Quantitative Analysis of Camptothecin Derivatives in *Nothapodytes foetida* Using ^{1}H-NMR Method

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A quantitative analysis using ^{1}H-NMR has been developed for the determination of camptothecin derivatives and trigonelline in *Nothapodytes foetida* root, stems and leaves. In the region of δ 9.5—5.5, the signals of H-7 of camptothecin (1), H-10 of 9-methoxycamptothecin (2), H-19 of pumiloside (3) and H-2 of trigonelline (4), were well separated from each other in DMSO-d_6. The quantity of the compounds was calculated by the ratio of the intensity of each compound to the known amount of internal standard 3,4,5-trimethoxybenzaldehyde. These results were compared with the conventional HPLC method. The advantages of the method are that no reference compounds are required for calibration curves, the quantification could be directly realized on a crude extract, an overall profile of the preparation could be directly obtained, and a very significant time-gain could be achieved, in comparison to conventional HPLC methods, for instance.

Key words quantitative analysis; ^{1}H-NMR; camptothecin derivative; trigonelline; *Nothapodytes foetida*

Camptothecin (**1**), a potent antitumor pyrrolo-[3,4-*b*]-quinoline alkaloid originally isolated from the wood and bark of Chinese *Camptotheca acuminata* (Nyssaceae),[1)] is an inhibitor of the DNA-replicating enzyme topoisomerase I and is believed to act by stabilizing a topoisomerase I-induced single strand break in the phosphodiester backbone of DNA.[2)] This alkaloid was also found to occur in some other plant species, such as *Ervatamia heyneana* (Apocynaceae)[3)] and *Nothapodytes foetida* (Wight) Sleumer (Icacinaceae) [formerly, *Mappia foetida* Miers].[4)] Amoung these, the stem of *N. foetida* is considered to be a rich source of the potent alkaloids camptothecin (**1**) and 9-methoxycamptothecin (**2**). Up to now HPLC has been used more often for the camptothecin alkaloids analysis. HPLC, however, is not always satisfactory due to the numerous variables that must be considered for the analyses of camptothecins. For better controlling of this pharmaceutically important alkaloid from *N. foetida*, a suitable method for the analysis of camptothecin alkaloids from *N. foetida* would be highly desirable.

Recently, nuclear magnetic resonance (NMR) spectroscopy has been developed into an important tool in quality control of phyto-preparations[5—8)] and in clinical diagnosis and monitoring of treatment.[9)] The advantages of ^{1}H-NMR method are manifold, *viz.* it is rapid and noninvasive. In addition, no standard compounds are required for preparing the calibration curves and it detects all the organic components present in herbal preparations simultaneously in a single measurement. Therefore, we hypothesize that NMR spectroscopy may be superior to the conventional HPLC for the analysis of camptothecin alkaloids. In this paper, we describe the quantitative analysis of camptothecin, 9-methoxycamptothecin, pumiloside (**3**) and trigonelline (**4**) from *N. foetida* by using ^{1}H-NMR spectroscopy (Fig. 1). Besides, the results obtained from ^{1}H-NMR method were compared with those from conventional HPLC method.

Experimental

Plant Material The root, stems and leaves of *N. foetida* were collected from Taiton Hsien (No. TSWU030815), Taiwan in October 2003 and verified by Prof. C. S. Kuoh. A voucher specimen is deposited in the Herbarium of Cheng Kung University, Tainan, Taiwan.

Chemicals and Instrument First grade methanol, acetonitrile, 3,4,5-trimethoxybenzylaldehyde were purchased from E. Merck. Dimethylsulfoxide-d_6 (99.9%) was obtained from Aldrich. 1,3,5-Trimethoxybenzene was prepared by methylation of phloroglucinol (Sigma).[8)] The reference compounds (camptothecin, 9-methoxycamptothecin, pumiloside and trigonelline) were isolated from the stem of *N. foetida* in a prior study.[10)] The purity of all these reference compounds was established by NMR and HPLC methods. ^{1}H-NMR spectra were recorded in dimethylsulfoxide-d_6 (99.9%) using a Varian UNITY plus 400 MHz spectrometer. For each sample, 100 scans were recorded with the following parameters: 0.187 Hz/point; spectra width, 14400 Hz; pulse width, 4.0 μs; relaxation delay, 1 s; acquiring time, 2.67 μs; temperature, 25 °C. For quantitative analysis, peak area was used and the start and end point of the integration of each peak were selected manually. HPLC was performed on a Shimadzu LC-10AT$_{VP}$ (Japan) system using a LiChroCART RP-18 endcapped column (4.6×250 mm, 5 μm, Merck). The mobile phase using gradient elution was a solvent mixture of water (mobile phase A) and acetonitrile (mobile phase B). The gradient elution started with 0% B for 5 min. The percentage of mobile phase B was increased to 20% at 10 min, 50% at 25 min and finally to 100% at 35 min. The flow rate was 1.0 ml/min with a sample volume of 20 μl and UV detection at 254 nm.

Extraction One gram of powdered plant material [the root, stem batch 1 (5 cm i.d.), stem batch 2 (0.5 cm i.d.) and leaf] were weighed out and mixed with 50 ml MeOH and refluxed for 30 min (three times). The combined extracts were separated to two equal volume fractions. One fraction was evaporated to dryness after addition of internal standard (3,4,5-trimethoxybenzylaldehyde). The dried sample was dissolved in 0.5 ml DMSO-d_6 and used for ^{1}H-NMR measurement. The other fraction was evaporated to dryness under reduced pressure and used for HPLC measurement.

Recovery Test Each standard of camptothecin, 9-methoxycamptothecin,

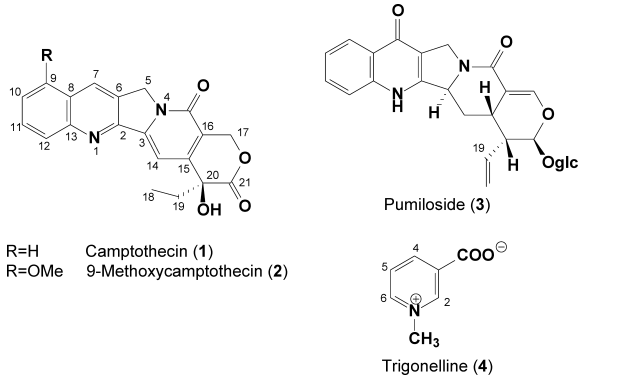

Fig. 1. Structures of the Major Constituents in *N. foetida*: Camptothecin, 9-Methoxycamptothecin, Pumiloside and Trigonelline

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pumiloside and trigonelline (3.0 mg) was spiked into the crude extracts (contained internal standard) and quantified by the ^{1}H-NMR method.

Results and Discussion

Since camptothecins are highly insoluble in organic solvents, DMSO-d_6 was used as the NMR solvent to ensure all the extract can be dissolved. The proton NMR spectra of **1** and **2** are well documented in DMSO-d_6 (Fig. 2). The analysis of the NMR spectra of **1** and **2** revealed that the proton H-7 of **1** and **2**, resonating in a no crowded region of the spectra as a singlet, could be used for quantification. However, in the total extract's NMR spectra, H-7 signals of **1** and **2** interfere with the other constituent, trigonelline, one of the major constituents of *N. foetida*. Accounting to the NMR spectra of total extracts, the suitable target signals can be selected. The H-7 of **1** and H-10 of **2**, resonating in a non-interfered region of the spectra and were selected as target signals for quantification. In addition, the signals of H-19 for pumilisode (**3**) and H-2 for trigonelline (**4**) were selected as target signals for analysis.

A suitable internal standard should be preferably a stable compound with a sharp signal in a non crowded region of the ^{1}H-NMR spectrum. For the purpose, 3,4,5-trimethoxybenzylaldehyde, with a singlet at δ 9.85 and the integral value maintaining constant within 12 h, has been chosen. The ^{1}H-NMR spectra obtained for 0.5 g of the four parts of samples of *N. foetida* extracted by MeOH are shown in Fig. 3. In the case of ^{1}H-NMR quantitative analysis, calibration curves are not needed for quantification of the compounds because integration of the peaks is always proportional to the amount of the compound and the same for all compounds in ^{1}H-NMR. However, calibration curves for each compound were determined in the range 0.1—8.0 mg/ml in order to check the accuracy of this method. The linearity for each target compound was found to be higher than 0.999. The accuracy of the method was also checked by adding a known amount of

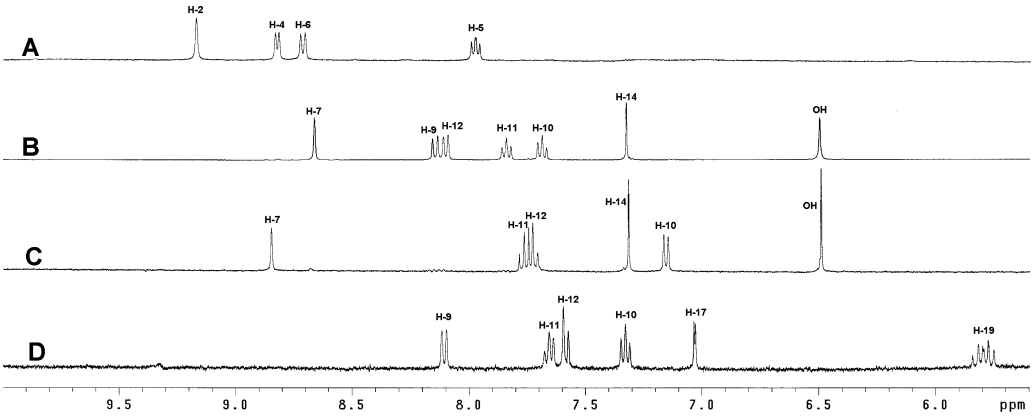

Fig. 2. ^{1}H-NMR Spectra of (A) Trigonelline (**4**), (B) Camptothecin (**1**), (C) 9-Methoxycamptothecin (**2**), and (D) Pumiloside (**3**) in DMSO-d_6 in the Range of δ 10.0—5.6

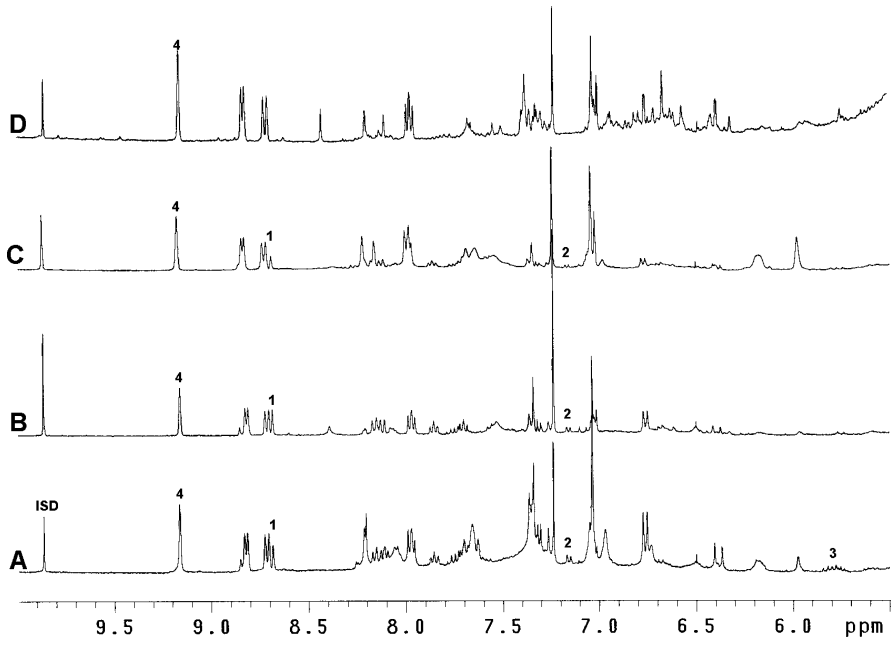

Fig. 3. ^{1}H-NMR Spectra of *N. foetida* Extracts in the Range of δ 10.0—5.5

(A) Root, (B) stem batch 1, (C) stem batch 2 and (D) leaves. (**1**: camptothecin; **2**: 9-methoxycamptothecin; **3**: pumiloside; **4**: trigonelline; ISD: internal standard, 3,4,5-trimethoxybenzylaldehyde).

reference compound (3.0 mg) to extract samples. The peak areas corresponding to each target constituent were found to increase proportionally with the added concentration of the standard. The results of the recovery tests were shown in Table 1. The concentrations of these target compounds in the root, stems and leaves of *N. foetida* determined by this ^{1}H-NMR method were shown in Table 2. The root of *N. foetida* was found to be a rich source of **1**—**4**. Different diameters of stems contain similar amount of **1** and **2**, but **4** was rich in stem batch 2 (i.d. *ca.* 0.5 cm). The leaves contain trace amounts of camptothecins **1**—**3**.

An HPLC method was used to confirm the analysis results obtained by ^{1}H-NMR method. Figure 4 showed the HPLC chromatogram of the plant material extracts at UV 254 nm. The target compounds **1**—**4** were eluted at retention time 21.6, 22.8, 17.3 and 2.6 min, respectively. The 1,3,5-trimethoxybenzene was used as an internal standard (retention time＝26.2 min) and the calibration curves for each compound using the ratio of peak areas of the reference compound and the internal standard were prepared. The linearity of **1**—**4** were found to be larger than 0.999. The concentrations of these target compounds in *N. foetida* were summarized in Table 2 which showed similar results (relative error values <3%) as with the ^{1}H-NMR method.

The NMR method is simple and rapid, specific, no reference compounds are needed, and an overall profile of the preparation can be obtained directly. Using this method the contents of camptothecin alkaloids can be determined in much shorter time than the conventional HPLC measurements. In conclusion, the described ^{1}H-NMR method is a rapid and simple method for the identification and quantification of camptothecin, 9-methoxycamptothecin, pumiloside and trigonelline in *N. foetida* plant materials or cell suspension cultures.

Table 1. The Recoveries of Camptothecin (**1**), 9-Methoxycamptothecin (**2**), Pumiloside (**3**) and Trigonelline (**4**) by the ^{1}H-NMR Method

Compound	Camptothecin (**1**)	9-Methoxy-camptothecin (**2**)	Pumiloside (**3**)	Trigonelline (**4**)
Recovery	98.4±2.8%	97.3±3.1%	96.8±2.5%	97.1±1.2%

Mean±% RSD. All experiments were based on triplicate measurement.

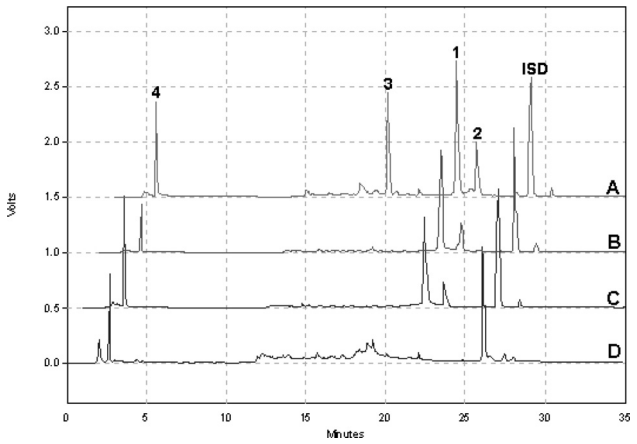

Fig. 4. HPLC Spectra of *N. foetida* Extracts

(A) Root, (B) stem batch 1, (C) stem batch 2 and (D) leaves. (**1**: camptothecin; **2**: 9-methoxycamptothecin; **3**: pumiloside; **4**: trigonelline; ISD: internal standard, 1,3,5-trimethoxybenzene).

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Table 2. The Concentrations (%w/w) of Camptothecin (**1**), 9-Methoxycamptothecin (**2**), Pumiloside (**3**) and Trigonelline (**4**) in *N. foetida* Root, Stems and Leaves

Compound	Root	Stem batch 1	Stem batch 2	Leaf
Camptothecin (**1**)	4.85±0.13$^{a)}$ 4.87±0.09$^{b)}$	3.86±0.09$^{a)}$ 3.90±0.06$^{b)}$	3.65±0.11$^{a)}$ 3.66±0.05$^{b)}$	—$^{a)}$ —$^{b)}$
9-Methoxycamptothecin (**2**)	4.73±0.11$^{a)}$ 4.75±0.11$^{b)}$	2.96±0.07$^{a)}$ 3.00±0.05$^{b)}$	2.25±0.06$^{a)}$ 2.30±0.05$^{b)}$	—$^{a)}$ —$^{b)}$
Pumiloside (**3**)	8.68±0.26$^{a)}$ 8.71±0.20$^{b)}$	—$^{a)}$ 0.54±0.02$^{b)}$	—$^{a)}$ —$^{b)}$	—$^{a)}$ —$^{b)}$
Trigonelline (**4**)	6.68±0.13$^{a)}$ 6.69±0.08$^{b)}$	3.25±0.05$^{a)}$ 3.29±0.06$^{b)}$	8.16±0.15$^{a)}$ 8.18±0.16$^{b)}$	6.97±0.09$^{a)}$ 7.00±0.12$^{b)}$

Mean±RSD. All experiments were based on triplicate measurement. *a*) ^{1}H-NMR method results. *b*) HPLC method results.