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Note

Partition chromatography of gibberellins and related diterpenes on columns of Sephadex LH-20

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Many chromatographic methods have been described for the separation of the gibberellin (GA) group of plant hormones. Partition chromatography has proved useful particularly for closely related GAs*, such as the pairs GA_1/GA_3 and GA_4/GA_7, which are difficult to separate by adsorption chromatography. For example, MacMillan and Suter[1] and Kagawa *et al.*[2] showed that these pairs can be separated by partition thin-layer chromatography. The pair GA_1/GA_3 can also be separated on a silica gel column by partitioning between pH 6.2 phosphate buffer and diethyl ether although the method is rather cumbersome[3]. Partition chromatography on a silica gel column using formic acid–ethyl acetate–hexane, was suggested by Powell and Tautvydas[4]. The latter method was evaluated by Durley *et al.*[5], who reported that the resolution was very sensitive to the grade of silica gel used. Pitel *et al.*[6] and Vining[7] have described excellent procedures for the separation of the pairs GA_1/GA_3 and GA_4/GA_7, and some of their closely related derivatives, by partition chromatography on columns of Sephadex G-25. However, Durley *et al.*[5] have indicated that these Sephadex columns do not separate other groups of GAs.

For biosynthetic studies of fungal GAs with mutants of *Gibberella fujikuroi* we required chromatographic methods which would separate metabolites of widely differing polarities. Using partition chromatography in which the aqueous phase is held in Sephadex LH-20 we have developed such a method covering the wide range of polarity from *ent*-kaur-16-ene to GA_3. This wide range column is supplemented by similar procedures for metabolites of relatively low polarity (narrow range column) and for non-polar metabolites (non-polar range column).

EXPERIMENTAL

Solvent systems

Bi-phasic solvent systems were devised starting from a mixture of light petroleum (b.p. 60–80°), methanol, and acetic acid. To this mixture were added ethyl acetate and sufficient water to maintain two phases until a suitable partition coefficient ($K_{a/o}$) between the aqueous and organic phases was obtained for a selected standard.

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* For structures of GA_1 to GA_{29} see ref. 8; for GA_{30} to GA_{36} see ref. 9; and for GA_{37} and GA_{38} see ref. 10.

For the wide range column a mixture of light petroleum–ethyl acetate–acetic acid–methanol–water (100:80:5:40:7) was selected in which the standard, GA_3, had a $K_{a/o}$ of 8 corresponding to 16 column elution volumes.

For the narrow range column, a mixture of light petroleum–ethyl acetate–acetic acid–methanol–water (50:15:10:10:2) was chosen in which the standard *ent*-7α-hydroxykaurenoic acid (4) had a $K_{a/o}$ of 3.25 giving 6 column elution volumes.

For the non-polar range column, the solvent mixture used was light petroleum–acetic acid–methanol (100:1:40).

The $K_{a/o}$ values were determined by gas–liquid chromatography (GLC) of methylated aliquots from each phase.

Packing of columns

Sephadex LH-20 was swollen in the aqueous phase of the solvent mixture for 3 h at 20°. Excess solvent was poured off and the thick slurry was added to a glass column (100 × 1.5 cm) containing aqueous phase (20 ml) and fitted with an extension column (100 × 1.5 cm). The gel was then vigorously dispersed by means of a plunger consisting of a jointed stainless-steel rod (0.4 cm diameter) and terminating in a stainless-steel disc (1 cm diameter). The column was eluted with aqueous phase until the Sephadex had formed a firm bed. At this stage the evenness of packing was tested by applying azobenzene in aqueous phase (100 μl) to the top of the gel bed from which excess aqueous phase had been drained off. If unsatisfactory the column could be readily re-packed at this stage.

The organic phase was then passed through the column until no more aqueous phase was eluted. The column packing was then re-tested by adding azobenzene in aqueous phase (100 μl) carefully through 3 mm of organic phase onto the column. Elution was then continued with organic phase. This technique, which was also used for mixtures of GAs and other samples dissolved in the minimum of aqueous phase, preserved the packing at the top of the column while applying the sample in a narrow band. For larger amounts of material, the sample was dissolved in aqueous phase and absorbed in Sephadex which was placed on the top of the column.

Wide range column. Sephadex LH-20 (75 g) gave a column bed (145 × 1.5 cm) with a column volume of *ca.* 75 ml and an unrestricted flow-rate of 50 $ml \cdot h^{-1}$.

Narrow range column. Sephadex LH-20 (50 g) was used giving a column bed (96 × 1.5 cm) with a column volume of 50 ml and a flow-rate of 100 $ml \cdot h^{-1}$.

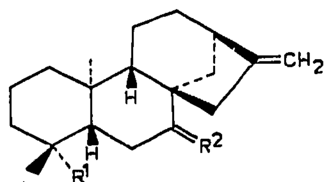
Non-polar range column. Column bed (180 × 1.5 cm) of Sephadex LH-20 (100 g) gave a column volume of *ca.* 100 ml and a flow-rate of 30 $ml \cdot h^{-1}$.

For each column, a 1-m extension tube was used to provide a head of organic phase.

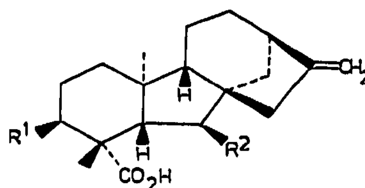
Elution of standard compounds

The mixtures, detailed below for each column, contained 160–330 μg of each compound. Fractions (10 ml) were collected and evaporated to dryness after addition of toluene to remove the acetic acid by azeotropic distillation. Aliquots were methylated and analysed by GLC except for [2-^{14}C]-mevalonic acid lactone (MVL) which was detected by liquid scintillation counting.

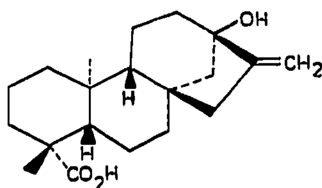
Wide range column. The following 29 compounds were used: *ent*-kaurene (1), *ent*-kaurenoic acid (3), *ent*-7α-hydroxykaurenoic acid (4), GA_{12} (6), GA_{12}-aldehyde



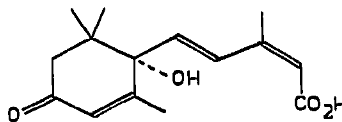
(1) $R^1 = CH_3$, $R^2 = H_2$
(2) $R^1 = CH_2OH$, $R^2 = H_2$
(3) $R^1 = CO_2H$, $R^2 = H_2$
(4) $R^1 = CO_2H$, $R^2 = \beta OH, H$
(5) $R^1 = CO_2H$, $R^2 = \alpha OH, H$



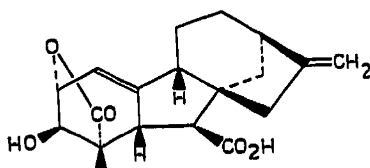
(6) $R^1 = H$, $R^2 = CO_2H$
(7) $R^1 = H$, $R^2 = CHO$
(8) $R^1 = H$, $R^2 = CH_2OH$
(9) $R^1 = OH$, $R^2 = CO_2H$
(10) $R^1 = OH$, $R^2 = CHO$



(11)



(12)



(13)

(7), GA_{12}-alcohol (8), GA_{14}-aldehyde (10), GA_9, steviol (11), abscisic acid (12), [2-^{14}C]-MVL, GA_1, GA_2, GA_3, GA_4, GA_5, GA_7, iso-GA_7 (13), GA_8, GA_{13}, GA_{14}, GA_{15}, GA_{16}, GA_{17}, GA_{24}, GA_{25}, GA_{28}, GA_{36}, and GA_{37}.

Narrow range column. The first eight compounds listed above were used. The mixture of *ent*-7α- and -7β-hydroxykaurenoic acids (4) and (5) was examined in a separate experiment.

Non-polar range column. A mixture of *ent*-kaurene (1), *ent*-kaurenol (2), *ent*-kaurenoic acid (3) and cholesterol was used.

Gas–liquid chromatography

For the less polar fractions, glass columns (152.5 cm × 3.2 mm I.D.) were packed with 2% SE-33 on Gas-Chrom Q (80–100 mesh) and temperature programmed from 200° at 5° min^{-1} with a nitrogen flow-rate of 30 $ml \cdot min^{-1}$. For more polar fractions, glass columns (152.5 cm × 6.4 mm I.D.) were packed with 3% QF-1 on Gas-Chrom Q (80–100 mesh); the temperature was held at 210° for 15 min and then programmed at 5° min^{-1} with a nitrogen flow-rate of 70 $ml \cdot min^{-1}$. For both columns a flame ionization detector (FID) was used.

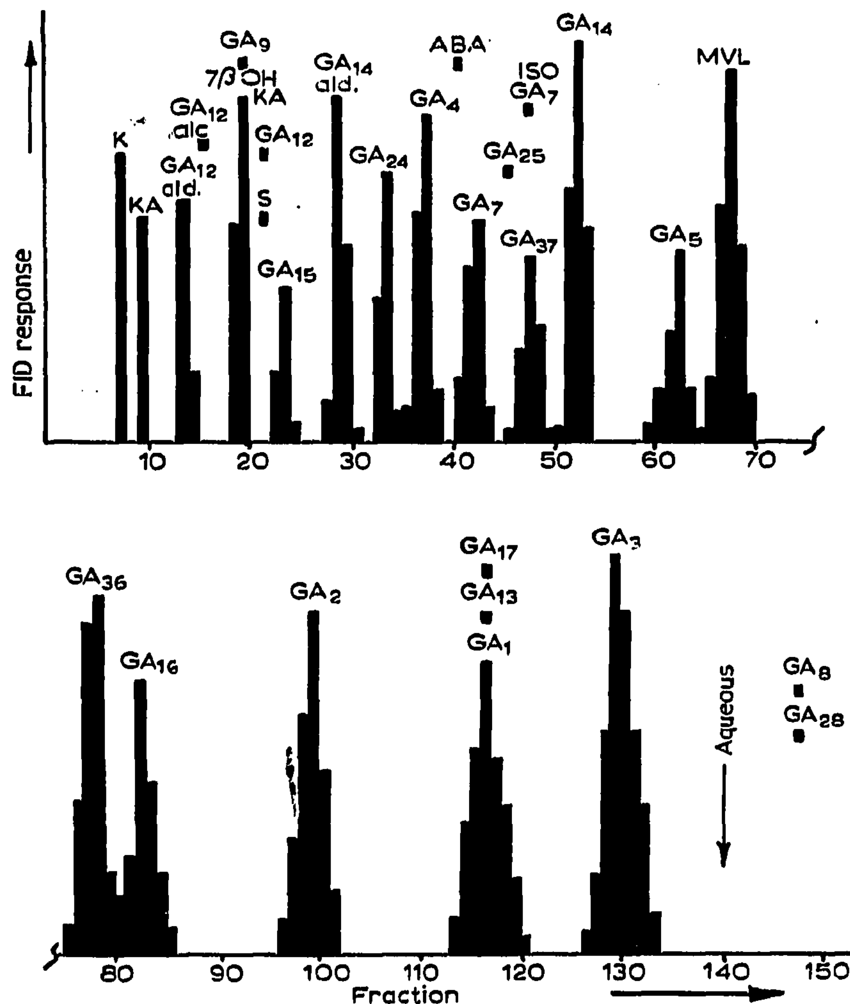


Fig. 1. Wide range column. Separation of mixture of standards on Sephadex LH-20 column (147 × 1.5 cm) with the solvent system, light petroleum–ethyl acetate–acetic acid–methanol–water (100:80:5:40:7). Number of theoretical plates for GA_3, 5,500. K = *ent*-kaurene (1); KA = *ent*-kaurenoic acid (3); GA_{12} ald. = GA_{12}-aldehyde (7); GA_{12} alc. = GA_{12}-alcohol (8); 7βOH KA = *ent*-7α-hydroxykaurenoic acid (4); 7αOH KA = *ent*-7β-hydroxykaurenoic acid (5); S = steviol (11); GA_{14} ald. = GA_{14}-aldehyde (10); ABA = abscisic acid (12).

RESULTS AND DISCUSSION

The separation, obtained with the wide range column, is shown in Fig. 1. The technique of packing the column in aqueous phase is superior to that using the organic phase[6]; it is more convenient and gives a more even packing. The resolution of this column was 5,500 theoretical plates for GA_3, compared with 800 for GA_3 calculated for the GA_1/GA_3 columns of Pitel *et al.*[6] from their data. Despite the high resolution, there are some overlaps. For example the group GA_{13}, GA_{17}, and GA_1 and the group GA_{25}, GA_{37}, and iso-GA_7 (13) are not resolved. Nor are GA_7 and abscisic acid (12).

The condensed region between GA_{12}-aldehyde (7) and GA_{14}-aldehyde (10) from the wide range column can be expanded by using the narrow range column (Fig. 2). This column provides also an excellent separation of the 7α- and 7β-hydroxy epimers (4) and (5) of *ent*-kaurenoic acid; this separation is difficult to achieve by adsorption chromatography on columns or thin layers of silica gel. The narrow range column has proved useful for the purification of the [6-^{3}H]-derivatives of GA_{12} (6), GA_{12}-aldehyde (7), and GA_{12}-alcohol (8)[11] for biosynthetic studies.

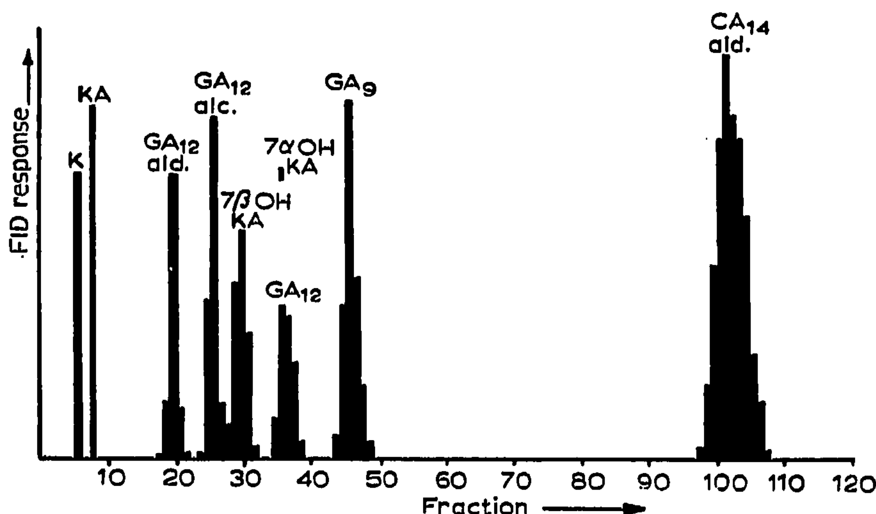


Fig. 2. Narrow range column. Separation of mixture of standards on Sephadex LH-20 column (96×1.5 cm) with solvent system, light petroleum–ethyl acetate–acetic acid–methanol–water (50:15:10:10:2). Number of theoretical plates for GA_{14}-aldehyde, 1,500. Abbreviations are explained in the legend of Fig. 1.

The column for non-polar compounds provides complete separation of *ent*-kaurene (1), *ent*-kaurenol (2), and *ent*-kaurenoic acid (3) which are eluted in the first few fractions from the wide range column. This column also separates these diterpenes from cholesterol.

The capacity of these columns depends upon the nature of the mixture and upon the solubility of the mixture in the organic phase. It also depends upon the acceptable resolution. The number of theoretical plates quoted above, were obtained with loadings of 5–6 mg of mixtures of pure standards. With these columns 100–200-mg loadings have been used with little loss in resolution. In cases where lower resolution is acceptable, considerably greater loading can be used. For example 2.5 g of crude extract of metabolites from *G. fujikuroi* have been adequately resolved using a column diameter of 5 cm.

These columns have been evaluated principally for the separation of the diterpenoid metabolites of *G. fujikuroi* and examples of this application will be presented in forthcoming papers. However, preliminary experiments with crude plant extracts have shown that the wide range column effects useful purification of GA-containing fractions.

The Sephadex LH-20 columns described here, together with the Sephadex G-25 columns described by Pitel *et al.*[6], provide a comprehensive range of partition chromatographic procedures for the separation of GAs and related diterpenes.

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