Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 4, pp. 309–314, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00
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Short Communication

Analysis and pharmacokinetics of a new prodrug *N-l*-leucyldoxorubicin and its metabolites in plasma using HPLC with fluorescence detection

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Keywords: *Prodrug; anthracyclines; metabolism; pharmacokinetics; solid-phase extraction.*

Introduction

Anthracyclines, with doxorubicin (Dox) as their main representative, have proven their importance in cancer chemotherapy. Cardiotoxicity, however, is dose limiting on prolonged treatment [1]. Many analogues have been developed in the past to diminish this major side effect [2].

An alternative way to improve the therapeutic index of a drug is the administration of a prodrug, from which the active compound is more selectively released at the target organ. To this end, amino acid derivatives of anthracyclines were synthesized [3]. It is assumed that they become active upon hydrolytic release of the core anthracycline by tissular peptidases, such as cathepsins, which have been demonstrated in tumour tissue [4, 5]. The *l*-leucine derivative of Dox (see Table 1 for structural formulae) emerged as the most promising analogue. In rabbits and mice it was shown that the area under the concentration vs time curve of Dox in heart tissue was lower after administration of the prodrug than after administration of an equimolar dose of Dox itself [6, 7]. Accordingly, a diminished cardiotoxicity was observed in rabbits [6]. Combined with a higher antitumour effect after administration of an equitoxic dose of *N-l*-leucyldoxorubicin (Leu-Dox) as compared to Dox, as found in preclinical studies, an improved therapeutic index is also expected in patients.

During the clinical Phase I study of Leu-Dox, plasma pharmacokinetics of Leu-Dox and its metabolites are being studied [8]. Because Dox is formed *in vivo* from Leu-Dox, it was expected that all known metabolites of Dox also would be present. Most important in that respect is the 13-dihydro derivative doxorubicinol (Dol) [9]. Analogous to Dox, Leu-Dox can first be metabolized to leucyldoxorubicinol (Leu-Dol) before it is hydrolysed to Dol. The purpose of the present study was to improve and optimize our isocratic HPLC procedure for Dox and its metabolites [10] to include the two new compounds Leu-Dox and Leu-Dol. The modified assay was applied to plasma samples of a patient who received Leu-Dox at two dose levels.

Experimental

Materials

N-l-leucyldoxorubicin (Leurubicine®) and *N-l*-leucyldoxorubicinol were kindly provided by Medgenix Group (Fleurus, Belgium); all other anthracyclines by Farmitalia Carlo Erba (Milan, Italy). Acetonitrile, chloroform, sodium dihydrogen phosphate and orthophosphoric acid were obtained from Merck (Amsterdam, The Netherlands) and methanol

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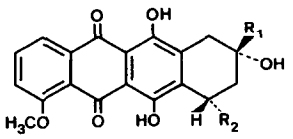
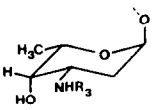
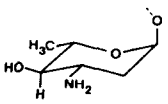
Table 1
Structures of test compounds



Compound	Abbreviation	R_1	R_2	
N-l-leucyldoxorubicin	Leu-Dox	$COCH_2OH$		*l*-leucyl $COCH(NH_2)CH_2CH(CH_3)_2$
N-l-leucyldoxorubicinol	Leu-Dol	$CH(OH)CH_2OH$		*l*-leucyl
Doxorubicin	Dox	$COCH_2OH$		H
Doxorubicinol	Dol	$CH(OH)CH_2OH$		H
Doxorubicin aglycon	Doxon	$COCH_2OH$	OH	
Doxorubicinol algycon	Dolon	$CH(OH)CH_2OH$	OH	
7-deoxydoxorubicin aglycon	7*d*-Doxon	$COCH_2OH$	H	
7-deoxydoxorubicinol aglycon	7*d*-Dolon	$CH(OH)CH_2OH$	H	
4′-Epidoxorubicin (I.S.)	Epi-Dox	$COCH_2OH$		

from Baker (Deventer, The Netherlands); triethylamine from Pierce Chemical Co. (Rockford, IL, USA). All reagents were of analytical grade.

Separate stock solutions of Leu-Dox and metabolites, prepared in methanol at 10^{-4} M, were combined in order to obtain an equimolar mixture of the compounds. From this mixture further dilutions, ranging from 1.0×10^{-8} M to 2.5×10^{-6} M, were prepared in methanol and stored at −20°C.

Before use, C_{18} Sep-Pak® cartridges (Waters–Millipore, Etten-Leur, The Netherlands) were equilibrated with 5 ml of methanol and 5 ml of water according to the manufacturer's instructions. Polypropylene tubes and vials were used throughout to minimize adsorption of anthracyclines.

Blood samples were obtained before, at 0, 5, 10, 15, 30 min and 1, 2, 4, 6, 9, 12, 24 and 48 h after infusion of Leu-Dox using polypropylene heparin-coated Sarstedt Monovette® tubes. The tubes were put on ice and centrifuged (10 min at 3000*g*) with 1 h of collection. Thereafter, plasma was stored at −20°C until analysis.

Analysis

Samples were put in an ultrasonic bath for 10 min to allow thawing and (partial) redissolution of particulate matter. After vortexing, the protein precipitate was spun down (10 min at 3000*g*) and 1.0 ml of the supernatant was added to 50 µl 1.0×10^{-6} M of 4′-epidoxorubicin (internal standard solution, I.S.). Following 15 min of vortexing, the anthracyclines were extracted from plasma using C_{18} Sep-Pak cartridges according to the procedure outlined in ref. 11. Briefly, the activated Sep-Pak was eluted with 2 ml of 20 mM NaH_2PO_4 (pH 4)–acetonitrile (9:1, v/v), loaded with 1.0 ml plasma provided with I.S., washed with another 2 ml of the buffer–acetonitrile mixture and dried with compressed air. Anthracyclines were desorbed from the Sep-Pak using 4 ml chloroform–methanol (1:3, v/v). The eluate was evaporated at 50°C under a stream of nitrogen. The residue was dissolved in 200 µl of buffer (20 mM NaH_2PO_4 (pH 2.5)–acetonitrile (4:1, v/v)) and transferred to an autosampler vial. The vials were centrifuged for 5 min at 10,000*g* to precipitate particulate matter. Of the supernatant, 60 µl was injected onto the HPLC. Calibration samples were prepared by evaporating methanol from 100 µl of each stock solution (50°C, N_2) and adding 50 µl of I.S. solution and 1.0 ml of blank heparin plasma to each residue. Further processing took place as described above. Calibration lines were constructed by plotting peak height ratios of Leu-Dox or its meta-

bolites to the I.S. against the known concentrations in plasma. Unknown anthracycline concentrations were determined from the calibration line by interpolation.

Chromatography

The HPLC-system consisted of an Applied Biosystems Spectroflow 400 pump (Separations Analytical Instruments, H.I. Ambacht, The Netherlands) and a Gilson 232-401 autosampler fitted with a 100 μl injection loop (Meyvis, Bergen op Zoom, The Netherlands). A Merck-Hitachi (Amsterdam, The Netherlands) F1000 fluorescence detector, set at λ_{ex} = 480 nm and λ_{em} = 580 nm, was used for peak monitoring. Data processing was performed with a Jones Chromatography JCL 6000 data system (Meyvis) operated on an Olivetti M240 personal computer (Olivetti, Rotterdam, The Netherlands). A 3 μm Microspher C_{18} analytical column (Chrompack, Middelburg, The Netherlands) 200 (2 × 100) × 4.6 mm (i.d.) was used together with a 10 × 2.0 mm (i.d.) Chromsep (Chrompack) reversed-phase guard column. The mobile phase, 0.5 mM triethylamine in 13 mM NaH_2PO_4 (pH 4)–acetonitrile (2:1, v/v), was passed over a 0.45 μm filter before use and delivered at a flow-rate of 1.0 ml min^{-1}.

Pharmacokinetics

Areas under the plasma concentration vs time curve (AUCs) (up to 48 h) were determined for each anthracycline using the trapezoidal rule, and half-life times of elimination were calculated by linear least-squares fitting of the final plasma concentrations (>8 h when possible).

Results and Discussion

Analysis

Figure 1(A) shows a chromatogram obtained after injecting an extract from plasma spiked with 50 nM of the test compounds. It demonstrates the base-line separation of Leu-Dox and six metabolites. Under the isocratic conditions used, all compounds elute within 15 min. The applicability to real samples is illustrated in Fig. 1(B), which shows the chromatogram of an extract from plasma obtained from a patient at 1 h after receiving Leu-Dox at 100 mg m^{-2}. The accompanying blank chromatogram, which was obtained after processing plasma of the same patient sampled before drug administration, clearly demonstrates the absence of interferences after our extraction procedure. The presence of a few unidentifiable peaks in the 1 h sample indicates the existence of one or more unknown metabolites.

Our plasma work-up procedure routinely used in the past for anthracycline analysis [10], needed a few adaptations to include Leu-Dox and Leu-Dol. Occasionally, it was observed that the sample solvent affected the HPLC separation. Therefore, redissolution of the residue obtained after evaporation of the Sep-Pak eluate was further investigated. As expected, the amount of organic modifier proved critical. The same or a higher percentage of acetonitrile in the sample as com-

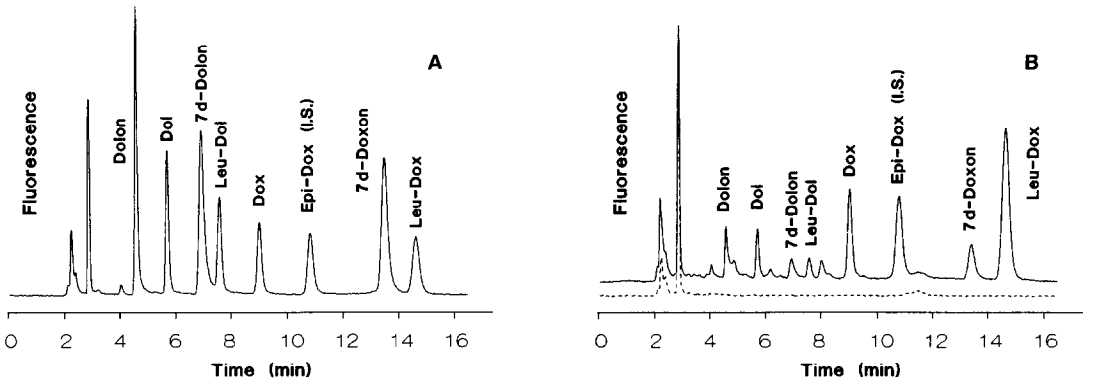


Figure 1
(A) Chromatogram of a human plasma extract, spiked with 5.0 × 10^{-8} M of each anthracycline. (B) Chromatograms of human plasma obtained from a patient before (---) and 1 h after (———) administration of leucyldoxorubicin (100 mg m^{-2}). [I.S.] = 5.0 × 10^{-8} M. Column: Microspher C_{18} analytical column (200 (2 × 100) × 4.6 mm i.d.) fitted with a Chromsep reversed-phase guard column (10 × 2.0 mm i.d.). Mobile phase: 0.5 mM triethylamine in 13 mM NaH_2PO_4 (pH 4)–acetonitrile (2:1, v/v). Flow-rate: 1.0 ml min^{-1}. Detection: fluorescence with λ_{ex} = 480 nm and λ_{em} = 580 nm. For peak identification, see Table 1.

pared to the mobile phase sometimes caused peak broadening, while too little acetonitrile in the sample (≤10%, v/v) produced solubility problems (resulting in a reduced repeatability). Increasing this percentage from 10 to 20% appeared optimal. Another important aspect of the sample solvent was the buffer pH. The use of a 20 mM phosphate buffer–acetonitrile mixture with the buffer pH adjusted to 4 (from ref. 10) occasionally resulted in a deterioration of the peak shape. This problem could be eliminated by lowering the pH to 2.5. At this pH, buffering of the sample is more adequate since the pK_a of H_3PO_4–H_2PO_4 is also about 2.5.

With respect to the mobile phase, triethylamine was added in order to improve peak shape and control retention behaviour. As can be seen from Table 1, part of the compounds contain an amino-group, and are thus prone to interaction with residual silanol groups on the stationary phase. This was only partly counteracted by 0.5 mM triethylamine. Nevertheless, retention times remained reproducible over months. Increasing the phosphate concentration had the same effect, perhaps because ion-pairs are formed with a reduced silanol interaction. Neither triethylamine nor phosphate affected the retention behaviour of the three aglycons, thus offering the possibility to 'tune' the separation.

As the number of compounds put severe demands on the separation efficiency, it appeared impossible to obtain acceptable resolution between 7*d*-Dolon and Doxon. From Dox pharmacokinetics, studied in the past [9], the latter was known to be only a minor metabolite. Therefore, it was decided not to include Doxon in the metabolite mix.

Since the present method had been validated for Dox, Epi-Dox and their metabolites [10], only a few key analytical data were determined for Leu-Dox and Leu-Dol, i.e. recovery, linearity, and detection limits. Recoveries for Leu-Dox and Leu-Dol (at 10 mM) were 74 ± 2 and 80 ± 2%, respectively. These values were higher than those obtained for Dox (63 ± 8% at 12.5 nM [10]). In Table 2 the slopes, intercepts and r^2-values of the calibration lines for the peak height ratio vs the concentration are presented for all seven anthracyclines. In all cases, r^2 was better than 0.998. Intercepts were always less than the ratio determined at the lowest calibration concentration, indicating no measurable deviations from linearity. However, the highest concentration of the aglycons (2.5×10^{-7} M) was omitted from the calculations since a considerable decrease of the slope became apparent when this value was included. This phenomenon has also been observed earlier [10] and can probably be attributed to the formation of molecular aggregates (oligomers) [12]. Since aglycon plasma concentrations are always in the nanomolar range, this observation does not hamper the pharmacokinetic analysis of the aglycons.

The requirement of calibration lines for each individual compound is obvious from the large inter-compound variation in the slopes of the calibration lines. These dissimilarities are principally caused by differences in fluorescence quantum yield, retention time, and recovery between the compounds. The detection limits (at a signal to noise ratio of 3) ranged from 0.2 nM for Dolon to 1.3 nM for Leu-Dox (Table 2), thus allowing pharmaco-

Table 2
Calibration lines and detection limits for Leu-Dox and metabolites

Compound	Calibration line*		r^2	Limit of detection† (nM)
	Slope	Intercept		
Leu-Dox	2.089	−0.081	0.9983	1.3
Leu-Dol	3.205	−0.072	0.9993	0.7
Dox	2.660	−0.085	0.9991	1.0
Dol	5.078	−0.142	0.9995	0.6
Dolon	8.997	−0.004	1.0000	0.2
7*d*-Doxon	3.961	0.032	0.9998	0.4
7*d*-Dolon	5.177	0.004	1.0000	0.4

*Linear least-squares fit of the peak height ratio (anthracycline/I.S.) vs concentration in the range of 1.0×10^{-9} M to 2.5×10^{-7} M, except for aglycons (range 1.0×10^{-9} M to 1.0×10^{-7} M).

†Determined at S/N = 3.

kinetic studies of Leu-Dox at the lowest dose level (18 mg m^{-2}).

Pharmacokinetics

Figure 2 shows the plasma concentration–time curves for all seven anthracyclines from a patient who received 100 mg m^{-2} Leu-Dox as a 5 min i.v. bolus injection. The plasma concentration of Dox reached a maximum at 10 min after the end of the injection. The concentration of Dox at $t = 0$ was only slightly lower than the peak value. Three possible explanations were apparent: firstly, the formation of Dox from Leu-Dox during the injection time was considerable, secondly, Dox was already present in the injection fluid and/or thirdly, the possible formation of Dox from Leu-Dox in the blood after sampling (R. Baurain, personal communication). HPLC analysis of the injection fluid showed that ≈0.5–1% of the fluorescence was accounted for by Dox.

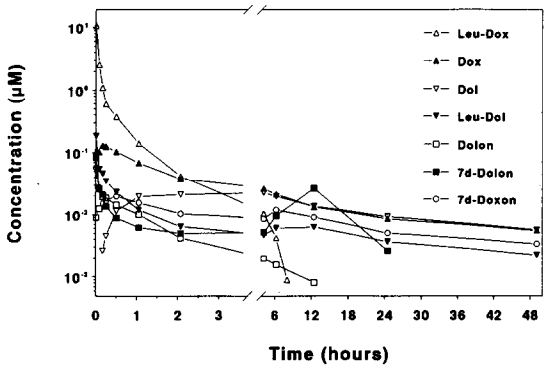


Figure 2
Plasma concentration vs time curves of Leu-Dox and six metabolites during 48 h in a patient who received 100 mg m^{-2} Leu-Dox by i.v. bolus. For abbreviations, see Table 1.

Table 3
Areas under the plasma concentration–time curve (0–48 h) for Leu-Dox (100 mg m^{-2}) and Dox (50 mg m^{-2}) including their metabolites

Metabolite	Leu-Dox (n = 1) (μM min) 100 mg m^{-2}†	Dox (n = 7)* (μM min) 50 mg m^{-2}
Leu-Dox	100.5 (60.0)	
Leu-Dol	14.4 (8.6)	
Dox	42.7 (25.5)	132.5 ± 26.0
Dol	34.6 (20.7)	45.6 ± 15.6
Dolon	2.5 (1.5)	1.8 ± 1.9
7*d*-Doxon	18.9 (11.3)	5.9 ± 3.1
7*d*-Dolon	24.2 (14.4)	18.4 ± 8.8

*AUCs of Dox are taken from ref. 9.
†Values between parentheses are normalized on a molar base to the dose of Dox (86.2 μmol m^{-2} or 50 mg m^{-2}).

The areas under the plasma concentration vs time curves (AUCs) of Leu-Dox and its metabolites are presented in Table 3 and compared with the already available data obtained after administration of Dox (from ref. 9). Assuming linear pharmacokinetics of Leu-Dox and its metabolites, the AUC values obtained after the administration of Leu-Dox were normalized to the dose of Dox on a molar basis (86 μmol m^{-2}) to allow a valid comparison between both drugs. The normalized AUC for Dox was much lower after the administration of Leu-Dox than after Dox (≈26 vs 133 μM min). If Leu-Dox itself is a genuine prodrug and thus not toxic, the figures obtained in this patient would suggest that the maximum tolerated dose of Leu-Dox (on a molar base) will be five times higher than that of Dox. Another advantage of Leu-Dox is that high peak concentrations of Dox are avoided, which may contribute to a reduction of toxicity.

Elimination of Leu-Dox was very fast, as illustrated by the final half-life time of 1.1 h vs 38.8 h for Dox. The terminal half-life of Dox after Leu-Dox administration is comparable with that after Dox (28.3 h in ref. 9).

Conclusions

Using this assay Leu-Dox and all its metabolites can be determined in a single isocratic run within 15 min. The sensitivity allows the quantification of Leu-Dox and all its known metabolites at the lowest administered dose (18 mg m^{-2}).

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[*Received for review 8 May 1991; revised manuscript received 23 July 1991*]