Pergamon

0731-7085(95)01301-6

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 4/5, pp. 625–633, 1995
Elsevier Science Ltd
Printed in Great Britain
0731-7085/95 $9.50 + 0.00

A sensitive procedure for the quantitation of free and N-(2-hydroxypropyl)methacrylamide polymer-bound doxorubicin (PK1) and some of its metabolites, 13-dihydrodoxorubicin, 13-dihydrodoxorubicinone and doxorubicinone, in human plasma and urine by reversed-phase HPLC with fluorimetric detection*

D. FRAIER,†‡ E. FRIGERIO,‡ E. PIANEZZOLA,‡ M. STROLIN BENEDETTI,‡ J. CASSIDY§ and P. VASEY§

‡ *Pharmacia-Farmitalia Carlo Erba, Milan, Itlay*
§ *Cancer Research Campaign, Phase 1/11 Committee, Beatson Oncology Centre, Glasgow, UK*

Abstract: A high-performance liquid chromatographic assay has been developed and validated for the determination in plasma and urine of doxorubicin (DXR) and some of its metabolites released *in vivo* from an N-(2-hydroxypropyl)methacrylamide (HPMA) polymer containing DXR linked through its aminosugar moiety to the polymer via an oligopeptide spacer (PK1). The method also allows measurement of the DXR still bound to the polymer. Following addition of two internal standards, the free compounds were extracted twice with isopropanol–chloroform (25:75, v/v). The first extraction was performed at physiological pH and the second after buffering at pH 8.4, in order to extract the aglycones and the glycosides, respectively. Determination of total DXR (polymer-bound plus free DXR) was performed, after quantitative acid hydrolysis to release doxorubicinone from free or polymer-bound DXR, by extraction with the same solvent mixture at pH 7.4. In both cases the organic phase was evaporated to dryness; the compounds were then separated by reversed-phase high-performance liquid chromatography (HPLC) under isocratic conditions and quantitated by fluorimetric detection.

In the chromatograms all the analytes appeared to be separated at the baseline and no interference from blank human plasma and urine was observed. The suitability of the method for *in vivo* samples was checked by the analysis of plasma and urine samples obtained from a cancer patient who had received a single intravenous dose of the test compound.

Keywords: *HPMA polymer-bound doxorubicin; doxorubicin and metabolites; HPLC separation; fluorimetric detection; plasma; urine.*

Introduction

Anthracyclines constitute a class of compounds with potent antineoplastic properties and include DXR, a clinically important anticancer agent which is effective against a broad spectrum of malignancies [1]. The clinical value of anthracyclines is, however, limited by a potentially fatal cardiomyopathy which becomes clinically significant above a certain cumulative dose [2]. When anthracycline cardiotoxicity becomes clinically important, treatment with these agents must cease, irrespective of the status of the disease [3]. Numerous attempts have been made to improve the therapeutic index of DXR by modifying its mode of delivery, including attempts to optimize kinetics of drug administration [4]; alternatively a variety of drug delivery systems (liposomes [5], microspheres [6], antibodies [7], polyaminoacids [8, 9] and soluble synthetic polymers [10]) has been used.

HPMA polymer-bound DXR (code name PK1) (I) (Fig. 1) is a macromolecule (M_w about 28 000) containing DXR (about 8.5% w/w) linked through its aminosugar moiety to the polymer via an oligopeptide spacer (Gly-Phe-Leu-Gly) specially designed to permit intratumoural cleavage by lysosomal enzymes [11]. In preliminary preclinical studies I was shown

*Presented at the Fifth International Symposium on Pharmaceutical and Biomedical Analysis, Stockholm, Sweden, September 1994.

†Author to whom correspondence should be addressed.

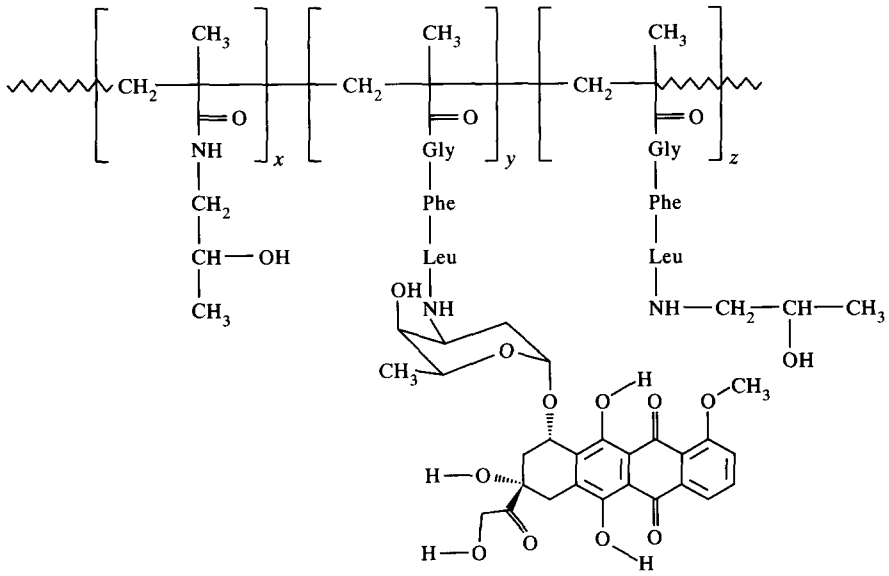

Figure 1
Structure of N-(2-hydroxypropyl)methacrylamide (HPMA) polymer-bound doxorubicin.

to display promising antitumour activity *in vivo* against L1210 leukaemia [12] and a number of solid tumour models such as M5076, P388, B16 melanoma, Walker sarcoma and the colon xenograft LS 174T, but it had lower toxicity than DXR [11, 12]. Owing to these interesting properties, I is now under clinical evaluation. Determination of free or HPMA polymer-bound DXR in tissues and plasma by HPLC has been previously investigated [11, 13, 14], but data on its bioanalytical validation are lacking.

Seymour *et al.* [13] found virtually no free drug (DXR) in plasma after administration of a single intravenous dose of I to DBA_2 mice (dose expressed as DXR equivalent was 5 mg kg^{-1}) whereas high and persistent plasma levels of I were found up to at least 24 h after dosing. In the same study, mice given an equivalent dose of free DXR showed very high levels of the compound in plasma, liver and heart compared to those obtained after a single intravenous dose of I. These findings were in good agreement with the antitumour activity and toxic effects observed in preliminary pre-clinical studies in rats and mice carried out with I, and suggested that this compound could be a suitable delivery system for DXR.

To better understand the activity of I, however, it was considered interesting to measure not only the plasma concentration of DXR but also the levels of some of its possible metabolites since some of them might be correlated with the pharmacological activity [15, 16].

In this study two groups of compounds were considered: those which could have originated from DXR when it was still bound to the polymer matrix (that is doxorubicinone and 13-dihydrodoxorubicinone); and those which could have originated from DXR after enzymic hydrolysis from the polymer (that is 13-dihydrodoxorubicin, doxorubicinone, 13-dihydrodoxorubicinone, 7-deoxydoxorubicinone (DXR-DONE) and 13-dihydro-7-deoxydoxorubicinone (DOL-DONE)) [17] (Fig. 2).

The hydrophilicity of I prevents its extraction in organic solvents. In addition, since I is only weakly fluorescent, for its sensitive quantitation it was necessary to release doxorubicinone from the polymeric moiety by acid hydrolysis. The hydrolysis indeed yielded a highly fluorescent aglycone that could be easily quantitated by HPLC [17]. A sensitive and selective HPLC method for the determination of free and polymer-bound DXR and some of its metabolites in human plasma and urine has been developed and subsequently validated.

This analytical method was used in a preliminary determination of these compounds in plasma and urine samples obtained from a cancer patient who received I in the first phase I study.

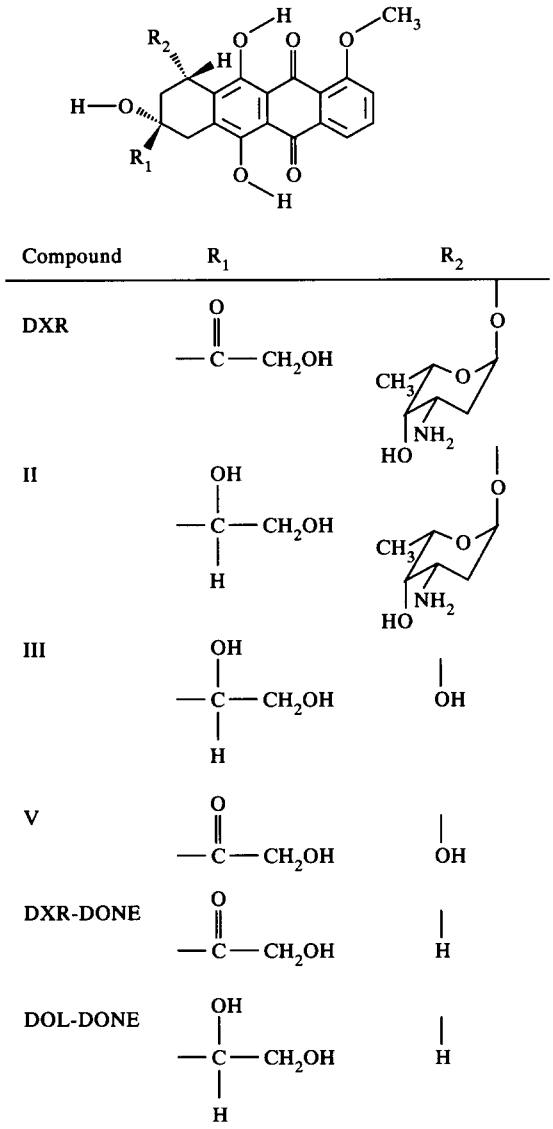

Figure 2
Structure of doxorubicin (DXR) and its metabolites 13-dihydrodoxorubicin (II), 13-dihydrodoxorubicinone (III), doxorubicione (V), 7-deoxydoxorubicinone (DXR-DONE) and 13-dihydro-7-deoxydoxorubicinone (DOL-DONE).

Experimental

Chemicals and solutions

I, 13-dihydrodoxorubicin (II), 13-dihydrodoxorubicinone (III), DXR, doxorubicinone (V) and the two internal standards daunorubicin (VI) and daunorubicinone (VII) were supplied by the Chemical Development Department of Pharmacia-Farmitalia Carlo Erba. All reference standards used in this study were at least 92% pure. DXR-DONE and DOL-DONE were not available as pure reference standards. I contained less than 1.1% of free DXR as an impurity. All other chemicals were analytical grade from Carlo Erba Reagents (Milan, Italy).

Stock solutions of the glycosides (in water) and the aglycones (in methanol) were prepared, protected from light and stored at 4°C. Under these conditions stock solutions of all analytes are stable for at least three weeks. Working solutions were prepared daily by dilution with double-distilled water or methanol.

All glassware was silanized before use by treatment with a dimethyldichlorosilane–toluene solution (7:93, v/v), followed by rinsing with ethanol, to prevent drug complexation and degradation on the active catalytic centres (free silanol groups) of the glassware.

Liquid chromatography

The HPLC system used in this study comprised a pump (model Isochrom), a refrigerated autosampler (model AS 3000) with a 200-μl loop, and a fluorescence detector (model FP 820). Data collection was carried out using a ChromJet integrator connected to a Spectra 386 computer with Winner 386 autolab software. All these instruments were supplied by Thermo Separation Products (Santa Clara, CA, USA) except the detector, which was purchased from Jasco (Hachioji, Japan). The detector was set at 480 and 560 nm (excitation and emission wavelengths, respectively) and wired to send a 1V signal to the integrator. The gain was set at ×10 and ×100. The chromatographic separation was performed on a 150 × 3.9 mm i.d. reversed-phase column packed with 4-μm Nova-Pak C18 (Waters, Milford, MA, USA) with a survival pre-column packed with pellicular 30–38 μm ODS (Whatman, Clifton, NJ, USA) wiht 15-μm pore size frits. The mobile phase was methanol–acetonitrile–phosphate buffer (pH 1.4; 0.01 M) (10:25:65, v/v/v). The separation of the analytes was performed at a flow-rate of 0.58 ml min^{-1}. Typical back-pressure was 950 lb in^{-2}.

Sample preparation

Quantitation of free DXR and metabolites. This determination involves quantitation of the free DXR released from the polymeric carrier by enzymic hydrolysis as well as that derived from the small percentage present as an impurity in the pharmaceutical preparation. The determination also involves the quanti-

tation of the free metabolites II, III and V. Owing to the different physico-chemical properties of the two classes of compounds to be determined (glycosides and aglycones) two extraction steps were performed, at physiological pH and after buffering to pH 8.4, and two different internal standards were used (VI and VII).

A 1.0 ml aliquot of the plasma or urine sample was placed in a 15-ml silanized glass stoppered test tube and, after addition of the two internal standards, extracted with 5 ml of isopropanol–chloroform (25:75, v/v) by mechanical shaking for 30 min. After centrifugation for 15 min at 1200*g* and 4°C the lower organic phase was transferred into another glass tube and evaporated under a stream of nitrogen at room temperature. The remaining aqueous phase was mixed with 2 ml of 0.05 M (for plasma) or 0.5 M (for urine) borate buffer (pH 8.4) and re-extracted as described above. The lower organic phase was separated, added to the residue of the first extraction and evaporated to dryness. The residue was dissolved in 600 μl of methanol–0.5 M H_3PO_4 (50:50, v/v) by vortex-mixing for 0.5 min. The solution obtained was washed with 2 ml of n-hexane (vortex mixer for 0.5 min) and after centrifugation the upper organic phase was discarded. The aqueous phase was then transferred into plastic vials in the autosampler and an aliquot (200 μl) was injected on to the column. For urine samples the washing step with n-hexane was omitted and the redissolved residue injected on to the column.

Quantitation of polymer-bound DXR. This determination is performed afer acid hydrolysis to release the aglycones from free and polymer-bound DXR. Aliquots of plasma or urine (from 0.1 to 1 ml according to the expected sample concentration) were placed in a 15-ml glass tube and 1 ml of 1 M HCl was added. Hydrolysis was performed at 85°C for 20 min. After cooling to room temperature, 1 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of VII (internal standard) and 1 ml of 1 M NaOH were added in sequence, each addition being immediately followed by thorough mixing of the sample. The sample was then extracted once with 5 ml of isopropanol–chloroform (25:75, v/v) and the organic phase was evaporated to dryness under nitrogen. Finally the residue was dissolved in 2 ml of methanol–0.5 M H_3PO_4 (50:50, v/v) and an aliquot (200 μl) was injected on to the column.

Bound DXR was then estimated by subtraction of free DXR from total V determined after acid hydrolysis. For this subtraction the total V concentrations were transformed into DXR equivalent concentrations. In theory, bound DXR should be estimated by subtraction of both free DXR and free V from the total V, but the small amounts of free V measured in human plasma from preliminary experiments proved that this was not strictly necessary (Fig. 4A and C).

Quantitation of quality control and calibration samples. Blank human plasma and urine samples spiked with known amounts of II, III, DXR, V and the two internal standards VI and VII (free DXR) or I and VII (bound DXR) were analysed using the above procedures. The linearity was evaluated from six calibration curves (five calibration points each) prepared and run on six different days. In plasma samples, the concentration range was 0.39–97.74 ng ml^{-1} for II, 0.54–107.5 ng ml^{-1} for III, 0.31–122 n g ml^{-1} for DXR and 0.38–150 ng ml^{-1} for V (free DXR); the range was 5.10–1020 ng ml^{-1} (low curve) and 510–204 000 ng ml^{-1} (high curve) for I (as DXR equivalent) (bound DXR). In urine samples, the concentration range was 9.78–391 ng ml^{-1} for II, 10.76–430 ng ml^{-1} for III, 12.20–488 ng ml^{-1} for DXR and 15–600 ng ml^{-1} for V (free DXR), the range was 25.50–5100 g ml^{-1} (low curve) and 510–306 000 ng ml^{-1} (high curve) for I (as DXR equivalent) (bound DXR). The precision and accuracy were evaluated by repeated analyses of all the compounds at three concentrations (low, mid and high) in three replicate samples analysed on six different days. All chromatograms obtained were evaluated by peak area measurement.

To evaluate the absolute extraction recovery, the peak area of extracted plasma samples was compared with the peak area obtained with the unextracted standard solution injected directly on to the chromatograph.

Chromatographic performance

The suitability of the chromatographic system for the analysis of the six compounds was checked on each day of the validation assay by calculating the column efficiency, the peak symmetry and the resolution factor of the

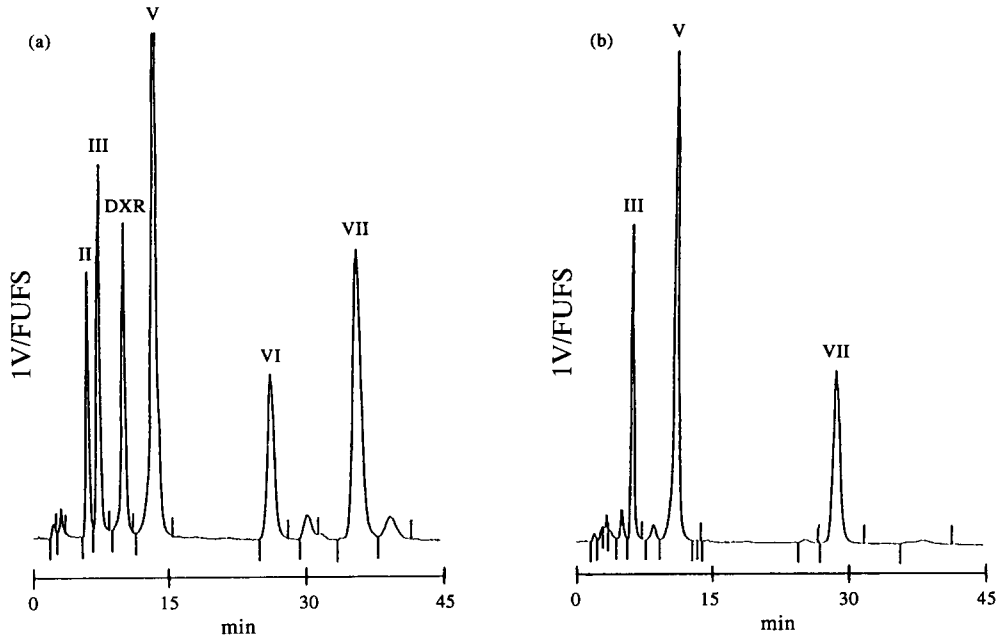

Figure 3
Chromatograms of (A) human plasma (1 ml) spiked with 48.87 ng of 13-dihydrodoxorubicin (II), 53.75 ng of 13-dihydrodoxorubicinone (III), 61 ng of doxorubicin (DXR), 75 ng of doxorubicinone (V) and with the two internal standards daunorubicin (VI) and daunorubicinone (VII) (quantitation of free DXR), and (B) human plasma (1 ml) spiked with 656 ng of 13-dihydrodoxorubicin (II), 1020 ng of HPMA polymer-bound DXR (I) and with the internal standard daunorubicinone (VII) (quantitation of bound DXR) and processed as described in the Experimental. No interfering peaks were observed in plasma and urine blanks. FUFS = fluorescence units full scale.

Table 1
Mean standard curve parameters* of doxorubicin compounds in human plasma

Compound	Linearity range (ng ml^{-1})	Regression estimates		
		Slope	*y*-intercept	Slope RSD (%)
13-Dihydrodoxorubicin	0.39–97.74	0.7417	1.6×10^{-3}	1.35
13-Dihydrodoxorubicinone	0.54–107.50	0.6279	3.2×10^{-3}	4.78
Doxorubicin	0.31–122	0.8501	1.1×10^{-3}	7.06
Doxorubicinone	0.38–150	0.8574	3.8×10^{-3}	4.67
HPMA polymer-bound DXR (low curve)	5.10–1020†	0.9054	2.6×10^{-3}	6.63
HPMA polymer-bound DXR (high curve)	510–204000†	0.9886	7.6×10^{-3}	3.03

*$n = 6$.
†As doxorubicin equivalent.

Table 2
Mean standard curve parameters* of doxorubicin compounds in human urine

Compound	Linearity range (ng ml^{-1})	Regression estimates		
		Slope	*y*-intercept	Slope RSD (%)
13-Dihydrodoxorubicin	9.78–390.96	0.3829	-1.01×10^{-2}	10.45
13-Dihydrodoxorubicinone	10.76–430	0.5080	1.38×10^{-2}	13.78
Doxorubicin	12.20–488	0.4069	-1.70×10^{-2}	7.37
Doxorubicinone	15–600	0.4465	-1.49×10^{-2}	8.96
HPMA polymer-bound DXR (low curve)	25.50–5100†	0.8679	4.51×10^{-3}	4.60
HPMA polymer-bound DXR (high curve)	510–306000†	0.9213	-3.45×10^{-2}	22.80

*$n = 6$.
†As doxorubicin equivalent.

Table 3
Accuracy and precision of the method* for the determination of doxorubicin compounds in human plasma

Compound	Concentration range (ng ml^{-1})	Accuracy		Precision	
		% Recovery range (intra-day)	Pooled % recovery range (inter-day)	% RSD (intra-day)	Pooled % RSD range (inter-day)
13-Dihydrodoxorubicin	1.17–61.09	89.30–109.87	96.58–102.64	<13.95	2.95–10.62
13-Dihydrodoxorubicinone	1.61–69.09	77.50–112.43	81.56–97.12	<21.53	5.20–13.90
Doxorubicin	0.92–76.25	83.83–115.67	88.11–106.23	<16.67	7.77–10.79
Doxorubicinone	1.13–93.75	94.30–108.20	98.51–105.96	<9.43	4.41–6.37
HPMA polymer-bound DXR	51–81600†	79.00–108.61	88.33–102.03	<15.76	6.34–10.72

*6 days; 3 concentrations in triplicate.
†As doxorubicin equivalent.

Table 4
Accuracy and precision of the method* for the determination of doxorubicin compounds in human urine

Compound	Concentration range (ng ml^{-1})	Accuracy		Precision	
		% Recovery range (intra-day)	Pooled % recovery range (inter-day)	% RSD (intra-day)	Pooled % RSD range (inter-day)
13-Dihydrodoxorubicin	19.54–244.36	87.83–106.43	96.46–98.45	<9.95	6.45–7.57
13-Dihydrodoxorubicinone	21.75–276.36	80.03–112.73	91.63–92.98	<14.86	7.82–14.05
Doxorubicin	24.40–305	91.63–114.37	100.97–104.74	<16.59	5.59–8.89
Doxorubicinone	30–375	88.77–111.67	100.66–106.37	<13.32	6.10–8.65
HPMA polymer-bound DXR	51–81600†	90.00–116.83	105.80–108.72	<15.38	6.01–13.96

*6 days; 3 concentrations in triplicate.
†As doxorubicin equivalent.

peaks of the six analytes. This evaluation was carried out according to the USP [18] using the System Suitability Test software supplied by Thermo Separation Products. The column efficiency was expressed as the number of theoretical plates. This value must be higher than 1500 for all the analytes. The tailing factor must be less than 1.7 for all the compounds. The resolution factor between the analyte peaks must be ≥ 1.

Results and Discussion

The chromatogram obtained from a standard solution of the analytes shows that all the compounds are fully resolved. The resolution factor between the six compounds was better than 1.95 and the tailing factor was better than 1.7. The parameters are characteristic of the day-to-day performance of the separation and can be used to evaluate column ageing or

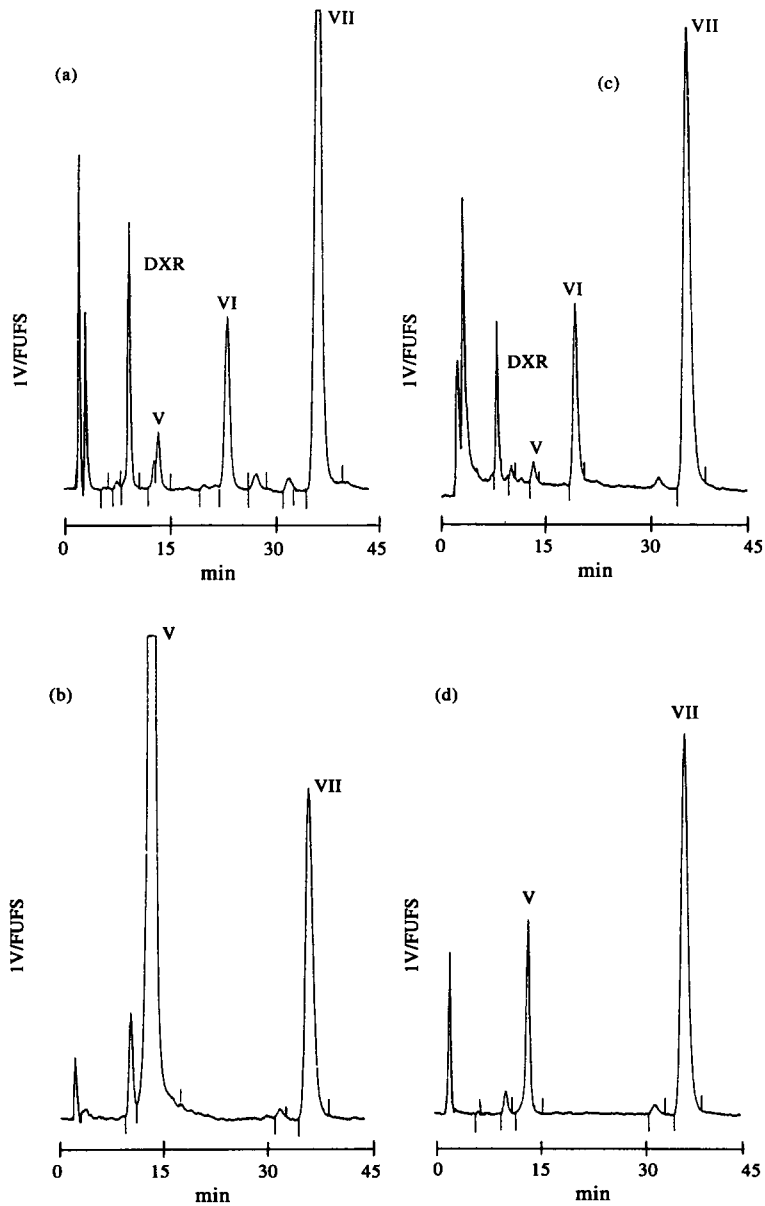

Figure 4
Typical chromatograms obtained from a patient who had received a single dose of 20 mg m^{-2} I (as DXR equivalent) as a short intravenous infusion: (A) and (B) plasma samples (0.5 and 0.25 ml) at 5 min after the end of the infusion (quantitation of free and bound DXR); (C) and (D) urine samples (0.1 ml) at 0–8 h after administration (quantitation of free and bound DXR). FUFS = fluorescence units full scale.

conditions and proper mobile phase preparation. The day-to-day reproducibility of the analytical conditions was good. The retention times of II, III, DXR, V, VI and VII were about 5, 7, 9, 13, 25 and 35 min, respectively. The overall specificity of the assay for free and total DXR in plasma and urine, resulting from the combination of the extraction step and the selectivity of chromatographic separation and fluorimetric detection, enabled chromatograms free of interference to be obtained in the assay of blank human plasma and urine. In addition, this assay procedure can also be applied to mouse, rat, dog and monkey plasma and urine since the relative chromatograms obtained from these species were free from interfering peaks at the retention times of the compounds of interest.

The linearity of this HPLC assay was evaluated from six separate calibration curves carried out on different days in the concentration ranges mentioned above. Two typical chromatograms obtained are shown in Fig. 3(A) and (B). The data were analysed by weighted ($1/y^2$) least squares linear regression of the peak-area ratio (peak area of each analyte/peak area of internal standard) versus the concentration ratio (concentration of each analyte/internal standard concentration). Correlation coefficients (r) for the regression were always better than 0.99. The mean calibration curves obtained in plasma and urine are summarized in Tables 1 and 2. Results for the intra-day and inter-day precision and accuracy obtained in plasma and urine are shown in Tables 3 and 4. The mean (n = 4 or 6) extraction recovery from plasma calculated for each compound at three concentrations was better than 72.1% for II, 89.4% for III, 67.8% for DXR and 81.4% for V (free DXR) and better than 78.6% for I (bound DXR). In plasma, the limit of quantitation was 0.39 for II, 0.54 for III, 0.31 for DXR and 0.38 ng ml^{-1} for V (free DXR) and 5.10 ng ml^{-1} for I (bound DXR). In urine, the corresponding value was 9.78 for II, 10.76 for III, 12.20 for DXR and 15.00 ng ml^{-1} for V (free DXR) and 25.50 ng ml^{-1} for I (bound DXR). At these concentrations the signal-to-noise ratio was higher than 5 and the RSD for replicate analyses (n = 6) was less than 16.67% for all compounds. The method was applied to the determination of plasma and urinary levels of I and its possible metabolites in a cancer patient who had received a single dose of 20 mg m^{-2} I (as DXR equivalent) as a short intravenous infusion. Typical chromatograms of plasma and urine samples obtained from this patient at 5 min after the end of the infusion and at 0–8 h after administration are shown in Fig. 4.

Conclusions

The proposed method is sensitive and selective for the determination of I, DXR and some of its possible metabolites in plasma and urine. It has been shown to be linear, precise and capable of accurately quantifying all the analytes. This method is the first analytical methodology validated for these compounds and can be very useful in clinical pharmacological studies attempting to correlate plasma concentrations with the clinical response and/or the toxic effects observed.

Acknowledgements — The authors wish to thank L. Magné and S. Cozzi for typing the manuscript.

References

[1] R.C. Young, R.F. Ozols and C.E. Myers, *N. Eng. J. Med.* **305**, 139–153 (1981).
[2] D.D. Von Hoff, M.W. Layard, P. Basa, H.L. Davis, A.L. Von Hoff, M. Rozencweig and F.M. Muggia, *Ann. Intern. Med.* **91**, 710–717 (1979).
[3] E. Saltiel and W. McGuire, *Western J. Med.* **139**, 332–341 (1983).
[4] J. Robert, *Cancer Drug Delivery* **4**, 191–199 (1987).
[5] A. Rahman, A. Fumagalli, B. Barbieri, P. Schein and A.M. Casazza, *Cancer Chemother. Pharmacol.* **16**, 22–27 (1986).
[6] N. Willmott, J. Cummings, J.F.B. Stuart and A.T. Florence, *Biopharm. Drug Dispos.* **6**, 91–104 (1985).
[7] R.O. Dillman, D.L. Shawler, D.E. Johnson, D.L. Meyer, J.A. Koziol and J.M. Frinkle, *Cancer Res.* **46**, 4886–4891 (1986).
[8] W.A.R. van Heeswijk, C.J.T. Hoes, T. Stoffer, M.J.D. Eenink, W. Potman and J. Feijen, *J. Cont. Rel.* **1**, 301–315 (1985).
[9] G. Pratesi, G. Sari, G. Pezzoni, O. Bellini, S. Penco, S. Tinelli and F. Zunino, *Br. J. Cancer* **52**, 541–548 (1985).
[10] F. Zunino, G. Pratesi and A. Micheloni, *J. Cont. Rel.* **10**, 65–74 (1989).
[11] R. Duncan, L.W. Seymour, K.B. O'Hare, P.A. Flanagan, S. Wedge, I.C. Hume, K. Ulbricht, J. Strohalm, V. Subr, F. Spreafico, M. Trandi, M. Ripamonti, M. Farao and A. Suarato, *J. Cont. Rel.* **19**, 331–346 (1992).
[12] R. Duncan, I.C. Hume, P. Kopeckova, K. Ulbrich, J. Strohalm and J. Kopecek, *J. Cont. Rel.* **10**, 51–63 (1989).
[13] L.W. Seymour, K. Ulbrich, J. Strohalm, J. Kopecek and R. Duncan, *Biochem. Pharmacol.* **39**, 1125–1131 (1984).
[14] S. Wedge, PhD Thesis, Keele University, UK (1992).
[15] B. Schott and J. Robert, *Biochem. Pharmacol.* **38**, 4069–4074 (1989).

[16] T. Bordoni, B. Barbieri, C. Geroni, G. Cassinelli, M. Grandi and F.C. Giuliani, 7th International Symposium on Future Trends in Chemotherapy, Pisa, May 1986, Abstract Book p. 107.
[17] J. Cummings, J.F.B. Stuart and K.C. Calman, *J. Chromatogr.* **311**, 125–133 (1984).
[18] *U.S. Pharmacopeia XXII*, p. 1566. U.S. Pharmacopeial Convention Inc., Rockville, M.D. (1990).

[Received for review 21 September 1994; revised manuscript received 29 November 1994]