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Note

Adsorption and reversed-phase partition high-performance liquid chromatography of gibberellins

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We have previously described the separation of gibberellins in the form of their *p*-nitrobenzyl esters by high-performance liquid chromatography (HPLC) on a silver nitrate-impregnated silica column[1] and the identification of these derivatives by thin-layer chromatography on silver nitrate-impregnated silica plates[2]. In the course of applying these methods to a study of the biosynthesis of gibberellins by *Giberella fujikuroi*[3], we became aware of the gibberellin separation by reversed-phase HPLC accomplished by Barendse *et al.*[4]. Having found their method useful for our study, we have modified it for work with radioactive gibberellins[5] and also developed a new HPLC method, based on adsorption on untreated silica gel. The present report describes the new adsorption and the modified reversed-phase HPLC methods, both of which are useful complementary techniques for purifying radioactive gibberellins.

EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. The adsorption column was a 250 × 4.6 mm I.D. stainless-steel chromatography tube (Altex, Berkeley, CA, U.S.A.), packed with Zorbax BP-SIL (7–8 μm; DuPont, Wilmington, DE, U.S.A.). The reversed-phase column had the same dimensions, but it was packed with Zorbax BP-ODS (7–8 μm, DuPont). Both columns were packed in our laboratory. The packing method, detector, recorder, solvents[6], pump, and injection valve[7] were as previously described. The chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

Our results are summarized in Table I. Having double bonds, gibberellins can be detected at 203 nm near their $\lambda_{max.}$. In our work on the metabolism of gibberellins in *G. fujikuroi*[3], we have observed no significant interference at this wavelength from the contaminants in the acidic fraction of fungal extracts.

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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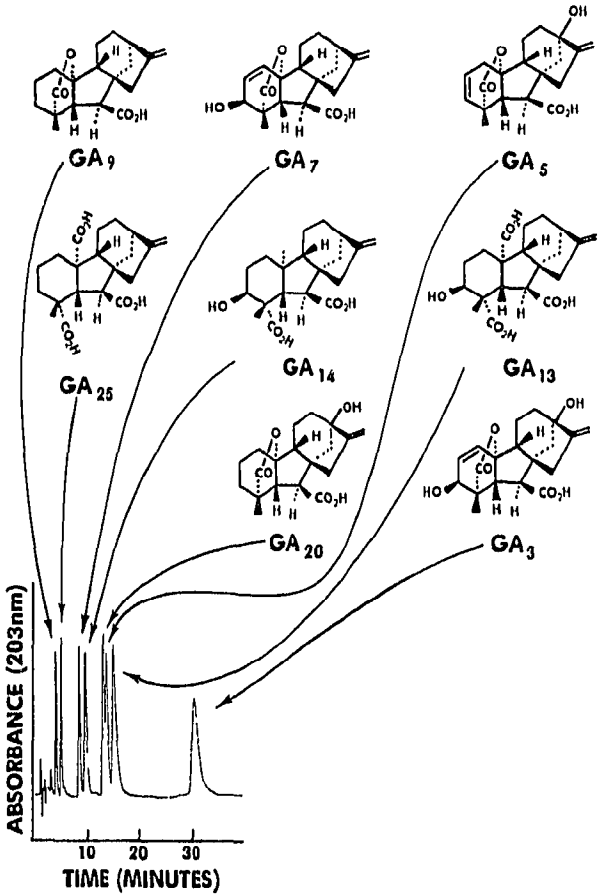

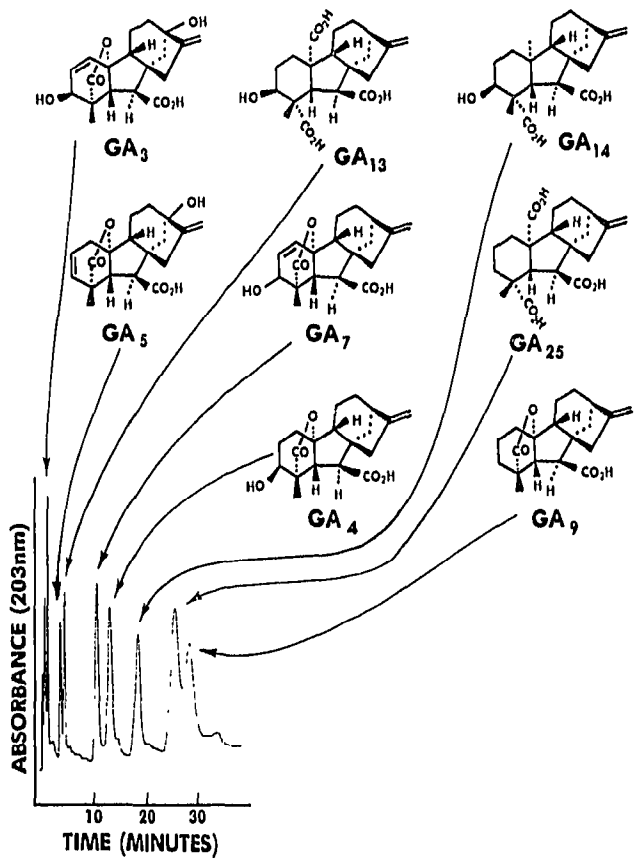

Fig. 1. Adsorption chromatogram of gibberellins. Between 1 μg (GA_9) and 5 μg (GA_3) of gibberellins, dissolved in about 50 μl of the eluent, were chromatographed on a column of Zorbax BP-SIL, 250 × 4.6 mm I.D. Eluent, *n*-hexane–ethanol–acetic acid (93:7:0.05); flow-rate, 2 ml/min; pressure, 1000 p.s.i.; detector, 203 nm, range 0.2, time constant 1.0; recorder, speed 12 cm/h, span 10 mV.

Fig. 2. Reversed-phase partition chromatogram of gibberellins. Between 2 μg (GA_3) and 10 μg (GA_9) of gibberellins, dissolved in about 50 μl of the eluent, were chromatographed on a columns of Zorbax BP-ODS, 250 × 4.6 mm I.D. Eluent, methanol–water–formic acid (55:45:0.05); pressure, 1000 p.s.i.; other conditions as in Fig. 1.

TABLE I

RETENTION TIMES OF GIBBERELLINS

Conditions: 1: see Fig. 1; 2: see Fig. 2; 3: see Fig. 3; 4: see Fig. 2, except that acetonitrile–water–formic acid (30:70:0.05) was used as the eluent; 5: see Fig. 2, except that acetonitrile–water–formic acid (20:80.0.05) was used as the eluent.

Gibberellin	*Retention time (min)*				
	1	*2*	*3*	*4*	*5*
GA_9	4	28.5		>40*	
GA_{25}	5	25.75		15.25*	
GA_4	8.5	13		16.25	
GA_7	8.5	10.5		15.25	
GA_{14}	9.5	18.25		14.25	
GA_{20}	12.75	4.5	23	5	21.75
GA_5	13.5	4	19.25	5	20.5
GA_{13}	15	4.5	26.5	3.5	18.5
GA_1	29	1.75	5.5	1.75	4.5
GA_3	30.5	1.75	4.5	1.75	4.5

* GA_9 and GA_{25} are eluted at 14 and 4.5 min, respectively, when acetonitrile–water–formic acid (40:60:0.05) is used as the eluent.

The eluents used contained 0.05% of glacial acetic acid in the adsorption HPLC and 0.05% of 88% formic acid in reversed-phase partition HPLC as ionic suppressors. Both acids can be removed easily by evaporation, while the phosphate buffer used by Barendse *et al.*[4] cannot. Moreover, because we use isocratic elution, there is no baseline shift and no gradient maker is required.

The degree of hydroxylation is the most important factor in determining the elution order of gibberellins in both adsorption and reversed-phase partition HPLC, as shown in Table I. Thus, the dihydroxy compounds GA_1 and GA_3 are more polar than the monohydroxy compounds GA_4 and GA_{20}, and the latter compounds are more polar than GA_9 and GA_{25}, which have no hydroxyl groups. The location of the hydroxyl groups is also an important factor. Thus, the C-13 hydroxylated gibberellin, GA_{20}, is more polar than its C-3 hydroxylated isomer, GA_4, in both adsorption and reversed-phase partition HPLC.

Fig. 1 shows the separation of eight gibberellins in one chromatogram by adsorption HPLC and isocratic elution. Pairs of gibberellins, differing from each other only by the presence or absence of a double bond were not as well separated as by argentation HPLC[1]. GA_4 and GA_7 were inseparable by adsorption HPLC, but the pairs GA_5/GA_{20} and GA_1/GA_3 were partially resolved (Table I). Better resolution was obtained by reversed-phase partition HPLC (Table I). Where they separated, the gibberellin having a double bond was more polar than the saturated analogue.

The gibberellins in Table I are arranged in the order of elution in adsorption HPLC. This order is not strictly reversed in reversed-phase partition HPLC. Also, the order of elution is not precisely the same in the methanol–water and acetonitrile–water systems. Thus, the three HPLC methods we have described complement each other and enable us to identify all the gibberellins available to us. These HPLC systems

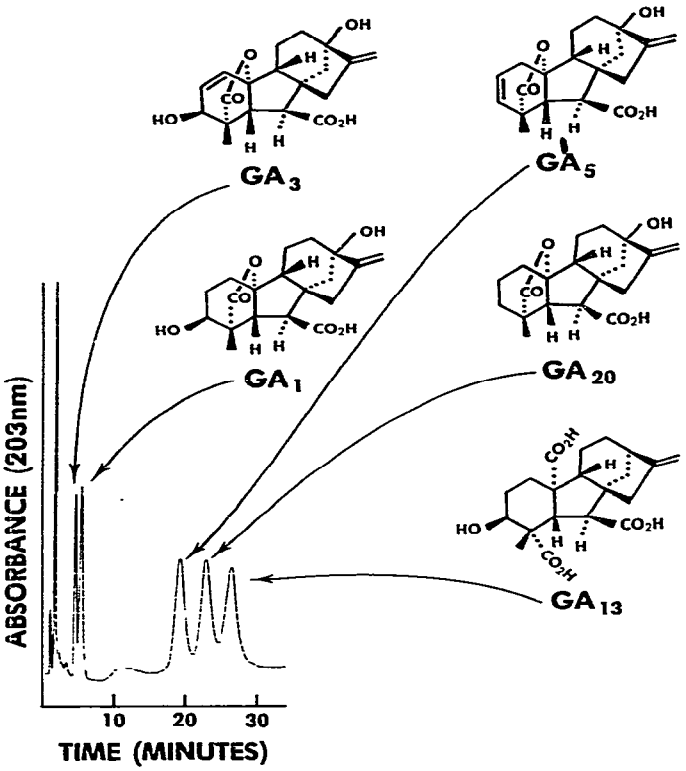

Fig. 3. Reversed-phase partition chromatogram of some more polar gibberellins. Conditions as in Fig. 2, except that methanol–water–formic acid (40:60:0.05) was used as the eluent.

are also suitable for purifying gibberellins prior to mass spectrometry. The detection limit of the method is *ca.* 0.1 μg and the recovery of radioactive GA_9-[^{14}C-17][5] was found to be 95%.

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