ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Chromatography B, 795 (2003) 309–318

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of 9-nitrocamptothecin by precolumn derivatization and its metabolite 9-aminocamptothecin in a biological fluid using reversed-phase high-performance liquid chromatography with fluorescence detection

Junhong Zhang, Thomas G. Burke, Lori J. Latus*

Tigen Pharmaceutical, Inc., 235 Bolivar Street, Lexington, KY 40508, USA

Received 19 May 2003; accepted 21 July 2003

Abstract

A novel insoluble topoisomerase I inhibitor, 9-nitrocamptothecin (9-NC), is in advanced stages of clinical development and has been used to treat a diverse array of tumor types, including breast, ovarian, pancreatic and haematological malignancies. We have established a sensitive high-performance liquid chromatography method using fluorescence detection for the quantitation of 9-NC. Non-fluorescent 9-NC is converted to fluorescent 9-aminocamptothecin (9-AC) via a one-step pre-column derivative reaction. The quantitative limit of 9-NC was 1 ng/ml and the method was reproducible with the respective intra- and inter-day variability falling below 5.0 and 9.0%. The determination of both 9-NC and its metabolite 9-AC in dog plasma was also achieved using the same chromatographic and detection conditions. In dog plasma, the quantitative limits of 9-AC and 9-NC were 0.25 and 1 ng/ml, respectively. The presence of 9-AC in the samples yielded no interference with the determination of 9-NC. However, individual matrices can affect the conversion efficiency of 9-NC, thus indicating that standard samples should be run for each matrix.
© 2003 Elsevier B.V. All rights reserved.

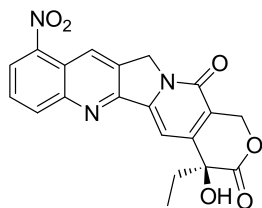
Keywords: Derivatization, LC; 9-Nitrocamptothecin; 9-Aminocamptothecin

1. Introduction

9-Nitro-20(S)-camptothecin (9-NC, Rubitecan) is an analogue of the alkaloid camptothecin, with a nitro group substituted at the C_9 position of the A-ring (Fig. 1). It is an orally available, water insoluble camptothecin congener and, similar to other camptothecins, 9-NC is a highly potent inhibitor of DNA topoisomerase I [1]. 9-NC has broad in vitro and in vivo antitumor activity [2–5] and, as a result, has undergone Phase I and II clinical evaluation in patients with breast, ovarian, pancreatic, and haematological malignancies [6–12]. Unlike other camptothecins, 9-NC is non-fluorescent, thus the determination of 9-NC is usually achieved by HPLC with UV detection [13]. However, even with solid-phase extraction

* Corresponding author. Tel.: +1-859-361-9004; fax: +1-859-258-2561.
E-mail address: ljlatus@earthlink.net (L.J. Latus).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1570-0232(03)00599-3



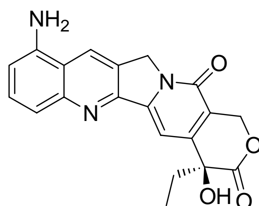


Fig. 1. Structures of 9-nitrocamptothecin (9-NC) and 9-aminocamptothecin (9-AC).

processing with a C_{18} column to concentrate the analytes, this method is restricted by its relatively high limit of quantification (10 ng/ml), which is typical of UV detection. Another reported method involves the conversion of non-fluorescent 9-NC to fluorescent 9-aminocamptothecin (9-AC, Fig. 1) by reacting 9-NC with iron carbonyl during sonication [14,15]. Unfortunately, this method is not readily reproducible as it results in a relatively high standard deviation. Moreover, measurement of 9-NC in biological samples is confounded by the fact that 9-NC can be partially metabolized to 9-AC in vivo [14,16,17]. Discerning both 9-AC and 9-NC levels in pharmacokinetics studies is important since both camptothecin congeners can effect significant antitumor activity [6,18,19].

In the present study, we explored two distinct issues. First, our goal was to establish a sensitive and reliable assay for the determination of 9-NC in plasma using fluorescence detection. Our approach involved optimizing 9-NC conversion in plasma to 9-AC, hereafter denoted as 9-AC^{con}. Secondly, we sought a method to distinguish 9-NC from its bioconversion metabolite 9-AC, henceforth referred to as 9-AC^{bio}, in a biological fluid sample. The procedures detailed herein are complementary and should facilitate the analyses of drug plasma samples.

2. Experimental

2.1. Chemicals and reagents

9-Nitrocamptothecin and 9-AC samples were obtained from the Stehlin Foundation and the National Cancer Institute, respectively. Recovered human plasma was obtained from Central Kentucky Blood Center (Lexington, KY) and stored at −20 °C. Dog plasma was obtained from Xenobiotic Laboratories, Inc. and stored at −20 °C. Carbonyl iron was purchased from Sigma Chemical (St. Louis, MO). HPLC-grade methanol and ammonium formate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). High purity water was provided by a Milli-Q UV Plus purification system (Millipore, Bedford, MA, USA). Stock solutions of 9-NC and 9-AC were prepared in ACS spectrophotometric grade dimethylsulfoxide (DMSO; Aldrich, Milwaukee, WI, USA) at a concentration of 2×10^{-3} M and stored in the dark at −20 °C until use.

2.2. Chromatographic conditions

The HPLC system consisted of a Waters Alliance 2690 Separations Module with a Waters™ 474 Scanning Fluorescence Detector. Separations were carried out on a Rainin Microsorb MV-C_8 column. Excitation and emission detector settings of 380 and 450 nm, respectively, and gain = 100 were used. Mobile phase flow rates of 1 ml/min were used. The mobile phase consisted of 37% methanol and 63% 10 mM ammonium formate (10 mM ammonium formate in water, adjusted to pH 2.0 with concentrated HCl). The mobile phase was degassed by filtration through a membrane (0.45 μm, Millipore). The fluorescence output signal was monitored and integrated using Millennium[32] Chromatography Manager software.

2.3. Method development for 9-NC determination in human plasma

In order to determine total 9-NC concentration (lactone and carboxylate forms) in human plasma,

non-fluorescent 9-NC is converted to fluorescent 9-AC^{con}, since the 9-AC congener is strongly fluorescent and can be readily quantitated. In our studies, we converted 9-NC to 9-AC^{con} by heating and studied a series of temperature and time combinations: 80 °C for 1 h, 70 °C for 30 min, 60 °C for 30 min and 50 °C for 30 min. Heating at 70 °C for 30 min was selected as the best condition.

2.3.1. Calibration curve

A stock solution of 9-NC (2 mM) was prepared in DMSO. The stock solution was diluted with DMSO to give standard solutions with concentration of 200, 100, 50, 20, 2, 0.4 and 0.2 μM. Calibration samples were prepared by adding the following volume of standard solution to 600 μl drug-free plasma: 3.82 μl (0.2 μM), 3.82 μl (0.4 μM), 3.82 μl (2 μM), 1.91 μl (20 μM), 3.82 (20 μM), 3.06 μl (50 μM), 3.06 μl (100 μM) or 3.82 μl (200 μM). In this manner, calibration samples were obtained with the concentrations of 0.5, 1, 5, 25, 50, 100, 200 and 500 ng/ml.

2.3.2. Sample preparation

To a 2.0 ml polypropylene eppendorf tube containing 400 μl cold methanol (−20 °C), 100 μl of human plasma with drug was added. The tube was vortexed for 10 s and centrifuged for 1 min at 8000 rpm. The supernatant (350 μl) was transferred into a new eppendorf tube. To the supernatant 25 μl concentrated HCl (12N) and 50 μl carbonyl iron suspension (25 mg/ml in water) were added, the mixture was mixed for 10 s and heated at 70 °C for 30 min. Finally, this solution was mixed with 700 μl water, 100 μl of which was analyzed by HPLC.

2.3.3. Validation

The method was validated with respect to precision (intra- and inter-day), linearity, limit of quantitation (LOQ) and limit of detection (LOD), recovery and stability of the analytes. Intra-day precision of the assay was assessed by performing three separate runs on five replicates of spiked samples at high, middle, and low concentrations in plasma against a calibration curve on the same day. Inter-day precision was assessed with the same standards on five different days. The mean and R.S.D. were evaluated at the three different concentrations using five replicates for each concentration. Moreover, three separate HPLC runs were performed for the three different concentrations.

LOD was determined as the lowest concentration for which the peak area had a signal-to-noise ratio of 3:1. LOQ was determined as the lowest concentration for which the precision in measuring the peak area response was lower than 20%.

For the determination of the recovery of 9-NC from human plasma, triplicate HPLC runs on five replicates were processed as detailed above in Section 2.3.1 with the exception that water was used instead of plasma. The concentration of 9-NC in the spiked plasma samples was calculated by means of a calibration curve, which consisted of six standard 9-NC concentrations prepared in water that were subjected to the same pre-treatment processing as the 9-NC plasma samples. The recovery was determined at each test concentration by comparing the measured concentration with the nominal concentration.

In this study, we determined 9-NC by precolumn derivatization to 9-AC^{con}. To enable accurate quantification of the biological samples, the stability of the 9-AC^{con} derivatives was studied. The analytes were tested for their stability in the autosampler at 4 °C for 20 h. HPLC analysis was performed on these samples using the same running conditions as detailed in Section 2.2.

2.4. Determination of both 9-NC and 9-AC in dog plasma

2.4.1. Calibration curve of 9-AC and 9-NC

The calibration curve of 9-NC in dog plasma was generated in the same manner as detailed in Section 2.3.1. For the calibration curve of 9-AC, a stock solution of 9-AC (2 mM) was prepared in DMSO and subsequently diluted with DMSO to give standard solutions with concentrations of 40, 20, 4, 2, 0.4 and 0.2 μM. Calibration samples in dog plasma were prepared containing drug concentrations of 0.25, 1, 5, 10, 25 and 100 ng/ml.

2.4.2. Determination of 9-AC levels

To a 2.0 ml polypropylene eppendorf tube containing 400 μl cold methanol (−20 °C), 100 μl of dog plasma with drug was added. The tube was vortexed for 10 s and centrifuged for 1 min at 8000 rpm. The supernatant (350 μl) was transferred into a fresh

eppendorf tube. Twenty-five microliters concentrated HCl (12N) was added to the transferred supernatant and this solution was mixed with 750 μl water. Hundred microliters of the sample was subsequently analyzed by HPLC.

2.4.3. Determination of total drug (9-AC + 9-NC) levels

To a 2.0 ml polypropylene eppendorf tube containing 400 μl cold methanol (−20 °C), 100 μl of dog plasma was added. The tube was vortexed for 10 s and centrifuged for 1 min at 8000 rpm. The supernatant (350 μl) was transferred into a fresh eppendorf tube. Twenty-five microliters of concentrated HCl (12N) and 50 μl carbonyl iron suspension (25 mg/ml in water) were added, the sample was mixed for 10 s and heated at 70 °C for 30 min. Finally, this solution was mixed with 700 μl water, 100 μl of which was analyzed by HPLC.

2.4.4. Determination of 9-NC in the presence of a fixed amount of 9-AC (10 ng/ml) in dog plasma

A stock solution of 9-NC (2 mM) was prepared in DMSO. The stock solution was diluted with DMSO to give standard solutions with a final concentration of 200, 40, 10, 4, 2 and 0.4 μM. Calibration samples were prepared by first adding 2.76 μl of a 4 μM 9-AC stock to 400 μl of drug-free dog plasma such that the concentration of 9-AC was 10 ng/ml. Subsequently, 9-NC calibration samples were prepared containing 1, 5, 10, 25, 100 and 500 ng/ml of 9-NC with a fixed amount of 9-AC (10 ng/ml) in each sample. The purpose was to determine the accuracy of determining 9-NC levels when 9-AC is present, a scenario that models actual dog plasma samples that are given to us for analysis.

2.5. Effect of different plasma components on the conversion rate of 9-NC to 9-AC

Five different plasma components from either human or dog were used to investigate their effect on the conversion rate of 9-NC to 9-AC^{con}. To individual vials containing 9-NC stock solution an aliquot of the following solutions, each at a physiologically relevant final concentration, was added: human serum albumin (30 mg/ml), dog albumin (30 mg/ml), human γ-globulin (30 mg/ml), human low-density lipoprotein (6.5 mg/ml) and human high-density lipoprotein (3 mg/ml). In each sample the final 9-NC concentration was 1 μM. Each solution of a plasma component was prepared in PBS (pH 7.4). Sample preparation occurred according to the procedure detailed in Section 2.3.2 and the chromatographic conditions are detailed in Section 2.2. The conversion rate was calculated by comparing the peak area for each component with the calibration curve of 9-AC.

3. Results and discussion

3.1. Method development and validation for determination of 9-NC

3.1.1. Method development

To find the best condition for the conversion of 9-NC to 9-AC^{con} we explored five different conditions, which consisted of either sonication at room temperature for 15 min or incubation at the following temperature and time period: 80 °C for 1 h, 70 °C for 30 min, 60 °C for 30 min and 50 °C for 30 min. The calibration curve of 9-AC^{con} in human plasma yielded variable results depending on the different reaction conditions. Sonication at room temperature resulted in poor linearity ($r = 0.9647$) and a high relative standard deviation (R.S.D., up to 40%). Relative to sonication at room temperature, reacting at 80 °C for 1 h resulted in worse linearity in the concentration response curve ($r = 0.5306$). It is possible that at this elevated temperature the carbonyl iron reacts with HCl very quickly and therefore the 9-NC conversion to 9-AC^{con} is incomplete. At 70 °C for 30 min, the calibration curve yields good linearity ($r = 0.9999$) and less R.S.D. Interestingly, with a decrease in temperature, the R.S.D. changes little but the linearity is negatively affected, with r being 0.9977 and 0.9930 at the temperatures of 60 and 50 °C, respectively. Based on these data, we selected for our study the condition of heating at 70 °C for 30 min.

3.1.2. Chromatography

Fig. 2 shows typical chromatograms for plasma alone and for plasma spiked with 9-NC. The retention time of 9-AC^{con}, which has been derived from 9-NC using the selected reaction conditions described above, was 5.9 min. As shown in the plasma control, no interfering peaks of endogenous compounds

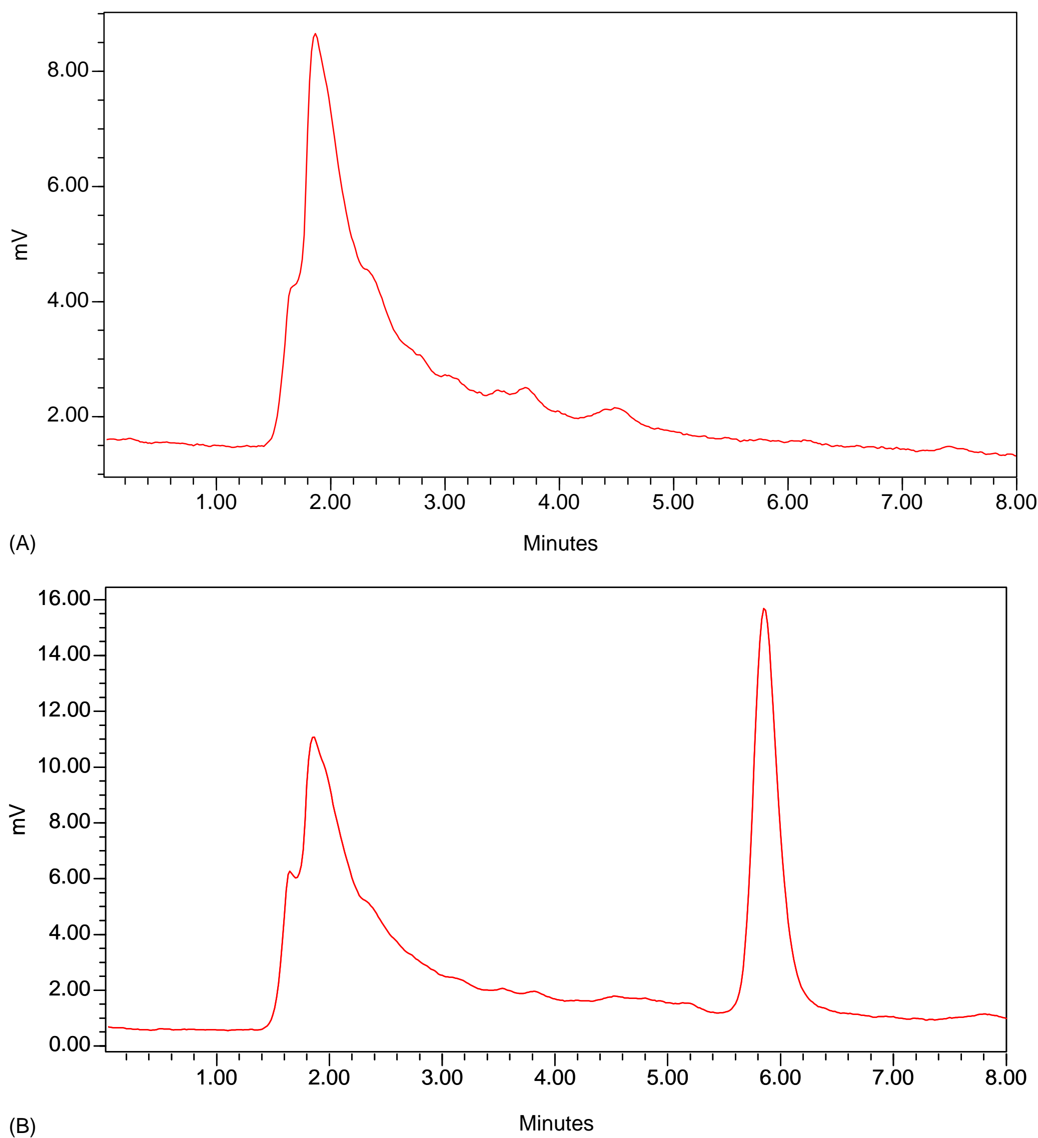


Fig. 2. Chromatograms for blank plasma (A) and for plasma spiked with 50 ng/ml 9-NC (B).

appeared at this retention time. The LOD was found to be 0.25 ng/ml while the limit of quantification was 1 ng/ml.

3.1.3. Calibration curve

Calibration curves for 9-AC^{con} in human plasma were linear over the range of 1–500 ng/ml and the coefficient of determination (r) was 0.9999. The standard curve equation of 9-AC^{con} was $y = 4212.1x - 1015.6$, where x and y were the concentration (ng/ml) and peak area, respectively.

3.1.4. Precision, recovery and stability

As shown in Table 1, the precision of the method was validated on the basis of intra- and inter-day assays according to international regulations. At the three different concentrations, the relative standard deviation was found to be less than 5% for the intra-day

Table 1
Intra- and inter-day precision and accuracy of HPLC determination of total 9-NC in human plasma ($n = 5$)

	Theoretical concentration (ng/ml)	Mean measured concentration (ng/ml)	R.S.D.[a] (%)	Accuracy (%)
Within-day	5.00	5.48	2.24	109.52
	50.00	53.35	2.98	106.70
	500.00	521.44	4.17	104.29
Between-day	5.00	4.88	8.14	97.60
	50.00	48.79	6.11	97.57
	500.00	512.90	6.61	102.58

[a] Relative standard deviation.

assay and below 9% for the inter-day assay. The calibration curve of 9-AC^{con} in water (using the selected reaction conditions implemented for human plasma) is shown in Fig. 3. The recovery of 9-AC^{con} in human plasma (Table 2) was calculated as follows: the spiked concentration in plasma divided by the measured concentration, which was calculated by the calibration

Table 2
Recovery of total 9-NC in human plasma ($n = 5$)

Concentration (ng/ml)	Recovery (%) (mean ± S.D.)
5	52.5 ± 1.3
50	56.5 ± 1.7
500	58.8 ± 0.7

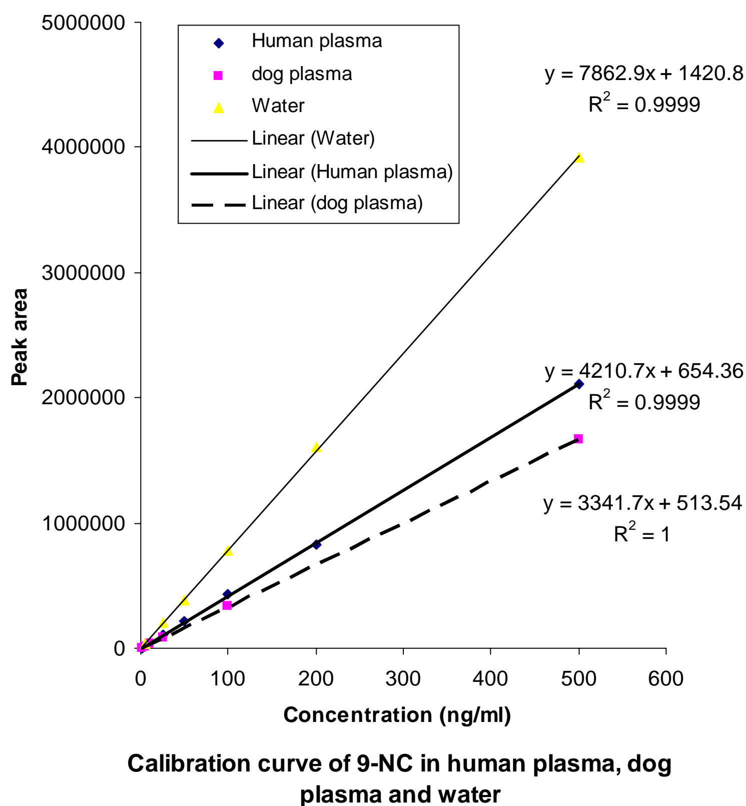


Fig. 3. Calibration curve of 9-NC in different matrices: water, human plasma and dog plasma. Each point in the graph is the mean value of three analyses.

curve in water. The average recoveries at the concentrations of 5, 50, 500 ng/ml were respectively 52.5, 56.5 and 58.8%. These values are somewhat low, yet additional studies suggest that it is unlikely that the high loss of 9-AC^{con} is due to the extraction procedure, which involves protein precipitation with cold methanol. First, we spiked plasma with 9-NC, precipitated the proteins with cold methanol, then reacted with carbonyl iron to convert 9-NC to 9-AC^{con}. Secondly, the protein was precipitated from plasma using cold methanol, 9-NC was added, then the mixture was treated with carbonyl iron under the selected reaction conditions to convert to 9-AC^{con}. Both of these procedures resulted in the same peak area of 9-AC^{con} with the same concentration, thus suggesting that protein precipitation with cold methanol is complete and the main difference between human plasma and water may involve some component in the plasma that negatively impacts the conversion of 9-NC to 9-AC^{con}. Finally, with respect to analyte stability, 9-AC^{con} was tested for its stability in the autosampler at 4 °C and, as indicated by the resulting chromatographs (data not shown), was found to be stable for at least 20 h.

3.2. Determination of both 9-NC and 9-AC in dog plasma

Above we describe a new method for converting non-fluorescent 9-NC to fluorescent 9-AC^{con} by creating a dispersion of iron carbonyl in the 9-NC sample. As 9-NC is partially converted to 9-AC (9-AC^{bio}) in vivo, a method distinguishing between the two drug species would be useful. Due to the fluorescent nature of 9-AC under acidic conditions, the 9-AC^{bio} concentration can be determined directly. However, as quantitation of 9-NC involves conversion to 9-AC^{con}, our protocol for establishing 9-AC^{con} relative to 9-AC^{bio} plasma levels is outlined below.

Standard curves were constructed by directly adding 9-AC to dog plasma and HPLC analysis was performed. To determine the total drug in dog plasma, 9-NC was converted to 9-AC^{con} and the total peaks (metabolically derived 9-AC^{bio} and iron carbonyl-derived 9-AC^{con}) were measured. We have determined that the iron carbonyl method is inefficient at converting the 9-NC contained in dog plasma to 9-AC^{con} (i.e. the efficiency of conversion is in the 27–47% range). As a result, the determination of total 9-AC levels is essential and these data need to be corrected for the 9-AC^{bio} already present in the sample. Without correction for the presence of 9-AC^{bio} in the sample, a the 9-NC concentration will be significantly overestimated as its quantitation is founded on the total 9-AC (9-AC^{con} + 9-AC^{bio}) levels in the sample. We now propose a revised method to determine 9-NC and 9-AC^{bio} levels in dog plasma.

3.2.1. Calibration curves for 9-AC and 9-NC

A calibration curve for 9-AC in dog plasma was constructed using samples with the following six concentrations of 9-AC: 0.25, 1, 5, 10, 25 and 100 ng/ml. The linear regression equation was as follows: area $= 10188 \times$ drug concentration (ng/ml) $- 284$ ($r = 0.9999$, $n = 3$).

In this procedure, the conversion of 9-NC to 9-AC^{con} was accomplished by sample heating to 70 °C for 30 min. The calibration curve for 9-AC^{con} in dog plasma was constructed using samples at six different drug concentrations: 1, 5, 10, 25, 100 and 500 ng/ml (Fig. 3). The linear regression equation was: area $= 3341.7 \times$ drug concentration (ng/ml) $+ 513$ ($r = 1$, $n = 3$).

The determination and calculation of 9-NC and 9-AC levels in dog plasma was accomplished using the following steps. First, we determined 9-AC levels in each dog plasma sample (no iron carbonyl employed in the analysis) and subsequently determined the total drug levels (using iron carbonyl in the analysis). Comparison of the two determinations yields the actual amount of 9-NC that was converted to 9-AC^{con} using the iron carbonyl conversion step. In the samples in which no pre-column carbonyl iron conversion has occurred, we calculate 9-AC^{bio} concentration by comparing 9-AC peak areas with the 9-AC calibration curve. By extension, we were able to back calculate the 9-NC concentration by subtracting the 9-AC^{bio} peak area from the 9-AC^{total} peak area in the parallel samples that had undergone carbonyl iron conversion. The resulting value reflects the 9-AC^{con} value, which can then be converted to the 9-NC concentration via comparison to the 9-NC calibration curve. So, the determination of both 9-NC and its metabolite 9-AC in dog plasma could be achieved using the same chromatographic and detection conditions. The

quantitative limits of 9-AC and 9-NC were 0.25 and 1 ng/ml, respectively.

3.2.2. Determination of 9-NC in the presence of a fixed concentration of 9-AC (10 ng/ml)

It has been reported previously that 9-NC can be converted to 9-AC in dogs and both 9-NC and 9-AC^{bio} are likely to be present in dog plasma samples following the administration of 9-NC to dogs. To demonstrate that we could accurately determine 9-NC levels in the presence of 9-AC^{bio}, we constructed a calibration curve for 9-NC in the presence of 9-AC in order to determine if the presence of 9-AC changes the conversion efficiency of 9-NC to 9-AC^{con} in dog plasma. From this study, we found that we obtained similar concentrations for 9-NC in the absence and presence of 9-AC (Table 3). Thus, we concluded from this study that the conversion efficiency of 9-NC to 9-AC^{con} is not altered by the presence of 9-AC spiked in the sample. Thus, it appears that our method of determining 9-NC concentration from the 9-NC calibration curve (following correction for the spiked quantity of 9-AC) is an accurate method of determining 9-NC levels.

3.2.3. Inter- and intra-species variability

The slopes of the calibration curves of 9-AC^{con} in water and in representative samples of human plasma and dog plasma clearly reveal differences in how the matrix can affect the conversion efficiency of 9-NC to 9-AC^{con}. For example, in one run the conversion efficiency is 84% in water, yet in samples of human plasma and dog plasma the respective efficiencies were 45 and 36%. Thus, we not only observed differences in the conversion efficiency between water and plasma, we also noted that different conversion efficiencies occur between different species (human versus dog plasma). To more fully investigate the impact of matrix on conversion, we analyzed the conversion rate of 9-NC to 9-AC^{con} in 17 different dog plasmas and discovered the conversion rates ranged between 27 and 47%. The higher conversion rate value (47%) parallels the rate observed in humans (45%), yet, the data set from the 17 different dogs clearly indicates that the conversion rate among the dogs varies substantially (Table 4). The data reveal that the duplicate determinations within a sample are close but there is reproducible variance among the distinct dog samples. Also, two calibration curves of 9-NC in two separate dog plasma samples are shown in Fig. 4; the difference in the conversion efficiency between the two animals is obvious and it is noteworthy that both calibration curves were linear ($R^2 = 0.9999$ or above) with a y-intercept of near 0 over the drug concentration range of interest. Interestingly, the differences in the 9-NC conversion rate among the individual dog plasmas can exceed the difference in conversion rate between dogs and human. It is important to note that, as the human plasma used for our studies was derived from a single individual, we can only speculate that human plasma

Table 3
Determination of 9-NC with and without the presence of a fixed concentration of 9-AC (10 ng/ml, where $n = 5$)

Nominal 9-NC concentration (ng/ml)	Mean measured concentration (9-NC without 9-AC) (ng/ml)	Mean measured concentration (9-NC with 10 ng/ml 9-AC) (ng/ml)
1	1.14	0.85
5	4.97	4.75
10	9.91	9.79
25	24.75	24.23
100	100.06	108.23
500	503.65	509.87

Table 4
Peak area of 9-NC standard (25 ng/ml) in individual $t = 0$ blank plasma samples

Animal ID	Peak area		
	Trial 1	Trial 2	Mean
1M2553	96551	95160	95856
1M2559	105121	104308	104715
1M2560	108228	106891	107560
1F2512	95717	94500	95109
1F2515	83752	82285	83019
2M2564	95126	99787	97457
2M2558	112622	110226	111424
2M2552	88763	90118	89441
2F2514	97235	98924	98080
2F2516	98189	98994	98592
3M2562	96236	98381	97309
3M2557	91555	91355	91455
3M2556	88769	89859	89314
3F2504	95553	92989	94271
3F2505	100765	98279	99522
Dog 1	105281	104222	104752
Dog 2	64824	64027	64426

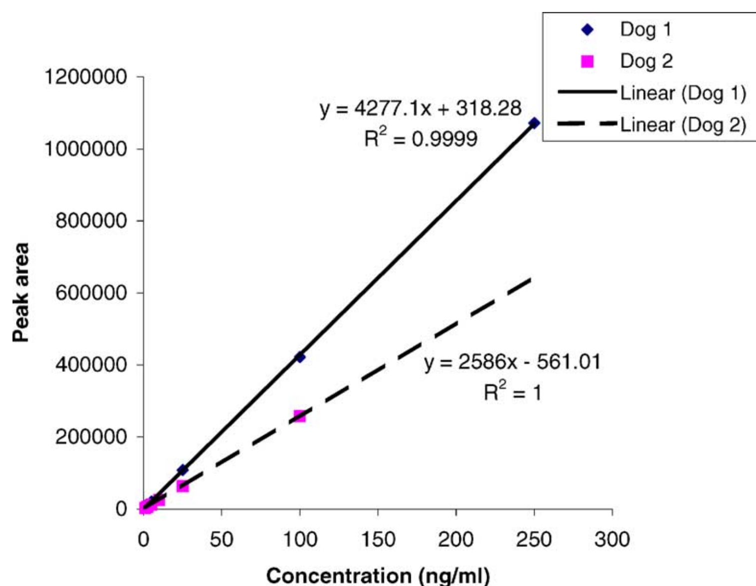


Fig. 4. Calibration curve of 9-NC in two different dog plasmas. Each point in the graph is the mean value of three analyses.

will likely display the same intra-species variability in drug conversion that was observed in dogs.

Further investigation on the effect of different blood components on the conversion rate of 9-NC to 9-AC^{con} was carried out. The individual conversion rates in physiologically relevant concentrations of human serum albumin, dog albumin, human γ-globulin, human low-density lipoprotein and human high-density lipoprotein were 63, 60, 79, 39 and 62%, respectively. These data indicate that different components of human plasma may exert a differential and negative effect on the conversion of 9-NC to 9-AC^{con}. As various blood components (such as those tested above) show inter-individual variation, this could explain the intra-species differences in conversion rate that we observed.

Given the intra-species differences in the conversion rate of 9-NC to 9-AC^{con}, a single calibration curve does not suffice for the determination of the 9-NC concentration. As the calibration curves in human plasma, dog plasma and water were linear ($R^2 = 0.9999$ or above) with a y-intercept near 0 over the drug concentration range of interest, a standard for each individual was made and the following equation was used to solve the unknown concentration of 9-NC: 25 ng/(peak area for $t = 0$ sample containing 25 ng/ml 9-NC) = (x or unknown 9-NC concentration in ng/ml)/(peak area for the given sample). From these results, we note there is a variance in the conversion efficiency of 9-NC to 9-AC^{con} in the $t = 0$ blank plasma samples. We have used these determinations to correct for differences in conversion efficiencies between individual dog samples.

A precise and sensitive HPLC assay has been developed for the quantification of 9-NC in human and dog plasma. The reaction conditions described herein (at 70 °C for 30 min) are a significant improvement over the previously reported sonication method in terms of sensitivity, precision and range of linearity. This method has been validated for a linear range of 1–500 ng/ml with the LOD being 0.25 ng/ml and the limit of quantification being 1ng/ml. The intra- and inter-day precisions were less than 5 and 9%, respectively. The autosampler stability of the analyte, 9-AC^{con}, also displayed excellent stability for at least 20 h. However, due to variability in the conversion efficiency of 9-NC to 9-AC^{con}, a standard sample should be performed with each individual animal and calibrations should be made for each individual standard. This method should prove to be a valuable tool for the clinical monitoring of drug plasma levels and for detailed pharmacokinetic studies.

References

[1] C. Jaxel, K.W. Kohn, M.C. Wani, M.E. Wall, Y. Pommier, Cancer Res. 49 (1989) 1465.
[2] P. Pantazis, J.A. Early, A.J. Kozielski, J.T. Mendoza, H.R. Hinz, B.C. Giovanella, Cancer Res. 53 (1993) 1577.
[3] P. Pantazis, J.T. Mendoza, J.A. Early, A.J. Kozielski, E.A. Natelson, B.C. Giovanella, Eur. J. Haematol. 50 (1993) 81.
[4] P. Pantazis, A. Kozielski, R. Rodriguez, E. Petry, M. Wani, M. Wall, B. Giovanella, Melanoma Res. 4 (1994) 5.
[5] J.G. Liehr, A.E. Ahmed, B.C. Giovanella, Ann. N.Y. Acad. Sci. 803 (1996) 157.
[6] E.A. Natelson, B.C. Giovanella, C.F. Verschraegen, K.M. Fehir, P.D. De Ipolyi, N. Harris, J.S. Stehlin, Ann. N.Y. Acad. Sci. 803 (1996) 224.
[7] C.F. Verschraegen, E.A. Natelson, B.C. Giovanella, J.J. Kavanagh, A.P. Kudelka, R.S. Freedman, C.L. Edwards, K. Ende, J.S. Stehlin, Anticancer Drugs 9 (1998) 36.
[8] J.S. Stehlin, B.C. Giovanella, E.A. Natelson, P.D. De Ipolyi, D. Coil, B. Davis, D. Wolk, P. Wallace, A. Trojacek, Int. J. Oncol. 14 (1999) 821.
[9] 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, Anticancer Drugs 10 (1999) 375.
[10] M.M. Konstadoulakis, P.T. Antonakis, B.G. Tsibloulis, G.P. Stathopoulos, A.P. Manouras, D.B. Mylonaki, B.X. Golematis, Cancer Chemother. Pharmacol. 48 (2001) 417.
[11] J.A. Ellerhorst, A.Y. Bedikian, T.M. Smith, N.E. Papadopoulos, C. Plager, O. Eton, Anticancer Drugs 13 (2002) 169.
[12] P. Schoffski, A. Herr, J.B. Vermorken, J. Van den Brande, J.H. Beijnen, H. Rosing, J. Volk, A. Ganser, S. Adank, H.J. Botma, J. Wanders, Eur. J. Cancer 38 (2002) 807.
[13] 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.
[14] H.R. Hinz, N.J. Harris, E.A. Natelson, B.C. Giovanella, Cancer Res. 54 (1994) 3096.
[15] Z. Cao, N. Harris, A. Kozielski, D. Vardeman, J.S. Stehlin, B. Giovanella, J. Med. Chem. 41 (1998) 31.
[16] P. Pantazis, N. Harris, J. Mendoza, B. Giovanella, Eur. J. Haematol. 53 (1994) 246.
[17] P. Pantazis, N. Harris, J. Mendoza, B. Giovanella, Eur. J. Haematol. 55 (1995) 211.
[18] P.L. de Souza, M.R. Cooper, A.R. Imondi, C.E. Myers, Clin. Cancer Res. 3 (1997) 287.
[19] C.H. Takimoto, J. Wright, S.G. Arbuck, Biochim. Biophys. Acta 1400 (1998) 107.