Journal of Chromatography, 257 (1983) 146–150
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

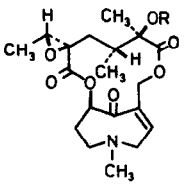
CHROM. 15,443

Note

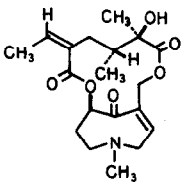
Separation and determination of macrocyclic pyrrolizidine alkaloids of the otonecine type present in the edible plant *Petasites japonicus* by reversed-phase high-performance liquid chromatography

HARUKI NIWA, HIROYUKI ISHIWATA and KIYOYUKI YAMADA*
Department of Chemistry, Faculty of Science, Nagoya University, Chikusa, Nagoya 464 (Japan)
(Received October 12th, 1982)

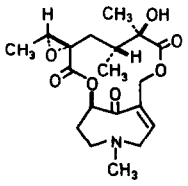
During the last 10 years considerable attention has been paid to pyrrolizidine alkaloids (PAs) of plant origin owing to their remarkable hepatotoxic and, in certain instances, carcinogenic properties from the viewpoints of veterinary and human medicine[1,2]. PAs have been found to be present in a variety of plants such as Compositae, Leguminosae and Boraginaceae, some of which are used as medicinal herbs and as foods and forage. High-performance liquid chromatography (HPLC) has been increasingly employed in recent years for the analysis of pyrrolizidine alkaloids[3–12]. We have recently investigated the carcinogenic constituent(s) of *Petasites japonicus* (Compositae family), which is used as folk medicine and foodstuff in Japan, isolated two new PAs, petasitenine (1)[13,14] and neopetasitenine (2)[13,14] together with senkirkine (3)[15], and established the carcinogenic activity[16] of petasitenine. These compounds are PAs of the otonecine type. Previous studies[3–12] on the HPLC of PAs have been performed solely on PAs of the retronecine type such as senecionine (5) and jacobine (6), and we have found that the previous systems[3–12] could not be applied satisfactorily to the analysis of PAs of otonecine type such as compounds 1–3 owing to the lack of good separation.



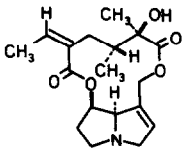
1 R= H
2 R= CH_3CO



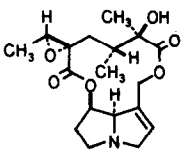
3



4



5



6

0021-9673/83/0000–0000/$03.00

This paper describes the efficient separation of these macrocyclic PAs of the otonecine type and their determination in the plant by reversed-phase HPLC. Further, results are presented on the effect of 'processing the plant material" (a particular cooking procedure, see below) on the content of the carcinogenic PA (1) in the plant material by HPLC analysis.

EXPERIMENTAL

Standard specimens of three PAs, petasitenine (1), neopetasitenine (2) and senkirkine (3), were available in our laboratory[13–15], and a pure sample of otosenine (4) was supplied by Dr. C. C. J. Culvenor, CSIRO, Australia. All reagents and solvents were of analytical-reagent grade.

Isolation of pyrrolizidine alkaloids

Young flower stalks of *Petasites japonicus* were collected in late March, 1980, in Gifu prefecture, Japan.

Procedure I. The dried and powdered plant material (10 g) was extracted with ethanol in a Soxhlet apparatus for 24 h, the extract filtered and the solvent removed under reduced pressure. The residue was diluted with water, acidified to pH 2 with 1 *N* sulphuric acid and extracted three times with diethyl ether. The aqueous phase was basified to pH 9 with concentrated ammonia solution. The aqueous solution was then extracted five times with chloroform. The combined organic extracts were dried over anhydrous sodium sulphate. After filtration the solvent was removed under reduced pressure to give a dark brown tar, which was further purified by TLC on aluminium oxide [150 F_{254} (Type T); Merck, Darmstadt, G.F.R.] with ethyl acetate–methanol (97:3), affording an amorphous alkaloidal mixture (4.7 mg). The mixture was dried over sodium hydroxide *in vacuo*. The alkaloidal mixture was dissolved in 100 μl of methanol for injection. Peaks were collected and the eluents evaporated under reduced pressure to give individual pure alkaloids. Each alkaloid isolated was identified by comparison of the HPLC retention time with that of the known standard and by mass spectral analysis.

Procedure II. The dried powdered plant material (10 g) was boiled with water (300 ml) for 1 h. After cooling, the mixture was filtered and the filtrate concentrated under reduced pressure below 40°C to give an oily residue. The residue was diluted with water, and from this mixture there was obtained an amorphous alkaloidal mixture (1.9 mg) as described under *Procedure I.* The alkaloidal mixture was subjected to HPLC analysis.

Liquid chromatography

The HPLC system used for separation consisted of a Trirotar high-pressure liquid chromatograph (Jasco, Tokyo, Japan) equipped with a UV detector (215 nm) (Jasco Uvidec-100-II spectrometer), a refractive index (RI) detector (Shodex RI SE-II differential refractometer) and a six-port valve injector (Jasco VL-611). Separations were performed at ambient temperature on a pre-packed Cosmosil 5 Ph (5 μm) stainless-steel column (150 × 4.6 mm I.D.) (Nakarai Chemicals, Kyoto, Japan). The solvent system was isocratic methanol–0.02 *M* ammonium carbonate (45:55) (pH 8.2) and the flow-rate was 1.0 ml/min.

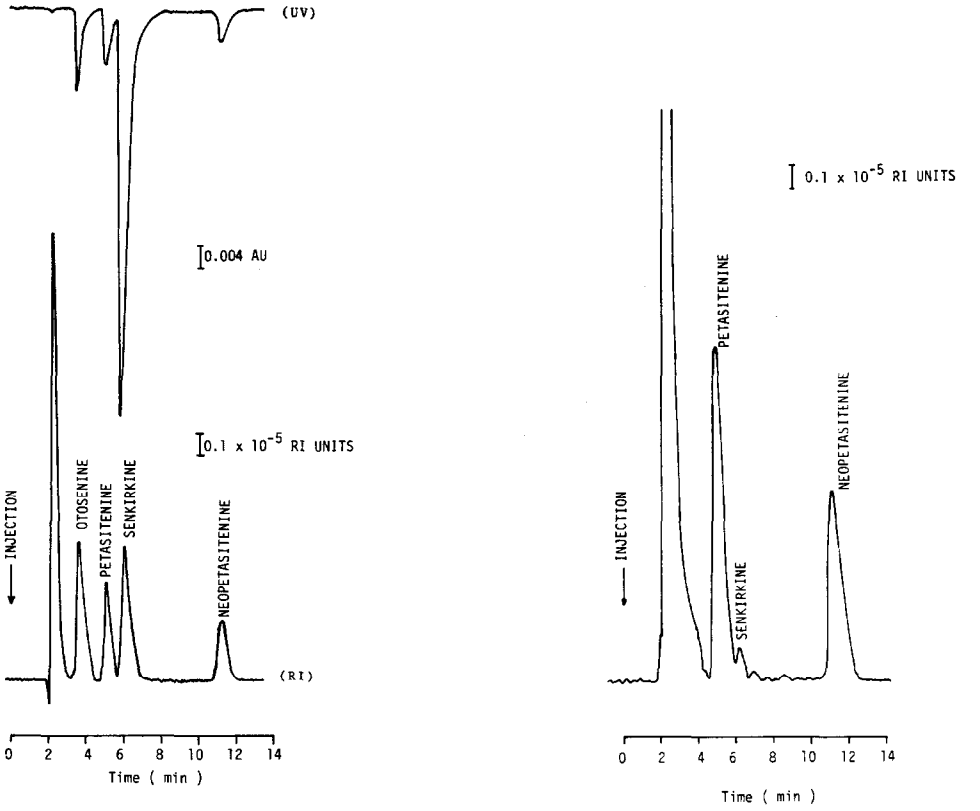


Fig. 1. Separation of a mixture of pure PAs. Column, 150 × 4.6 mm I.D., packed with Cosmosil 5 Ph. Sample: a mixture of 20 μg of each of pure PAs in 2 μl of methanol. Solvent, methanol–0.02 *M* $(NH_4)_2CO_3$ (45:55) (pH 8.2); flow-rate, 1 ml/min. Column temperature, 25°C. Detectors, Uvidec-100-II UV spectrometer operated at 215 nm and Model SE-II RI differential refractometer set at a sensitivity of $4 \cdot 10^{-5}$ RI units.

Fig. 2. Separation of an alkaloidal mixture obtained from ethanolic extracts of *P. japonicus*. Sample, 470 μg of the mixture in 10 μl of methanol. Conditions as in Fig. 1.

Mass spectrometry

Mass spectra were determined on a Hitachi RMU-6C mass spectrometer equipped with a direct inlet system operating with an ionization energy of 70 eV.

RESULTS AND DISCUSSION

Optimal separation of a mixture of compounds 1–4 was obtained using the reversed-phase Cosmosil 5 Ph column and a methanol–0.02 *M* ammonium carbonate eluent as shown in Fig. 1. Although otosenine (4) was not an alkaloidal component of *P. japonicus*, we added it to a mixture of compounds 1–3 and examined whether or not separation of otosenine from petasitenine could be achieved because of the close resemblance of their structures (otosenine is the stereoisomer of petasitenine with respect to the epoxy group). Fig. 1 shows the first example of the successful separation

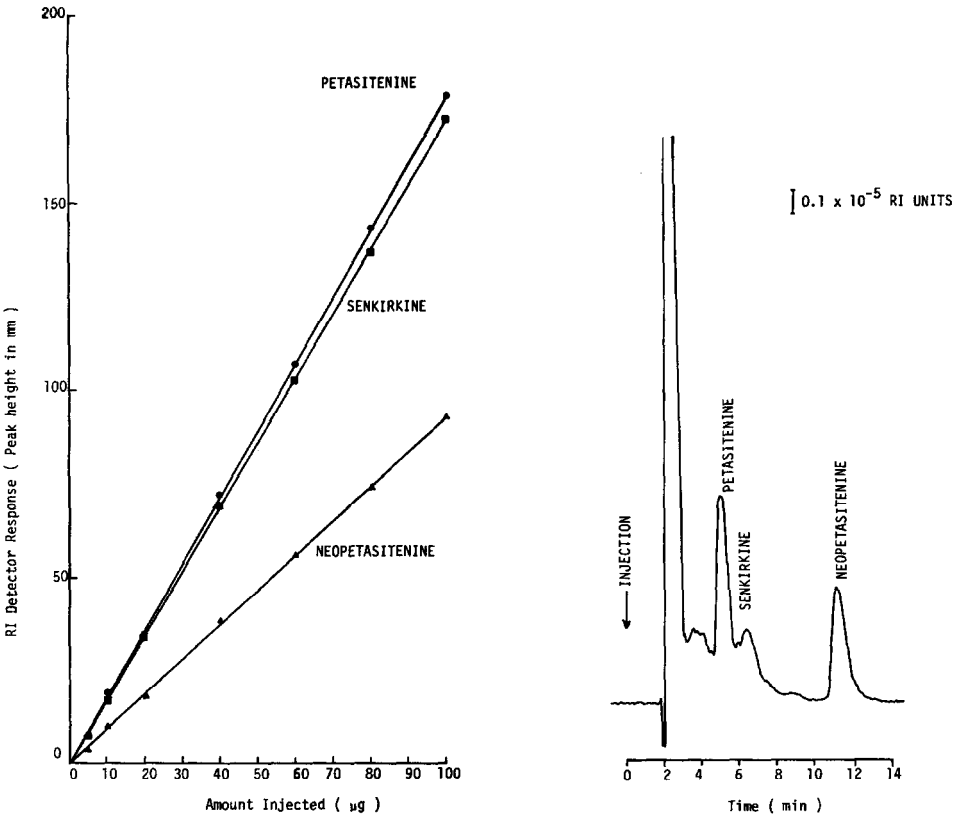


Fig. 3. RI Detector response *versus* amount injected. Conditions as in Fig. 1. Each data point represents a single determination.

Fig. 4. Separation of an alkaloidal mixture isolated from an aqueous extracts obtained by "processing" *P. japonicus*. Sample, 190 μg of the mixture in 10 μl of methanol. Conditions as in Fig. 1.

of PAs of the otonecine type. Previous systems[3–12] utilized for separation of PAs of the retronecine type did not give as good a resolution as the present system. For example, the use of a μBondapak C_{18} (10 μm) column and methanol–0.01 *M* potassium dihydrogen phosphate (1:1) as the mobile phase[5] gave an inadequate separation of petasitenine and senkirkine.

Fig. 2 shows the chromatogram of an alkaloidal mixture from a *P. japonicus* extract. Individual peaks were identified by comparison of the mass spectra with those of standard specimens. Fig. 3 illustrates the RI detector response curves obtained for compounds 1–3, which were used for the determination of the contents of PAs in *P. japonicus*. The response (peak heights) is linear over a 20-fold range of PAs injected. The contents of these PAs in *P. japonicus* (μg/g dry weight) were 56 for petasitenine (1), 60 for neopetasitenine (2) and 2 for senkirkine (3).

In order to investigate the effect of storage of the plant materials on the contents of PAs, dried *P. japonicus* stored for 1 year at room temperature was subjected

to HPLC analysis. It was found that there was essentially no variation in the PA contents during this period.

Customarily in Japan, young flower stalks of *P. japonicus* are boiled with water in the initial stage of cooking in order to remove harshness (harsh taste): this is called "processing". From the boiled water obtained by "processing" *P. japonicus* an alkaloidal mixture was isolated by the extraction procedure described above.

This alkaloidal mixture was subjected to the described HPLC analysis (Fig. 4), and it was found that more than half of the PAs (54% of petasitenine and 55% of neopetasitenine) were extracted with boiling water. Thus the content of the carcinogenic petasitenine in the "processed" *P. japonicus* was less than half of that in the unprocessed materials. It should be emphasized that treatment of the plant materials with boiling water, *i.e.*, "processing" is very effective for the purpose of removing PAs.

ACKNOWLEDGEMENT

Financial support from the Ministry of Education, Science and Culture, Japan (Grant-in-aid for Cancer Research No. 501026, 56010028 and 57010020) is gratefully acknowledged.

REFERENCES

1 L. B. Bull, C. C. J. Culvenor and A. T. Dick, *The Pyrrolizidine Alkaloids*, North-Holland, Amsterdam, 1968.
2 A. R. Mattocks, in J. B. Harborne (Editor), *Phytochemical Ecology*, Academic Press, London, New York, 1972, p. 179.
3 C. W. Qualls, Jr. and H. J. Segall, *J. Chromatogr.*, 150 (1978) 202.
4 H. J. Segall, *Toxicol. Lett.*, 1 (1978) 279.
5 H. J. Segall, *J. Liquid Chromatogr.*, 2 (1979) 429.
6 H. J. Segall, *J. Liquid Chromatogr.*, 2 (1979) 1319.
7 M. A. Adams and K. Nakanishi, *J. Liquid Chromatogr.*, 2 (1979) 1097.
8 G. Tittel, H. Hinz and H. Wagner, *Planta Med.*, 37 (1979) 1.
9 H. J. Segall and T. P. Krick, *Toxicol. Lett.*, 4 (1979) 193.
10 G. P. Dimenna, T. P. Krick and H. J. Segall, *J. Chromatogr.*, 192 (1980) 474.
11 H. S. Ramsdell and D. R. Buhler, *J. Chromatogr.*, 210 (1981) 154.
12 H. J. Huizing, F. de Boer and Th. M. Malingré, *J. Chromatogr.*, 214 (1981) 257.
13 K. Yamada, H. Tatematsu, M. Suzuki, Y. Hirata, M. Haga and I. Hirono, *Chem. Lett.*, (1976) 461.
14 K. Yamada, H. Tatematsu, Y. Hirata, M. Haga and I. Hirono, *Chem. Lett.*, (1976) 1123.
15 K. Yamada, H. Tatematsu, Y. Kyotani, Y. Hirata, M. Haga and I. Hirono, *Phytochemistry*, 17 (1978) 1667.
16 I. Hirono, H. Mori, K. Yamada, Y. Hirata, M. Haga, H. Tatematsu and S. Kanie, *J. Nat. Cancer Inst.*, 58 (1977) 1155.