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ANALYSIS OF GIBBERELLINS AND GIBBERELLIN CONJUGATES BY ION-SUPPRESSION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

EINAR JENSEN
Institute of Biology and Geology, University of Tromsö, N-9001 Tromsö (Norway)
and
ALAN CROZIER* and ANA MARIA MONTEIRO*
Department of Botany, The University, Glasgow G12 8QQ (U.K.)
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SUMMARY

Ion-suppression reversed-phase high-performance liquid chromatography of 42 gibberellins and 20 gibberellin glucosides and glucosyl esters has been investigated using a C_{18} support eluted isocratically with a range of methanol concentrations in aqueous phosphoric acid at pH 3.0. Detection was with an absorbance monitor operating at 208 nm. The data obtained enables correlations to be made between chromatographic retention properties and gibberellin structure.

INTRODUCTION

Since the first reports on the analysis of gibberellins (GAs) by high-performance liquid chromatography (HPLC) in the 1970s[1–4] the technique has been used with ever increasing frequency in GA research and is now a routine procedure in many laboratories. In metabolism studies, reversed- and normal-phase HPLC have been used in conjunction with a radioactivity monitor[5] to analyse radiolabelled GAs as either underivatized structures[6–10] or as benzyl[2,3] or methoxycoumaryl esters[11]. However, by far the most extensive use of HPLC has been as a reversed-phase purification step for endogenous extracts[12–14] or samples containing ^2H- or ^{13}C-labelled GAs[15,16], prior to methylation, silylation and analysis by combined gas chromatography–mass spectrometry. Despite the widespread use of this technique there are only a few reports on the retention properties of GAs and GA conjugates on reversed-phase supports[17–21]. The most comprehensive investigation has been that of Koshioka *et al.*[21]. In this useful study detection of some of the GAs and their conjugates involved collecting fractions for subsequent analysis by bioassay and in other instances retentions were extrapolated from the data of Jones *et al.*[19]. As a consequence, chro-

* Permanent address: Departmento de Fisiologia Vegetal, Universidade de Campinas, 13100 Campinas, São Paulo, Brazil.

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matographic resolution was affected adversely and the relative retentions of many GAs, especially those with similar k' values, can be no more than approximate.

This paper reports on ion-suppression reversed-phase HPLC of a range of GAs and GA conjugates on a 5-μm C_{18} support. Chromatographic resolution was maintained through the use of isocratic rather than gradient elution and in all instances detection was on-line with an absorbance monitor operating at 208 nm.

EXPERIMENTAL

High-performance liquid chromatography

A Waters Assoc. liquid chromatograph was used which consisted of Model 510 and 6000A pumps, a Model 680 automated gradient controller, a Model U6K injector and a Lambda-Max Model 480 LC spectrophotometer, operating at 208 nm, linked to a Hewlett-Packard Model 3390A integrator. Ion-suppression reversed-phase HPLC utilised a 250 × 4.6 mm I.D. 5-μm Supelcosil LC 18 column eluted isocratically at a flow-rate of 1.0 ml min^{-1} with varying ratios of methanol in aqueous phosphoric acid at pH 3.0.

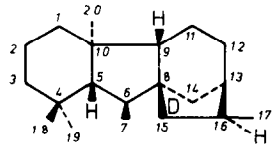
Gibberellins and gibberellin conjugates

Standards of GA_1, GA_{3-10}, GA_{12}, GA_{12}-aldehyde, GA_{13-24}, GA_{26}, GA_{28-31}, GA_{33-35}, GA_{37-41}, GA_{44}, GA_{47}, GA_{51} and GA_{53} (see Fig. 1) and the conjugates, GA_1-3-O-glucoside (gluc), GA_1-13-O-gluc, GA_1-glucosyl ester (GE), GA_3-3-O-gluc, GA_3-13-O-gluc, GA_3-GE, GA_4-GE, GA_5-13-O-gluc, GA_5-GE, GA_7-3-O-gluc, GA_7-GE, GA_8-2-O-gluc, GA_{20}-13-O-gluc, GA_{20}-GE, GA_{26}-2-O-gluc, GA_{29}-2-O-gluc, GA_{35}-11-O-gluc, GA_{37}-GE, GA_{38}-GE and gibberellenic acid-3-O-gluc (see Fig. 2), were dissolved in methanol at concentrations which enabled 5-μl volumes to be analysed with absorbance monitor attenuation at 0.05–0.1 a.u.

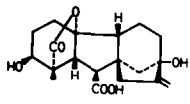
RESULTS AND DISCUSSION

Retention data obtained with ion-suppression reversed-phase HPLC of GAs are presented in Table I. The information in Table II can be used in conjunction with the data in Table I as a basis for assessing the influence of oxidation at C-20 on GA retention properties. In the case of deoxy- and 3β- and 3β,13-hydroxylated GAs the elution order is 20-CHO > 20-COOH > δ-lactone = γ-lactone > 20-CH_3. γ- and δ-lactonic GAs have similar, intermediate retentions with their elution sequence being determined by the substituent hydroxyl groups. 20-CHO GAs have the shortest retentions, usually eluting marginally before their 20-COOH analogues, while 20-CH_3 GAs are the most highly retained structures. However, this elution profile does not apply to 13-hydroxylated GAs as the aldehyde, GA_{19}, and its carboxylated analogue GA_{17} are well separated and elute after the γ- and δ-lactones GA_{20} and GA_{44}.

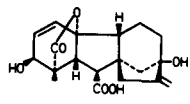
The effect on HPLC retentions of hydroxyl groups on the *ent*-gibberellane skeleton can be gauged by examining the elution patterns of the following pairs of GAs: GA_{16}/GA_4 (1α-hydroxylation); GA_{40}/GA_9, GA_{47}/GA_4 (2α-hydroxylation); GA_8/GA_1, GA_{27}/GA_{37}, GA_{29}/GA_{20}, GA_{34}/GA_4, GA_{51}/GA_9 (2β-hydroxylation);



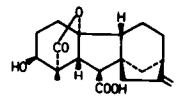
ent-gibberellane



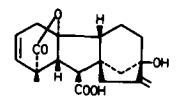
GA_1



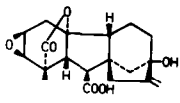
GA_3



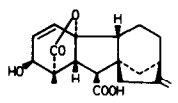
GA_4



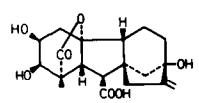
GA_5



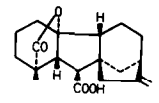
GA_6



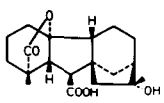
GA_7



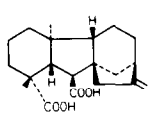
GA_8



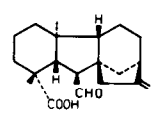
GA_9



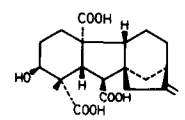
GA_{10}



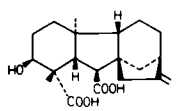
GA_{12}



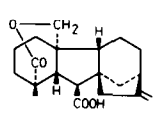
GA_{12} aldehyde



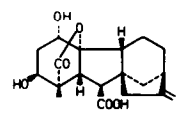
GA_{13}



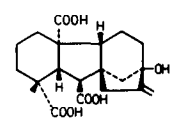
GA_{14}



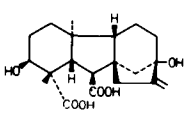
GA_{15}



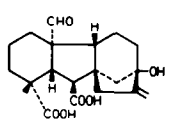
GA_{16}



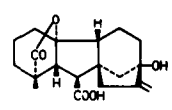
GA_{17}



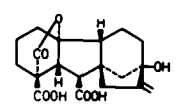
GA_{18}



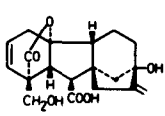
GA_{19}



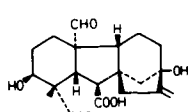
GA_{20}



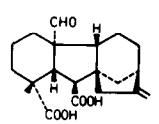
GA_{21}



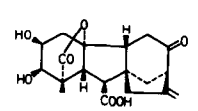
GA_{22}



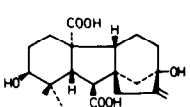
GA_{23}



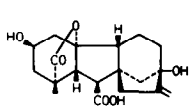
GA_{24}



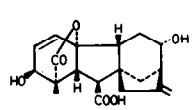
GA_{26}



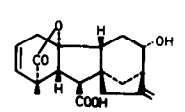
GA_{28}



GA_{29}



GA_{30}



GA_{31}

Fig. 1.

(*Continued on p. 380*)

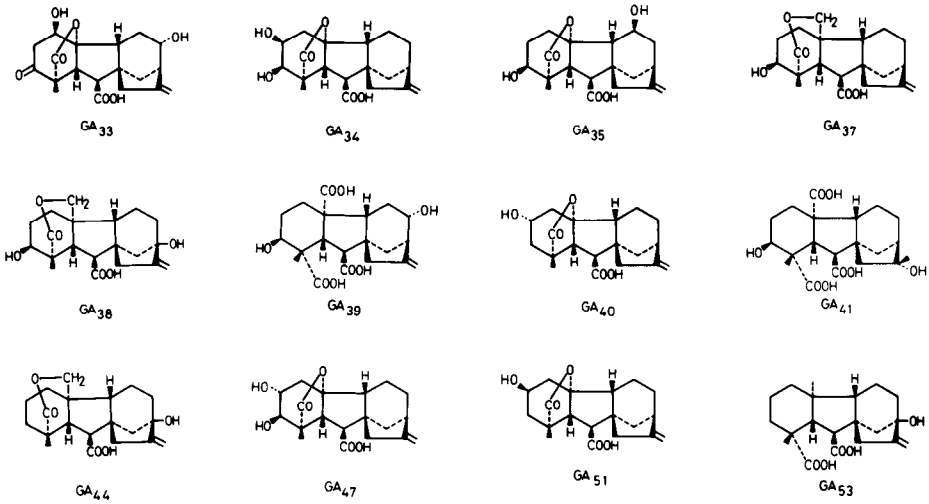


Fig. 1. *Ent*-Gibberellane skeleton and GA structures.

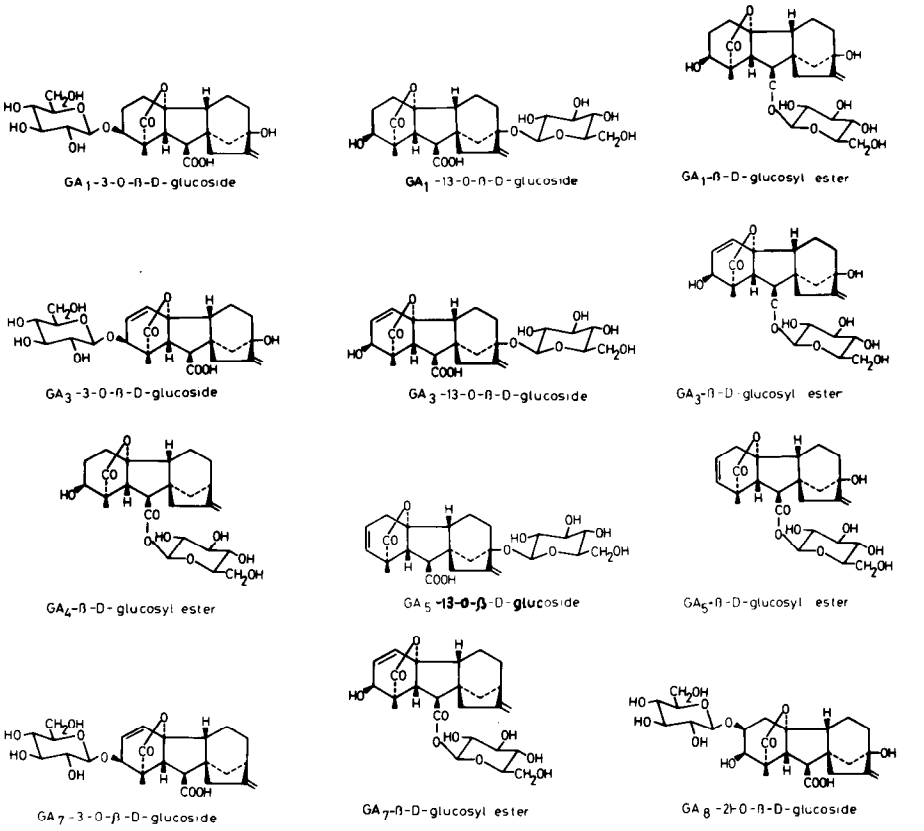


Fig. 2.

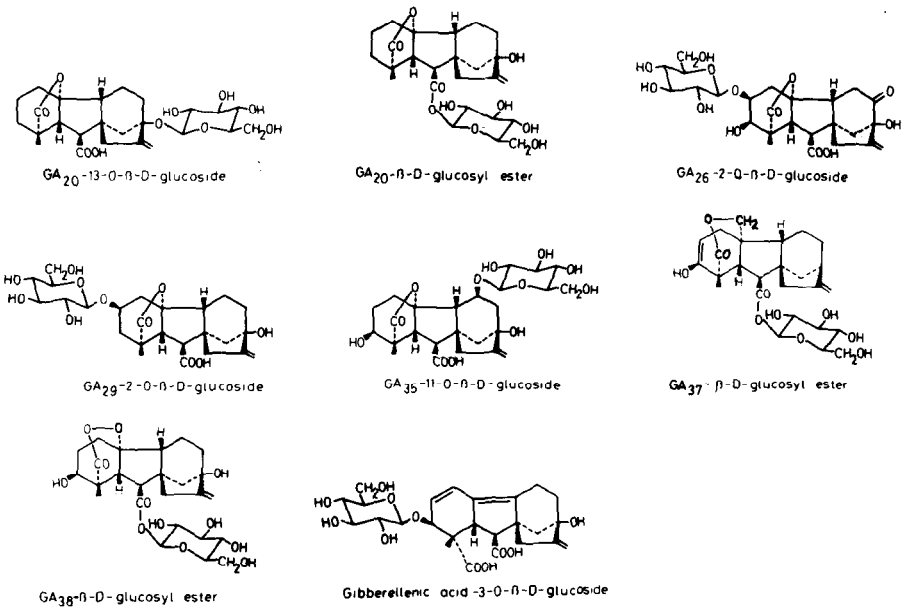


Fig. 2. Structures of GA conjugates.

GA_4/GA_9, GA_8/GA_{29}, GA_{14}/GA_{12}, GA_{36}/GA_{24}, GA_{37}/GA_{15} (3β-hydroxylation); GA_{35}/GA_4 (11β-hydroxylation); GA_{30}/GA_7, GA_{39}/GA_{13} (12α-hydroxylation); GA_1/GA_4, GA_3/GA_7, GA_{19}/GA_{24}, GA_{20}/GA_9, GA_{44}/GA_{15} (13-hydroxylation); GA_{10}/GA_9, GA_{41}/GA_{13} (16α-hydroxylation).

The more hydroxyl groups the less retained the GA. The degree to which an hydroxyl group enhances elution is dependent upon its point of attachment to the GA molecule. Although there are occasional exceptions, hydroxylation of the C- or D-rings at the 11β-, 12α, 13- and 16α-positions reduces retention to a greater extent than the introduction of an hydroxyl group to the 1α-, 2α-, 2β- and 3β-positions of the A-ring. In general, 3β-hydroxylation has less affect on GA retentions than hydroxylation at other loci.

The elution of GA_{12} and GA_{12}-aldehyde indicates that the presence of a 7-CHO rather than a 7-COOH group increases the retention of C_{20}-GAs. The data in Table I on GA_3/GA_1, GA_7/GA_4 and GA_5/GA_{20} show that both $\Delta^{1,2}$ and $\Delta^{2,3}$ GAs elute earlier than their saturated derivatives while the elution of GA_{22}/GA_5 and GA_{21}/GA_{20} demonstrate that oxidation of the 18-CH_3 group to CH_2OH and COOH functions also results in decreased retentions.

The reversed-phase HPLC retentions of twenty GA conjugates are presented in Table III. Comparison of these data with those in Table I show that all the conjugates are well separated from their respective aglycones. In all instances the elution order was GA-13-O-gluc > GA-3-O-gluc > GA-GE > GA. 11-O- and 2-O-glucosides also eluted more rapidly than the corresponding free GA. The resolution is thus somewhat better than that obtained by Koshioka *et al.*[21] who reported only minimal separation of GAs and their glucosyl ester conjugates.

TABLE I

ION SUPPRESSION REVERSED-PHASE HPLC RETENTION PROPERTIES OF GIBBERELLINS

Gibberellins analysed on a 250 × 4.6 mm I.D. 5-μm Supelcosil LC 18 column eluted isocratically at 1 ml min^{-1} with methanol in aqueous phosphoric acid at pH 3.0. Detection with an UV absorbance monitor at 208 nm. Data expressed as retention times in min.

	Methanol (%)												
	20	*25*	*30*	*35*	*40*	*45*	*50*	*55*	*60*	*65*	*70*	*75*	*80*
GA_8	11.5	8.2											
GA_{29}	14.8	10.3	8.2										
GA_{39}	18.0	11.5	8.3										
GA_{33}	17.8	11.9	9.0										
GA_{30}	21.7	13.1	9.6										
GA_{23}	26.3	16.0	11.3										
GA_{28}		18.4	12.2	8.0									
GA_{38}		19.1	12.4	8.2									
GA_{41}		21.7	13.7	9.1									
GA_{26}		22.3	14.0	9.1									
GA_3		23.0	14.0	9.2									
GA_1		26.5	16.5	10.8									
GA_6			24.6	15.2	10.1								
GA_{18}			24.8	15.4	10.0								
GA_{35}			24.2	15.8	10.5								
GA_{22}			33.7	19.8	12.1	8.1							
GA_{21}				22.0	14.7	9.7							
GA_{31}				28.7	16.4	10.6							
GA_5					24.3	14.6	9.6						
GA_{10}					24.9	15.3	9.8						
GA_{16}					25.6	16.2	11.0	8.2					
GA_{20}					28.0	17.2	11.2	8.4					
GA_{27}					31.2	19.3	11.7	9.1					
GA_{47}					30.5	19.5	12.6	9.4					
GA_{36}						22.1	13.8	10.1	8.1				
GA_{13}						22.6	14.3	10.0	8.0				
GA_{40}						23.0	14.3	10.0	8.4				
GA_{44}						25.7	15.2	11.0					
GA_{19}						32.9	19.0	13.4	9.5				
GA_{34}						32.0	21.0	14.9	10.7				
GA_{51}							22.8	15.3	10.8				
GA_{17}							24.8	16.2	11.0				
GA_{37}							24.2	16.8	11.6				
GA_7							30.0	20.6	13.3	8.4			
GA_4							36.8	25.0	15.3	9.7			
GA_{14}								38.0	21.6	12.6	8.1		
GA_{53}										13.9	9.5		
GA_{24}								42.2	23.6	13.8	10.0		
GA_9								43.0	24.6	15.0	10.4		
GA_{15}									26.7	17.6	11.1		
GA_{12}											25.5	17.5	11.5
GA_{12}-ald											33.0	21.5	14.1

TABLE II

GIBBERELLIN STRUCTURES BASED ON VARIATION IN THE OXIDATION STATE AT C-20 AND THE PRESENCE OR ABSENCE OF HYDROXYL GROUPS AT C-3 AND C-13

Oxidation at C-20		*Hydroxylation*			
		None	*3β*	*13*	*3β,13*
C_{20}-GAs	CH_3	GA_{12}	GA_{14}	GA_{53}	GA_{18}
	δ-lactone	GA_{19}	GA_{37}	GA_{44}	GA_{38}
	CHO	GA_{24}	GA_{36}	GA_{19}	GA_{23}
	COOH	GA_{25}	GA_{13}	GA_{17}	GA_{28}
C_{19}-GAs	γ-lactone	GA_9	GA_4	GA_{20}	GA_1

Although reversed-phase HPLC provides good separation of individual GAs from their conjugates, it does not separate GAs as a group from GA conjugates. This can however be achieved by steric exclusion chromatography[22] which simplifies greatly the use of HPLC-based techniques in the analysis of radiolabeled GAs and GA conjugates in biosynthesis and metabolism studies[11].

TABLE III

ION SUPPRESSION REVERSED-PHASE HPLC RETENTION PROPERTIES OF GIBBERELLIN CONJUGATES

Gibberellin conjugates analysed on a 250 × 4.6 mm I.D. 5-μm Supelcosil LC 18 column eluted isocratically at 1 ml min^{-1} with methanol in aqueous phosphoric acid at pH 3.0. Detection with an UV absorbance monitor at 208 nm. Data expressed as retention times in min.

	Methanol (%)									
	10	*15*	*20*	*25*	*30*	*35*	*40*	*45*	*50*	*55*
GA_{29}-2-O-Gluc	19.9	11.2	7.4							
GA_8-2-O-Gluc	23.8	12.6	8.0							
Gibberellenic acid-2-O-Gluc		25.7	13.6	8.9						
GA_3-13-O-Gluc		26.1	13.9	8.9						
GA_1-13-O-Gluc		30.0	15.9	10.0						
GA_3-3-O-Gluc			22.1	12.8	8.4					
GA_1-3-O-Gluc			23.3	13.5	8.7					
GA_{38}-GE			23.9	14.5	9.2					
GA_{26}-2-O-Gluc			25.1	15.6	9.6					
GA_3-GE			26.4	16.0	9.8					
GA_1-GE			31.1	17.8	11.2					
GA_{35}-11-O-Gluc				19.6	11.3	8.0				
GA_5-13-O-Gluc					24.4	14.5	9.2			
GA_{20}-13-O-Gluc					25.2	15.2	9.8			
GA_5-GE					44.9	23.1	13.1			
GA_{20}-GE						24.3	14.0	9.4		
GA_{37}-GE						25.3	17.3	11.2		
GA_7-3-O-Gluc							20.1	12.6	8.4	
GA_4-GE							24.5	15.3	9.8	
GA_7-GE								22.6	13.6	8.9

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