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Determination of a peptide–doxorubicin, prostate-specific antigen activated prodrug, and its active metabolites in human plasma using high-performance liquid chromatography with fluorescence detection Stabilization of the peptide prodrug with EDTA

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Abstract

A method for the determination of **I**, a peptide–doxorubicin conjugate that was evaluated for the treatment of prostate cancer, and two of its active metabolites, doxorubicin and leucine–doxorubicin is described. Blood samples were chilled immediately after being drawn in order to prevent ex vivo entry of the metabolites into red blood cells. EDTA (10 mg/ml final concentration) was used to prevent plasma-mediated degradation of the peptide portion of the prodrug. After the addition of internal standard, plasma was prepared for analysis using a C-8 solid-phase extraction column. In order to overcome secondary ionic interactions with the silica-based extraction column, the analytes were eluted with ammonium hydroxide in methanol. The extracts were evaporated to dryness, reconstituted, and assayed by step change, gradient, reverse phase HPLC with fluorescence detection. Two interfering metabolites found in post dose plasma were chromatographically separated by an adjustment of the mobile phase pH. The within-day reproducibility of the doxorubicin and leucine–doxorubicin chromatographic retention times was improved by a brief washing of the analytical column with 90% acetonitrile after each injection. The range of the standard curve was 12.5–1250 ng/ml for doxorubicin and 25–2500 ng/ml for **I** and leucine–doxorubicin.

Keywords: Peptide–doxorubicin; Doxorubicin; Leucine–doxorubicin

1. Introduction

Prostate cancer is the second leading cause of cancer deaths among men in the United States [1]. The clinical cytotoxic regimens now in use are limited by systemic toxicity. Compound **I** (Fig. 1), a peptide–doxorubicin conjugate, was designed to deliver the conventional cytotoxic drugs doxorubicin (**II**, Fig. 1) and leucine–doxorubicin (**III**, Fig. 1) selectively to prostate cancer cells. The peptide portion of the drug is cleaved by prostate specific antigen (PSA), a protease expressed by prostate cells [2]. PSA-mediated cleavage of the peptide allows the active metabolites doxorubicin and leucine–doxorubicin to enter the cells and exert cytotoxic effects. Compound **I** was shown to selectively kill PSA producing prostate cancer cells both in tissue culture and in nude mice with xenografts [3].

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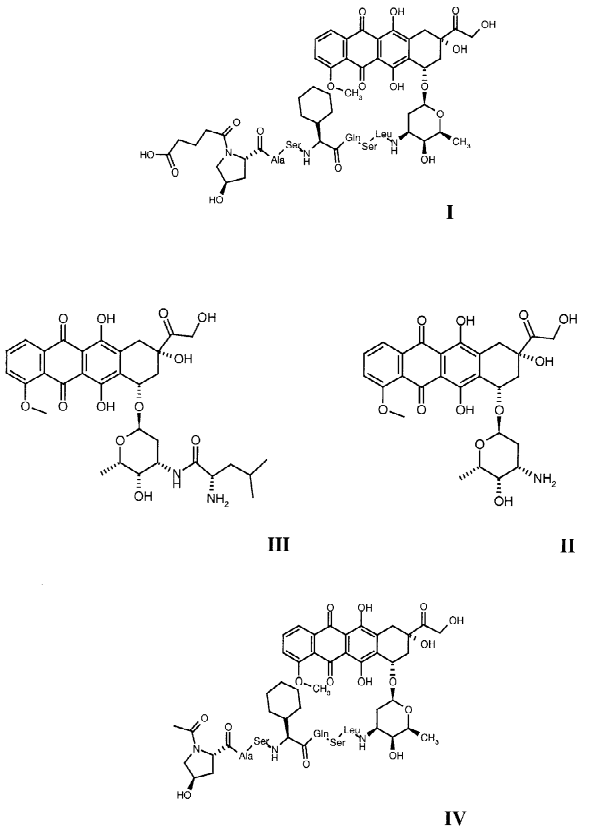


Fig. 1. Chemical structures of doxorubicin–conjugate (**I**), doxorubicin (**II**), leucine–doxorubicin (**III**), and internal standard (**IV**).

A method for the determination of **I**, **II**, and **III** in plasma after intravenous doses was needed to support human pharmacokinetic studies. Although a method for the analysis of **II** and **III** in human plasma utilizing online solid-phase sample preparation and high-performance liquid chromatography (HPLC) with fluorescence detection has been reported [4], the simultaneous assay of the prodrug conjugate **I** along with the two metabolites **II** and **III** presented several unique problems. First, was the apparent instability of **I** in human plasma. Incubation of **I** spiked into control human plasma caused the hydrolysis of **I** and the release of compound **III** as a degradant. In the presence of a large excess of prodrug **I** (such as during i.v. dosing), degradation of **I** might significantly elevate the apparent plasma concentration of **III**.

A second experimental difficulty was in developing an adequate and reliable chromatographic separation of the three analytes from themselves and from unidentified plasma metabolites. Although the chromatographic conditions initially developed for the separation of the three analytes spiked into control human plasma where highly adequate for their quantification, post dose plasma samples contained additional unknown metabolites, some of which were not fully separated from the analytes of interest. Modification of the chromatographic conditions was necessary to achieve the necessary chromatographic separation and to assure the selectivity of the method in post dose plasma samples.

This paper describes the analytical methodology for the determination of **I** and two major metabolites **II** and **III** in human plasma in the presence of other metabolites of **I**. The method includes stabilization of **I** in human plasma by the addition of EDTA, the isolation of analytes **I**, **II**, and **III** from plasma by solid-phase extraction, and gradient high-performance chromatographic separation of the analytes from unidentified metabolites prior to fluorescence detection.

2. Experimental

2.1. Materials

Compound **I** was obtained obtained from the Chemical Data Department of Merck Research Laboratory (Rahway, NJ). The HCl salt of **II** was purchased from Sigma (St. Louis, MO, USA). Internal standard (**IV**, Fig. 1) and the HCl salt of **III** were synthesized by Dr Garsky (Medicinal Chemistry, MRL, West Point, PA, USA). Tripotassium EDTA was purchased from Aldrich. Drug-free plasma was purchased from Biological Specialties (Colmar, PA, USA). All HPLC solvents were obtained from Fisher Scientific. All other reagents were of ACS grade and were used as received.

2.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA, USA) 1090 chromatograph connected to a Perkin-Elmer (Norwalk, CT, USA) LC 240 fluorescence detector. The analog output of the

detector was connected to a PE-Nelson (San Jose, CA, USA) Access-Chrom data acquisition system via a PE-Nelson model 941 interface.

An API-300 triple quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with a turbo ionspray interface was used to obtain mass spectra of the analytes. The absorption and fluorescence spectra were taken with a diode-array spectrophotometer (HP 8452) and Hitachi (San Jose, CA, USA) F-4500 fluorescence spectrophotometer, respectively.

2.3. Absorption and fluorescence spectra and relative fluorescence quantum efficiencies

Both ultraviolet (UV) absorption and fluorescence spectra of **I**–**III** were taken in a 1-cm quartz cell after subtracting the contribution of the solvent. The compounds were dissolved in mobile phase A (a mixture of 1 *M* ammonium acetate (pH 4.5)–water–acetonitrile (1:75:24, v/v) at a concentration of 10 μ*M*. The relative quantum efficiencies (ϕ_f) of **I** and **III** were determined relative to doxorubicin (**II**). Since fluorescence spectra of all three compounds were practically the same in terms of the positions of peak maxima and peak widths, uncorrected spectra were utilized for the determination of relative quantum efficiencies. At 10 μ*M*, all three compounds exhibited similar absorbance at the wavelength of excitation (480 nm). The measurements of the relative ϕ_f were performed using a similar procedure as described earlier [5].

2.4. Chromatographic conditions

A C-8 Betasil Basic (Keystone Scientific, State College, PA, USA) analytical column (100×3 mm, 5 μm) was used at a flow-rate of 1.0 ml/min and a temperature of 35 °C. A three-step gradient was used for the analysis: (A) a 1-*M* ammonium acetate (adjusted to pH 4.5 with 1 *M* acetic acid)–water–acetonitrile (1:75:24, v/v) mixture; (B) a 1-*M* ammonium acetate (adjusted to pH 5.0 with 1 *M* acetic acid)–water–acetonitrile (1:68:31, v/v) mixture; and (C) water–acetonitrile (10:90, v/v). A, B, and C were pumped for 0–2.5, 2.5–7.5, and 7.5–8.8 min, respectively, post injection. From 8.8 to 12.5 min, the column was re-equilibrated in mobile phase A.

The sample injection volume was 50 μl. The fluorescence detector was set at an excitation wavelength of 480 nm and an emission wavelength of 560 nm.

2.5. Preparation of standards

Using DMSO as solvent, 1 mg/ml stock solutions of **I,II**, **III** and **IV** (internal standard) were made and stored at −70 °C. Stock solutions of **II** and **III** were made as 1 mg/ml free base. A combined working standard containing **I** (125 μg/ml), **II** (62.5 μg/ml) and **III** (125 μg/ml) was made by adding stock solutions of 125 μl **I**, 62.5 μl **II**, and 125 μl **III** to 688 μl DMSO. Using DMSO, additional dilutions of this working standard were made to give working standards containing 50, 25, 12.5, 5, 2.5, and 1.25 μg/ml **I** and **III,** and 25, 12.5, 6.25, 2.5, 1.25, and 0.625 μg/ml **II.** All working standards were stored at −70 °C and thawed by brief warming at 35 °C before use. Internal standard working solutions (10 μg/ml) were made by adding 40 μl stock standard to 3.96 ml mobile phase A.

Plasma standards were made by adding 10 μl of the above working standards to 500 μl control human plasma containing 10 mg/ml tripotassium EDTA. The resulting standards were used to quantitate samples containing 25–2500 ng/ml **I** and **III**, and 12.5–1250 ng/ml **II**. Samples containing higher concentrations were diluted with control plasma prior to analysis.

2.6. Clinical plasma collection procedure

Seven ml blood was collected in an EDTA Vacutainer and immediately placed on ice. The blood was transferred (within 1 h) to an ice-cold tube containing an additional 35 mg EDTA. The tube was inverted several times and centrifuged at 2200 *g* for 10 min at 0–5 °C. The plasma was then transferred to a polypropylene tubes and frozen at −70 °C.

2.7. Plasma extraction procedure

Frozen plasma samples were thawed at room temperature and mixed by inversion. Following centrifugation (2000 *g* for 1–2 min), a 0.5-ml aliquot was pipetted into a 12×75 mm polypropylene test tube. After addition of 25 μl internal standard

working solution and 0.5 ml water, the tube was vortexed. A 0.9-ml aliquot of this solution was pipetted into a C-8 solid-phase extraction (SPE) column (1 ml column containing 50 mg packing, Varian, Walnut Creek, USA) that had been conditioned with 1 ml methanol and 1 ml water. Using a vacuum manifold, sufficient vacuum was applied to pull the sample through in 1–2 min. The column was then washed with 1 ml water, 1 ml 10% methanol and placed in a 12×75 mm tube (the column was suspended in the tube by its flange). Residual wash solvent was removed by centrifugation at 2000 *g* for 1–2 min. After transfer to a 13×100 mm glass tube, 1 ml elution solvent (0.5 *M* ammonium hydroxide in methanol) was added and the column was centrifuged at 500 *g* for 1–2 min. The column was then discarded and the eluate evaporated in a Turbo-Vap evaporator (Zymark, Hopkinton, MA, USA) at a temperature of 40 °C using a stream of nitrogen. After 10 min, the tube was promptly removed from the evaporator. The residue was dissolved in 150 μl sample solvent mobile phase A–water (90:10, v/v) and transferred to an auto-sampler vial containing a glass, limited volume insert. The vial was capped and placed in an autosampler tray for injection.

2.8. Plasma and whole blood stability testing

2.8.1. Plasma stability

Initially, **I** was spiked into ten lots of untreated control human plasma (final concentration 100 ng/ml) and incubated at 37 °C for 3 h. In follow up experiments, 1 ml aliquots of human control plasma spiked with 250 ng/ml **I** were incubated at 37 °C for 80 min. Two to 50 μl of the following reagents were added to the plasma to test their use as preservatives: (A) EDTA (1 g/ml), (B) 5 *M* phosphoric acid, (C) 1 *M* phosphate buffer (pH 7), (D) EDTA plus 5 *M* phosphoric acid, and (E) EDTA plus 1 *M* phosphate buffer. At the end of the incubation, the plasma was extracted by SPE and assayed for **I** and **III,** as described above.

2.8.2. Whole blood stability

Compounds **I** and **II** were added to ice-cold fresh human blood containing 10 mg/ml EDTA (final concentration about 1 μg/ml blood) and aliquots incubated at 37 and 4 °C. Samples were removed at time points between 0 and 4 h, centrifuged at 4 °C, and the plasma removed. The plasma was extracted by SPE and assayed as described above.

3. Results and discussion

3.1. Absorption, fluorescence and positive ion mass spectra

The UV absorption spectrum of **I** had absorption bands with maxima at 254 and 480 nm (26 800 and 13 800 M^{-1} cm^{-1}, respectively). The UV spectra of **II** and **III** were similar in terms of the positions of absorption bands and the intensity of the maxima of absorption. At a fluorescence excitation wavelength of 480 nm, two broad peaks with maxima at 560 and 590 nm were observed in the fluorescence emission spectra of **I–III**. The relative quantum efficiencies (ϕ_f) of **I** and **III** relative to doxorubicin (**II**) were 1.04 and 0.96, respectively, indicating that intramolecular quenching of the fluorescence originating from the doxorubicin chromophore did not occur, allowing sensitive fluorescence detection of both compounds.

Preliminary studies suggested that using an excitation wavelength of 480 nm and an emission wavelength of 560 nm, a limit of quantification (LOQ) in the range of 10–25 ng/ml in human plasma would be possible for compound **I**. The excitation–emission wavelengths selected were similar to those used in a plasma assay for doxorubicin and leucine–doxorubicin [4].

Compound **I** was also infused into the turboionspray interface of a API-300 tandem mass spectrometer. Positive ion Q1 spectra contained sodium and potassium adducts of the molecule (*m*/*z* 1419 and 1435, respectively), but no pseudomolecular ion ($[M+H]^+$) was observed. Attempts to obtain product ions of the adducts useful for quantification were not successful.

Because of its simplicity, selectivity and sensitivity, fluorescence detection using an excitation wavelength of 480 nm and an emission wavelength of 560 nm was chosen as the optimal method of detection for **I** and metabolites **II** and **III**.

3.2. Extraction procedure

C-8 SPE columns were used to optimize the conditions for solid-phase extractions. Preliminary experiments indicated that the SPE columns retained both **I** and **II**. Acetonitrile or methanol eluted **I** from the extraction column, but not **II**. This was presumably due to secondary ionic interactions between the amino group of **II** and the stationary phase of the SPE column. Acetonitrile containing 0.1 or 0.05% TFA eluted **II**, but caused degradation of **I** during drydown of the elution solvent.

Chloroform–methanol (2:1, v/v), ammonium hydroxide in acetonitrile, and ammonium hydroxide in methanol were evaluated as alternative SPE elution solvents. Optimal recoveries were obtained with 0.5 *M* ammonium hydroxide in methanol. Centrifugation of the SPE column was performed prior to elution in order to remove residual water. Failure to remove residual water resulted in degradation of the analytes during drydown of the SPE column eluate. The SPE solvent was evaporated in a TurboVap at 40 °C for 10 min. Significant degradation of drug was noted if the plasma extract was left in the TurboVap for longer periods of time.

3.3. Chromatographic conditions

The difference in polarity between **I** and the two metabolites required the use of gradient HPLC. A ''step'' gradient was developed to separate **I**, **II**, **III**, and internal standard (**IV**) in 10 min using a 3-mm diameter C-8 column. The use of a 3-mm diameter column provided equivalent separation to a 4.6-mm column with better sensitivity and lower solvent consumption. Samples were dissolved in a solution containing 10% less acetonitrile than the initial mobile phase in order to focus the analytes on the head of the column and avoid the peak broadening sometimes associated with large volume injections on small diameter columns.

Attempts to validate the plasma assay using a two-step gradient resulted in slowly drifting retention times for doxorubicin and leucine–doxorubicin but not for **I** (Fig. 2). The addition of a 1-min column washout with 90% acetonitrile–10% water at the end of each run, stabilized the retention times. Highly reproducible retention times were important in the analysis of post dose samples due to the presence of other metabolites of **I** eluting near the analytes of interest (see Section 3.5).

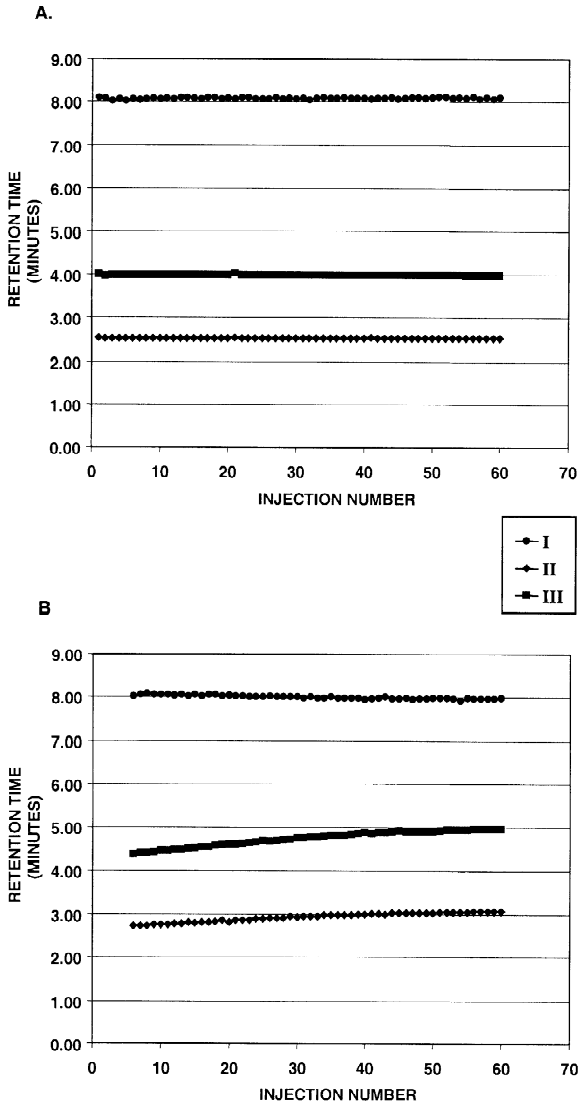


Fig. 2. The effect of a column washout on the retention times of **I**, **II**, and **III**. (A) With 1-min column washout step at the end of each run, (B) without washout step.

3.4. The stability of *I* in control human plasma and whole blood

Preliminary experiments demonstrated that the incubation of control human plasma spiked with **I** at

Table 1
Hydrolysis of **I** to **III** in human plasma incubated for 80 min at 37 °C

Donor number	Hydrolysis to **III** (%)	
	Untreated plasma	Plasma containing EDTA/phosphoric acid
1	4.8	1.2
2	2.2	0.0
3	2.9	0.0
4	1.7	0.4
5	2.7	0.0
6	31.4	0.0
7	23.8	0.6
8	0.7	0.0
9	0.7	0.6
10	1.8	0.0

37 °C for 3 h resulted in significant degradation of drug, primarily through hydrolysis to **III**. The extent of hydrolysis from plasma to plasma was quite variable and two batches of plasma from the same donor exhibited the highest level of hydrolytic activity (Table 1). EDTA, phosphoric acid, and phosphate buffer (alone and in combination) were screened as preservatives using the highest activity plasma. The results demonstrated that EDTA alone was about as effective in stabilizing **I** as in combination with phosphoric acid or phosphate buffer (Table 2). After incubation at 37 °C for 80 min, drug **I** in untreated plasma and plasma containing 10 mg/ml EDTA was 24 and 0.5%, respectively, hydrolyzed to **III**.

Spiked into fresh human blood containing 10 mg/ml EDTA, prodrug **I** was stable for up to 4 h at room temperature or at 4 °C. There was no evidence of hydrolysis to leucine–doxorubicin at either temperature. Doxorubicin, on the other hand was stable at 4 °C, but declined to about 50% of its starting concentration within 1 h of incubation at room temperature (Fig. 3). The most likely explanation is the entry of doxorubicin into the red blood cells. Subsequent analysis of washed red cell lysates confirmed the presence of doxorubicin (data not shown).

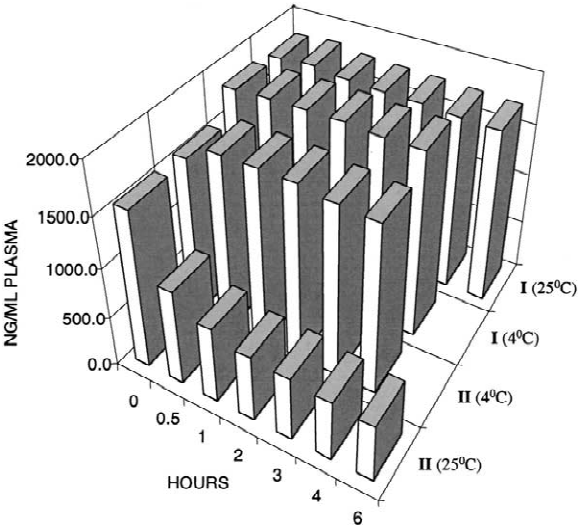


Fig. 3. Stability of **I** and doxorubicin (**II**) in fresh human blood at 4 °C and at room temperature (25 °C).

Based on the experimental results, the clinical blood collection procedure outlined in the methods section was designed to prevent hydrolysis of **I** and/or ex vivo entry of metabolites into red blood cells.

Table 2
Effect of EDTA, phosphoric acid, and phosphate buffer on the hydrolysis of **I** to **III** in human plasma incubated for 80 min at 37 °C

Volume of reagent (μl) added[a]	Hydrolysis to **III** (%)				
	EDTA[b]	Phosphoric acid (5 *M*)	EDTA[b]+ Phosphoric acid (5 *M*)	Phosphate buffer (1 *M*, pH 7)	EDTA[b]+phosphate buffer (1 *M*, pH 7)
0	24.3	23.0	24.5	22.3	21.7
2	12.9	18.9	6.3	18.2	11.4
10	0.5	5.9	1.7	10.9	0.0
50	0.0	1.3	0.0	2.2	0.0

[a] Per 1 ml of plasma.
[b] EDTA in water (1 g/ml).

3.5. Chromatographic separation of interfering metabolites

Initially, adequate separation of analytes spiked into control plasma was achieved using a pH of 5.0 for mobile phase components A and B. However, when this gradient was applied to post dose plasma samples, the appearance of shoulders on the leucine–doxorubicin (**III**) and compound **I** peaks indicated the presence of unknown coeluting metabolites (Fig. 4B). In order to determine the effect of mobile phase pH on chromatographic separation, the pH of mobile phase A was adjusted 0.5 pH units above and below the initial value. Separation of the interfering metabolites was achieved by lowering the pH of mobile phase A from 5.0 to 4.5 (Fig. 4A). Using the modified gradient conditions, the retention times of **II**, **III**, **I**, and **IV** were 2.5, 3.9, 8.4, and 9.3 min, respectively.

3.6. Extraction recovery

Extraction recoveries were determined by comparing the response of neat standards injected directly to those of extracted plasma standards. Mean recoveries across the range of the standard line were 81.3, 72.7,

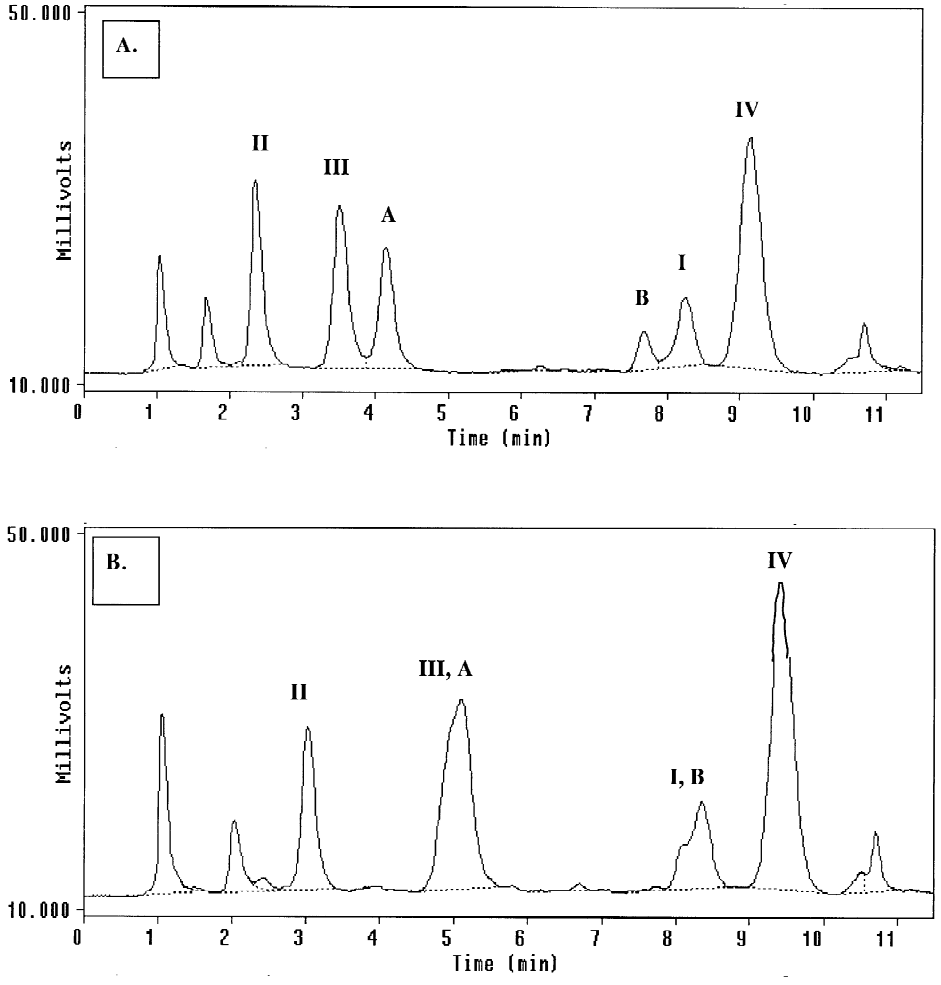


Fig. 4. The effect of mobile phase pH on the separation of **I** and **III** from unidentified human plasma metabolites A and B. (A) pH 4.5 mobile phase, (B) pH 5.0 mobile phase.

73.7, and 87.3% for compounds **I**, **II**, **III**, and **IV**, respectively.

3.7. Assay selectivity

Chromatograms of extracted drug-free plasma containing 500 ng/ml **IV** and a plasma standard containing 50 ng/ml **II**, 100 ng/ml **III**, 100 ng/ml **I**, and 500 ng/ml **IV** are shown in Fig. 5A and B, respectively. Chromatograms of extracted clinical plasma samples taken from a subject 0.75, 2.5 and 6.5 h after receiving 120 mg/m^2 compound **I** intravenously are shown in Fig. 5C, D, and E, respectively. Comparison of Fig. 5A with B demonstrates that there was no interference from endogenous plasma components. As mentioned in Section 3.5, an adjustment to the pH of the mobile phase was made during assay development in order to separate two unidentified metabolites (A and B) from compounds **III** and **I**, respectively, in chromatograms of post dose plasma extracts. Unlike compounds **I** and **III**, which reached peak plasma concentration (C_{max}) at the end of the intravenous infusion period and then declined rapidly, metabolites A and B achieved C_{max} at later time points (usually 6–12 h post dose). Failure to chromatographically separate them from the analytes would have led to significant errors in the determination of the plasma concentrations of **I** and **III**, and in subsequent calculations of pharmacokinetic parameters such as area under curve (AUC), and half-life.

3.8. Linearity

Weighted (weighting factor$=1/x$, where $x=$ concentration) least-squares regression calibration curves, constructed by plotting the peak height ratio of analytes **I**, **II**, and **III** to internal standard versus the standard concentrations yielded coefficients of regression typically greater than 0.999 over the concentration ranges of the standard lines. The use of weighted least-squares regression resulted in less than a 10% (15% at the lowest concentration) deviation between the nominal concentration and the experimentally determined concentration calculated from the regression equation.

3.9. Precision and accuracy

Replicate ($n=5$) standards containing **I**, **II,** and **III**, were analyzed to assess the within-day variability of the assay. The mean accuracy of the assayed concentrations as well as the standard deviation (RSD) of the plasma replicate standards are presented in Table 3.

Plasma quality control (QC) samples containing drug and metabolites at low (20 ng/ml for **II** and 40 ng/ml for **I** and **III**), middle (100 ng/ml for **I** and 200 ng/ml for **I** and **III**), and high (1000 ng/ml for **II** and 2000 ng/ml for **I** and **III**) concentrations were prepared and frozen at −70 °C in 0.75-ml aliquots. Two pairs of quality control samples were assayed with each of 30 standard curves over a 2-year period. The results (Table 4) indicated that the long term inter-day variability (RSD) of the method was under 10%. The results also demonstrated that drug and metabolites were stable for a period of at least 2 years in plasma stored at −70 °C.

3.10. Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded a within-day RSD of less than 10% and accuracy between 85 and 115% of nominal was 25 ng/ml for **I** and **III** and 12.5 ng/ml for **II**.

4. Conclusions

The development of a simultaneous method for the determination of peptide prodrug **I** and its metabolites doxorubicin (**II**) and leucine–doxorubicin (**III**) required careful design of the sample collection procedure in order to avoid degradation of the prodrug and ex vivo partitioning of the metabolites into red blood cells. Rapid chilling of the blood samples ensured that plasma/red blood metabolite concentrations did not change during sample processing and the addition of EDTA effectively prevented the hydrolysis of the prodrug to leucine–doxorubicin (**III**) in plasma. The observation that unidentified metabolites coeluted with **I** and **III** demonstrated the importance of optimizing the chromatographic conditions on post dose rather than

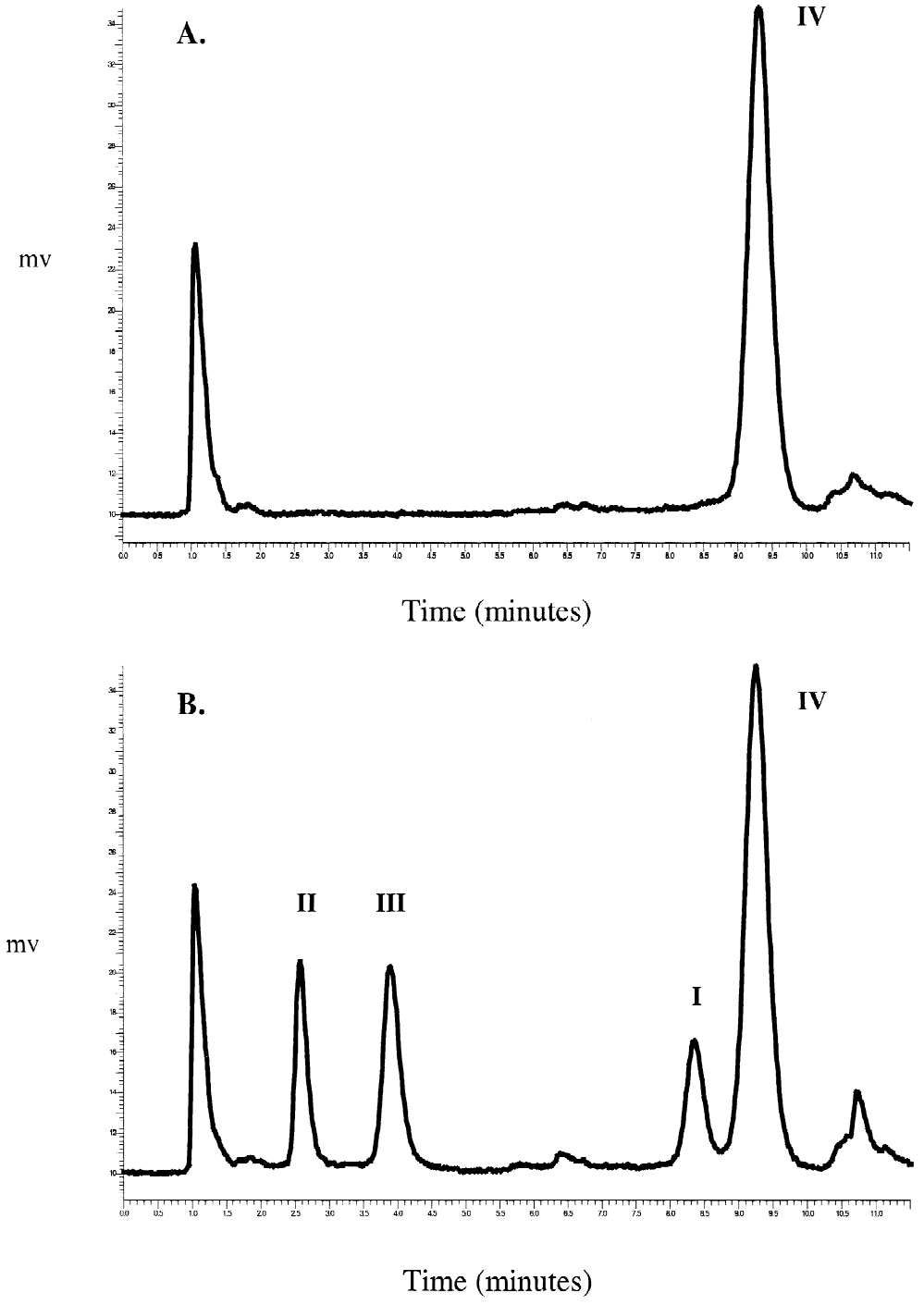
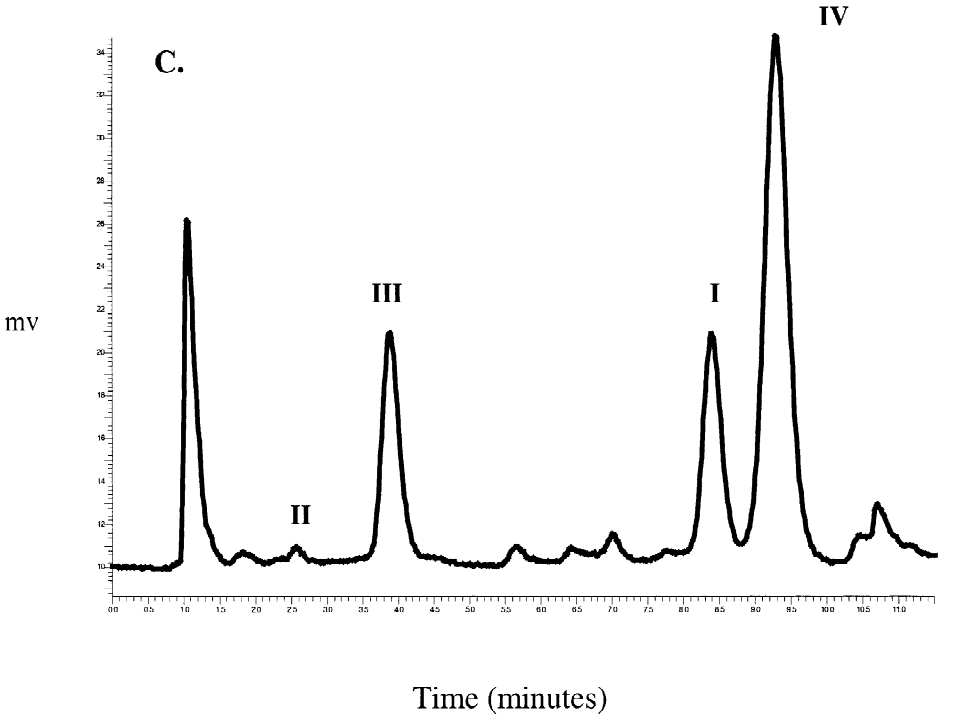


Fig. 5. Representative chromatograms of **I**–**IV** in spiked and post-dose human plasma. (A) Control plasma containing 500 ng/ml **IV** (internal standard), (B) control plasma containing 50 ng/ml **II**, 100 ng/ml **I** and **III**, and 500 ng/ml **IV**, (C) 45 min post dose plasma (diluted ten-fold with control plasma) from a patient dosed intravenously with 120 mg/m^2 **I**, (D) 2.5-h post dose plasma, (E) 6.5-h post dose plasma.



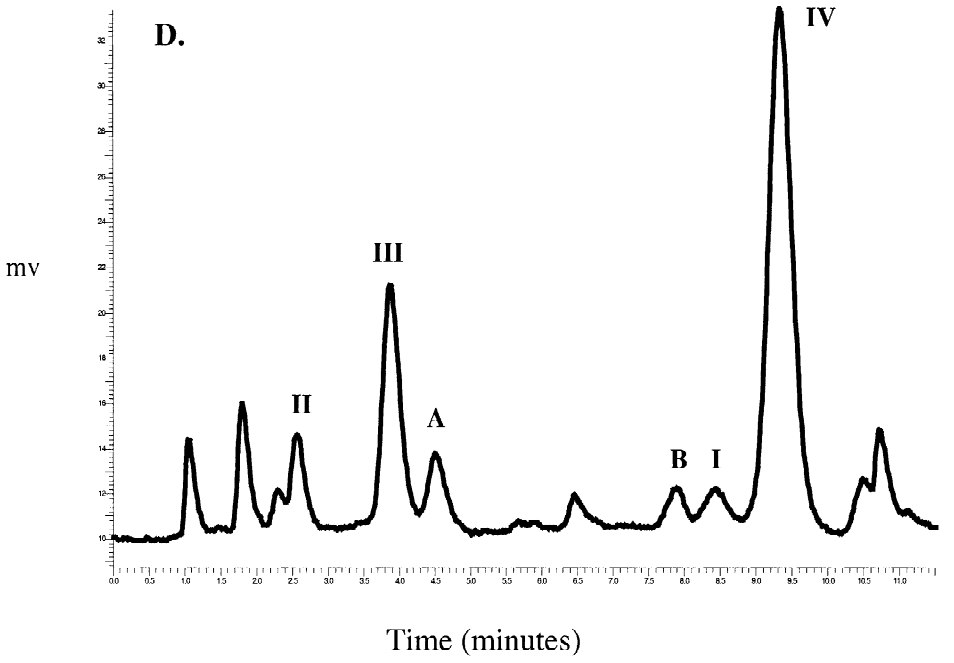


Fig. 5. (*continued*)

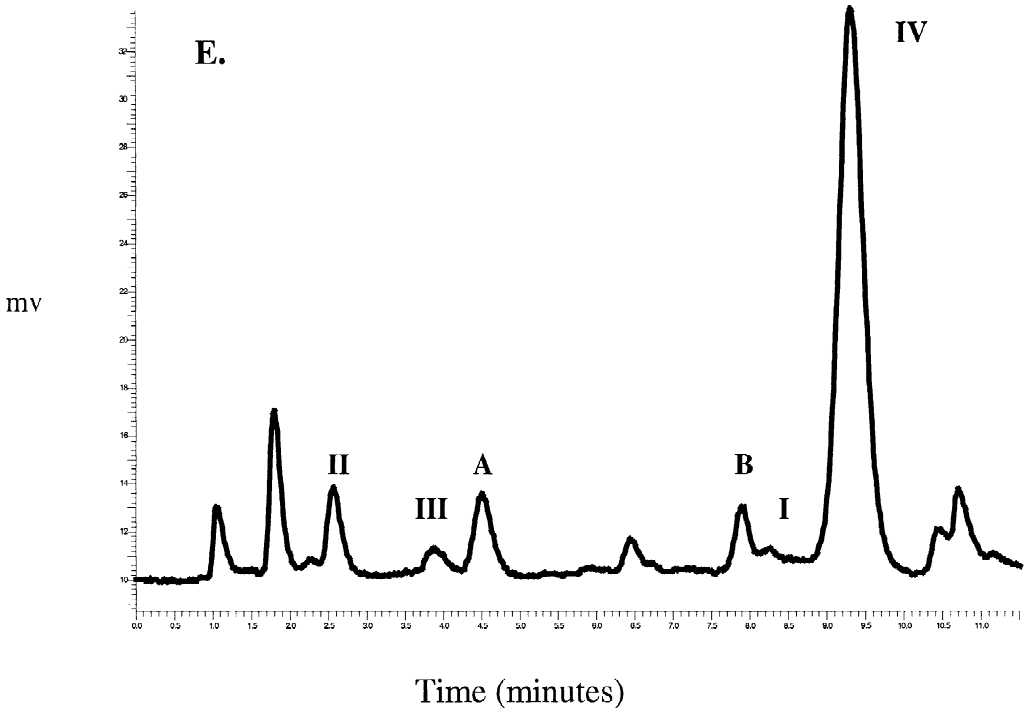


Fig. 5. (*continued*)

spiked plasma samples. Separation of the interfering metabolites was achieved by a simple adjustment of the mobile phase pH. A 1-min column washout step (using 90% acetonitrile) at the end of each chromatographic analysis improved the long term retention time reproducibility of the two basic metabolites, insuring that those analytes eluted within narrow retention time windows.

Table 3
Within-day precision and accuracy data for the determination of **I**, **II**, and **III** in human plasma as assessed by the replicate ($n=5$) analysis of standards

Analyte	Concentration range (ng/ml)	Accuracy[a] (%)	Precision[b] (%)
I	25–2500	92.8–104.2	2.2–5.7
II	12.5–1250	91.8–104.5	2.9–6.9
III	25–2500	86.6–105.8[c]	4.2–6.0

[a] Expressed as [(mean observed concentration)/(nominal concentration)]·100.

[b] % Coefficient of variation of peak height ratios of the drug to internal standard.

[c] At the limit of quantification (LOQ, 25 ng/ml).

With the above sample preparation procedures and modifications, the method was found suitable for the analysis of plasma samples from clinical studies of **I** at intravenous doses of 20–315 mg/m^2 [6].

Table 4
Inter-day variability of the plasma assay for the determination of **I**, **II**, and **III**, as assessed by the analysis of low, middle, and high concentration quality control (QC) samples

Analyte	QC concentration (ng/ml)	Accuracy[a] (%)	Precision[b] (%)
I	40	99.5	7.1
	200	102.6	5.6
	2000	100.0	5.1
II	20	99.2	8.0
	100	100.2	7.6
	1000	102.0	6.8
III	40	95.6	6.9
	200	101.4	5.6
	2000	99.8	5.4

[a] $n=30$, over a 2-year period, expressed as [(mean observed concentration)/(nominal concentration)]·100.

[b] % Coefficient of variation of daily analyzed QC concentrations.

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