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REVERSED-PHASE C_{18} AND NORMAL-PHASE SILICA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GIBBERELLINS AND THEIR METHYL ESTERS

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SUMMARY

Twenty-three gibberellins and their methyl esters were chromatographed by gradient reversed-phase C_{18} partition and isocratic normal-phase silica adsorption high-performance liquid chromatography (HPLC). These four complementary HPLC systems allowed for the necessary separation to resolve these gibberellins. The four HPLC systems can be used to identify radioactive metabolites in metabolism studies by co-chromatography of radioactive labelled gibberellins with authentic standards using UV and radioactive flow detectors. The highest separation efficiency for these gibberellins was achieved with the reversed-phase C_{18} HPLC of their methyl esters.

INTRODUCTION

High-performance liquid chromatography (HPLC) is now a routine procedure for the purification and separation of gibberellins, a group of plant hormones. Reversed-phase C_{18} partition HPLC of gibberellins has been the most frequently used and reported HPLC system[1–5]. The HPLC of some gibberellin derivatives have also been reported[6–8]. Very little of the C_{18} HPLC of methyl esters of gibberellins and the silica HPLC of free gibberellins and their methyl esters have been reported.

Lin *et al.*[9,10] have previously used both normal-phase silica adsorption and reversed-phase C_{18} partition HPLC of twenty-six androgens ($C_{19}O_2$ stereoisomers) and their acetates in metabolism studies to identify radioactive metabolites by co-chromatography with standards. The four HPLC systems used complemented each other and all of the twenty-six $C_{19}O_2$ androgens available to us were resolved. There are more than seventy gibberellins that have been identified in plants and the fungus *Gibberella fujikuroi*, while the number of gibberellins in particular plants is limited[11]. The structures among the twenty-six $C_{19}O_2$ androgens[12] are more similar than those of gibberellins[11]. It was therefore easier to separate these gibberellins than to separate the $C_{19}O_2$ androgens by HPLC. We report here the separation of gibberellins using both reversed-phase C_{18} partition and normal-phase silica adsorption HPLC of free (underivatized) gibberellins and their methyl esters. We also report here the elution properties of gibberellins in these HPLC systems.

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Gas chromatography–mass spectrometry (GC–MS) has been used to detect labelled isotopic ions of the HPLC purified gibberellin metabolites when gibberellins labelled with ^{13}C, ^{14}C or ^{2}H were used as precursors[13,14]. Gas chromatography–selected ion monitoring (GC–SIM) or GC–MS also has been used to identify HPLC purified radioactive metabolites without the detection of isotopic ions when the gibberellins labelled with ^{3}H or ^{14}C were used as precursors[15,16]. Reversed-phase C_{18} HPLC alone without GC–MS or GC–SIM has been used to tentatively identify the radioactive metabolites[17]. One or more HPLC systems described here can be used to purify the metabolites with identification of the metabolites by GC–MS or GC–SIM. Gibberellins from these HPLC systems can be derivatized for GC–MS or GC–SIM identification. These four HPLC systems sequentially also can be used to identify radioactive metabolites by co-chromatography with authentic standards using UV and on-line radioactive flow detectors. The radioactive flow-detector can very accurately match the UV and radioactive peaks of a single HPLC run.

EXPERIMENTAL

A Waters Assoc. liquid chromatograph was used which consisted of two pumps (M510), a multiwavelength detector (M490), and a data and chromatography control station (M840). The injector was a Rheodyne Model 7125. The columns were a silica column (25 cm × 0.46 cm, 5 μm, Spherisorb S5W, Alltech/Applied Science, Deerfield, IL, U.S.A.) and a C_{18} column (25 cm × 0.46, 5 μm, Ultrasphere ODS, Beckman, San Ramon, CA, U.S.A.). Gibberellins were methylated with diazomethane. The eluents for the HPLC of free gibberellins needed 0.05% of glacial acetic acid as ion repressor, while the eluents for the HPLC of the methyl esters of gibberellins needed no glacial acetic acid. The eluent mixture for the silica HPLC of free gibberellins needs premixing, to avoid air bubble formation which interferes with UV detection. The chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

Results are summarized in Table I. Chromatograms from four HPLC systems are shown in Figs. 1–4 and are direct photocopies of printouts from HPLC runs. The gibberellins in Table I are arranged in the order of elution (decreasing polarity) in the C_{18} HPLC of free gibberellins. The elution orders are not the same or exactly the reverse among these four HPLC systems. Some gibberellins in Table I cannot be adequately separated by one HPLC system. However, they can be adequately separated by at least one of the three remaining HPLC systems. The C_{18} HPLC of up to 42 gibberellins has been reported[1]. We include in Table I, GA_{55}, GA_{32} and GA_{54} for which HPLC results have not been reported previously[1–5].

The four chromatograms (Figs. 1–4) showed that the separation efficiency (theoretical plate number illustrated by peak width and retention time) in decreasing order is as follows: C_{18} HPLC of methyl esters of gibberellins (system 2) > C_{18} HPLC of free gibberellins (system 1) > silica HPLC of methyl esters of gibberellins (system 4) > silica HPLC of free gibberellins (system 3). Both C_{18} HPLC systems are very good for the separation of gibberellins. The peak widths in Fig. 2 are slightly smaller than those in Fig. 1 and therefore the separation efficiency of system 2 is the best among

TABLE I

RETENTION TIMES OF GIBBERELLINS IN HPLC

Conditions: (1) C_{18} HPLC, free gibberellins, see Fig. 1; (2) C_{18} HPLC, methyl esters of gibberellins, see Fig. 2; (3) silica HPLC, free gibberellins, see Fig. 3; (4) silica HPLC, methyl esters of gibberellins, see Fig. 4.

Gibberellins	*Retention time (min)*			
	1	*2*	*3*	*4*
GA_{55}	5.03	6.64	12.21	12.57
GA_8	5.08	7.36	11.69	12.49
GA_{32}	6.50		12.61	12.21
GA_3	9.38	10.32	9.34	9.73
3epi-GA_1	9.41	11.51	12.68	12.31
GA_1	10.51	11.41	8.99	9.24
GA_5	17.86	18.67	5.75	4.87
GA_{20}	18.97	19.41	5.73	4.70
GA_{36}	20.44	24.56	4.73	3.31
GA_{13}	20.47	26.10	6.74**	3.05
GA_{44}*	21.4			
GA_{19}	22.41	23.54	5.81	3.93
GA_{54}	22.58	22.85	5.32	4.45
GA_{34}	22.83	22.80	4.93	4.30**
GA_{17}	23.74	25.35	6.67**	3.89
GA_{37}	24.07	22.94	4.84	4.38
GA_7	24.92	23.69	4.03	3.50
GA_4	26.07	24.60	4.00	3.36
GA_{53}	27.81	27.34	4.96	3.13
GA_{14}	28.17	28.64	4.36	2.74
GA_{24}	28.86	29.91	2.90	1.84
GA_9	29.36	27.79	2.77	2.08
GA_{25}	29.54	31.21	3.31	1.74
GA_{15}	29.76	27.87	3.20	2.38

* Retention time of GA_{44} was obtained with extract from wheat seedlings[20].

** Broad peaks, the peak width is about twice of that of other peaks at similar retention times.

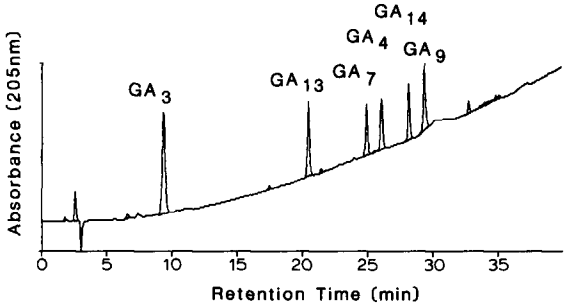

Fig. 1. Chromatogram of C_{18} HPLC of free gibberellins (in elution order GA_3, GA_{13}, GA_7, GA_4, GA_{14} and GA_9). The standards (about 1–5 μg each) dissolved in less than 25μl of methanol were chromatographed on a column of Ultrasphere ODS. Eluent, linear gradient from 35% methanol in water (containing 0.05% of acetic acid) to 100% methanol (containing 0.05% of acetic acid) in 40 min; flow-rate, 1 ml/min; pressure, 3000 p.s.i.

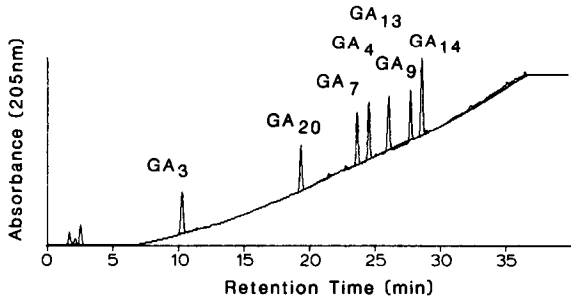

Fig. 2. Chromatogram of C_{18} HPLC of the methyl esters of gibberellins (in elution order, GA_3, GA_{20}, GA_7, GA_4, GA_{13}, GA_9 and GA_{14}). The standards (about 1–5 μg each) dissolved in less than 25 μl of methanol were chromatographed on a column of Ultrasphere ODS. Eluent, linear gradient, from 40% methanol to 100% methanol in 30 min, then 100% methanol for additional 10 min; flow-rate, 1 ml/min; pressure, 3000 p.s.i.

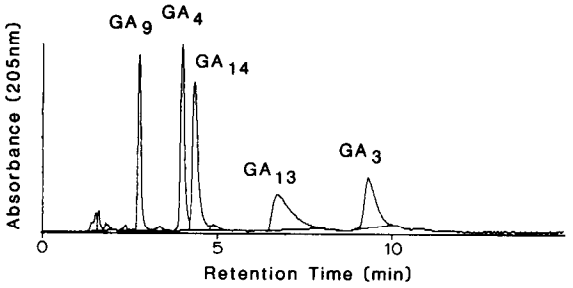

Fig. 3. Chromatogram of silica HPLC of free gibberellins (in elution order, GA_9, GA_4, GA_{14}, GA_{13} and GA_3). The standards (about 1–5 μg each) dissolved in less than 25 μl of the eluent were chromatographed on a column of Spherisorb S5W. Eluent, *n*-hexane–ethanol (90:10) containing 0.05% of acetic acid, pre-mixed; flow-rate, 2 ml/min; pressure, 800 p.s.i.

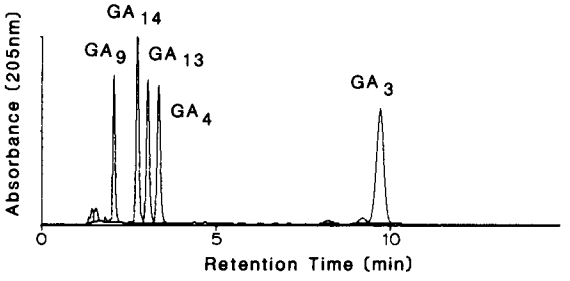

Fig. 4. Chromatogram of silica HPLC of the methyl of gibberellins (in elution order, GA_9, GA_{14}, GA_{13}, GA_4 and GA_3). The standards (about 1–5 μg each) dissolved in less than 25 μl of the eluent were chromatographed on a column of Spherisorb S5W. Eluent, *n*-hexane–ethanol (92:8); flow-rate, 2 ml/min; pressure, 800 p.s.i.

these four HPLC systems. Even though the separation efficiency of C_{18} HPLC of methyl esters of gibberellins (system 2) is the best among these four HPLC systems, it has only been reported previously by Birnberg *et al.*[18] using acetonitrile–water as the eluent for purification. The silica HPLC of methyl esters of gibberellins has been reported[16,18] with the retention times of GA_{53}, GA_4, GA_{20}, $GA_{17/19}$ given[16]. Lin and Heftmann[3] have previously reported the only silica HPLC of free gibberellins. This is adsorption chromatography and is different from the widely used silica partition chromatography[19].

The factors affecting the polarity (or retention time) of gibberellins in HPLC are as follows: number of hydroxyl groups, position and orientation of hydroxyl groups, functional groups at the C-10 position, lactone form and double bond. The more polar compounds elute later from normal-phase silica HPLC and sooner from reversed-phase C_{18} HPLC.

Some gibberellin retention properties of reversed-phase C_{18}[1–5] and normal-phase silica[3] HPLC of free gibberellins have been previously reported but not those of the other two HPLC systems. The more hydroxyl groups, the more polar the gibberellins will be and therefore will elute sooner from C_{18} HPLC and elute later from silica HPLC for both gibberellins and the methyl esters of gibberellins. Examples of the effect of hydroxyl group on polarity in HPLC from the present study are the following pairs of gibberellins: $GA_{55} > GA_1$, $GA_{54} > GA_4$ (C-1β); $GA_8 > GA_1$, $GA_{34} > GA_4$ (C-2β); 3epi-$GA_1 > GA_{20}$ (C-3α); $GA_1 > GA_{20}$, $GA_4 > GA_9$, $GA_{36} > GA_{24}$, $GA_{37} > GA_{15}$ (C-3β); $GA_{32} > GA_3$ (C-12α); $GA_1 > GA_4$, $GA_3 > GA_7$, $GA_{17} > GA_{25}$, $GA_{19} > GA_{24}$, $GA_{20} > GA_9$, $GA_{44} > GA_{15}$, $GA_{55} > GA_{54}$ (C-13).

The orientation and location of hydroxyl groups are also an important factor affecting the polarity of gibberellins and their HPLC retention times. The conclusions given here are derived in part from the retention times of gibberellins of Jensen *et al.*[1] which are not given in Table I. The ring A α-hydroxylated gibberellins are in general more polar than the ring A β-hydroxylated gibberellins in the C_{18} HPLC of free gibberellins (system 1, Table I). The examples are as follows: $GA_{16} > GA_{54}$ (C-1), $GA_{40} > GA_{51}$ (C-2), 3epi-$GA_1 > GA_1$ (C-3). Based on C_{18} HPLC of free gibberellins the polarity of gibberellins with hydroxyl groups at different locations (system 1, Table I) is, in general, $12\alpha > 16\alpha > 13 > 11\beta > 1\alpha > 2\alpha > 1\beta > 2\beta > 3\alpha > 3\beta$. Examples are as follows: $12\alpha > 16\alpha$; $GA_{39} > GA_{41}$, $GA_{31} > GA_{10}$. $16\alpha > 13$; $GA_{10} > GA_{20}$, ($GA_{41} < GA_{28}$). $13 > 11\beta$; $GA_1 > GA_{35}$. $11\beta > 1\alpha$; $GA_{35} > GA_{16}$. $1\alpha > 2\alpha$; $GA_{16} > GA_{47}$. $2\alpha > 1\beta$; $GA_{47} > GA_{54}$. $1\beta > 2\beta$; $GA_{55} > GA_8$, $GA_{54} > GA_{34}$. $2\beta > 3\alpha$;$GA_{29} >$ 3epi-GA_1. $3\alpha > 3\beta$; 3-epi-$GA_1 > GA_1$. These retention properties affected by the orientation and location of a hydroxyl group of free gibberellins in C_{18} HPLC have not been previously reported. The elution order of free gibberellins in the C_{18} HPLC in Table I (system 1) is consistent with that of Jensen *et al.* except GA_{53}–GA_{14}.

In these four HPLC systems, C-13 hydroxygibberellins in general are more polar than the C-1β, C-2β, C-3β hydroxygibberellins, The examples are as follows: $13 > 1\beta$; $GA_1 > GA_{54}$. $13 > 2\beta$; $GA_1 > GA_{34}$. $13 > 3\beta$; ($GA_{19} > GA_{36}$), $GA_{53} > GA_{14}$, $GA_5 > GA_7$, $GA_{20} > GA_4$, ($GA_{17} > GA_{13}$). Some examples shown here with the parenthesis are the exception only with C_{18} HPLC of C_{20} gibberellins (system 1). In these four HPLC systems 1β-hydroxyl gibberellins are in general more polar than 2β-hydroxyl gibberellins such as $GA_{54} > GA_{34}$, $GA_{55} > GA_8$.

Monohydroxygibberellins can be more polar than the dihydroxygibberellins depending on the locations of hydroxyl groups. In the C_{18} HPLC of free gibberellins (system 1), 12α-monohydroxygibberellin (GA_{31}) and 16α-monohydroxygibberellin (GA_{10}) are more polar than the dihydroxygibberellins (GA_{16}, GA_{27}, GA_{47}, GA_{34}). 13-Monohydroxygibberellin (GA_{20}) is more polar than the dihydroxygibberellins (GA_{27}, GA_{47}, GA_{34}). In the other three HPLC systems (Table I) 13-hydroxygibberellin (GA_{20}) is more polar than the dihydroxygibberellin (GA_{34}).

The polarity of the free gibberellins with different groups at the C-10 position in the C_{18} HPLC (system 1) is –CHO > –COOH > $-CH_3$, while in silica HPLC (system 3) –COOH > –CHO > $-CH_3$. The polarity of the methyl esters of gibberellins with different groups at the C-10 position is –CHO > $-COOCH_3$ > $-CH_3$ in both C_{18} and silica HPLC (systems 2 and 4). The examples are as follows: GA_{24} > GA_{25} > GA_{12}, GA_{19} > GA_{17} > GA_{53} and GA_{36} > GA_{13} > GA_{14}.

The polarity of the gibberellins with γ-lactone or δ-lactone at ring A in silica HPLC of both free gibberellins and methyl esters of gibberellins (systems 3 and 4) is in general δ-lactone > γ-lactone, such as GA_{37} > GA_4, GA_{15} > GA_9. The polarity of gibberellins in the C_{18} HPLC (systems 1 and 2) is γ-lactone > δ-lactone when there is no hydroxyl group at ring A, such as GA_9 > GA_{15}, GA_{20} > GA_{44}, and δ-lactone > γ-lactone when there is a hydroxyl group at C-3β position, such as GA_{37} > GA_4, GA_{27} > GA_{34}, GA_{38} > GA_1. This retention property of γ-lactone and δ-lactone has not been previously reported.

Gibberellins with a double bond are more polar than those without the double bond in both silica and C_{18} HPLC of both free gibberellins and the methyl esters of gibberellins (systems 1–4). The examples are as follows: GA_3 > GA_1, GA_7 > GA_4, GA_5 > GA_{20}. The C_{18} HPLC can separate these pairs of gibberellins better than the silica HPLC.

One or more of the HPLC systems described can be used sequentially either with preparative or analytical column to purify gibberellins from plant extracts. They also can be used for identification together with GC–MS or GC–SIM. In metabolism studies using radioactive precursors, the radioactive metabolites can be identified by co-chromatography with authentic standards using the four HPLC systems sequentially.

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