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Short Communication

Determination of the neurolathyrogen β-N-oxalyl-L-α,β-diaminopropionic acid using high-performance liquid chromatography with fluorometric detection

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ABSTRACT

This paper describes a sensitive spectrofluorometric HPLC method suitable for determining picogram levels of β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), a neurotoxin. Aqueous extracts of powdered *Lathyrus sativus* seeds were treated with 9-fluorenylmethyl chloroformate (FMOC) and the ODAP-FMOC derivative analyzed by reversed-phase chromatography using a μBondapak C_{18} column. The excitation and emission wavelengths were 254 and 315 nm, respectively. The mobile phase was sodium acetate buffer (0.05 *M*, pH 6.35)–acetonitrile (72:28, v/v) at a flow-rate of 1 ml/min. This method represents a major advance over the standard spectrophotometric assays used currently.

INTRODUCTION

Lathyrism [1,2], a motor-neurone disease in humans, is associated with excess consumption of β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), also known as BOAA [1] or by the preferred IUPAC name L-2-amino-3-oxalyl-aminopropanoic acid [3–6]. This compound is present in the seed of *Lathyrus sativus* L., the grass pea, a legume cultivated in India, Bangladesh and Ethiopia. It grows in poor soil and is resistant to salt, flood and drought [7–9]. It is a staple food which is rich in protein. Lathyrus constitutes a health hazard when alternative food is in limited supply [4,10–12]. The concentration of ODAP in seed is genetically controlled and modified by environmental factors. It can range from O.l-0.4% in the dry seed [13].

Several methods have been described for determination of ODAP including electrophoresis, spectroscopy and high-performance liquid chromatography (HPLC) [14–18]. In this paper a simple and precise method for the estimation of ODAP in Lathyrus seed is presented based on a HPLC-spectrofluorometric technique reported earlier [18] which used gradient chromatography. The method reported here is sensitive, reprodu-

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cible and utilizes precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC) [19] followed by reversed-phase HPLC with fluorescence detection. The system is suitable for determination of ODAP in seeds and could be used to assay ODAP in animal tissue at sub-microgram levels.

MATERIALS AND METHODS

The reference standard of ODAP was provided by Dr. P. Nunn, Kings College, London, UK. *Lathyrus sativus* seeds were obtained from Dr. C. Campbell, Agriculture Canada Research Station, Morden, Manitoba, Canada. A standard amino acid mixture was obtained from Sigma (St. Louis, MO, USA). FMOC was purchased from Pierce (Rockford, IL, USA). All other chemicals were analytical grade and solvents were HPLC grade from Fisher Scientific, Canada. Purified water was produced using a Millipore Milli-Q unit. Purified water was produced using a Millipore Milli-Q unit. Aqueous solvents were filtered through a 0.45-μm membrane prior to use.

HPLC analysis

All chromatographic studies utilized a Waters HPLC system with a M45 pump and a U6K injector. A 300 x 3.9 mm stainless steel μBondapak C_{18} column, 10 μm particle size, (Waters Chromatography Division, Millipore) was used. A Shimadzu RF-535 variable-wavelength spectrofluorometer with a CR 501 integrating recorder was used for collection of data and their analysis. The excitation and emission wavelengths were 254 and 315 nm, respectively. The FMOC derivatives of sample and standard were eluted using sodium acetate buffer (0.05 *M*, pH 3.65)–acetonitrile (72:28, v/v) as the mobile phase at a flow-rate of 1 ml/min at ambient temperature of 23°C.

Preparation of derivative

A known amount of ODAP or sample was derivatized by the addition of 1 ml borate buffer (0.025 *M*, pH 9.6), 1 ml acetone and 0.1 ml FMOC (0.1 *M*, freshly prepared in acetone). The tube was vortex mixed for 2 min and derivatization was complete in 30 min at room temperature. A 2-ml volume of hexane-ethyl acetate (1:1) was added, vortex mixed for 30 s and 1.3 ml of aqueous layer was collected. Since the ODAP-FMOC derivative is soluble in acetone and insoluble in hexane, diethyl ether, ethyl acetate and chloroform, the aqueous layer was taken for HPLC injection. To optimize the derivatization conditions, concentrations of FMOC up to 1.0 *M* were studied. Similarly, 2,3-diaminopropanoic acid (DAP) and a standard mixture of 17 amino acids were also derivatized and analyzed.

Preparation of lathyrus seed extract

Aqueous extract of seed material was prepared from 10–100 mg of seed powder. A 2-ml volume of water was added and the sample was placed on a mechanical shaker for 12 h. The aqueous solution was separated after centrifugation (10 min at 3000 g in an IEC clinical centrifuge) and subsequently filtered through a 5-μm membrane; 5-10 μl of the extract were used for derivatization and 10 μl of the product were taken for HPLC analysis.

RESULTS AND DISCUSSION

As shown in Fig. 1, the retention time for the ODAP-FMOC derivative was 8.4 min and for unreacted FMOC 48.4 min. Peak areas corresponding to ODAP-FMOC were recorded. The precision of the method was assessed by repeated analyses of samples containing known concentrations of ODAP. The relative standard deviation for within-day precision ranged from 6.3 to 8.7% (n = 4). The day-to-day variation observed in peak area was in the range of 8.1 to 11.5%. There was also some variation in the retention time of the ODAP-FMOC peak ranging from 8.1 to 8.6 min. Hence standard samples of known ODAP concentration were required on each day. The ODAP-FMOC derivative eluted before the DAP-FMOC or any of the amino acid-FMOC derivatives from a lathyrus extract or standard plant amino acid mixture. The reason for selecting DAP and the amino acid mixture was to identify any possible interference

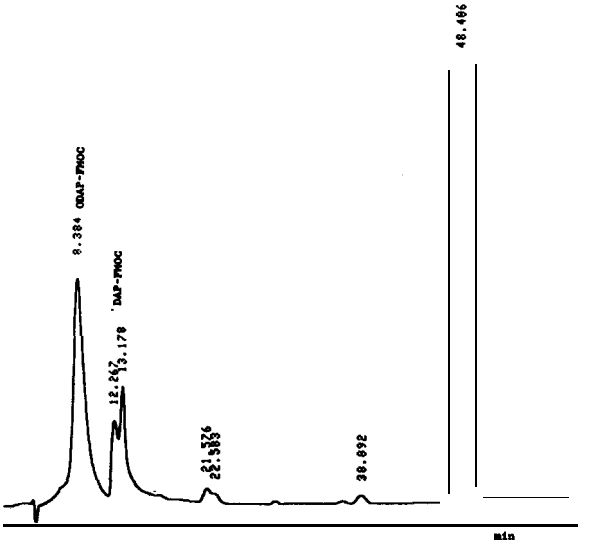

Fig. 1. A typical chromatogram of ODAP-FMOC and DAP-FMOC derivatives using a μBondapak C_{18} column.

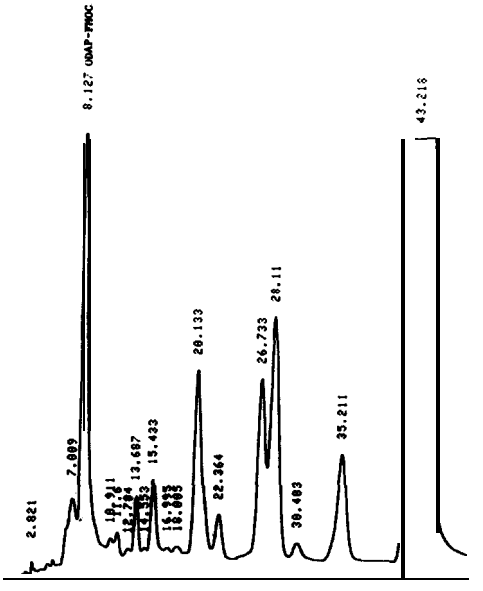

Fig. 2. A typical chromatogram of the FMOC-derivatized seed extract using μBondapak C_{18} column.

from these compounds with the ODAP-FMOC peak. Furthermore, DAP is the hydrolysis product of ODAP and any aqueous plant extract would contain amino acids as normal constituents. DAP exists in two isomeric forms and the doublet peaks at 12.3 and 13.2 min possibly correspond to their FMOC derivatives.

The amount of FMOC selected for derivatization was based on preliminary experiments which showed that the molar concentration of FMOC should be at least 100 times that of ODAP. Hence, 0.1 ml of a 0.1 *M* solution was used and found adequate for the reaction to be complete.

A typical chromatogram of the derivatized seed extract is shown in Fig. 2. It shows the presence of a number of peaks including the FMOC derivatives of amino acids, secondary metabolites and other normal constituents of an aqueous seed extract [20,21]. To reduce the total elution time of these compounds the column was flushed with pure acetonitrile 20 min after injection. The system was then re-equilibrated with 45 ml of the mobile phase before the next injection. Concentrations were determined by estimation of peak areas with reference to calibration curve for derivatized ODAP (correlation coefficient = 0.9745, intercept = -32.191 and the slope = 27543.6, linear up to 14 nmol of ODAP). The detection limit was found to be 15 pmol on column.

This HPLC-spectrofluorometric procedure showed that the average ODAP content of the *Lathyrus sativus* seed samples studied was 0.33 g/100 g of seed material. Breeding programs designed to eliminate ODAP from lathyrus seed may adopt this method for analysis of seed with very low levels of ODAP.

CONCLUSIONS

HPLC combined with spectrofluorometry can be used for the detection and quantitative estimation of pmol amounts of ODAP in Lathyrus seed. This technique is suitable for estimation of neurotoxic content of individual seeds from a single pod which is desirable for selection and breeding Lathyrus varieties with low ODAP content. This represents a major advance over spectrophotometric methods conventionally used to assay this compound in plant material.

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