Journal of Chromatography, 481 (1989) 368–372
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 748

Note

Determination of panaxytriol, a new type of tumour growth inhibitor from Panax ginseng, by capillary gas chromatography

HISASHI MATSUNAGA, MITSUO KATANO, HIROSHI YAMAMOTO, MASATO MORI and KATSUMI TAKATA*

Hospital Pharmacy and Department of Surgery, Saga Medical School, Nabeshima-Sambonsugi, Nabeshima-machi, Saga 840-01 (Japan)

and

MASATOSHI NISHI

Faculty of Pharmaceutical Science, Setsunan University, Nagaotouge-cho, Hirakata, Osaka 573-01 (Japan)

(First received March 13th, 1989; revised manuscript received June 26th, 1989)

Panax ginseng is known for its unique antitumour therapeutical effect[1]. During a series of studies aimed at isolation of the tumour growth inhibitory substance from Panax ginseng, we found a substance, a polyacetylenic alcohol, which inhibits tumour cell growth in a dose-dependent fashion *in vitro*[2,3]. Data from infrared (IR), proton and carbon-13 nuclear magnetic resonance and high-resolution mass spectra were identical with those of authentic panaxytriol, heptadec-1-ene-4,6-diyne-3,9,10-triol, previously described by several investigators[4,5].

In order to examine the mechanism of cell growth inhibition caused by panaxytriol, it is important to develop an assay method for panaxytriol. We describe in this paper a gas chromatographic (GC) method for its quantitation by silylation.

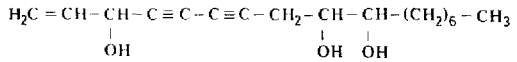

Fig. 1. Chemical structure of panaxytriol.

EXPERIMENTAL

Materials and reagents

A powder of the root of Panax ginseng C. A. Meyer was provided by Nikkan Korai Ninjin (Kobe, Japan). The root material is commonly used in Japan for treatment of various diseases as a commercial medicinal drug by the name of Korean Red Ginseng Powder. The isolation of panaxytriol from Korean Red Ginseng Powder has been described previously[2,3]. Briefly, the powder was extracted with ethyl acetate, and the extracts were separated by silica gel chromatography. Panaxytriol-rich fractions were collected and panaxytriol was purified by crystallization from distilled water. The chemical structure of isolated panaxytriol was verified by comparison with IR, proton and carbon-13 nuclear magnetic resonance and high-resolution mass

0021-9673/89/$03.50

spectra in the literature[4,5]. The purity of panaxytriol was confirmed by two-dimensional thin-layer chromatography (TLC). Briefly, TLC was performed on high-performance silica gel 60 plates (10 cm × 10 cm; E. Merck, Darmstadt, F.R.G.). The plate was first developed with ethyl acetate. When the solvent front had migrated about 8 cm, the plate was dried in air for 15 min and developed in the second dimension with ethyl acetate–*n*-hexane (1:1, v/v); the solvent front was again allowed to move about 8 cm. The spots were detected by spraying the plates with concentrated H_2SO_4 and by heating.

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Gasukuro Kogyo (Tokyo, Japan). The internal standard, 1-docosanol, was obtained from Gasukuro Kogyo and a solution containing 20 μg/ml in chloroform was prepared.

Panaxytriol was dissolved in RPMI 1640 culture medium containing 10% foetal calf serum (Gibco Lab., NY, U.S.A.).

All other chemicals were of reagent grade and were used without further purification.

Instrumentation

An Hitachi gas chromatograph Model 663-30 equipped with a flame-ionization detector (Hitachi, Tokyo, Japan) and moving needle solvent-cut sample injection (MNSCSI) (Gasukuro Kogyo) was used for all analyses[6]. MNSCSI was used for increasing the sensitivity by concentrating the sample on the tip of the needle.

The column used was a flexible fused-silica capillary coated with OV-1701 (50 m × 0.25 mm I.D., d_f 0.15 μm; theoretical plates, 3806 per metre; Gasukuro Kogyo). The injection and detector temperatures were set at 250°C, while the column temperature was kept at 250°C. Helium was used as the carrier gas and make-up gas at flow-rates of 1.1 and 35 ml/min, respectively. The flow-rates of air and hydrogen were adjusted to 400 and 40 ml/min, respectively. A splitting ratio of 68:1 was used.

Gas chromatography–mass spectrometry (GC–MS) measurements were carried out with a JEOL JMS-D300, operated in the electron impact (EI) mode. The ionization voltage and ionization current were 30 eV and 0.3 mA, respectively; the injection port and separator temperatures were 250°C, the interface temperature 200°C and the electron multiplier voltage 1500 V, respectively. EI mass spectra were taken by continuously scanning the mass range *m/z* 50–500 every 1.0 s.

Extraction and derivatization

A 50-μl volume of internal standard (1-docosanol, 20 μg/ml, in chloroform) was transferred to extraction tubes and dried at 40°C under a stream of nitrogen. A 1.0-ml panaxytriol-containing culture medium was added to the residue, and mixed. Ethyl acetate (5 ml) was added and vortex-mixed for 30 s. The mixture was vigorously shaken mechanically at approximately 280 strokes per min for 10 min at room temperature. After centrifugation at 1500 *g* for 10 min, the organic phase was transferred to a 10-ml glass tube and concentrated under a stream of nitrogen at 40°C. The concentrated material was transferred to a micro-glass tube and dried. Then, 10 μl of triethylamine and 10 μl of BSTFA were added to the dry residue for derivatization of panaxytriol and 1-docosanol. The micro-glass tube was sealed and heated at 60°C for 60 min. After silylation, an 1.0-μl aliquot was adhered to the needle surface of the injector. After evaporating the solvent, the sample was injected onto the column.

Calibration graph

Various amounts (0.25, 0.5, 1.0 and 2.0 μg) of panaxytriol were added to 1.0-ml aliquots of blank culture medium. All the samples were extracted and analyzed using the procedure described above. The calibration graph was obtained by plotting the peak height ratio of the silylated panaxytriol derivative to the silylated internal standard.

Reproducibility

Six samples of culture medium each containing 0.5, 1.0 or 2.0 μg/ml of panaxytriol were prepared. All the samples were extracted and analyzed using the procedure described above. The intra- and interassay coefficients of variation (C.V.) were determined by replicate analysis.

RESULTS AND DICUSSION

The reaction time of the silylation was examined over the range of 30–120 min at 60°C. Many extraneous peaks were observed using 120 min, and only weak peaks for 30 min. Thus, it was determined that the optimum reaction time was 60 min.

In order to verify the structure of the trimethylsilyl (TMS)-panaxytriol derivative, we measured its spectrum by use of GC–MS (Fig. 2). The weak molecular ion m/z 494 (M^+) and m/z 479 ($M^+ - 15$) were observed. Consequently, silylated panaxytriol was shown to contain three trimethylsilyl groups. Similarly the structure of the internal standard, 1-docosanol, derivative was measured by use of GC–MS. The weak molecular ion m/z 398 (M^+) and m/z 383 ($M^+ - 15$) were observed. The silylated internal standard was shown to contain one trimethylsilyl group.

Chromatograms obtained from blank culture medium and culture medium containing 2.0 μg of silylated panaxytriol derivative and 1.0 μg of silylated internal standard are shown in Fig. 3. The retention times of silylated panaxytriol and silylated internal standard were 9.4 and 11.4 min, respectively.

No interfering peaks were observed in the extracts of culture medium containing

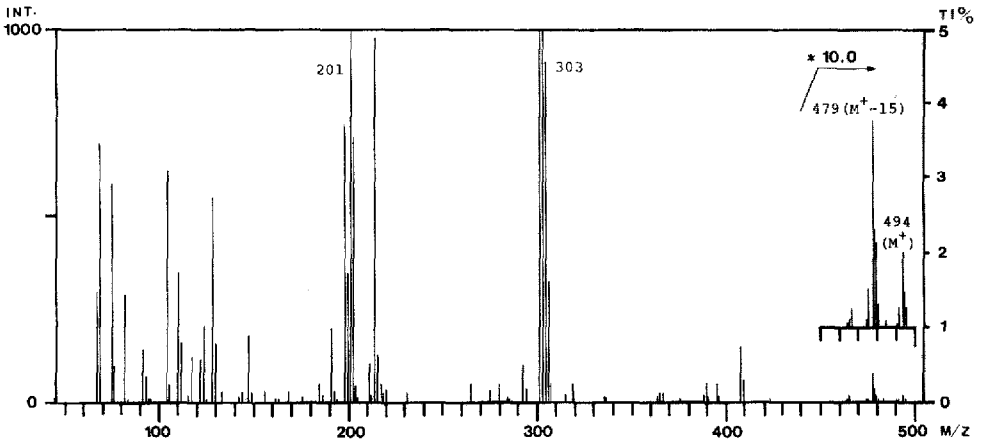

Fig. 2. Mass spectrum of the silylated panaxytriol.

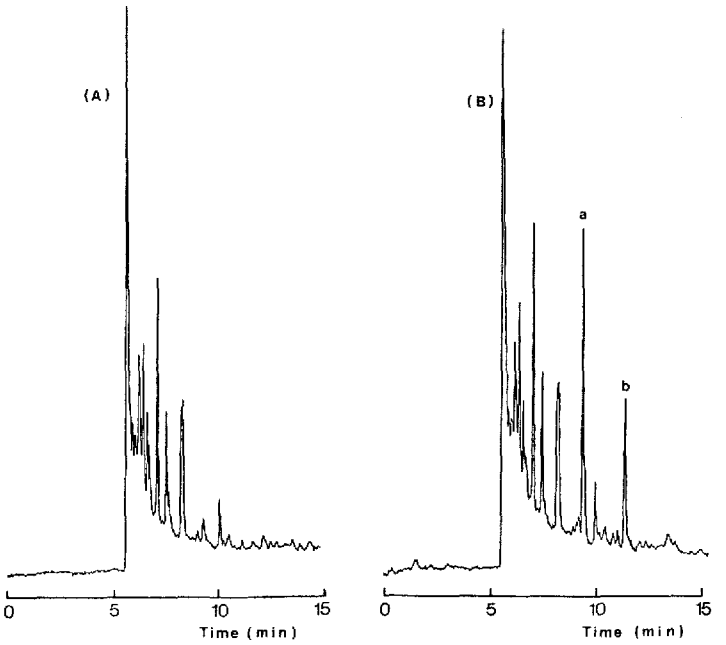

Fig. 3. Chromatograms of (A) blank culture medium and (B) culture medium containing 2.0 μg silylated panaxytriol and 1.0 μg silylated 1-docosanol. Peaks: a = silylated panaxytriol; b = silylated 1-docosanol, internal standard.

foetal calf serum and silylated panaxytriol and silylated internal standard.

This method allowed us to detect the silylated panaxytriol derivative at a concentration as low as 0.125 μg/ml (signal-to-noise ratio >3).

The ratio of the peak height of silylated panaxytriol to that of the silylated internal standard plotted against the drug concentration was linear between 0.25 and 2.0 μg/ml, with a correlation coefficient of 0.998.

The intra- and interassay data are shown in Table I. The intraassay reproducibility of silylated panaxytriol (n = 6, C.V. = 6.5–9.5%) was satisfactory in the concentration range of 0.5–2.0 μg/ml. On the other hand, the interassay reproducibility of silylated panaxytriol (n = 6, C.V. = 1.4–8.0%) was also satisfactory in the

TABLE I

THE INTRA- AND INTERASSAY COEFFICIENTS OF VARIATION FOR PANAXYTRIOL (n = 6)

Concentration (μg/ml)	*Intraassay*		*Interassay*	
	Mean ± S.D.	*C.V. (%)*	*Mean ± S.D.*	*C.V. (%)*
0.5	0.66 ± 0.063	9.5	0.55 ± 0.044	8.0
1.0	1.02 ± 0.068	6.5	0.95 ± 0.059	6.2
2.0	2.16 ± 0.015	7.0	2.03 ± 0.029	1.4

concentration range of 0.5–2.0 μg/ml. The recovery of panaxytriol from culture medium to which 2.0 μg/ml had been added was 89.56 ± 2.99% ($n = 5$). The recovery of 1-docosanol from culture medium was 98.4 ± 5.3% ($n = 5$).

In conclusion, a specific GC method has been developed for the analysis of panaxytriol in solution containing serum. The use of a fused-silica capillary column coated with OV-1701 permitted the selective determination of panaxytriol as its silylated derivative. It was also shown that MNSCSI is very useful for the straightforward and reproducible determination of panaxytriol. In addition, the sensitivity of silylated panaxytriol determination with MNSCSI, under the optimum conditions, was about five times higher than that with a conventional split injector system.

The present method of analysis of panaxytriol may be useful for studies of the biological properties of this compound such as its antitumour action and metabolism.

ACKNOWLEDGEMENT

The authors are indebted to Dr. J. Tadano and Mr. S. Kawasaki for analytical data.

REFERENCES

1 K. D. Lee and R. P. Huemer, *Jpn. J. Pharmacol.*, 21 (1971) 299.
2 M. Katano, H. Matsunaga and H. Yamamoto, *J. Jpn. Surg. Soc.*, 89 (1988) 971.
3 M. Katano, H. Yamamoto and H. Matsunaga, *Proc. 5th International Ginseng Symposium*, Korea Ginseng and Tobacco Research Institute, Seoul, 1989, in press.
4 I. Kitagawa, M. Yoshikawa, M. Yoshihara, T. Hayashi and T. Taniyama, *Yakugaku Zasshi*, 103 (1983) 612.
5 S. C. Shim, H. Y. Koh and B. H. Han, *Phytochemistry*, 22 (1983) 1817.
6 P. M. J. van den Berg and Th. P. H. Cox, *Chromatographia*, 5 (1972) 301.