The Identification of Three Human Metabolites of a Peptide–Doxorubicin Conjugate Using HPLC–MS–MS in Positive and Negative Ionization Modes

R.B. Desai*, M.S. Schwartz, and **B.K. Matuszewski**
Merck Research Laboratories, Sumneytown Pike, West Point, PA 19486

Abstract

A peptide–doxorubicin conjugate (I) is a drug candidate that has been evaluated for the treatment of prostate cancer. During the high-performance liquid chromatographic (HPLC)–fluorescence analysis of clinical samples for compound I and its two known metabolites [doxorubicin (II) and leucine–doxorubicin (III)], additional metabolites are observed in postdose human plasma extracts. Using neutral loss, precursor, and product ion tandem mass spectrometric (MS–MS) experiments, two of these metabolites are identified as doxorubicinol (IV) and leucine–doxorubicinol (V), the active 13-hydroxy metabolites of doxorubicin and leucine–doxorubicin, respectively. A third metabolite, 7-deoxydoxorubicinol aglycone (VI), is detected using single-ion monitoring at *m/z* 399 in the negative ionization mode. The product ion mass spectrum of this metabolite contains a major fragment at *m/z* 351, resulting from the loss of water and formaldehyde from the pseudomolecular ion. An HPLC–MS–MS method for simultaneous analysis of II, III, IV, V, and VI is developed utilizing gradient HPLC with a combination of positive/negative ionization MS in the multiple reaction monitoring mode and monitoring the appropriate MS–MS transitions. Using this methodology, rat, dog, and human plasma metabolite profiles are compared and found to be qualitatively similar. Simultaneous fluorescence and MS detection experiments confirm that the peaks observed in the HPLC–fluorescence chromatograms of plasma extracts correspond to each of the five metabolites (II–VI).

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 (Figure 1), a peptide–doxorubicin conjugate was designed to deliver the conventional cytotoxic drugs doxorubicin and leucine–doxorubicin (II and III, respectively, Figure 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. PSA-mediated cleavage of the peptide allows the active metabolites doxorubicin and leucine–doxorubicin to enter the cells and exert cytotoxic effects. The peptide conjugate (I) was shown to selectively kill PSA producing prostate cancer cells both in tissue culture and in nude mice with xenografts (2) and was evaluated for the treatment of prostate cancer (1).

A high-performance liquid chromatographic (HPLC)– fluorescence method for the determination of I, doxorubicin (II), and leucine–doxorubicin (III) in plasma was developed for supporting human pharmacokinetic studies (3). During the analysis of postdose plasma samples, chromatographic peaks from three unknown metabolites (peaks A, B, and C in Figure 2) were observed. Unlike other minor metabolite peaks, metabolites A, B, and C were observed consistently and appeared to have long half

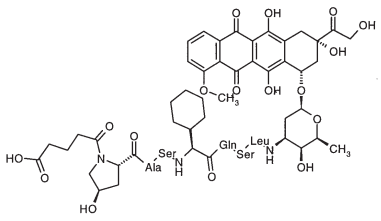

Figure 1. Peptide doxorubicin conjugate (I) and its active metabolites, doxorubicin (II) and leucine–doxorubicin (III).

* Author to whom correspondence should be addressed: email rajesh_desai@merck.com.

lives. Two potential difficulties in the identification of metabolites A, B, and C by tandem mass spectrometry (MS–MS) were low plasma concentrations and the unavailability of purified metabolites from *in vitro* sources or synthetic reference standards.

Published methods have described the analysis of II, III, and metabolites in biological fluids utilizing HPLC with fluorescence detection (3,5,6). In one publication (5), leucine–doxorubicin (III) and six human plasma metabolites of III were simultaneously assayed by an HPLC–fluorescence method. The metabolites were separated by isocratic HPLC and identified by HPLC retention time. The difficulty with this approach was that not all the potential metabolites were chromatographically separated and that structural information about metabolites was not obtained during the analysis to confirm the identities of the metabolites. Another paper (7) reported the analysis of II and a single metabolite in human plasma using HPLC–MS, but the structure of the metabolite was not confirmed by MS–MS.

The aim of this study was to obtain direct structural information on the three unidentified plasma metabolites of compound I through the use of HPLC–MS–MS and to determine if they corresponded to the doxorubicin or leucine–doxorubicin metabolites reported in the mentioned papers. Also described is the development of a gradient HPLC–MS–MS methodology used to compare human metabolite profiles with those of animals (rats and dogs) used in preclinical safety studies.

Experimental

Chemicals

Compound I was obtained from the Chemical Data Department of Merck Research Labs (Rahway, NJ). The HCl salt of II was purchased from Sigma (St Louis, MO) and of III was synthesized by Dr. Garsky (Medicinal Chemistry, MRL, West Point, PA). Doxorubicin aglycone, a potential metabolite, was synthesized by mild acid hydrolysis of doxorubicin (4). Ten milligrams of doxorubicin was dissolved in 2 mL of 1% HCl and heated for 6 h at 65°C. The insoluble aglycone was washed with water, dried, and redissolved in dimethyl sulfoxide. Acid hydrolysis of I and III yielded the same product when assayed by HPLC with fluorescence detection. All HPLC solvents were obtained from Fisher Scientific (Hampton, NH). All other reagents were of ACS grade and were used as received.

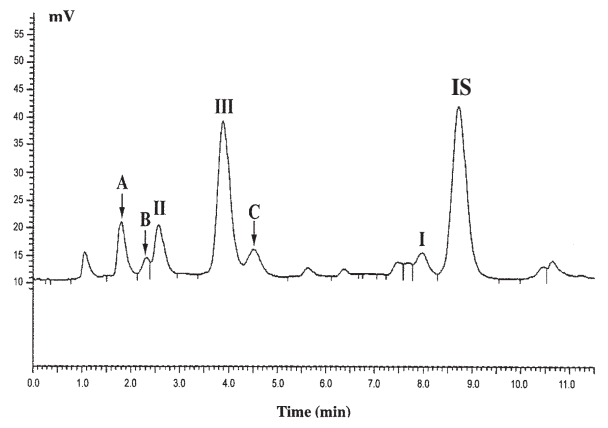

Figure 2. HPLC–fluorescence chromatogram of a 2.5-h postdose human plasma extract after I.V. dosing with I under chromatographic conditions described in the literature. Unknown metabolites are indicated as peak A, B, and C; IS is the internal standard used in method.

Plasma samples

Human plasma samples (0–24 h postdose) were obtained from 2 patients dosed intravenously with I (225 mg/M^2). Plasma from 3 rats and 2 dogs dosed intravenously with 40 mg/kg and 5 mg/kg, I respectively, was obtained from Preclinical Drug Metabolism (West Point, PA). All plasma samples were prepared for analysis by C-8 solid-phase extraction (SPE) as described in the literature (3).

Instrumental

Chromatography for HPLC–MS–MS analysis (A)

The HPLC system consisted of a PerkinElmer (Norwalk, CT) series 200 autosampler and two microflow pumps. Chromatography was performed on a Keystone Scientific (Bellefonte, PA) Betabasic C-8 (2.1 × 100 mm) column at a flow rate of 0.2 mL/min and a temperature of 35°C. A linear gradient composed of two components was used for the analysis. Component A was 10mM ammonium acetate buffer (pH 4.5) and component B was 90:10 acetonitrile–water (v/v). The following gradient profile was used: 100% A (0–1 min), linear gradient from 100% A to 60% A (1–7 min), and 60% A (7–9 min). Component B was pumped at 100% for 1 min at the end of each run to wash out the column. The sample injection volume was 20 µL.

Chromatography for the combined HPLC–fluorescence and MS–MS experiments (B)

The HPLC system in these experiments consisted of a PerkinElmer series 200 autosampler and 2 microflow pumps. A PerkinElmer LC240 fluorescence detector was used. Chromatography was performed on a Keystone Scientific Betabasic C-8 (3 × 100 mm) column at a flow rate of 1 mL/min and a temperature of 35°C. An 80:20 (fluorometer–MS) flow split was maintained at the column outlet. Fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 560 nm. A step gradient composed of two components was used for the analysis. Component A was 10mM ammonium acetate buffer (pH 4.5) and component B was 90:10 acetonitrile–water (v/v). Component A was pumped for 5 min and the column was washed with 90% acetonitrile (component B) for 1 min. The 1-min wash was needed to elute I and to maintain consistent retention times. The column was equilibrated for 5 min before the next injection. The sample injection volume was 20 µL.

MS conditions

MS analyses were performed on PE Sciex (Toronto, Canada) API-3000 MS using a turbo ionspray interface (TISP) at a source temperature of 300°C. Positive ion analyses typically employed ionizing, orifice, and collision voltages of 4800, 30, and –16.5 V, respectively. In the negative ionization mode, the ionizing, orifice, and collision voltages were –3280, –80, and 28 V, respectively.

Sample preparation

Plasma was prepared for analysis using C-8 SPE columns. One

half milliliter of plasma was diluted with an equal volume of water and applied to a conditioned 50-mg, 1-mL C-8 SPE cartridge (Varian, Walnut Creek, CA). After washing with 1 mL water and 1 mL 10% methanol, residual wash solvent was removed by centrifugation of the cartridge at 2000 × g for 1–2 min. After transfer to a 13- × 100-mm glass tube, 1 mL elution solvent (0.5M 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) 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 (90:10, v/v, mobile phase A–water) and transferred to an autosampler vial containing a glass, limited-volume insert. The vial was capped and placed in an autosampler tray for injection.

Results and Discussion

Reduced metabolites of doxorubicin and leucine–doxorubicin

Initially, extracts of human plasma were screened for peptide–doxorubicin conjugate metabolites by HPLC–MS in the positive ionization mode. In order to maximize sensitivity, Q1 scanning was not used. Instead, specific ions corresponding to known doxorubicin and leucine–doxorubicin metabolites (5,6) were monitored. Total ion chromatograms of predose plasma extracts contained many nondrug-related peaks, and no obvious differences were noted between extracts of pre- and postdose samples. Because neutral loss of the aglycone moiety (MW = 414) dominated the (positive) product ion spectrum of III (Figure 3, *m/z* 657 to 243) and II (*m/z* 544 to 130, mass spectrum not shown), neutral loss (of 414) experiments were performed on the extracts as a more selective screen for metabolites. Only two metabolites corresponding in molecular weight and HPLC retention time to doxorubicin (II) and leucine–doxorubicin (III) were detected in this experiment. Product ion mass spectra of the protonated molecules of these metabolites were practically the same as those of synthetic standards. Metabolites of II and III had been reported in which the 13-keto group of the aglycone was metabolically reduced to an alcohol (doxorubicinol and leucine– doxorubicinol) (Figure 4, IV and V, respectively) (5,6). Because metabolites of I containing a reduced aglycone moiety might be expected to undergo a neutral loss of 416, additional HPLC–MS–MS experiments (neutral loss of 416) were performed to selectively detect this type of molecule. In this case, two new metabolites were observed (Figure 5). The metabolites, M-1 and M-2, eluted at 3.02 and 3.96 min, respectively. Examination of the Q1 spectrum of M-1 revealed that the (*m/z*) value of the protonated molecule was 2 amu greater than that of II. Likewise, the (*m/z*) value of the protonated molecule of M-2 was 2 amu greater than that of III, suggesting that the metabolites detected in the neutral loss experiment were indeed doxorubicinol (IV) and leucine– doxorubicinol (V).

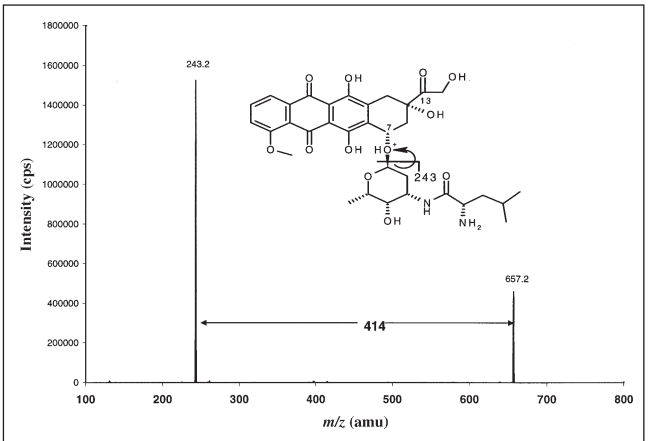

Figure 3. Product (positive) ion mass spectrum of synthetic leucine–doxorubicin (III).

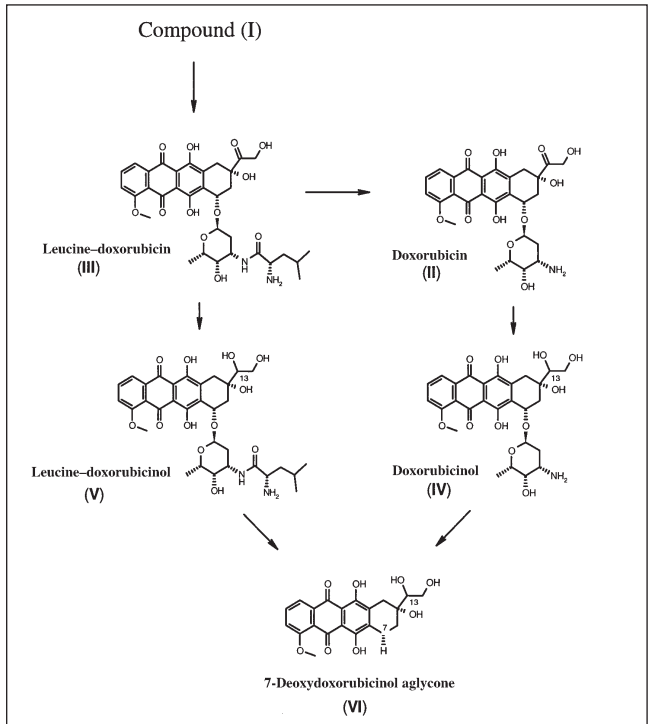

Figure 4. Chemical structures of metabolites of compound I and proposed metabolism pathways.

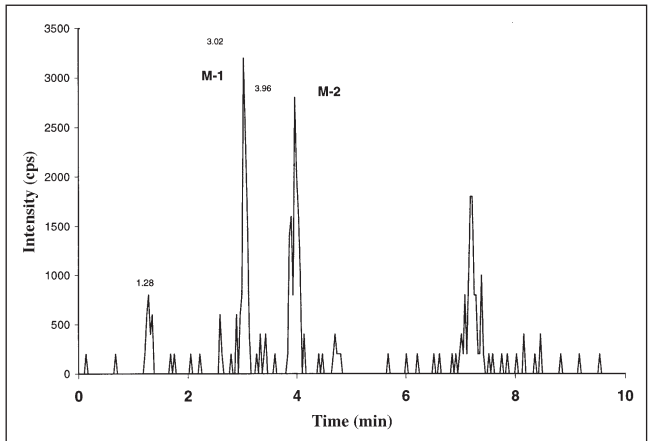

Figure 5. Analysis of human postdose extract by gradient HPLC–MS–MS in the positive ion, neutral loss mode. Total ion chromatogram; masses of M-1 and M-2 were 546.5 and 659.3, respectively.

In order to confirm the identities of the metabolites, product ion MS of M-1 (*m/z* 546) and M-2 (*m/z* 659) were recorded. The MS of M-1 contained fragment ions at *m/z* 399 and 130 (Figure 6A). The fragment ion at *m/z* 130 corresponded to the (unchanged) amino sugar portion of doxorubicinol (IV) and the fragment at *m/z* 399 corresponded to the reduced aglycone. Similarly, the product ion MS of M-2 (Figure 6B) contained a major fragment at *m/z* 243 corresponding to the amino sugar–leucine portion of leucine–doxorubicinol (V) and a fragment at *m/z* 399 consistent with a reduced aglycone.

Stereochemistry of doxorubicinol

The metabolic reduction of doxorubicin to doxorubicinol by a mammalian aldo-keto reductase creates a new chiral center at carbon 13 (IV, Figure 4). This enzymatic reduction was reported to be stereoselective, producing only one of the two possible stereoisomers (8). A high resolution HPLC–fluorescence method was used in our laboratory to confirm that compound I was metabolized to a single diastereomer of doxorubicinol (unpublished data).

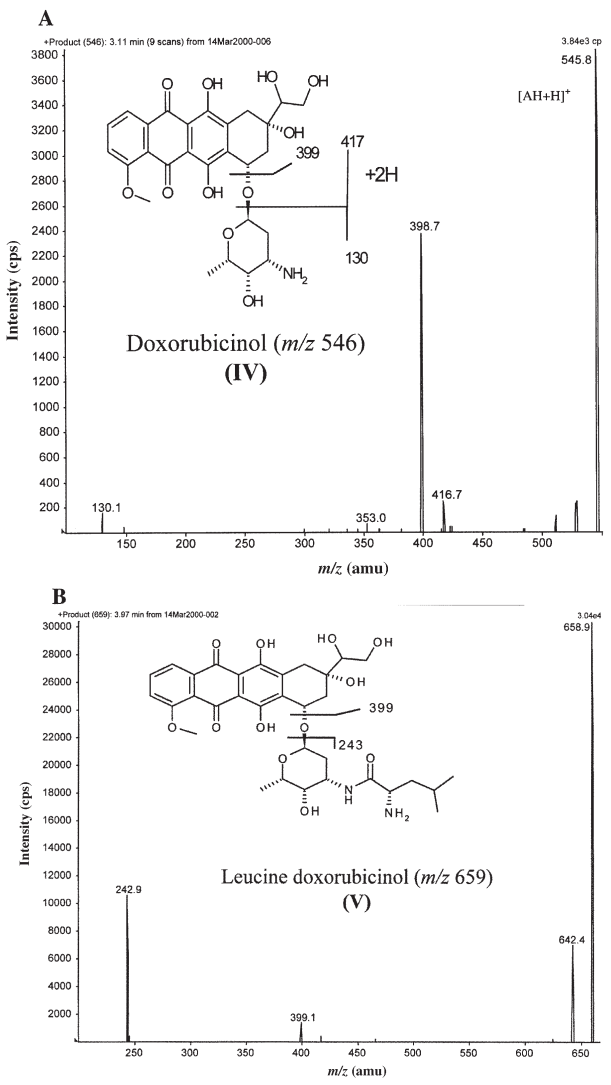

Figure 6. Positive product ion mass spectra of M-1 (Peak A, Figure 2) and M-2 (Peak B, Figure 2) and proposed structure of M-1 and M-2.

Aglycone metabolites

As mentioned in the Introduction section, three unidentified metabolites of I (Figure 2, peaks A, B, and C) were observed in postdose plasma assayed by a clinical HPLC–fluorescence assay. Although synthetic doxorubicin aglycone (Figure 7) coeluted with peak C under the same chromatographic conditions, this was insufficient evidence to establish the identity of the metabolite. Because of the low metabolite concentration in plasma, conditions for the sensitive detection of doxorubicin aglycone by HPLC–MS were optimized before attempting to identify the metabolite by this technique. Q1 spectra of the synthetic aglycone were recorded in the positive and negative ionization modes. The positive ion MS contained a pseudomolecular ion at *m/z* 415 of low intensity. By comparison, the negative ion Q1 spectrum had a more intense $[M-H]^-$ at *m/z* 413. This suggested that the negative ionization mode would be better suited for the detection of aglycone metabolites. The improved stability of the negative pseudomolecular ion was attributed to the acidity of the molecule's two phenolic hydroxyl groups.

Human plasma extracts were then analyzed by gradient HPLC (see Instrumental section, chromatographic condition A) with negative ion MS detection. In order to maximize sensitivity and selectivity, the instrument was not operated in the scanning mode. Instead, four selected ions were monitored (*m/z* 413, 415, 397, and 399), corresponding to doxorubicin aglycone, doxorubicinol aglycone, 7-deoxydoxorubicin aglycone, and 7-deoxydox-

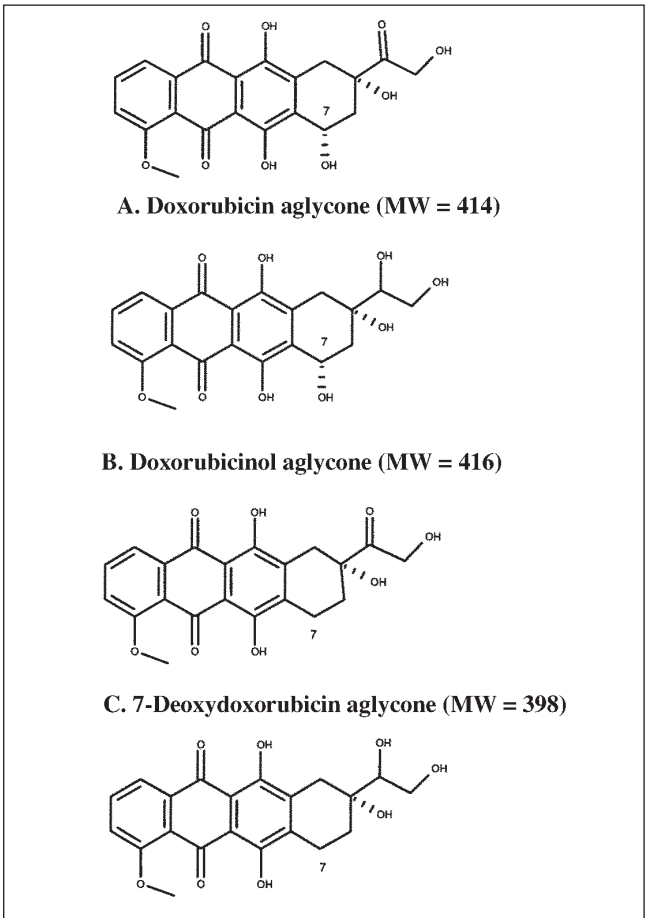

Figure 7. Structures of possible aglycone metabolites.

orubicinol aglycone, respectively (Figure 7). These molecules were previously reported as aglycone metabolites of II and III (5,6) and were considered here as potential metabolites of compound I.

Extracted ion chromatograms of pre- and postdose samples were compared in order to identify drug related peaks. No peak corresponding to doxorubicin aglycone was found in extracted ion chromatograms at *m/z* 413 of post dose plasma, indicating that doxorubicin aglycone was not a metabolite of doxorubicin–peptide conjugate I. However, a single drug-related peak corresponding in molecular weight to 7-deoxydoxorubicinol aglycone was observed in the extracted ion chromatogram at *m/z* 399 of postdose plasma (M-3, Figure 8A). The product ion MS of M-3 (*m/z* 399) was recorded and is shown in Figure 9. The spectrum contained a base peak at *m/z* 351 consistent with the loss of water and formaldehyde (48 amu) from VI. It was concluded that 7-deoxydoxorubicinol (M-3, VI) was the metabolite that corresponded to chromatographic peak C observed in the clinical HPLC–fluorescence assay (Figure 2). This was later confirmed in dual HPLC–MS–MS–fluorescence detection experiments (see Combined HPLC–fluorescence and MS–MS experiments section). Overall, these experiments confirmed that metabolite identification based on HPLC retention time alone is not sufficient and that an MS–MS technique or other confirmatory technique should be used to provide positive confirmation of the presence of metabolites.

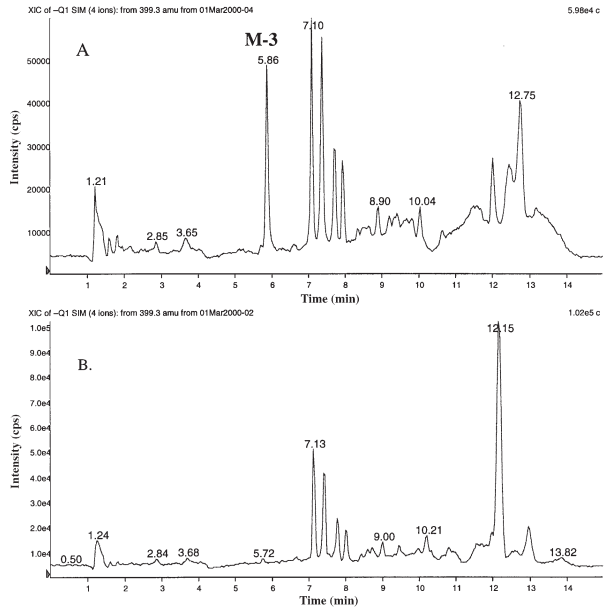

Figure 8. Extracted negative ion mass chromatograms (m/z 399.3) of human plasma extracts: postdose (A) and predose (B).

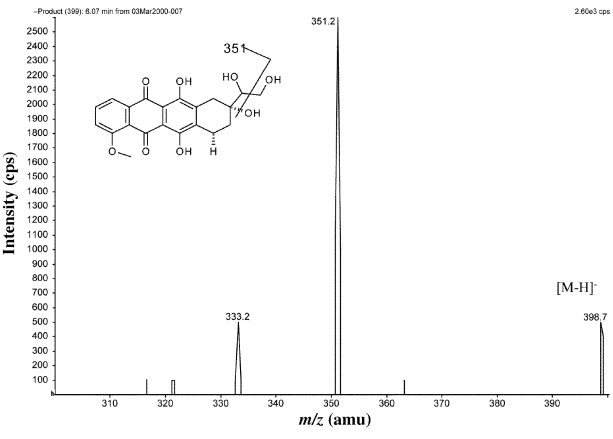

Figure 9. Product ion mass spectrum (*m/z* 399) and proposed structure of metabolite M-3 (7- deoxydoxorubicinol aglycone, VI).

HPLC–MS–MS analysis of metabolites in plasma extracts using positive/negative ion MRM detection

To achieve sensitive and selective detection of all metabolites in a single chromatographic run, multiple reaction monitoring (MRM) was used. The ionization mode was changed from positive to negative ionization midway through the run so that 7-deoxydoxorubicinol aglycone (VI) could be detected in the negative ion-

Table I. MRM Transitions and Ionization Modes Used for the Detection of Metabolites of Compound I

Metabolite	HPLC retention time* (min)	Ionization mode	MRM transitions (m/z)
IV	3.01	Positive	546.2 → 399.3
V	3.88	Positive	659.2 → 243.3
II	4.59	Positive	544.2 → 397.3
III	5.56	Negative	655.0 → 395.4
VI	5.82	Negative	399.1 → 351.2

* Using chromatographic system A described in the Instrumental section.

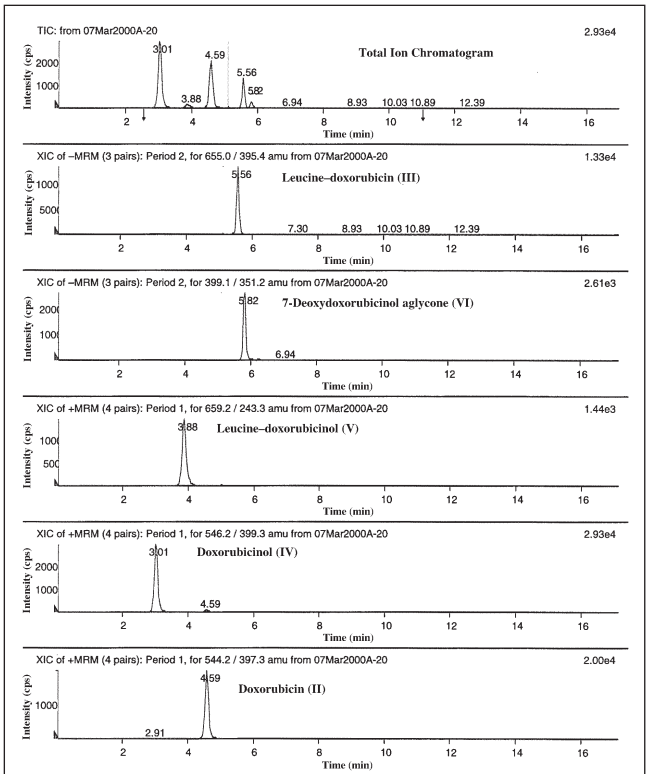

Figure 10. Total and extracted ion MRM chromatograms of metabolites of I in 6.5 h postdose human plasma.

ization mode. The retention times of the metabolites and the MRM transitions used for MS–MS detection are shown in Table I. Total and extracted ion chromatograms of a (pooled) 6.5 h postdose human plasma extract are shown in Figure 10.

Comparison of human, rat, and dog plasma metabolite profiles

Postdose human, rat, and dog plasma extracts of 1, 2, 4, 6, and 24 h were assayed by HPLC–MS–MS under the conditions described previously. Relative to doxorubicin, the human samples contained the highest concentrations of doxorubicinol (IV), followed by the dog and rat samples. 7-Deoxydoxorubicinol aglycone (VI) was found in the 2–6-h human and rat extracts, but was barely detectable in dog plasma.

Combined HPLC–fluorescence and MS–MS experiments

In order to fully establish the correspondence between the three metabolites observed in HPLC–fluorescence chromatograms from clinical studies (Figure 2, A–C) and those identified by MS–MS, HPLC analysis with simultaneous fluorescence and MS–MS detection was utilized (see Instrumental section, chromatographic conditions B). The same MRM transitions as described in Table I were utilized. Fluorescence and total ion chromatograms obtained from a pooled 6.5 h postdose human plasma extract were recorded and compared. Peaks observed in the HPLC–fluorescence chromatograms corresponded in retention time to each of the metabolites detected by the MS. This confirmed that the plasma metabolites identified by MS–MS corresponded to peaks observed in HPLC–fluorescence chromatograms.

Conclusion

HPLC–MS–MS analysis in the neutral loss mode is a useful technique for the selective detection of metabolites in complex biological matrices if characteristic neutral loss masses can be identified. In this work, MS–MS scanning for the neutral loss of doxorubicinol aglycone (MW = 416) led to the detection of two previously unknown reduced metabolites (IV and V) of compound I in post dose human plasma extracts.

A third metabolite, 7-deoxydoxorubicinol aglycone (VI), was detected using HPLC–MS in the negative ionization mode. Compound VI and doxorubicin aglycone (a potential metabolite) coeluted with a metabolite peak observed in HPLC–fluorescence chromatograms of post dose plasma extracts, but HPLC–MS analysis demonstrated that the samples contained compound VI, but no doxorubicin aglycone.

An HPLC–MS–MS method for the separation and detection of five metabolites of I was developed using MRM detection. Metabolites II, IV, and V were monitored in the positive ionization mode, while compounds III and VI were detected utilizing MS–MS in the negative ionization mode. The method enabled a qualitative comparison and profiling of metabolites present in human, rat, and dog plasmas.

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Manuscript accepted May 24, 2004.