ELSEVIER

Journal of Chromatography B, 758 (2001) 221–228

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantification of daunorubicin and daunorubicinol in plasma by capillary electrophoresis

Georg Hempel[a,b,*], Petra Schulze-Westhoff[b], Silke Flege[b], Joachim Boos[b]

[a]*Institut für Pharmazeutische Chemie, Universität Münster, Hittorfstrasse 58–62, 48149 Münster, Germany*
[b]*Universitäts-Kinderklinik, Abt. Haematologie/Onkologie, Albert-Schweitzer-Strasse 33, 48129 Münster, Germany*

Received 10 November 2000; received in revised form 16 March 2001; accepted 19 March 2001

Abstract

Capillary electrophoresis (CE) with laser-induced fluorescence detection was applied to quantify daunorubicin and daunorubicinol in plasma. Separation was carried out in a 47 cm×50 μm I.D. fused-silica capillary, with a running buffer, pH 5 containing 60 μ*M* spermine and 70% acetonitrile. Sample preparation was done either by protein precipitation with acetonitrile or by liquid–liquid extraction. The assay can be applied in a concentration range from 40 mg/l down to 2 μg/l for daunorubicin and from 1 mg/l to 2 μg/l for daunorubicinol. Precision and accuracy were between 2.9 and 14.5% ($n=6$) on 1 day and between 1.0 and 14.7% from day to day ($n=6$) for both analytes. Thus, the CE method enables precise and accurate quantification of daunorubicin and daunorubicinol in small sample volumes over a wide concentration range.

Keywords: Daunorubicin; Daunorubicinol

1. Introduction

Anthracyclines are a very important part of many treatment protocols in paediatric oncology. Daunorubicin (Fig. 1) is mainly used for the treatment of acute lymphatic and myeloic leukaemia in combination with cytarabine [1].

Although daunorubicin is necessary to achieve high response rates, a main problem is the cardiotoxicity of the anthracycline antibiotics which can cause congestive heart failure even years after treatment [2]. There is an ongoing discussion about the best schedule of administration to reduce the incidence of cardiotoxic events [3]. Two studies have shown that in adults prolonged infusions are less cardiotoxic than bolus injections [4,5]. Another approach to reduce anthracycline-related cardiotoxicity is liposomal encapsulation. Studies with Daunoxome, a liposomal formulation of daunorubicin in adults with acquired immune deficiency syndrome (AIDS)-related karposi sarcoma showed no clinically manifested cardiotoxicity even at high cumulative doses [6].

In this context pharmacokinetic studies, especially in children, are necessary to investigate the role of the pharmacokinetics and the importance of the main metabolite, daunorubicinol (Fig. 1) for both the cytotoxic effect and adverse events. To conduct studies in children, analytical methods requiring only small sample volumes are necessary as well as pharmacokinetic approaches such as nonlinear mixed

*Corresponding author. Fax: +49-251-8347-828.
E-mail address: hempege@uni-muenster.de (G. Hempel).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00185-2

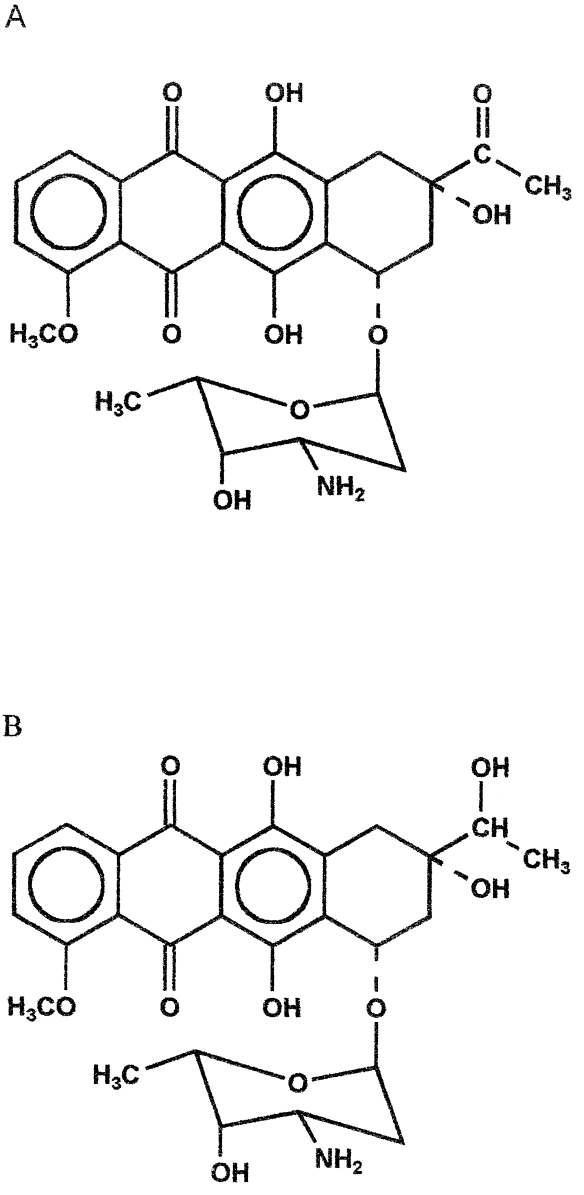

Fig. 1. Daunorubicin (A) and daunorubicinol (B).

effect modelling (NONMEM) to reduce the patient's burden due to intense blood sampling [7].

High-performance liquid chromatography (HPLC) methods for the determination of daunorubicin usually require a sample volume of 1 ml plasma, which is too much for our purpose [8]. Capillary electrophoresis (CE) is an attractive technique for this purpose, because theoretically the method requires only a sample volume of a few nanolitres. However, with UV detection, the sensitivity of CE is often not sufficient to monitor drugs in biological fluids given in low mg amounts [9]. Hence, as first shown by Reinhoud et al., laser-induced fluorescence (LIF) detection can be used for the detection of anthracyclines [10]. Recently, we have developed methods for the quantification of idarubicin and doxorubicin and their main metabolites in plasma [11,12]. On the basis of this work, we have developed and validated an assay for the quantification of daunorubicin and daunorubicinol in biological fluids. For the quantification of daunorubicin, especially after application of the liposomal formulation (Daunoxome) several modifications were necessary to be able to quantify the drug and its main metabolite over a higher concentration range.

2. Materials and methods

2.1. Drugs and reagents

Daunorubicin, daunorubicinol and idarubicin were kindly supplied by Farmacia (Erlangen, Germany). All chemicals used were of analytical grade. Acetonitrile, chloroform and sodium hydroxide solution (0.1 *M*) were purchased from Malinckrodt Baker (Deventer, The Netherlands); spermine tetrahydrochloride from Aldrich (Steinheim, Germany) and sodium dihydrogenphosphate from Merck (Darmstadt, Germany). Purified water was prepared on a Millipore-Q-UF system.

2.2. Patient samples

All patients or their parents gave informed consent to the sample collection. Samples after administration of free daunorubicin were derived from 17 patients treated for acute lymphatic leukaemia receiving 30 mg/m^2 as a 1- or 2 h-infusion. Patient characteristics were: age 6.0 (0.5–14) years, height 117 (52–168) cm, mass (4.0–57.8) 21.0 kg and body surface area 0.80 (0.2–1.6) m^2 (median, range in parentheses). Samples after administration of Daunoxome were from 19 patients treated for relapsed acute lymphoblastic leukaemia and received

30 to 60 mg/m^2 as a 1-h infusion. Patient demographics were as follows: age 14.6 (2.8–21.5) years, height 157 (89–183) cm, mass 38 (14–76.5) kg, body surface area 1.28 (0.58–1.98) m^2 (median, range in parentheses). Blood samples were taken either intravenously (i.v.) with heparinised or EDTA-tubes or from the fingertip and were immediately centrifuged at 8°C to separate the plasma fraction. Samples were stored at −20°C until analysis.

2.3. Sample preparation

Three different schemes for sample preparation were used depending on the expected concentration:

(1) Daunorubicin and daunorubicinol up to 50 μg/l: 100 μl of plasma is mixed with 50 μl of a solution of 10 μg/l of the internal standard (I.S.) and 100 μl 50 m*M* phosphate buffer, pH 7.4. The mixture is extracted with 1 ml of chloroform. After centrifugation 900 μl of the organic phase is evaporated to dryness and the residue is dissolved in 50 μl of acetonitrile with 10% water for CE analysis.

(2) Between 50 and 1000 μg/l of daunorubicin and daunorubicinol: 10 μl of plasma (patient plasma, calibration plasma or quality control samples) is mixed with 90 μl of a solution of idarubicin in acetonitrile 50 μg/l using a vortex mixer and ultrasound both for 60 s. Subsequently, the mixture is centrifuged at 1500 *g* for 3 min. At least 35 μl of the supernatant is transferred to a polycarbonate vial for CE analysis.

(3) Daunorubicin higher than 1 mg/l: 20 μl of plasma is diluted to 100 μl with water. To 10 μl of this dilution 90 μl of a solution of the I.S. (idarubicin 50 μg/l) in acetonitrile is added and vortex mixed for 60 s following ultrasound treatment for 60 s.

2.4. Capillary electrophoresis

A Model 5510 P/ACE (Beckman Coulter, Munich, Germany) equipped with an air-cooled argon ion laser operating at 488 nm was used (5 mW, Beckman Coulter). Detection was carried out with a Beckman LIF detector equipped with a 520 nm band pass filter. Fused-silica capillaries of 40 cm effective length×50 μm I.D.×375 μm O.D. (Beckman Coulter) were used. Separation was done at 532 V/cm (25 kV) applied voltage with the cathode at the detection end of the capillary. The running buffer was prepared by adjusting a solution of 100 m*M* sodium dihydrogenphosphate with 100 m*M* sodium hydroxide to a pH value of 5.0. Subsequently, spermine was added to obtain a concentration of 60 μ*M* and the solution was mixed with acetonitrile to a final concentration of 70% (v/v). All solutions for CE were filtered through a 0.45-μm filter.

Samples were applied to the capillary by electrokinetic injection at 10 kV for 7 s. For concentrations above 50 μg/l pressure injection for 7 s with 3447 Pa (0.5 p.s.i.) was applied. Between runs, the capillary was rinsed with sodium hydroxide 100 m*M* for 1 min and the running buffer for 2 min. Every day before analysing the first sample, the capillary was rinsed with sodium hydroxide 100 m*M* for 20 min and the running buffer for 10 min.

2.5. Preparation of standard solutions

Stock solutions containing 20 mg/l daunorubicin and daunorubicinol were prepared in acetonitrile. Idarubicin was dissolved in acetonitrile to obtain a concentration of 5.7 mg/l. The stock solutions were stored at −20°C and could be used for up to 12 weeks. Blank plasma for the preparation of calibration solutions were taken from healthy donors from the department of transfusion medicine, University of Münster.

Standards were prepared by serial dilution of the stock solutions with blank plasma to obtain the desired concentrations of both daunorubicin and daunorubicinol; quality control samples were prepared in the same manner.

2.6. Quantification

All calculations were done using the corrected peak area (peak area/migration time). Calibration graphs were calculated by analysing seven different standard solutions from 2 to 50 μg/l for daunorubicin and daunorubicinol using the internal standard method and weighted linear regression ($1/x$). In the higher concentration ranges from 50 to 1000 μg/l and from 1 to 40 mg/l eight different

standards were used and analysed in the same manner. Within every series, two to three quality control samples were analysed (concentrations 2, 30, 50, 200, 700, 1000, 5000 and 30 000 μg/l).

Precision and accuracy was assessed by repeated analysis of quality control samples in one series (intra-day) or on subsequent days (inter-day). To estimate the recovery of the method, the peak areas of the analytes in a similar matrix (acetonitrile with 10% water) were compared with the peak areas of plasma samples after sample preparation.

3. Results

3.1. Separation

The separation of daunorubicin, daunorubicinol and the I.S. in a patient plasma after liquid–liquid extraction is shown in Fig. 2A. The conditions for the separation are the same as developed in our group for the separation of idarubicin and doxorubicin [11,12]. As anthracyclines have the tendency to interact with the silanol groups of glass surfaces [1], the addition of acetonitrile and spermine is necessary to reduce the adsorption of the analytes to the capillary wall.

In blank plasma no peaks interfering with the analytes were observed (Fig. 2B). This is due to the selective detection of the analytes based on their fluorescence when excited at 488 nm. There are only a few chemicals showing fluorescence under the same conditions. Thus, the CE–LIF combination provides a highly selective and sensitive device for the identification and quantification of anthracyclines.

Idarubicin was chosen as the internal standard because of its structural similarity to the analytes. The addition of an internal standard is necessary to adjust for deviations during electrokinetic injection, deviations due to the extraction procedure and due to evaporation of the sample in the autosampler before injection.

3.2. Extraction procedure

To quantify daunorubicin in a low concentration range, we found that a liquid–liquid extraction procedure is necessary to remove proteins and other compounds from plasma. Chloroform was chosen because it gave the best results regarding to reproducibility. Attempts to increase the recovery by adding 10% isopropanole to the chloroform, as reported in the literature [13], resulted in problems with current stability during CE analysis. The recovery of the procedure was determined by comparing the peak areas of stock solutions in acetonitrile with 10% water and extracted plasma samples containing the same concentrations (Table 1). However, it must be noted that sample matrix effects during electrokinetic injections can occur. Therefore,

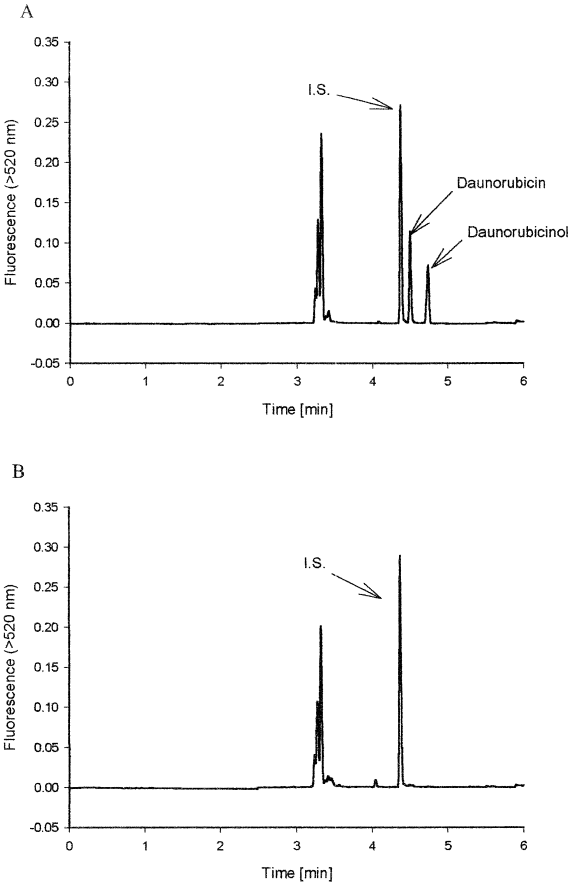

Fig. 2. (A) Electropherogram of human plasma 48 h after administration of 30 mg/m^2 daunorubicin as a 2-h infusion. The sample was prepared by liquid–liquid extraction as described above. (B) Blank plasma prepared in the same manner.

Table 1
Recovery of the analytes after liquid–liquid extraction with chloroform

Analyte	Concentration (μg/l)	Recovery (%)	RSD (%)	*n*
Daunorubicin	30	72.8	7.86	8
Daunorubicin	10	67.7	7.34	5
Daunorubicinol	30	64.4	5.80	8
Daunorubicinol	10	62.1	6.11	5

the recovery data give only a rough estimate of the extraction efficacy.

For concentrations above 50 μg/l it was found that protein precipitation with acetonitrile is sufficient to prepare the samples for the analysis. As anthracyclines show a high fluorescence intensity the dilution of the samples was necessary to keep the detector signal in a linear range. Fig. 3 shows the electropherograms of a plasma sample with a higher concentration of the analytes as well as blank plasma prepared in the same way. We found that this procedure provided reproducible data for concentrations up to 1000 μg/l for daunorubicin and daunorubicinol.

For concentrations of daunorubicin higher than 1 mg/l, which occur after administration of liposomal daunorubicin, a second dilution step was found to be necessary. Fig. 4 shows an electropherogram of a peak plasma sample after administration of Daunoxome and a blank plasma analysed with this method.

The extraction efficacy of the protein precipitation procedure with acetonitrile was determined in the same manner and the results are shown in Table 2. From the data it can be seen that co-precipitation of the analytes with the proteins does not occur in higher amounts.

3.3. Reproducibility

With a sample volume of 100 μl, the limit of quantification was found to be 2 μg/l. The accuracy and precision data of the method, determined in one series, are shown in Table 3. It is apparent that the method meets the generally accepted requirements

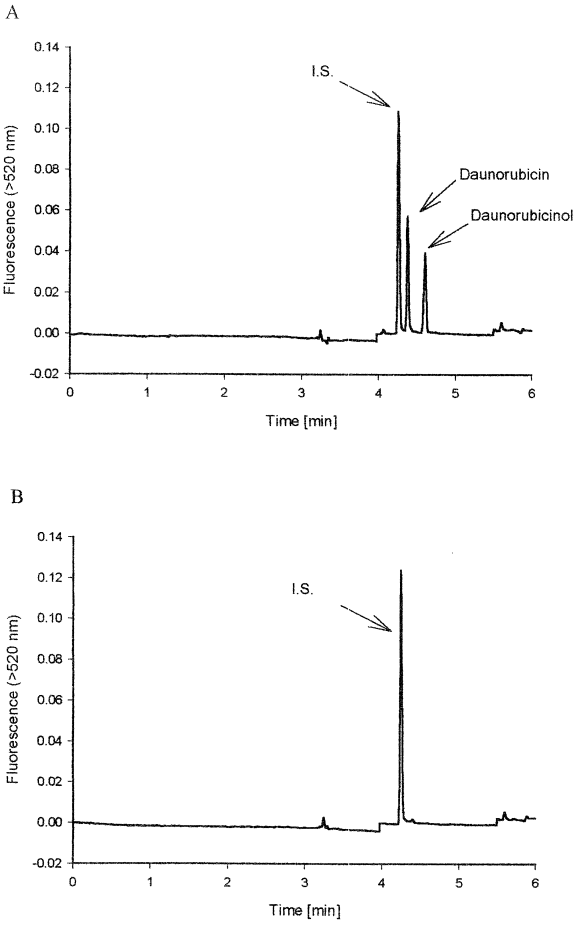

Fig. 3. (A) Electropherogram of a plasma sample containing 209 μg/l daunorubicin and 199 μg/l daunorubicinol prepared by protein precipitation with acetonitrile (peak plasma sample after administration of 30 mg/m^2 daunorubicin as a 1-h infusion). (B) Blank plasma prepared in the same manner.

for bioanalytical methods [14]. Table 4 shows the results for precision and accuracy determined on consecutive days. The method was robust over a long time period.

4. Discussion

For the determination of anthracyclines in biological fluids, several HPLC methods are described

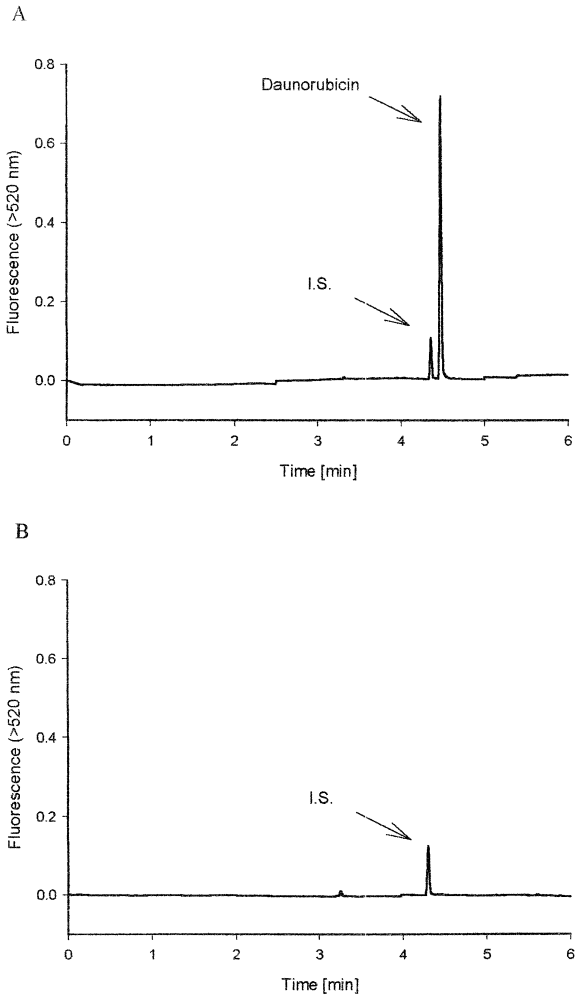

Fig. 4. (A) Typical peak plasma sample of a patient receiving 60 mg/m^2 Daunoxome as a 1-h infusion (39.4 mg/l daunorubicin). (B) Blank plasma prepared in the same manner.

Table 2
Recovery of the analytes after protein precipitation with acetonitrile

Analyte	Concentration (μg/l)	Recovery (%)	RSD (%)	*n*
Daunorubicin	40 000	79.6	8.25	5
Daunorubicin	5000	78.4	10.20	6
Daunorubicin	50	77.5	12.30	5
Daunorubicinol	200	92.1	10.88	7
Daunorubicinol	50	84.5	5.60	5

[8,13,15–17]. Due to the sensitivity to proteins and other compounds from plasma often time-consuming sample preparation procedures are necessary. In addition, HPLC methods usually require plasma volumes between 500 and 1000 μl. For pharmacokinetic studies in children, these amounts of plasma are not acceptable, especially with infants. Therefore, we investigated the potential of CE to reduce the required sample volume for the analysis of drugs in biological fluids. For the anthracyclines, a very sensitive detection system is available, as an LIF detector with an Ar ion laser emitting at 488 nm can be used to excite the analytes. The limit of quantification of 2 μg/l is sufficient for our purpose. However, for other application it can be lowered by applying a higher plasma volume than 100 μl. Another advantage is that for concentrations above 50 μg/l sample preparation can be done using simple protein precipitation with acetonitrile.

In 17 children treated with daunorubicin for acute lymphatic leukaemia we analysed the peak plasma concentrations (C_{max}) during a 1- or a 2-h infusion (Fig. 5). Substantial variability was observed in the C_{max} with relative SDs of 59% and 79% while the mean C_{max} is in accordance to pharmacokinetic parameters from the literature in adults [18].

To date, there are only a few studies describing the pharmacokinetics of daunorubicin after application of Daunoxome [19,20]. In children, we are only aware of one study with a pharmacokinetic investigation of liposomal daunorubicin [21]. We received and analysed a series of samples from children receiving Daunoxome for the treatment of relapsed acute lymphoblastic leukaemia. The total peak plasma concentrations of up to 39.4 mg/l (Fig. 4) are about two orders of magnitude higher than after administration of the free drug. Daunorubicinol was not detectable in the samples during or shortly after the end of infusion due to the high excess of daunorubicin. Currently, an alternative sample preparation procedure is developed to enable quantification of daunorubicinol in the presence of high concentrations of daunorubicin. Another question to be addressed is the importance of the amount of the free drug in plasma for the cytotoxic effect. Work is in progress to separate the liposomal encapsulated daunorubicin from the free drug in plasma samples.

Table 3
Precision and accuracy of the assay in one series (intra-day)

Concentration added (μg/l)	Mean concentration found (μg/l)	Accuracy (%)	Precision (%)	*n*
Daunorubicin				
30 000	32 060	6.9	8.0	6
5000	5200	40	6.9	6
1000	975	−2.5	12.5	6
700	712.3	1.8	5.7	6
200	206.8	3.4	5.0	6
50	51.8	3.7	8.6	8
30	30.7	2.2	8.0	8
2	1.9	−3.5	2.9	6
Daunorubicinol				
200	209.0	4.5	8.4	6
50	55.0	10.0	10.0	8
30	29.8	−0.7	?11.0	8
2	1.7	−14.5	5.7	6

Therefore, several questions need to be answered about the pharmacokinetics of liposomal daunorubicin, especially in children. For this purpose, CE offers several advantages over chromatographic methods, i.e., the smaller sample volume required and the shorter analysis time.

Acknowledgements

This work was supported by the Federal Department of Research and Technology (1EC9401). We thank Karen Kranz for her excellent technical assistance.

Table 4
Precision and accuracy of the assay on consecutive days (inter-day)

Concentration added (μg/l)	Mean concentration found (μg/l)	Accuracy (%)	Precision (%)	*n*
Daunorubicin				
30 000	34 400	14.67	3.76	10
5000	4990	−0.20	9.02	12
1000	1010	1.00	10.7	11
700	713.0	1.9	6.2	6
200	206	3.00	7.4	6
50	56.4	12.80	6.4	6
30	29.4	−2.0	6.6	8
2	1.9	−5.0	10.6	6
Daunorubicinol				
700	735.0	5.0	8.0	6
200	198.0	−1.0	6.2	6
50	52.4	4.8	9.7	8
30	29.3	−2.3	8.2	8
2	2.1	4.0	13.7	6

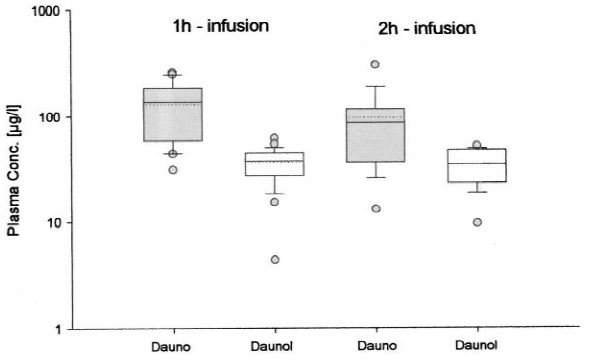

Fig. 5. Peak plasma concentrations of daunorubicin (Dauno) and daunorubicinol (Daunol) after adminstration of 30 mg/m^2 either as a 1- or 2-h infusion. Boxes represent the 15th and 75th percentiles (1-h infuion: nine patients, 2-h infusion: 12 patients).

References

[1] J. Robert, in: L.B. Grochow, M.M. Ames (Eds.), A Clinician's Guide To Chemotherapy: Pharmacokinetics and Pharmacodynamics, Williams and Wilkins, Baltimore, MD, 1998, p. 93.
[2] S.S. Bielack, R. Erttmann, B. Kempf-Bielack, K. Winkler, Eur. J. Cancer A 32 (1996) 1652.
[3] K. Shan, M. Lincoff, J.B. Young, Ann. Intern. Med. 125 (1996) 47.
[4] S.S. Legha, R.S. Benjamin, B. Mackay, M. Ewer, S. Wallace, M. Valdiviesco, Ann. Intern. Med. 96 (1982) 133.
[5] J. Shapira, M. Gotfried, M. Lishner, M. Ravid, Cancer 65 (1990) 870.
[6] E.A. Forssen, M.E. Ross, J. Liposome Res. 4 (1994) 481.
[7] R.E. Kauffman, G.L. Kearns, Clin. Pharmacokinet. 23 (1992) 10.
[8] J. De Jong, P.A. Maessen, A. Akkerdaas, S.F. Cheung, H.M. Pinedo, W.J. F Van der Vigh, J. Chromatogr. 529 (1990) 359.
[9] G. Hempel, Electrophoresis 21 (2000) 691.
[10] N.J. Reinhoud, U.R. Tjaden, H. Irth, J. van der Greef, J. Chromatogr. 574 (1992) 327.
[11] G. Hempel, S. Haberland, P. Schulze-Westhoff, G. Blaschke, J. Boos, J. Chromatogr. B 698 (1997) 287.
[12] G. Hempel, P. Schulze Westhoff, S. Flege, N. Laubrock, J. Boos, Electrophoresis 19 (1998) 2939.
[13] J.H. Bejnen, P.L. Meenhorst, R. Van Guin, M. Fromme, H. Rosing, W.J.M. Underberg, J. Pharm. Biomed. Anal. 9 (1991) 995.
[14] V.P. Shah, K. Midha, S. Dighe, I.J. Mcgilveray, J.P. Skelly, A. Yacobi et al., Eur. J. Drug Metab. Pharmacokinet. 4 (1991) 249.
[15] S. Eksborg, B. Nilsson, J. Chromatogr. 488 (1989) 427.
[16] C.M. Camaggi, P. Carisi, E. Strocchi, F. Pannuti, Cancer Chemother. Pharmacol. 30 (1992) 303.
[17] S. De Graaf, C.A. Riley-Stewart, W.E. Evans, J. Chromatogr. 491 (1989) 501.
[18] S.O. Nilsson, B. Andersson, S. Eksborg, M. Beran, H. Ehrsson, Cancer Chemother. Pharmacol. 5 (1981) 261.
[19] P.S. Gill, B.M. Espina, F. Muggia, S. Cabriales, A. Tulpule, J.A. Esplin et al., J. Clin. Oncol. 13 (1995) 996.
[20] W. Yeo, K.K. Chan, G. Mukwaya, M. Ross, W.T. Leung, S. Ho et al., Cancer Chemother. Pharmacol. 44 (1999) 124.
[21] R. Bellott, A. Auvrignon, T. Leblanc, Y. Pérel, V. Gandemer, Y. Bertrand et al., Cancer Chemother. Pharmacol. 47 (2001) 15.