Journal of Chromatography, 574 (1992) 273–281
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6172

Fully automated determination of a new anthracycline N-*l*-leucyldoxorubicin and six metabolites in plasma by high-performance liquid chromatography with on-line sample handling

J. de Jong*, C. N. Munniksma and W. S. Guérand

Department of Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam (Netherlands)

A. Bast

Department of Pharmacochemistry, Faculty of Chemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam (Netherlands)

W. J. F. van der Vijgh

Department of Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam (Netherlands)

(Received September 19th, 1991)

ABSTRACT

N-*l*-Leucyldoxorubicin (Leu-Dox) was developed as a prodrug of doxorubicin (Dox) in order to diminish the cardiotoxic side-effect associated with repeated anthracycline treatment. To study the pharmacokinetics of Leu-Dox, Dox and other metabolites a sensitive and selective assay was needed. Leu-Dox and six of its known metabolites were extracted from plasma using an in-line reversed-phase precolumn (40–50 μm C_8 particles). The trapped analytes were subsequently flushed to the analytical column (3 μm C_{18}) using 0.5 ml of phosphate buffer (pH 3.5)–acetonitrile (2:1, v/v), which also served as the isocratic mobile phase. Within 12 min, a clean baseline-resolved chromatogram is obtained by fluorescence detection. Recoveries were almost quantitative and highly reproducible, with standard deviations $\leqslant$ 5.4% and $\leqslant$ 2.7% at spiked concentrations of 10 and 100 n*M*. Using 300 μl of plasma, detection limits ranged from 0.3 to 0.8 n*M* at a signal-to-noise ratio of 3. The calibration curves were linear from 1 to 300 n*M* ($r^2 \geqslant 0.999$) for each of the seven compounds. The between-day accuracy was in the range 91–99% and 99–105% at 10 and 100 n*M*, respectively, with standard deviations of 1–4%. Application of the assay to the analysis of plasma from patients after administration of Leu-Dox proved successful.

INTRODUCTION

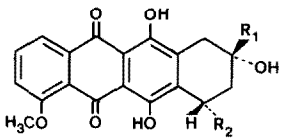
Doxorubicin (Dox), the main representative of the anthracycline antibiotics, is frequently used in the treatment of various solid tumours. Its applicability is limited by the cardiotoxicity that can develop upon cumulative treatment. Many analogues have been developed to diminish this major side-effect [1]. In an alternative approach, N-*l*-leucyldoxorubicin (Leu-Dox, see Table I for molecular structures) was synthesized as a prodrug from which the active compound Dox is released after administration. Supposedly, this hydrolysis occurs more specifically in tumour tissue [2], and an increased therapeutic index is thus expected.

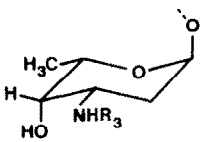
To obtain insight into the release of Dox from Leu-Dox, the plasma pharmacokinetics of Leu-Dox were studied in patients participating in a Phase I clinical trial [3]. Because Dox is formed *in vivo* from Leu-Dox, all known metabolites of Dox may also be present. Most important in this respect is the 13-dihydro derivative doxorubicinol (Dol) [4], and also the (7-deoxy)-aglycones, which are formed by removal of the daunosa-

0378-4347/92/$05.00

TABLE I

MOLECULAR STRUCTURES OF TEST COMPOUNDS



Compound	Abbreviation	R_1	R_2	
N-*l*-Leucyldoxorubicin	Leu-Dox	$COCH_2OH$		R_3 = *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 aglycone	Doxon	$COCH_2OH$	OH	
Doxorubicinol aglycone	Dolon	$CH(OH)CH_2OH$	OH	
7-Deoxydoxorubicin aglycone	7d-Doxon	$COCH_2OH$	H	
7-Deoxydoxorubicinol aglycone	7d-Dolon	$CH(OH)CH_2OH$	H	

mine side-chain (Table I). Analogous to Dox, the 13-dihydro derivative of Leu-Dox can be formed (Leu-Dol). Although the metabolites probably do not contribute to the antitumour effect, there are strong indications that the 13-dihydro derivatives in particular play a role in the cardiotoxicity [5,6].

In order to measure the parent compound and the metabolites during a 48-h time period after administration (necessary for a reliable assessment of the pharmacokinetic data) a method is required which not only can discriminate between all possible metabolites, but also has a high sensitivity. Since the introduction of anthracyclines, numerous methods for their analysis in pharmaceutical formulations and body fluids have appeared in the literature [7]. Most of these employed liquid–liquid or solid-phase extraction in combination with reversed-phase liquid chromatography. The main disadvantage of liquid–liquid extraction is that, owing to their wide range of polarities, not all known metabolites can be extracted with sufficient recovery. Therefore, in our department methods were set up employing C_{18} Sep-Pak cartridges for the extraction of Dox, epidoxorubicin [8], daunorubicin [9] and Leu-Dox [10] including all their known metabolites from plasma. Although highly selective and sensitive, with detection limits in the subnanomolar range, the procedures were quite laborious owing to their off-line nature. To our knowledge, only one paper has described a method employing on-linc sample pretreatment [11] for the analysis of Dox and three metabolites from plasma. However, this method did not meet our demands regarding selectivity and sensitivity. Therefore, our purpose was to set up a rapid on-line procedure for the analysis of Leu-Dox and its known metabolites, enabling pharmacokinetic monitoring at all dose levels used in the Phase I dose-escalation study.

EXPERIMENTAL

Materials

N-*l*-Leucyldoxorubicin (Leurubicine) and N-*l*-leucyldoxorubicinol were kindly provided by Medgenix Group (Fleurus, Belgium), and all other anthracyclines by Farmitalia Carlo Erba (Milan, Italy). Acetonitrile, sodium dihydrogenphosphate and phosphoric acid were obtained from Merck (Amsterdam, Netherlands), methanol from Baker (Deventer, Netherlands) and triethylamine from Pierce (Rockford, IL, USA). All reagents were of analytical grade. Phosphate-buffered saline (PBS), composition (m*M*) disodium hydrogenphosphate (9.0), sodium dihydrogenphosphate (3.6), and NaCl (140) (pH 7.4), was supplied by the Free University Hospital Pharmacy Department (Amsterdam, Netherlands). Blank heparinized plasma was obtained from healthy volunteers.

Separate stock solutions of Leu-Dox and metabolites, 100 μM in methanol, were combined in order to obtain an equimolar standard mixture (10 μM) of the compounds. From this mixture further dilutions (10, 30, 100, 300 nM, 1 and 3 μM) were prepared monthly in methanol and stored at −20°C.

Polypropylene tubes and vials were used throughout to minimize adsorption of anthracyclines.

Calibration samples were prepared by successively pipetting 35 μl of a standard mixture in a 1.5-ml Eppendorf vial (Hamburg, Germany), evaporating the methanol by heating to 40°C under a stream of nitrogen, adding 350 μl of blank plasma, and vortexing for 15 min to obtain an equilibrium between free and macromolecule-bound anthracycline. Thus, starting with standard mixtures ranging from 10 nM to 10 μM, spiked plasma concentrations of 1–1000 nM were obtained.

Recoveries were determined by comparing the peak heights obtained after injection of 30 μl of standard mixture with those after preconcentration and chromatography of 300 μl plasma spiked with the same absolute amount of anthracyclines.

Accuracy samples, used for the determination of between-day accuracy and precision, were prepared by evaporating methanol from 1.0 ml of the 100 nM, 1000 nM and 10 μM standard solutions, respectively, and redissolving each residue in 10 ml of blank plasma by vortexing for 15 min (resulting in spiked concentrations of 10, 100 and 1000 nM, respectively). Aliquots of 1 ml were transferred to 1.5-ml vials and stored at −20°C.

Chromatography

The high-performance liquid chromatography (HPLC) system consisted of an ABI Spectroflow 400 pump (Separations Analytical Instruments, H.I. Ambacht, Netherlands) and a Gilson 232-401 autosampler, fitted with a 5-ml syringe and 2800-μl tubing (Meyvis, Bergen op Zoom, Netherlands). A specially developed Eppendorf injection needle was used (Meyvis). The injection valve was fitted either with a 100-μl injection loop (for recovery measurements) or a Chromsep 40–50 μm C_8 reversed-phase preconcentration column (10 × 2.0 mm I.D., Chrompack, Middelburg, Netherlands). A Merck-Hitachi (Amsterdam, 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 (version 5.01, Meyvis) operated on an Olivetti M240 personal computer (Olivetti, Rotterdam, Netherlands). A Chromsep 3 μm Microspher C_{18} analytical column (Chrompack) 20 cm × 4.6 mm I.D. (two 10-cm cartridges in series) was used, together with a standard 10 × 2.0 mm I.D. reversed-phase guard column (Chrompack). An in-line 3-μm filter was placed between the injection valve and the column. The column was thermostatted at 32°C by a water jacket. The mobile phase, 3.5 m*M* triethylamine in 28 m*M* sodium dihydrogenphosphate (pH 3.5–acetonitrile (2:1, v/v), was passed through a 0.45-μm filter before use and delivered at a flow-rate of 1.0 ml/min.

Sample pretreatment

Frozen plasma samples were put in an ultrasonic bath for 10 min to allow thawing and (partial) redissolution of particulate matter. After vortex-mixing, the remaining precipitate was centrifuged (5 min, 12 000 *g*). In a 1.5-ml vial, 350 μl of the supernatant were mixed (5 s vortex) with 700 μl of an acidic phosphate buffer, consisting of acetonitrile–1 *M* phosphoric acid in PBS–PBS (7.5:15:77.5, v/v/v). Thus, the final mixture contained 5% acetonitrile and 0.1 *M* phosphoric acid. For plasma, the resulting pH is *ca.* 3. In the case of calibration samples (for calibration and recovery experiments), 700 μl of buffer were added to 350 μl of spiked plasma, also followed by a 5 s vortex. After centrifugation (5 min, 12 000 *g*) the vials were transferred to the autosampler and thermostatted at 4°C. Routinely, 900 μl of the diluted plasma were injected on the precolumn.

Preconcentration and clean-up procedure

Breakthrough volumes of all anthracyclines were measured in duplicate, essentially as described before [12], using a Gilson Dilutor 401 syringe pump as solvent-delivery system (Meyvis). Anthracycline solutions of 0.05 μM were prepared by evaporating methanol from 25 μl of a 100 μM stock solution and dissolving the residue in 50 ml of acetonitrile and phosphoric acid in PBS (5%, v/v and 0.1 *M*, respectively). The resulting pH was *ca.* 2. The breakthrough volume V_B of each anthracycline was defined as the volume of a solution that could be loaded on the precolumn before the fluorescence intensity of the effluent increased to 1% of the total fluorescence of the solution. Except where otherwise indicated, a flow-rate of 1.5 ml/min was used.

The whole sample handling procedure, outlined in Table II, was performed with a Dilutor 401 as part of Autosampler 232-401. The regeneration solvent (Dilutor-mix) was aspirated from

TABLE II

SAMPLE HANDLING PROCEDURE USING THE GILSON 232-401 AUTOSAMPLER IN COMBINATION WITH THE DILUTOR 401

(1)	Condition the precolumn by flushing with 2.5 ml of phosphate buffer (20 m*M*, pH 2.9, 0.75 ml/min)
(2)	Trap the anthracyclines from the 900 μl of diluted (plasma) sample on the precolumn (0.36 ml/min)
(3)	Wash the precolumn with 1.5 ml of phosphate buffer (20 m*M*, pH 2.9, 0.36 ml/min)
(4)[a]	Desorb (forward flush) the anthracyclines from the precolumn towards the analytical column using 500 μl of mobile phase (with the HPLC flow-rate of 1.0 ml/min)
(5)	Clean the injection valve with 1 ml of "Dilutor-mix" [acetonitrile–phosphoric acid in water (70:30, v/v, pH 4, 12 ml/min)]
(6)	Clean the Dilutor tubing and injection needle with 5 ml of Dilutor-mix (48 ml/min)
(7)	Regenerate the precolumn with 1.5 ml of Dilutor-mix (1.5 ml/min)

[a] After desorption step 4, the next sample is processed while the separation of the former takes place.

the Dilutor reservoir, while the washing and conditioning buffer were placed in beakers located within the sample tray boundaries. Consecutively aspirated liquids were separated from each other in the tubing by an air bubble to prevent mixing. Using the amounts and flow-rates listed in Table II, the whole procedure takes *ca.* 14 min. After the desorption step, the next sample is processed while the chromatographic separation of the former takes place.

Details concerning the 232-401 program will be made available upon request.

RESULTS AND DISCUSSION

Chromatography

Fig.1 shows a chromatogram obtained after the on-line pretreatment of 300 μl of plasma spiked with 30 nM of the anthracyclines. It demonstrates the baseline separation of Leu-Dox and six metabolites. Under the isocratic conditions used, all compounds elute within 12 min. The applicability to real samples is illustrated in Fig. 1B, which shows the chromatogram of 300 μl of plasma obtained from a patient 4 h after receiving Leu-Dox at 180 mg/m^2. The accompanying blank chromatogram, which was obtained from plasma from the same patient sampled before drug administration, clearly demonstrates the absence of interferences from endogenous compounds after our on-line extraction procedure. It also suggests that small amounts of possible unknown metabolites are present in the 4-h sample.

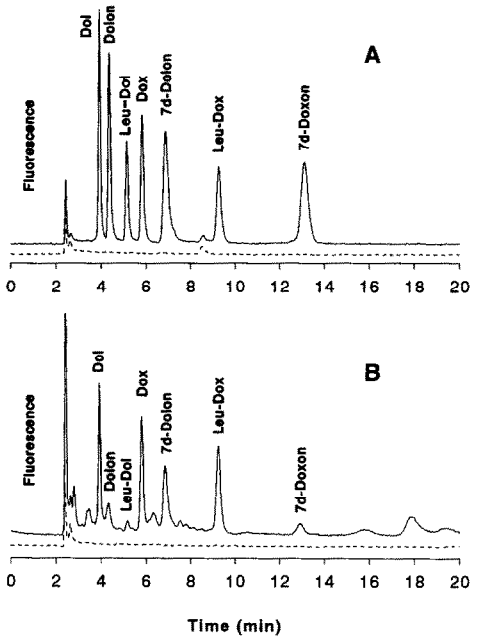


Fig. 1. (A) Chromatograms of 300 μl of human plasma after automated on-line preconcentration/clean-up. The broken and solid lines represent blank and spiked plasma (30 nM of each anthracycline), respectivley. (B) Chromatograms of 300 μl of patient plasma obtained before (- - -) and 4 h after (——) administration of leucyldoxorubicin (180 mg/m^2). Column, Microspher C_{18} analytical column [20 (2 × 10) cm × 4.6 mm I.D.] fitted with a Chromsep reversed-phase guard column (10 × 2.0 mm I.D.); mobile phase, 3.5 mM triethylamine in 28 mM NaH_2PO_4 (pH 3.5)–acetonitrile (2:1, v/v); flow-rate, 1.0 ml/min; detection, fluorescence with λ_{ex} = 480 nm and λ_{em} = 580 nm. For peak identification, see Table I.

In order to obtain this result, a few problems had to be overcome. Perhaps the most critical part in the process of changing from an off-line to an on-line procedure is the optimization of the desorption eluent, which generally is the mobile phase. In order to minimize peak broadening it has to be strong enough to elute the adsorbed analytes in a small volume, whereas it also has to provide resolution of, in this instance, eight compounds.

First a suitable mobile phase was developed to enable isocratic analysis of all anthracyclines of interest. As noted before [13], increasing the concentrations of triethylamine and/or phosphate result in decreasing retention times of the compounds containing the daunosamine side-chain, leaving the capacity factor (k') of the aglycones virtually unaffected. Triethylamine exerts this effect through ionic binding to the residual silanol groups of the C_{18} material, thus reducing their interaction with anthracycline amino groups. The negatively charged phosphate is capable of forming ion-pairs with these amino moieties, thus also reducing their silanol binding.

As the number of compounds demands high separation efficiency, it appeared impossible to obtain acceptable resolution between 7d-Dolon and Doxon. From Dox pharmacokinetics, studied in the past [4], the latter was known to be only a minor metabolite, Therefore, it was decided to exclude Doxon from the anthracycline mixture. When Doxon would be present in plasma, only a slight increase of the 7d-Dolon peak is to be expected.

Increasing the temperature from 20°C to 40°C resulted in the expected reduced retention of the aglycones. The k' values of Dox and Leu-Dox decreased much less, whereas the k' values of Dol and Leu-Dol showed an increase. This phenomenon could be used advantageously to "tune" the separation.

Finally, using a solution containing 28 m*M* sodium dihydrogenphosphate and 3.5 m*M* triethylamine, buffered at pH 3.5 and mixed 2:1 (v/v) with acetonitrile, we were able to obtain baseline separation of seven compounds. Furthermore, it appeared possible to effect instantaneous desorption of the anthracyclines from the precolumn using this mobile phase. This was proved by comparing the chromatograms obtained upon straight-flush *vs.* back-flush desorption, which did not show any difference with regard to peak heights and shapes. Because of the risk of flushing dirt, trapped at the top of the precolumn, to the analytical column when using back-flush, straight-flush was used throughout the study.

Extraction of anthracyclines using an in-line precolumn

The commercially available 10 × 2 mm I.D. reversed-phase precolumn, packed with 40–50 μm C_8 material, was used from the start of the experiments. Since Dolon and Leu-Dox are the first and last, respectively, to elute from a reversed-phase column (when no triethylamine is present in the mobile phase), we initially determined values of V_B for these two compounds. A solution in PBS (pH 7.4) was used, as this roughly represents the ionic conditions present in plasma and urine. For both compounds, breakthrough did not occur after loading 15 ml on the precolumn. Since plasma sample volumes will normally not exceed 1 ml, this would be amply sufficient. Indeed, both for Dolon and Leu-Dox, the recovery from 1 ml of a 50 n*M* solution in PBS was *ca.* 100%. However, when 1 ml of spiked plasma was loaded on the precolumn a loss of 35% and 50% was observed for Leu-Dox and Dolon, respectively. The most plausible explanation for this discrepancy may be protein binding, *e.g.* 74% in the case of Dox and 76% for Dol [14], which hampers solid-phase extraction of anthracyclines. Apparently, the re-equilibration between free and protein-bound anthracycline on removal of free compound from the plasma is not fast enough.

Huber and Zech [15] suggested several approaches to cope with this problem. We found that acidification of the plasma to pH 3 by diluting with an equal volume of 0.2 *M* phosphoric acid resulted in a recovery for Leu-Dox of 70%. The addition of acetonitrile only (10%, v/v) increased the extraction efficiency to 90% (from 1 ml of plasma spiked with Leu-Dox at 50 n*M*). The combination of phosphoric acid and acetonitrile resulted in the desired quantitative recovery. However, breakthrough curves showed that 10% acetonitrile was rather critical: Dolon already started to elute from the precolumn at a loading volume of 1 ml. Lowering the percentage of acetonitrile to 5% again yielded safe breakthrough volumes, while maintaining the almost quantitative recoveries. Therefore, we determined V_B by using a solution of the anthracylines in the mixture described above, *i.e.* acetonitrile–1 *M* phosphoric acid in PBS–PBS (7.5:15:77.5, v/v/v). Breakthrough volumes ranged from 5.1 ml for Dol, via 9.6, 13.7, 18.2 and 23.7 ml for Leu-Dol, Dolon, Dox and 7d-Dolon, respectively, to more than 28.5 ml (the maximum volume loaded) for Leu-Dox and 7d-Doxon. Furthermore, it could be demonstrated that the value of V_B more than doubled when the Dilutor flow-rate was set at 0.36 ml/min instead of 1.5 ml/min (determined for Dol only). Since the lower rate is used in the actual analysis, the value of V_B in plasma, although not measurable directly, will be amply sufficient. Thus, the final sample concentrations of acetonitrile and phosphoric acid were set at 5% (v/v) and 0.1 *M* in PBS, respectively.

Final procedure

It appeared necessary to equilibrate the precolumn with 2.5 ml of phosphate buffer (20 m*M*, pH 2.9) prior to loading of the (acidified) plasma to prevent early elution of the polar compounds Dol and Leu-Dol in particular. Apparently, their retention on the precolumn is greatly influenced by the interaction of the protonated amino group with residual silanol groups, which decreases at pH values near the pK_a of the NH_2 moiety (8.1 for Dox).

TABLE III

RECOVERIES AND WITHIN-DAY PRECISION FOR LEU-DOX AND SIX METABOLITES

Values are percentage of spiked concentration ± S.D. (n = 5), obtained from 300 μl of plasma and based on peak heights.

Anthracycline	Recovery (%)		
	10 nM	100 nM	1000 nM
Leu-Dox	95.4 ± 5.0	96.1 ± 2.1	94.1 ± 2.3
Dol	107.6 ± 3.5	106.6 ± 2.7	
Dolon	109.1 ± 3.2	101.4 ± 1.1	
Leu-Dol	107.6 ± 4.3	100.1 ± 1.7	
Dox	109.6 ± 4.8	102.5 ± 2.2	
7d-Dolon	105.9 ± 3.6	106.7 ± 1.9	
7d-Doxon	100.9 ± 5.4	100.7 ± 1.9	

Incidentally, loading of plasma samples on the precolumn resulted in over-pressure (more than 8 bar). Therefore, plasma samples were diluted with two volumes of acetonitrile–1 M phosphoric acid in PBS–PBS (7.5:15:77.5, v/v/v), resulting in final concentrations equal to those in the preceding section (*i.e.* 5% acetonitrile and 0.1 M phosphoric acid). After loading of the diluted plasma, the precolumn was flushed with 1.5 ml of 20 mM phosphate buffer (pH 2.9) to remove proteins. Subsequent desorption of the anthracyclines was achieved with 0.5 ml of the mobile phase. This appeared to be amply sufficient and was used throughout.

Although it appeared possible to regenerate and use one precolumn for several hundred analyses, it was renewed after every 100 samples during routine operation.

General performance

Blank plasma samples were spiked with the anthracycline mixture to obtain a concentration of 10 or 100 nM. Five plasma samples of each concentration were analysed once within a day to determine the recovery and within-day precision of the assay. For Leu-Dox, an additional plasma sample concentration of 1000 nM was evaluated, because of the expected peak concentrations in the pharmacokinetic study. The results are summarized in Table III. Recoveries, determined by comparing peak heights of the anthracyclines in the plasma sample after preconcentration with the direct injection of the same absolute amount in 30 μl of mobile phase, were quantitative for all compounds except Leu-Dox (95%). The precision was highly satisfactory, with a standard deviation (S.D.) of less than 5.4% at 10 nM, 2.7% at 100 nM and 2.3% at 1000 nM (n = 5). Although extraction methods are available with (near) quantitative recoveries for Dox and Dol [16,17], they do not include Leu-Dox and the remaining aglycone metabolites. All calibration lines showed good linearity over a concentration range of 1 to 300 nM, as shown by the r^2 values $\geqslant$0.999 (Table IV). The intercepts were generally below the lowest calibration concentration, being

TABLE IV

CALIBRATION LINES AND DETECTION LIMITS FOR LEU-DOX AND SIX METABOLITES

Anthracycline	Calibration curves[a] (mean ± S.D., n = 5)			Detection limit (nM)
	Slope (A.U. × nM^{-1})	Intercept (A.U.)	r^2	
Leu-Dox	18 ± 1.6	0 ± 11	0.9998 ± 0.0001	0.8
Dol	50 ± 4.8	56 ± 25	0.9995 ± 0.0004	0.3
Dolon	39 ± 5.1	37 ± 19	0.9997 ± 0.0003	0.4
Leu-Dol	24 ± 2.2	5 ± 6	0.9998 ± 0.0002	0.6
Dox	29 ± 2.4	23 ± 7	0.9997 ± 0.0002	0.5
7d-Dolon	22 ± 4.5	2 ± 4	0.9999 ± 0.0001	0.6
7d-Doxon	17 ± 2.3	−1 ± 5	0.9998 ± 0.0002	0.8

[a] Six concentrations ranging from 1 to 300 nM.

close to the detection limit. The sensitivity of the assay is comparable with that of the off-line method [8], *i.e.* 0.3 n*M* for Dol to 0.8 n*M* for Leu-Dox and 7d-Doxon (based on a signal-to-noise ratio of 3, Table IV). This is low enough for the pharmacokinetics of Leu-Dox and its metabolites in plasma to be determined even at the lowest dose (18 mg/m^2) in the Phase I study [3]. It should be noted that the present assay uses only 300 μl of plasma instead of 1 ml. This three-fold gain not only permits a valuable reduction in blood sample volumes, which is always an important consideration in a clinical Phase I study, but it also allows the preparation of calibration samples in blank plasma obtained from the same patient. Thus unanticipated differences in recovery, for instance resulting from variable protein binding, lipid content, etc., are prevented from influencing the quantification. As can be concluded from the breakthrough volumes, larger plasma samples can also be loaded onto the precolumn without the risk of sample loss when a higher sensitivity is required. Indeed, quantitative recovery was maintained when 1 ml of spiked plasma was assayed (results not shown).

Validation

Calibration curves showed a good day-to-day repeatability, as illustrated by the S.D. values of the slopes (Table IV). The between-day precision of the assay was determined over 5 days by the duplicate analysis of accuracy samples spiked with an anthracycline mixture to obtain concentrations of 10 and 100 n*M*. The results for the between-day precision and accuracy of the assay, as determined by interpolating peak heights of the spiked samples on the calibration line of each individual metabolite, are summarized in Table V. The variation was very low at both concentrations, 0.8–2.7% and 1.9–4.1% for the 10 and 100 n*M* samples, respectively. The deviations from the spiked concentrations were quite satisfactory, with values ranging from −9% to −1% and from −1% to +5% at 10 and 100 n*M*, respectively.

TABLE V

BETWEEN-DAY ACCURACY AND PRECISION OF THE AUTOMATED ASSAY FOR LEU-DOX AND SIX METABOLITES

Values are percentage of spiked concentration ± S.D. (n = 5), obtained from 300 μl of plasma.

Anthracycline	Accuracy (%)	
	10 n*M*	100 n*M*
Leu-Dox	91.3 ± 2.5	99.3 ± 2.7
Dol	92.6 ± 0.8	102.8 ± 3.3
Dolon	94.7 ± 1.9	102.8 ± 3.4
Leu-Dol	94.0 ± 2.4	100.8 ± 2.6
Dox	90.4 ± 2.6	99.1 ± 3.8
7d-Dolon	98.7 ± 2.7	104.6 ± 3.9
7d-Doxon	98.8 ± 2.6	104.9 ± 4.1

CONCLUSIONS

A sophisticated on-line preconcentration/clean-up method was developed for the determination of leucyl-doxorubicin and six metabolites in plasma. Using minimal sample pretreatment, anthracyclines with a wide range of polarities can be analysed at the sub-nanomolar level in one run. The sensitivity and selectivity allow pharmacokinetic studies of leucyl-doxorubicin in cancer patients at the lowest dose level.

ACKNOWLEDGEMENT

Separations Analytical Instruments is gratefully acknowledged for the loan of a column thermostat.

REFERENCES

1 R. B. Wein, G. Sarosy, K. Clagett-Carr, M. Russo and B. Leyland-Jones, *Cancer Chemother. Pharmacol.*, 18 (1986) 185.
2 P. Briozza, M. Morriset, F. Capony, C. Rougeot and H. Rochefort, *Cancer Res.*, 48 (1988) 3688.
3 J. de Jong, G. J. Geijssen, C. N. Munniksma, J. B. Vermorken, A. Bast and W. J. F. van der Vijgh, *Proc. Am. Assoc. Cancer Res.*, 32 (1991) 1046.
4 K. Mross, P. A. Maessen, W. J. F. van der Vijgh, H. Gall, E. Boven and H. M. Pinedo, *J. Clin. Oncol.*, 6 (1988) 517.
5 R. D. Olson, P. S. Mushlin, D. E. Brenner, S. Fleischer, B. J. Cusack, B. K. Chang and R. J. Bousek, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 3585.
6 J. de Jong, P. R. Schoofs, W. S. Guérand, A. Bast and W. J. F. van der Vijgh, *Proc. Am. Assoc. Cancer Res.*, 31 (1990) 2036.

7 U. R. Tjaden and E. A. de Bruijn, *J. Chromatogr.*, 531 (1990) 235.
8 P. A. Maessen, H. M. Pinedo, K. Mross and W. J. F. van der Vijgh, *J. Chromatogr.*, 417 (1987) 339.
9 J. de Jong, P. A. Maessen, A. Akkerdaas, S.-F. Cheung, H. M. Pinedo and W. J. F. van der Vijgh, *J. Chromatogr.*, 529 (1990) 359.
10 J. de Jong, J. B. Vermorken and W. J. F. van der Vijgh, *J. Pharm. Biomed. Anal.*, in press.
11 C. A. Riley, W. R. Crom and W. A. Evans, *Ther. Drug Monit.*, 7 (1985) 455.
12 C. E. Werkhoven-Goewie, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 53 (1981) 2072.
13 J. de Jong, W. S. Guérand, P. R. Schoofs, A. Bast and W. J. F. van der Vijgh, *J. Chromatogr.*, 570 (1991) 209.
14 R. F. Greene, J. M. Collins, J. F. Jenkins, J. L. Speyer and C. E. Myers, *Cancer Res.*, 43 (1983) 3417.
15 R. Huber and K. Zech, in R. W. Frei and K. Zech (Editors), *Selective Sample Handling and Detection in High-Performance Liquid Chromatography*, Part A, Elsevier, Amsterdam, 1988, pp. 81–143.
16 S. Eksborg, H. Ehrsson and I. Andersson, *J. Chromatogr.*, 164 (1979) 479.
17 S. Shinozawa, Y. Mimaki and Y. Araki, *J. Chromatogr.*, 196 (1980) 463.