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REVERSED-PHASE C_{18} HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ACIDIC AND CONJUGATED GIBBERELLINS

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SUMMARY

The retention times of gibberellins and their glucosyl esters and glucoside conjugates on C_{18} reversed-phase high-performance liquid chromatographic (HPLC) columns were determined using gradient or isocratic elution with methanol–acetic acid 1%. The separation of double-bond gibberellin isomers was accomplished without the need for derivatization or the addition of salts. A combined HPLC–radiocounting with flow-through scintillation spectrometric procedure was suitable for the routine radioassay of acidic and conjugate-like metabolites from [^{3}H] gibberellin feeds. Similarly, HPLC–bioassay detection was suitable for purified plant extracts. The totally volatile methanol–1% acetic acid solvent in the gradient and/or isocratic mode should be capable of separating virtually any of the known gibberellins, their conjugates and their catabolites. However, retention time alone is inadequate and definitive detection techniques must be utilized.

INTRODUCTION

We have been using reversed-phase C_{18} high-performance liquid chromatography (HPLC) for the separation of acidic gibberellin(s) (GAs) since 1976. For the most part our use of the technique has involved biological[1] or radiochemical assay as a detection method prior to gas chromatography–mass spectrometry (GC–MS) and/or gas chromatography–radiocounting. During this time we have utilized gradients of methanol–water or methanol–1% acetic acid and/or an isocratic mode with the same solvents. We have found the technique to be very useful with relatively highly purified

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extracts [*e.g.*, after polyvinylpolypyrrolidone (PVPP)[2] and silica gel partition chromatography[3], or after the use of a purification procedure involving C_{18} Sep-Pak material[4,5]].

Since 1976 several groups have reported the application of HPLC to the separation of acidic GAs[4,6–9], naturally occurring GA conjugates[10] and derivatized GAs[11–13]. For acidic and/or derivatized GAs, high resolution silica gel partition systems[6,7,11], silver nitrate-impregnated silica gel[12], or cyanopropyl-silica gel[13] have been utilized. The use of reversed-phase C_{18} HPLC columns has been reported[4,8–13]. For naturally occurring GA conjugates, a method was also developed utilizing octadecylsilanized and dimethylsilanized columns eluted with inorganic buffers or their mixtures with methanol[10]. Unfortunately, many of these techniques have not yet been shown to be practical for the isolation and identification of GAs from plant material.

It has been our preference, and the preference of other groups[4,8,9], to utilize C_{18} reversed-phase HPLC columns and methanol–water or methanol–1 % acetic acid for the separation of acidic GAs. We have found that other alcohols or acetonitrile did not give good separations, and wished to avoid introducing a residue of salt into fractions that were destinated for subsequent bioassay, GC–radiocounting and/or GC–MS. In this paper we expand the data of Jones *et al.*[8] and Barendse *et al.*[4] to show with standard compounds, endogenous GAs and metabolites from plants fed [^{3}H]GAs, that the use of C_{18} HPLC columns with a linear gradient of methanol in 1 % acetic acid can separate a large number of acidic GAs and GA glucosyl conjugates.

EXPERIMENTAL

Apparatus

The following were used: a Waters Assoc. ALC/GPC R-401 liquid chromatograph with two Model 6000 pumps, a Model 660 solvent flow programmer and a Model U6K universal injector, Schoeffel Models GM 770 and SF 770 UV monitors and Models GM 970 and FS 970 LC fluorimeters, a Berthold HPLC radioactivity monitor (LB 503), a Nuclear Chicago liquid scintillation spectrometer, a Packard Model 430 gas chromatograph and a Packard Model 884 gas proportional counter.

Columns

Waters Assoc. μBondapak C_{18} (300 × 7.9 and 3.9 mm I.D.) and Radial-Pak A cartridge C_{18} (100 × 8 mm I.D.) columns and a Whatman Partisil M9 10/50 ODS-2 (500 × 9 mm I.D.) column were used.

Solvents

Water and methanol were purified by fractional distillation, followed by filtration through 0.45-μm (HATF) and 0.5-μm (FHUP) pore-size Millipore filters, respectively. Solvents consisting of aqueous components (1 % acetic acid) were allowed to equilibrate, degassed and kept under vacuum with magnetic stiring to prevent gas accumulation.

Standard samples

^{3}H-Labeled GA_1 (structure 5), epi-GA_1 (27), GA_1 methyl ester (6), GA_4 (4),

epi-GA_4 (26), GA_4 methyl ester (2), GA_5 (11), GA_7 (9), iso-GA_7 (12), GA_8 (8), GA_9 (1), GA_{12} aldehyde (13), GA_{13} (22), GA_{14} (15), GA_{18} (16), GA_{20} (3), GA_{43} (23), kaurenoic acid (33); ^{14}C-labeled GA_3 (10), kaurene (32); GA_{19} (17), GA_{34} (53), GA_{53} (47), $\Delta^{1(10)}GA_1$ counterpart (29), $\Delta^{1(10)}GA_1$ counterpart half methyl ester (48), GA_{29} catabolite (28), gibberellenic acid (30), allogibberic acid (31), epi-allogibberic acid (54); GA_1-O(3)-, GA_1-O(13)-, GA_3-O(3)-, GA_3-O(13)-, GA_4-O(3)-, GA_5-O(13)-, GA_8-O(2)- and GA_{26}-O(2)-glucosides (51, 42, 50, 41, 45, 44, 46 and 39, respectively); GA_1-, GA_3- and GA_4-glucosyl esters (52, 43 and 40, respectively); abscisic acid (ABA, 37), t-ABA (38), ABA-methyl estér (49), indoleacetic acid (IAA, 34), indolebutyric acid (IBA, 35) and indolelactic acid (ILA, 36) were used as standards.

Each sample was filtered [pore size 0.5 μm (FHLP)] after being solubilized in a small amount of absolute methanol, concentrated under nitrogen and injected in 1–100 μl of methanol solution. Substances eluted from HPLC columns were detected with an in-line radioactivity monitor, UV monitor or fluorescence monitor, or analysed subsequently by GC, GC–radiocounting, liquid scintillation spectrometry or bioassay[1].

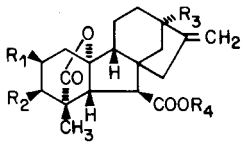
Extracts of plant materials

(1) Samples of 2 g of freeze-dried maize (*Zea mays* L.) apical meristem tissue from intact plants that had been fed [^{3}H]GA_{20}, or that contained only native GAs, were extracted with 100 ml of 80% methanol. After removal of the methanol *in vacuo* at 35°C, to the aqueous phase (*ca.* 20 ml), 20 ml of 0.5 *M* phosphate buffer (pH 8.0) were added, the pH was adjusted to 9.0 by the addition of 1 *M* potassium hydroxide solution and the aqueous solution was partitioned six times against equal volumes of diethyl ether. The aqueous phase was then adjusted to pH 3.0 with 1 *M* hydrochloric acid and partitioned six times against equal volumes of ethyl acetate and the combined ethyl acetate extract was evaporated to dryness after removal of water at −70°C. The residue was subjected to silica gel partition column chromatography[3] and/or HPLC and bio- or radioassay.

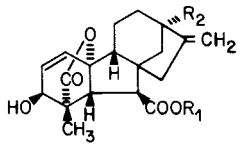
(2) Samples of 2 g of freeze-dried tissue from apple (*Malus domestica* Borkh.) leaves that had been fed [^{3}H]GA_4 (via the stem), or that contained only native GAs, were extracted with 80 ml of 80% methanol. The methanol extract was forced through a column (3 × 2.5 cm I.D.) of Waters Assoc. C_{18} Sep-Pak (3 g of C_{18} per gram dry weight of tissue) to remove pigments, such as chlorophyll and carotenoids[14]. The eluate was diluted to 50% methanol by addition of water and the solution was forced through a second column of C_{18} material to remove less polar compounds such as kaurene and kaurenoic acid. This eluate was evaporated to dryness *in vacuo*, and the residue was extracted in succession with (I) 40 ml of ethyl acetate–methanol (1:1) or water–saturated ethyl acetate, (II) 40 ml of methanol and (III) 40 ml of water. Fraction I was subjected to silica gel partition column chromatography and the methanol wash of the silica gel column (*e.g.*, GA glucosyl conjugates which are not eluted in hexane–ethyl acetate) was subjected to HPLC (Fig. 5). The HPLC of fractions II and III, which will also contain GA glucosyl conjugates[5], are not shown. The extract containing native apple GAs was processed in a manner similar to maize tissue.

HPLC conditions

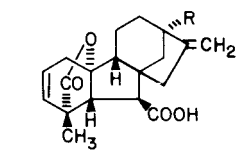
The conditions were as follows: pump A, 10% methanol in 1% acetic acid; pump B, methanol: standard linear gradient program, 0–10 min (pump A, 100%),



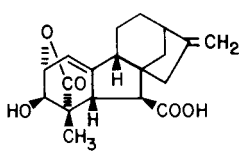
(1) R_1 = H R_2 = H R_3 = H R_4 = H
(2) R_1 = H R_2 = H R_3 = H R_4 = CH_3
(3) R_1 = H R_2 = H R_3 = OH R_4 = H
(4) R_1 = H R_2 = OH R_3 = H R_4 = H
(5) R_1 = H R_2 = OH R_3 = OH R_4 = H
(6) R_1 = H R_2 = OH R_3 = OH R_4 = CH_3
(7) R_1 = OH R_2 = H R_3 = OH R_4 = H
(8) R_1 = OH R_2 = OH R_3 = OH R_4 = H
(40) R_1 = H R_2 = OH R_3 = H R_4 = Glc
(42) R_1 = H R_2 = OH R_3 = O-Glc R_4 = H
(45) R_1 = H R_2 = O-Glc R_3 = H R_4 = H
(46) R_1 = O-Glc R_2 = OH R_3 = OH R_4 = H



(9) R_1 = H R_2 = H
(10) R_1 = H R_2 = OH
(41) R_1 = H R_2 = O-Glc
(43) R_1 = Glc R_2 = OH



(11) R = OH
(44) R = O-Glc



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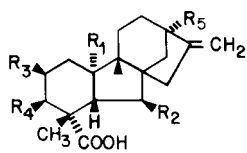
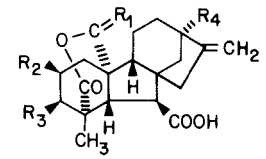
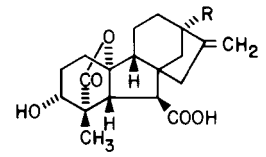


(13) R_1 = CH_3 R_2 = CHO R_3 = H R_4 = H R_5 = H
(14) R_1 = CH_3 R_2 = COOH R_3 = H R_4 = H R_5 = H
(15) R_1 = CH_3 R_2 = COOH R_3 = H R_4 = OH R_5 = H
(16) R_1 = CH_3 R_2 = COOH R_3 = H R_4 = OH R_5 = OH
(17) R_1 = CHO R_2 = COOH R_3 = H R_4 = H R_5 = OH
(18) R_1 = CHO R_2 = COOH R_3 = H R_4 = OH R_5 = H
(19) R_1 = CHO R_2 = COOH R_3 = H R_4 = OH R_5 = OH
(20) R_1 = COOH R_2 = COOH R_3 = H R_4 = H R_5 = H
(21) R_1 = COOH R_2 = COOH R_3 = H R_4 = H R_5 = OH
(22) R_1 = COOH R_2 = COOH R_3 = H R_4 = OH R_5 = H
(23) R_1 = COOH R_2 = COOH R_3 = OH R_4 = OH R_5 = H

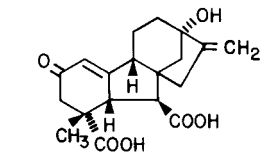
(47) R_5 = OH on 14
(48) C-6 = $COOCH_3$ on 29
(49) R_2 = $COOCH_3$ on 37
(50) C-3 = O-Glc on 10
(51) R_2 = O-Glc on 5
(52) R_4 = Glc on 5
(53) R_1 = OH on 4
(54) C-13 = β-OH on 31



(24) R_1 = H,H R_2 = H R_3 = H R_4 = OH
(25) R_1 = H,H R_2 = H R_3 = OH R_4 = H



(26) R = H
(27) R = OH



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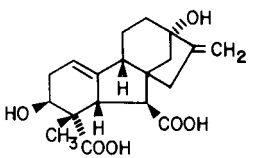

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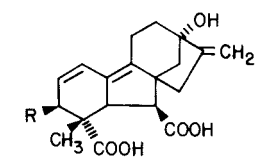
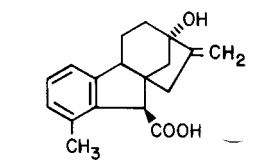


(30) R = OH



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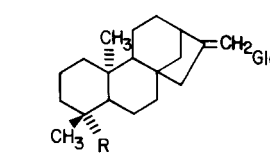
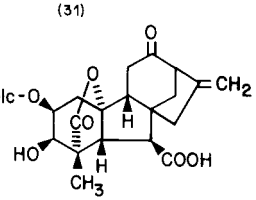


(32) R = CH_3
(33) R = COOH



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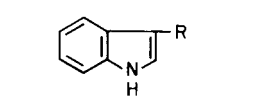
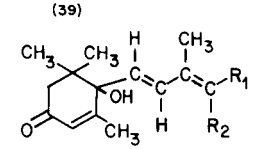


(34) R = CH_2 COOH
(35) R = $CH_2CH_2CH_2$ COOH
(36) R = CH_2 CH(OH) COOH



(37) R_1 = H R_2 = COOH
(38) R_1 = COOH R_2 = H

10–40 min (pump B, 0–70%), 40–50 min (pump B, 70%), 50–80 min (pump B 100%); temperature, 22–25°C.

HPLC on-line detector conditions

The conditions were as follows: UV monitor, wavelength 254 nm, range 0.1; fluorescence monitor, emission wavelength 370 nm, excitation wavelength 285 nm, range 0.1 (A); radioactivity monitor, counting time 1.00 min, threshold factor 1.00; background 30 cpm; peak reject, 20 counts; rate meter range, 300 cpm.

GC conditions

GAs were derivatized with diazomethane and N,O-bis(trimethylsilyl)trifluoroacetamide (1% trimethylchlorosilane). The columns were 1% XE-60 (2 m × 2.5 mm I.D.), 2% SE-30 (2 m × 2 mm I.D.) and 3% OV-101 (2 m × 2 mm I.D.); column temperature 205°C, detector temperature, 250°C, injector temperature, 230°C. The carrier gas was helium at a flow-rate of 50 ml/min; splitting ratio, 25:1 (radiocounting/FID). GA conjugates that had already been converted into the methyl ester with diazomethane or metabolized to the glucosyl ester were derivatized to form permethylated derivatives according to the method of Rivier *et al.*[15]. The column was 3% OV-101 (2 m × 2 mm I.D.); column temperature, 285°C, detector temperature 300°C, injector temperature 295°C. The carrier gas was helium at a flow-rate of 50 ml/min; splitting ratio, 10:1 (radiocounting/FID).

GC on-line radiocounting conditions

The flow-rates were as follows: carrier gas (helium), 100 ml/min; hydrogen, 6 ml/min; and quench gas, 8 ml/min. The inlet temperature was 300°C and furnace temperature 750°C. Other conditions were time constant 10, background offset 2 and range 500 cpm.

Bioassay

The "Tan-ginbozu" dwarf rice micro-drop bioassay was used in serial dilution for each silica gel and HPLC fraction[1].

RESULTS

Graphs of retention times of various gibberellins

Curves of GA retention times t_R were required for the determination of an optimum separation system. The GAs and two of their logical precursors (*e.g.*, kaurene and kaurenoic acid) had similar elution patterns (Fig. 1). For the gradient chosen, the retentions of the gibberillins increased with increasing water concentration, and the elution curves did not intersect. It can thus be expected that the elution order will be constant on reversed-phase columns as long as the same solvent systems are used. The elution order is related to functional groups on the ent-gibberellin skeleton[8,9].

Isocratic and gradient systems

A variety of isocratic systems could be selected from Fig. 1. By drawing an appropriate vertical line on Fig. 1 the retention time of any gibberellin could be

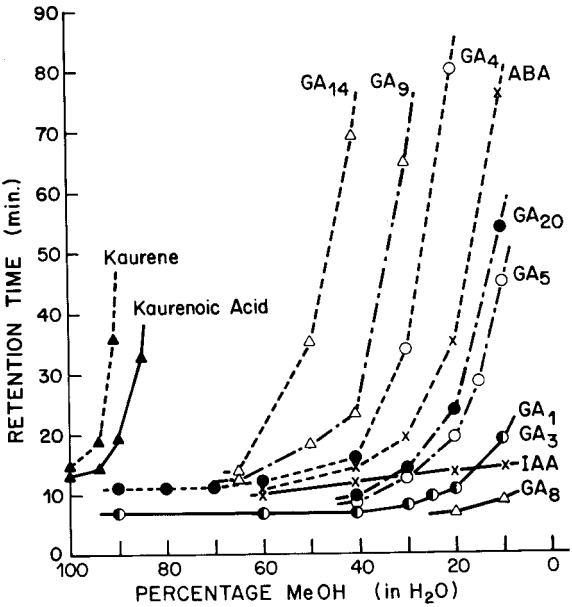


Fig. 1. Elution times of various gibberellins and gibberellin precursors on reversed-phase C_{18} HPLC (300 × 3.9 „ I.D. column), isocratically eluted with methanol–water (flow-rate 0.5 ml/min). ABA was detected by UV absorption; all other compounds were radioactive and were detected by liquid scintillation spectrometry. MeOH = Methanol.

determined at the corresponding methanol concentration. For example, four isocratic chromatograms are recorded in Table I. A variety of gradient systems could be selected by drawing an oblique line on Fig. 1. Using this approach, an "appropriate" linear gradient system was found (see Figs. 4–6). By using this gradient the retention

TABLE I

RETENTION TIMES (IN MINUTES) IN REVERSED-PHASE (C_{18}) HPLC OF GIBBERELLINS WITH SEVERAL ISOCRATIC SYSTEMS (METHANOL–1% ACETIC ACID)

Column, μBondapak C_{18} (300 × 3.9 mm I.D.); flow-rate, 0.5 ml/min.

Gibberellin	*Compound No.*	*Methanol concentration in 1% acetic acid (%)*			
		60	*40*	*20*	*40**
$[^3H]GA_8$	8	–	–	7	–
$[^3H]GA_1$	5	7	7	11.5	3.2
$[^3H]GA_3$	10	7	7	11.5	–
$[^3H]GA_5$	11	–	8.5	19.5	–
$[^3H]GA_{20}$	3	–	9.5	23.5	–
$[^3H]GA_4$	4	12	16	80	7.5
$[^3H]GA_9$	1	14	23.5	–	12
$[^3H]GA_{14}$	15	22	–	–	34.5

* Flow-rate 1.0 ml/min; detection by HPLC–radiocounting.

TABLE II

RETENTION TIMES (IN MINUTES) IN REVERSED-PHASE HPLC WITH GRADIENT ELUTION FOR THE SEPARATION OF ACIDIC GIBBERELLINS, RELATED COMPOUNDS, ABSCISIC ACID AND THREE INDOLE AUXINS

Standard program (Figs. 4, 5 and 6) used for all compounds except those marked***. Flow-rate, 2 ml/min; methanol–1% acetic acid. Detection of compounds 28, 30, 31, 35, 49 and 54 was by UV absorption, compounds 34, 37 and 38 by UV absorption or fluorescence, compounds 29 and 48 by GC–FID, compounds 17, 47 and 53 by bioassay; all other compounds were detected by HPLC–radiocounting.

Compound	*No.*	*Analytical column**	*Preparative column***
[^{3}H]GA_8	8	12–13	6–7
GA_{29}	7	(16–20)***	
Gibberellenic acid	30	17–18	
GA_{23}	19	(21–25)***	
ILA	36	21–22	20–21
[^{3}H]epi-GA_1	27	25–26	
[^{14}C]GA_3	10	25–26	20–21
[^{3}H]GA_1	5	25–25	20–21
GA_{29} catabolite	28	25–26	
IAA	34	25–26	29–30
$\Delta^{1(10)}GA_1$ counterpart	29	26–27	
[^{3}H]GA_{18}	16	27–28	
t-ABA	38	27–28	
[^{3}H]GA_1 methyl ester	6	28–29	
$\Delta^{1(10)}GA_1$ counterpart half methyl ester	48	28–29	
ABA	37	29–30	
[^{3}H]GA_{43}	23	30	
[^{3}H]GA_5	11	30–31	29–30
[^{3}H]GA_{20}	3	30–31	29–30
GA_{44}	24	(31–32)***	
GA_{53}	47	32–33	
GA_{19}	17	33–34	
[^{3}H]GA_{13}	22	33–34	
ABA methyl ester	49	33–34	
Allogibberic acid	31	33–34	
GA_{34}	53	33–35	
GA_{36}	18	(34–35)***	
epi-Allogibberic acid	54	35–36	
GA_{17}	21	(35–36)***	
GA_{37}	25	(36–37)***	
[^{3}H]epi-GA_4	26	36–37	
[^{3}H]GA_7	9	37–38	40–41
iso-[^{3}H]GA_7	12	37–38	
[^{3}H]GA_4	4	37–38	40–41
IBA	35	37–38	40–41
[^{3}H]GA_9	1	38–39	43–44
[^{3}H]GA_{14}	15	39–40	46–49
GA_{25}	20	(39–40)***	
[^{3}H]GA_4 methyl ester	2	40	
GA_{12}	14	(45–47)***	
[^{3}H]GA_{12} aldehyde	13		60–63
[^{3}H]Kaurenoic acid	33	56–57	60–63
[^{14}C]Kaurene	32	58–61	

* Column μBondapak C_{18} (300 × 3.9 mm I.D.); flow-rate, 2 ml/min; methanol–1% acetic acid.
** Column μBondapak C_{18} (300 × 7.8 mm I.D.); flow-rate, 3 ml/min; methanol–1% acetic acid.
*** These compounds should be eluted here, estimates based on data from Jones *et al.*[8].

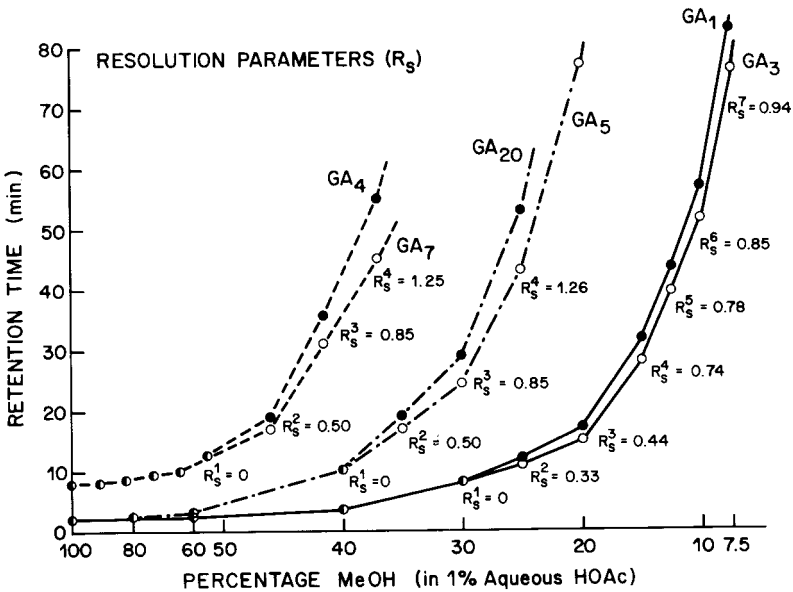


Fig. 2. Separation and resolution parameters of gibberellin unsaturated analogs on reversed-phase C_{18} HPLC–radiocounting (300 × 3.9 mm I.D. column), isocratically eluted with methanol–1% acetic acid (HOAc) (flow-rate 2 ml/min).

times of various gibberellins were determined on the analytical and preparative columns (Table II).

As expected, the elution order of the gibberellins was the same on both columns, although differences were observed in retention times. Many other gradient curves were tried, but gave inferior separations.

The separation of certain unsaturated gibberellin isomers (*e.g.*, GA_1/GA_3, GA_4/GA_7 and GA_5/GA_{20}) was not readily achieved with a gradient system. However, by using isocratic systems, good separations can be achieved (Figs. 2 and 3). Thus GA_1/GA_3, GA_4/GA_7 and GA_5/GA_{20} were separated from each other at meth-

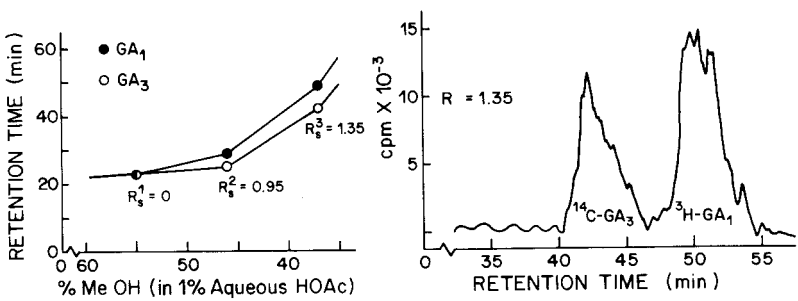


Fig. 3. Separation of [^{3}H]GA_1 and [^{14}C]GA_3 on a Whatman Partisil M9 ODS-2 column (500 × 9 mm I.D.), isocratically eluted with methanol–1% acetic acid (flow-rate 2 ml/min); detection by HPLC–radiocounting. The figure on the right is adopted from Rood *et al.*[27].

anol concentrations below 30%, 55%, and 40%, respectively. At a methanol concentration of 37% the resolution parameter R_S [$R_s = 2(t_{R_B} - t_{R_A})$/(peak width of A + peak width of B)] between GA_4 and GA_7 was 1.25 and the R_s between GA_5 and GA_{20} was 1.26 at methanol concentration of 25%. Attempts to separate GA_1 and GA_3 on the analytical column were not successful, even at lower methanol concentrations (*e.g.*, at 7.5% methanol, $R_s = 0.94$). However, when the preparative column was used, these GAs separated well provided that the methanol concentration was below 55%. Their R_s value was 1.3 at a methanol concentration of 37% (Fig. 3). The GAs containing an endo-double bond (*e.g.*, GA_3, GA_5 and GA_7) were eluted earlier than the corresponding GAs without an endo-double bond (*e.g.*, GA_1, GA_{20} and GA_4)[4].

TABLE III

ARRANGEMENT OF GIBBERELLINS IN ORDER OF ELUTION IN HPLC (C_{18})

GA	*Compound No.*	*Positions of functional groups*					
		2	*3*	*13*	*4*	*6*	*10*
20-C gibberellins							
GA_{23}*	19		OH	OH	COOH	COOH	CHO
GA_{18}	16		OH	OH	COOH	COOH	CH_3
GA_{43}	23	OH	OH		COOH	COOH	COOH
GA_{44}*	24			OH		COOH	OCO–
GA_{53}	47			OH	COOH	COOH	CH_3
GA_{19}	17			OH	COOH	COOH	CHO
GA_{13}	22		OH		COOH	COOH	COOH
GA_{36}*	18		OH		COOH	COOH	CHO
GA_{17}*	21			OH	COOH	COOH	COOH
GA_{37}*	25		OH			COOH	OCO–
GA_{14}	15		OH		COOH	COOH	CH_3
GA_{25}*	20				COOH	COOH	COOH
GA_{12}*	14				COOH	COOH	CH_3
19-C gibberellins							
GA	*Compound No.*	*Positions of functional groups*					
		2	*3*	*13*	*6*	*Δ*	
GA_8	8	OH	OH	OH	COOH		
GA_{29}*	7	OH		OH	COOH		
GA_3	10		OH	OH	COOH	$\Delta^{1,2}$	
GA_1	5		OH	OH	COOH		
GA_5	11			OH	COOH	$\Delta^{2,3}$	
GA_{20}	3			OH	COOH		
GA_{34}	53	OH	OH		COOH		
GA_7	9		OH		COOH	$\Delta^{1,2}$	
GA_4	4		OH		COOH		
GA_9	1				COOH		

* Adapted from Jones *et al.*[8].

Thus, the unsaturated GA isomers were well separated on the C_{18} reversed-phase columns without derivatization or the use of buffer salt elution systems.

Elution order of acidic gibberellins

Functional groups as –OH, –COOH, –CHO, $-CH_3$ and $-C=C-$ affect the elution order (Table III). The relationship between the elution order and the functional group was as follows:

(I) The more hydroxyl groups, the faster the GA will generally be eluted*:

C_{20}-GA: (GA_{23}, GA_{18}, GA_{43}) > (GA_{44}, GA_{53}, GA_{19}, GA_{13}, GA_{36}, GA_{17}, GA_{37}, GA_{14}) > (GA_{25}, GA_{12}).

C_{19}-GA: GA_8 > (GA_{29}, GA_3, GA_1) > (GA_5, GA_{20}, GA_7, GA_4) > GA_9.

(II) A hydroxyl group at the C-13 position will generally speed up the elution to a greater extent than one at the C-2 or C-3 position*:

C_{20}-GA: GA_{44} > GA_{37}; GA_{19} > GA_{36}; GA_{53} > GA_{14}.

C_{19}-GA: GA_{29} > GA_{36}; GA_1 > GA_{34}; GA_5 > GA_7; GA_{20} > GA_4.

(III) A hydroxyl group at the C-2 position will accelerate the elution more than will hydroxylation at the C-3 position*:

C_{19}-GA: GA_{29} > GA_1.

(IV) An aldehyde group or a carboxyl group at the C-10 position will generally cause faster elution than a methyl group at the C-10 position*:

C_{20}-GA: GA_{23} > GA_{18}; GA_{13} > GA_{14}; GA_{36} > GA_{14}; GA_{25} > GA_{12}.

(V) A double bond group will also speed up the elution (as mentioned above):

C_{19}-GA: GA_3 > GA_1; GA_5 > GA_{20}; GA_7 > GA_4.

(VI) Specific differences between C_{19} GAs and C_{20} GAs were not observed, although a difference between the ent-gibberellane and kaurene skeletons was observed (see also Table II). Kaurene and kaurenoic acid are eluted much later than GA_9, or even GA_4-Me.

Elution order of gibberellin glucosyl conjugates

The gradient retention times of eleven GA-glucosyl conjugates were determined for the methanol–1 % acetic acid gradient system (Table IV). The GA-glucosyl conjugates had the same or slightly shorter retention times than the corresponding acidic GAs. The unsaturated GAs and the C-13 glucosides were also eluted faster than the corresponding saturated analogs and C-3 glucosides. Their elution patterns were thus the same as those of GAs:

GA_3-O(13)-G > GA_1-O(13)-G; GA_3-O(3)-G > GA_1-O(3)-G.

GA_3-O(13)-G > GA_3-O(3)-G; GA_1-O(13)-G > GA_1-O(3)-G.

Separation and identification of GAs and GA conjugates in plant extracts

Metabolites of [^{3}H]GA_{20} and [^{3}H]GA_4. When [^{3}H]GA_{20} was fed to *ca.* 20-day-old maize plants, the meristem tissue showed one major radioactive metabolite in the acidic ethyl acetate extract (Fig. 4). This metabolite had the same retention time as GA_1. This was further confirmed by isocratic HPLC (Fig. 4) or by packed column GC–radiocounting ([^{3}H]GA_1: retention time 13.7 min on QF-1 and 15.8 min on XE-60). Both GA_1 and GA_{20} are native to maize[16].

* Order of elution, as noted above, adapted from Jones *et al.*[8] for $GA_{12,17,23,25,29,36,37,44}$.

TABLE IV

RETENTION TIMES (IN MINUTES) IN REVERSED-PHASE HPLC OF GIBBERELLIN GLUCOSYL CONJUGATES

Standard program system (Figs. 4–6) was used. Flow-rate, 2 ml/min; methanol–1 % acetic acid. Retention times of gibberellin glucosyl conjugates were determined by the dwarf rice immersion bioassay, except that for GA_{26}-O(2)-glucoside, which was determined by UV absorption at 290 nm.

Compound	*No.*	*Conjugate*	*Corresponding GA*
GA_8-O(2)-glucoside*	46	11–12	12–13
GA_3-O(13)-glucoside*	41	20–21	25–26
GA_1-O(13)-glucoside*	42	21–22	25–26
GA_3-O(3)-glucoside*	50	22–23	25–26
GA_1-O(3)-glucoside*	51	23–24	25–26
GA_1-glucosyl ester*	52	24–25	25–26
GA_3-glucosyl ester*	43	25	25–26
GA_5-O(13)-glucoside*	44	30	30–31
GA_4-O(3)-glucoside*	45	33–34	37–38
GA_4-glucosyl ester*	40	36–37	37–38
GA_{26}-O(2)-glucoside**	39	46–49	

* μBondapak analytical column (300 × 3.9 mm I.D.).
** μBondapak preparative column (300 × 7.8 mm I.D.).

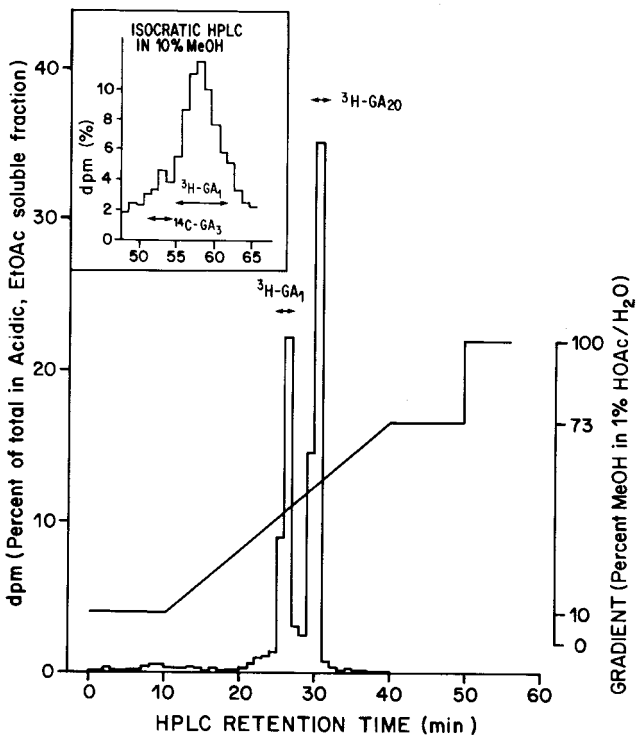


Fig. 4. HPLC–radiocounting elution pattern of a purified (by partitioning then PVPP chromatography) acidic, ethyl acetate (EtOAc) soluble fraction from maize meristems[23] (plants were fed [^{3}H]GA_{20} at age *ca.* 20 days) on an analytical C_{18} μBondapak column (300 × 3.9 mm I.D.). The inset shows that the majority of the peak which eluted coincidentally with ^{3}H-GA_1 on gradient-elution HPLC also eluted with ^{3}H-GA_1 on an isocratic system that separates GA_1 from its unsaturated analog, GA_3.

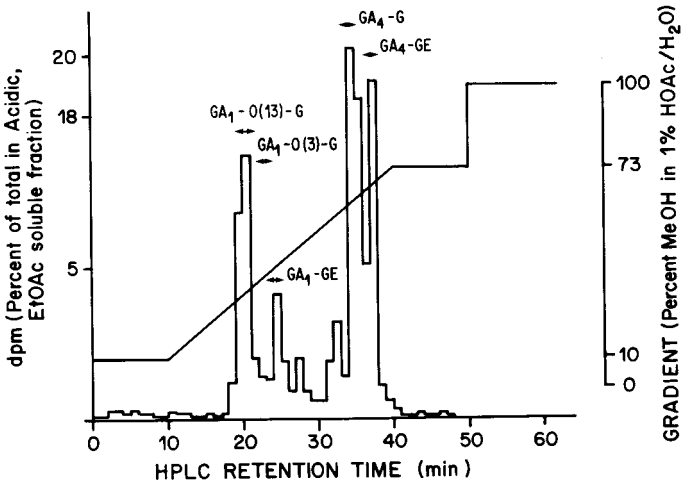


Fig. 5. HPLC–radiocounting elution pattern of [^{3}H]GA conjugate-like metabolites (see Experimental) from apple leaf tissue[24] (trees were fed [^{3}H]GA_4 via the stem during the period of flower bud initiation) on an analytical C_{18} μBondapak column (300 × 3.9 mm I.D.).

When [^{3}H]GA_4 was fed to apple propagules, four major radioactive peaks were observed on HPLC (Fig. 5) of a methanol wash from the silica gel partition column (*e.g.*, conjugate-like substances that are not eluted by hexane–ethyl acetate from the silica gel column can be eluted with methanol). These peaks had retention times similar to those of GA_1-O(13)-glucoside, GA_1-glucosyl ester, GA_4-glucosyl ester and GA_4-O(3)-glucoside (Fig. 5). Both GA_1 and GA_4 are native to apple seeds[17]. The retention times of the purported [^{3}H]GA-glucosyl conjugates from apple (Fig. 5) were the same as those of GA-glucosyl conjugate metabolites isolated from anise and carrot suspension cultures fed [^{3}H]GA_4[18,19]. Further characterization of the anise and carrot [^{3}H]GA_4-glucosyl conjugate-like metabolites and their hydrolysis products was accomplished by HPLC–radiocounting followed by GC–radiocounting. The results[18,19] were consistent with the identifications noted above and in Fig. 5.

Endogenous gibberellin-like substances. Tissues from young shoots of apple and maize meristems showed a large "polar" peak in the $GA_1/GA_3/GA_{19}$ region of a silica gel partition column (see inserts in Figs. 6 and 7). The peak was resolved by HPLC–bioassay for apple into several small and one large peak, the latter coinciding with the actual retention time of GA_{19} on C_{18} μBondapak (Fig. 6). For maize it was resolved into two peaks, the minor one coinciding with GA_1 and the major one with GA_{19} (Fig. 7), on reversed-phase radial compression HPLC (however, see comments below on Radial-Pak A columns). Both GA_{19} and GA_1 are native to maize[16]. Thus, HPLC subsequent to silica gel partition chromatography can be used to resolve a C_{20}-like GA (purported GA_{19}) from a C_{19}-like GA (purported GA_1).

Radial compression columns

For a brief period we used the Waters Assoc. radial compression system (Radial-Pak A cartridges), as described by Barendse *et al.*[4]. Initially we found it almost as good as C_{18} analytical columns, and much faster. Unfortunately, most of the Radial-Pak A columns "bled off" a milky white substance (C_{18} phase?), the

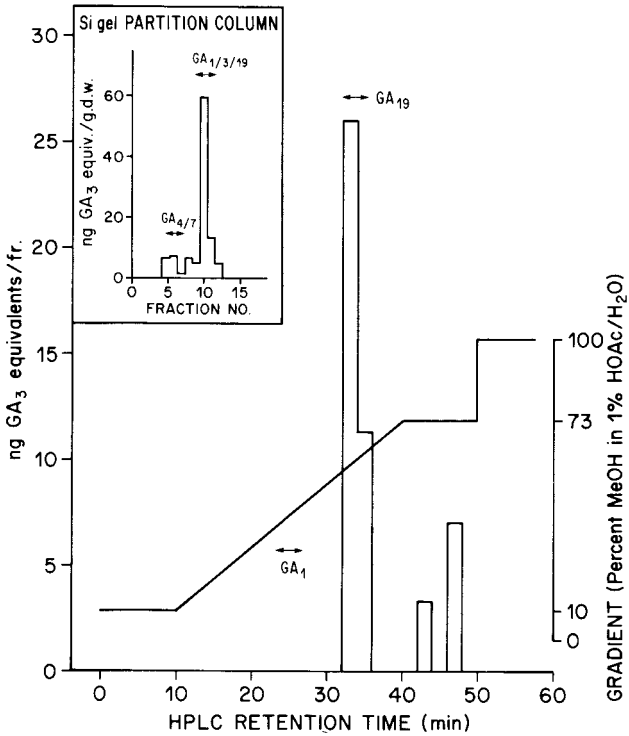


Fig. 6. C_{18} μBondapak HPLC (300 × 3.9 mm I.D. column) after silica gel partition column chromatography (inset) of endogenous acidic, gibberellin-like substances from apple shoot tissue[25]; detection by dwarf rice micro-drop bioassay[1].

retention time became variable and the plate efficiency decreased (to 50% of initial efficiency). This is an unsolved problem, also experienced with Radial-Pak A cartridges by other groups using methanol–water solvents. However, preliminary tests with columns designed for Waters Assoc. "Z Module" radial-compression separation system indicated no such problems and gave equivalent separations.

DISCUSSION

Reversed-phase C_{18} HPLC is an effective method for separating a large number of underivatized acidic GAs and GA conjugates, particularly when gradient elution is applied to a purified extract followed by re-chromatography of each GA peak under isocratic conditions. Unpurified (*e.g.*, methanol) extracts sometimes yield non-polar compounds, apparently artifacts, which, upon further purification and/or silica gel partition chromatography, would, when re-chromatographed on C_{18}, elute at their "proper", more polar retention times.

Although only four examples ([^{3}H]GA_1 methyl ester, [^{3}H]GA_4 methyl ester, $\Delta^{1(10)}GA_1$ counterpart half methyl ester, ABA methyl ester) are shown, the system can also be used to effect additional purification by subsequent re-chromatography of the acidic GA or GA conjugate in derivatized form (*e.g.*, methyl ester, permethyl ester, benzyl ester, *p*-nitrobenzyl ester). However, the retention time alone is too variable to serve as a basis for collecting specific fractions. Also, derivatization to

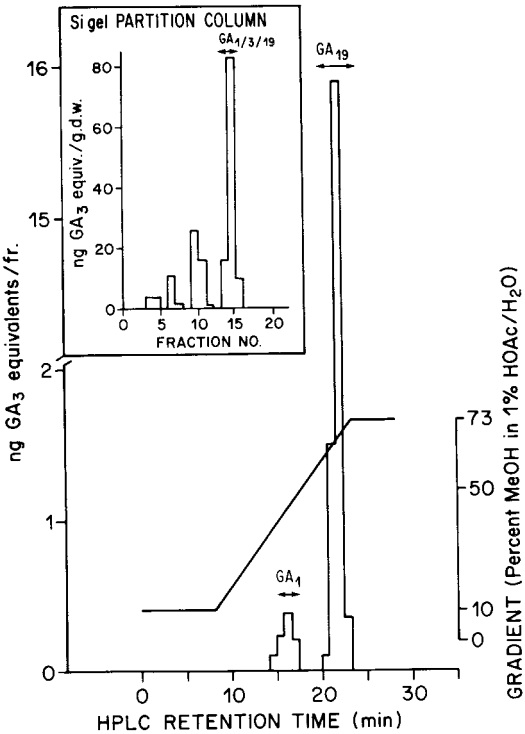


Fig. 7. C_{18} radial compression HPLC (100 × 8.0 mm I.D. column) after silica gel partition column chromatography of endogenous acidic, gibberellin-like substances from maize meristems[26]; detection by dwarf rice micro-drop bioassay[1].

UV-absorbing and/or fluorescing compounds, followed by C_{18} HPLC alone, is an insufficiently rigorous detection method, as numerous contaminants present in plant extracts also absorb and/or fluoresce in the UV region in free and/or derivatized form. Hence, the key to the effective use of HPLC (or any other chromatographic system) is to utilize a definite qualitative assay. For endogenous GAs, this would entail use of a bioassay specific for GAs and GA conjugates, such as the dwarf rice or dwarf maize assay, or specific physical methods [*e.g.*, GC–MS, GC–mass fragmentometry based on at least six characteristic ions, or GC–selected ion monitoring (SIM)[20]]. Where radioactive GAs and their metabolites are analyzed, HPLC with gradient elution, followed by isocratic elution and GC–radiocounting would be reasonably specific, although the use of GC–SIM–MS with ^{2}H- or ^{13}C-labeled precursors of high specific activity would be most desirable. Because we have experienced with crude extracts, and even with relatively purified extracts, "double peaking" and elution of known GAs at a more "non-polar" retention time than would be expected, we recommend the use of reversed-phase C_{18} HPLC only on highly purified extracts. For acidic GAs we strongly recommend, as did Jones *et al.*[8], that silica gel partition[3] and/or silica gel or charcoal adsorption[21] chromatography be used prior to reversed-phase C_{18} HPLC. Discrete fractions from the charcoal and/or silica gel system may then be chromatographed on preparative[8,21,22] and/or analytical reversed-phase C_{18}

HPLC. Such a sequential technique can also offer additional information (*e.g.*, see Figs. 6 and 7) and can yield fractions suitable for GC–MS or GC–SIM–MS[21,22].

Finally, we recommend the methanol–1 % acetic acid gradient and isocratic solvent mixture to other workers because it is easily handled, the eluent is completely volatile, there is flexibility in terms of solubilization of acidic GAs, GA conjugates and GA derivatives and there is the ability to effect good separations of various GAs/GA conjugates/GA catabolites by either gradient or isocratic elution without the addition of salts.

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