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HPLC determination of doxorubicin, doxorubicinol and four aglycone metabolites in plasma of AIDS patients*

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Abstract: A high-performance liquid chromatographic (HPLC) assay has been developed for the determination of the anticancer drug doxorubicin and the metabolites doxorubicinol, doxorubicinone, 7-deoxydoxorubicinone, doxorubicinolone and 7-deoxydoxorubicinolone in plasma of AIDS patients. Samples can be heated at 60°C for 30 min to inactivate the human immunodeficiency virus. The sample pre-treatment involves a liquid–liquid extraction of the buffered plasma sample (pH 9) with a chloroform–1-propanol (4:1, v/v) mixture. The chromatographic analysis is performed on a Lichrosorb RP-8 (5 μm) column and by isocratic elution with a mobile phase of acetonitrile–tetrahydrofuran–phosphate buffer (pH 2.2) (800:5:200, w/w/w) with fluorescence detection (excitation wavelength: 460 nm; emission wavelength: 550 nm). The proposed method has been validated and, subsequently, implemented in a pharmacokinetic study of doxorubicin in AIDS patients with Kaposi's sarcoma who are treated with the combination regimen doxorubicin, vincristine and bleomycin.

Keywords: *Doxorubicin; metabolites; HPLC; bio-analysis; AIDS.*

Introduction

Doxorubicin (Dx) is an anthracycline glycosidic antibiotic which possesses profound antitumour activity in several human malignancies [1, 2]. The drug has been used for more than 20 years, in the treatment of patients with, for example, soft tissue sarcomas, breast and gastric cancer. It does appear that Dx is also one of the most effective agents for Acquired Immuno-Deficiency Syndrome (AIDS)-related Kaposi's sarcoma and trials are ongoing with the drug as single agent or in combination with other antineoplastic drugs [3]. Laubenstein *et al.* [4] reported a response percentage of 84 with the triple combination regimen doxorubicin (40 mg m^{-2}), bleomycin (15 units) and vinblastine (6 mg m^{-2}). Unfortunately, the clinical use of Dx is limited by its toxicity, such as myelosuppression, cumulative dose-related cardiotoxicity and the development of drug resistance. The major concern about the use of Dx in AIDS-related epidemic Kaposi's sarcoma is that this cytotoxic treatment will further immunosuppress the patient. Therefore, lower dosages of Dx are being tested in the clinic [5].

So far, attempts to correlate the pharmacokinetics of Dx with therapeutic efficacy and toxicity have been mostly unsuccessful. Anthracyclines, *in vivo*, undergo extensive biotransformation and the role of this metabolism in drug-induced toxicity has not been fully elucidated as yet. It has been hypothesized that the metabolites, rather than the parent drug, correlate with toxicity [6]. The main metabolic pathway consists of carbonyl reduction, mediated by cytoplasmic NADPH-dependent aldo-keto reductases, yielding doxorubicinol (Dx-ol) which has cytotoxic activity. Dx-ol and Dx

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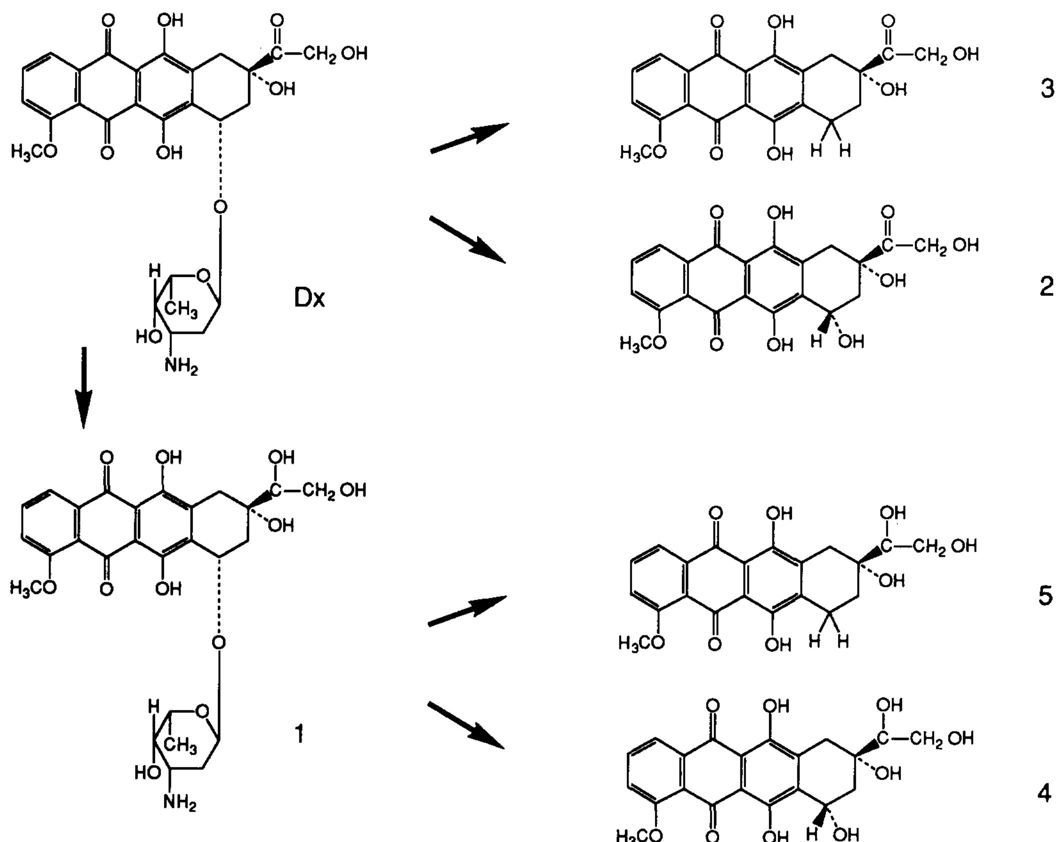

Figure 1
Chemical structures and metabolic routes for doxorubicin. Dx, doxorubicin; 1, doxorubicinol (Dx-ol); 2, doxorubicinone (Dx-one); 3, 7-deoxydoxorubicinone (7-deoxyDx-one); 4, doxorubicinolone (Dx-olone); 5,7-deoxydoxorubicinolone (7-deoxyDx-olone).

are converted to their respective 7-deoxy- and 7-hydroxyaglycones. The different metabolic pathways are shown in Fig. 1. *O*-Demethylation and conjugation with sulphate and glucuronic acid have been described as consecutive metabolic routes of the aglycones [7]. In cancer patients there are marked inter-individual differences in anthracycline biotransformation to the C13 hydroxy derivatives and aglycones [8, 9]. For AIDS patients this subject has not been studied as yet.

In our hospital a clinical research program has been initiated focusing on metabolism and pharmacokinetics of Dx in AIDS patients and to correlate this with efficacy and toxicity. For this purpose a high-performance liquid chromatographic (HPLC) method was developed which is able to detect the parent drug and low concentrations of Dx-ol and aglycones in human plasma. In comparison with other assays for the bioanalysis of anthracyclines [7, 10, 11] the proposed method has the advantage that it is simple, sensitive and rapid; Dx, five metabolites and the internal standard are determined after a single extraction step and with an isocratic HPLC run of 25 min.

Experimental

Chemicals and reagents

Dx, Dx-ol, doxorubicinone (Dx-one), doxorubicinolone (Dx-olone), 7-deoxydoxorubicinone (7-deoxyDx-one) and 7-deoxydoxorubicinolone (7-deoxyDx-olone) were kindly provided by Dr S. Penco, Farmitalia Carlo Erba (Milan, Italy) and daunorubicin (Dr) was obtained through Rhône Poulenc BV (Amstelveen, The Netherlands). Dx, Dx-ol and Dr were supplied as hydrochloride salts. Chloroform, 1-propanol, tetrahydrofuran (THF) and borax (all 'pro analyse' quality) were purchased from Merck (Darmstadt, Germany). Acetonitrile was HPLC grade and obtained from Promochem GmbH (Wesel, Germany). A borax buffer solution pH 9.3 was prepared by dissolving 6 g borax, under heating, in 100 ml of water. A perchloric solution (pH 2.2)

was prepared by adding 70% perchloric acid to distilled water to reach a final pH of 2.0. The extraction solvent was a mixture of chloroform–1-propanol (4:1, v/v). Distilled water was used throughout the study.

Chromatography

The chromatographic system consisted of a solvent delivery system, type 6000A (Waters Assoc., Milford, MA, USA), a model SP 8880 automatic injection device (Spectra Physics, Santa Clara, CA, USA), a LS40 fluorescence detector (Perkin–Elmer Corp., Norwalk, CT, USA) and a SP 4290 integrator (Spectra Physics, Santa Clara, CA, USA). The analyses were performed on a Lichrosorb RP-8 column (particle size 5 μm, 125 mm long × 4 mm i.d.; Merck). The analytical column was protected by a LiChroCART® 4–4 (4 mm long × 4 mm i.d.) pre-column packed with LiChrospher® 100 RP-8 (5 μm) material (Merck). The columns were eluted with a mobile phase of phosphate buffer (pH 2.2)–acetonitrile–THF (800:200:5, w/w/w) at ambient temperature. The phosphate buffer pH 2.2 was prepared by adding 85% phosphoric acid to distilled water to reach a final pH of 2.2. The mobile phase was degassed by using an ultrasonic bath. The flow rate was 1.5 ml min^{-1}. The fluorescence detector operated with excitation wavelength 460 nm and emission wavelength 550 nm. The slit width of the fluorescence detector is 10 nm.

Standard solutions

Stock solutions of Dx, Dx-ol and the internal standard Dr were prepared in a perchloric acid solution pH 2.0. The aglycones are very poorly soluble in water and, therefore, were dissolved in chloroform–1-propanol (4:1, v/v). The stock solutions are stable for at least 1 month, when stored at 4°C.

Sample preparation

The plasma sample size for Dx and Dx-ol analysis for the concentration range of 1–10 ng ml^{-1} was 1.0 ml and for the range 10–1000 ng ml^{-1} it was 0.1 ml. Analysis of the aglycones was performed in the concentration range 1–10 ng ml^{-1} with 1.0 ml samples and in the range 10–100 ng ml^{-1} with 0.1-ml plasma samples. The 0.1-ml plasma samples were stored in polypropylene Eppendorf vials (1.5 ml) originating from Treff Lab (Degersheim, Switzerland) and the 1.0 ml plasma samples were stored and processed in TR95PP polypropylene test tubes from International Medical Products BV (Zutphen, The Netherlands).

Calibration samples

Calibration curves were prepared by spiking 0.1 or 1.0 ml drug-free plasma with the appropriate amounts of the glycosidic antibiotics and the aglycones. For the analysis of Dx and Dx-ol in the concentration range of 100–1000 ng ml^{-1}, 100-μl drug-free plasma samples were spiked with 10–100 μl of a standard solution containing both 1 μg Dx and 1 μg Dx-ol ml^{-1}, 100 μl of the internal standard solution (1 μg Dr ml^{-1}), 100 μl buffer solution (pH 9.3) and a perchloric acid solution pH 2.0 to a final volume of 400 μl. This solution was mixed with 1.0 ml of the extraction solvent. After vortex mixing for 10 s, and centrifugation for 5 min (12 000 rpm) the aqueous supernatant and protein interface were discarded by suction under reduced pressure. The resulting clear organic extract was transferred to a clean vial and evaporated to dryness under a gentle stream of nitrogen at 50°C. The residue was dissolved in 150 μl of the mobile phase and an aliquot of 40 μl injected into the chromatograph.

The calibration samples in the concentration range of 10–100 ng ml^{-1} for Dx and Dx-ol were combined with the four aglycone metabolites in the concentration range 10–100 ng ml^{-1}. For this purpose 100-μl drug-free plasma samples were spiked with 10–100 μl of a standard solution containing 0.1 μg Dx and 0.1 μg Dx-ol ml^{-1}, 100 μl of the internal standard solution (0.2 μg Dr ml^{-1}), 10–100 μl of a standard solution containing 0.1 μg Dx-one, 0.1 μg 7-deoxyDx-one, 0.1 μg Dx-olone and 0.1 μg 7-deoxyDx-olone ml^{-1}, 100 μl buffer solution (pH 9.3) and a perchloric solution (pH 2.0) to a final volume of 500 μl. This solution was mixed with 1.0 ml of the extraction fluid. The samples were further processed as described above except for the reconstitution step. After evaporation of the organic solvent the residue was dissolved in 30 μl of the mixture acetonitrile–THF (200:5, w/w) with vortex mixing for 30 s. Next, 120 μl of a phosphate buffer (pH 2.2) was added and, after mixing, the solution centrifuged and a 100-μl aliquot of the clear solution injected into the chromatograph.

In the concentration range of 1–10 ng ml^{-1} for Dx, Dx-ol and the aglycones 1.0-ml

samples of drug-free plasma were spiked with 10–100 μl of a standard solution containing 0.1 μg Dx and 0.1 μg Dx-ol ml^{-1}, 100 μl of the internal standard solution (0.2 μg Dr ml^{-1}), 10–100 μl of a standard solution containing 0.1 μg Dx-one, 0.1 μg 7-deoxyDx-one, 0.1 μg Dx-olone and 0.1 μg 7-deoxyDx-olone ml^{-1}, 1 ml of a buffer solution (pH 9.3) and a perchloric solution (pH 2.0) to a final volume of 2.3 ml. The resulting solution was mixed with 5.0 ml of the extraction solvent and the samples further processed as described above except for the centrifugation procedure which was, in this case, 5000 rpm for 10 min. The reconstitution step was, again, performed with 30 μl of the mixture acetonitrile–THF (200:5, w/w) and, subsequently, with 120 μl of the phosphate buffer (pH 2.2). An aliquot of 100 μl was injected into the chromatograph.

Clinical samples

Two AIDS patients with Kaposi's sarcoma were treated with the multi-agent combination: vincristine (2 mg), bleomycin (15 units m^{-2}) and doxorubicin (patient 1: 20 mg m^{-2}; patient 2: 15 mg m^{-2}). The drugs were dissolved in 100 ml 0.9% sodium chloride infusion and given, in this order, as 30-min intravenous infusions. Periodically, blood samples were collected from an indwelling intravenous cannula in the arm contralateral to that receiving the drugs. Blood samples were collected in EDTA tubes prior to the start of the infusions, during the Dx infusion and at specific times post-infusion. Plasma was immediately isolated by centrifugation and stored at −30°C prior to analysis. Stability experiments revealed that the plasma samples are stable for at least 1 month at this temperature.

Validation

Recoveries of Dx, Dx-ol and the four aglycone metabolites have been calculated by comparison with spiked plasma samples and standard solutions of the compounds with known concentrations. In the concentration range 1–10 ng ml^{-1} peak height measurements were performed and in the range 10–1000 ng ml^{-1} peak areas were determined. Linearity, precision and accuracy were determined repeatedly by analysing spiked plasma samples. For the determination of the detection limit the fluorescence was set at a response equal to three times the average noise level. The stability of the analytes in plasma under conditions (30 min at 60°C) which inactivate HIV have been investigated.

Results and Discussion

General aspects on the bioanalysis of the anticancer anthracyclines have been described extensively [7, 10–13]. Therefore, only the critical parameters, which appeared to be important for the development of the presented assay, are discussed here.

Chromatography and detection

In the past decade HPLC with fluorescence detection has developed into the most powerful tool in terms of selectivity, sensitivity and simplicity for anthracycline bioanalysis [7]. Reversed-phase HPLC (RP-HPLC) is to be preferred over normal phase chromatography as this technique allows separation between the aglycone metabolites [7]. In Fig. 2(A) it is demonstrated that the presented isocratic RP-HPLC system provides separation between Dx, Dx-ol, four aglycone metabolites and the internal standard Dr (Fig. 3) within one run of 25 min. The addition of only 0.5% THF to the mobile phase gave an important improvement of the peak shapes of all compounds without affecting the fluorescence yields. The presented assay was developed with a commercially available Lichrosorb RP-8 (particle size: 5 μm) column. Columns of different batches were tested and there was hardly any inter-column variability. When the optimal separation could not be achieved with a new column and the standard eluent, simple adaptations in terms of minor variations of the pH of the aqueous component of the mobile phase or variation of the water–organic modifier ratio appeared to be sufficient to obtain the desired separation. The retention times of the glycosidic antibiotics increase with a higher pH of the aqueous phase of the eluent at a constant water–organic modifier ratio while the retention times of the aglycones remain constant in this case. The retention of the aglycones can be manipulated by changing the water–organic modifier ratio [14].

Sample pretreatment

Liquid–liquid extraction is often used successfully for the isolation of glycosidic anthracyclines and the aglycone metabolites from biological matrices [7]. The pH of the matrix is an important factor to obtain high recoveries of

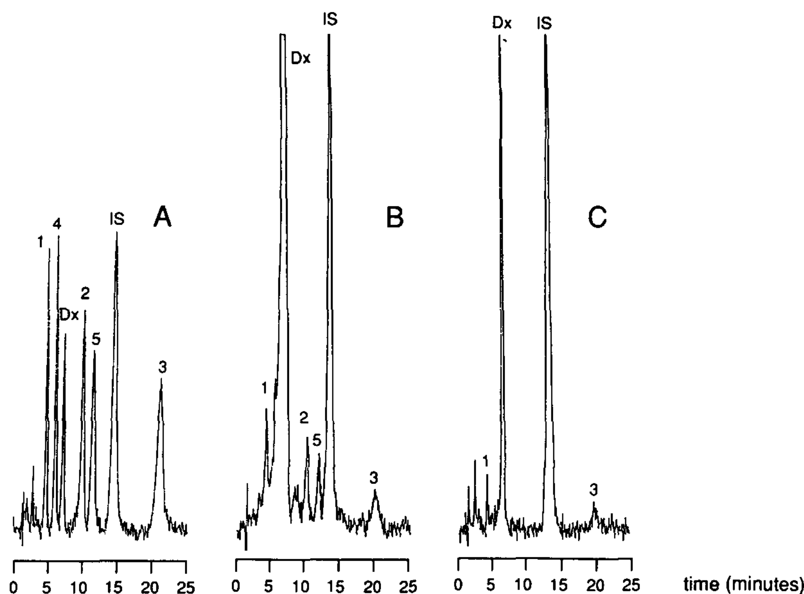

Figure 2
A: HPLC chromatogram of a mixture of reference compounds. Dx, doxorubicin; 1, doxorubicinol (Dx-ol); 2, doxorubicinone (Dx-one); 3,7-deoxydoxorubicinone (7-deoxyDx-one); 4, doxorubicinolone (Dx-olone); 5,7-deoxydoxorubicinolone (7-deoxyDx-olone); IS, internal standard daunorubicin (Dr). B: HPLC chromatogram of a plasma sample of a cancer patient treated with Dx (dose 50 mg m^{-2}) at t = 12 min after intravenous bolus administration. C: HPLC chromatogram of a plasma sample of an AIDS patient treated with Dx (dose 20 mg m^{-2}) at t = 50 min after intravenous administration (30-min infusion).

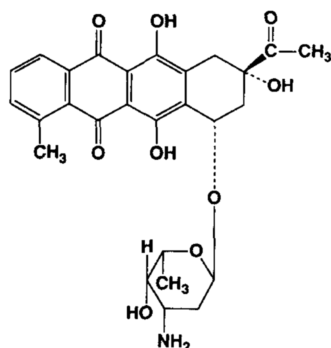

Figure 3
Chemical structure of the internal standard daunorubicin (Dr).

Dx, Dx-ol and Dr. The pH of the samples was, therefore, adjusted to 9 [7]. During the development of this method it was noticed that the recoveries of the compounds were strongly dependent on the quality of the chloroform used for the extraction. It could not be traced whether an impurity was the cause of this phenomenon and this will be subject for further studies.

Different polypropylene test tubes were investigated as tubes from different manufacturers may contain interfering fluorescent compounds. The TR95PP tubes used here did not contain these interfering components.

Reconstitution, after evaporation of the extraction fluid, from the residues of Dx and Dx-ol from plasma samples in the concentration range 100–1000 ng ml^{-1} was performed quantitatively with the mobile phase. In the other samples the reconstitution procedure was done in two steps: first, 30 μl of a mixture of acetonitrile–THF was added and, subsequently, a phosphate buffer (pH 2.2). This procedure was necessary in order to obtain a complete dissolution of the aglycones.

Validation

The presented analytical methodology has been validated in terms of precision, accuracy and linearity for the parent drug Dx, the glycosidic metabolite Dx-ol and the four aglycone metabolites as shown in Tables 1 and 2. The recoveries of Dx, Dx-ol and the four aglycones have been determined at a plasma concentration of 50 ng ml^{-1} and were for all compounds >90% (±8%). The detection limits for Dx and Dx-ol were 1 ng ml^{-1} and the aglycones 0.5 ng ml^{-1} (using 1.0 ml plasma samples). The chemical stabilities of the analytes in the biological matrix were investi-

Table 1
Equations of calibration lines for the analysis of Dx and metabolites in plasma

Concentration range	Equation	r^2	n
Doxorubicin (Dx)			
1–10 ng ml^{-1}	$y^1 = -0.011\ (\pm 0.016) + 0.124\ (\pm 0.002)\ x$	0.9989	10
10–100 ng ml^{-1}	$y^2 = -0.011\ (\pm 0.026) + 0.007\ (\pm 0.0003)\ x$	0.9917	10
100–1000 ng ml^{-1}	$y^2 = -0.028\ (\pm 0.048) + 0.001\ (\pm 0.00006)\ x$	0.9991	10
Doxorubicinol (Dx-ol)			
1–10 ng ml^{-1}	$y^1 = -0.012\ (\pm 0.106) + 0.262\ (\pm 0.012)\ x$	0.9913	10
10–100 ng ml^{-1}	$y^2 = 0.0127\ (\pm 0.020) + 0.0083\ (\pm 0.0002)\ x$	0.9970	10
100–1000 ng ml^{-1}	$y^2 = 0.0130\ (\pm 0.0267) + 0.0011\ (\pm 0.00003)\ x$	0.9969	10
Doxorubicinone (Dx-one)			
1–10 ng ml^{-1}	$y^1 = 0.0019\ (\pm 0.033) + 0.1377\ (\pm 0.0067)\ x$	0.9919	10
10–100 ng ml^{-1}	$y^2 = -0.0299\ (\pm 0.026) + 0.0173\ (\pm 0.0005)\ x$	0.9965	10
7-Deoxydoxorubicinone (7-deoxyDx-one)			
1–10 ng ml^{-1}	$y^1 = 0.0216\ (\pm 0.0256) + 0.234\ (\pm 0.009)\ x$	0.9934	10
10–100 ng ml^{-1}	$y^2 = -0.0243\ (\pm 0.068) + 0.0429\ (\pm 0.0025)\ x$	0.9899	10
Doxorubicinolone (Dx-olone)			
1–10 ng ml^{-1}	$y^1 = -0.034\ (\pm 0.0522) + 0.294\ (\pm 0.012)\ x$	0.9934	10
10–100 ng ml^{-1}	$y^2 = 0.0171\ (\pm 0.028) + 0.0161\ (\pm 0.0007)\ x$	0.9936	10
7-Deoxydoxorubicinolone (7deoxyDx-olone)			
1–10 ng ml^{-1}	$y^1 = -0.007\ (\pm 0.0233) + 0.348\ (\pm 0.008)\ x$	0.9979	10
10–100 ng ml^{-1}	$y^2 = 0.0120\ (\pm 0.0414) + 0.0305\ (\pm 0.0014)\ x$	0.9916	10

Where x is the concentration of the analyte in ng ml^{-1} and y^1 is the peak height ratio between the analyte and the internal standard Dr and y^2, y^3 are ratios between the peak area integrator units of the analytes and the internal standard Dr.

Table 2
Accuracy and precision for the bioanalysis of Dx and metabolites

Theoretical conc. (ng ml^{-1})	Measured conc. (ng ml^{-1})	Accuracy (%)	RSD* (%)	n
Doxorubicin (Dx)				
4.78	4.87	102	7	5
47.8	51.1	107	9	5
478.0	454.1	95	5	5
Doxorubicinol (Dx-ol)				
4.95	4.80	97	6	5
49.5	48.5	98	5	5
495.0	470.3	95	3	5
Doxorubicinone (Dx-one)				
2.88	2.78	97	9	5
28.8	30.0	104	8	5
7-Deoxydoxorubicinone (7-deoxyDx-one)				
3.1	2.97	96	10	5
31.0	28.2	91	9	5
Doxorubicinolone (Dx-olone)				
2.50	2.40	96	5	5
25.0	25.5	102	6	5
7-Deoxydoxorubicinolone (7deoxyDx-olone)				
3.41	3.21	94	10	5
34.1	33.42	98	5	5

* RSD: relative standard deviation.

Table 3
Pharmacokinetic parameters of Dx in two AIDS patients using an open two-compartment model with 30-min infusion time

	Patient 1	Patient 2
$t_{½}$ (α)	0.15 h	0.09 h
$t_{½}$ (β)	49.9 h	15.2 h
Clearance	48.5 l h^{-1}	55.9 l h^{-1}
AUC	0.7 h mg l^{-1}	0.43 h mg l^{-1}
Dose (total dose)	20 mg m^{-2} (36 mg)	15 mg m^{-2} (24 mg)

AUC: area under the plasma concentration–time curve; clearance = dose/AUC.

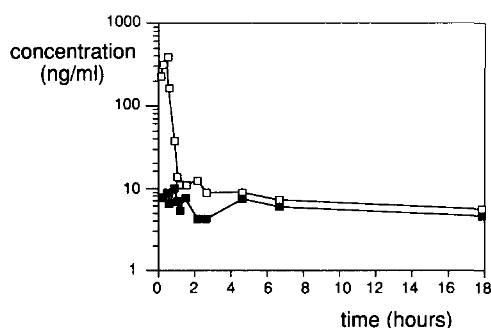

Figure 4
Plasma concentration–time curve of doxorubicin (□) and doxorubicinol (■) in an AIDS patient with Kaposi's sarcoma (dose: 15 mg m^{-2} given as a 30-min infusion).

gated by warming spiked plasma samples (drug concentration: 50 ng ml^{-1}) at 60°C for 30 min. These conditions have been advocated to inactivate the human immunodeficiency virus (HIV) [15]. These experiments revealed that Dx, Dx-ol and the four aglycones are stable under these conditions and that the clinical samples can be pretreated in this manner which is important in view of safe handling with the samples.

Pharmacokinetics

Interestingly, the only aglycone metabolite found in plasma of the treated AIDS patients was 7-deoxyDx-one, in very low concentrations. At the end of the 30-min infusion period the concentration of this metabolite reached the maximal concentration of 1.5 ng ml^{-1} and 1 h after the start of infusion the concentration decreased to 0.5 ng ml^{-1} and it was not detectable at later time points. This pattern was observed in both AIDS patients. In plasma of cancer patients treated with Dx, the aglycones 7-deoxyDx-one and 7-deoxyDx-olone were detected [8]. As a comparison, the HPLC chromatograms of a plasma sample (12 min after 50 mg Dx per m^2, intravenously as a bolus injection) of a cancer patient and an AIDS patient (50 min after 20 mg Dx per m^2, intravenously as a 30-min infusion) are shown in Fig. 2B and C, respectively. The HPLC chromatogram of the plasma sample of the cancer patient shows the presence of the aglycones Dx-one, 7-deoxyDx-one and 7-deoxyDx-olone.

The plasma concentration time curves for Dx and Dx-ol in an AIDS patient (Fig. 4) show a bi-exponential decline and can be described by an open two-compartment model. The pharmacokinetic parameters for Dx are listed in Table 3.

In conclusion, a simple, sensitive and validated HPLC method for the analysis of Dx and five metabolites in plasma has been developed which can be used for pharmacokinetic research.

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