J Plant Growth Regul (1985) 4:91–100

Journal of
Plant
Growth
Regulation

Auxin Levels in Single Half Nodes of *Avena fatua* Estimated Using High Performance Liquid Chromatography with Coulometric Detection

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Received December 11, 1984; accepted March 26, 1985

Abstract. A high performance liquid chromatography (HPLC) based micro-method for estimation of indole-3-acetic acid (IAA) levels in single half nodes from the flowering stalks of *Avena fatua* has been developed; this features a dual electrode coulometric electrochemical detector operating at a detection limit of c. 2 pg. Samples were prepared by solvent partitioning and preliminary fractionation with C^{18} Sep-Pak cartridges. Two stages of reversed phase ion pair HPLC were employed; the first was gradient elution with fluorescent detection, the second, isocratic elution with coulometric detection. The lower limit for estimation of IAA levels in purified extracts was c. 5 pg.

Investigation of the mode of action of indole-3-acetic acid (IAA) in plants is greatly benefited by the use of an experimental system in which the hormone can be shown to control a developmental process. A system which shows this clearly is found in the grass node, where estimated levels of endogenous IAA from pooled gravistimulated nodes can be directly related to the extent of growth (Wright et al. 1978). Relating the growth rates of the upper and lower halves of a single node (Wright and Osborne 1977) to the IAA contents of those halves has not been possible because of the limitations of current methods of IAA analysis which rely on gram amounts of tissue. For practical routine analysis on individual half nodes (fresh weight c. 10 mg) a highly sensitive method of auxin analysis is required.

This paper contains the description of such a method, followed by a discussion of its application to the nodal system.

The method is HPLC-based and uses a highly sensitive mode of electro-

chemical detection. Because of the diversity of plant extracts, extensive sample preparation is usually needed to ensure sufficient resolution of IAA to validate high sensitivity measurement. Sample preparation has been considerably simplified by using C^{18} Sep-Pak cartridges (Jensen and Junttila 1982, Law and Hamilton 1982, Akiyama et al. 1983). In the procedure now reported, the separation of IAA from compounds of differing polarity has been optimized for these cartridges.

The efficient resolution of IAA during HPLC has been achieved by combining ion pairing with gradient elution; ion pairing has been used to increase the retention of IAA (Mousdale 1981, Sandberg and Dunberg 1982) and to give improved resolution from interfering compounds (Blakesley et al. 1984); gradient elution has also been used to improve resolution of IAA (Crozier et al. 1980, Jensen and Junttila 1982), this having the advantage of inherently shorter analysis times. However, there are no previous reports of their combined usage for IAA separation.

Electrochemical detection was employed (in addition to fluorescence detection), as it appears to have a high sensitivity for IAA in plant extracts (Sweetser and Swartzfager 1978, Law and Hamilton 1982) and because of the potential advantages of working in a field with such a rapidly expanding technology (McClintock and Purdy 1984). Furthermore, the development of highly sensitive fluorescence detection for routine use may be limited by the necessity for extensive purification of solvents (Crozier et al. 1980).

For this work the coulometric type of electrochemical detector was chosen; this can electrolyze all of the solute rather than the 1–10% typically electrolyzed by amperometric detectors. In the detector used here (Coulochem model 5100A, Environmental Sciences Associates Inc.) complete electrolysis is achieved because the solute passes through porous graphite electrodes rather than over the surface of the carbon electrodes as in amperometric detection. This has the inherent advantages of higher sensitivity (Roe 1983) and the capacity to subject the eluent to successive quantifiable electrolytic steps, by using multi-electrode systems (Dutrieu and Delmotte 1983). The application of these aspects of coulometric detection to the measurement of IAA is described in this paper.

The coulometric detector has been used in series with a fluorescence detector for a second isocratic stage of HPLC. To simplify the procedure, minimize sample losses, and take full advantage of the purification achieved by an initial gradient separation the same ion pairing agent has been used throughout. Using this technique, the fluorescent, electrochemical, and chromatographic properties of putitive IAA from plant extracts were found to correspond to those of standard IAA. Within these limitations IAA can now be estimated in purified extracts from single half nodes.

Materials and Methods

Preparation of Plant Material

Seeds of wild oat (*Avena fatua*) were decoated, pricked to break dormancy, and sprouted in a lighted incubator at 20°C. After 5–6 days the sprouted seeds

were planted in 12-inch pots (5/pot), in a greenhouse provided with supplementary heating and lighting during the winter. The nodes of the flowering stalks were harvested, bisected longitudinally, and the halves of the leaf sheath base were removed (Wright 1981). These were frozen in liquid N_2 and then stored at −20°C.

Procedure for IAA Estimation

Throughout this procedure all glassware was acid washed. The water used for the final rinsing of glassware and making up solutions was purified by a Labro reverse osmosis unit (Elga), followed by deionization and double distillation (final conductivity 0.7 μS).

Extraction and Sample Preparation. Half nodes (15–50) were ground in a porcelain pestle and mortar containing liquid nitrogen. The homogenate, together with the liquid nitrogen, were poured into a beaker to which was added 20 ml of methanol (HPLC grade) and 500 μl of methanol containing ^{3}H-IAA (c. 2000 dpm) (CEA, Gif-sur-Yvette, France). The presence of liquid N_2 around the plant tissue was maintained throughout grinding and until after the methanol was added. Single half nodes were ground in methanol (200 μl) in a precooled conical glass grinder. The homogenate plus three 1-ml methanol rinses were then increased to 20 ml with methanol and the ^{3}H-IAA, as were the liquid N_2 homogenates. The extract was then shaken and left overnight at −20°C. Five milliliters of 0.1 M phosphate buffer (pH 9.0), containing 200 mg/liter of sodium diethyldithiocarbamate (DDC), was then added; the mixture was shaken and left for a further 1 h at −20°C. It was then centrifuged at 10,000 *g* for 20 min at −5°C and the supernatant reduced to a 4-ml aqueous fraction by rotary evaporation at 35°C. This was cooled in ice, increased to 5 ml with water, then partitioned against 3 × 5 ml aliquots of re-distilled ether containing 1 mg/10 ml of butylated hydroxytoluene (BHT) at 0°C. The ether was discarded and further ether removed by evaporation under a stream of N_2 at 35°C for 5 min. The aqueous fraction was then acidified to pH 2.5–3.0 with c. 500 μl of M HCl. Sep-Pak C^{18} sample preparation cartridges (Waters Associates) were preconditioned by flushing with 30 ml of methanol over a period of at least 1 h. (A sufficiently long conditioning time was found to be critical for the optimal functioning of the cartridges.) The cartridges were rinsed with 15 ml of 0.01 M phosphate buffer (pH 3.0), containing 200 mg/liter of DCC; the samples were loaded onto the cartridges together with a 5-ml rinse of the same 0.01 M phosphate buffer. The cartridges were then eluted with 5 ml of a mixture of the same phosphate buffer and methanol (90:10, v/v) followed by 3 ml of the phosphate buffer and methanol (50:50, v/v). The final 3 ml of eluent (constituting the central portion of the IAA containing peak, equivalent to 90% of the applied IAA) was reduced to its aqueous component by rotary evaporation at 35°C. It was then cooled in ice and extracted with three aliquots (1 ml) of ice-cooled re-distilled ether containing BHT (1 mg/10 ml). The ether extracts were pooled in silanized tubes (Glas-Treet, Phase Separations Ltd), frozen in liquid

nitrogen for c. 2 min, then thawed with gentle shaking, resulting in separation of the aqueous component as a single frozen globule. The ether was decanted, the process repeated, and the ether fraction evaporated to a residue under a stream of N_2. The residue was taken up in 50 μl of 100% methanol and stored in a similarly silanized Wheaton v-vial at −20°C.

Immediately prior to the first stage of HPLC each sample was reduced to 20 μl under N_2, and 80 μl of 10 mM tetrabutylammonium phosphate (TBAP), pH 6.5, was added. This was prepared from 40% tetrabutylammonium hydroxide (Fisons, HPLC grade) and phosphoric acid, (BDH, Aristar grade). The 100-μl samples were filtered with 0.2 μm regenerated cellulose filters using a BAS centrifugal filtration system (Anachem Ltd.).

Gradient HPLC Stage. The HPLC equipment was comprised of the following: two 6000A pumps, a UK6 injector and 720 system controller (all Waters Associates), an FS-970 fluorescence detector set at an exitation wavelength of 280 nm and fitted with a 375-nm band pass emission filter (Kratos), and a Spherisorb 250 × 4.6 mm 5-μm, OD S2 reverse phase column (Phase Separations).

The sample (100 μl) was run in the ion pair mode in a mixture of methanol (Rathburn Chemicals) and 10 mM TBAP (pH 6.5) (20:80, v/v) at 100 μl/min. The elution was isocratic for the first 5 min and then by a linear gradient to 100% methanol over a period of 120 min. The IAA containing peak was collected and c. 10% taken for measurement of radioactivity. The remainder was evaporated under N_2 to a predominantly aqueous fraction, then made up to 50 μl with 10 mM TBAP (pH 6.5) and methanol to give a final methanol concentration of 40%. This was used for the next stage of HPLC.

Isocratic HPLC Stage. For this the model 5100A Coulochem Electrochemical Detector, fitted with a model 5020 guard cell and model 5011 analytical cell (ESA), together with a high efficiency membrane pulse damper (Kratos) and a guard column (Waters Associates), were incorporated into the HPLC equipment already described. The flow sequence was as follows: pump, pulse damper, guard column, guard cell, injector, analytical cell, and finally the fluorescence detector.

Prior to using the coulometric detector, special care was needed to minimize interference from electrochemically active contaminants. The system was cleaned with 6 N nitric acid and glacial acetic acid as described in the manufacturers instructions. Water used for making up the TBAP was filtered (0.2 μm) and degassed. The TBAP (10 mM, pH 6.5) and methanol were both filtered and degassed before and after mixing (60:40, v/v); the system was then run using a single pump at 1 ml/min. The coulometric detector was used in the screen mode; for this the first of the two electrodes in the analytical cell was set at 0.45 V, which was marginally above the minimum oxidation voltage for IAA, and the second electrode at 0.65 V, which was marginally above the optimum oxidation voltage (Fig. 4). The guard cell was set at 0.75 V to elim-

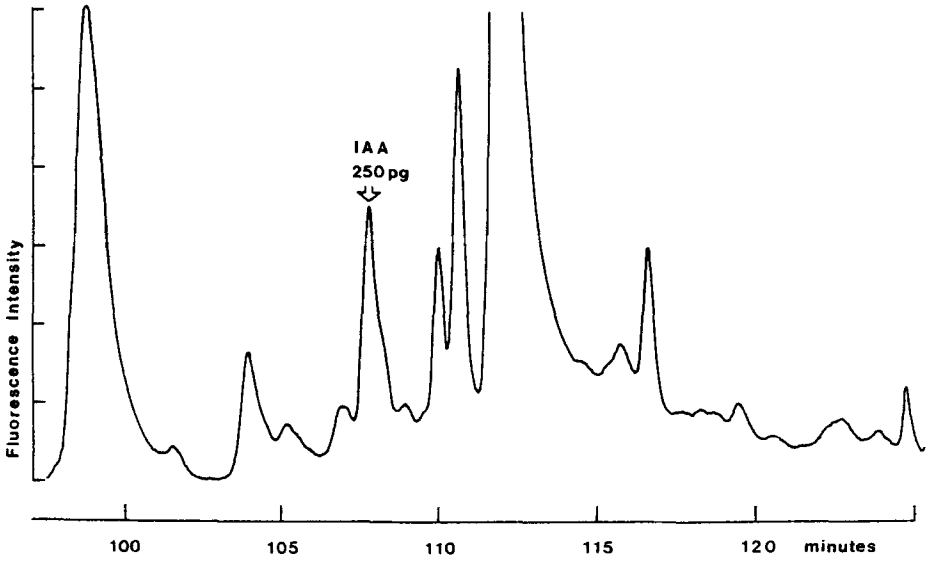

Fig. 1. HPLC chromatogram of an extract from 15 half nodes showing the eight main fluorescent peaks which are resolved between 95 and 125 min during gradient elution, and indicating the IAA level based on the peak height.

inate electrochemical interference from the eluent, and the system was left running overnight to equilibrate.

Results

A scan of the peaks eluting from the gradient separation of a nodal extract is shown in Fig. 1. The IAA-containing peak eluted typically between 106 and 110 min according to ambient temperature (20–25°C). Its position was assessed from the co-elution of ^{3}H-IAA and standard IAA recrystallized from chloroform. The major peaks all show some degree of tailing; this may partly explain the size of the shoulder on the IAA-containing peak. However, the height of the sample peak was additive with that of standard IAA. It is unlikely, therefore, that the compound responsible for the shoulder effects peak height as this could occur only in the event of its fluorescence intensity and chromatographic properties being similar to those of IAA. The overall peak height rather than the peak area was therefore taken to be the most reasonable estimate of IAA levels in the sample.

The peak containing the IAA fraction was re-run isocratically. Electrochemical and fluorescence scans for a 25 half node (850 pg) and a 1 half node (30 pg) sample are shown in Fig. 2. The IAA eluted between 10 and 11 min depending on ambient temperature. The relative peak heights from the two modes of detection were constant, for both extracts and standard re-crystallized IAA. This is illustrated in Fig. 3 in which the calculated IAA levels, based on stan-

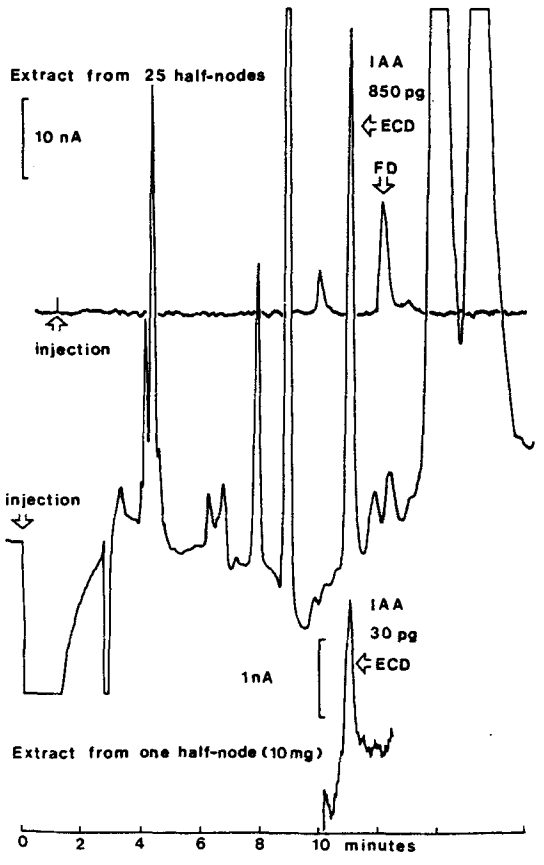

Fig. 2. HPLC chromatogram of IAA extracts re-run isocratically following purification by gradient elution: fluorescent (FD) and electrochemical (ECD) scans are shown for the 25 half node extracts, and an electrochemical scan for the single half node extract; IAA levels indicated are based on peak heights.

dard IAA peak heights, are the same for both electrochemical and fluorescence detection.

The estimations of IAA levels in the original extracts based on the recoveries of the ^{3}H-IAA at the two HPLC stages are compared (Table 1); estimates are similar from both stages, further confirming that the IAA-containing fraction collected from the gradient is predominantly IAA (recoveries of the original ^{3}H-IAA spike were typically 50–60% from the gradient and 25–30% from the isocratic stage).

The selectivity of the electrochemical detector and the resolution of the isocratic elution can be seen from a scan of a standard mixture of 5-hydroxy-indole-3-acetic acid (5-OH-IAA), IAA, and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Fig. 4). Similar relative retention times were observed by Blakesley et al. (1984) using natural fluorescence and UV detection. The same figure shows the scan for the first electrode (detector 1) in the analytical cell. At this potential (0.45 V) a relatively high proportion of the 5-OH-IAA and a very low proportion of IAA and 4-CL-IAA were oxidized, illustrating how such screening may be used to distinguish compounds of different oxidation poten-

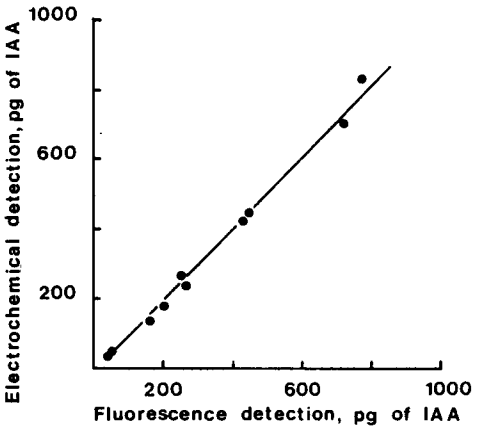

Fig. 3. A comparison of fluorescent and electrochemical estimations of IAA levels in a series of extracts. The estimations are calculated from the peak heights of the IAA containing peaks of extracts in relation to those of standard IAA.

Table 1. Estimation of the endogenous IAA levels (pg) of a set of four unmatched samples of nodes, calculated from the recovery of ^{3}H-IAA (added during methanol extraction) at each of the two successive HPLC stages

Wt of sample (mg)	Gradient separation fluorescence detection	Isocratic separation Electrochemical detection	Ratio of estimated IAA levels
190	590	625	1.06
190	722	695	0.97
200	517	520	1.01
220	724	602	0.84

tials. For the latter two compounds proportionally smaller signals are produced at detector 2. However, it was considered that this reduction in amplitude was outweighed by the accompanying greater potential selectivity in such a complex mixture as a plant extract.

The sensitivity of the electrochemical detector is shown from the scan of an extract purified from a single half node (Fig. 2) and from the detection limits for standard IAA (Fig. 5). Both scans are at maximum sensitivity. Baseline fluctuations reflect pressure pulses caused by the reciprocating pump. The recovery of 30 pg of IAA from a single half node extract correlates closely with the recovery of 34 pg per half node from the 25 half node extracts (Fig. 2).

Discussion

The procedure for IAA estimation described was developed primarily as a micro-method applicable to extracts from single half nodes of *Avena fatua* or

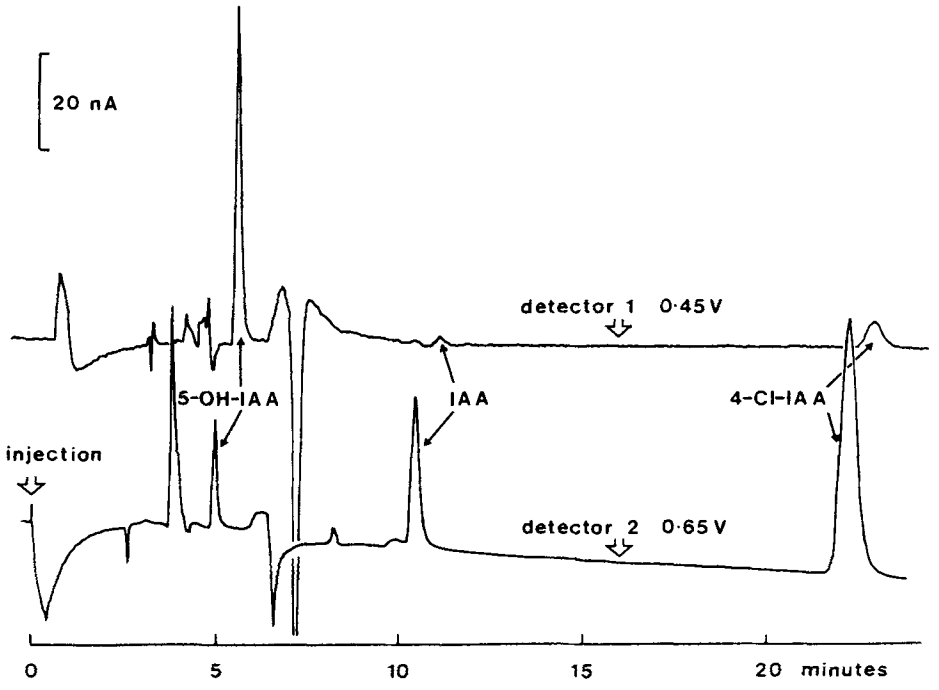

Fig. 4. HPLC chromatogram of a standard mixture of IAA, 5-OH-IAA, and 4-Cl-IAA showing the scans of the screening (detector 1) and analytical (detector 2) electrodes.

other very small (c. 10 mg) pieces of plant tissue. Initially, to enable comparisons to be made between electrochemical and the less sensitive fluorescent mode of detection, extracts from larger amounts of tissue (c. 500 mg) from nodes of *Avena fatua* were used. The purification of these extracts by solvent partitioning, C^{18} Sep-Pak, and gradient elution HPLC proved adequate for subsequent reproducible and accurate estimations of IAA on a routine experimental basis. This has enabled confirmation (Wright, in preparation) of the relationship between cell growth and estimated endogenous IAA levels, which was established in gravitropically stimulated grass nodes with the 2-methyl indolo-α-pyrone assay (Wright et al. 1978). The exact correlation of the electrochemical and fluorescent properties of the IAA-containing fraction with those of standard IAA (Fig. 3) provides evidence for the purity of the IAA and the validity of the method.

For experiments in which large numbers (15–50) of pooled nodes were used, the gradient elution combined with fluorescence detection was sufficient to give a reliable IAA estimate, comparable with that obtainable using a second isocratic stage of HPLC (Table 1). This probably derives from the very high column efficiency (equivalent to 240,000 plate counts, calculated from Fig. 1) and correspondingly high resolution of the IAA-containing peak during the initial gradient elutions. The very low flow rates (100 μl/min) used in this elution gave considerably enhanced efficiency of fluorescence detection (cf.

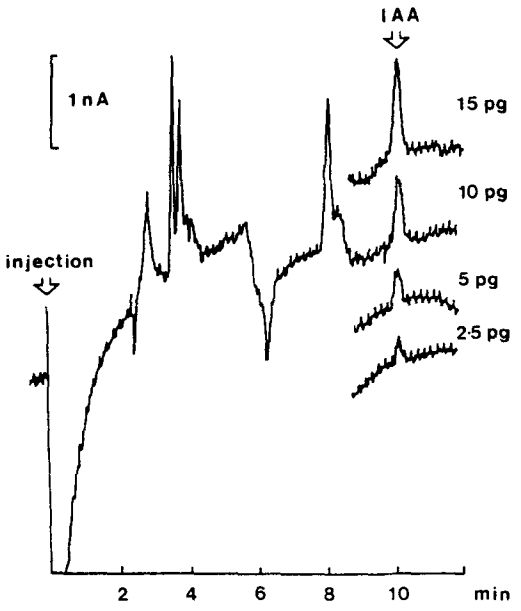

Fig. 5. HPLC chromatograms of IAA standards to illustrate the detection limits of the coulometric detector at maximum sensitivity.

Figs. 1 and 2) as was previously found for fluorescent peptide derivatives (Frei et al. 1976). This factor does not appear to have been exploited by other workers, but in this work it proved invaluable for peak detection (c. 50 pg of IAA) when the gradient elution was used as a purification step for extracts of single half-nodes.

The subsequent use of the procedure as a micro-method has proved highly successful; the mean recovery of ^{3}H-IAA following extraction of a set of four single half nodes was 59% (standard error ± 1.3%) from the first stage of HPLC and 31% (standard error ± 1.0%) from the second stage. Thus accurate estimations of the IAA levels in single half node extracts are well within its capability (Fig. 2). This use of coulometric detection offers the highest sensitivity of any method so far reported for measurement of IAA in plant extracts. It can now be applied to a study of IAA regulated growth using extracts made from individual upper and lower halves of nodes, rather than from pooled nodes (Wright et al. 1978). It is hoped in this way to take advantage of the biochemical variability of individual nodes, and establish more precise relationships between their IAA levels and growth, during different stages of development and gravitropic stimulation.

Preliminary work has indicated that the method may be of general application for other plant tissues; thus coulometric detection could well become the method of choice for IAA measurement in the future.

Acknowledgments. The authors wish to thank David Blakesley (School of Life Sciences, Leicester Polytechnic) for his gift of 4-Cl-IAA, Drs. Daphne J. Osborne and Richard Hooley (Developmental Botany, Weed Research Organization) for their advice in the preparation of the manuscript, and Dr. Stephen Mitchell (Severn Analytical) for his invaluable help in this work.

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