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Note

Separation of conjugates and oxidative metabolites of indole-3-acetic acid by high-performance liquid chromatography

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Indole-3-acetic acid (IAA) is a natural plant hormone involved in various aspects of plant growth and development. In plants, it is metabolized at varied rates by conjugation with sugars and amino acids and by oxidation. Formation of such metabolites is likely to be an integral part of homeostatic control of IAA levels[1] and thus its function. Although conjugation and oxidation of IAA have been extensively studied, high-performance liquid chromatography (HPLC) has been used in only a few instances for the separation of IAA metabolites. Hollenberg *et al.*[2] reported the separation of several amino acid conjugates of IAA and Michalczuk and Bandurski[3] described the separation of indole-3-acetyl-myo-inositol from indole-3-acetylglucose by HPLC, but mixtures of amino acid and sugar conjugates of IAA were not analyzed. Crozier and Reeve[4] reported the HPLC separation of IAA and some related compounds but certain important oxidation products of IAA, such as 3-methylene oxindole[5] and oxindole-3-acetic acid[6], and IAA conjugates were not included in their study. Recently Nonhebel *et al.*[7] demonstrated the use of HPLC in the analysis of [^{14}C]IAA metabolites from maize roots: eleven unidentified radioactive peaks were observed.

This paper presents procedures for the separation of several major sugar and amino acid conjugates and oxidative metabolites of IAA by reversed-phase HPLC.

EXPERIMENTAL

HPLC equipment and procedures

Reversed-phase HPLC was carried out on 30 cm × 3.9 mm I.D. μBondapak C_{18} and μBondapak Phenyl columns (Waters Scientific, Mississauga, Canada), separately or in combination, using a Waters system composed of Model 6000A and Model 45 pumps, a Model U6K sample injector, a Model 720 system controller and a Model 440 UV detector operated at 254 nm in conjunction with a Shimadzu Model C-R1B data integrator.

Acetonitrile–water or methanol–water mixtures containing acetic acid or ammonium acetate at controlled pH were used for isocratic or gradient chromatography (details given in the figure captions). All chromatographic runs were performed at room temperature.

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Chemicals

The structures of the compounds used for this study are shown in Fig. 1. N-(Indole-3-acetyl)aspartic acid and N-(indole-3-acetyl)glutamic acid were synthesized by the method of Mollan *et al.*[8]. Indole-3-acetyl-myo-inositol was synthesized from IAA and myo-inositol by the method of Nowacki *et al.*[9] with the exception that the reaction was stopped by dilute hydrochloric acid. Water was added and the solution was extracted with diethyl ether. After adjustment of the pH to 9.0 with dilute ammonium hydroxide, the solution was re-extracted with butanol and the extracted material was chromatographed on a 70 × 2 cm Sephadex G-10 column[10]. Fractions were analyzed by silica gel TLC (Polygram Sil G/UV 254) using ethyl acetate–2-butanone–ethanol–water (5:3:1:1) for development and the Van Urk-Salkowski reagent[11] for visualization. Fractions 38–52 contained material showing two spots with R_F values (0.30 and 0.35) similar to those reported by Michalczuk and Chisnell[12] for authentic indole-3-acetyl-myo-inositols. These fractions were combined and recrystallized from water. 1-O-(Indole-3-acetyl)-β-D-glucopyranose was prepared by the method of Keglević and Pokorny[13].

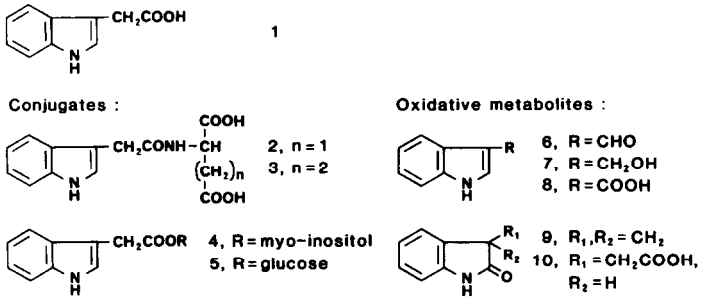

Fig. 1. Structures of IAA and IAA metabolites. 1 = indole-3-acetic acid; 2 = N-(indole-3-acetyl)aspartic acid; 3 = N-(indole-3-acetyl)glutamic acid; 4 = indole-3-acetyl-myo-inositol; 5 = indole-3-acetyl-β-D-glucopyranose; 6 = indole-3-aldehyde; 7 = indole-3-methanol; 8 = indole-3-carboxylic acid; 9 = 3-methylene oxindole; 10 = oxindole-3-acetic acid.

Indole-3-aldehyde, indole-3-methanol and indole-3-carboxylic acid were purchased from Sigma (St. Louis, MO, U.S.A.). Indole-3-methanol was recrystallized from benzene yielding plates, m.p. 95–99°C. Oxindole-3-acetic acid and 3-methylene oxindole were synthesized by the methods of Hinman and Bauman[14,15].

RESULTS AND DISCUSSION

Separation of conjugates of IAA

Since conjugates of IAA are not commercially available, we have synthesized the major known conjugates of IAA by published procedures. The indole-3-acetyl-myo-inositol preparation, m.p. 170–177°C, consisted of one major and three minor isomers as revealed by HPLC on μBondapak C_{18} or μBondapak Phenyl columns (Fig. 2). Silica gel thin-layer chromatography (TLC) showed that the major isomer was the most polar with an R_F value comparable to that observed for B_2 from maize tissue[16] which has been assigned the structure 2-O-(indole-3-acetyl)-myo-inositol[10].

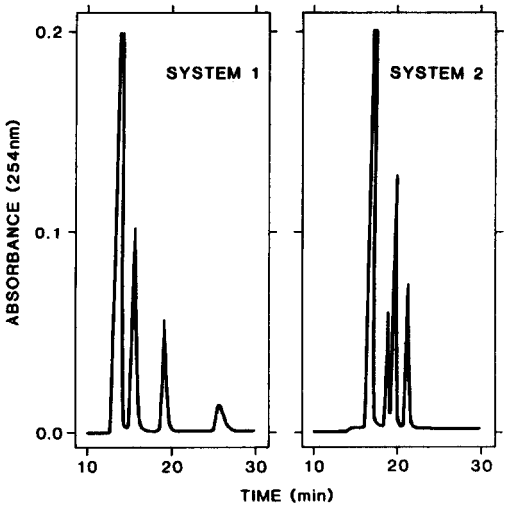

Fig. 2. Separation of indole-3-acetyl-myo-inositol isomers by reversed-phase HPLC. System 1 = μBondapak C_{18} column; system 2 = μBondapak Phenyl column. The chromatographic conditions are the same as described for Fig. 3 (system 1) and Fig. 4 (system 2).

The three minor isomers were inseparable on silica gel TLC and the R_F value for the mixture was similar to that reported for B_1 from maize tissue[16]. HPLC separations also indicated that the major isomer was the most polar but the sequence of elution for the three minor isomers appeared to vary with the column (Fig. 2). The four components of indole-3-acetyl-myo-inositol observed here probably are comparable to the four peaks reported in gas chromatograms of an acetylated indole-3-acetyl-myo-inositol preparation[17].

The separation of amino acid and sugar conjugates of IAA and IAA itself on a μBondapak C_{18} reversed-phase column eluted with a methanol–1% acetic acid gradient is shown in Fig. 3. The first three peaks were the isomers of indole-3-acetyl-myo-inositol. However, the fourth isomer, which comprised about 5% of the total and had a retention time of 26.2 min (Fig. 2), was not separated from N-(indole-3-acetyl)glutamic acid. The separation was improved when a μBondapak Phenyl reversed-phase column and acetonitrile were used. As shown in Fig. 4, the four isomers of indole-3-acetyl-myo-inositol were eluted before the other compounds in the mixture; all components were well separated.

Attempts to find a satisfactory isocratic solvent system for the separation of these sugar and amino acid conjugates of IAA using either the C_{18} or Phenyl column were not successful. Using the two columns in series, which produced excellent results for the oxidative metabolites of IAA (Fig. 5), improved the separation of indole-3-acetylglucose and N-(indole-3-acetyl)aspartic acid but yielded poor separation of amino acid conjugates and the isomers of indole-3-acetyl-myo-inositol.

Separation of oxidative metabolites of IAA

Isocratic HPLC of the oxidative metabolites of IAA on a μBondapak C_{18} or μBondapak Phenyl column with acetonitrile–water or methanol–water solvent sys-

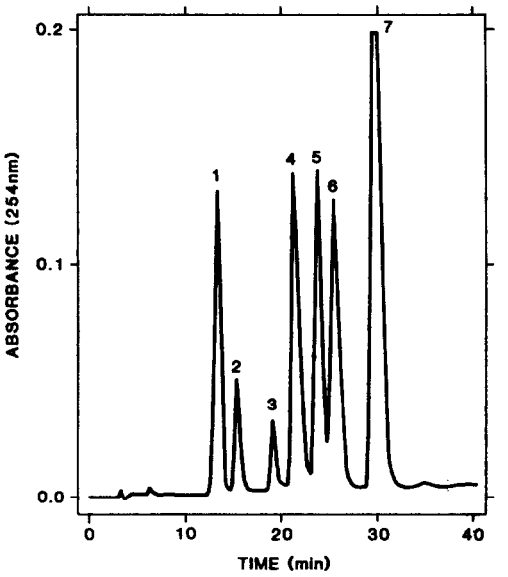

Fig. 3. Chromatogram of IAA and several IAA conjugates on a μBondapak C_{18} reversed-phase column. Solvent A: 1% acetic acid (pH 2.9); solvent B: methanol. Conditions: A for 6 min, a linear increase from 0 to 30% B during 30 min, followed by 30% B for 4 min. Flow-rate: 1 ml/min. Peaks: 1, 2, 3 = indole-3-acetyl-myo-inositol isomers; 4 = N-(indole-3-acetyl)aspartic acid; 5 = indole-3-acetylglucose; 6 = N-(indole-3-acetyl)glutamic acid; 7 = IAA.

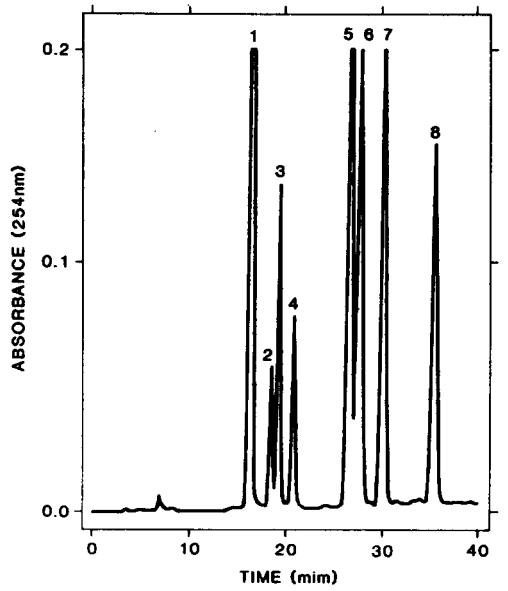

Fig. 4. Chromatogram of IAA and several IAA conjugates on a μBondapak Phenyl reversed-phase column. The chromatographic conditions are the same as described for Fig. 3 with the exception that solvent B is acetonitrile. Peaks: 1, 2, 3, 4 = indole-3-acetyl-myo-inositol isomers; 5 = N-(indole-3-acetyl)aspartic acid; 6 = indole-3-acetylglucose; 7 = N-(indole-3-acetyl)glutamic acid; 8 = IAA.

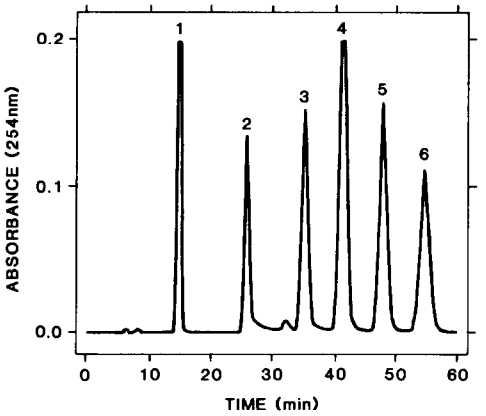

Fig. 5. Chromatogram of IAA and five oxidative metabolites on a μBondapak C_{18} and a μBondapak Phenyl column in series. Eluent: acetonitrile–20 m*M* ammonium acetate (pH 4.5) (1:9). Flow-rate: 1.0 ml/min. Peaks: 1 = oxindole-3-acetic acid; 2 = indole-3-methanol; 3 = IAA; 4 = indole-3-carboxylic acid; 5 = indole-3-aldehyde; 6 = 3-methylene oxindole.

tems gave poor separation of indole-3-carboxylic acid and indole-3-aldehyde, and of oxindole-3-acetic acid and indole-3-methanol. The gradient solvent system, which was used successfully for the separation of the sugar and amino acid conjugates of IAA (Fig. 4), was ineffective for the separation of the oxidative metabolites of IAA. The isocratic separation was greatly improved when the two columns were used in series; one example with acetonitrile–20 m*M* ammonium acetate (pH 4.5) (1:9) as the eluent is shown in Fig. 5. Addition of ammonium acetate to the mobile phase was necessary for suppressing tailing of indole-3-methanol. In this respect, acetic acid was less effective.

Metabolism of IAA in plants is complex and the mechanisms as well as the chemical identity of the metabolites are not completely known. Although the compounds tested here are well known metabolites formed by conjugation and oxidation of IAA, some may be less significant than others in individual plant species or at certain stages of growth. In practice, it will be difficult to separate a complete mixture of IAA metabolites by one HPLC system because of the complex nature of IAA metabolism. A simple solution to this problem is to separate the two classes of metabolites before HPLC by solvent fractionation, a procedure which has often been used for isolation of IAA metabolites. IAA and all the oxidative metabolites studied can be separated from the IAA conjugates by extraction of the aqueous solution with diethyl ether. Oxindole-3-acetic acid is the only compound in this group requiring relatively prolonged extraction to achieve a complete isolation.

CONCLUSION

Reversed-phase HPLC offers a useful technique for the analysis of sugar and amino acid conjugates and oxidative metabolites of IAA. Because of the diversity in chemical properties, the two broad classes of metabolites require different separation conditions. For analyzing a mixture of IAA metabolites, a simple solvent fraction-

ation can be used as an initial step before HPLC to separate the conjugates from the oxidative metabolites. Then the amino acid and sugar conjugates, including four isomers of indole-3-acetyl-myo-inositol, and the oxidative metabolites can be readily analyzed by HPLC using appropriate conditions. The solvent system used in this study will be convenient for the isolation of IAA metabolites for further characterization since all components are easily removed during lyophilization.

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