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Development of a bioanalytical liquid chromatography method for quantitation of 9-nitrocamptothecin in human plasma

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Abstract

9-Nitrocamptothecin (9-NC) is an orally administered camptothecin (CPT) that is under evaluation in clinical trials. This compound is not fluorescent, which has hampered development of a sensitive high-performance liquid chromatographic (LC) assay for measurement of drug concentrations in clinical trials. We now report development of an assay that involves reduction of 9-NC to the fluorescent compound 9-aminocamptothecin (9-AC). The method is based on enzymatic reduction of 9-NC using bovine liver S-9 fraction. This method is validated to quantitate 9-NC and 9-AC in patient samples, and yields results comparable to those obtained with an LC/MS method.

Keyword: 9-Nitrocamptothecin

1. Introduction

Camptothecin (CPT) is a naturally occurring alkaloid that specifically targets the nuclear enzyme DNA topoisomerase I (Top 1) [1]. Identification of Top 1 as anti-cancer drug target led to synthesis and testing of numerous synthetic analogs of CPT as potential anti-cancer agents. Substitutions at the 9-position (Fig. 1) are associated with enhanced antitumor activity relative to the parent compound [2]. 9-NC demonstrated a high degree of antitumor activity in human cancer xenografts of colon, lung, breast, and melanoma in nude mice and in cell lines that were resistant to doxorubicin and 5-FU [3]. In phase II studies, responses to 9-nitrocamptothecin (9-NC) were reported in patients with pancreatic carcinoma, ovarian carcinoma, breast carcinoma, and acute myelogenous leukemia [4]. Studies of 9-NC metabolism in cell lines and in liver, kidney, lung and other tissues indicate significant conversion of the drug to the active metabolite 9-aminocamptothecin (9-AC) [5,6]. Indeed, we identified an enzymatic activity in bovine and human liver subcellular fractions that is capable of rapidly reducing 9-NC to 9-AC. We now report the use of this enzymatic activity in the development of a simple, sensitive, and accurate enzymatic-liquid chromatographic (LC) method for the quantitation of total (lactone and carboxylate forms) 9-NC in human plasma.

2. Experimental

2.1. Chemicals

9-Nitrocamptothecin and 9-aminocamptothecin, along with the internal standard (IS), 12-nitrocamptothecin (12-NC), were supplied by SuperGen Pharmaceutical Research

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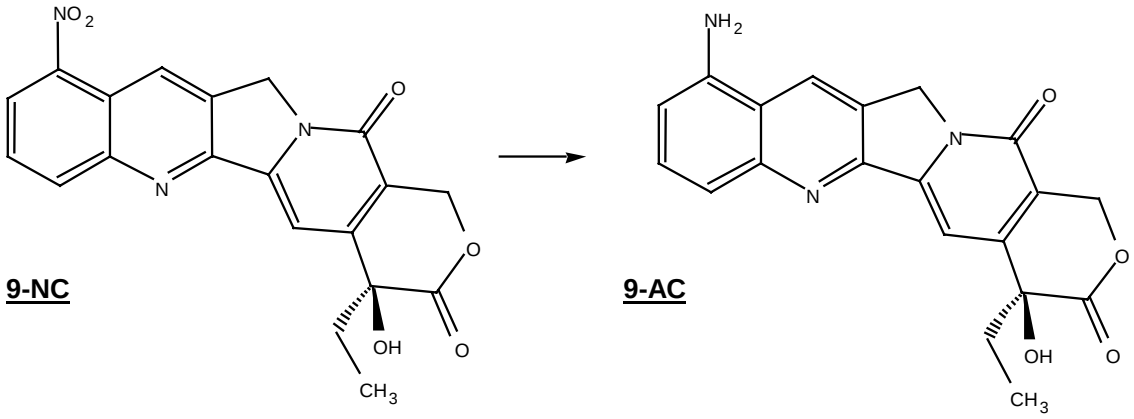

Fig. 1. Chemical structures of 9-nitrocamptothecin and its metabolite 9-aminocamptothecin.

Institute (Dublin, CA, USA). Flavin adenine dinucleotide (FAD), glucose-6-phosphate, glucose-6-phosphate dehydrogenase (G-6-PDH)) (EC 1.1.1.49 Type XV Bakers yeast), nicotine adenine dinucleotide phosphate (β-NADP), and $MgCl_2$, dimethyl sulfoxide (DMSO) were obtained from Sigma. Bovine serum albumin and protein assay reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). HPLC grade solvents methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals (ACS grade) were purchased from JT Baker (Phillipsberg, NJ, USA). Human plasma was obtained from New Brunswick Affiliated Hospitals Blood Center (New Brunswick, NJ, USA) and from Biochemed Pharmacologicals (Winchester, VA, USA). SPEC PLUS™ 96-WELL C18 extraction plates were obtained from Ansys® Diagnostics Inc. (Lake Forest, CA, USA).

2.2. Bovine liver S-9 fractions preparation

Fresh bovine liver tissues were collected on dry ice. All tissue processing was carried at 0–4 °C. The tissue was cut into small pieces and stored at –80 °C until further use. For the preparation of liver S-9 fraction, the tissue was homogenized in 100 mM phosphate buffer pH 7.4 (1:3 w/v) and centrifuged at 800 × *g*, with the resulting supernatant further centrifuged at 10,000 × *g* for 30 min. The supernatant (S-9 fraction) was collected and stored at –80 °C until further use. The protein content in the S-9 fractions was quantitated according to the Bradford method [7] using bovine serum albumin as a reference standard.

2.3. Preparation of standards solutions

2.3.1. Preparation of stock and working standard solutions for HPLC analysis

Stock solutions of 9-NC (3.935 mg/ml) and 9-AC (3.635 mg/ml) were prepared in DMSO and stored at −80 °C. From the primary stock solution an intermediate stock solution 100 μg/ml was prepared in DMSO. From the intermediate stock a set of eight calibration standards of 9-AC at concentrations 0.98, 1.96, 3.9, 7.8, 15.6, 31.25, 62.5, and 125 ng/ml were prepared in100 mM phosphate buffer pH 7.4, and in donor plasma by serial dilution of stock solutions. All solutions were stored at −80 °C. The standard solutions were stored at −80 °C. Working standards stored at −80 °C were stable for at least 3 months.

2.3.2. Preparation of stock and working standard solutions for LC/MS analysis

Two individual aliquots of each test article were used to prepare the calibration standards and quality control (QC) samples. Stock solutions of 9-NC, 9-AC, and 12-NC were prepared in dimethyl sulfoxide at concentrations of 1.0 mg/ml. Equal volumes of 9-NC and 9-AC stock solutions were combined followed by successive dilutions with methanol to provide a set of either working standard or QC solutions. All stock and working solutions were stored at −20 °C prior to use. The internal standard, 12-NC working solution was freshly prepared daily in 10% formic acid aqueous solution at a concentration of 5 ng/ml.

2.4. Enzymatic conversion of 9-NC to 9-AC

2.4.1. Preparation of reaction buffer

The enzymatic reduction of 9-NC to 9-AC required cofactors NADPH and FAD. Hence, all studies involving conversion of 9-NC to 9-AC employed an NADPH-generating system (10 mM NADP, 33 mM glucose-6-phosphate, 33 mM $MgCl_2$, 50 U/ml of glucose-6-phosphate dehydrogenase, according to Desta et al. [8], with 10 mM FAD (reaction buffer). In our preliminary studies both FAD and FMN gave similar results (data not shown). Hence, FAD was used in subsequent studies. The reaction buffer stored at −80 °C was stable at least a month. All the reactions were carried out in 1.5 ml amber colored Eppendrof polypropylene tubes.

2.4.2. 9-NC assay conditions and sample preparations

The patient plasma samples (200 μl) were pipetted into 1.5 ml polypropylene tubes containing 140 μl of the reaction buffer and the enzymatic reaction was started by addition of

20 μl of cold S-9 fraction (about 1.2 mg protein) to each tube. The samples were vortexed for 10 s, centrifuged at 700 × *g* for 10 s, and incubated at 37 °C for 60 min. The reaction was terminated by addition of 1140 μl of 20 mM ice cold acidified methanol (1.66 ml HCl diluted in methanol to 1 l). The tubes were vortexed for 20 s and left on ice for 5 min and then centrifuged at 10,000 g for 10 min. The supernatant was diluted (1:1) with mobile phase transferred to autosampler vials and then injected into the HPLC system. To check the assay efficiency, known concentrations of 9-NC standards were spiked in buffer, and in control donor plasma and were processed similarly to assess the quantitative conversion of 9-NC to 9-AC.

For the preparation of calibration standard curve, 9-AC calibration standards (200 μl) were mixed with 160 μl phosphate buffer pH 7.4, and 1140 μl of cold acidified methanol, vortexed briefly and processed for HPLC analysis similarly as in Section 2.4.2. The calibration curves were constructed based on the peak heights versus nominal standard concentrations ratios. Linear regression model was used to determine the concentrations of the analyte in the sample.

2.5. High-performance liquid chromatography

The high-performance liquid chromatographic systems (Hitachi 7000 series, Hitachi Ltd., Tokyo, Japan) consisted of Hitachi L-7200 autosampler with 500 μl loop, L-7100 quaternary pump, L-7480 fluorescence detector, and D-7000 interface data processing software. The chromatographic analysis of 9-NC and 9-AC in samples was performed using modification of a previous method [9]. Briefly, the separation of analyte in the samples were performed using reverse phase YMC ODS AQ 150 mm × 4.6 mm 5 μm particle column (Waters Corp., Milford, MA, USA) maintained at 45 ± 1 °C; protected by a μBondapak C18 precolumn inserts (Waters Corp., Milford, MA, USA). The mobile phase 25 mM potassium dihydrogenphosphate pH 2.5 ± 0.05: acetonitrile: methanol (79:15:6) was delivered at 2.0 ml/min under isocratic solvent delivery mode. To achieve highest sensitivity, the detector lamp energy and spectral bandwidth were set at high and 15 nm, respectively. The fluorescence detector was set at excitation and emission wavelengths of 365 and 440 nm, respectively. Samples were diluted with mobile phase buffer 1:1 to decrease band broadening as well as to decrease the asymmetry of the 9-AC peak. Standards and sample solutions (225 μl) were injected into the system and run for 13 min. The retention time of 9-AC was about 9.5 min. When the samples were prepared in clear glass autosampler vials, the chromatograms showed a large degraded FAD peak eluting before the 9-AC peak. Hence, all the samples were analyzed using amber vials.

2.6. LC/MS/MS assay

Liquid chromatography–mass spectrometer (LC/MS/MS) analyses of 9-NC in patient samples were performed by the previously described method (Gu et al., 2002, unpublished). Briefly human plasma calibration standards and QC samples were prepared by spiking 100 μl of a working standard or QC solution into control human plasma to make 2 ml of each standard or QC sample. A set of seven calibration standards was prepared at concentrations of 0.2, 0.5, 1, 5, 10, 15, and 20 ng/ml to establish a calibration curve in a range of 0.2–20 ng/ml. A 500 μl aliquot of each human plasma sample was combined with 200 μl of 12-NC IS working solution, was then purified by solid phase extraction using a SPEC PLUS™ 96-WELL C18 extraction plate. Following a washing step (500 μl of deionized water) to remove plasma proteins, the analytes and IS were recovered by eluting the plate with 500 μl of 0.2% formic acid methanol solution followed by 500 μl of 0.2% formic acid acetonitrile solution. The methanol and acetonitrile eluates were combined and evaporated to dryness using an EvapArray™ system, followed by reconstitution in 200 μl of methanol:2% aqueous formic acid (1:1, v/v), and analysis by LC/MS/MS.

2.6.1. LC/MS instrumentation and conditions

The chromatographic system consisted of a Waters 2690 Separations Alliance HPLC system (Milford, MA, USA) with a Zorbax XDB-C18 column (150 mm × 4.6 mm i.d., particle size 3.5 μm, Rockland Technologies Inc., Newport, DE, USA). The eluting solvent, which consisted of 1% aqueous formic acid and 1% methanolic formic acid solutions (43:57, v/v), was pumped through the column at a flow rate of 0.8 ml/min. The autosampler tray was thermostatically controlled at about 4 °C and the column was maintained at about 35 °C. The sample injection volume was 35 μl.

Detection was performed using an API-365 liquid chromatography–tandem mass spectrometer system (LC/MS/MS, PE Sciex, Concord, Toronto, Canada) with APCI interface operating in the positive ion mode. The heated nebulizer was maintained at a temperature of 500 °C with a discharge ionization of 3 μA. Nitrogen, with a flow rate of 14 l/min and pressure of 90 psi, was used as both an auxiliary and nebulizing gas. Quantitation was achieved using the multiple reaction monitoring (MRM) mode with ion transitions of *m/z* 394 → 350 for 9-NC, *m/z* 364 → 320 for 9-AC, and *m/z* 394 → 350 for 12-NC. Data were collected and processed using PE Sciex MacQuan™ 1.5 data collection and integration software, run on a G3 Power Macintosh personal computer (Apple Computer Inc., Brea, CA, USA). The calibration curve was constructed based on the ratios of analyte peak to IS peak areas versus nominal standard concentrations. A linear regression model was used with a weighting factor of $1/(\text{concentration})^2$.

2.7. Quantitation of 9-NC concentrations in patient plasma samples

The method was applied in a phase I non-randomized pharmacokinetic study of oral 9-NC (Rubitecan) and

cisplatin for patients with advanced malignacies. The patients received a single intravenous administration of cisplatin (40 mg/m^2) on day 1 and escalating doses of oral 9-NC 1.5, 1.75, 2, and 2.25 mg daily × 5, every week × 3. On day 15, blood samples were collected prior to the first dose of 9-NC (predose) and 1, 2, 3, 4, 6, 8, 10, and 24 h after the first dose as part of an institutionally approved phase I clinical trial of 9-NC and cisplatin. The measurement of 9-NC in the plasma was performed in two steps. In step one, 400 μl aliquots of patient plasma were extracted with 1100 μl of ice cold acidified methanol and analyzed by LC. 9-AC in the samples was quantitated using similarly prepared 9-AC calibration standards. In step two, another set of 200 μl aliquots of patient plasma samples was processed by the enzymatic method to convert 9-NC to 9-AC and processed as described in Section 2.4.2. 9-AC in the samples were quantitated using similarly prepared 9-AC calibration standards. Since the sample contained 9-NC and it metabolite 9-AC, the amount of 9-NC (total) in plasma samples was determined by the method of difference using following equation:

the amount of 9-NC (total) in plasma
= amount of 9-AC measured by enzymatic method in plasma − amount of 9-AC in plasma.

2.8. Statistical methods

The agreement of patient plasma concentrations obtained by the enzymatic-LC method and by the LC/MS/MS method was assessed in three steps. The first step was based on the within-patient correlation analyses [10] that would assess the strength of the linear correlation between the two methods. The second step was based on the agreement between the summary measurement area under the curve (AUC) using the LC and the LC/MS/MS methods, which was assessed using the Bland–Altman method of 'limits of agreement' [11] and using Lin's 'concordance correlation coefficient' (CCC) [12]. The AUCs were estimated using the trapezoidal rule. The 95% confidence interval and the 90% of limits of agreement for the difference of log(AUC) were constructed, together with the estimated CCC of log(AUC) between LC and LC/MS/MS methods and the corresponding 95% confidence interval of CCC. In the last step, the mixed effect regression models [13] were used for the serial measurements of concentration levels. The response variable is the difference of the concentration of 9-NC by the two methods, LC and LC/MS/MS. The covariates were time and concentration measured by LC method. The compound symmetry covariance structure was used to account for the within-patient variation. To assess the consistency of the two measurement methods in the mixed effect model, we tested if the regression coefficients are all zero and checked if the between- and within-patient's variation are small.

3. Results and discussion

3.1. Enzymatic reduction of 9-NC

Our previous studies indicated that human and bovine liver tissues rapidly convert in vitro 9-NC to 9-AC. Since many reductases use FAD as a cofactor, we studied the effect of FAD on reduction 9-NC by liver S-9 fraction. In the absence of FAD only a very small amount of 9-NC (∼4%) was reduced at 60 min, indicating that under our reaction conditions, FAD is essential for the conversion of 9-NC to 9-AC.

3.2. LC analysis

We developed an invitro system in which bovine liver S-9 fraction is used to reduce 9-NC to 9-AC in human plasma samples. Accurate quantitation of 9-AC in the reaction mixture required separation of 9-AC from added cofactors NADP, FAD, and the components. When spiked in plasma, 9-AC eluted at about 9.7 min under our LC conditions (Fig. 2A). In control plasma no peak at 9.7 was seen. 9-NC spiked plasma sample processed by the enzymatic method showed a peak in the chromatogram at the same retention time of 9-AC (Fig. 2B), the resolution between 9-AC and the nearest peak FAD was about 3. When samples were prepared using clear glass vials, a major peak of FAD with a retention time of about 7.5 min eluted before 9-AC and interfered with quantitation of 9-AC. Hence, low actinic amber containers were used for sample preparation and analysis. The resolution between flavin nucleotide peak and 9-AC was greater than 3. The column performance was unaffected by the operating temperature (45 °C) and acidic mobile phase. Column performance parameters, such as peak tailing 1.2, theoretical plates 12,000 m^{-1} and the retention time 9.7 remained unchanged over a period of 18 months. The ruggedness of the method was tested using another Hitachi HPLC system with a Waters 474 scanning fluorescence detector and a different batch of column. The retention time, resolution, and tailing factor were similar in both the HPLC systems.

3.3. Effects of time and enzyme concentration on 9-NC conversion by liver S-9 fraction

To optimize the time required for the complete conversion of 9-NC to 9-AC, the reactions were performed from 5 to 180 min using control donor plasma spiked with 9-NC (1 μg/ml) and fixed amount of S-9 fraction (∼1.2 mg protein). Nearly 95% of 9-NC was converted to 9-AC in 30 to 60 min. Beyond this time almost 100% of 9-NC was converted to 9-AC (Fig. 3A).

To optimize the amount of S-9 fraction liver protein required for the conversion of 9-NC to 9-AC in plasma, using the assay method 9-NC (1 μg/ml) was spiked in donor control plasma and treated with S-9 protein (0–2.4 mg). The

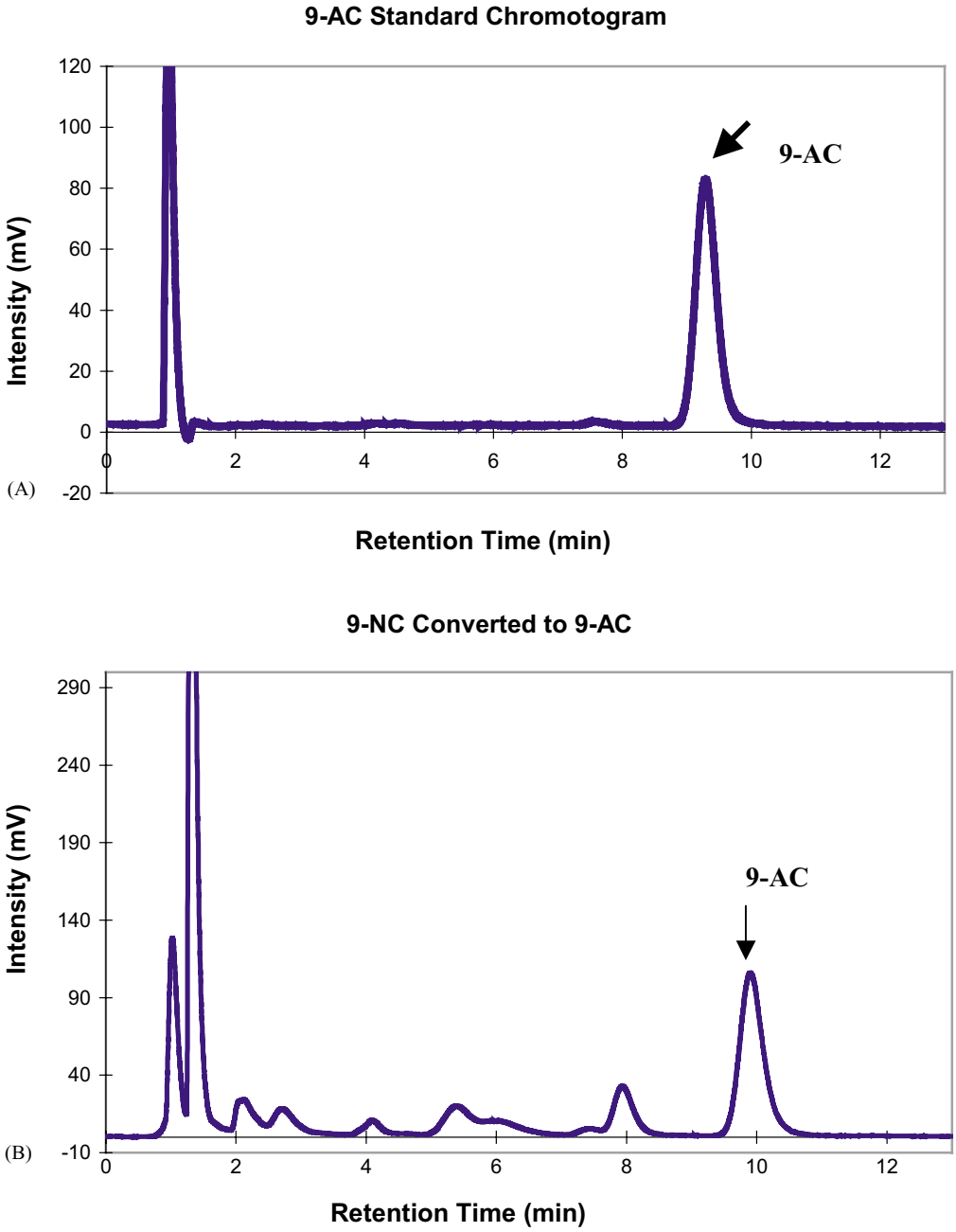

Fig. 2. Representative chromatograms of 9-AC and 9-NC. (A) Extract of human plasma spiked with 125 ng/ml 9-AC. (B) Extract of human plasma spiked with 125 ng/ml 9-NC and then converted to 9-AC by the enzymatic method. The peak at 8 min is due to FAD.

results showed that for 100% conversion of 9-NC to 9-AC, a minimum of 0.75 mg protein was necessary (Fig. 3B). 9-NC reducing activity of the S-9 fraction was unaffected even after repeated thawing and freezing for several times.

3.4. Characterization of the kinetics of 9-NC reduction

The kinetics of 9-NC reduction to 9-AC were studied using varying amounts of 9-NC in buffer (Fig. 4). The results suggest that the reduction of 9-NC to 9-AC by bovine liver S-9 fraction follows Michaelis–Menten kinetics. V_{max} and K_m were estimated graphically to be about 920 nmol/s mg protein and 36.3 μM, respectively.

3.5. Assay validation

3.5.1. Standard linearity

Calibration standard curves (eight points), for 9-AC were plotted using concentrations ranging from 0.98 to 125 ng. Several calibration standard curves were prepared and the

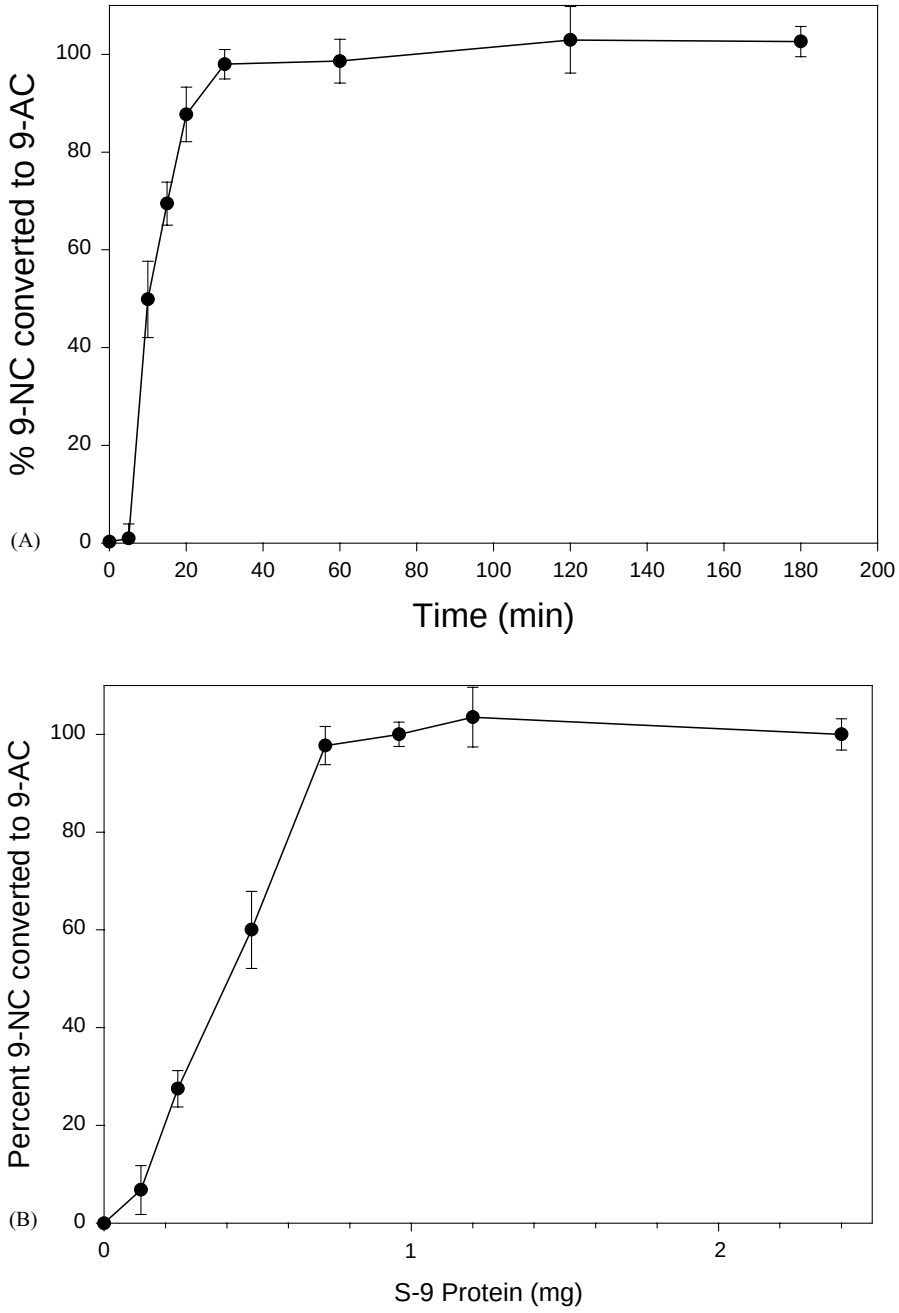

Fig. 3. Kinetics of 9-NC conversion to 9-AC. (A) Time kinetics of 9-NC conversion to 9-AC. Two hundred microliters aliquots of control donor plasma containing 1 μg/ml of 9-NC were pre-incubated at 37 °C for 5 min with reaction buffer before the addition of S-9 fraction (1.2 mg per tube). The reactions were carried out as described in Section 2.4, except that the reactions were terminated at 5, 10, 15, 20, 30, 60, 120, and 180 min. The samples were processed and analyzed by the method for 9-AC. Percent 9-NC converted to 9-AC was calculated against 9-AC (1 μg/ml) reference standard. Each point represents mean ± S.D. of triplicate analyses. (B) Effect of S-9 liver protein concentration on 9-NC reduction to 9-AC in plasma. Varying amount of bovine liver protein (S-9 fraction) was added to plasma containing 1 μg/ml of 9-NC and the reactions were carried out as described in Section 2.4. Each point represents mean ± S.D. of triplicate analyses.

correlation coefficient (r) (mean ± S.D., $n = 6$) for 9-AC is 0.9990 ± 0.0009.

A signal to noise ratio 10 ± 2 was used to define the lower limit of quantitation (LOQ) for 9-AC. The relative standard deviation (R.S.D.) of replicate analyses was calculated and R.S.D. less than 20% was accepted [14]. The lowest LOQ was 0.95 ng/ml and the lowest detection limit was 0.5 ng/ml. Based on volume of injection, the actual instrument detection limit was 15 pg.

3.5.2. 9-AC assay accuracy and precision

Accuracy and precision of the assay for 9-AC was determined by spiking four different concentrations (125, 31.25, 7.8, and 1.95 ng/ml) of 9-AC in water, in buffer,

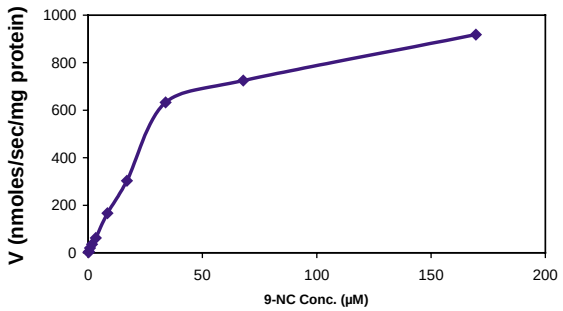

Fig. 4. Kinetics of the formation of 9-AC (nmol/mg protein of S-9) from 9-NC. The kinetics of 9-NC conversion to 9-AC were performed using varying concentrations of 9-NC prepared in 100 mM phosphate buffer pH 7.4 and a fixed amount of bovine liver S-9 protein (0.88 mg). The reactions were carried out as described in Section 2.4, except that the incubations were terminated at 30 min. Each point represents average of duplicate analyses.

and in control donor plasma. The accuracy and precision of the assay in plasma (Table 1), in buffer, in water (data not shown) were almost the same. Since the conversion of 9-NC to 9-AC required buffer (pH 7.4), we also performed the accuracy and precision of 9-AC in buffer. Overall mean accuracy and precision of the 9-AC assay in plasma ranged from 102.2 to 107.6% and in buffer 95.8 to 101.4%. The results indicate that almost all of 9-AC is extracted from the plasma. The method showed good within-day and between-day assay precision and accuracy as %R.S.D. were within the required 20% (14).

3.5.3. 9-NC assay accuracy and precision

The accuracy and precision of the assay for 9-NC was determined using four different concentrations of 9-NC (125, 31.25, 7.8, and 1.95 ng/ml) spiked in control donor plasma. The samples were converted to 9-AC by the enzymatic assay method and quantitated using a 9-AC calibration standard curve. The results indicated that the under the assay conditions almost 100% of 9-NC was converted to 9-AC (Table 2). The precision and accuracy of the assay for all the concentrations tested were within 10% R.S.D., except at 1.95 ng/ml, which was around 15%. The enzymatic conversion of 9-NC to 9-AC in human plasma was also validated for within-day and between-day variation. Overall mean accuracy of 9-NC assay ranged from 97.4 to 108.9%, and the precision (%R.S.D.) ranged from 3.7 to 13, thus indicating a reproducible assay method.

3.5.4. Analysis of 9-AC in the presence of 9-NC in control plasma

To quantitate of 9-AC in the presence of 9-NC, a fixed concentration of 9-AC (3.12 ng/ml) was spiked into four different concentrations of 9-NC (125, 31.25, 7.8, and 1.95 ng/ml) in control donor plasma. We chose 3.12 ng/ml of 9-AC for the study because this was in the middle range of concentrations observed in patient plasma. 9-AC in the samples was extracted in acidified methanol and quantitated for 9-AC. Since 9-NC is non-fluorescent, the signals obtained in the chromatograms were that of 9-AC only. The results indicate that 9-AC can be estimated quantitatively in the presence of 9-NC (Table 3).

3.5.5. Analysis of 9-NC in the presence of 9-AC in control plasma

Plasma samples from patients treated with 9-nitrocamptothecin contain both 9-NC and its metabolite 9-AC. Therefore, plasma samples were prepared to contain four different concentrations (125, 31.25, 7.8, 1.95 ng/ml) of 9-NC and a fixed concentration (3.12 ng/ml) of 9-AC. The samples were processed using enzymatic method and quantitated for 9-AC. The amount 9-NC in the samples were quantitated by subtracting the measured 9-AC (Table 3) from the total 9-AC measured in the assay sample. The results indicate that the quantitation of 9-NC by the enzymatic method remains accurate and precise in the presence of 9-AC (Table 4).

3.6. Comparison of the enzymatic assay to an LC/MS method for quantitation of 9-NC in patient plasma

We compared 9-NC plasma concentrations obtained with our enzymatic assay to those obtained with a validated LC/MS/MS method in samples from eight patients receiving 9-NC as part of a phase I clinical trial (Fig. 5). The mean value of within-patient Pearson correlation coefficients was 0.983, with a standard deviation of 0.017 and a 95% confidence interval from 0.971 to 0.994, indicating that the two assay methods were highly linearly correlated. In terms of the ratios of the measurements by the LC and the LC/MS/MS methods, a 95% confidence interval of the ratio was from 90.7 to 106.7% (well within the FDAs bioequivalence criterion of 80–125%). Using Bland–Altman's method [11] the 90% limits of agreement were from −0.21 to 0.18 (showing a narrow interval containing 0). In terms of ratios of the measurements by the LC and LC/MS/MS

Table 1
Average accuracy and precision of 9-AC assay in human plasma

9-AC, theory (ng/ml)	Amount of 9-AC measured (ng/ml)			Accuracy (% of theory)	Within-run precision %R.S.D.	Between-run precision %R.S.D.
	Mean	±S.D.	%R.S.D.			
125.0	128.2	3.6	2.8	102.6	2.6	5.1
31.25	32.9	0.8	2.5	105.3	1.1	4.1
7.8	8.4	0.4	4.3	107.7	1.6	8.4
1.95	2.1	0.2	10.3	107.7	3.9	10.8

Number of observations: 15; R.S.D.: relative standard deviation.

Table 2
Average accuracy and precision of 9-NC assay

9-AC, added, theory (ng/ml)	Amount of 9-AC measured (ng/ml)			Accuracy (% of theory)	Within-run precision %R.S.D.	Between-run precision %R.S.D.
	Mean	±S.D.	%R.S.D.			
125.0	125.5	6.0	4.8	99.9	4.8	5.7
31.25	30.8	2.1	6.7	98.6	3.7	6.7
7.8	8.5	0.6	7.3	108.9	6.0	7.6
1.95	1.9	0.3	15.2	97.4	8.0	13.3

Number of observations: 15; R.S.D.: relative standard deviation. Percent of theory was calculated based on the amount of 9-AC measured in the samples against 9-NC spiked in the samples.

Table 3
Assay of 9-AC in the presence of 9-NC in control plasma

9-AC, theory (ng/ml)	9-NC, theory (ng/ml)	Amount of 9-AC measured (ng/ml)			% of theory
		Mean	±S.D.	%R.S.D.	
3.12	125.0	2.91	0.25	8.3	93.3
3.12	31.25	3.05	0.38	12.6	97.8
3.12	7.8	3.24	0.28	8.8	103.8
3.12	1.95	3.13	0.17	5.3	100.0

Number of observations: 15; R.S.D.: relative standard deviation. Percent of theory was calculated based on the amount of 9-AC measured/amount of 9-AC spiked in the samples. 9-NC does not fluorescence therefore, 9-AC peak in the chromatograms was due only to 9-AC in the samples.

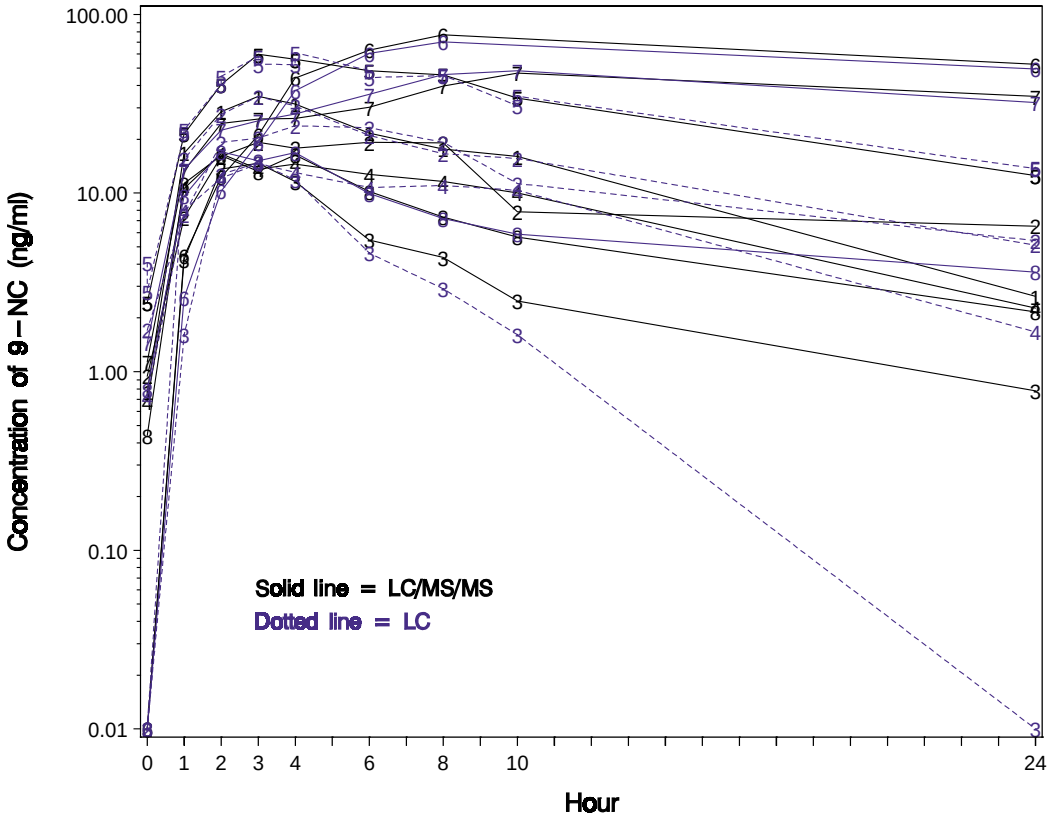

Fig. 5. Plasma concentration–time curves (semilogarithmic) of patients treated with 2 mg (fixed dose) oral 9-NC. Numbers on each curve represent individual patient. Results obtained by the LC/MS/MS method are represented in solid lines and the enzymatic-LC method in dotted lines. The values represent plasma concentrations of 9-NC on day 15 of the treatment cycle.

Table 4
Assay of 9-NC in the presence of 9-AC in control plasma

9-NC, theory (ng/ml)	9-AC, theory (ng/ml)	Amount of 9-NC measured (ng/ml)[a]			% of theory
		Mean	±S.D.	%R.S.D.	
125	3.12	129.8	7.6	5.7	103.8
31.25	3.12	32.76	1.6	4.8	104.8
7.8	3.12	8.54	0.6	6.4	109.5
1.95	3.12	1.92	0.7	13.9	98.5

Number of observations: 15; R.S.D.: relative standard deviation. Percent of theory was calculated based on the amount of measured 9-NC × 100/amount of 9-NC spiked in the sample.

[a] 9-NC measured = total 9-AC measured − 9-AC measured (Table 3).

Table 5
Estimated log(AUC) by patient and method

Patient ID	Log(AUC) (ng/ml × h)[a]		
	LC	LC/MS/MS	Difference
1	5.885	5.865	0.020
2	5.692	5.540	0.152
3	4.312	4.551	−0.239
4	5.260	5.334	−0.074
5	6.620	6.626	−0.006
6	7.120	7.202	−0.082
7	6.777	6.759	0.018
8	5.157	5.079	0.078

[a] Log(AUC) was calculated using trapezoidal rule.

methods, the 90% limits of agreement were from 0.81 to 1.19 (showing a narrow interval containing 1).

AUCs for 9-NC in the samples were also calculated using each data set and the trapezoidal rule. The mean of the differences of log(AUC) between the two methods was −0.0166 with a standard deviation of 0.1178 (Table 5). Furthermore, the 95% confidence interval of the difference of log(AUC) was from −0.098 to 0.065 (showing narrow interval containing 0). The Lin's concordance correlation coefficient [12] of log(AUC) between the two measurement methods was 0.992 with a 95% confidence interval from 0.962 to 0.998 (i.e., very close to 1). This indicates the agreement of the AUC between the two measurement methods. Furthermore, using a mixed effects model for the difference of the serial measurements by the two methods, with response variable and time and LC as covariates, both time effect and the LC effect were not significant ($P = 0.8822$ and 0.282, respectively). Also within-patient variation was 1.69 and the between-patient variation was 4.95. Together these analyses indicate that the two measurement methods yield very similar results.

4. Conclusion

For quantitation of 9-NC in human plasma, previous HPLC-based methods used a simple and straight forward assay involving ultraviolet detection [15] and a chemical method that reduces 9-NC to 9-AC, a fluorescent derivative. The chemical conversion was based on reduction of 9-NC using metallic organic iron (pentacarbonyl) and hydrochloric acid [5]. However, the harsh conditions of chemical method may yield products in addition to 9-AC. Indeed, our attempts to use the chemical reduction assay for measurement of 9-NC in plasma were complicated by a large variation (30–80%) in the amount of 9-AC produced from a fixed quantity of 9-NC (data not shown).

We therefore developed a enzymatic-HPLC assay method to measure 9-NC in patient plasma. In essence, 9-NC in the sample is quantitatively reduced by bovine liver S-9 fraction to 9-AC. This validated enzymatic-HPLC assay is precise, sensitive, and accurate. The method yields results comparable to an LC/MS/MS method and can be applied for the quantitative analysis of total 9-NC and 9-AC in plasma of patients.

Studies to identify the enzyme(s) responsible for reduction of 9-NC are in progress, and may yield results important in further optimization of this assay method, as well as improving the current understanding of the human metabolism of 9-NC.

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References

[1] Y.H. Hsiang, L.F. Liu, Cancer Res. 48 (1988) 1722.
[2] B.C. Giovanella, J.S. Stehlin, M.E. Wall, M.C. Wani, A.W. Nicholas, L.F. Liu, R. Silber, M. Potmesil, Science 246 (1989) 1046.
[3] B.C. Giovanella, H.R. Hinz, A.J. Kozielski, J.S. Stehlin Jr., R. Silber, M. Potmesil, Cancer Res. 51 (1991) 3052.
[4] C.F. Verschraegen, E. Gupta, E. Loyer, J.J. Kavanagh, A.P. Kudelka, R.S. Freedman, C.L. Edwards, N. Harris, M. Steger, V. Steltz, B.C. Giovanella, J.S. Stehlin, Anti-Cancer Drugs 10 (1999) 375.
[5] H.R. Hinz, N.J. Harris, E.A. Natelson, B.C. Giovanella, Cancer Res. 54 (1994) 3096.
[6] P. Pantazis, J. Mendoza, A. DeJesus, J. Early, M. Shaw, B.C. Giovanella, Anti-Cancer Drugs 5 (1994) 473.
[7] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[8] Z. Desta, T. Kerbusch, N. Soukhova, E. Richard, J.W. Ko, D.A. Flockhart, J. Pharm. Exp. Ther. 285 (1998) 428.
[9] C.H. Takimoto, R.W. Klecker, W.L. Dahut, L.K. Yee, J.M. Strong, C.J. Allegra, J.L. Grem, J. Chromatogr. B: Biomed. Appl. 655 (1994) 97.
[10] R. Woolson, Statistical Methods for the Analysis of Biomedical Data, Wiley, New York, 1987.

[11] J.M.A. Bland, D.G. Altman, Lancet (1986) 307.
[12] L.I. Lin, A.S. Hedayat, B. Shinha, M. Yang, Biometrics 45 (1989) 255.
[13] R.C. Littell, G.A. Miliken, W.E. Stroup, R.D. Wolfinger, SAS System for Mixed Models, SAS Institute Inc., Cary, NC, USA, 1996.
[14] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, Eur. J. Drug Metab. Pharmacokinetics 16 (1991) 249.
[15] N.E. Schoemaker, H. Rosing, S. Jansen, P. Schoffski, J. Rizzo, J.H. Schellens, J.H. Beijnen, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 775 (2002) 231.