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Analysis of dencichine in *Panax notoginseng* by gas chromatography–mass spectrometry with ethyl chloroformate derivatization

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Abstract

Dencichine (β-*N*-oxalyl-L-α,β-diaminopropionic acid) is a haemostatic agent present in well-known traditional Chinese medicinal herbs such as *Panax notoginseng*, as well as other *Panax* species. It is also a reported neurotoxic agent found in *Lathyrus sativus* (grass pea seed) and cycad seeds. A method was developed for quantitative determination of the non-protein amino acid, dencichine, in plant samples of *P. notoginseng* and the adventitious roots directly from the explants of *P. notoginseng* after derivatization with ethyl chloroformate (ECF) by gas chromatography–mass spectrometry (GC–MS). L-2-chlorophenylalanine was used as an internal standard. Calibration curves were linear ($r^2 = 0.9988$, $n = 6$) in the range of 10–800 μg/ml for dencichine. Limit of detection and quantification for dencichine were 0.5 μg/ml and 2 μg/ml, respectively. This rapid and specific method may be applied to the quantification of dencichine in complex medicinal plants and their products.

Keywords: Gas chromatography–mass spectrometry; Dencichine; *Panax notoginseng*; Adventitious roots; Ethyl chloroformate (ECF)

1. Introduction

Panax notoginseng (Burk.) F.H. Chen (commonly known as Tianqi or Sanqi) is a highly valued and important Chinese medicinal herb produced mainly in Yunnan Province, China. Some of its chemical constituents are similar to those present in two other well-known species in the same plant genus—*Panax ginseng* and *Panax quinquefolium*. For decades, the raw *P. notoginseng* has been highly valued for its wide ranging therapeutic properties which include the induction of blood clotting for bleeding conditions, promotion of blood circulation, relief of swelling, alleviation of pain. Currently, the roots of *P. notoginseng* are used to treat coronary heart disease, cardiac angina, apoplexy and atherosclerosis in clinics [1,2]. Recently, much of the research effort has been focused on the numerous bioactive saponins (such as ginsenosides, notoginsenosides) present in these *Panax* species.

Dietary non-protein amino acids have been implicated as potential factors interfering with fundamental biochemical processes and causing clinical disorders [3]. β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) (Fig. 1), a neuro-excitatory non-protein amino acid, was first isolated from the seeds of *Lathyrus sativus* (grass pea seeds) [4]. It was identified as the main cause of a neurological disorder known as neurolathyrism, a condition with typical and acute neurotoxic symptoms such as the inability to stand, head retraction, stiffening of the neck and extensor paralysis of the legs [5,6]. Besides being present in the seeds of *L. sativus*, β-ODAP was also found in cycad seeds [7]. The mechanisms whereby β-ODAP causes neurotoxicity have been researched by some investigators.

Interestingly, dencichine, a trivial name for β-*N*-oxalyl-L-α,β-diaminopropionic acid, was also found to be present in a very different *Panax* species, the only non-leguminous plants in which β-ODAP is known to be present [8]. Furthermore, it is a bioactive therapeutic amino acid component in *P. notoginseng*. Studies have reported dencichine as the compound responsible for the medicinal herb's main haemostatic and platelet-increasing properties in vivo [9] and found that the haemostatic effect was present at a low dose of dencichine, while neurotoxicity occurred at higher doses [9]. Therefore an accurate, sensitive, rapid and simple analytical method for detection of dencichine must be developed.

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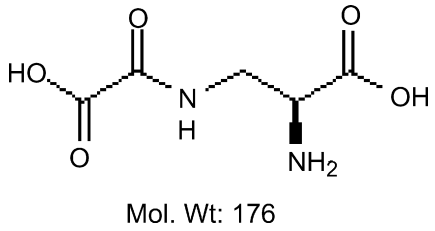

Fig. 1. Chemical structures of dencichine, i.e., β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP).

Several methods have been developed to address the determination of β-*N*-oxalyl-L-α,β-diaminopropionic acid including a colorimetric method which utilized the reaction of *o*-phthalaldehyde (OPT) with α,β-diaminopropionic acid [10,11], high-performance thin-layer chromatography (HPTLC) [12], HPLC with pre-column derivatization methods [8,13–18] and hydrophilic interaction chromatography with positive electrospray ionization tandem mass spectrometry (HILIC/ESI-MS/MS) [19]. However, GC–MS with ethyl chloroformate (ECF) derivatization for the determination of dencichine from *P. notoginseng* has been not reported.

The aim of this study is to develop a GC–MS method for the quantification of dencichine with ethyl chloroformate derivatization in complex plant matrices of *P. notoginseng*. The method reported here is sensitive, reproducible and rapid, which is suitable for the determination of β-ODAP in *P. notoginseng*. Additionally, amino acids were also identified in the adventitious roots directly from the explants of the *P. notoginseng* by the developed method in order to examine whether the adventitious roots have dencichine also.

2. Experimental

2.1. Chemicals and materials

The dencichine standard was purified from *P. notoginseng* by our laboratory (Section 2.2), its purity was confirmed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) [20,21] and through ^{1}H NMR, ^{13}C NMR, gas chromatography (GC). The structure was confirmed as β-*N*-oxalyl-L-α,β-diaminopropionic acid. Ethyl chloroformate and all of the amino acids for confirmation and internal standard ware purchased from Sigma–Aldrich, including alanine, glycine, valine, leucine, sercine, isoleucine, threonine, proline, asparagine, aspartic acid, methionine, glutamine acid, phenylalanine, cysteine, glutamine, 2-chlorophenylalanine (IS), lysine, histamine, tyrosine, tryphtothan, cystine.

Analytical cation-exchange resin 732 was purchased from Huazhen science Co., Ltd., Shanghai, China. Analytical-grade reagents were from Shanghai Lin Feng chemical reagent Co., Ltd., China. HPLC water was prepared with the Millipore Milli-Q SP water purification system (18.2 MΩ, Milipore, Bedford, MA, USA). All aqueous solutions were prepared with HPLC water.

Genuine *P. notoginseng* was collected from Wenshan, Yunnan, China. They were verified by professor of Shanghai Jiao Tong University of China as the dried rhizoma of *P. notoginseng* (Burk.) F.H. Chen.

2.2. Standard dencichine preparation

The air dried roots of *P. notoginseng* (10 g) were powdered and then extracted with methanol (3 × 100 ml) at 40–50 °C with occasional stirring. After filtration, the residue was air dried and extracted with water (3 × 100 ml) at 40–50 °C with occasional stirring. The combined water extracts were concentrated under reduced pressure to about 10 ml. The concentrate was extracted with *n*-butanol (3 × 10 ml). The combined aqueous phase, after being freed of solvents was percolated through a 3 cm × 60 cm column of resin 732 (H^+), (200–400 mesh) and first eluted with Millipore water and then eluted with ammonium hydroxide (0.05 M). The pooled ninhydrin-positive fractions were concentrated to about 10 ml at 40–50 °C in a rotary evaporator and treated with an excess of acetone (400 ml) and stirred. The precipitated compound was collected and re-precipitated from water. Yield, 0.4–0.5 g. The compound was recrystallized from water; m.p. 207 °C, decomp $[\alpha]_D{}^{20} = -31.92^\circ$ (H_2O):

- ^{13}C NMR (D_2O) δ_C: 56.3 (CH), 42.1 (CH_2);
- ^{1}H NMR (D_2O) δ_C: 3.87 (1H, ABd, J = 15.0, 4.15 Hz), 3.96 (1H, ABd, J = 15.0, 4.15 Hz), 4.24 (1H, dd, J = 6.8, 4.2 Hz);
- ESI *m/z*: 176 (M) (100%).

A 1 mg/ml dencichine standard stock solution in water was prepared and kept at 4 °C for later use.

2.3. Sample preparation of P. notoginseng

The air dried roots of *P. notoginseng* (10 g) were powdered and then extracted with methanol (3 × 100 ml) at 40–50 °C with occasional stirring. After filtration, the residue was air dried and extracted with water (3 × 100 ml) at 40–50 °C with occasional stirring. The combined water extracts were concentrated under reduced pressure to about 10 ml. The concentrate was extracted with *n*-butanol (3 × 10 ml). The combined aqueous phase, after being freed of solvents, was dissolved with water by ultrasonication (250 W) and transferred to 25 ml volumetric flask. An aliquot of 50 μl of the solution was used for derivatization.

2.4. Sample preparation of adventitious roots

Adventitious roots were obtained by the methods of our laboratory [22]. The sample was prepared according to the procedure of Section 2.3. An aliquot of 200 μl of the sample solution was used for derivatization.

2.5. Ethyl chloroformate derivatization procedure

A 500 μl samples (balanced with water if sample volume was less than 500 μl) and 100 μl L-2-chlorophenylalanine internal standard, 0.1 μg/μl were placed in a screw glass tube (5 ml). A 400 μl alcohol, 100 μl pyridine and 50 μl ethylchloroformate