Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 9, pp. 793–802, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

A liquid chromatographic method for the determination of the enantiomeric purity of the anticancer drug, 9-amino-20(*S*)-camptothecin*

JOHN R. KAGEL, VALENTINO J. STELLA and CHRISTOPHER M. RILEY†

Department of Pharmaceutical Chemistry and the Higuchi Biosciences Center, University of Kansas, 3006 Malott Hall, Lawrence, KS 66045, USA

Abstract: A method was developed for determining the enantiomeric purity of 9-amino-20(*S*)-camptothecin (9-A-20(*S*)-CAM). The chiral derivatizing reagent, 1-(1-naphthyl)ethyl isocyanate (NEI) was used to derivatize the enantiomers of 9-A-CAM, and ^{1}H-NMR, LC–MS, and LC–UV were used to identify and quantitate the two diastereomers produced. During the first 24 h, derivatization was exclusively at the 9-amino nitrogen. The much slower reaction involving reaction of NEI with the 20-hydroxy oxygen could be prevented by quenching the reaction within the first 24 h with methanol. NMR analysis provided useful information about the site of derivatization; however, the partial separation of the signals was insufficient for quantitative analysis of the two diastereomers. Whereas baseline resolution of the two diastereomers was achieved by reversed-phase LC, the reproducibilities of the resolution and the peak area ratios were dependent on the nature and composition of the mobile phase, the flow rate, the column temperature, sample concentration and sample preparation.

Keywords: *9-Amino-20(S)-camptothecin; liquid chromatography; enantiomer separation; derivatization; 1-(1-naphthyl)-ethyl isocyanate; NMR chiral shift reagent.*

Introduction

20(*S*)-Camptothecin (CAM), a natural product with anti-cancer activity, was first reported in 1966 [1]. The recent discovery of the unique mechanism for the anti-cancer activity of CAM, via its stabilization of the complex of DNA topoisomerase I and DNA, has prompted a renewed interest in CAM and its analogues. One of the most promising synthetic analogues of CAM to emerge from structure–activity studies is 9-amino-20(*S*)-camptothecin (9-A-20(*S*)-CAM) [2–5].

The stereochemistry at the 20 position of CAM and its analogues, such as 9-A-20(*S*)-CAM, is crucial for the anti-cancer activity, which resides exclusively with the 20(*S*) enantiomer [5]. Contamination from the inactive 20(*R*) enantiomer can be anticipated for 9-A-20(*S*)-CAM (Fig. 1), which is not a natural product, as there are two reasonable sources for its formation. One is through a total synthesis of 9-A-20(*S*)-CAM that proceeds with incomplete stereocontrol [5]. Alternatively, the 20(*R*) enantiomer might be formed via the potentially epimerizable nature of the tertiary, benzylic alcohol at the chiral centre (Fig. 1). Although 9-A-20(*S*)-CAM can be prepared from CAM via partial synthesis, the high cost of CAM itself (approx. \$20,000 g^{-1}) and low yields (approx. 10%) [3] of the reactions introducing the 9-amino group makes partial synthesis an unattractive option. Therefore, large-scale preparation of 9-A-20(*S*)-CAM via a total synthesis is a reasonable alternative, despite the additional problem regarding stereocontrol at the 20 position.

Given the importance of the chiral centre in 9-A-CAM, establishing the ratio of the active 9-A-20(*S*)-CAM to the inactive 20(*R*) enantiomer in bulk drug samples will allow a more accurate assessment of sample activity and purity. Determination of sample purity should be reproducible and accurate for minimum levels of contamination of 1% or less. Because the optical rotation of 9-A-20(*S*)-CAM is too low (16°) (W. Waugh and C.M. Riley, University of Kansas, unpublished results, 1991) to enable minor contamination to be detected, a more sensitive assay was required.

* Presented at the Seventh Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists (Analysis & Pharmaceutical Quality Section), San Antonio, TX, USA, 15–19 November 1992.

† Author to whom correspondence should be addressed.

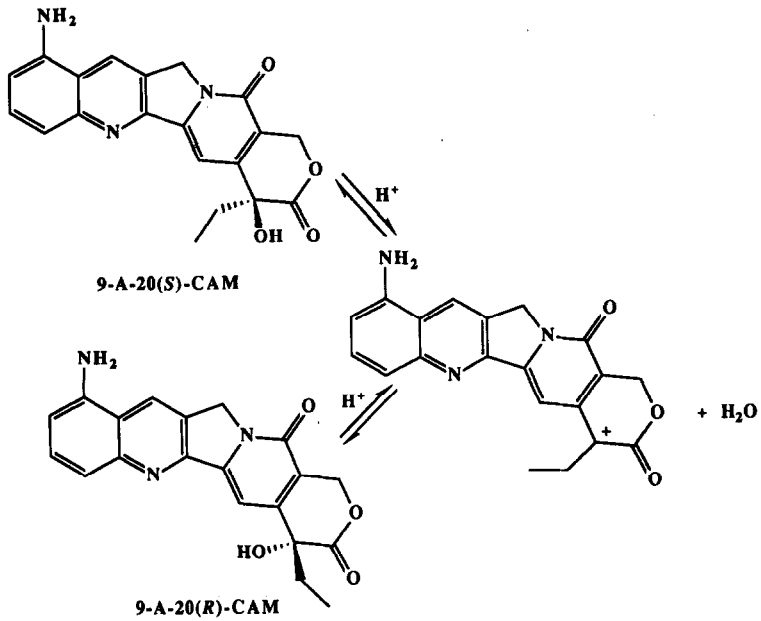

Figure 1
Proposed epimerization of 9-amino-20(*S*)-camptothecin.

Experimental

Chemicals and reagents

9-A-CAM (racemic, 20(*S*)-, and 20(*R*)-) were obtained from the National Cancer Institute, Bethesda, MD, USA. NMR solvents, (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol (98%+), (*R*)-(+)-1-(1-naphthyl)ethyl isocyanate (98%), (*S*)-(−)-1-(naphthyl)ethyl isocyanate (99%), were purchased from Aldrich Chemical Company and used as received. HPLC organic solvents (HPLC grade) were purchased from Fisher Scientific.

Apparatus

^{1}H-NMR spectra were recorded on a Bruker AM 500, and obtained in pyridine-d_5 using the most upfield signal of contaminating pyridine (7.19 ppm) as an internal reference. Liquid chromatography experiments were conducted on a modular LC system consisting of the following components: a Beckman Model 110B pump, a Rheodyne Model 7125 fixed loop injector (20 μl), and a Kratos Spectroflow 757 monitor (370 nm). The LC column was ODS Hypersil column (5 μm, 150 × 4.6 mm), with temperature control using a heater from Jones Chromatography at 25°C. The mobile phase was methanol–water (50.5:49.5, v/v). Chromatographic data were collected and reduced with a Shimadzu C-R3A data station; relative peak heights were used to determine the enantiomeric purity (*EP*) of the drug:

$$EP = \frac{H_2}{H_1 + H_2} \times 100\%, \qquad (1)$$

where H_1 and H_2 are the peak heights of the first and second peaks to elute, respectively. In the final method the first peak was derived from the *R*-enantiomer and the second peak was from the *S*-enantiomer.

On-line mass spectra (LC–MS) were obtained using a Vestec Model 201A LC–MS with thermospray ionization; the control temperature was 166°C, tip temperature was 215°C, and block temperature was 268°C.

^{1}H-NMR assignments of 9-A-CAM in pyridine-d_5

(500 MHz) δ 9.03 (s, 1 H, H-7), 8.65 (brs, 1 H, -OH), 7.92 (d, J = 8 Hz, 1 H, H-12), 7.70 (m, 1 H, H-11), 7.18 (obscured by solvent, 1 H, H-10), 6.67 (brs, 2 H, -NH_2), 5.90 (d, J = 16.1 Hz, 1 H, H-17$_\beta$), 5.57 (d, J = 16.1 Hz, 1 H, H-17$_\alpha$), 5.33 (d, J = 18.5 Hz, 1 H, H-5), 5.27 (d, J = 18.5 Hz, 1 H, H-5), 2.10 (q, 2 H, H-19), 1.13 (t, 3 H, H-18).

Sample handling

Due to the severe toxicity of 9-A-CAM, all samples were handled with gloves, and

destroyed by treatment for several hours with a considerable excess of 0.2 N NaOH.

NEI derivatization reactions. General procedures

All manipulations (prior to derivatization) and reactions were performed using anhydrous conditions; i.e. in dry apparatus and under an atmosphere of argon. For NMR analysis, approximately 1–2 mg (2.7–5.4 μM) of 9-A-CAM was dissolved in 1 ml of pyridine-d_5 in an NMR tube, and 30 μl (171 μM) of NEI were added. The reactions were analysed after 12 h, and after 7 days. For HPLC analysis, approximately 1–2 mg (2.7–5.4 μM) of 9-A-CAM was dissolved in 2 ml of pyridine, and 20 μl (114 μM) of NEI were added. After 12 h, 100 μl of methanol were added, after a further 12 h, 50 μl of the sample was added to 950 μl of methanol, and analysed.

Calibration and analysis of samples

To 0.5 ml solutions of 9-A-20(*S*)-CAM (2.0 mg ml^{-1}) in pyridine were added 0.5 ml of pyridine solutions of 9-A-20(*R*)-CAM (0.0063, 0.0125, 0.050, 0.10 or 0.20 mg ml^{-1}). (*S*)-NEI (20 μl) was added and the reactions processed according to the general procedure.

Results and Discussion

Preliminary studies with non-covalent chiral shift reagents

Early on in the present study, ^{1}H-NMR studies involved (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (TAE), a non-covalent chiral shift reagent established as resolving chiral lactones [6]. A concern was the poor solubility of 9-A-CAM, which precluded use of all but the most polar solvents (DMSO, DMA, DMF). Use of polar NMR solvents was disadvantageous because the shift reagents can coordinate to the polar moiety of the solvent, rather than to the polar group of the enantiomers resulting in minimal signal differences between the transient reagent–enantiomer complexes.

Pyridine was the least polar solvent that dissolved a sufficient concentration of 9-A-CAM for these studies. However, no difference of ^{1}H-NMR signals was observed upon addition of TAE to a pyridine solution of 9-A-CAM. Although 9-A-CAM was not soluble in chloroform, a significantly less polar solvent than pyridine, 9-A-CAM could be dissolved in chloroform by using a substantial excess of TAE; the strategy was not useful because the 9-A-CAM signals were obscured by the signals of the shift reagent. Because analogous problems were anticipated for all other non-covalent shift reagents in similar studies with 9-A-CAM, non-covalent shift reagents were not pursued further.

Table 1
Analysis of samples of 9-A-20(*S*)-CAM containing various amounts of the *R*-enantiomer

[9-A-20(*S*)-CAM]*				
Added (% w/w)	Found (% w/w)	SD	*n*	RSD (%)
10.0	12.2	0.09	7	0.77
5.0	7.70	0.11	6	1.56
2.5	4.0	0.09	7	2.22
1.25	2.57	0.15	6	5.76
0.63	2.11	0.14	8	6.56
0.32	1.87	0.14	7	7.49
0.00	1.50	0.21	6	14.2

*Ratio of 9-A-20(*S*)-CAM to 9-A-20(*R*)-CAM expressed as a percentage. The initial concentration of 9-A-20(*S*)-CAM prior to derivatization was 1 mg ml^{-1}.

Derivatization strategies

The enantiomers were resolved and quantitated following derivatization with the chiral shift reagent 1-(1-naphthyl)ethyl isocyanate (NEI). ^{1}H-NMR was used to confirm that derivatization occurred, and RP-LC was used to quantitate the results. Pyridine was used as the reaction solvent because the ^{1}H-NMR signals of 9-A-CAM in pyridine-d_5 demonstrated superior resolution relative to those reported in other solvents, the solubility of 9-A-CAM (2 mg ml^{-1}) was acceptable and the reaction proceeded more rapidly with fewer side products in pyridine than in DMA. A reasonable explanation for some of the effects of solvent on spectra involved the C-20 alcohol. The alcohol can hydrogen-bond to the adjacent C-19 carbonyl group in non-hydrogen bonding solvents (pyridine), resulting in a restricted movement of the 9-A-CAM ring system, and more significant signal differences. When 9-A-CAM is in a hydrogen bonding solvent such as DMSO that can break up this intramolecular hydrogen bond, the ring is not fixed in such a rigid position, which simplifies the spectrum.

Two concerns with the use of very polar solvents (e.g. pyridine) for these derivatization reactions were H_2O absorbed by the hygroscopic solvent and contamination from D_2O,

used in the manufacture of deuterated solvents. Excess of the NEI was added to ensure the availability of sufficient reagent for reaction with the enantiomers because water reacts rapidly with electrophilic derivatizing reagents. Another concern of using hygroscopic (i.e. wet) pyridine as a solvent was the instability of the lactone ring of CAM and its analogues. This lactone undergoes a facile ring opening under aqueous, basic conditions, assisted by an intramolecular hydrogen bond from the adjacent hydroxyl group [2, 7–11] (Fig. 2). This opening creates two new potential sites for reactions with the derivatizing reagent. However, control studies demonstrated that the 9-A-CAM lactone ring did not undergo opening after 24 h in wet pyridine.

9-A-CAM contains two nucleophilic sites for reaction with NEI, the 9-amino group, and the 20-hydroxyl group. The addition of NEI to a solution of 9-A-CAM in pyridine resulted first in reaction of the derivatizing reagent with contaminating water. Subsequent reaction (completed overnight) occurred at the 9-amino rather than the 20-hydroxyl group, as evidenced by the addition of one equivalent of the derivatizing reagent, disappearance of the $-NH_2$ signal, differences (0.01 ppm) of the chemical shifts for protons at C17 and at C7 (the C5 signals were obscured by the excess reagent), and no change of the 20-ethyl signals. Subsequent derivatization of the hydroxyl group at C-20 was detected (FABMS) after approximately 2 weeks reaction with excess NEI. Although ^{1}H-NMR was used to confirm site of the initial derivatization reaction as the 9-amino nitrogen and not the 20-hydroxy oxygen, the diastereomers of 9-A-CAM were not sufficiently resolved by ^{1}H-NMR for quantitative measurements of chiral purity (Fig. 3).

A conformation possible for the amine-

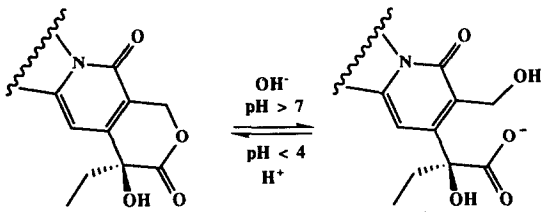

Figure 2
Hydrolysis of the lactone ring of camptothecin analogues.

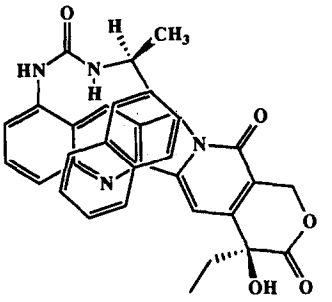

Figure 4
Possible conformation of the (*R*)-NEI adduct of 9-amino-20(*S*)-camptothecin.

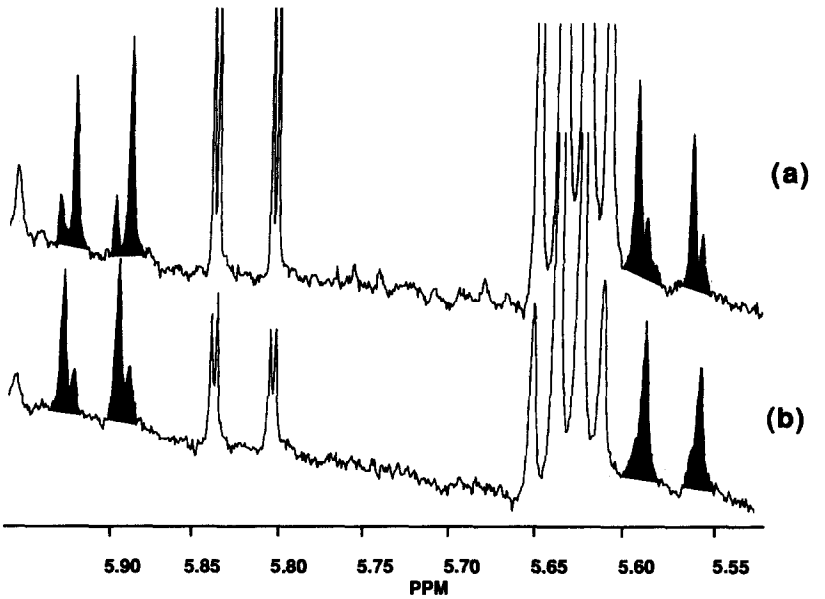

Figure 3
Effect of derivatization with 9-A-CAM NEI on the ^{1}H-NMR spectrum of 9-A-CAM in pyridine-d_5. The shaded, split peaks at 5.92, 5.90, 5.58 and 5.56 ppm appeared as singlets in the spectra of the underivatized samples. The ratio of the *R*:*S* isomers of 9-A-CAM was 1:3 and the derivatizing agent was (a) (*R*)-NEI and (b) (*S*)-NEI.

derivatized product suggested in Fig. 4 shows how derivatization might influence the protons whose NMR signals were altered. The large separation between the two chiral centres is consistent with the small difference in the chemical shifts observed.

In principle, the lactone of 9-A-CAM could be opened deliberately (Fig. 2) and the resulting carboxylic acid reacted with a chiral amine or alcohol. This would form products bringing the two chiral centres in much closer proximity to each other, and probably improve resolution. However, concerns regarding the reproducibility of such reactions, given the reversible nature of this facile reaction [8] indicated that an alternative would be desirable. The mono-derivatized 9-A-CAM enantiomers were resolved by RP-HPLC (Fig. 5) and characterized by LC–MS analysis (Figs 6 and 7). The two diastereomers produced identical mass spectra (Fig. 7), each with the molecular ion (*m/z* 561) required for mono-derivatization with NEI. Comparable tracking of important fragments (*m/z* 346, 320) using selective ion monitoring (Fig. 6) also was consistent with the two compounds being very similar.

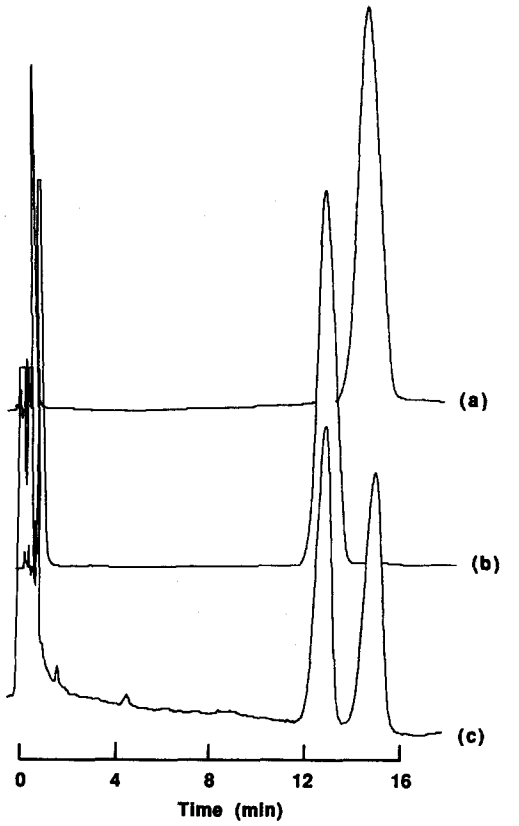

Figure 5
Reversed-phase LC separation of 9-amino-camptothecin after reaction with NEI. Chromatographic conditions: columns: ODS Hypersil column (5 μm, 150 × 4.6 mm); mobile phase: methanol–water 1:1 (v:v); temperature: 35°C; flow rate 2.5 ml min^{-1}; detection: 370 nm. Key: (a) 9-Amino-20(*S*)-camptothecin + (*S*)-NEI; (b) 9-amino-20(*S*)-camptothecin + (*R*)-NEI; (c) racemic 9-amino-camptothecin + (*S*)-NEI.

Factors affecting chromatographic resolution and reproducibility

Choice of the stereochemistry of the derivatizing reagent. Derivatization of each purified enantiomer was performed to prepare analytical standards of each diastereomer. Selection of the (*S*)-NEI for derivatization of racemic 9-A-CAM afforded diastereomers in which the derivatized 9-A-20(*R*)-CAM (the contaminant) eluted before the derivatized 9-A-20(*S*)-CAM (Fig. 5), the prefered elution order for the trace analysis of impurities.

Choice of detection wavelength. The greatest sensitivity for the detection of the diastereomers in the reaction mixture was via a deuterium lamp using absorbance measurements at 280 nm. However, other constituents of the reaction mixture also absorbed at that wavelength and interfered with the analytes of interest. The diastereomers were detected with much greater selectivity by using a tungsten lamp at 370 nm, which corresponds to the λ_{max} of the camptothecin ring system. Consequently the signal-to-noise ratio for the detection of the analytes of interest was much greater when detected at 370 nm compared with 280 nm even though the sensitivity was about 2 orders of magnitude less. The interferences that were only detected at 280 nm were presumed to contain a naphthyl ring system and were therefore probably degradation products of the chiral derivatizing agent, NEI. Camptothecin is highly fluorescent. However, the 9-amino-camptothecins have little or no fluorescence and no improvement in sensitivity or signal-to-noise ratio was observed using fluorescence detection with excitation at 370 nm.

Effects of temperature on resolution. Initially, the chromatography was characterized at ambient temperature. However, substantial variability in retention times was observed as a result of fluctuations in room temperature. Furthermore, resolution was found to decrease with elevated temperature

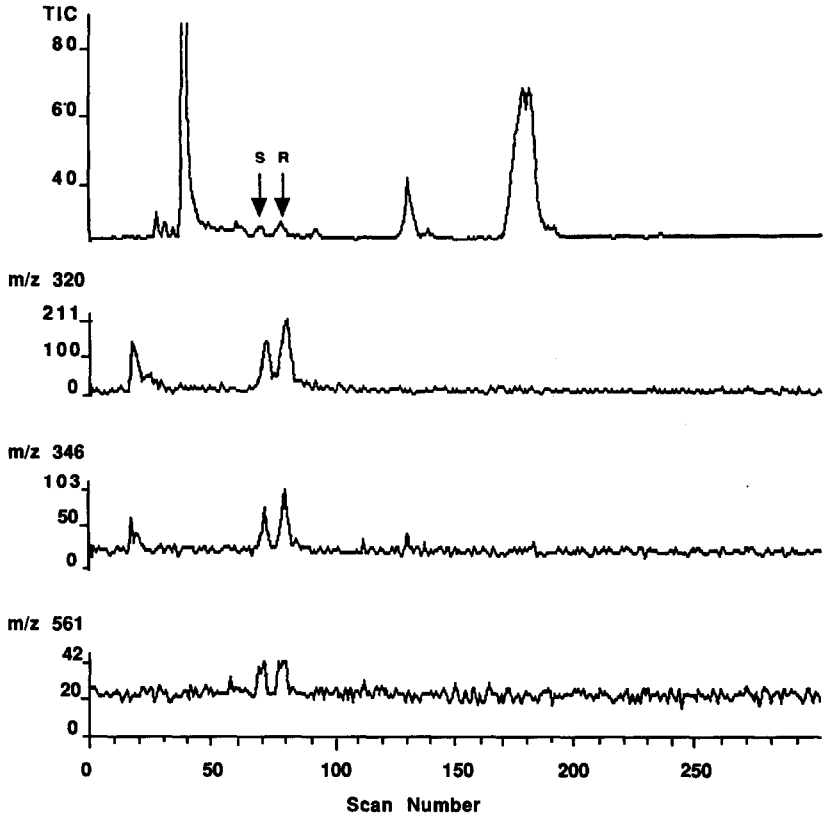

Figure 6
Reversed-phase liquid chromatographic separation with total ion-current (TIC) and single-ion monitoring of racemic 9-amino-camptothecin after reaction with (*R*)-NEI. Chromatographic conditions: column: ODS Hypersil column (5 μm, 150 × 4.6 mm); mobile phase: methanol–ammonium acetate (0.1 M) (7:3, v/v); temperature: ambient; flow rate 1.5 ml min^{-1}; detection: thermospray MS.

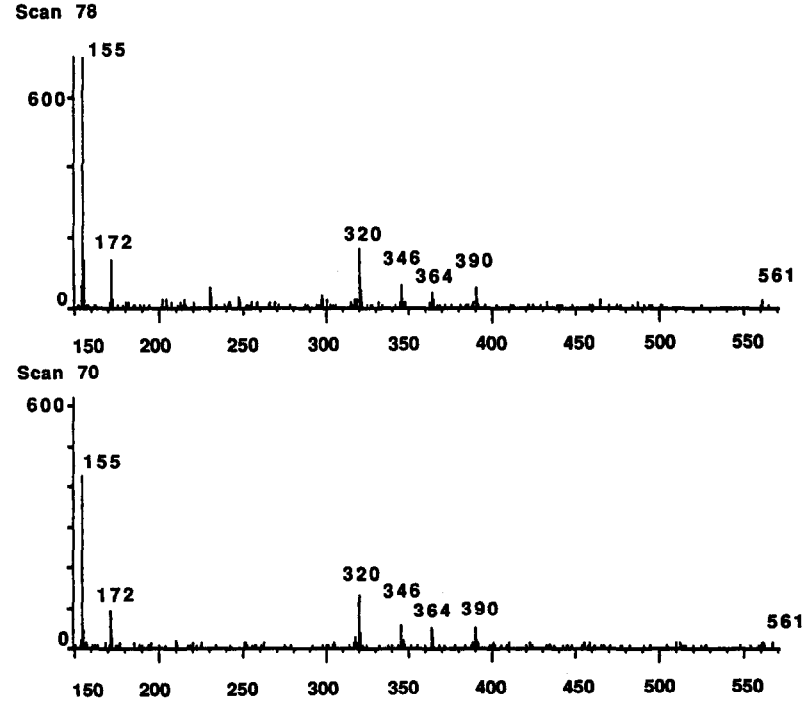

Figure 7
Mass spectra of the two diasereomers of racemic 9-amino-camptothecin after reaction with (*R*)-NEI adducts obtained by LC–MS. See Fig. 6 for conditions and positions of scan numbers.

due to concomitant decreases in retention and selectivity. Therefore, variations in retention and resolution due to fluctuations in room temperature were avoided by thermostating the column slightly above room temperature, at 25°C.

Mobile phase effects. Resolution of the diastereomers was affected greatly by the organic modifier added to the mobile phase. Under isoelutropic conditions (t_r (9-A-20(*S*)-CAM) = 14 min) baseline resolution (R_s = 1.34) was obtained using methanol–water (1:1), whereas acetonitrile–water (3:7) afforded partial resolution (R_s = 0.7), and tetrahydrofuran–water (3:2) failed to produce any resolution at all. Addition of 2-propanol (2%) to the methanol–water system did not produce any further improvement in resolution.

Resolution was sensitive to changes in flow rate and the concentration of methanol in the mobile phase. At a flow rate of 2.5 ml min^{-1}, a 1% increase in methanol concentration decreased the retention times of both diastereomers by approximately 1 min. The separation of the two diastereomers was optimized under conditions of normalized retention by simultaneously varying the flow rate and the concentration of methanol in the mobile phase. The flow rate was varied from 0.5 to 2.5 ml min^{-1} and the retention time of 9-A-20(*S*)-CAM was maintained at 14 min by changing the concentration of methanol in the mobile phase. The greatest resolution was obtained at a flow rate of 2.5 ml min^{-1} with a mobile phase of methanol–water (1:1, v/v). However, flow rates greater than 2.5 ml min^{-1} could not be used for long periods of time because of the high pressure drop that was generated.

Quenching the reaction. When crude reaction mixtures (containing excess NEI) were diluted with mobile phase to quench the reaction, the retention time of each successive sample decreased substantially as a function of the cumulative amount of reaction mixture injected. This decrease in retention was attributed to a component from the derivatization reaction mixture binding tightly to the column and blocking sites for interaction with the diastereomers. This component was generated by reaction of NEI with the water in the mobile phase to afford an amine, which then reacted further with NEI to give a urea (Fig. 8). Formation of the urea was avoided by quenching the excess NEI with methanol prior to analysis, to form a more stable carbamate (Fig. 8). The stable carbamate did not disturb the chromatography of the analytes and gave reproducible retention times for the diastereomers of interest. The proposed carbamate was isolated from the reaction mixture and its structure confirmed by independent synthesis. The ^{1}H-NMR spectra of the isolated compound and the synthetic standard were identical.

Quenching of the reaction also prevented the production of the double derivatives of 9-A-CAM, which eluted at approximately 140 min. Samples contaminated with di-derivatized 9-A-CAM required either a 140 min run time, or washing and re-equilibration of the column between injections. The solvent used to dilute prior to injection also affected the stability and the solubility of the derivative. The derivatized diastereomers (0.10 mg ml^{-1}) precipitated and decomposed rapidly when the concentration of water in the injection solvent was greater than 10%. Thus the samples could not be diluted with mobile phase prior to analysis and the best solvent for injection was methanol.

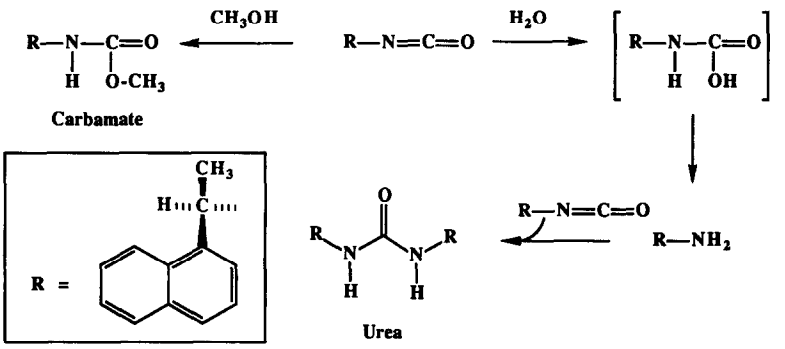

Figure 8
Proposed mechanisms for the degradation of the derivatizing reagent in water and in methanol.

Assay validation

Reproducibility of peak area ratio. Quenching the monoderivatization reaction with methanol after 12 h introduced the possibility that the reaction was not complete at the time of the quench. Therefore, experiments were conducted to confirm that the two diastereomers were produced at equal rates. Racemic 9-A-CAM was derivatized with either (*S*)-NEI or (*R*)-NEI. Aliquots were removed at various times over the time period which corresponded to 40–95% of completion of the reaction. Each aliquot was quenched immediately with methanol and the ratio of derivatized 9-A-20(*S*)-CAM:derivatized 9-A-20(*R*)-CAM determined. That ratio was 1:1 for each aliquot, indicating that the two diastereomers of derivatized 9-A-CAM were produced at equal rates.

Linearity, precision and limit of detection for the method. The region of linearity for the assay was determined to be 0.0002 to 0.1 mg ml^{-1} for each enantiomer in the reaction mixture ($n = 6$, $r > 0.999$). Whereas 0.1 mg ml^{-1} of each diastereomer could be injected without peak distortion, the resolution of the two peaks of interest was influenced by the amount of sample injected (Fig. 9). The best compromise between optimum sensitivity and resolution was obtained when the reaction medium was diluted with methanol to a concentration equivalent to 0.05 mg ml^{-1} 9-A-CAM (Figs 9–11).

Calibration curves to determine the enantiomeric purity of the bulk drug were prepared by adding 0.32 to 10% w/w 9-A-20(*R*)-CAM to a solution containing 1.0 mg ml^{-1} 9-A-(*S*)-CAM. The method was linear (Table 1) from 0 to 10% w/w 9-A-20(*R*)-CAM added; however, a positive intercept equivalent to 1.5% w/w 9-A-20(*R*)-CAM was observed. The positive intercept can be attributed to either residual 9-A-20(*R*)-CAM in the sample of 9-A-20(*S*)-CAM, contamination of the (*S*)-NEI by its *R*-enantiomer or both. When the sample of 9-A-20(*S*)-CAM was reacted with the (*R*)-NEI the apparent enantiomeric purity was found to 2.5%. The manufacture's claims for purity of the (*R*)-NEI and the (*S*)-NEI are 98 and 99%, respectively. Therefore, it is reasonable to conclude that the positive intercept (Figs 10 and 11) arose from the contamination of the (*S*)-NEI by its *R*-enantiomer because control studies showed that the derivatives did not racemize under the conditions of the reaction.

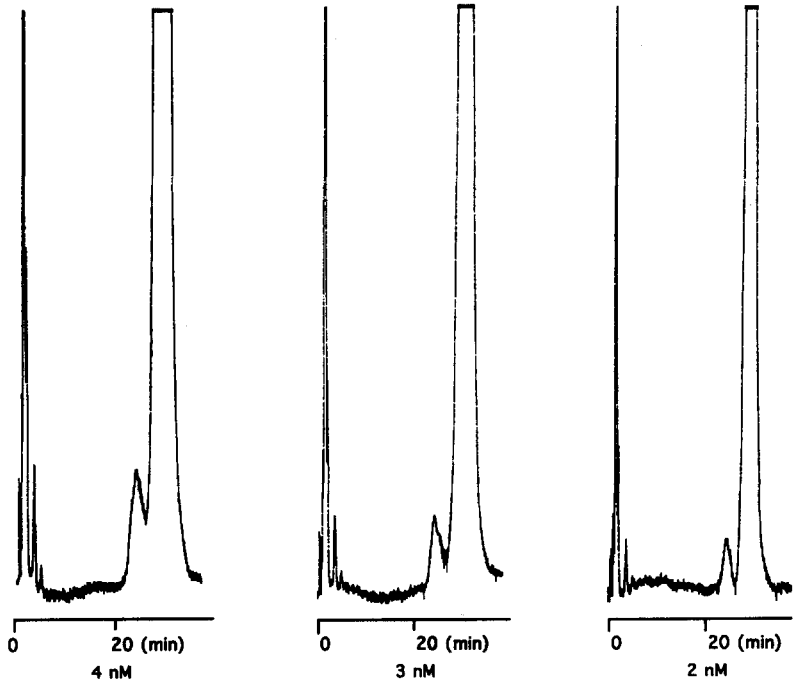

Figure 9
Effect of the amount of sample injected on the resolution of the diastereomers. The concentrations of the *S*-enantiomers are shown below each chromatogram. Chromatographic conditions: column: ODS Hypersil column (5 μm, 150 × 4.6 mm); mobile phase: methanol–water (50.5:49.5, v/v); temperature: 25°C; flow rate 2.1 ml min^{-1}; injection volume 20 μl: detection: 370 nm.

Conclusions

The two enantiomers of 9-A-CAM were reacted at the amino group with the chiral derivatizing reagent (*S*)-NEI, and the resulting diastereomers were resolved by reversed-phase liquid chromatography. One novel aspect of this study arose from the high insolubility of 9-A-CAM in non-polar solvents. Thus a very polar, hygroscopic solvent, pyridine had to be used for the reaction instead of a non-polar solvent conventionally used for the reaction of nucleophiles with isocyanates. The second problem experienced in this study was the presence of two nucleophilic sites (9-amino, 20-hydroxyl) on the analyte of interest. Whereas a substantial excess of NEI was used to prevent the derivatization reagent being consumed by the water in the solvent, quenching of the reagent was necessary to prevent di-derivatization of the analytes. This two-step derivatization procedure did not introduce any bias into the method because the diastereomers were produced at equal rates. The limit of quantification of the *R*-isomer in samples of 9-A-20(*S*)-CAM could not be reliably determined because the sample of *S*-isomer available was not pure. Whereas, the RSD for the determination of the *R*-isomer after spiking at the 0.32% w/w level was 14.2, the apparent concentration of *R*-isomer in the blank was 1.50% w/w. The signal-to-noise ratio of the

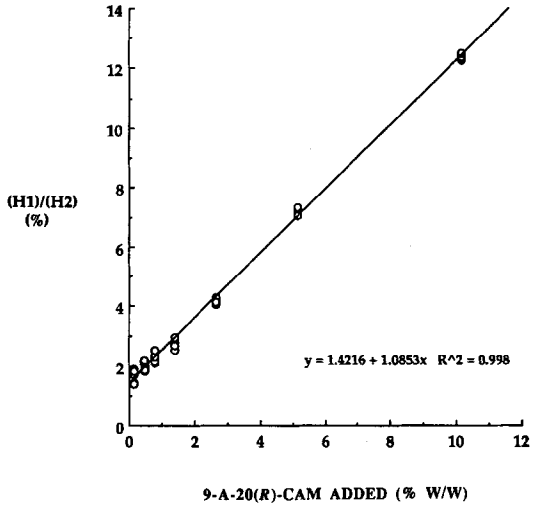

Figure 10
Relationship between the peak height ratio of the two derivatives (H1/H2) and the amount of 9-A-20(*R*)-CAM added. The initial concentration of 9-A-20(*S*)-CAM, prior to derivatization, was 1 mg ml^{-1}.

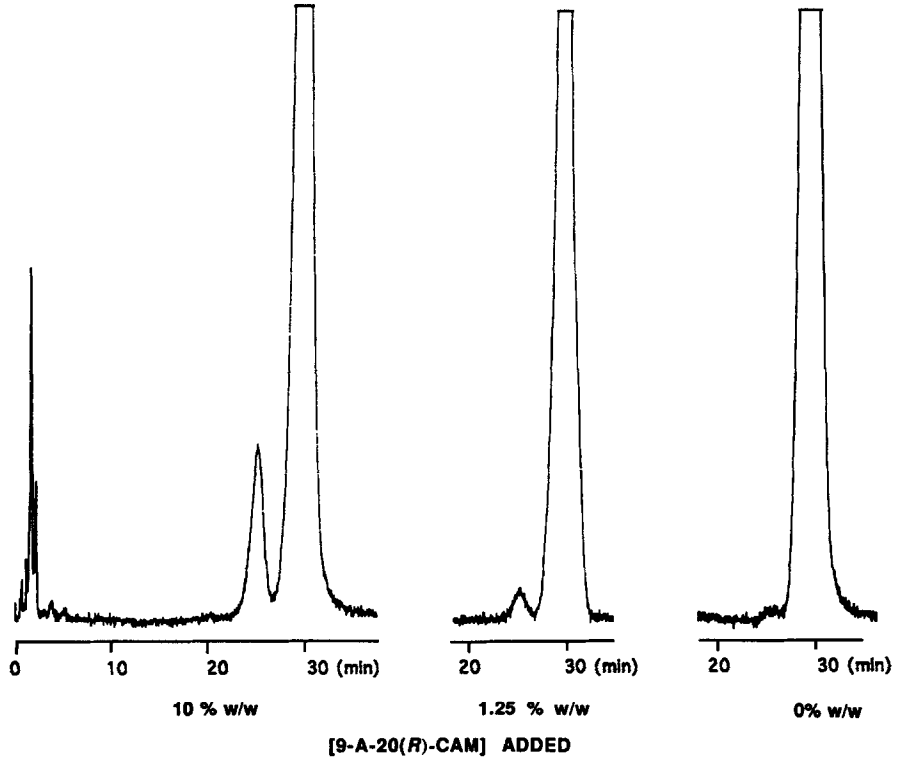

Figure 11
Chromatograms of 9-A-20(*S*)-CAM containing various amounts of the *R*-isomer. Chromatographic conditions: see Fig. 9. The initial concentration of 9-A-20(*S*)-CAM, prior to derivatization, was 1 mg ml^{-1}.

peak corresponding to the *R*-isomer in the blank was 5:1. This suggests that the limit of detection for the determination of 9-A-20(*R*)-CAM in bulk drug samples of 9-A-20(*S*)-CAM is approximately 1%, which is substantially better than methods based on optical rotation (C.M. Riley and W. Waugh, unpublished data) or NMR (this study). Considering the large distance between the two chiral centres, it is quite surprising that the diastereomers produced by the reaction of 9-A-CAM with NEI were well separated (R_s = 1.5) by reversed-phase liquid chromatography (Fig. 4). However, that degree of resolution ultimately limits the amount of the *R*-isomer that can be detected in samples of 9-A-20(*S*)-CAM to about 1% w/w.

Acknowledgements — The authors thank Dr D. Bindra and W. Waugh for assistance with the HPLC analysis, Dr K. Shorno for performing the LC–MS analysis, Dr D. Vander Velde for performing the 2-D NMR analysis, and Dr G. Georg for helpful discussions. Support for this research by NCI Training Grant CA 09242 and the Wesley Foundation Scholars Program is gratefully acknowledged.

References

[1] M.C. Wani, A.W. Nicholas and M.E. Wall, *J. Am. Chem. Soc.* **94**, 3888–3890 (1966).
[2] W.D. Kingsbury, J.C. Boehm, D.R. Jakas, K.G. Holden, S.M. Hecht, G. Gallagher, M.J. Caranfa, F.L. McCabe, L.F. Faucette, R.K. Johnson and R.P. Hertzberg, *J. Med. Chem.* **34**, 98–107 (1991).
[3] M.C. Wani, A.W. Nicholas and M.E. Wall, *J. Med. Chem.* **29**, 2358–2363 (1986).
[4] M.C. Wani, A.W. Nicholas, G. Manikumar and M.E. Wall, *J. Med. Chem.* **30**, 1774–1779 (1987).
[5] M.C. Wani, A.W. Nicholas and M.E. Wall, *J. Med. Chem.* **30**, 2317–2319 (1987).
[6] W.H. Pirkle, D.L. Sikkenga and M.S. Pavlin, *J. Org. Chem.* **42**, 384–387 (1977).
[7] J.H. Beijnen, B.R. Smith, W.J. Keijer, R.V. Gijn, W.W.T.B. Huinik, L.T. Vlasveld, S. Rodenhuis and W.J.M. Underberg, *J. Pharm. Biomed. Anal.* **8**, 789–794 (1990).
[8] J. Fassberg and V.J. Stella, *J. Pharm. Sci.* **81**, 676–684 (1992).
[9] J.P. Loh and A.E. Ahmed, *J. Chromatogr.* **530**, 367–376 (1990).
[10] D.O. Scott, D.S. Bindra and V.J. Stella, *Pharm. Res.*, submitted.
[11] J.G. Supko and L. Malspeis, *J. Liq. Chromatogr.* **14**, 1779–1803 (1991).

[*Received for review 1 November 1992; revised manuscript received 2 December 1992*]