Separation of 9-Methoxycamptothecin and Camptothecin from *Nothapodytes foetida* by Semipreparative HPLC

S.C. Puri*, G. Handa, B.A. Bhat, V.K. Gupta, T. Amna, N. Verma, R. Anand, K.L. Dhar, and **G.N. Qazi**
Regional Research Laboratory (CSIR), Jammu-180 001, India

Abstract

The present work describes the isolation of camptothecin and 9-methoxycamptothecin from the aerial parts of *Nothapodytes foetida* by semipreparative high-performance liquid chromatography because the separation of compounds by conventional procedures is tedious and cumbersome. The purity of the isolates is determined by physicochemical data and liquid chromatography–mass spectrometry.

Introduction

Nothapodytes foetida Sleumer or *Mappia foetida* Miers (commonly called "Kalgur") belongs to the family *Icacinaceae*. This small tree, whose extract shows promising anticancer activity, is distributed in the western Indian peninsula from Konkan southwards in the areas of the Nilgiris and Konkan Ghats. *N. foetida*, along with other species, is a rich source of camptothecin (CPT) (Figure 1) and minor camptothecinoids (1–3). CPT has regained its position as a leading molecule in cancer chemotherapy with the development of certain derivatives [such as irinotecan, topotecan, exetecan, 9-amino, and 9-nitro-20 (S) CPT], which are under clinical trails and have promising chemotherapeutic efficacy. CPT and its analogues have a unique mechanism of action; they produce DNA damage in the presence of topoisomerase-I by binding to and stabilizing a covalent DNA-topoisomerase-I complex in which one strand of DNA gets broken.

N. foetida plants have been successfully raised from their seeds in the agro-climatic region of the northwestern Himalayas (4). Earlier reported procedures for isolation of 9-methoxycamptothecin from the crude extract involved cumbersome procedures encompassing high-speed counter-current chromatography and repeated crystallizations (1,5). In the present methodology, 9-methoxycamptothecin has been isolated in 95% purity by semipreparative high-performance liquid chromatography (HPLC) from the mother liquor after crystallization of CPT from the crude extract of *N. foetida*.

* Author to whom correspondence should be addressed.

Experimental

Chemicals

HPLC-grade acetonitrile and water were obtained from Ranbaxy (Mohali Chandigar, India).

Chromatographic conditions

Analytical HPLC instrumentation

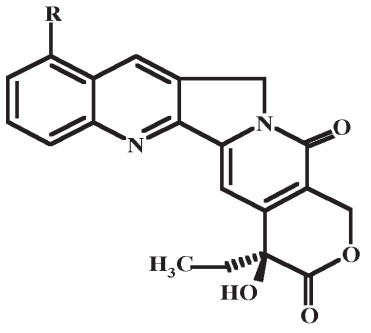
Analytical HPLC was performed on a Gilson (Villiers Le Bel, France) HPLC that included a 305 pump and 10SC pump head, 306 manometric module, 115 UV detector set at 256 nm, 7725i injector with a 50-µL sample loop (Rheodyne, Cotati, CA), and Lichrosphere RP-18 column (4- × 100-mm, 5-µm particle size) (Merck, Darmstadt, Germany) to separate the compounds.

Semipreparative HPLC instrumentation

A Gilson semipreparative HPLC system consisting of 305 pump and 25SC pump head, 306 manometric module, Rheodyne 7725i injector with 300-µL sample loop, 116 UV detector set at 272 nm, and Rainin Dynamax semiprep C-18 column (100- × 5-mm, 2-µm particle size) was used along with Gilson 201 fraction collector.

Liquid chromatography–mass spectrometry

Liquid chromatography (LC)–mass spectrometry (MS) experi-



CPT **I R = H**
9-Methoxycamptothecin **II R = OMe**

Figure 1. The structure of CPT.

ments were performed on a Bruker (Bremen, Germany) Esquire 3000 ion trap MS with an electrospray ionization (ESI) interface and connected to an Agilent (1100 series) binary pump, photodiode array detector, automatic sample injection module, and thermostatic column oven (Agilent, Palo Alto, CA).

Isolation of 9-methoxycamptothecin and CPT and processing of collected fractions

Plant material was collected from RRL (J) botanical garden (Jammu, India) and cultivated from the seeds obtained from the Mahabaleshwar forests of India. A finely powdered aerial portion of plant material (100 g) was extracted with methanol in a Soxhlet apparatus. The extract was filtered and concentrated on a rotatory evaporator. The crude extract (9.5 g) was suspended in 100 mL of water, which was further partitioned with petroleum ether (3 × 50 mL) and chloroform (3 × 50 mL), successively. The chloroform extract was dried over anhydrous sodium sulphate, concentrated on a rota-evaporator, and dissolved in boiling chloroform–methanol (80:20 v/v). As a clear solution standing for 4–6 h at 5°C, CPT precipitates out as a fine, yellow powder.

Mother liquor (10 mg) was dissolved in 10 mL of a chloroform–methanol mixture (80:20 v/v) and subjected to an analytical HPLC system with UV detector set at 256 nm (Figure 2). The mobile phase consisting of premixed water–acetonitrile (25:75) [filtered and degassed on Millex HV filter (0.45 µm, Millipore, Billerica, MA)] was injected and eluted at a flow rate of 1 mL/min. The chromatogram of the filtered extract was plotted on an HP 3395 integrator (Palo Alto, CA). The peaks of CPT and 9-methoxycamptothecin were detected at retention times (t_R) of 13.079 and 22.13 min, respectively, and confirmed by cospiking with their corresponding standards.

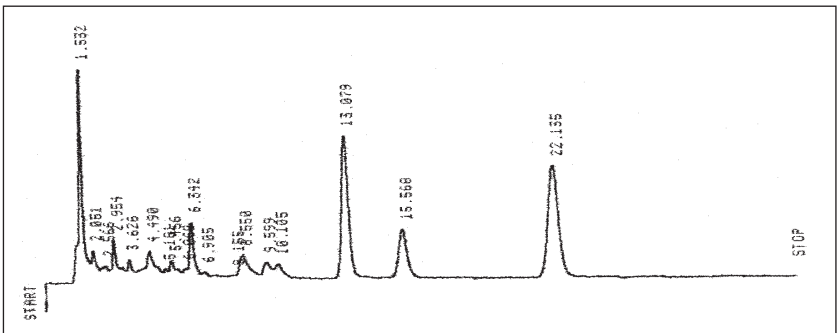


Figure 2. HPLC chromatogram of the crude extract of *N. foetida*. The t_R of CPT is 13.079 min and 9-methoxycamptothecin is 22.135 min.

Semipreparative HPLC of the extract

The mother liquor of the extract (432 mg) was subjected to semipreparative HPLC. A premixed solvent system consisting of water–acetronitrile (25:75) was isocratically pumped at a flow rate of 3 mL/min. Extract (300 µL) was loaded on the Rheodyne injector. Two peaks were collected in clean, preweighed flasks. CPT (I) was collected from 14.5 to 16 min and 9-methoxycamptothecin (II) was collected from 23 to 25 min (Figure 3). After 20 such collections, the pooled eluates in an Erlenmeyer flask were visualized under UV showing blue fluorescence in fraction (I) and yellow in fraction (II). The azeotrope phase was removed by rotatory evaporation under reduced pressure. Removal of the organic phase from the respective pooled fractions yielded CPT (15 mg) and 9-methoxycamptothecin (42 mg), respectively.

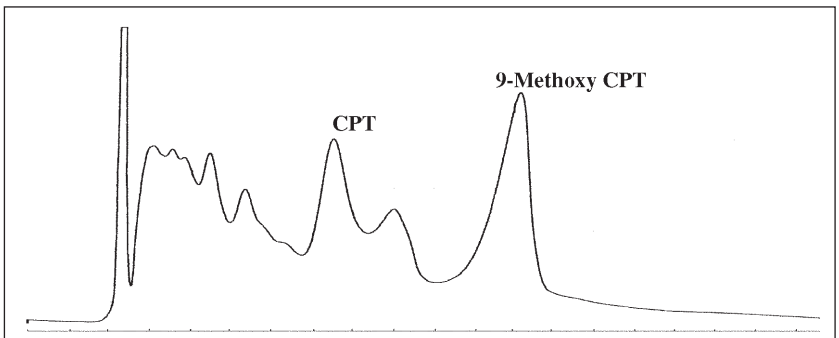


Figure 3. Semipreparative HPLC profile of crude extract of *N. foetida*. Each division on the *x*-axis depicts a time interval of 2 min.

Purity check of the collected fractions

To check the purity, the residue was dissolved in chloroform–methanol (80:20 v/v). The prepared solution was analyzed on an HP-100 HPLC system (Agilent) linked to a Bruker Daltonics Esquire 3000 MS with an ESI source (LC–ESI-MS–MS) scanned over a mass range between *m/z* 100 and 900. Fraction (I) and (II) solutions (10 µL) were injected separately with an autoinjector on a Merck Chromolith C_{18} column (100 × 4 mm) and eluted with water–acetonitrile (25:75) at a flow rate of 0.8 mL/min. The fraction (I) eluting at a t_R of 13.08 min exhibited molecular adduct $(M+H)^+$ at *m/z* 349.1 (Figure 4). The fraction (II) eluting at a t_R of 22.14 min exhibited molecular adduct $(M+H)^+$ at *m/z* 379.1.

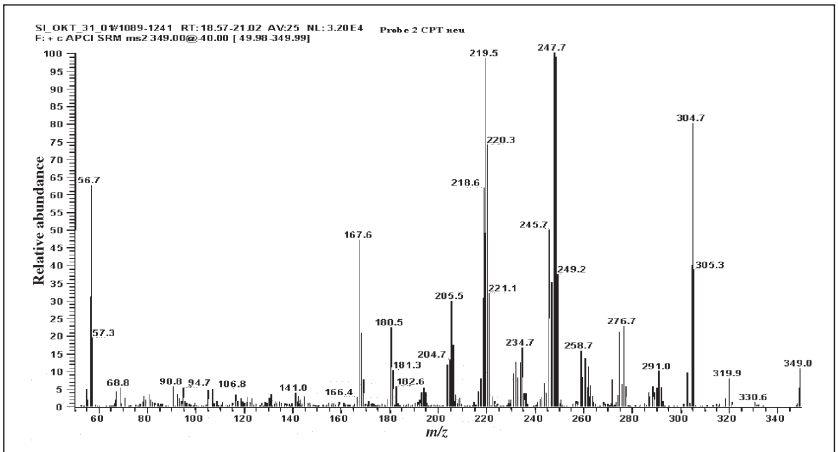


Figure 4. Mass spectrum of CPT.

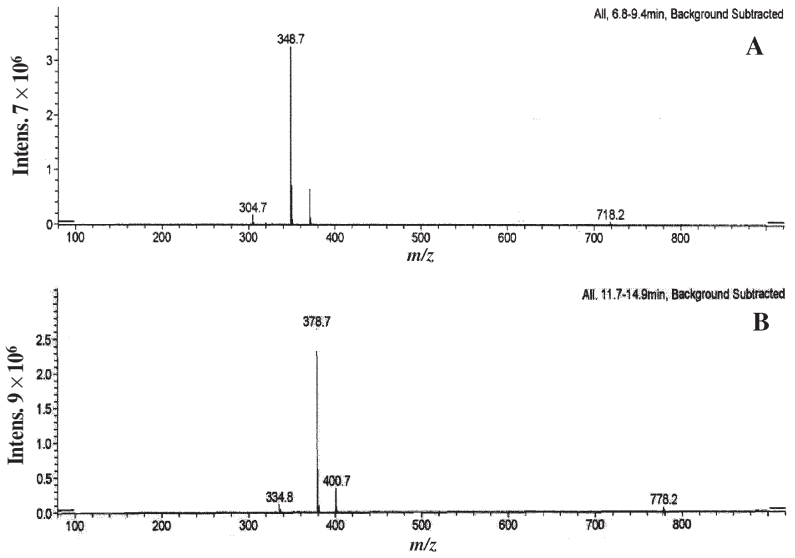


Figure 5. Mass spectra and fragmentation of CPT (A) and 9-methoxycamptothecin (B).

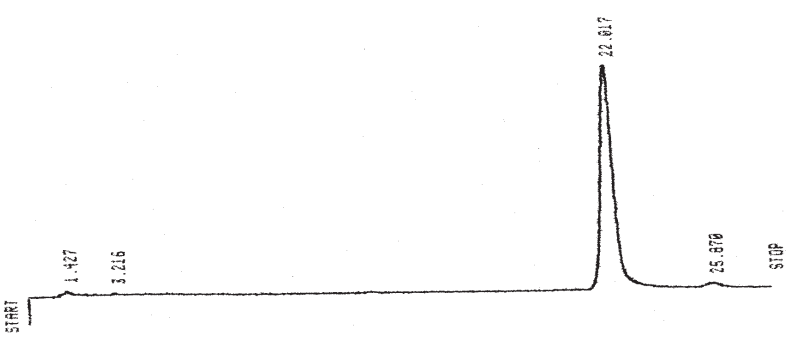


Figure 6. HPLC profile of isolated 9-methoxycamptothecin.

Results and Discussion

The purity of collected fractions was determined separately on the basis of LC runs. CPT and 9-methoxycamptothecin eluted at a t_R of 13.08 and 22.14 min, respectively, were confirmed simultaneously by comparison of a total ion chromatogram (TIC) with a LC–UV chromatogram. Complete overlap of the TIC and UV chromatograms pointed out the purity of the collected compounds.

Fraction (I) CPT (95% pure) was crystallized from chloroform–methanol (80:20 v/v) to give light yellow crystals with a melting point of 273–274°C, $C_{20}H_{16}N_2O_4$; $[M+H]^+$ 349.1 (calculated CHN 348.11), $[a]_D^{25}$ = +34.8 (*c* 0.40, 8:2 $CHCl_3$–MeOH; literature +35°). Analytical and spectral data were in agreement with those reported in the literature (6,7). Fraction (II) on crystallization from chloroform–methanol (80:20 v/v) gave yellow crystals: melting point 266–269°C, $C_{21}H_{18}N_2O_5$; M^+ 378 (calculated CHN 378.38) $[a]_D^{25}$ = –98.54 (concentration of compounds in pyridine 0.29). Thin-layer chromatography, UV, HPLC, and LC–MS data (Figure 5) confirmed its identity and purity (95%) as 9-methoxycamptothecin (Figure 6).

References

1. T.R. Govindachari and N. Vishwanathan. Alkaloids of Mappia foetida. *Phytochemistry* **11:** 3529 (1972).
2. M. Arisawa, S.P. Gunasekera, G.A. Cordell, and N.R. Farmsworth. Plant anticancer agents XXI constituents of Merrillodendeon megacarpum. *Planta Med.* **43:** 404 (1981).
3. X. Zhang and J. Bao. Extracting Camptothecin from *Nothapodytes*. Faming Zhuanli Shenquing Cronkai Shuomingshu CN 1,045,266 (Cl.C07D491/147), 12 September 1990. CA. 114 1644601v.
4. S.N. Sharma, S.C. Puri, T. N. Srivastava, G. Handa, and B.L. Kaul. Enhancement of seed germination in *Nothapodytes foetida*. *JMAPS* **22:** 206–10 (2000).
5. S. Broglia, L. Verotta, and M. Battislini. Application of high speed countercurrent chromatography to the separation of Camptothecin and 9-methoxycamptothecin. *Fitoterapia*. **LXV(6):** 522–23 (1994).
6. M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPhail, and G.A. Sim. Plant antitumor agents I. The isolation and structure of camptothecin, a novel alkaloidal leukaemia and tumor inhibitor from Camptotheca accuminata. *J. Amer. Chem. Soc.* **88:** 3888–90 (1966).
7. Z.-L. Long and G.A. Cordell. ^{13}CNMR Assignments of Camptothecine and 10-hydroxycamptothecine. *J. Nat. Prod.* **53:** 186–89 (1990).

Manuscript received April 28, 2004;
revision received May 5, 2005.