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SIMULTANEOUS DERIVATIZATION OF GIBBERELLIC, INDOLYL-3-ACETIC AND ABSCISIC ACIDS BY α-BROMO-2′-ACETONAPHTHONE AND SEPARATION OF DERIVATIVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1]

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Abstract

An efficient method was developed for simultaneous determination of gibberellic, indolyl-3-acetic and abscisic acids. Plant growth regulators were derivatized with α-bromo-2′-acetonaphthone in the presence of crown ether. Derivatization was designed to follow the common extraction step of plant growth regulators with organic solvents at low pH. Derivatives can be readily separated by reversed-phase high performance liquid chromatography with low-end detection limits at the 10 pmole levels.

Key words: Plant growth regulators; GA_3; IAA; ABA; derivatization; α-bromo-2′-acetonaphthone; HPLC.

Introduction

Plant growth regulators are major factors involved in the dynamic process of plant development (MacMillan, 1980). Determination of plant growth regulators during plant growth and morphogenesis of tissue cultures have received great attention (Hiraga *et al.*, 1974; Koshioka *et al.*, 1983).

Gas chromatography-mass spectrometry (GC-MS), the most powerful tool for identification of plant growth regulators, is not convenient for the simultaneous determination of multiple components on a routine basis. With the advent of high performance liquid chromatography (HPLC), methods for determination of plant growth regulators are developed rapidly (Crozier and Reeve, 1977). In general,

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liquid chromatographic analysis of gibberellins (GAs) is more difficult in comparison with indolyl-3-acetic acid (IAA) and abscisic acid (ABA). GAs lack characteristic chromophore with proper wavelength for on-line detection (Barendse *et al.*, 1980; Lin and Heftmann, 1981). As a result, simultaneous determination of GAs, IAA and ABA in higher plants by HPLC was not well established although isolation conditions of these growth regulators are so similar that extraction can be performed in one preparation (Knegt *et al.*, 1981; Durley *et al.*, 1982; Saunders, 1978; Takahashi *et al.*, 1975).

We like to report an efficient method for simultaneous derivatization of gibberellic acid (GA_3), IAA and ABA through their common functionalities, namely the carboxyl groups. Derivatization was carried out with α-bromo-2′-acetonaphthone (ANT-Br) (Distler, 1980) in the presence of crown ether. Naphthacyl derivatives of GA_3, IAA and ABA have strong UV absorption and are highly fluorescent for HPLC detection. Derivatives are more hydrophobic and manageable in reversed-phase liquid chromatography than underivatized plant growth regulators. We also like to show that derivatives are readily separable in simple isocratic elution. Monitoring by UV at 247 nm, low-end detection limits are at 10 pmole levels. Derivatization is designated to follow the step of organic solvent extraction of these three classes of plant growth regulators (Fig. 1).

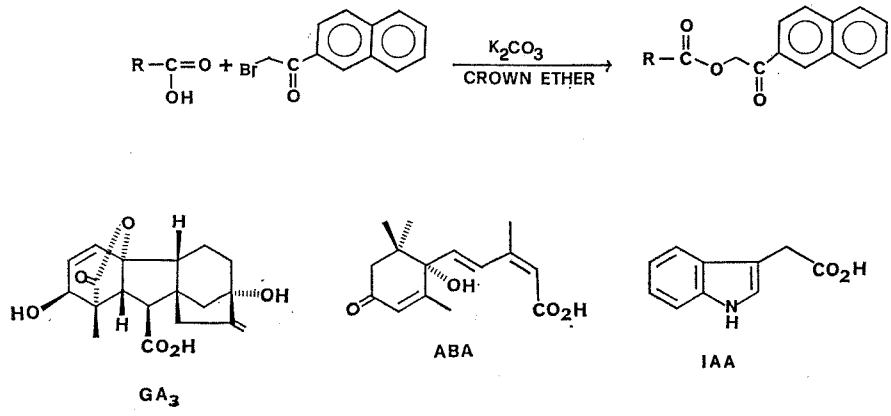

Fig. 1. Derivatization of GA_3, IAA and ABA with α-bromo-2′-acetonaphthone.

Materials and Methods

Chemicals

GA_3 (90% purity calculated), IAA and (±)-cis, trans-abscisic acid (ABA, racemic mixture) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). α-Bromo-2′-acetonaphthone and 18-crown-6 were obtained from Aldrich Chem. Co. (Milwaukee,

WI, USA). Pre-coated thin layer chromatography (TLC) plates (Silica gel, 20×20 cm, 250 μ) and preparative layer chromatography (PLC) plates (Silica gel, 20×20 cm, 1,000 μ) are poducts of Analtech Inc. (Newark, DE, USA).

High Performance Liquid Chromatography

HPLC was performed on a Waters model 6,000A high pressure pump equipped with a model 450 variable wavelength UV detector (Waters Assoc., Milford, MA, USA). Samples, cleaned by Waters Sep-Pak C-18 catridges, were introduced into a 25-cm C-18 column (Yamamura Co., Japan) through a U6K injector.

Spectroscopic Characterization of Hormonal Derivatives

Naphthacy derivatives of GA_3, IAA and ABA were confirmed by NMR, UV and IR spectroscopic methods.

Preparation of Derivatives

Each plant growth regulator was dissolved in fresh THF to a final concentration of 0.01 M. After putting K_2CO_3 (anhydrous powder, 2 eq. excess), α-bromo-2′-acetonaphthone (1.5 mol. eq.) and 18-crown-6 (0.2 mol. eq.) were also added. In the preparation of GA_3 derivative, 18-crown-6 was brought up to one molar equivalent to facilitate hindered acylation. Reaction was carried out under N_2 in the darkness at room temperature. Derivatization is complete within 3 hours for IAA and ABA. GA_3 reacts slower and process usually takes 16 hours. Each crude preparation was worked up by passing through a layer of glasswool in disposable pipette. Precipitates were thoroughly rinsed with EtOAc and washes were combined with filtrate for rotary evaporation in the darkness. Crude products were dissolved in $CHCl_3$ and applied for PLC purification (CH_2Cl_2:Et_2O=1:1 for GA_3 and ABA derivatives; C_6H_6:Hexane:EtOAc=5:5:1 for IAA derivative, v/v). Derivatives were recovered from PLC plates by extracting the corresponding bands of silica gel with EtOAc. Further purification through recrystallization ($CHCl_3$/petroleum ether) provided pure derivatives as HPLC standards and for spectroscopic characterization.

Condition for GA_3 derivatization was extended for simultaneous derivatization of three growth regulators. In microscale preparation, fresh solution of α-bromo-2′-acetonaphthone and 18-crown-6 (1:1 mol. ratio) in THF was added in large excess to mini-vials containing growth regulators/K_2CO_3 in THF and the reaction proceeded by sonication.

Results and Discussion

We have demonstrated that GA_3, IAA and ABA can be simultaneously derivatized with α-bromo-2′-acetonaphthone. The yields and TLC behavior of these

derivatives are summarized in Table 1. As examples, UV spectra of GA_3 derivative (designated as GA_3-ANT) and GA_3 free acid in methanol are shown in Fig. 2 to illustrate the contribution of the naphthacy chromophore.

With higher molar ratio of crown ether and derivatization carried out at 25°C,

Table 1. *Derivatization yields of GA_3, IAA and ABA with α-bromo-2'-acetonaphthone and R_f values of derivatives in thin layer chromatography*

Growth regulators	Yields[a] (%)	R_f in TLC (Silica Gel)	
		CH_2Cl_2:Et_2O=1:1	φH:Hexane:EtOAc=5:5:1
GA_3	89	0.4	—
IAA	87	—	0.15
ABA	94	0.6	—

[a]: Means of three experiments.

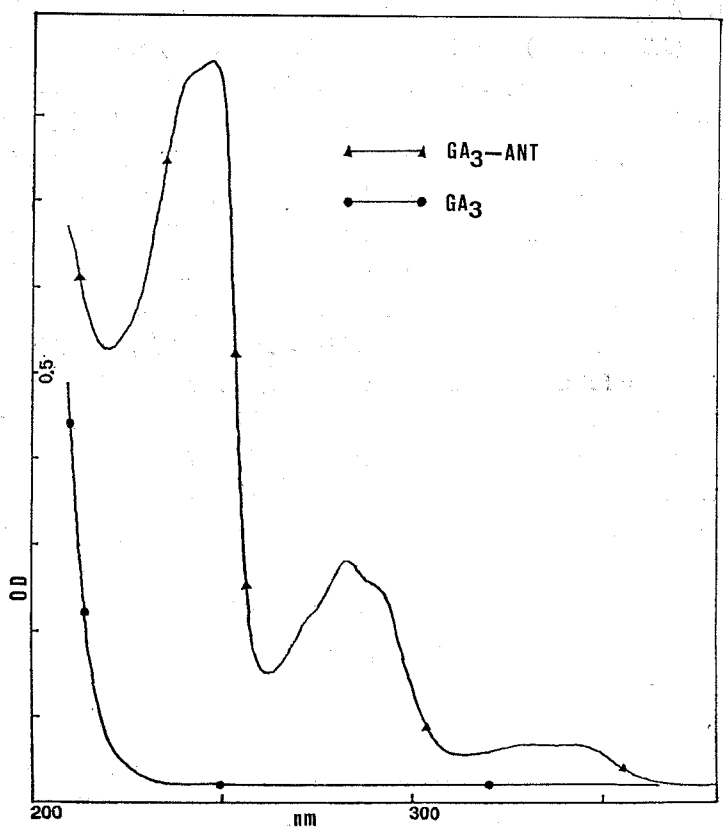

Fig. 2. Ultra-violet spectra of GA_3-ANT and GA_3 free acid in methanol.

decomposition of growth regulators was greatly minimized. NMR spectra of GA_3-ANT showed all characteristic chemical shifts of an intact GA_3 skeleton. No cis-trans isomerization at C-2 of ABA was observed if derivatization was performed in the darkness under inert gas. Although acetonitrile and methanol were commonly used as solvents for derivatizing organic acids with *p*-bromophenacyl bromide (Ahmed *et al.*, 1980), tetrahydrofuran was found superior in our study.

Advantages for derivatization of GA_3, IAA and ABA with *α*-bromo-2′-acetonaphthone are multiple. Naphthacyl derivatives absorb UV strongly at 247 nm in methanol and still can be easily monitored at 254 nm. It provides common source for on-line spectrophotometric detection in HPLC. Determination of GA_3 at 5 ng level is now easily achieved as shown in Fig. 3. As illustrated by chromatograms in Fig. 4, GA_3, IAA and ABA derivatives were readily separated by reversed-phase liquid chromatography. Base-line separation was obtained in less than 12 minutes

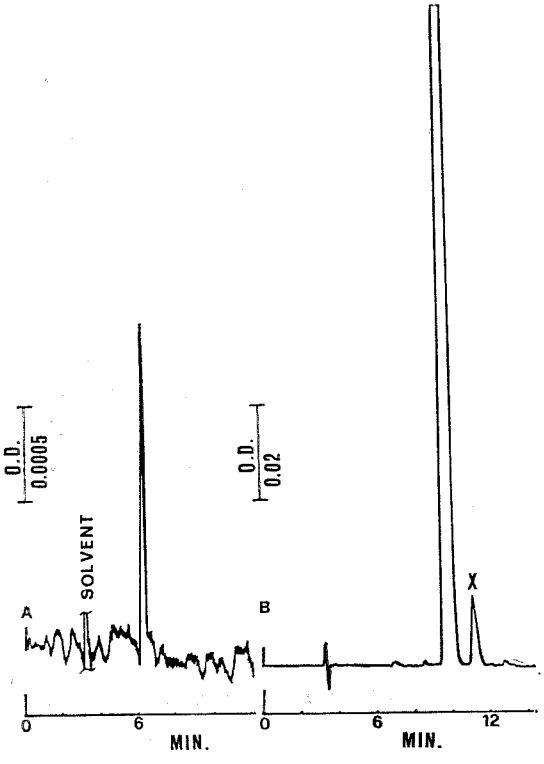

Fig. 3. Liquid chromatograms of GA_3-ANT. (A) 5 ng; (B) 4 μg; Peak X is impurity.
LC conditions: Column, RP C-18, 25 cm; Flow Rate, 0.8 ml/min; Detection, UV at 247 nm; Chart Speed, 0.5 cm/min; Eluting Solvents, (A) MeOH: H_2O = 80: 20, v/v, (B) MeOH: H_2O = 70: 30, v/v.

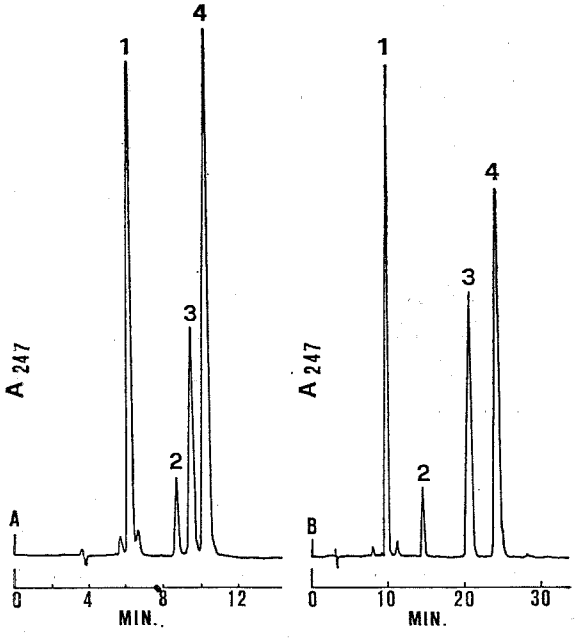

Fig. 4. High performance liquid chromatographic profiles of derivatives of GA_3, IAA and ABA. Chromatographic coditions are identical with thosein Fig. 3 except chart speed.
Peak identification: (1) GA_3-ANT, (2) ANT-Br, (3) IAA-ANT, (4) ABA-ANT.

Table 2. *Correlation between solvent compositions and retention times in reversed-phase high performance liquid chromatography of naphthacyl derivatives of GA_3, IAA and ABA*

Values in the parentheses indicate relative retention time of derivatives to α-bromo-2'-acetonaphthone (ANT-Br).

Solvents ($MeOH:H_2O$, v/v)	Retention time, min.			
	GA_3	IAA	ABA	ANT-Br
80:20	6.1 (0.70)	9.4 (1.08)	10.2 (1.17)	8.7 (1.0)
75:25	7.4 (0.68)	13.0 (1.20)	14.8 (1.37)	10.8 (1.0)
70:30	10.1 (0.68)	20.6 (1.39)	24.2 (1.64)	14.8 (1.0)

with isocratic elution. Relation between retention time and solvent composition is also shown in Table 2.

We intend to establish methodology using derivatization and microbore HPLC for simultaneous determination of multiple plant growth regulators. Detailed study is now underway.

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Literature Cited

Ahmed, M.S., R.H. Dobberstein, and N.R. Fransworth. 1980. *Stevia rebaudiana*. 1. Use of *p*-bromophenacyl bromide to enhance ultraviolet detection of water-soluble organic acid (steviobioside and rebaudioside B) in high-performance liquid chromatographic analysis. J. Chromatogr. **192**: 387-393.

Barendse, G.W.M., P.H. Van de Werken, and N. Takahashi. 1980. High-performance liquid chromatography of gibberellins. J. Chromatogr. **198**: 449-455.

Crozier, A. and D.R. Reeve. 1977. The application of high performance liquid chromatography to the analysis of plant hormones. *In* P.E. Pilet (ed.), Plant Growth Regulation. Proc. 9th. Int. Conf. Plant Growth Substances. Springer-Verlag, Brelin, Heidelberg, New York, pp. 67-76.

Distler, W. 1980. Modified preparation of 2′-naphthacyl fatty acid esters and their fluorescence detection by high-performance liquid chromatography. J. Chromatogr. **192**: 240-246.

Durley, R. C., T. Kannangara, and G. M. Simpson. 1982. Leaf analysis for abscisic, phaseic and 3-indolylacetic acids by high-performance liquid chromatography. J. Chromatogr. **236**: 181-188.

Hiraga, K., S. Kawabe, T. Yokota, N. Murofushi, and N. Takahashi. 1974. Isolation and characterization of plant growth substances in immature seeds and etiolated seedlings of ***Phaseolus vulgaris***. Agric. Biol. Chem. **38**: 2521-2527.

Knegt E., E. Vermeer, and J. Bruinsma. 1981. The combined determination of indolyl-3-acetic and abscisic acids in plant materials. Anal. Biochem. **114**: 362-366.

Koshioka, M., A. Jones, M. N. Koshioka, and P. Pharis. 1983. Metabolism of [^{3}H]-gibberellin A_4 in somatic suspension cell cultures of carrot. Phytochem. **22**: 1585-1590.

Lin, J.-T. and E. Heftmann. 1981. Adsorption and reversed-phase partition high-performance liquid chromatography of gibberellins. J. Chromatogr. **213**: 507-510.

MacMillan, J. 1980. Hormonal Regulation of Development I. Molecular Aspects of Plant Hormones. Springer-Verlag, Brelin, Heidelberg, New York, 681 pp.

Saunders, P. F. 1978. The identification and quantitative analysis of abscisic acid in plant exextracts. ***In*** J. R. Hillman (ed.), Isolation of Plant Growth Substances. Cambridge University Press, London, pp. 115-134.

Takahashi, N., I. Yamaguchi, T. Kono, M. Igoshi, K. Hirose, and K. Suzuki. 1975. Characterization of plant growth substances in *Citrus unshiu* and their change in fruit development. Plant Cell Physiol. **16**: 1101-1111.

使用溴乙基萘酮製備徒長素、吲哚乙酸及離層素之衍生物，並配合高效液相層析法作一併檢測

蕭 明 熙　郝 蕓 蕓

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徒長素、吲哚乙酸、離層素三者可與溴乙基萘酮反應，在冠醚的催化作用下製成衍生物，三種衍生物具共同强紫外光吸收，可配合高效液相層析法作一併檢測。衍生物均比原植物生長調節素更具疏水性，使用簡易逆相層析條件，即可完全分離，檢測下限爲 10 pmole。