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Communication

Measurement of Indolebutyric Acid in Plant Tissues by Isotope Dilution Gas Chromatography-Mass Spectrometry Analysis[1]

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ABSTRACT

An internal standard, [^{13}C][indole-2]-indole-3-butyric acid, was synthesized from indole-2[^{13}C] and was shown to be effective for the quantitative determination of indole-3-butyric acid from plant tissue. When this standard was used along with [$^{13}C_6$]indole-3-acetic acid, both indolic auxins could be quantified from the same tobacco (*Nicotiana tabacam*) leaf sample by isotope dilution analysis using selected ion monitoring gas chromatography-mass spectrometry for detection.

Auxins are important hormonal factors affecting adventitious root formation. In propagation systems, auxins, particularly in the form of IBA,[2] have been used to improve the percentage of rooting and quality of roots formed in both herbaceous and woody plants (3). IAA has been considered the primary endogenous auxin closely involved in adventitious root formation and has been found to be positively correlated with adventitious root formation (14). IBA, until recently thought of only in terms of its use as a synthetic growth regulator, has been found to be considerably more effective as an exogenous agent than IAA (1). Several hypotheses have been proposed to explain the greater effectiveness of IBA over that of IAA including (a) that IBA may be less susceptible to degradative enzymes than is IAA and (b) that over time IBA may be slowly converted to IAA, thus providing a steady supply of free IAA (2, 9). More recent studies by Nordstrom *et al.* (12) indicated that the greater effectiveness of IBA was due, in part, to its greater longevity within tissues.

Concepts about the relationship between IAA and IBA have changed with the recent demonstration that IBA is a native auxin occurring in maize, tobacco (*Nicotiana tabacam*) tumors, and carrot (4, 8, 10). To extend these findings, it has become important to devise accurate procedures to determine how much endogenous IBA contributes toward adventitious root formation as well as other physiological processes thought to be mediated by endogenous auxins. Thus, we set about the establishment of a method that would enable us to study the physiological involvement of both auxins by accurate quantitation of the levels of both IAA and IBA in plant tissues. In this report, we describe the production of stable isotope-labeled IBA and its application, together with [$^{13}C_6$] IAA, to the analysis of plant-derived IAA and IBA using stable isotope dilution GC-SIM-MS.

MATERIALS AND METHODS

Plant Material

Plants of tobacco (*Nicotiana tabacum* cv SRI) were grown from seed. When the plants were 4 weeks old, shoot tips were removed, surface sterilized using 0.05% sodium hypochlorite, and then placed in Murashige and Skoog nutrient medium containing 0.1 mg/L BA. Shoots were multiplied *in vitro* and were transferred every 4 weeks.

Synthesis of [^{13}C]Indole-[ring 2]-3-Butyric Acid

We used a modification of the method of Cohen and Schulze (7), described for the synthesis of ^{14}C-labeled IBA, for production of [^{13}C]IBA. Twelve milligrams of indole-2[^{13}C] (Cambridge Isotope Laboratories,[3] CLM-1863) were placed in a 1-mL (4 mL capacity) freeze-drying tear bulb (A.H. Thomas, 5136–610) fitted with a condenser collar. Freshly broken NaOH pellets (0.75 g) and 1.5 mL of γ-butyrolactone (Sigma) were added to the bulb. The tear bulb was placed in a heating mantle, brought to a temperature of 220°C at the rate of 2°C/min, and then refluxed for an additional 23-h period. The reaction was stopped by the addition of water and the reaction mixture was cooled to a hardened, white, solid glass. The product was then dissolved slowly by adding

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[2] Abbreviations: IBA, indole-3-butyric acid; SIM, selected ion monitoring.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products or vendors that may be suitable.

2- to 5-mL aliquots of distilled water and shaking the mixture on a Vortex mixer each time. The final volume was 50 mL.

After the reaction mixture was dissolved, it was partitioned twice against an equal volume of chloroform. The aqueous phase was then adjusted to pH 2.5 with 6 N HCl and partitioned three times against diethyl ether. The ether extracts were combined and checked by TLC (7) for complete extraction of IBA into the ether phase. The chloroform and aqueous fractions were also checked for product. The chloroform fraction was found to contain substantial amounts of product, which was then partitioned against water and subsequently reextracted with diethyl ether. The ether fractions were combined and then dried over anhydrous sodium sulfate for 1 h, filtered, and evaporated to near dryness in a Buchi rotovaporator. The residue was applied to a 2.5 × 48 cm column of Sephadex LH-20 after being dissolved in 2 mL of 50% 2-propanol (aqueous, v/v). The IBA was eluted with 50% 2-propanol and collected in 16-mL fractions. IBA eluted at 276 to 294 mL as determined by TLC. These fractions were combined, dried *in vacuo*, and 2 mL of 2-propanol was added. The product was run on silica gel TLC (chloroform:methanol:water, 85:14:1 [v/v]; EM Science No. 5719–2 plates) against standards containing known amounts of IBA to determine approximate concentration and yield (30%, based on indole). Final concentration was determined by reverse isotope dilution analysis using a mixture of a known volume of the product solution and a known amount of unlabeled IBA, followed by methylation and GC-SIM-MS analysis (see below).

Determination of IBA and IAA Levels in Tobacco Tissues

Between 0.7 and 1.0 g fresh weight of leaf tissue was obtained from tobacco shoots grown *in vitro*. The tissue was ground in a liquid nitrogen-chilled mortar to a fine powder and 4 mL of imidazole buffer (35% 0.2 M imidazole, 65% 2-propanol, pH 7) per g of material was added. The ground plant material and buffer were transferred to a 13-mL Corex centrifuge tube and the following standards were added: 100,000 dpm [^{3}H]IBA (7 Ci/mmol, a gift from Dr. Ephraim Epstein, Volcani Center, Israel), 100,000 dpm [^{3}H]IAA (29 Ci/mmol, Amersham), and 50 ng each of [$^{13}C_6$]IAA (synthesized as in ref. 6) and [^{13}C][ring 2]-IBA. The extract was allowed to equilibrate with the added isotopes for 1 h at 4°C. The sample was centrifuged for 5 min at 2000*g* and was washed two additional times with extraction buffer. Extracts were combined and reduced *in vacuo* to one-third of their original volume.

The extract was transferred quantitatively to a Mixxor vessel (Lida, Israel) or to a small separatory funnel and partitioned once against one-half volume hexane. The aqueous phase was brought to pH 2.5 to 3.0 and was partitioned two times against diethyl ether, saving the ether phase. The ether portions were combined and dried over one-third volume anhydrous sodium sulfate for 1 h at 4°C. The ether phase was evaporated to dryness *in vacuo* and the residue dissolved in 100 μL of 15% acetonitrile.

HPLC Analysis

Samples were further purified by reverse-phase HPLC using a system consisting of two Waters 6000A pumps with a model 680 controller and fitted with a C_{18} reverse-phase, 3-μm Microsorb column (Rainin, 4.6 mm i.d. × 100 mm) and an Upchurch guard column packed with Whatman Co:Pell ODS. The gradient used was a linear change of acetonitrile/1% acetic acid from 15 to 50% acetonitrile over 25 min at 1 mL/min. Under these conditions, the retention volume of

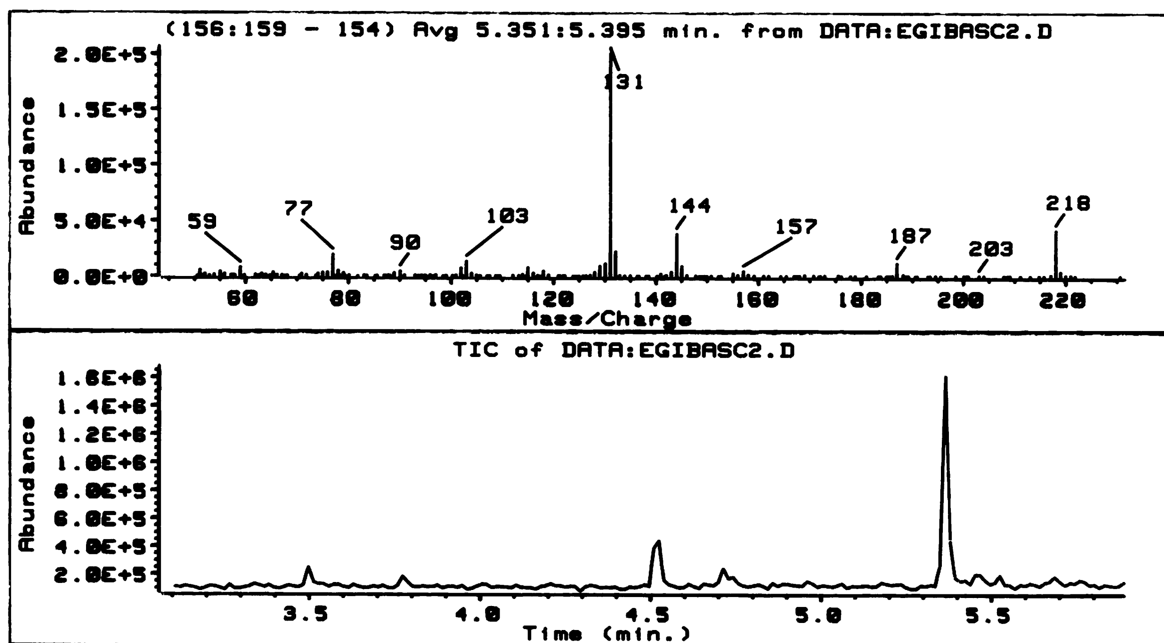

Figure 1. Mass spectrum and total ion chromatogram of synthesized [$^{13}C_1$]IBA methyl ester. The ions at m/z 131 and 218 are from the quinolinium ion and molecular ions, respectively.

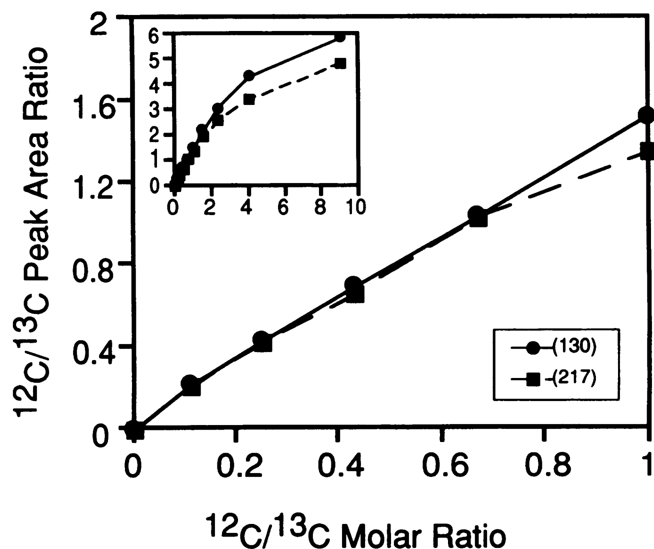

Figure 2. Calibration plot of [$^{13}C_1$]IBA showing the relationship between the molar ratio for methyl [$^{12}C/^{13}C$]IBA and the peak area ratios for methyl [$^{12}C/^{13}C$]IBA derived from the molecular and quinolinium ions.

IAA was 7.8 mL and that of IBA was 13.5 mL. Samples containing the radioactive peaks were combined, keeping the IAA and IBA samples separate. Each sample was evaporated to dryness *in vacuo* and 100 μL of methanol was added immediately. The samples were methylated with ethereal diazomethane (5), dried under nitrogen, and dissolved in 20 μL of ethyl acetate for analysis by GC-MS.

Analysis by GC-MS

GC-MS was performed using a Hewlett-Packard 5890 GC equipped with a 15 m × 0.21 mm i.d. DB-1701 fused silica column (J&W Scientific). Helium was used as the carrier gas at a flow rate of 1 mL/min. IAA and IBA were analyzed separately; however, the GC conditions were identical. The injector was at 250°C and the initial column temperature was 140°C. After a 1-min hold, the temperature was programmed to increase at a rate of 20°C/min. Under these conditions, methyl-IAA had a retention time of 4.7 min and methyl-IBA had a retention time of 6.5 min. On older columns, these times were reduced somewhat due to shortening of the column length (we routinely remove 0.33 m of column at the injector side periodically to maintain column performance) and a decrease in sample retention.

MS was performed with a Hewlett-Packard 5971A mass selective detector coupled to the GC using SIM with a dwell time of 50 ms for each ion. The selected ions monitored were at m/z 130, 136, 189, and 195 for IAA and its $^{13}C_6$ standard and m/z 130, 131, 217, and 218 for IBA and its $^{13}C_1$ standard.

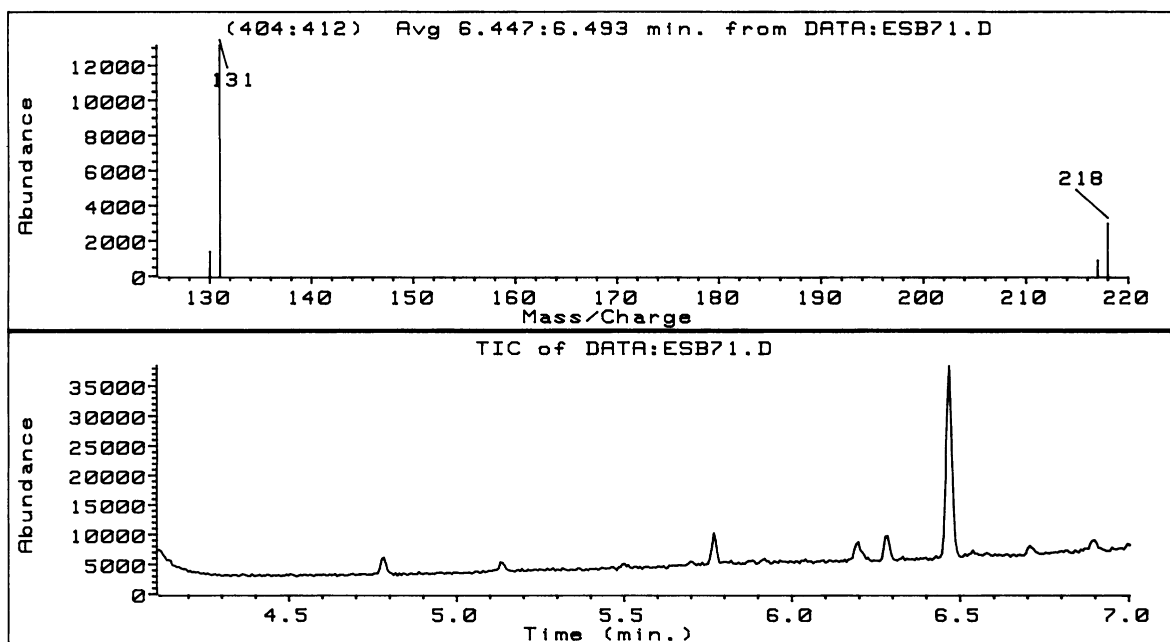

Figure 3. Mass spectrum and selected ion chromatogram of IBA fraction from partially purified and methylated extracts of tobacco leaf tissue using 100 ng of [$^{13}C_1$]IBA as internal standard.

RESULTS AND DISCUSSION

The identity of the synthesized $^{13}C_1$ standard was confirmed by GC-MS. The quinolinium ion at m/z 131 and the molecular ion at m/z 218 were present as expected with no significant ^{12}C isotopic contribution at m/z 130 and 217 (Fig. 1). In addition, an essentially linear relationship was observed between the molar ratio and area ratio of $^{12}C/^{13}C$ (Fig. 2). One potential problem with using a stable isotope-labeled standard with only one heavy atom is that it overlaps with the naturally occurring heavy isotope cluster on the plant-produced compound (11, 13). Thus, when the internal standard was significantly less than the sample, nonlinearity occurred (Fig. 2, inset). This was primarily due to the contribution of ^{13}C that occurs naturally in unlabeled IBA. The amount of ^{13}C in unlabeled IBA accounts for 10.28% of the ion at m/z 130 (13). However, because the internal standard we produced contains no significant amount of unlabeled IBA, near linearity is obtained as long as the ratio of internal standard to endogenous IBA in the sample is high. Under conditions in which the amount of internal standard added is high relative to what was in the plant sample, only a minor correction (*i.e.* subtraction of 10.28% of the m/z 130 value from the m/z 131 abundance) is required to account for the small overlap at m/z 131 and 218. In other situations in which a higher endogenous level of IBA is found than was expected, the quantitation can be calculated using the known relationship (see ref. 11) or, more conveniently, the standard curve can be used for quantitation.

The mass spectrum of the tobacco leaf sample showed a clear peak at the retention time for IBA (Fig. 3). The selected ion mass spectrum of the peak had m/z values at 130, 131, 217, and 218, confirming that the peak was IBA. For the sample shown, the values obtained for IAA were 26 ng/g fresh weight free and 52 ng/g fresh weight total; for IBA they were 9 ng/g fresh weight free and 37 ng/g fresh weight total.

The use of [$^{13}C_1$]IBA as an internal standard is similar to the previous use of [$^{13}C_6$]IAA (6) for measurement of IAA, except as noted above. Previously, no heavy-labeled internal standard was available of analysis of IBA. The availability of [$^{13}C_1$]IBA now allows the determination of the amount of IBA in plant tissues with the precision and reliability inherent in the stable isotope dilution GC-SIM-MS technique.

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