Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 677–685, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00

High-performance liquid chromatographic bioanalysis of anthracycline cytostatic drugs*

J. H. BEIJNEN†‡ and W. J. M. UNDERBERG§

† Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands
§ Department of Pharmaceutical Analysis, Faculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

Abstract: In the past decade high-performance liquid chromatography (HPLC) has developed into the most powerful tool available for the analysis of anthracycline antitumour drugs in all kinds of biological matrices. The results of drug level monitoring studies have contributed significantly to a better understanding of the pharmacokinetics and metabolic disposition of these drugs. Some aspects of anthracycline HPLC analysis in plasma samples are discussed, including the chromatographic separation and detection, sampling procedures, storage of plasma samples and sample pre-treatment.

Keywords: *Anthracyclines; doxorubicin; metabolites; HPLC; bioanalysis.*

Introduction

Doxorubicin (Dx), is an anthracycline compound currently in widespread routine clinical use. It is among the most active of anticancer drugs. Its therapeutic efficiency has been proven in the treatment of a diverse array of human malignancies [1–5]. However, the drug is not devoid of serious side effects, the major dose-limiting toxicities being myelosuppression and cumulative dose-related cardiotoxicity [5–7]. Therefore, in the past years, derivatives of Dx have been synthesized in an attempt to find analogs with a more favourable therapeutic index [1, 2, 8, 9]. Several compounds have been studied in clinical trials [10–18] and as a result, 4′-epidoxorubicin (4′-epiDx) has already been registered in several countries. Structures of several important anthraquinone glycosides are shown in Fig. 1.

Anthracyclines in the body undergo extensive metabolism [19–29]. The main metabolic pathway consists of C13 carbonyl reduction mediated by cytoplasmic NADPH-dependent aldo-keto reductases yielding doxorubicinol (Dx-ol) in the case of Dx. To a minor degree glycosidases convert Dx and Dx-ol into their respective 7-OH aglycones whereas microsomal reductive deglycosidation results in the formation of 7-deoxy aglycones [19–29]. These transformations are illustrated by Fig. 2. Aglycone

*Presented at the "International Symposium on Pharmaceutical and Biomedical Analysis", September 1987, Barcelona, Spain.

‡To whom correspondence should be addressed.

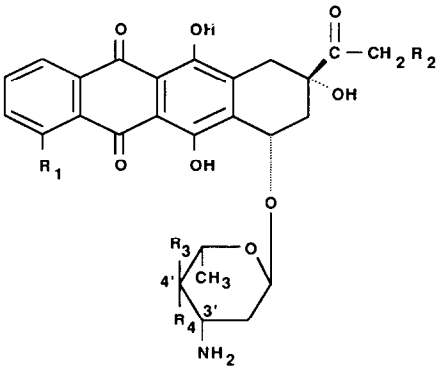

Figure 1
Structures of anthracyclines

	R_1	R_2	R_3	R_4
Doxorubicin	OCH_3	OH	H	OH
4′-Epidoxorubicin	OCH_3	OH	OH	H
4′-Deoxydoxorubicin	OCH_3	OH	H	H
4′-*O*-methyl-4-demethoxy-doxorubicin	H	OH	H	OCH_3
Daunorubicin	OCH_3	H	H	OH
4-Demethoxydaunorubicin	H	H	H	OH

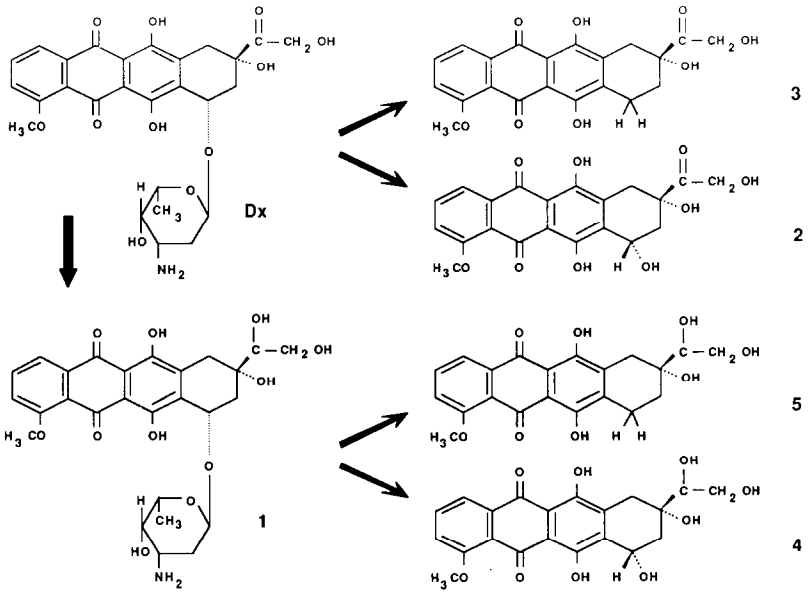

Figure 2
Metabolic routes for doxorubicin. Dx, doxorubicin; 1, doxorubicinol; 2, doxorubicinone; 3, 7-deoxydoxorubicinone; 4, doxorubicinolone; 5, 7-deoxydoxorubicinolone.

formation also can be due to hydrolytic and/or photolytic degradation [30–35]. C4 *O*-demethylation and conjugation with sulphate and glucuronic acid have been described as consecutive metabolic routes of the aglycones [19, 20, 22, 23, 36, 37]. Interestingly, in urine and plasma of patients undergoing 4′-epiDx treatment 4′-*O*-β-D-glucuronyl-4′-epiDx and 4′-*O*-β-D-glucuronyl-4′-epi-doxorubicinol have been detected [38–41]. Analogous glucuronides for related anthracyclines have never been observed, hitherto. Apparently, this unique metabolic pathway in terms of C4′-OH glucuronidation is to be correlated with the equatorial position of the C4′-hydroxyl function in 4′-epiDx and 4′-epidoxorubicinol.

C13 hydroxy metabolites retain antitumour action whilst aglycones lack this potency, however they are suspected of eliciting cardiotoxicity [1, 42, 43].

This implies that in order to get a full picture of the metabolic fate and pharmacokinetic profile of Dx and its congeners, the availability of an analytical method capable of analysing the anthraquinone glycosides as well as their aglycones is a prerequisite. Several analytical techniques have been used but, unquestionably, HPLC is the first choice [34, 44, 45]. Extensive HPLC methods have been published for Dx [34, 44–50], 4′-epiDx [38–41, 51–54], 4′-deoxydoxorubicin [51, 55, 56], 4′-*O*-methyl-4-demethoxydoxorubicin [57], daunorubicin [51, 58–60], 4-demethoxydaunorubicin [12, 51, 52, 61–63], *N*-trifluoroacetyl-doxorubicin-14-valerate (AD-32) [64], carminomycin [65], rubidazone [66], etc. [34, 44]. This paper deals with several aspects of the importance of HPLC anthracycline bioanalysis, including chromatographic separation and detection, sampling procedures, storage of samples and sample pre-treatment.

Chromatography and detection

In anthracycline analysis a tremendous number of HPLC systems have been reputed, as in recent reviews by Aszalos [45], Eksborg and Ehrsson [44] and Bouma *et al.* [34]. In reviewing the available literature, it is clear that reversed-phase (RP)-HPLC is preferred to normal phase (NP)-HPLC. The major drawback of the NP–HPLC systems, apart from the general disadvantages of NP–HPLC in comparison with RP–HPLC, is the inability of these systems to achieve separation of aglycone fractions [34, 67, 68]. Also, ion-pair (IP) chromatography and gradient elution offer no real advantages over the available simple isocratic RP–HPLC systems, except for the situation where C4-*O*-sulphates and C4-*O*-glucuronides need to be analysed [36]. In the authors' pharmacokinetic studies, the analysis of Dx, 4′-epiDx, their C13 hydroxy metabolites, 7-hydroxy- and 7-deoxy-aglycones was achieved with a simple isocratic system involving a C8 alkyl bonded silica stationary phase and a phosphoric acid/acetonitrile eluent within 20 min (Fig. 3). Simple adaptions of this system in terms of minor variations of the composition of the mobile phase appeared in most cases sufficient to obtain a system capable of analysing related anthracyclines and their metabolites. This is demonstrated here for the analysis of the new anthracycline derivative 4-demethoxydaunorubicin (4-DD), its C13 hydroxy metabolite, 7-hydroxy and 7-deoxy-aglycones (Fig. 4) [61]. Systematic studies on the chromatographic behaviour of the anthraquinone glycosides suggest that to a certain extent ion-exchange, involving the free residual silanol groups of the stationary phase, contributes to the elution process since the retention of the 3′-aminoglycosides, unlike the aglycones, changed in accordance to variations of pH or ionic strength of the mobile phase at constant water/organic modifier ratios [30, 34, 69, 70]. Changes in intramolecular hydrogen bonding which in turn change the solvation of the glycosides

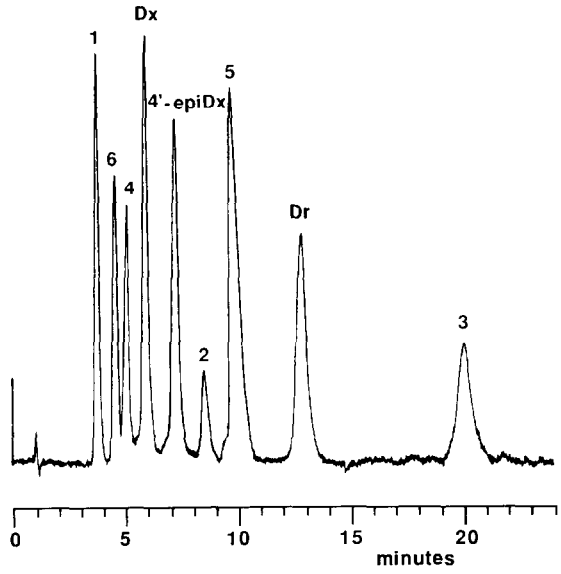

Figure 3
Chromatogram of a mixture of reference compounds. HPLC conditions: column: Lichrosorb RP-8 (5 μm) (125 × 4 mm i.d.); mobile phase: phosphate buffer, pH 2.2 + acetonitrile (80 + 20, m/m); flow: 1.5 ml/min.; fluorescence detection with excitation at 460 nm and emission at 550 nm. Dx, doxorubicin; 4′-epiDx, 4′-epidoxorubicin; Dr, daunorubicin (internal standard); 1, doxorubicinol; 2, doxorubicinone; 3, 7-deoxydoxorubicinone; 4, doxorubicinolone; 5, 7-deoxydoxorubicinolone; 6, 4′-epidoxorubicinol.

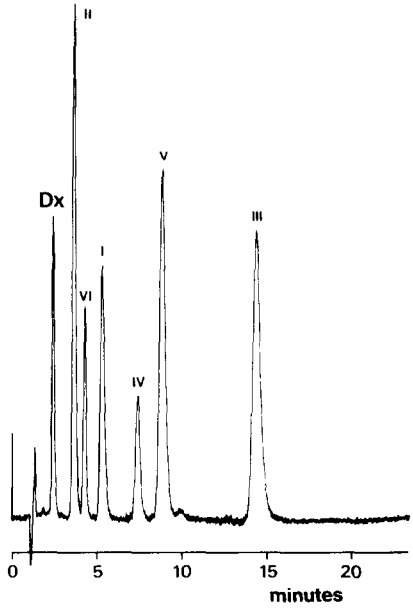

Figure 4
Chromatogram of a mixture of reference compounds. HPLC conditions: column: Lichrosorb RP-8 (5 μm) (125 × 4 mm i.d.); mobile phase: phosphate buffer, pH 2.4 + acetonitrile (70 + 30, m/m); flow: 1.5 ml min^{-1}; fluorescence detection with excitation at 460 nm and emission at 540 nm. Dx = doxorubicin (internal standard); I, 4-demethoxydaunorubicin; II, 4-demethoxydaunorubicinol; III, 7-deoxy-4-demethoxydaunorubicinone; IV, 4-demethoxydaunorubicinone; V, 7-deoxy-4-demethoxydaunorubicinolone; VI, 4-demethoxy-daunorubicinolone.

may also contribute. Probably a complex of chromatographic mechanisms is involved [34, 70].

So far, detection in anthracycline HPLC bioanalysis has involved: (i) fluorescence detection based on the native fluorescence of the compounds [34, 44]; (ii) UV–VIS detection based on the absorptivity of the anthracyclines in the UV and visible regions [34, 44]; (iii) oxidative electrochemical detection based on the oxidability of the hydroquinone moiety of the anthracyclines [34, 44, 71, 72].

From a practical point of view, fluorescence detection is to be preferred. This detection mode offers the advantages of high sensitivity, selectivity and reliability. Detection limits as low as 0.5 ng ml^{-1} plasma can be achieved [51], which is sufficient for therapeutic drug monitoring purposes. By using laser induced fluorescence detection the sensitivity is enhanced [73].

Sampling procedures

Anthracyclines have a strong tendency to adsorb onto container walls thereby causing substantial losses [34]. This phenomenon can lead to erroneously low recovery results in bioanalysis, especially with low concentration samples. However, as long as acidic solutions or organic solvents, together with polypropylene container materials are used, the extent of adsorption is negligible and will not jeopardize the analytical results. Eksborg [59] recommended the addition of desipramine to samples in order to reduce the adsorption of analytes on to glass surfaces [59].

Blood samples should be collected in polypropylene tubes containing EDTA as anticoagulant instead of heparin, due to the existence of an ionic interaction between the negatively charged mucopolysaccharide heparin and anthracycline cations at physiological pH [74, 75]. Such interactions have not been observed in the case of EDTA. Heparine interference has also been noticed recently in the bioanalysis of the anticancer anthraquinone oxantrazole [76].

After collection of the blood sample it is important to separate immediately the plasma fraction from the blood cells, since these cells concentrate the anthracyclines and convert them into the C13 hydroxy metabolites [34].

Storage of samples

Knowledge about the chemical stability of the anthracyclines is pertinent when the biological samples are stored prior to analysis [34, 77–80]. In the authors' studies, storage of Dx and 4-DD plasma and urine samples at −20°C for one month did not result in any detectable degradation. The same results have been obtained for 4′-epiDx [40] and carminomycin [65]. However, literature data show different opinions for Dx on this matter [48, 51, 81]. Blood samples containing AD-32 must be handled very specifically in order to prevent plasma-esterase catalyzed hydrolysis [82].

Anthracycline standard solutions can be stored for several weeks in 10^{-2}M phosphoric acid in the refrigerator, protected from light [80].

Sample pre-treatment

Sample pre-treatment procedures in anthracycline bioanalysis include liquid–solid extraction, protein precipitation, liquid–liquid extraction or combinations of these

procedures. Urine and bile samples may be injected directly into the chromatographic system [34, 44]. Recently, an on-line loop-extraction method has been reported for analysing Dx and metabolites in plasma [83]. Liquid–liquid extractions, however, are usually preferred by most researchers. The pH of the aqueous phase plays an important role in obtaining high recoveries [84]. The anthracycline molecule contains three prototropic functions: the 3′-amino group and the two phenolic functions. The pKa values for the amino group and the first phenolic deprotonation overlap. This implies that five different species can be distinguished, namely a mono-cation, a neutral and a zwitter ionic form, the mono-anionic form and the di-anionic form [31, 34]. For optimal extraction the compound must exist in its neutral form. The fractions of each species can be calculated from the macro-ionization constants [31, 32]. The graphic representations of such calculations for daunorubicin (pK_1 = 7.92; pK_2 = 10.09; pK_3 = 13.30 [31]) are shown in Fig. 5. From these graphs it is obvious that the pH for optimal extraction of the glycosides is 9. The extractability of the aglycones is optimal at pH values ≤9.

Many extraction schemes include a back-extraction of the glycoside from the organic extract into an acidic aqueous phase (pH = 2) [34]. This extract is then injected into the HPLC system. By using this procedure the glycosides are effectively separated from endogenous plasma components. However, the aglycones remain in the organic phase and are not analysed. Back-extraction is not necessary when HPLC with fluorescence detection is used, as demonstrated in Fig. 6. In this example a 100 μl plasma sample from a rabbit treated with 4-DD, was extracted at pH 9 with 1.0 ml chloroform/1-propanol (4:1). The extract was evaporated to dryness and the residue was reconstituted with 100 μl methanol from which 40 μl was injected into the HPLC system. Reconstitution of the residue with the mobile phase was found to be irreproducible and incomplete. Dissolution with methanol turned out to be an improvement and it was found that a maximum of 40 μl of methanol could be injected without affecting the

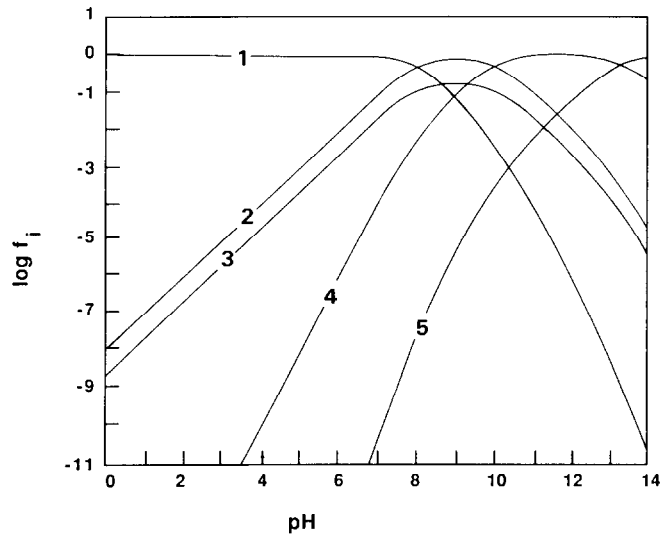

Figure 5
Logarithm of the fraction of daunorubicin species as function of pH. 1, monocation; 2, neutral species; 3, zwitter ionic species; 4, mono-anion; 5, di-anion.

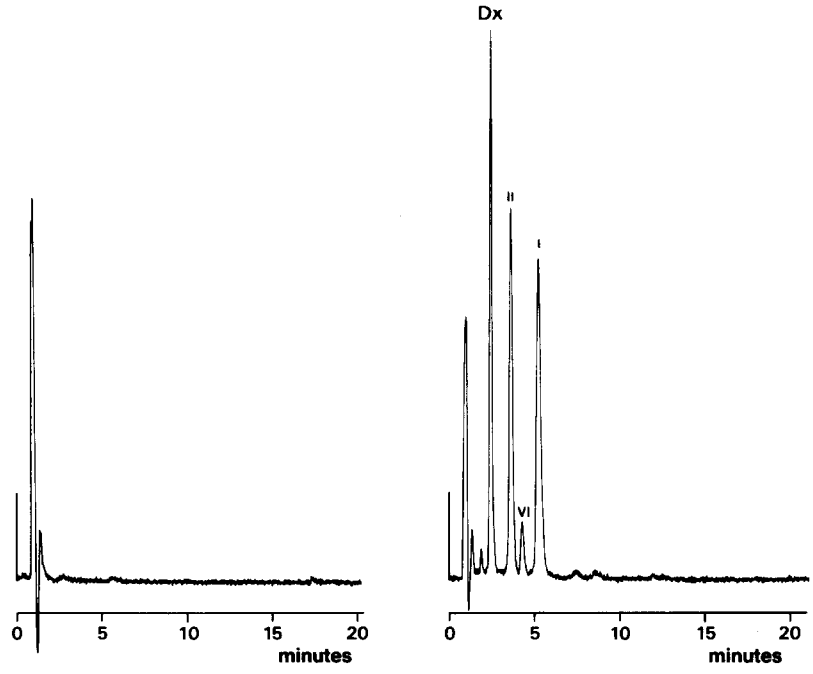

Figure 6
Chromatograms of a blank plasma sample (left) and a plasma sample (right) of treated rabbit (dose: 5.5 mg 4-demethoxydaunorubicin) at t = 20 min.

chromatography of the analytes. The applicability of the method has been demonstrated in a pharmacokinetic study of 4-DD in rabbits [61].

The solvent used for anthracycline extraction is mostly a chloroform/alcohol (propanol or pentanol or heptanol) mixture and extraction recoveries of >90% are obtainable. The presence of the alcohol appears to be crucial since the recovery yield strongly decreases when the alcohol is omitted.

Isolation of the 4′-*O*-β-D-glucuronide metabolites of 4′-epiDx and 4′-epidoxorubicinol is at an optimal when a liquid–solid extraction is employed with C18 Sep-Pak cartridges [39] rather than a liquid–liquid extraction [38, 40].

References

[1] F. Arcamone, *Doxorubicin, Anticancer Antibiotics*. Academic Press, New York (1981).
[2] C. E. Myers, in *The Cancer Chemotherapy Annual 6* (H.M. Pinedo and B.A. Chabner, Eds), pp. 58–84. Elsevier, Amsterdam (1984).
[3] S. K. Carter, *J. Natl. Cancer Inst.* **55**, 1265–1273 (1975).
[4] H. L. Davis and T. E. Davis, *Cancer Treat. Rep.* **63**, 809–815 (1979).
[5] R. C. Young, R. F. Ozols and C. E. Myers, *N. Engl. J. Med.* **305**, 139–153 (1981).
[6] J. G. Henderson and E. Frei, *Am. Heart. J.* **99**, 671–674 (1980).
[7] A. DiMarco, *Drugs Exp. Clin. Res.* **9**, 751–765 (1983).
[8] F. Arcamone, *Cancer Res.* **45**, 5995–5999 (1985).
[9] A. M. Casazza, *Cancer Treat. Rep.* **63**, 835–844 (1979).
[10] S. Kaplan, C. Sessa, Y. Willems, M. A. Pacciarini, V. Tamassia and F. Cavalli, *Invest. New Drugs* **2**, 281–286 (1984).
[11] V. Bonafante, L. Ferrari, F. Villani and G. Bonadonna, *Invest. New Drugs* **1**, 161–168 (1983).

[12] A. N. Daghestani, Z. A. Arlin, B. Leyland-Jones, T. S. Gee, S. J. Kempin, R. Mertelsmann, D. Budman, P. Schulman, R. Baratz, L. Williams, B. D. Clarkson and C. W. Young, *Cancer Res.* **45**, 1408–1412 (1985).
[13] J. Robert, P. Vrignaud, T. Nguyen-Ngoc, A. Illiadis, L. Mauriac and P. Hurteloup, *Cancer Treat. Rep.* **69**, 633–640 (1985).
[14] F. Ganzina, N. DiPietro and O. Magni, *Tumori* **71**, 233–240 (1985).
[15] G. Bonnadonna (Ed.), *Advances in Anthracycline Chemotherapy: Epirubicin*. Masson Italia Editori, Milano (1984).
[16] R. Keiling, P. P. Armand, P. Hurteloup and P. Cappelaere, *Onkologie* **9**, (Suppl. 1), 8–10 (1986).
[17] L. Ferrari, A. Rossi, C. Brambilla, V. Bonfante, F. Villani, F. Crippa and G. Bonadonna, *Invest. New Drugs* **2**, 287–295 (1984).
[18] E. Berman, R. E. Wittes, B. Leyland-Jones, E. S. Casper, R. J. Gralla, J. Howard, L. Williams, R. Baratz and C. W. Young, *Cancer Res.* **43**, 6096–6101 (1983).
[19] S. Takanashi and N. R. Bachur, *Drug Metab. Dispos.* **4**, 131–134 (1976).
[20] S. Takanashi and N. R. Bachur, *J. Pharmacol. Exp. Ther.* **195**, 41–49 (1975).
[21] N. R. Bachur, *Cancer Treat. Rep.* **63**, 817–820 (1979).
[22] M. A. Asbell, E. Schwartzbach, F. J. Bullock and D. W. Yesair, *J. Pharmacol. Exp. Ther.* **182**, 63–69 (1972).
[23] H. S. Schwartz and B. Paul, *Cancer Res.* **44**, 2480–2484 (1984).
[24] H. S. Schwartz and N. B. Parker, *Cancer Res.* **41**, 2343–2348 (1981).
[25] S. Penco, G. Cassinelli, A. Vigevani, P. Zini, G. Rivola and F. Arcamone, *Gazz. Chim. Ital.* **115**, 195–197 (1985).
[26] D. A. Gewirtz and S. Yanovich, *Biochem. Pharmacol.* **35**, 4059–4064 (1986).
[27] J. Cummings, N. Willmott, I. More, D. J. Kerr, J. G. Morrison and S. B. Kaye, *Biochem. Pharmacol.* **36**, 1521–1526 (1987).
[28] J. Cummings and R. Milroy, *Anticancer Res.* **6**, 1177–1180 (1986).
[29] J. Cummings, R. Milstead, D. Cunningham and S. Kaye, *Eur. J. Cancer Clin. Oncol.* **22**, 991–1001 (1986).
[30] J. H. Beijnen, G. Wiese and W. J. M. Underberg, *Pharm. Weekbl. Sci. Ed.* **7**, 109–116 (1985).
[31] J. H. Beijnen, O. A. G. J. van der Houwen, M. C. H. Voskuilen and W. J. M. Underberg, *Int. J. Pharm.* **31**, 75–82 (1986).
[32] J. H. Beijnen, O. A. G. J. van der Houwen and W. J. M. Underberg, *Int. J. Pharm.* **32**, 123–131 (1986).
[33] J. H. Beijnen, R. P. Potman, R. D. van Ooijen, R. J. Driebergen, M. C. H. Voskuilen, J. Renema and W. J. M. Underberg, *Int. J. Pharm.* **34**, 247–257 (1987).
[34] J. Bouma, J. H. Beijnen, A. Bult and W. J. M. Underberg, *Pharm. Weekbl. Sci. Ed.* **8**, 109–133 (1986).
[35] P. J. Gray and D. R. Phillips, *Photochem. Photobiol.* **33**, 297–303 (1981).
[36] P. A. Andrews, D. E. Brenner, F. T. Chou, H. Kubo and N. R. Bachur, *Drug Metab. Disp.* **8**, 152–156 (1980).
[37] P. E. Deesen and B. Leyland-Jones, *Drug Metab. Disp.* **12**, 9–13 (1984).
[38] H. Weenen, A. P. R. M. Osterop, S. E. J. M. van der Poort, J. Lankelma, W. J. F. van der Vijgh and H. M. Pinedo, *J. Pharm. Sci.* **75**, 1201–1204 (1986).
[39] P. A. Maessen, K. B. Mross, H. M. Pinedo and W. J. F. van der Vijgh, *J. Chromatogr.* **417**, 339–346 (1987).
[40] H. Weenen, J. Lankelma, P. G. M. Penders, J. G. McVie, W. W. Ten Bokkel Huinink, M. M. de Planque and H. M. Pinedo, *Invest. New Drugs* **1**, 59–64 (1983).
[41] H. Weenen, J. M. S. van Maanen, M. M. de Planque, J. G. McVie and H. M. Pinedo, *Eur. J. Clin. Oncol.* **20**, 919–926 (1984).
[42] M. Del Tacca, R. Danesi, M. Ducci, C. Bernardini and A. Romanini, *Pharmacol. Res. Commun.* **17**, 1073–1084 (1985).
[43] R. Mhatre, E. Herman, A. Huidoboro and V. Waravdekar, *J. Pharmacol. Exp. Ther.* **178**, 216–222 (1971).
[44] S. Eksborg and H. Ehrsson, *J. Chromatogr.* **340**, 31–72 (1985).
[45] A. Aszalos, *J. Liq. Chromatogr.* **7**(S-1), 69–125 (1984).
[46] A. M. B. Bots, W. J. van Oort, J. Noordhoek, A. van Dijk, S. W. Klein and Q. G. C. M. van Hoesel, *J. Chromatogr.* **272**, 421–427 (1983).
[47] S. Shinozawa, Y. Mimaki, Y. Araki and T. Oda, *J. Chromatogr.* **196**, 463–469 (1980).
[48] H. Ichiba, M. Morishita, T. Yajima, Y. Kawatsu, M. Kawamura, H. Komatsu and T. Ikegami, *Chem. Pharm. Bull.* **33**, 3868–3874 (1985).
[49] J. Cummings, J. G. Morrison and N. Willmott, *J. Chromatogr.* **31**, 373–384 (1986).
[50] J. M. Gallo, C. T. Hung and D. G. Perrier, *J. Pharm. Biomed. Anal.* **4**, 483–490 (1986).
[51] M. J. M. Oosterbaan, R. J. M. Dirks, T. B. Vree and E. van der Kleijn, *J. Chromatogr.* **306**, 323–332 (1984).
[52] E. Moro, V. Bellotti, M. G. Jannuzzo, S. Stegnjaich and G. Valzelli, *J. Chromatogr.* **274**, 281–287 (1983).

[53] T. V. Sweatman and M. Israel, *Cancer Chemother. Pharmacol.* **19**, 201–206 (1987).
[54] B. Leyland-Jones, P. Deesen, R. Wittes and C. W. Young, *Clin. Pharmacol. Ther.* **33**, 244 (1982).
[55] J. Cummings, *J. Chromatogr.* **341**, 401–409 (1985).
[56] Y. M. Peng, D. S. Alberts, S. E. Salmon and T. P. Davis, *Invest. New Drugs*, **2**, 277–280 (1984).
[57] F. Formelli, R. Carsana and C. Pollini, *Cancer Chemother. Pharmacol.* **16**, 15–21 (1986).
[58] P. A. J. Speth, P. C. M. Linssen, J. B. M. Boezeman, J. M. C. Wessels and C. Haanen, *J. Chromatogr.* **377**, 415–422 (1986).
[59] S. Eksborg, H. Ehrsson, B. Andersson and M. Beran, *J. Chromatogr.* **153**, 211–218 (1978).
[60] S. Eksborg, *J. Chromatogr.* **149**, 225–232 (1978).
[61] J. H. Beijnen, S. Rodenhuis, A. C. A. Paalman and W. J. M. Underberg, *Proc. Am. Assoc. Cancer Res.* **28**, 434 (1987).
[62] G. Pizzorno, F. Trave, A. Mazzoni, O. Russello and A. Nicolin, *J. Liq. Chromatogr.* **8**, 2557–2566 (1985).
[63] J. Cummings, R. Milroy, S. W. Banham and S. B. Kaye, *Cancer Chemother. Pharmacol.* **19**, 296–300 (1987).
[64] M. Israel, P. M. Wilkinson, W. J. Pegg and E. Frei, *Cancer Res.* **38**, 365–370 (1978).
[65] J. Lankelma, P. G. M. Penders, J. G. McVie, A. Levya, W. W. Ten Bokkel Huinink, M. M. de Planque and H. M. Pinedo, *Eur. J. Cancer Clin. Oncol.* **18**, 363–367 (1982).
[66] J. J. Nguyen-Huu, P. Turk and B. Diquet, *J. Chromatogr.* **380**, 455–461 (1986).
[67] S. D. Averbuch, T. T. Finkelstein, S. E. Fandrich and S. D. Reich, *J. Pharm. Sci.* **70**, 265–270 (1981).
[68] D. Londos-Gagliardi, R. Baurain, J. Robert and G. Aubel-Sadron, *Cancer Chemother. Pharmacol.* **9**, 45–48 (1982).
[69] A. Alemanni, U. Breme and A. Vigevani, *Process Biochem.* 9–12 (1982).
[70] M. A. Van Lancker, H. J. C. F. Nelis and A. P. De Leenheer, *J. Chromatogr.* **254**, 45–52 (1983).
[71] C. M. Riley, A. K. Runyan and J. Graham-Pole, *Anal. Letters* **20**, 97–116 (1987).
[72] C. M. Riley and A. K. Runyan, *J. Pharm. Biomed. Anal.* **5**, 33–43 (1987).
[73] M. J. Sepaniak and E. S. Yeung, *J. Chromatogr.* **190**, 377–383 (1980).
[74] E. Confrancesco, A. Vigo and E. Pogliani, *Thromb. Res.* **18**, 743–746 (1980).
[75] J. Cummings, J. F. B. Stuart and K. C. Calman, *J. Chromatogr.* **311**, 125–133 (1984).
[76] S. K. Frank, D. A. Mathiesen, L. R. Whitfield and M. M. Ames, *J. Chromatogr.* **419**, 225–232 (1987).
[77] A. G. Bosanquet, *Cancer Chemother. Pharmacol.* **17**, 1–10 (1986).
[78] D. M. Maniez-Devos, R. Baurain, M. Lesne and A. Trouet, *J. Pharm. Biomed. Anal.* **4**, 353–365 (1986).
[79] D. M. Maniez-Devos, R. Baurain, A. Trouet and M. Lesne, *J. Pharmacol.* **17**, 14–20 (1986).
[80] S. Eksborg and H. Ehrsson, *J. Pharm. Biomed. Anal.* **2**, 297–303 (1984).
[81] S. Eksborg, H. Ehrsson, I. Wallin and A. Lindfors, *Acta Pharm. Suec.* **18**, 215–220 (1981).
[82] B. Barbieri, R. Abbruzzi, A. Benigni, M. Rizzardini, M. G. Donelli, S. Garattini and M. Salmona, *J. Chromatogr.* **163**, 195–200 (1979).
[83] C. A. Riley, W. R. Crom and W. E. Evans, *Ther. Drug Monitor.* 7, 455–460 (1985).
[84] S. Eksborg, *J. Pharm. Sci.* **67**, 782–785 (1978).

[*Received for review 7 October 1987*]