Journal of Chromatography, 481 (1989) 421–427
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 851

Note

Reversed-phase high-performance liquid chromatographic assay for camptothecin and related alkaloids

B. L. POEHLAND*, N. TROUPE, B. K. CARTÉ and J. W. WESTLEY

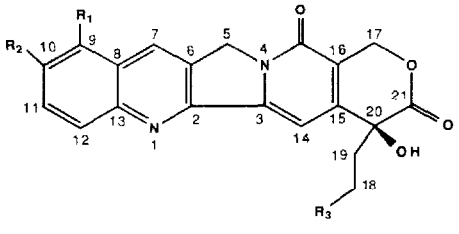
Research and Development Division, Department of Biomolecular Discovery, Smith Kline and French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939 (U.S.A.)

(First received March 6th, 1989; revised manuscript received July 20th, 1989)

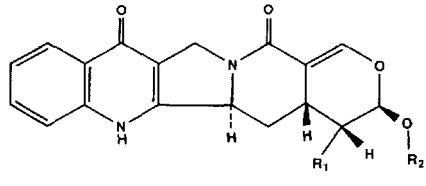
The potent antitumor alkaloid camptothecin **1** (Fig. 1), a component of *Camptotheca acuminata* (family Nyssaceae), a tree indigenous to China, was first described over twenty years ago[1]. Since then a number of hydroxyl and methoxyl derivatives have been reported, which exhibit similar levels of activity[2,3], and in addition a number of structurally related compounds have been isolated[4]. Despite problems of insolubility and toxicity there persists a significant level of interest in the mechanism of action of camptothecin[5].

In conjunction with our interest in water-soluble derivatives of camptothecin antineoplastics[6] approximately 2 l of alkaloid-enriched extract of *C. acuminata* were obtained for the purpose of investigating the possible presence of naturally occurring polar camptothecins which might exhibit appreciable aqueous solubility. We were also interested in the identification and isolation of any known campthotecin analogues which would be useful as bioassay standards or starting material for synthetic transformations. A suitable high-performance liquid chromatographic (HPLC) assay was essential for achieving these objectives.

Due to anticipated requirements for high sensitivity and solvent programming, a reversed-phase assay employing acetonitrile–water was selected because of its reduced baseline displacement of the gradient profile compared to methanol–water



1. $R_1 = R_2 = R_3 = H$
2. $R_1 = R_3 = H$, $R_2 = OH$
3. $R_1 = OCH_3$, $R_2 = R_3 = H$
4. $R_1 = R_3 = H$, $R_2 = OCH_3$
8. $R_1 = R_2 = H$, $R_3 = OH$



5. $R_1 = CH{=}CH_2$, $R_2 = \beta$-D-(+)glucose
6. $R_1 = CH_2CH_3$, $R_2 = \beta$-D-(+)glucose
7. $R_1 = CH{=}CH_2$, $R_2 = CH_3$

Fig. 1. Structures discussed in the text.

0021-9673/89/$03.50

systems. We wish to report the chromatographic analysis of the compounds we encountered in our work, including a novel analogue whose structure elucidation is reported elsewhere[7].

EXPERIMENTAL

Apparatus

Liquid chromatography was carried out on a Beckman/Altex instrument (San Ramon, CA, U.S.A.) equipped with two 110A solvent metering pumps, a 420/421 gradient programmer, a Hewlett-Packard (Palo Alto, CA, U.S.A.) 3390A integrator, a Perkin-Elmer (Norwalk, CT, U.S.A.) ISS-100 autosampler with a 50-μl sample loop and custom timing interface[9], and a Schoeffel/Applied Biosystems (Westwood, NJ, U.S.A.) SF770 Spectroflow monitor set at 270 nm (0.04 u.a.f.s.). A Whatman (Hillsboro, OR, U.S.A.) Partisil-10 ODS-3 C_{18} reversed-phase column (25 cm $\times$ 4.6 mm I.D.) was used at ambient temperature with a flow-rate of 2.0 ml/min and nominal pressure drop of 2000 p.s.i.g. (13.8 MPa). Standard materials were weighed on a Cahn (Cerritos, CA, U.S.A.) Model 29 electrobalance.

Starting material

Sample FB-12100B (1900 g) was received from Polysciences (Warrington, PA, U.S.A.) as an opaque viscous liquid containing suspended solids. This was the concentrate from the final one-third of the eluate from a large scale Amberlyst 15 column using 0.25% ammonia in isopropanol as the eluent. The starting material for this column (365 cm $\times$ 30 cm) was an isopropanol extract of sawdust from 24 000 pounds ($10.9 \cdot 10^3$ kg) of *C. acuminata* logs. The isopropanol extractions were performed by Madis Labs. (Hackensack, NJ, U.S.A.) using trees obtained from the USDA Plant Introduction Center (Chico, CA, U.S.A.).

Reagents and standards

Unless otherwise indicated, all chemicals used were ACS reagent grade. Water used in preparing the HPLC solvent system was obtained from an in-house Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Acetonitrile for the HPLC system was Baker HPLC grade used directly without filtration. Silica used for column chromatography was from Merck (Darmstadt, F.R.G.) silica gel 60 70–230 mesh, and Sephadex LH-20 chromatography gel was obtained from Pharmacia (Uppsala, Sweden). Authentic samples of camptothecin **1**, 10-hydroxycamptothecin **2**, 9-methoxycamptothecin **3**, and 10-methoxycamptothecin **4** were supplied by the National Cancer Institute. The 18-hydroxy analogue **8** was isolated and identified by comparison of its spectroscopic and physical properties with published data[8]. Glycoside **5** was isolated and structural assignment made by analysis of physical and spectroscopic data[7]. The hydrogenated derivative **6** and methoxy hydrolysate **7** were prepared synthetically from **5**.

HPLC of camptothecin standards

Camptothecin and its 10-hydroxy and 9- and 10-methoxy analogues were each dissolved in acetonitrile at a concentration of 100 μg/ml and filtered through a 0.45-μm filter before use. A 25-μl aliquot of each compound was injected in a variety

of HPLC systems employing various concentrations of acetonitrile–water and acetonitrile–water acidified with 0.1% trifluoracetic acid. The acidified solvent systems were quickly abandoned due to excessive retention.

Aqueous isocratic systems emplying more than about 40% acetonitrile caused the standards to elute close to the solvent front and failed to adequately resolve the individual components. Several linear gradient solvent programs were evaluated to increase retention and improve resolution, and the gradient program below was found optimal for best resolution within a reasonable analysis time: beginning time 0.0 min, 15% acetonitrile–water isocratic for 1.0 min; beginning time 1.0 min, 15% acetonitrile–water increasing linearly to 25% acetonitrile–water over 10 min; beginning time 11.0 min, 25% acetonitrile–water isocratic for 14 min; beginning time 25.0 min, 25% acetonitrile–water decreasing linearly to 15% acetonitrile–water over 1.0 min; at time 30.0 min, stop program (ready for next injection).

To establish a basis for quantitation the standard materials were dried under high vacuum for 48 h, and their purity assumed to be 100% since each standard gave only a single peak when individual injections were made under the HPLC conditions described above. A stock solution of the standards was prepared by weighing out 1.00 mg of each compound and dissolving in 10.0 ml acetonitrile to give a standard mixture with a concentration of 100 μg/ml of each component. This solution was filtered through a 0.45-μm filter and stored at 4°C. A suitable aliquot of the standard mix was placed in each of three autosampler vials which were sealed before injection.

The system was calibrated for single-point quantitation by analyzing the chromatograms produced by two injections from each vial using the HPLC conditions described above. Measured peak areas and retention times for each standard were averaged and divided by the concentration to generate a response factor whose inverse was programmed into the integrator as a multiplication factor. For subsequent injections the integrator quantitated the standards peaks and printed results directly in units of concentration (μg/ml). When the standards were injected following calibration, the reported concentrations were within 1% of the programmed values (external standard method). At the beginning and end of each day of routine assays, standard injections were made to monitor calibration accuracy. Calibration was observed to deteriorate gradually over a period of weeks; fresh standards were prepared and the system recalibrated when calibration error exceeded 10%.

HPLC assay of isolation fractions

The HPLC conditions established for the camptothecin standards were used to evaluate fractions generated by the isolation scheme summarized in Fig. 2. Sample solutions were filtered through a 0.45-μm filter before injection, and quantitative results were reported as the average of two injections per sample.

Unknown peaks appearing in the HPLC standard profile were roughly quantitated by comparing their peak areas to that of the closest standard. Concentrations of camptothecin **1** and the 10-hydroxy analogue **2** in isolation fractions were determined by direct readout of sample concentration. Sample solutions were diluted as necessary to bring peak response on scale. No peaks corresponding to the methoxy derivatives **3** and **4** were observed in any of our extracts, and all of the isolated compounds were obtained with estimated purity better than 98% (single peak using the standard HPLC conditions).

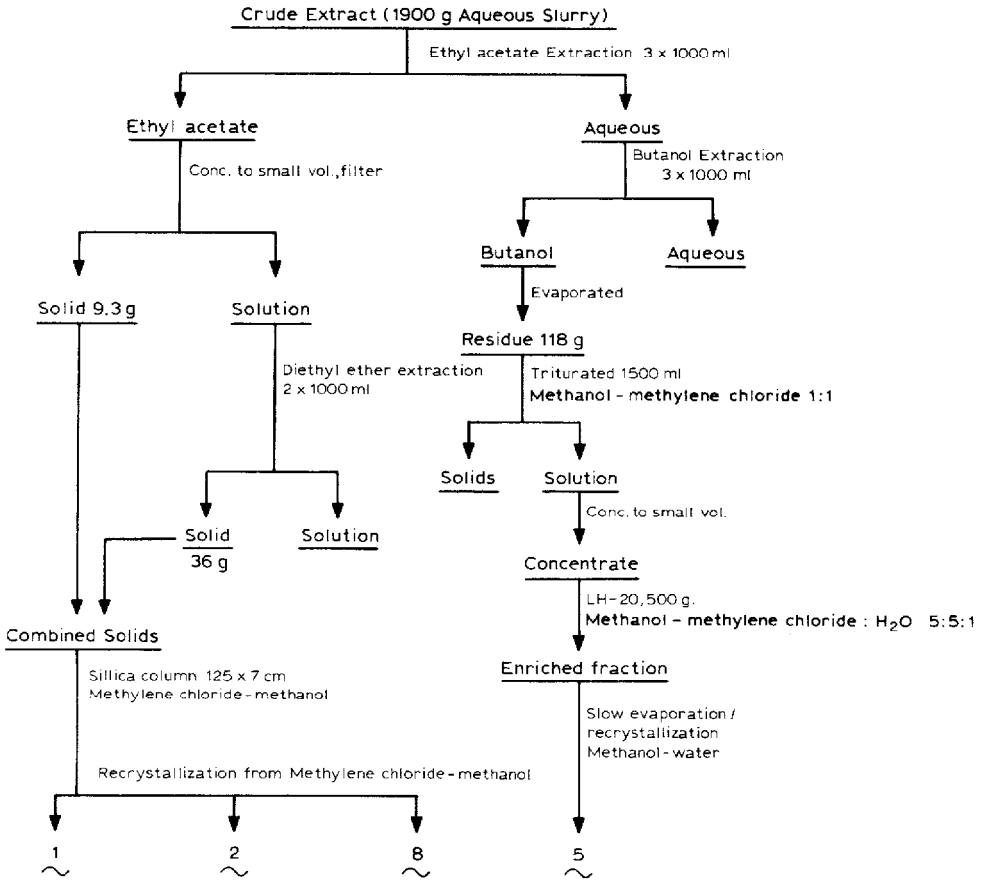


Fig. 2. Simplified purification scheme for camptothecin alkaloids. Numerals refer to structures in Fig. 1.

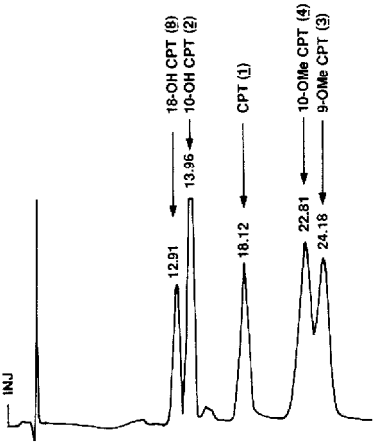


Fig. 3. Reversed-phase chromatographic profile of standard camptothecins.

The first unknown peak to appear in the reversed-phase HPLC profile of the standard camptothecins was the 18-hydroxy analogue **8**, obtained from a silica column fraction of the ethyl acetate soluble portion of the crude extract. This peak was monitored in the purification scheme until its isolation as a single component. The pure material had the same retention time as the peak isolated from partially purified fractions, and comparison of its UV, IR, NMR and mass spectra with published data[8] confirmed its identity. In later assays this compound was incorporated as a standard, as seen in the standard HPLC profile of Fig. 3.

Peaks with retention times corresponding to those of camptothecin **1** and 10-hydroxycamptothecin **2** were similarly monitored until their isolation as single components, and the isolated materials coeluted upon coinjection with the respective standard materials. Their identities were also confirmed spectroscopically.

Purification of the novel glucoside **5** {[3*S*-(3α,4β,4α,5*a*β)]-4-ethenyl-3-(β-D-glucopyranosyloxy)-4,4*a*,5,5*a*,6,12-hexahydro-3H-pyrano-[3′,4′:6,7]indolizino[1,2–6]-quinoline-11,14-dione} from the aqueous portion of the crude extract was easily followed using the standard HPLC conditions because it eluted well ahead of all other components subjected to the assay. However, upon injection of the purified material the HPLC profile exhibited two peaks (Fig. 4A) whose profile remained essentially unchanged despite additional recrystallization. Subsequently it was observed that when the sample was prepared in water or methanol (instead of acetonitrile) the profile collapsed into a single peak whose retention time coincided with that of the later-eluting material (Fig. 4B).

Chemical transformation and HPLC assay of glycoside derivatives

Hydrogenation. The glycoside **5** (100 mg, 20 mmol) was added to 10 ml methanol containing 10 mg of catalyst (10% palladium on activated carbon) and stirred at room temperature under hydrogen atmosphere for 20 h. Filtration of the solution

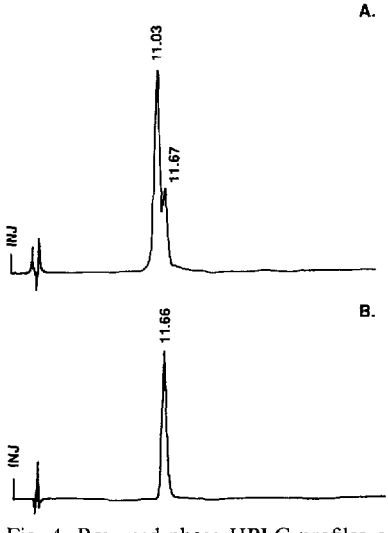


Fig. 4. Reversed-phase HPLC profiles of glycoside **5**. (A) Sample prepared in acetonitrile; (B) sample prepared in water.

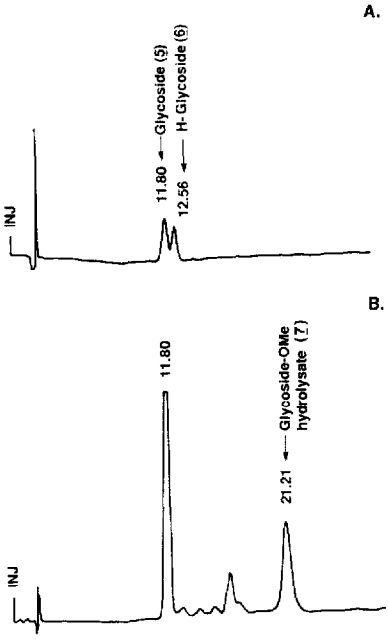


Fig. 5. Reversed-phase HPLC profiles of glycoside **5** reaction mixtures showing (A) the hydrogenated derivative **6** and (B) the O-methyl hydrolysate 7.

yielded 30 mg of hydrogenated glycoside **6** whose structure was confirmed by NMR spectroscopy. Progress of this reaction was monitored using the standard HPLC conditions. Although chromatographic resolution was not as good as for the following methanolysis reaction, it was still sufficient to distinguish product from reactant (Fig. 5A) without modification of the assay.

Methanolysis. The glycoside **5** (50 mg, 10 mmol) was added to 10 ml methanolic 2 *M* HCl and stirred for 80 h at 70°C. Following evaporation of reaction solvent under nitrogen, the residue was taken up in 20% isopropanol–methylene chloride and filtered through silica gel to give a crude product. Final purification via semi-preparative HPLC (Dynamax C_{18}, 23% acetonitrile–water, 2.0 ml/min) yielded 6 mg of the methoxy aglycone **7**, confirmed by NMR spectroscopy. Using the standard HPLC conditions the course of this reaction was easily followed due to the high degree of resolution between the starting material **5** and the methanolysis product **7** (Fig. 5B).

RESULTS AND DISCUSSION

Retention times of all the camptothecins and related alkaloids subjected to the described HPLC assay are summarized in Table I. The ability of the assay to resolve these compounds was of immeasurable value to the success of our laboratory in isolating both known and novel camptothecins.

Solubility of camptothecins in acetonitrile was generally poor. The relatively dilute sample concentrations required by the assay are close to the upper limit of sample solubility required for reasonably reproducible quantitation. Due to the vola-

TABLE I

RETENTION TIMES OF CAMPTOTHECIN ALKALOIDS IN THE REVERSED-PHASE HPLC ASSAY

Compound	*No.*	t_R *(min)*
Glycoside	**5**	11.7
Hydrogenated glycoside	**6**	12.5
18-Hydroxycamptothecin	**8**	12.9
10-Hydroxycamptothecin	**2**	13.9
Camptothecin	**1**	18.1
Glycoside methoxy hydrolysate	**7**	21.2
10-Methoxycamptothecin	**4**	22.8
9-Methoxycamptothecin	**3**	24.1

tility of acetonitrile it was difficult to maintain accurate sample volumes, and on a daily basis a 10% variation in quantitation was tolerated. Small amounts of water or dichloromethane as required were added to samples to improve solubility. As long as the amount was low ($<10\%$ of sample volume), no chromatographic problems were observed. Although methanol is a better solvent for the camptothecins, its use for sample preparation had to be judiciously applied as methanol tended to disrupt the chromatographic profile.

Although some additional investigation was undertaken to examine more closely the anomalous chromatographic behavior of the glycoside **5**, attempts to isolate and identify the two peaks were frustrated by their apparent interconversion according to the type of solvent to which the samples were exposed. However, these observations did not interfere with the ability of the assay to distinguish between the various camptothecins we sought to purify.

In summary, the HPLC assay reported here is a fast, convenient, and quantitative method for microscale analysis of camptothecins. The procedure resolves nine camptothecins including several novel structures and has proven useful for monitoring both natural product isolation and preparation of synthetic derivatives.

REFERENCES

1 M. E. Wall, M. C. Wani, C. E. Cook and K. H. Palmer, *J. Org. Chem.*, 88 (1966) 3888.
2 M. C. Wani and M. E. Wall, *J. Org. Chem.*, 34 (1969) 1364.
3 L. Long-ze, S. Chun-qing and X. Ren-sheng, *Act. Chim. Sin.*, 40 (1982) 86.
4 H. Xian-guo, J. Fu-xiang and Z. Qian-ru, *Act. Bot. Sin.*, 20 (1978) 76.
5 Y. H. Hsiang, R. Hertzberg, S. Hecht and L. F. Liu, *J. Biol. Chem.*, 260 (1985) 14873.
6 W. D. Kingsbury, R. P. Hertzberg, J. C. Boehm, K. G. Holden, D. R. Jakas, M. J. Caranfa, F. L. McCabe, L. F. Faucette, R. K. Johnson, R. W. Busby and S. M. Hecht, *Proc. Am. Assoc. Cancer Res.*, 30 (1989) 622–624.
7 B. K. Carté, C. DeBrosse, D. Eggleston, M. Hemling, B. L. Poehland, N. Troupe, J. W. Westley and S. M. Hecht, in preparation.
8 L. Z. Lin, J. S. Zhang, J. H. Shen, T. Zhou and W. Y. Zhang, *Act. Pharm. Sin.*, 23 (1988) 186.
9 B. L. Poehland, *Am. Lab. (Fairfield, Conn.)*, 16 (1984) 108.