma samples prepared for HPLC analysis as described above were stable for at least 10 h at room temperature.

In most of the experiments described, citrated plasma was used. However, citrated plasma, heparinized plasma, EDTA-treated plasma, serum, and Hanks buffered salt solution (pH 7.4) resulted in virtually identical extraction efficiencies in the concentration range studied.

Analysis of Urine Samples—Urine samples were analyzed without prior extraction. After shaking at 37 °C and ultrasonic treatment, the precipitate, practically always present in thawed urine samples, was dissolved or homogeneously dispersed. Quantitative errors due to the adsorption of drug and/or metabolites to the precipitate are thus avoided. This is particularly important when analyzing urine containing glucuronides of 2, since these tend to adsorb totally to the urine precipitate. The addition of a small volume of H3PO4 has the advantage that the peak shapes are significantly improved, increasing both the detection limit and the resolution. The best separation was obtained at pH 2.5. The recovery was virtually 100%. The calibration curves obtained showed a linear response for the glucuronides up to $2 \times 10^{-5}$ M and, for 2 and 4, to at least $5 \times 10^{-6}$ M ($r = 0.999$). This means that patient samples can be analyzed without prior dilution. The detection limits were $5 \times 10^{-6}$ M. The within run standard deviations were 1.5% (2), 2.2% (4), and 3.3% (10) measuring six samples, and the between day standard deviations (n = 3) were 1.6% (2), 2.5% (4), and 3.3% (10) measured at a concentration of $4.5 \times 10^{-7}$ M.

Samples, prepared as described above, were stable for 48 h at room temperature. Standard deviations of 18 determinations of identical urine samples, spread out over three consecutive days, were <9.5% for all compounds tested.

Analysis of Plasma and Urine from Two Patients Receiving 4‘-Epidoxorubicin—The plasma samples of two patients in this study contained relatively high levels of metabolites (Table I). The area under the plasma concentration versus time curve for 2 accounted for only ~40% of the total area under the curve for drug and metabolites in a 48-h period after drug administration. Reduction of the carbonyl moiety (4), glucuronidation (10 and 11), and reductive deglycosylidation (7 and 8) appear to be the major metabolic pathways. Hydrolytic deglycosylidation leading to 5 and 6 seems to be of minor importance, if not negligible. Urine samples contain only glycosidic metabolites in addition to 2, since the aglycones are polar and not water soluble. The glucuronides, being very polar metabolites, are present in urine in concentrations comparable with those of 2 and 4 (Fig. 3 and Table II). Pharmacokinetic parameters other than area under the curve and cumulative urine excretion (Table II) were not determined because of the limited number of patients.

Table I—Metabolites of 4‘-Epidoxorubicin in Plasma of Patients *

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Patient No. 1</th>
<th>Patient No. 2</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>40</td>
<td>39</td>
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<tr>
<td>4</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>15</td>
</tr>
</tbody>
</table>

*Expressed as percent of relative AUC (0–48 h) after a dose of 75 mg/m² of 4‘-epidoxorubicin.

Table II—Cumulative Urine Excretion of Metabolites *

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Patient No. 1</th>
<th>Patient No. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.2</td>
<td>6.3</td>
</tr>
<tr>
<td>4</td>
<td>2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>6.8</td>
<td>2.4</td>
</tr>
<tr>
<td>11</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Total</td>
<td>17.0</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*Expressed as percentage of the administered dose (0–48 h).
Discussion

The analytical method described combines simplicity, versatility, and selectivity. Both 1 and 2 and their metabolites can be analyzed with the same eluants. Other anthracyclines can also be analyzed with this solvent system, or with minor changes in the polarity of the eluant. Different eluants were used when analyzing plasma or urine because urine does not contain aglycones and can, therefore, be analyzed more efficiently with a less polar solvent to ensure complete resolution of the rather polar glucuronides from other urine components. Similarly, when analyzing 10 and 11 in plasma samples, the less polar solvent system II was used. We were able to separate 5 and 8 into completely resolved peaks, which so far has not been reported elsewhere. We observed that standard aqueous phase--organic phase extractions tend to result in very limited linearity for the 7-deoxyaglycones. This may easily lead to erroneously underestimated values, especially at high metabolite concentrations. We were able to overcome this problem to some extent by using a mixture of methanol and chloroform as the solvent for injecting the samples. However, under these circumstances, linearity was still limited; the upper concentration limit being 10^{-6} M for 7 (r = 0.996) and 5 \times 10^{-7} M for 8 (r = 0.998). We tentatively attribute the apparent decrease in fluorescence quantum yield of the 7-deoxyaglycones at high concentrations to self-association (see Results). The recoveries of the plasma extraction in the linear concentration range were >80% for all compounds investigated except for the glucuronide, which showed a recovery of only 12 ± 1%. The presence of the two sugar moieties in the anthracycline molecule apparently increases the water solubility to such an extent that the aqueous phase--organic phase partition coefficient increases 4–5 fold. Attempts to increase the recovery of the glucuronides using more polar organic solvents such as CH_3Cl_2,MeOH (1:4, v/v), resulted in only slightly higher recoveries (23 ± 2%). Moreover, such methods resulted in chromatograms with plasma impurities interfering with the glucuronide peaks. Standard samples prepared with citrated plasma, heparinized plasma, EDTA-treated plasma, serum, or Hanks buffered salt solution (pH 7.4) gave virtually identical recoveries. Apparently, extraction efficiencies are not affected by protein binding of anthracyclines. For patient plasma samples, the most practical choice therefore seems to be determined by the stability of plasma against protein denaturation under conditions of freezing and thawing. In this regard, we have had good experience with EDTA-treated and citrated plasma, but less with heparinized plasma.

A preliminary study of plasma and urine of two patients who received 2 as an iv bolus injection indicated that metabolism of 2 is quite significant; especially the formation of 4, 10, 11, 8, and, to a lesser extent, 7 (Tables I and II, Fig. 4). The metabolism of 2 to 5 and 6 seems rather insignificant.

References and Notes


Acknowledgments

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Figure 4—Plasma concentration versus time curve for patient no. 2 after receiving 75 mg/m² of 2 as an iv bolus. Namely 2 and metabolites (5, 6, 7, 8, 10, 11) are given in the Experimental Section.

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