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Fractionation of Gibberellins in Plant Extracts by Reverse Phase High Performance Liquid Chromatography[1]

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M. GEORGE JONES, JAMES D. METZGER, AND JAN A. D. ZEEVAART[2]
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

In studies on endogenous plant gibberellins (GAs), reverse phase (Bondapak C_{18}) high performance liquid chromatography (HPLC) has proved to be a useful method for the fractionation of plant extracts. The behavior of 18 authentic GAs in such a chromatographic system is described. The main factors determining chromatographic behavior are the degree and the position of hydroxylation of the GA. Generally, dihydroxylated GAs elute before monohydroxylated GAs, whereas 13-hydroxylated GAs elute before 3-hydroxylated GAs. The number of carboxyl groups and the degree of saturation of the A-ring have little effect. For 20-carbon GAs, the oxidation state at C-20 is only relevant insofar as GAs having a methyl group at this position elute later than those with other groups (lactone, aldehyde, or carboxyl).

As an illustration of the use of reverse phase HPLC, the endogenous GAs of immature seeds of *Pharbitis nil* L., strain "Violet," were reinvestigated. The presence of gibberellins A_3, A_5, A_{17}, A_{20}, and A_{29} was confirmed by gas-liquid chromatography-mass spectrometry. In addition, two other GAs, A_{19} and A_{44}, were also identified in extracts of this material.

The levels of endogenous GAs[3] in vegetative tissues are generally very low (1–10 μg/kg fresh weight). Consequently, plant extracts must be extensively purified before reliable analysis by bioassay or physicochemical means can be undertaken. Traditional methods of column chromatography and TLC are time-consuming and greatly limit the number of extracts which can be processed in any given period. In recent years, the speed and efficiency of HPLC in the purification of plant extracts and its utility in the study of endogenous plant growth substances have begun to be recognized by plant physiologists (2, 3, 12, 15). The two methods (8, 14) which have been published so far on HPLC of GAs appear to be of little practical use for the routine purification of crude plant extracts (see under "Discussion"). We report here on the use of a reverse phase HPLC system for this purpose, and describe the behavior of 18 authentic GAs in this system. To illustrate the effectiveness of this chromatographic system, we have reexamined the endogenous GAs of immature seeds of *Pharbitis nil.*

MATERIALS AND METHODS

HPLC. The preparative HPLC system consisted of a series of four stainless steel columns each 60 × 0.65 cm i.d., packed with Bondapak C_{18}/Porasil B (Waters Associates). Solvents used were 1% (v/v) aqueous acetic acid (solvent A) and redistilled 95% ethanol (solvent B). Samples were dissolved in 70% A-30% B, filtered through 0.45-μm type HA Millipore filters and loaded onto the column via a Waters U6K universal injector fitted with a 5-ml loop. A linear gradient of B (30–100% in 25 min) in A was delivered by two Waters 6000A pumps, controlled by a Waters 660 solvent programmer. The solvent flow rate was 9.9 ml min^{-1}, and the gradient was started 30 s after injection. Fractions were collected every min from the time of injection and dried in a fume hood.

For analytical separations, a column 30 × 0.4 cm i.d. packed with μBondapak C_{18} (Waters Associates) was used. Elution was by means of linear gradients of methanol in 1% aqueous acetic acid, with a flow rate of 2 ml min^{-1}. The gradient was started 2.5 min after injection and fractions were collected every min from the time of injection.

Bioassays. Gibberellins A_1, A_3, A_4, A_5, A_7, A_9, and A_{20} in the eluted fractions were determined using a modified lettuce hypocotyl assay (6), in which the beakers used to collect fractions from the HPLC were also used to grow the lettuce seedlings. Gibberellins A_{12}, A_{18}, A_{23}, A_{36}, and A_{37} were assayed with the d-5 corn assay (18).

GLC. The positions of gibberellins A_8, A_{13}, A_{14}, A_{17}, A_{25}, and A_{29} in the HPLC eluate were determined by GLC. Fractions from the HPLC were methylated with ethereal diazomethane and chromatographed on a glass column 183 × 0.3 cm i.d., packed with 4% SE-33 as described previously (19). Column temperature was 220 C (240 C for Me-GA_8) and detection was by flame ionization.

Seeds of *P. nil.* Seeds were harvested from plants of *P. nil* L., strain "Violet," 20–22 days after anthesis, frozen in liquid N_2, and lyophilized. Seeds (16 g dry weight) were extracted in 100 ml 80% (v/v) methanol. The residue was reextracted overnight with 100 ml methanol and the combined methanolic extracts taken to aqueous under reduced pressure at 35 C. The aqueous fraction was adjusted to pH 3 and loaded onto a column of 1 g charcoal and 2 g Celite. A volume of 250 ml 80% acetone was passed through the column. The eluate was reduced to aqueous, adjusted to pH 2.5, and partitioned five times against half-volumes of ethyl acetate. The resultant acidic fraction was subjected to HPLC on the columns described above. Appropriate fractions were methylated with ethereal diazomethane. The TMS ethers of the methyl esters were prepared by adding 50 μl of a solution containing pyridine-hexamethyldisilazane-trimethylchlorosilazane (9:3:1, v/v) to methylated samples dried in Reacti-vials.

GC-MS. Derivatized samples were chromatographed on a Hewlett Packard 5840A gas chromatograph, equipped with a glass column packed with 2% SP-2100 on 100/120 Supelcoport. The helium flow rate was 20 ml min^{-1} and the column temperature was programmed from 170 to 280 C at 10 C min^{-1}. The GC was connected to a Hewlett Packard 5985 mass spectrometer by a jet separator and mass spectra were collected every 4.5 s. The ionizing potential was 70 ev.

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[2] To whom correspondence should be addressed.

[3] Abbreviations: GA(s): gibberellin(s); HPLC: high performance liquid chromatography; Me-GA: methyl ester of gibberellin; TMS: trimethylsilyl.

RESULTS

Elution of Authentic GAs. The fractions in which authentic GAs eluted from the preparative HPLC system are shown in Table I. All of the GAs were found to elute over at least two, and usually three, fractions. Since the column used is reverse phase, the most polar compounds eluted first and the least polar ones last. The main factors determining the elution of any particular GA appear to be the degree and the position of hydroxylation (Fig. 1). With respect to degree of hydroxylation, two elution series can be seen in the data presented here: $A_8 < A_1 < A_{20} < A_9$ and $A_{18} < A_{14} < A_{12}$. GAs hydroxylated at the C-13 position generally eluted before 3-hydroxylated GAs (*e.g.* $A_{44} < A_{37}$, $A_{19} < A_{36}$, $A_{20} < A_4$). The one exception is in the case of A_{13} and A_{17}, which co-chromatographed in this system. Hydroxylation in the C-2 position increased the speed of elution to a greater extent than hydroxylation at C-3 (compare A_{29} with A_1). Factors which appeared to exert little influence on the chromatographic behavior included the number of carboxyl groups and the degree of saturation in the A-ring. The oxidation state at the C-20 position of the 20-carbon GAs affected their behavior only to the extent that GAs possessing a methyl group at this position eluted later than those with other groups (lactone, aldehyde, or carboxyl).

Table I. *Elution of GAs from Preparative Reverse Phase HPLC System*

Linear gradient of 95% ethanol (30–100%) in 1% aqueous acetic acid run in 25 min at 9.9 ml min^{-1}. Fractions collected every min. Underlining indicates fraction in which most of GA eluted.

Gibberellin	Fraction No.	Gibberellin	Fraction No.
A_8	10, 11, 12	A_{13}	19, 20
A_{29}	11, 12	A_{17}	19, 20
A_{23}	11, 12, 13	A_{36}	19, 20
A_1	12, 13, 14	A_{37}	20, 21
A_3	12, 13, 14	A_4	21, 22, 23
A_{18}	14, 15	A_7	21, 22, 23
A_5	16, 17, 18	A_{14}	23, 24
A_{20}	16, 17, 18	A_9	23, 24, 25
A_{44}[a, b]	17, 18, 19	A_{25}	23, 24
A_{19}[a]	17, 18, 19	A_{12}	27, 28, 29

[a] The data for A_{44} and A_{19} were obtained with extracts from *Pharbitis* seeds (see under "Results").

[b] ABA eluted in fractions 14, and 15.

A-ring structure		none	3	13	3,13	2,13	2,3,13
20-C GAs	CH_3	A_{12}	A_{14}		A_{18}		
	$O-CH_2$ / CO (lactone)		A_{37}	A_{44}			
	CHO		A_{36}	A_{19}	A_{23}		
	COOH	A_{25}	A_{13}	A_{17}			
19-C GAs	CO (saturated)	A_9	A_4	A_{20}	A_1	A_{29}	A_8
	CO (1,2-unsaturated)		A_7		A_3		
	CO (2,3-unsaturated)			A_5			

FIG. 1. A systematic arrangement, on the basis of structural features, of the GAs used in this study.

As would be expected, the position of elution of one GA relative to the others was the same in both the preparative and the analytical columns (Table II). However, due to the much greater resolving power of the microparticulate packing in the analytical column, the majority of GAs eluted within a single fraction.

While we have found it advantageous from a practical point of view to use the same gradient for all initial fractionation on the preparative column, various gradients can be applied to the analytical column. Table III illustrates the separation of three relatively polar GAs with a gradient different from the one used for preparative purposes. Other separations of different GAs might be obtained with solvents other than methanol (*e.g.* acetonitrile or tetrahydrofuran).

Identification of GAs in *P. nil* Seeds. The acidic extract was subjected to preparative HPLC. One-fiftieth of each fraction was assayed using the d-5 corn test. Two zones of biological activity were observed, in fractions 12–14, and 16–19. Fractions 11–15 were combined and designated as sample A; fractions 16–22 were combined and designated as sample B. Each sample was then chromatographed on the μBondapak C_{18} column, using a gradient of 10–70% methanol in 1% acetic acid for sample A and 30 to 100% methanol in 1% acetic acid for B. In both cases the gradient lasted for 30 min and the flow rate was 2 ml min^{-1}. Fractions were collected every min and one-fiftieth of each fraction was again assayed. In sample A, biological activity was observed in fractions 19 and 20; in B, activity was located in fractions 16–18. Various fractions were then methylated and silylated before being examined further by GC-MS (Table IV).

Although it had been reported (17) that GA_{29}-glucoside occurs in immature seeds of *P. nil,* there are no published data concerning the presence of free GA_{29}. Any GA_{29} eluting from the analytical HPLC of sample A would have been expected to occur in fractions 13–15 (Table III). GA_{29} has very low activity in the d-5 corn test (16), so bioassay of these fractions gave no indication of its presence. However, when the derivatized sample from these combined fractions was examined by GC-MS, a well resolved peak

Table II. *Elution of GAs from Analytical Reverse Phase HPLC System*

Linear gradient of methanol (30–100%) in 1% aqueous acetic acid, run in 30 min at 2 ml min^{-1}. Fractions collected every min. Underlining indicates fraction in which most of GA eluted.

Gibberellin	Fraction No.	Gibberellin	Fraction No.
A_1	10, 11	A_{17}	19, 20
A_{20}[a]	16, 17	A_{37}	20
A_{44}[b]	17	A_7	21
A_{19}[b]	18	A_9	24
A_{13}	18	A_{25}	24
A_{36}	18	A_{12}	28

[a] ABA eluted in fractions 14 and 15.

[b] The data for A_{44} and A_{19} were obtained with extracts from *Pharbitis* seeds (see under "Results").

Table III. *Elution of Polar GAs from Analytical Reverse Phase HPLC System*

Linear gradient of methanol (10–70%) in 1% aqueous acetic acid, run in 30 min at 2 ml min^{-1}. Fractions collected every min. Underlining indicates fraction in which most of GA eluted.

Gibberellin	Fraction No.
A_8	12, 13
A_{29}	13, 14, 15
A_1	19, 20

Table IV. *GC-MS Data Obtained with Samples from P. nil and with Authentic GAs*

Fraction numbers refer to fractions eluted from analytical HPLC system as described in text.

Sample	Time of Scan	Peaks in Mass Spectrum with Relative Abundances in Parentheses						
	min	*m/e values*						
A, fractions 13–15	12.2	506 (M^+, 100)	491 (16)	477 (10)	447 (14)	389 (22)	208 (37)	207 (90)
MeTMS-GA_{29}	12.1	506 (M^+, 100)	491 (12)	477 (5)	447 (9)	389 (15)	208 (12)	207 (41)
A, fractions 19 + 20	12.2	504 (M^+, 33)	489 (4)	445 (9)	208 (100)	207 (80)		
MeTMS-GA_3	12.1	504 (M^+, 34)	489 (4)	445 (6)	208 (100)	207 (45)		
B, fraction 16	10.8	416 (M^+, 100)	401 (23)	375 (51)	357 (25)	208 (53)	207 (87)	
MeTMS-GA_5	10.8	416 (M^+, 47)	401 (11)	375 (100)	357 (10)	208 (23)	207 (34)	
B, fraction 16	10.8	418 (M^+, 100)	403 (19)	359 (23)	208 (9)	207 (29)		
MeTMS-GA_{20}	10.7	418 (M^+, 100)	403 (17)	359 (16)	208 (10)	207 (28)		
B, fraction 17	13.1	432 (M^+, 17)	417 (4)	373 (10)	251 (6)	238 (34)	208 (45)	207 (100)
B, fraction 18	11.6	462 (M^+, 6)	447 (5)	434 (81)	402 (52)	375 (81)	374 (100)	208 (45)
B, fraction 19	11.4	492 (M^+, 8)	460 (11)	432 (11)	401 (9)	373 (18)	208 (100)	207 (82)
MeTMS-GA_{17}	11.4	492 (M^+, 19)	460 (25)	432 (21)	401 (16)	373 (26)	208 (100)	207 (84)

with a retention time of 12.2 min exhibited a mass spectrum virtually identical to that of an authentic MeTMS-GA_{29} sample (Table IV). The presence of free GA_{29} in immature seeds of *P. nil* is thus established.

Biological activity in the eluate from the analytical HPLC of sample A was located in fractions 19 and 20, corresponding to the dihydroxylated GAs A_1 and A_3. GC-MS of these combined fractions indicated the presence of MeTMS-GA_3 in a partially resolved peak with the same retention time as authentic MeTMS-GA_3. Murofushi *et al.* (10) used GLC alone to identify GA_3 in seeds of *P. nil.*

Sample B contained those fractions from the preparative HPLC in which monohydroxylated GAs would be expected to elute (Table I). In order to obtain as much information as possible from this sample, fractions eluting from the analytical column were derivatized and examined individually by GC-MS. Fraction 16 contained a well resolved peak at the same retention time as authentic MeTMS-GA_5 and MeTMS-GA_{20}, GAs previously shown to occur in *P. nil* seeds by Murofushi *et al.* (10). These two GAs, differing only by a double bond in the A-ring, were not resolved by the GLC system used in this study. However, when the GC-MS computer facility was used to compare the elution of m/e 416 (M^+ for MeTMS-GA_5) with that of m/e 418 (M^+ for MeTMS-GA_{20}), two overlapping peaks were resolved with the A_5 peak preceding the A_{20} peak. Full mass spectra obtained from the appropriate scans in these regions confirmed the presence of MeTMS-GA_5 and MeTMS-GA_{20} (Table IV). A similar situation was described for Me-GA_5 and Me-GA_{20} in extracts obtained from *Phaseolus multiflorus* seeds (9).

Although a large part of the biological activity in sample B was located in fraction 16, there was also activity in fractions 17 and 18. When these fractions were examined by GC-MS, however, no A_5 or A_{20} was found. In order to see if any related GAs were present in these fractions, use was again made of the GC-MS computer, to look for m/e 207 and 208, ions characteristic of C-13 hydroxylated MeTMS-GAs. In the GLC trace of fraction 17, a well resolved peak after 13.1 min was found to contain a high proportion of ions with m/e 207. In fraction 18, an incompletely resolved peak after 11.6 min coincided with a peak of the ion, m/e value 208. Although authentic samples were not available for comparison, the mass spectra for these two peaks closely resembled the published mass spectra for MeTMS-GA_{44} (7) and MeTMS-GA_{19} (1), respectively.

Murofushi *et al.* (11) found another 13-hydroxylated GA in *P. nil* seeds, namely GA_{17}. This compound is biologically inactive (4) and so would not have been detected in the d-5 corn assay of the fractions from analytical HPLC of sample B. However, the data in Table II indicated that, if present, A_{17} would occur in fractions 19 and 20. When fraction 19 was examined by GC-MS (Table IV), a single large peak with retention time and mass spectrum identical to that of MeTMS-GA_{17} was found, confirming the presence of GA_{17} in *P. nil.*

DISCUSSION

The results described above on the endogenous GAs of immature seeds of *P. nil* provide a clear illustration of the utility of reverse phase HPLC for the fractionation of plant extracts. Not only was the presence of five GAs confirmed, but two additional GAs, A_{44} and A_{19}, were also identified, even though the quantity of material used in the present study was very much less than that used by earlier workers (16 g dry weight, compared with 60 kg fresh weight in ref. 10). Extracts of seeds generally contain fewer impurities than extracts from vegetative tissues and we have found that silica adsorption and partition (5) chromatography are necessary, in addition to HPLC, for the identification of GAs in extracts from large quantities of vegetative material. However, before extracts from leaves and shoots can be fractionated by HPLC prior to bioassay, all that is required is partial purification by solvent partitioning and charcoal adsorption chromatography. Such a fractionation procedure allows the isolation of several other acidic plant growth substances (*e.g.* ABA, IAA, phaseic acid, dihydrophaseic acid) in addition to the endogenous GAs in an extract. In our experience over the last 2 years, such routine fractionations give very reproducible results and are faster and more efficient than TLC. We have found that ABA provides a useful UV-absorbing marker with which the performance of the HPLC columns can be periodically checked. The use of reverse phase HPLC for fractionation of GAs from vegetative material will be described in detail in subsequent papers.

In comparison with other methods described for HPLC of GAs, the reverse phase system described here has several advantages. The stationary phase is chemically bonded to the packing and so does not suffer from the problems of instability seen with silica partition HPLC columns (14). Silica adsorption HPLC (8, 14) is able to separate isomeric GAs differing only by one double bond, which partition HPLC cannot. However, adsorption columns are not suitable for routine chromatography of crude plant extracts due to the polar nature of the silica, with column deterioration resulting from the adsorption of polar impurities in the sample being chromatographed. Regeneration of such columns, if it is possible at all, is time-consuming. With reverse phase HPLC, it has been our experience that no material is retained on the column once the gradient has been run up to 100% organic solvent. Consequently, elaborate regeneration procedures are unnecessary and the interval between successive injections is only of the order of 40 min. Fractionation of plant extracts with reverse phase HPLC has the additional advantage of a large sample capacity.

When a sample is loaded onto the column in a solution of relatively high polarity, most of the compounds in the sample are retained and concentrated on the head of the column. Thus, samples of 4 to 5 ml can be injected with no effect on subsequent chromatographic separation.

Although the μBondapak column used in this study has been referred to, for convenience, as an "analytical" column, the identification of GAs in plant extracts cannot be made merely on the basis of HPLC data. GAs only exhibit UV absorption around 200 nm, wavelengths at which the choice of organic solvents would be very limited and at which there would also be severe interference from contaminants in the plant extract. Consequently, it is only possible to monitor GAs using currently available HPLC detectors if the GAs are first converted to derivatives absorbing at 260 nm (8, 13). Derivatization of the mixture of compounds present in plant extracts would almost certainly result in many spurious peaks, in addition to those due to any endogenous GAs. Moreover, derivatization of GAs generally renders them inactive in any subsequent bioassay. Perhaps the main argument against the use of HPLC data alone for GA analysis of plant extracts is the large number of GAs known. To date, 56 naturally occurring GAs have been given A-numbers, many of them having similar chromatographic properties, although displaying disparate biological properties. Clearly, the only method for conclusive identification of GAs in plant extracts is MS.

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