Phytochemistry, 1979, Vol. 18, pp. 1699–1702. Pergamon Press Ltd. Printed in England.

HIGH PRESSURE LIQUID CHROMATOGRAPHY OF CONJUGATED GIBBERELLINS

ISOMARO YAMAGUCHI, TAKAO YOKOTA, SHIGEO YOSHIDA and NOBUTAKA TAKAHASHI
Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

(*Received* 2 *November* 1978)

Key Word Index—High pressure liquid chromatography; *Quamoclit pennata*; Convolvulaceae; identification of gibberellin A_3 glucoside and gibberellenic acid glucoside.

Abstract—The application of high pressure liquid chromatography to the purification and identification of conjugated gibberellins was examined. Two kinds of reversed phase columns, octadesylsilanized and dimethylsilanized silica gel, were useful for the isolation and identification of gibberellin A_3 glucoside and gibberellenic acid glucoside from immature seeds of *Quamoclit pennata*.

INTRODUCTION

The progress in the isolation techniques and analyses of gibberellins has made it possible to characterize as many as 52 free gibberellins [1]. The data on paper, thin layer, column and gas–liquid chromatographic behaviour of free gibberellins are well documented and these methods are used for the purification and identification of gibberellins in plant extracts and in *Gibberella fujikuroi*. Moreover, the combination of a purification technique and an identification method, such as GC–MS including mass fragmentography, provides a powerful tool for the identification of extremely small amounts of naturally occurring free gibberellins.

Twelve conjugated gibberellins have been isolated [2–9] and they are found mainly in seedlings, immature and/or mature seeds thus indicating that they play important roles in seed maturation and germination [10, 11]. The purification and identification methods for conjugated gibberellins, however, have not been developed very well compared to those for free gibberellins.

This paper describes the application of high pressure liquid chromatography (HPLC) to the purification and identification of conjugated gibberellins. Conjugated gibberellins in immature seeds of *Quamoclit pennata* were efficiently purified and identified by this method.

RESULTS AND DISCUSSION

Two kinds of reversed phase partition column were used and inorganic buffers and their mixtures with methanol, which can be monitored at 200–210 nm end absorption, were chosen as solvents because conjugated gibberellins do not show any characteristic UV absorption.

The results are summarized in the tables. Table 1 gives the results obtained from the column packed with Wakogel ODS. Experiment A was performed with 10 mM NH_4Cl of pH 3.2 and its mixture with methanol*. With the pure buffer, samples were hardly eluted. Only gibberellin A_8 glucoside (A_8-2-G) and A_{29}-2-G were eluted at 17.5 and at 16.8 min respectively at the flow rate of 90 ml/hr. With the mixture containing 10–15% methanol in the buffer, gibberellin glucosides were well separated though A_{35}-11-G was eluted too slowly. An increase in the methanol content to 30% greatly shortened the retention time (R_t), but the separation was insufficient. In this condition, however, polar glucosyl esters such as gibberellin A_1 glucosyl ester (A_1GE), A_3GE and A_{38}GE began to be eluted, while less polar ones such as A_4GE and A_{37}GE were still retained ($R_t > 25$ min). In conditions VI and VII, A_4GE and A_{37}GE, which possess the same R_f values on TLC in several solvent systems, were eluted separately, while others came out at the solvent front.

Experiment B was performed with various concentrations of methanol in 10 mM NH_4Cl of pH 6.0 after repacking the column. In condition VIII, all the glucosyl ethers were sufficiently separated. Glucosyl esters, except for A_3GE and A_{38}GE, were well separated in condition X.

Experiment C was carried out to clarify the effect of pH on the retention time. The R_t of glucosyl ethers was greatly influenced. The lower the pH, the longer the glucosyl ethers were retained. It is noteworthy that the retention times of A_3-3-G and A_{35}-11-G were particularly influenced by pH and the order of elution was reversed; in condition XIII, the R_t of A_3-3-G was longer than that of A_{35}-11-G, while it was smaller in condition XIV. As expected, the R_t of glucosyl esters was hardly influenced by pH. Though glucosyl esters were retained a little longer at lower pH, this seemed to be due to the slight difference in methanol content which could be caused in the degassing process.

Table 2 contains the results obtained from the column packed with silanized Merksorb SI 60. Experiment D was performed with 10 mM NH_4Cl of pH 3.2 containing various concentrations of methanol and in experiment E the pH of the buffer was raised to 5.5. In condition XV, though A_8-2-G and A_{29}-2-G showed the same R_t, other glucosyl ethers were clearly separated. In condition

* This solvent system is convenient for recovery of samples because it is easily evaporated. However, it is corrosive and lines must be flushed with water immediately after use.

Table 1. Retention time (min) on Wakogel LC, ODS-10H

Experiment condition	A I	II	III	IV	V	VI	VII	B VIII	IX	X	XI	XII	C XIII	XIV
A_1-3-G	—	19.0	—	4.7	—	—	—	—	—	—	—	—	—	—
A_1-13-G	—	12.4	—	3.4	—	—	—	—	—	—	—	—	—	—
A_3-3-G	—	16.8	13.3	4.1	2.7	—	—	19.0	3.3	—	—	—	2.0	4.9
A_8-2-G	17.5	4.5	4.0	1.7	1.3	—	—	4.2	1.2	—	—	—	1.0	2.0
A_{26}-2-G	—	—	10.5	—	2.4	—	—	6.0	1.5	—	—	—	1.3	4.7
A_{29}-2-G	16.8	4.2	3.6	1.6	1.3	—	—	3.3	1.1	—	—	—	1.0	2.0
A_{35}-11-G	—	27.0	21.2	5.8	3.9	1.0	2.0	10.5	2.2	—	—	1.9	1.9	7.9
Gibb.G	—	9.5	7.6	—	—	—	—	4.9	1.4	—	—	—	1.0	2.5
A_1GE	—	—	—	—	3.9	1.0	2.0	—	15.2	2.8	1.0	1.9	7.3	7.5
A_3GE	—	—	—	—	3.4	1.0	1.9	—	13.0	2.3	0.8	1.8	6.3	6.5
A_4GE	—	—	—	—	>25.0	2.2	4.5	—	>25.0	18.0	2.2	4.6	>25.0	>25.0
A_{37}GE	—	—	—	—	>25.0	1.8	3.2	—	>25.0	12.2	1.9	3.2	>25.0	>25.0
A_{38}GE	—	—	—	—	3.3	1.0	1.9	—	13.0	2.3	0.8	1.8	6.0	6.1
A_1	—	—	—	—	—	—	—	—	—	—	—	—	2.2	8.9
A_3	—	—	—	—	—	—	—	—	—	—	—	—	2.2	7.8
A_4	—	—	—	—	—	—	—	—	—	—	—	—	>25.0	—
A_5	—	—	—	—	—	—	—	—	—	—	—	—	4.4	—
A_8	—	—	—	—	—	—	—	—	—	—	—	—	1.0	2.0
A_{27}	—	—	—	—	—	—	—	—	—	—	—	—	6.0	—
A_{36}	—	—	—	—	—	—	—	—	—	—	—	—	5.5	—

I: 10 mM NH_4Cl (pH 3.2), 90 ml/hr; II: 10% MeOH in 10 mM NH_4Cl (pH 3.2), 60 ml/hr; III: 15% MeOH in 10 mM NH_4Cl (pH 3.2), 60 ml/hr; IV: 20% MeOH in 10 mM NH_4Cl (pH 3.2), 60 ml/hr; V: 30% MeOH in 10 mM NH_4Cl (pH 3.2), 60 ml/hr; VI: 50% MeOH in 10 mM NH_4Cl (pH 3.2), 60 ml/hr; VII: 50% MeOH in 10 mM NH_4Cl (pH 3.2), 30 ml/hr; VIII: 5% MeOH in 10 mM NH_4Cl (pH 6.0), 60 ml/hr; IX: 15% MeOH in 10 mM NH_4Cl (pH 6.0), 60 ml/hr; X: 30% MeOH in 10 mM NH_4Cl (pH 6.0), 60 ml/hr; XI: 50% MeOH in 10 mM NH_4Cl (pH 6.0), 60 ml/hr; XII: 50% MeOH in 10 mM NH_4Cl (pH 6.0), 30 ml/hr; XIII: 20% MeOH in 10 mM NH_4Cl (pH 5.6), 60 ml/hr; XIV: 20% MeOH in 10 mM NH_4Cl (pH 3.2), 60 ml/hr.

Table 2. Retention time (min) on silanized Merksorb SI 60

Experiment condition	D XV	XVI	XVII	XVIII	E XIX	XX
A_1-3-G	—	—	—	—	—	—
A_1-13-G	—	—	—	—	—	—
A_3-3-G	5.0	2.6	2.1	4.2	—	—
A_8-2-G	2.6	2.0	—	1.8	1.6	1.6
A_{26}-2-G	4.7	2.5	—	2.0	—	—
A_{29}-2-G	2.6	2.0	2.0	1.6	—	—
A_{35}-11-G	5.6	2.9	—	2.4	1.6	1.6
Gibb.G	1.7	—	—	1.8	—	—
A_1GE	—	—	2.0	—	3.2	3.0
A_3GE	—	—	2.0	—	3.2	3.0
A_4GE	—	—	2.8	—	16.5	8.0
A_{37}GE	—	—	2.6	—	10.0	6.2
A_{38}GE	—	—	2.0	—	3.2	3.0
A_1	—	3.2	2.2	—	—	1.8
A_3	—	2.8	2.2	—	—	1.8
A_5	—	5.2	2.7	—	—	2.2
A_8	—	2.4	2.2	—	—	1.8
A_{27}	—	5.4	2.7	—	—	—
A_{36}	—	7.2	2.9			

XV: 10% MeOH in 10 mM NH_4Cl (pH 3.2), 40 ml/hr; XVI: 30% MeOH in 10 mM NH_4Cl (pH 3.2), 40 ml/hr; XVII: 50% MeOH in 10 mM NH_4Cl (pH 3.2), 40 ml/hr; XVIII: 10 mM NH_4Cl (pH 5.5), 40 ml/hr; XIX: 20% MeOH in 10 mM NH_4Cl (pH 5.5), 40 ml/hr; XX: 30% MeOH in 10 mM NH_4Cl (pH 5.5), 40 ml/hr.

XVIII, A_8-2-G and gibberellenic acid glucosyl ether (Gibb-G) showed the same R_t, but others were well separated. In experiments D and E, A_1GE, A_3GE and A_{38}GE could not be separated at all, while A_4GE and A_{37}GE were well separated in experiment E.

The minimum detectable amount was checked with A_{35}-11-G in condition IV, monitored at 200 nm. A clear peak was observed with 200 ng but a distorted peak was obtained with 20 ng. There is no doubt that the minimum detectable amount depends on the monitoring wavelength and R_t; with longer R_t more sample is required because a broad peak is obtained. However, HPLC can be a useful tool for the semi-quantitative analysis of conjugated gibberellins.

To test the potential use of this technique, we applied it to the final step of the isolation and identification of gibberellin glucosyl ethers in immature seeds of *Quamoclit pennata* (Bojer). The fraction, partially purified by the conventional techniques as described in the Experimental, was submitted to HPLC analysis by condition III monitored at 210 nm. As shown in Fig. 1, four peaks were observed; peaks B and D showed the same R_ts as those of Gibb-G and A_3-3-G, respectively. The effluent of each peak was concentrated separately at pH 7.0 and submitted to the second column with system XV. On the second column, the concentrate of peak B gave a single peak with the same R_t as that of Gibb-G. Peak D gave a single peak with the same R_t as that of A_3-3-G. Thus, the glucosyl ethers present in immature seeds of *Quamoclit pennata* were identified as A_3-3-G and Gibb-G but the latter is expected to be an artifact of the former. This

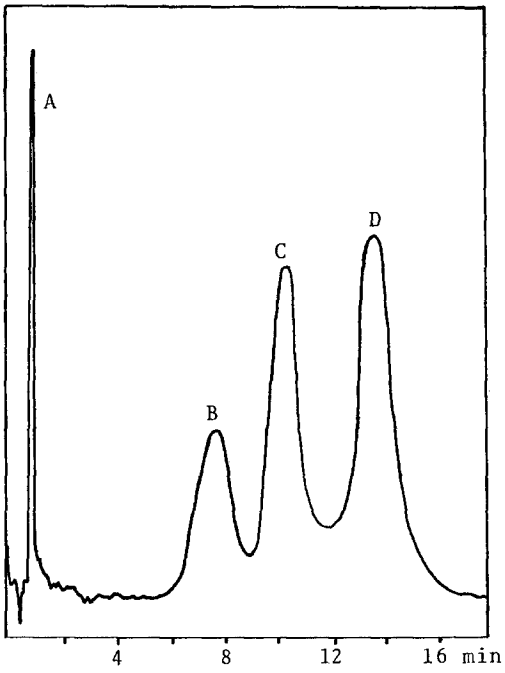

Fig. 1. HPLC of conjugated gibberellins from *Quamoclit pennata*. Wakogel LC, ODS. 15% MeOH in 10 mM NH_4Cl (pH 3.2), 60 ml/hr. A and C: unidentified (non-gibberellin-like); B: Gibb-G; D; A_3-3-G.

conclusion was further confirmed by MS of the methyl ester trimethylsilyl ethers of the concentrates of the HPLC column effluents [12], while MS analyses of the concentrates of peaks A and C showed these were not conjugated gibberellins.

This experiment proves the effective applicability of HPLC to the isolation and identification of naturally occurring conjugated gibberellins. Further simplification of pre-purification steps required prior to HPLC application may increase the utility of HPLC in this field.

EXPERIMENTAL

Samples. Most of the conjugated gibberellins used in this experiment were isolated or prepared in our laboratory [2, 5–7]; 3-*O*-*β*-glucosyl gibberellin A_1 (A_1-3-G) and 13-*O*-*β*-glucosyl gibberellin A_1 (A_1-13-G) were gifts from Professor Sembdner [13].

Instrument and column packing. A Varian Aerograph Model 8500 equipped with a programmer for gradient elution mode and connected to a detector unit, Uvi Dec-100 of JASCO, was used. Stainless steel columns (250 × 2 mm) purchased from NEVA were packed as follows. Wakogel LC, ODS-10H for HPLC (particle size 10 μm) and silanized Merksorb SI 60 for liquid chromatography under pressure (medium particle size, 5 μm) were suspended in $CHCl_3$ and packed into columns with the help of a packer purchased from NEVA. Prior to use, the columns were washed with 20 ml $CHCl_3$, 100 ml MeOH and 250 ml H_2O, successively.

Preparation of solvents and setting of experimental conditions. Buffer solns were prepared by adjusting the pH of 10 mM NH_4Cl with conc HCl or conc NH_4OH. For the gradient elution, solvent reservoir A was charged with pure buffer and reservoir B with 50% MeOH in the buffer, both of which were degassed by a water aspirator for 10–15 min. To find suitable concns of MeOH, programmed gradient elution modes were used and after finding appropriate MeOH concns, samples were developed isocratically; firstly, in a gradient method with the solvent system MeOH–aq. buffer, some ghost peaks appear to confuse peak identification; secondly, an isoclatic method is convenient to run samples in rapid repetition and gives high reproducibility compared to a gradient method, since with the latter method the column has to be reequibilated prior to each run.

All experiments were performed at room temp. However, solvents were warmed by the heat of the instrument and this influenced the R_f. To prevent this problem, the instrument was switched on for 3–4 hr prior to the experiments to be warmed up to a constant temp.

Isolation of conjugated gibberellins from immature seeds of Quamoclit pennata. (1) *Extraction and fractionation.* The immature seeds (1.7 kg) were homogenized in MeOH using a blender and extracted 3 × with MeOH. The extracts were combined and condensed *in vacuo* to give an aq. residue, which was first extracted 3 × at pH 7 with C_6H_6 to give a soluble neutral (NBz) fraction (7.13 g) and then with EtOAc to give a second soluble neutral (NE) fraction (2.07 g). The aq. residue was adjusted to pH 3 and extracted with EtOAc and *n*-BuOH successively, to give an EtOAc soluble acidic (AE) fraction (6.54 g) and a *n*-BuOH soluble (NAB) fraction. The AE and NAB fractions showed high gibberellin activity on rice seedlings.

(2) *Charcoal chromatography.* The NAB fraction, which was expected to contain conjugated gibberellins, was submitted to a charcoal column (30 g) eluted with Me_2CO–H_2O mixture increasing the Me_2CO concn by 10% steps in 500 ml fractions. The fractions eluted with 40 (500 mg), 50 (240 mg) and 60% (370 mg) Me_2CO in H_2O showed gibberellin activity. Charcoal chromatography was repeated on the fractions of 40 and 50% Me_2CO by using a charcoal column (10 g) eluted with Me_2CO–H_2O mixtures increasing the Me_2CO concn by 10% steps in 300 ml fractions. The fractions of 50 (45 mg) and 60% (8 mg) Me_2CO of the second column showed the strongest activity. Thus, the fractions of 60% Me_2CO of the first column and 50 and 60% Me_2CO of the second column were combined to give a gum (423 mg).

(3) *Partition chromatography and PLC.* The gum (423 mg) obtained in (2) was submitted to a partition column of Sephadex G-50 (6 g) impregnated with M phosphate buffer of pH 5.5 and eluted with EtOAc–*n*-BuOH (9:1) in 50 ml fractions. Each fraction was checked by TLC on Si gel in $CHCl_3$–MeOH–HOAc (50:20:3). Fractions 9–11 showed fluorescence characteristic of gibberellins at 3650 Å after spraying with 70% H_2SO_4 and heating. Fractions 9–11 were combined (110 mg) and submitted to PLC on Si gel developed in the same solvent system mentioned above. The zone from R_f 0.2 to 0.4 was scraped off and extracted with MeOH. The MeOH extract was concd to give a gum (24 mg) and submitted to a partition column of Sephadex LH-20 (2 × 40 cm) eluted with *n*-BuOH–H_2O (6:100) in 2 ml fractions. Fractions 14–22 showed the characteristic fluorescence mentioned above and were combined (1.2 mg) to be submitted to HPLC analysis.

REFERENCES

1. Takahashi, N. (1978) *Seikagaku* **50**, 184.
2. Yokota, T., Murofushi, N., Takahashi, N. and Tamura, S. (1973) *Agric. Biol. Chem.* **35**, 583.
3. Schreiber, K., Weiland, J. and Sembdner, G. (1970) *Phytochemistry* **9**, 189.
4. Harada, H. and Yokota, T. (1970) *Planta (Berlin)* **92**, 100.

5. Yamane, H., Yamaguchi, I., Murofushi, N. and Takahashi, N. (1974) *Agric. Biol. Chem.* **38**, 649.
6. Hiraga, K., Yokota, T., Murofushi, N. and Takahashi, N. (1974 *Agric. Biol. Chem.* **38**, 2511.
7. Hiraga, K., Kawabe, S., Yokota, T., Murofushi, N. and Takahashi, N. (1974) *Agric. Biol. Chem.* **38**, 2521.
8. Lorenzi, R., Horgan, R. and Heald, J. K. (1976) *Phytochemistry* **15**, 789.
9. Yokota, T., Kobayashi, S., Yamane, H. and Takahashi, N. (1978) *Agric. Biol. Chem.* **42**, 1811.
10. Yamane, H., Murofushi, N. and Takahashi, N. (1975) *Phytochemistry* **14**, 1195.
11. Yamane, H., Murofushi, N., Osada, H. and Takahashi, N. (1977) *Phytochemistry* **16**, 831.
12. Yokota, T., Hiraga, K., Yamane, H. and Takahashi, N. (1975) *Phytochemistry* **14**, 1569.
13. Sembdner, G., Borgmann, E., Schneider, G., Liebisch, H. W., Miersch, O., Adam, G., Lischewski, M. and Schreiber, K. (1976) *Planta (Berlin)* **132**, 249.