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Purification and Separation of Plant Gibberellins from Their Precursors and Glucosyl Conjugates[1]

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ABSTRACT

A procedure using two small preparative columns (in sequence) of C_{18} reverse phase Bondapak B material with methanolic extracts of plant tissue (*Pisum sativum* L., *Malus domestica* Borkh., *Pimpinella anisum* L.) yields two fractions: (i) gibberellin (GA) precursors, and (ii) free GA/GA methyl esters (GA-Me)/GA glucosyl conjugates. The discrete separation of (iii) free GA/GA-Me from (iv) GA glucosyl conjugates is then accomplished by a combination of differential solvent solubility and SiO_2 partition chromatography. All fractions are almost pigment free, and appreciable dry weight purification was accomplished for the GA precursor and free GA/GA-Me fractions. Solvent volumes can be kept low, no buffer salts are introduced, and each fraction (i, iii, iv) can be subjected directly to preparative or analytical reverse phase C_{18} high performance liquid chromatography without recourse to solvent partitioning, and often without further purification.

Conventional methods for purification and isolation of GA[3] and GA glucosyl conjugates from plant tissues involve partitioning of aqueous solutions against various organic solvents at one or more steps throughout the procedure. There exists in the literature of the past two decades numerous examples of various partitioning solvents used with a wide variety of plant tissues to extract or purify a structurally diverse range of acidic GA (2, 4, 13, 16, 17, 21, 31) and GA glucosyl conjugates (11, 27, 34, and numerous references cited in 26). However, partitioning techniques, as useful as they have been, do have a number of drawbacks. They are time consuming, especially where additional partitioning steps may be required through the use of PVPP (11) and/or charcoal (4, 36) purification procedures. They require a knowledge of the partition coefficient of each GA/GA precursor/GA glucosyl conjugate in question, for each of the various solvent systems used, in order to avoid variable and significant losses (5). Partitioning exposes the rather labile GA/GA conjugates to extremes of pH for varying periods of time, and partitioning may introduce salts into the organic phase containing the GA/GA conjugates. Finally, GA precursors, GA, and GA glucosyl conjugates have wide and overlapping ranges of partition coefficients for any one solvent system (5, 11, 26, 27), making it impossible to design a simple partitioning sequence that will allow their resolution into discrete classes.

The above reasons have prompted us and others (1) to utilize a reverse phase C_{18} material[4] in an attempt to avoid the many problems of conventional solvent partitioning, the inconvenience of PVPP and charcoal purification columns, and still obtain extracts which are properly fractionated (*e.g.* GA separated from GA precursors and from GA glucosyl conjugates) and are pure enough for subsequent chromatography by preparative or analytical C_{18} HPLC prior to more definitive characterization and quantitation. An earlier technique (using C_{18} Sep-Paks[4]) will remove appreciable amounts of nonpolar substances, including pigments (1, 9), but as used by Barendse *et al.* (1) does not solve the problem of separating free GA from GA precursors or GA glucosyl conjugates. This inability to separate free GA (as a class) from their precursors and conjugates prior to reverse phase C_{18} preparative or analytical HPLC columns is exacerbated by the fact that free GA and their glucosyl conjugates have very similar chromatographic behavior on reverse phase C_{18} HPLC (14, 32).

In the present paper, we describe a method using two small preparative columns of C_{18} reverse phase material on Bondapak B[4] (C_{18}-PC) in combination with differential solvent solubility and SiO_2 partition column chromatography to yield three discrete fractions: (i) GA precursors such as kaurene, kaurenoic acid; (ii) acidic GA/GA-Me; and (iii) GA glucosyl conjugates.

MATERIALS AND METHODS

Plant Materials. Eleven-d-old seedlings of Alaska pea (*Pisum sativum* L.) were fed [^{3}H]GA_{20} (2.36 Ci/mmol) at 1.88 μCi/plant by spreading it on the first trifoliate leaf in 5 μl of 95% ethanol, and harvested 11 d later. One-year-old potted propagules (about 2 m in height) of apple (*Malus domestica* Borkh.) were fed [^{3}H]GA_4 (1.3 Ci/mmol) at 8 μCi/propagule by injecting it in 400 μl of 0.6% ethanol into the stem at about 1 m height. Leaf tissue was harvested 7 d later. Suspension cultures of somatic cells of anise (*Pimpinella anisum* L.) were fed [^{3}H]GA_4 (1.3 Ci/mmol) in aqueous solution in varying amounts and harvested 2, 204, and 348 h later (15).

C_{18} HPLC Columns, HPLC Conditions, and HPLC-RC Conditions. A Waters Associates reverse phase C_{18} μ-Bondapak column (3.9 mm × 30 cm) was used with gradient elution at 2 ml/min as described previously (14). The HPLC was a Waters Associates ALC/GPC R-401 liquid chromatograph with two model 6000 pumps, a model 660 solvent flow programmer, and a model U6K universal injector. The manually implemented linear gradient program was 0 to 10 min (pump A, 100%), 10 to 40 min (pump B, 0–70%), 40 to 50 min (pump B, 70%), 50 to 80 min (pump B, 100%); solvents, pump A: 10% MeOH in 1% acetic acid/H_2O, pump B: 100% MeOH (see also Figs. 2 and 3). The temperature was 22 to 25°C. A Berthold HPLC radioactivity

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[3] Abbreviations: C_{18}-PC, small preparative columns of 55- to 105-μm particle size C_{18} monochloroctadecylsilane material on Porasil B[4]; d.w., dry weight; EtOAc, ethyl acetate; GA, gibberellin(s); GA Me, GA methyl ester(s); MeOH, methanol; PVPP, polyvinylpolypyrollidone; RC, radiocounting.

[4] Waters Scientific Ltd., Mississauga, Ontario, Canada.

monitor (LB 503) with either a flow-through cell (300 μl) or a pump delivering a Triton-X:xylene:MeOH:PPO (330 ml:630 ml:150 ml:10 g) scintillant to a mixing cell (200 μl, ratio 1:4 [effluent volume:scintillant volume]) was used for some $^3H/^{14}C$ standards and extracts, and was set at time constant 0.4 min, threshold factor 1.00, background 30 cpm, peak reject 20 counts, ratemeter range 300 cpm.

Bioassay. The Tan-ginbozu dwarf rice micro-drop assay (18) modified in that 0.5 μl drops were applied, and measurement took place at hour 48, was used in serial dilution on each HPLC fraction (Fig. 4). For determination of GA_3 glucoside and GA_4 glucosyl ester, the Tan-ginbozu immersion assay (19) was utilized, an aliquot of the C_{18}-PC eluate being incorporated into 2 ml of 0.5% aqueous agar medium, on which 10 germinated seeds were planted. Length of the second leaf sheath was measured 6 d after planting.

Solvents. The H_2O for HPLC, hexane and EtOAc for SiO_2 partition columns, MeOH for extraction, and C_{18}-PC were purified by fractional distillation. The MeOH for HPLC was purchased as HPLC grade solvent. The HPLC solvents were filtered through 0.45-μm (HATF for H_2O) and 0.5-μm (FHUP for MeOH) pore size filters, respectively. The HPLC solvents were allowed to equilibrate, degassed, mixed, and during the elution of the column were maintained under vacuum over a magnetic stirrer to prevent gas accumulation. Extraction and C_{18}-PC solvents were reagent grade or distilled.

SiO_2 Partition Column. The procedure varied from that described earlier (3) in that the H_2O partition on the Woelm SiO_2[5] was made by gently rotating (15 rph) the container containing 500 g SiO_2 for 2 weeks with 20% H_2O by weight, the H_2O being added over 30 min. The SiO_2 was then allowed to equilibrate a further 7 d before use. The sample was dissolved in EtOAc:MeOH (1:1) or varying percentages of MeOH:H_2O (depending on its solubility) and loaded onto GFA paper discs (Whatman) or adsorbed onto a small amount of the SiO_2 and dried in a warm air stream, prior to placing onto the column. After completion of the gradient (HCOOH saturated, hexane:EtOAc), which elutes the free GA, the column was washed with at least 100 ml of 100% MeOH (or the discs and SiO_2 removed with air pressure, slurried in MeOH, and washed with excess MeOH on a Büchner funnel), to remove GA glucosyl conjugates. The MeOH wash of the SiO_2 was neutralized with 3 N NH_4OH, then taken to dryness *in vacuo* at 35°C prior to subsequent HPLC, either alone, or combined with the MeOH- and H_2O-soluble fractions from the C_{18}-PC (Fig. 1A and below).

Use of the C_{18}-PC Method for Authentic GA, GA Precursors, and GA Glucosyl Conjugates, and for Plant Extracts. C_{18} reverse phase Bondapak material[4] was used to build small columns, either in glass tubing or syringe barrels (i.d. 2 cm, length 1.5–2.5 cm, weight of C_{18} material optimized at 4 times sample d.w.). Freeze-dried (or fresh) tissue (0.5–1.0 g d.w.) was homogenized with 40 ml of MeOH (MeOH:H_2O, 80:20 v/v) and)pH 6.7) filtered through Whatman No. 1 filter paper. The 80% MeOH filtrate (pH 6.7) (or 40 ml solution containing the standard compound; Table I) was passed under pressure at 2 to 4 ml/min through the first C_{18}-PC, the column then being washed with an additional 20 ml of 80% MeOH. An additional 36 ml of H_2O was added to the combined eluates of 80% MeOH and the resultant 50% MeOH (pH 6.5) solution (96 ml) was passed through a second C_{18}-PC (dimensions as noted above). This second column was then washed with an additional 20 ml of 50% MeOH.

Subsequent elution of the second C_{18}-PC with 60 ml 100% MeOH will remove the GA precursors (*e.g.* kaurene and kaurenoic acid) retained on the column. The combined 50% MeOH eluates (116 ml), which will contain all of the free GA and GA glucosyl conjugates (see "Results"), were taken to dryness in a rotary evaporator flask at 35°C by the addition of excess absolute MeOH, or by removing most of the MeOH *in vacuo* at 35°C, and freezing and freeze-drying the residue. The dried residue of the 50% MeOH eluate was then extracted with 90 ml (30 ml × 3) of EtOAc:MeOH (1:1) to dissolve all of the free GA. The residue remaining thereafter was further extracted with 90 ml (30 ml × 3) of MeOH. The final residue was dissolved in 40 ml of H_2O, from which aliquots could be taken for radio- or bioassay.

Although the above technique of sequential solvent extraction of the dried residue of the 50% MeOH eluate from the second C_{18}-PC worked well (see "Results") for tissue amounts up to 1 g for most extracts, some extracts high in sugars may, when taken to dryness *in vacuo*, yield a residue that might occlude free GA during the above washing process. These residues can be dissolved in 98% MeOH (or dissolved first in a small amount of H_2O, MeOH then being added to make up a 98% solution). An equal volume of EtOAc is then added, yielding a flaky precipitate. This precipitate is then washed with two additional volumes of EtOAc:MeOH (1:1), the solution filtered and taken to dryness in vacuo. This residue is dissolved in minimal amounts of EtOAc:MeOH (1:1) followed by a few drops of 80% MeOH, and loaded onto the SiO_2 partition column as noted above. An alternative procedure, especially useful with extract residues from small tissue amounts (1.0 g or less), involves dissolving the entire residue from the second C_{18}-PC in a minimal amount of 80% MeOH, followed by EtOAc:MeOH, and applying it to the SiO_2 partition column as noted above. However, care must be taken not to overload the GFA paper discs or SiO_2 particles if occlusion effects with the hexane:EtOAc solvents of the SiO_2 partition column are to be avoided. Since all of the highly H_2O-soluble conjugates will be present on this SiO_2 partition column, an excess (about 200 ml) of absolute MeOH should be used when washing the column after completion of the hexane:EtOAc gradient. When extracts have been rich in [^{3}H]GA glucosyl conjugates of a highly polar nature (*e.g.* [^{3}H]GA_8-glucoside), we have used an 80% MeOH wash of the SiO_2 after the wash with absolute MeOH. This second wash yielded up to 10% additional GA conjugate radioactivity, and allowed for a quantitative recovery of radioactivity. However, significant amounts of SiO_2 are dissolved in 80% MeOH, and this may limit its further use.

Analytical or preparative C_{18} reverse phase HPLC was accomplished on the various fractions (after taking to dryness) as follows: (i) 100% MeOH eluate of the second C_{18}-PC (GA precursors fraction)—dissolve in 100% MeOH and inject in 10 to 100 μl; (ii) EtOAc:MeOH-soluble fraction—dissolve in 50% MeOH and dilute if possible with H_2O to same MeOH percentage as initial solvent prior to injecting in 10 to 100 μl; (iii) MeOH-soluble (EtOAc-MeOH insoluble)—dissolve in 10% MeOH and inject in 100 to 500 μl; (iv) H_2O-soluble fraction (MeOH insoluble)—dissolve in 10% MeOH and inject in 100 to 500 μl; (v) MeOH wash of the SiO_2 partition column—neutralize as noted above prior to taking to dryness, then dissolve in 10% MeOH and inject in 100 to 500 μl.

Standard Samples. [^{3}H]GA_1[6], [^{3}H]GA_4 (6), [^{3}H]GA_7 (synthesized by H. Aoki, N. Murofushi, K. Ogiyama, M. Noma, and T. Sassa), [^{3}H]GA_8 (20), [^{3}H]GA_9 (35), [^{3}H]GA_{14} (8), [^{3}H]GA_{20} (20), the methyl esters of [^{3}H]GA_4, [^{3}H]GA_9, and [^{3}H]GA_{14}, [^{3}H] kaurenoic acid (23), [^{14}C]GA_3[7], [^{14}C]kaurene (synthesized by J-T. Lin and E. Heftmann), [^{3}H]GA_{12} aldehyde, GA_3-O (3) glucoside (synthesized by G. Schneider and G. Sembdner), and GA_4 glucosyl ester (synthesized by N. Murofushi and N. Takahashi) were used, and detected by radioassay (aliquots dissolved in 1 ml MeOH or MeOH:H_2O were assayed by liquid scintillation spectrometry) or immersion bioassay (GA glucosyl conjugates).

Solvent Partitioning. The procedure used (Fig. 1A, right side) was a modification of a number of traditional procedures (2, 4,

[5] Woelm SiO_2, Cat. No. 02766, purchased from ICN Cleveland, OH.

[6] Purchased from New England Nuclear, Lachine, Quebec, Canada.

[7] Purchased from Amersham, Oakville, Ontario, Canada.

Table I. *Elution of Authentic Standards of Gibberellins, Gibberellin Methyl Esters, Gibberellin Precursors, and Gibberellin Glucosyl Conjugates from C_{18}-PC*

	Fraction (MeOH Concn.)								
	1 (10%)	2 (20%)	3 (30%)	4 (40%)	5 (50%)	6 (60%)	7 (70%)	8 (80%)	9 (100%)
	% eluted[b]								
Sample I[a]									
[^{3}H]GA_1	92.2	6.3							
[^{14}C]GA_3	98.3	1.2							
[^{3}H]GA_4	97.5	2.5							
[^{3}H]GA_7	93.0	4.2	0.3						
[^{3}H]GA_8	94.5	2.9							
[^{3}H]GA_9	96.9	2.1							
[^{3}H]GA_{14}	97.0	2.1							
[^{3}H]GA_{20}	94.5	3.7							
[^{3}H]GA_4-Me	78.4	19.8	4.7	2.0	3.7				
[^{3}H]GA_9-Me			4.9	39.0	45.0	5.2			
[^{3}H]GA_{14}-Me	42.7	19.4	14.1	6.5	10.5	9.7	5.0		
[^{3}H]GA_{12} aldehyde				0.4	53.5	39.8	4.4	1.4	0.5
[^{3}H]Kaurenoic acid						1.6	5.5	86.7	3.3
[^{14}C]Kaurene						2.7	8.3	57.8	27.2
Sample II[c]									
GA_3 glucoside				97.7[d]	0.5	0.5	0.4	0.4	0.5
GA_4 glucosyl ester				92.0	3.1	0.9	1.1	1.1	1.8

[a] 2×10^5 to 6×10^6 dpm of standard (in 1–100 μl of methanolic solution) was diluted into 40 ml of 10% MeOH. Each solution was then passed through the column of C_{18} reverse phase material[4] (C_{18}-PC) (i.d., 1 cm; length, 1 cm; weight of C_{18} material, 0.35 g). The column was then eluted stepwise with 40 ml of each of varying percentages of MeOH:H_2O. at pH 6.3 (10% MeOH) to 6.7 (80% MeOH).

[b] Values are percentages of total radioactivity applied to column.

[c] One or 2 μg of GA_3 glucoside and GA_4 glucosyl ester, respectively, were dissolved in 40 ml of 40% MeOH. Each solution was passed through the C_{18}-PC as noted above in footnote a.

[d] Values based on assay (immersion) with dwarf rice var Tan-ginbozu.

13, 16, 17, 21, 31). The 80% MeOH extraction solvent was taken to the aqueous phase *in vacuo* at 35°C, and an equal volume of pH 9.0, 0.5 M KH_2PO_4:Na_2HPO_4 buffer added. This aqueous fraction was filtered through Whatman No. 1 paper on a Büchner funnel before adjusting the pH to 9.0 with 1 N KOH, and partitioning 3× against diethyl ether (3:5 ether:buffer). The aqueous phase was then adjusted to pH 3.0 with 1 N HCl and partitioned 5× against EtOAc (3:5 EtOAc:buffer). The acidic, EtOAc phase was then dried by freezing at −70°C and filtering out the ice on a Büchner funnel, prior to taking to dryness in a rotary evaporator flask at 35°C *in vacuo*. The dry residue was then placed in a freeze drier overnight to remove any traces of acetic acid. These acidic, EtOAc-soluble substances were then solubilized in as small an amount as possible (about 1–2 ml) of 0.5 M, pH 9.0 phosphate buffer (higher pH used if solubility was a problem), and loaded onto a PVPP column (10) eluted with 0.1 M pH 8.0 phosphate buffer. The 0.1 M buffer eluate was adjusted to pH 3.0 with 1 N HCl and partitioned as noted above with EtOAc. The acidic, EtOAc-soluble substances were dried as noted above, then dissolved in dry EtOAc:MeOH (1:1) for loading onto GFA (Whatman) discs for subsequent SiO_2 partition column chromatography.

RESULTS

Elution Characteristics from the C_{18}-PC of GA, GA Me, GA Precursors, and GA Glucosyl Conjugates. The elution/retention profiles of authentic GA with a wide variety of functional groups, and of representative GA-Me, GA precursors, and GA glucosyl conjugates were determined (Table I).

Eight GAs of widely varying polarity (5), three GA-Me, and three GA precursors were tested. After loading in 40 ml of 10% MeOH, all eight GAs were quantitatively eluted with the solvent (10% MeOH) followed by 40 ml 20% MeOH. Also, GA_9-Me, GA_4-Me, and GA_{14}-Me (GA-Me should not be overlooked as being potential native compounds in plants; [see 22, 31]), were quantitatively eluted by the elution sequence of 10 to 50% MeOH (Table I). However, two GA precursors, kaurene and kaurenoic acid, did not begin to elute until a 60% MeOH concentration was reached. A third GA precursor, GA_{12} aldehyde, was only half-eluted at a 50% MeOH concentration (Table I), the rest eluting at 60 and 70% MeOH concentration. As representatives of GA glucosyl conjugates, GA_3 glucoside and GA_4 glucosyl ester were dissolved in 40 ml of 40% MeOH, applied to the C_{18}-PC and eluted as above. The majority of each eluted with the first 40 ml of 40% MeOH (Table I; and would also be eluted by lower MeOH concentrations because of their high polarity).

These elution patterns from C_{18}-PC coincide with those noted on reverse phase C_{18} HPLC, namely, a large number of GA, GA-Me, and GA glucosyl conjugates were eluted by a linear gradient (10–73% MeOH:1% AcOH:H_2O), but kaurene, kaurenoic acid, and GA_{12} aldehyde are only eluted with MeOH concentrations greater than 73% (14). Thus, the data provided by Table I, combined with our knowledge of GA elution from reverse phase C_{18} HPLC (14), indicated that elution of the C_{18}-PC with 50% MeOH would remove GA, GA and GA glucosyl conjugates Me, and about one-half the GA_{12} aldehyde, leaving about 45% of GA_{12} aldehyde and most of the kaurene and kaurenoic acid (Table I). A complete retention of GA_{12} aldehyde could be obtained on the C_{18}-PC if 40% MeOH was used, but this is accomplished only by splitting the GA_9 Me fraction (Table I).

Effects of pH, in varying percentages of MeOH:H_2O, on elution of authentic GA from the C_{18}-PC. The influence of pH on the elution of [^{3}H]GA_4, [^{3}H]GA_{14}, and [^{3}H]GA_{12} aldehyde from the C_{18}-PC in various percentages of MeOH:H_2O was also examined (Table II). If the pH is decreased appreciably below that normally found in the MeOH:H_2O mixtures (*e.g.* pH 6.3–6.7

[Table I]), there was a greater retention of the GA on the C_{18}-PC. This retention effect was most pronounced at lower MeOH percentages (Table II). Therefore, the pH of methanolic plant extracts must be adjusted to approximately 6.5, if necessary, before performing the C_{18}-PC procedure to ensure proper elution of free GA while GA precursors are retained. Further, the pH of the eluate after the second C_{18}-PC should be checked and, if it has dropped below 6.0, then an additional volume of pH 6.5 MeOH:H_2O should be flowed through the second C_{18}-PC.

It is apparent from Table II that, while use of either 40% or 50% MeOH at pH 6.5 will elute 98+% of GA_4, the less polar GA_{14} is retained in part. Hence, if the tissue of interest contains GA_{14} (or GA_{14} aldehyde), a higher percentage (60%) of MeOH must be used if elution of GA_{14} or GAA_{14} aldehyde from the second C_{18}-PC is desired. Gibberellin Ag may also be partially retained on the second C_{18}-PC at 40% MeOH (*e.g.* its elution characteristics lie between GA_4 and GA_{14} [14].

Although the present work does not attempt to explore the ramifications of the use of various pH and MeOH:H_2O percentages on separation of GA (other than those shown in Table II), it is obvious that such a use could become a powerful tool in isolating specific GA of interest in a relatively highly purified fraction.

Removal of Plant Pigments by C_{18}-PC Columns. Leaf tissue harvested from apple propagules was processed through the C_{18}-PC two column sequence in Figure 1A, and an estimate of the removal of neutral plant pigments was obtained. Absorption at 640 and 436 nm (9) was considered indicative of pigment (Chl and carotenoids) levels. Virtually all of the Chl were removed as was much of the 436 nm absorbing pigments. Thus, as noted earlier (1, 9), the C_{18}-PC method can be used in place of conventional ether partitioning at high pH for removal of pigments and other nonpolar substances with similar C_{18} retention characteristics.

Ratio of C_{18} Reverse Phase Bondapak B Material to Tissue Dry Weight and Recovery of [^{3}H]GA and Their ^{3}H Metabolites, Including [^{3}H]GA Glucosyl Conjugate-like Compounds. Methanolic extracts from six samples of tissue (varying in d.w. from 0.5 to 1.46 g) containing variable amounts of the precursor [^{3}H] GA_4, ^{3}H-free GA metabolites, and ^{3}H highly water-soluble metabolites (*e.g.* glucosyl conjugate-like compounds) were obtained from suspension cultures of somatic embryos of anise (15). The 80% MeOH extracts of six samples were passed through two C_{18}-PC where the ratio of C_{18} material to tissue dry weight varied from 2.05 to 4.00. The recovery of total radioactivity varied between 86% and 96% (mean = 90.3 ± 4.4). The 5 to 14% of radioactivity retained on the columns may represent incomplete elution of GA and GA glucosyl conjugates due to differential response of GA to pH (Table II) or may be accounted for by nonpolar conjugates (see 30) of the applied [^{3}H]GA_4 or its metabolites.

Thus, the two column sequence of C_{18}-PC yields reproducibly high recoveries, and the amount of C_{18}-PC material, relative to the dry weight of tissue, does not significantly affect the recovery of GA or GA glucosyl conjugate-like substances.

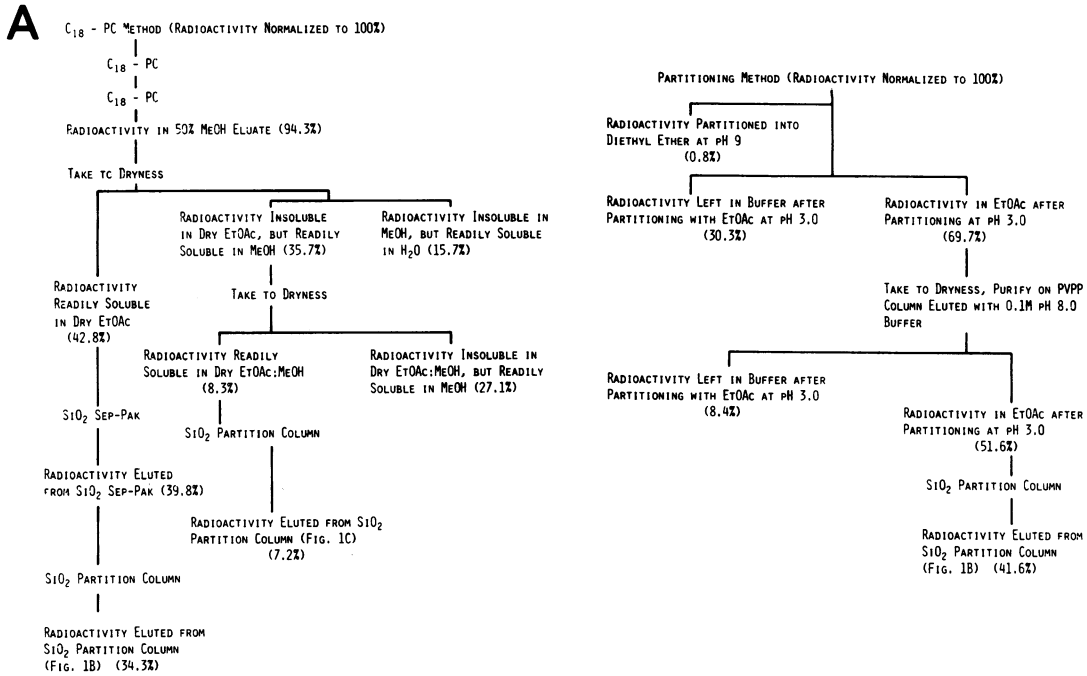
Removal of Dry Weight by the C_{18}-PC Method. Extraction of four apple leaf tissue samples with 80% MeOH yielded about 19% of the tissue d.w. (18.8–21.5%). The C_{18}-PC two column sequence was quite effective in removing d.w. from the fraction containing all of the GA/GA-Me (Fig. 5; EtOAc:MeOH soluble [ii and iii]); only 0.7 to 2.3% of the MeOH-extractable d.w. remained. The GA precursor fraction (Fig. 5i) was also effectively purified; it contained about 6 to 9% of the MeOH-extractable d.w. Elution of the first C_{18}-PC with 100% MeOH (after first eluting with 80% MeOH) yielded the plant pigments in a fraction that contained 24% of the MeOH-extractable d.w. Based on Figure 1, where conjugates formed by metabolism of [^{3}H]GA_4 were analyzed, only about one-third of the GA glucosyl conjugates will be present in the relatively low d.w. fraction (0.7–2.3% d.w.) that also contains all of the GA/GA-Me. The majority of conjugates will occur in the high d.w. residues that are dissolved only by MeOH (about one-half of conjugates and 34–41% of MeOH-extractable d.w.) → H_2O (about one-sixth of conjugates and 24–28% of the MeOH-extractable d.w.). Thus, the C_{18}-PC method does not significantly purify fractions containing the majority of GA glucosyl conjugates (and GA_{32} if present). If use of high capacity C_{18} reverse phase HPLC columns is not feasible, or use of aliquots is impractical, then some additional purification can be accomplished by partitioning into 1-butanol at pH 3.0 (25).

A Comparison of the C_{18}-PC Method with a Solvent Partitioning Procedure for [^{3}H]GA Metabolites and Endogenous GA-Like Substances. The solvent partitioning and C_{18}-PC methods (Fig. 1) were compared in their efficacy by analyzing metabolites in an extract from pea seedlings fed [^{3}H]GA_{20}. Both methods yielded 90% recovery of radioactivity after removal of nonpolar pigments (Fig. 1A). Highly buffer-soluble substances (presumably

Table II. *Elution of Authentic Gibberellins from the C_{18}-PC at pH 3.0 to 6.5 in 40 to 80% Aqueous Methanol.*

Eluent Solutions	GA[a]	Recovered Radioactivity at Following pH of Eluant Solution				
		3.0	4.0	5.0	6.9	6.5
				%		
80% MeOH	[^{3}H] GA_4	65.2	90.4	96.8	99.3	100.0
	[^{3}H] GA_{14}	84.0	99.8	100.0	100.0	100.0
	[^{3}H] GA_{12} aldehyde	62.4	97.8	98.7	99.2	99.6
60% MeOH	[^{3}H] GA_4	30.0	91.9	98.0	99.5	99.9
	[^{3}H] GA_{14}	19.1	86.2	96.9	99.3	99.7
	[^{3}H] GA_{12} aldehyde		1.1	7.6	26.4	74.2
50% MeOH	[^{3}H] GA_4	2.4	36.8	69.0	95.8	99.7
	[^{3}H] GA_{14}	2.9	8.0	16.3	47.8	98.3
	[^{3}H] GA_{12} aldehyde			1.4	11.8	33.7
40% MeOH	[^{3}H] GA_4		1.0	45.2	95.2	97.6
	[^{3}H] GA_{14}	1.0	2.3	8.6	26.8	52.7
	[^{3}H] GA_{12} aldehyde				1.4	5.2

[a] Radioactive GA standard (5×10^5 dpm) was diluted into 40 ml of pH 3.0 MeOH:H_2O solution. Each solution was then passed through the column of C_{18} reverse phase material[5] (C_{18}-PC) (i.d., 2.2 cm; length, 1.7 cm; weight of C_{18} material, 2.5 g). The column was then eluted stepwise with 40-ml volumes of MeOH:H_2O at varying percentages and pH.



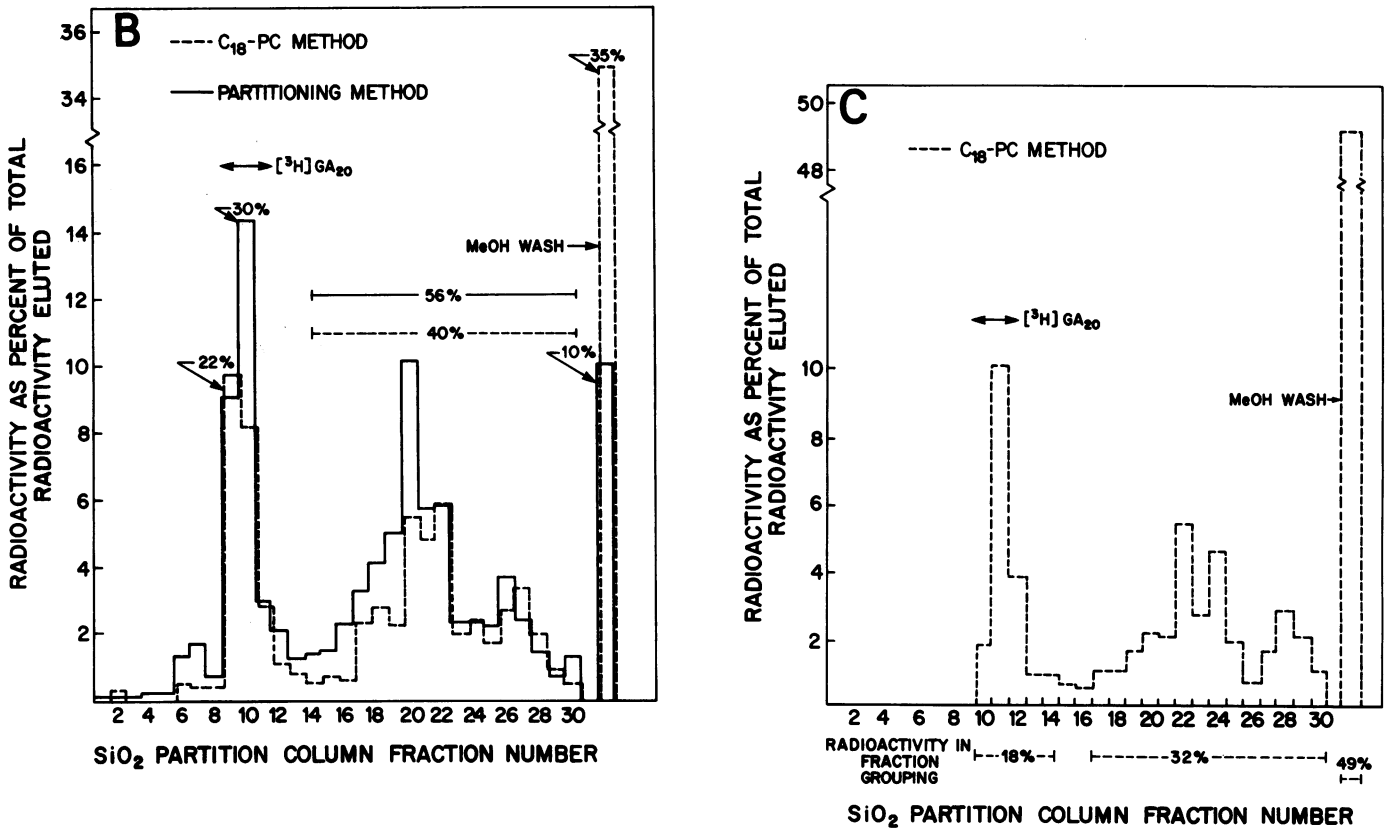


FIG. 1A. Purification and chromatography schemes for the C_{18}-PC method (left) and traditional solvent partitioning method (right) utilized for each of two halves of an 80% MeOH extract of 2.6 g d.w. of freeze-dried shoots of Alaska pea seedlings to which [^{3}H]GA_{20} (2.36 Ci/mmol) at the dosage of 1.58 μCi/plant had been applied (first trifoliate leaf 11 d prior to harvest). The initial radioactivity has been normalized to 100% for each half of the MeOH extract. Specific details of both schemes are given in "Materials and Methods." Two SiO_2 partition columns were developed for the C_{18}-PC method in order to determine the relative proportion of 'GA conjugate-like' radioactivity in residues that were sequentially soluble in (i) dry EtOAc (Fig. 1B) and (ii) dry EtOAc:MeOH (50:50) (Fig. 1C).

FIG. 1B. SiO_2 partition column profiles of radioactivity (extracted from pea seedlings fed [^{3}H]GA_{20}; see Fig. 1A) which is soluble in dry EtOAc (-----) from the C_{18}-PC method, and which is acidic, EtOAc soluble (——) from the solvent partitioning method. The precursor, [^{3}H]GA_{20}, elutes in fractions 9 to 11. A logical metabolite GA_{29} (27) would elute in fractions 20 to 22. A MeOH wash of the column, following completion of the hexane:EtOAc gradient, was used to remove radioactivity which was not eluted by the gradient solvents. Glucosyl esters and glucosides of GA will be washed from the SiO_2 partition column by MeOH (Figs. 2 and 3). See Figure 1A and "Materials and Methods" for further details on the extract, tissue, or purification scheme.

FIG. 1C. SiO_2 partition column profile of the radioactivity that is insoluble in dry EtOAc, but is soluble in a 1:1 mixture of dry EtOAc:MeOH (----). The dry EtOAc-soluble radioactivity profile is shown in Figure 1B. Additional details are given in Figure 1, A and B, and in "Materials and Methods."

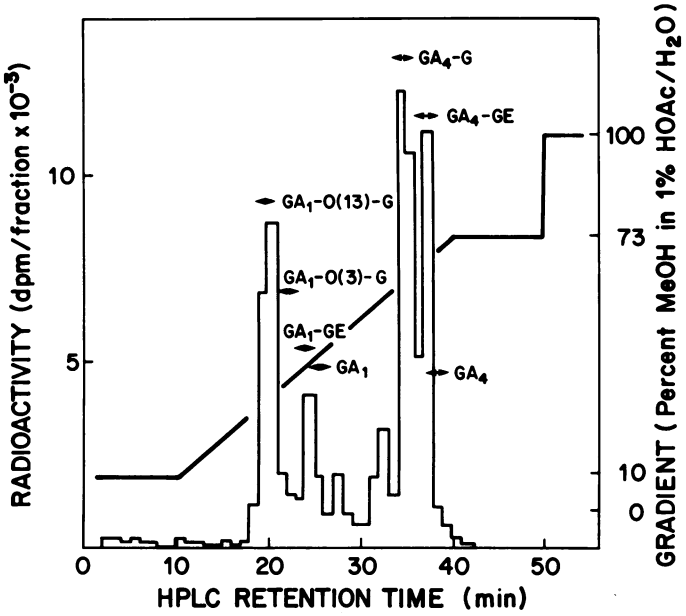


FIG. 2. Reverse phase analytical C_{18} HPLC profile of [^{3}H]GA glucosyl conjugate-like substances from the MeOH wash fraction of a SiO_2 partition column which had been loaded with the dry EtOAc:MeOH (1:1)-soluble substances from leaves of apple propagules which had been fed [^{3}H]GA_4 7 d prior to harvest (M Koshioka, G Edwards, J Taylor, and R Pharis, unpublished data). This profile is typical of similar fractions from extracts of other plants fed [^{3}H]GA_4, or other less polar [^{3}H]GA, in that GA glucosyl ester-like conjugates (especially) and GA glucoside-like conjugates (to a lesser degree) of the precursor tend to predominate, with conjugates of more polar metabolites being in the minority (due to differential solubility in the dry EtOAc:MeOH). Retention times of standard GA_1 and GA_4 glucosides (GA_1G, GA_4G) and GA_1 and GA_4 glucosyl esters (GA_1GE, GA_4GE) are shown by (↔).

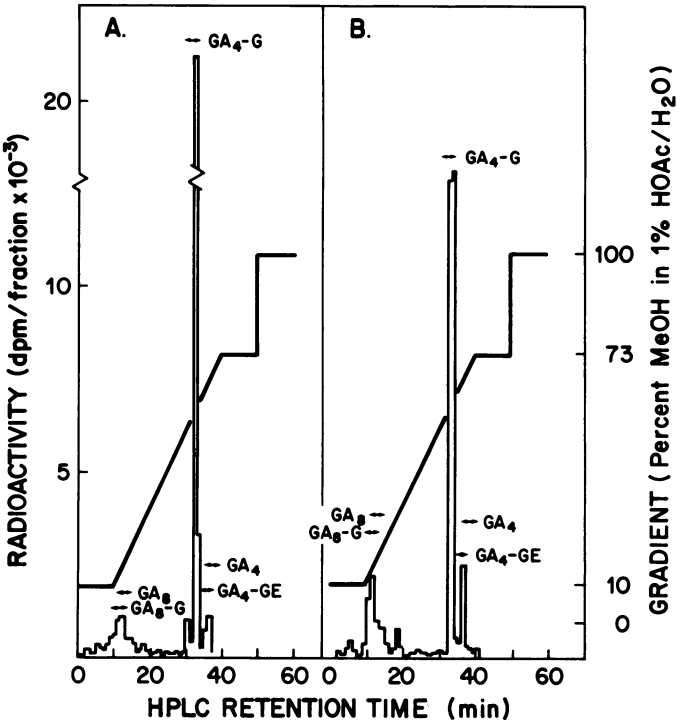


FIG. 3. Reverse phase analytical C_{18} HPLC profile of [^{3}H]GA glucosyl conjugate-like substances from the MeOH-soluble (*e.g.* dry EtOAc:MeOH-insoluble) fraction (A) and the H_2O-soluble (*e.g.* MeOH-insoluble) fraction (B) of extracts of somatic embryos of anise which had been fed [^{3}H]GA_4 348 h previously (15). The retention times of standards of GA_4 and GA_8, GA_4 glucosyl ester (GA_4G), and GA_4 and GA_8 glucosides (GA_4G and GA_8G) are also given (↔).

GA glucosyl conjugates) constituted only 38.7% of the radioactivity in the solvent partitioning method. To this amount should be added the radioactivity eluted in the MeOH wash of the SiO_2 partition column (Fig. 1B, normalized to 5.2% of the initial radioactivity), bringing the total of GA glucosyl conjugate-like substances to 43.9% for the solvent partitioning method. Substances which were insoluble in dry EtOAc, or a mixture of dry EtOAc:MeOH (50:50), constituted 42.8% of the radioactivity for the C_{18}-PC method, to which should be added the 19.1% (calculated on basis of initial radioactivity) radioactivity eluted in the MeOH wash of the two SiO_2 partition columns (Fig. 1B, 35% normalized to 15.0%; Fig. 1C, 49% normalized to 4.1%; total = 19.1%), bringing the total of GA glucosyl conjugate-like substances to 61.9% for the C_{18}-PC method. The solvent partitioning procedure also yielded an increased amount of a compound which is probably 'ring C/D rearranged GA_{20}' (Fig. 1B, fractions 6 and 7) and part or all of the increase in the fraction 6, 7 compound may originate from hydrolysis of a conjugate (7).

Thus, solvent partitioning, at least as used by us, may hydrolyze a significant portion of conjugate-like substances into free GA.

The qualitative spectrum of free [^{3}H]GA metabolites is similar between the two methods (Fig. 1B). Procedures used to dissolve the dry residue after C_{18}-PC were examined through the use of SiO_2 partition column chromatography and analytical C_{18} HPLC. The radioactivity readily soluble in dry EtOAc (42.8% of initial radioactivity, Fig. 1A, left) is 65% free GA, and 35% GA glucosyl conjugate-like substances (MeOH wash; Fig. 1B; see also 11, 26). The radioactivity insoluble in dry EtOAc, but readily soluble in MeOH (35.7% of initial radioactivity; Fig. 1A, left) can be further categorized by the sequential use of EtOAc:MeOH (8.3%) → MeOH (27.1%). The SiO_2 partition column of the EtOAc:MeOH-soluble fraction (8.3% of the initial radioactivity) yielded about half as free [^{3}H]GA, and half as [^{3}H]glucosyl conjugate-like compounds (*e.g.* MeOH wash of the SiO_2 partition column, Fig. 1C). Thus, a good solvent mixture for dissolving all of the free GA in a small volume of liquid is EtOAc:MeOH (1:1). However, one must be aware that appreciable amounts of GA glucosyl conjugates are also dissolved by EtOAc:MeOH (*e.g.* see Fig. 1, B and C, 'MeOH wash' fractions).

An indication of the qualitative spectrum of [^{3}H]GA glucosyl conjugate-like substances can be obtained by subjecting the purported conjugate fractions to analytical C_{18} HPLC (14). Thus, for apple tissue fed [^{3}H]GA_4, EtOAc:MeOH-soluble compounds that are eluted in the MeOH wash of a SiO_2 partition column (Fig. 1, B and C) include GA_4 glucoside- and GA_4 glucosyl ester-like conjugates and also conjugates of one of its metabolic products, [^{3}H]GA_1 (Fig. 2). Compounds that are sequentially soluble in MeOH → H_2O (but insoluble in dry EtOAc:MeOH) as per the C_{18}-PC method (Fig. 1A) are shown in Figure 3, A and B. They include not only the more polar conjugates (*e.g.* [^{3}H]GA_8 glucoside-like substance), but also appreciable amounts of less polar conjugates (*e.g.* [^{3}H]GA_4 glucoside- and [^{3}H]GA_4 glucosyl ester-like substances). Although we term these 'conjugate-like substances,' all of the above-mentioned [^{3}H]GA_4 metabolites were subsequently subjected to isocratic HPLC-RC before and after hydrolysis, with identification of the GA moiety after hydrolysis being made by isocratic HPLC-RC and/or GLC-RC (15). Virtually no free GA were present upon isocratic HPLC analysis before hydrolysis, thereby confirming that occlusion of free GA in the EtOAc:MeOH-insoluble residue was not occurring and that complete solubilization of free GA by the EtOAc:MeOH solvent is effected (however, see "Materials and Methods" and Fig. 5 in reference to use of H_2O → EtOAc:MeOH to prevent

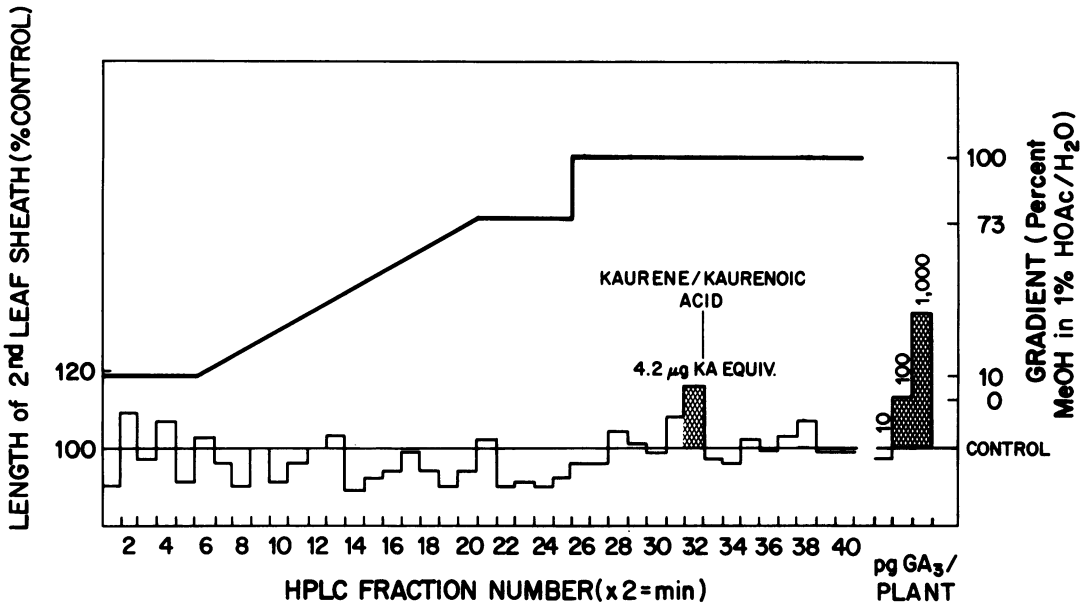


FIG. 4. Reverse phase C_{18} HPLC profile of the GA precursor fraction eluted with 100% MeOH from the C_{18}-PC (Fig. 5) of a MeOH (80%) extract of 0.88 g d.w. somatic grape embryos (29). Biological activity (4.2 μg kaurenoic acid eq/0.88 g tissue d.w.) had initially been detected on the dwarf rice microdrop bioassay (in serial dilution) in the bulked GA precursor fraction, and was similarly detected in HPLC fractions 31 and 32. Statistically significant activity is shown by cross-hatching (Rt 60–64 min). Fractions 31 and 32 were subsequently subjected to packed column GLC-MS; kaurene and kaurenoic acid were identified based on complete MS which were identical to MS of authentic standards.

possible occlusion effects).

Thus, while there is a relationship between polarity of the conjugate and its solubilization in the sequence of dry EtOAc → EtOAc:MeOH → MeOH → H_2O, appreciable amounts of the more polar GA conjugates are solubilized by dry EtOAc:MeOH (Fig. 2). However, even absolute MeOH will not completely solubilize all of the [^{3}H]GA_4 glucosyl ester-like conjugate (Fig. 3, A and B). Therefore, in order to have all of the GA glucosyl conjugates in one fraction prior to gradient-eluted reverse phase C_{18} HPLC, we recommend that the MeOH wash fraction from the SiO_2 partition column (*e.g.* on which is loaded substances soluble in dry EtOAc:MeOH) be combined with those residual substances that are soluble in MeOH → H_2O, or soluble in 10% MeOH (*e.g.* scheme shown in Fig. 5).

Use of the C_{18}-PC Method for Endogenous GA-Like Substances. Barendse *et al.* (1) have shown the usefulness of the C_{18} reverse phase Bondapak B material for obtaining biologically active GA-like substances, although their technique would have grouped free GA with at least a portion of GA precursors, and with GA glucosyl conjugates. We have used the C_{18} method successfully on a variety of plant materials, and describe one example below for endogenous GA precursors.

The second C_{18}-PC after elution with 50% MeOH will retain about 70% of the kaurene, almost all of the kaurenoic acid (Table I), about 46% of the GA_{12}-aldehyde, and many metabolites of kaurene. Significant biological activity was thus obtained in a 100% MeOH eluate of the second C_{18}-PC (after it had first been eluted with 50% MeOH; as in Fig. 1, A and C) for extracts of somatic embryos of grape (29). The 100% MeOH eluate (Fig. 5i) was subjected directly to a gradient-eluted analytical reverse phase C_{18} HPLC (14). Bioassay of the HPLC fractions showed a significant peak of activity in the kaurene/kaurenoic acid region (Fig. 4). This peak was subjected to GLC-MS and yielded MS identical to those of kaurene and kaurenoic acid (29). The C_{18}-PC→HPLC sequence may thus be useful in obtaining these and other GA precursors from plant extracts. However, one should keep in mind the possibility that nonpolar GA conjugate-like compounds (28), if present, may also elute in the 100% MeOH eluate of the second C_{18}-PC.

DISCUSSION

The C_{18}-PC method (as outlined in Figs. 1A and 5) can safely replace solvent partitioning techniques as an extraction/purification method for GA precursors, free GA, GA-Me, and GA glucosyl conjugates from the same tissue sample. When used in combination with a SiO_2 partition column (isocratic, step or gradient-eluted), GA and/or GA-Me can be readily separated from GA glucosyl conjugates, thus yielding three discrete fractions: (i) many GA precursors, including kaurene and kaurenoic acid, (ii) free GA/GA-Me, and (iii) GA glucosyl conjugates. All of fractions (i–iii) can subjected directly to preparative and/or analytical reverse phase C_{18} HPLC, with detection and quantitation by appropriate means (*e.g.* bioassay, radioassay, GLC-MS, GLC-MS-SIM). For use in bioassay, purity of free GA (ii) is as good or better than PVPP- and charcoal:celite-purified fractions from the solvent partitioning method. And, use of the bulked fraction of GA glucosyl conjugates (iii) in serial dilution on a combination of micro-drop (18) and immersion (19) bioassays on Tan-ginbozu dwarf rice has yielded significant activity without severe toxicity, indicating that these bioassays can also be used to assay fractions eluted from reverse phase C_{18} HPLC of (iii).

The C_{18}-PC method is of special interest for work on the metabolism of radioactive GA/GA precursor/GA glucosyl conjugates, and lends itself well to preliminary separation of these three categories of compounds prior to more definitive assay by analytical reverse phase C_{18} HPLC-RC and/or sequential GLC-RC.

Our recommended procedure using the C_{18}-PC method is detailed in the form of a flow chart (Fig. 5). Where isolation of only one or a few free GA/GA precursors/GA glucosyl conjugates is desired, the procedure can be simplified, purification enhanced, and indeed preliminary chromatography often can be accomplished by loading the extract onto the second (or a third) C_{18}-PC in a reduced volume of MeOH (0–50%), and monitoring the eluate fractions. The elution pattern from such C_{18}-PC will be the same as that noted earlier (12, 14), although it should be remembered that GA glucosyl conjugates, if present, will also be eluted with the GA if the SiO_2 partition column (or analogous

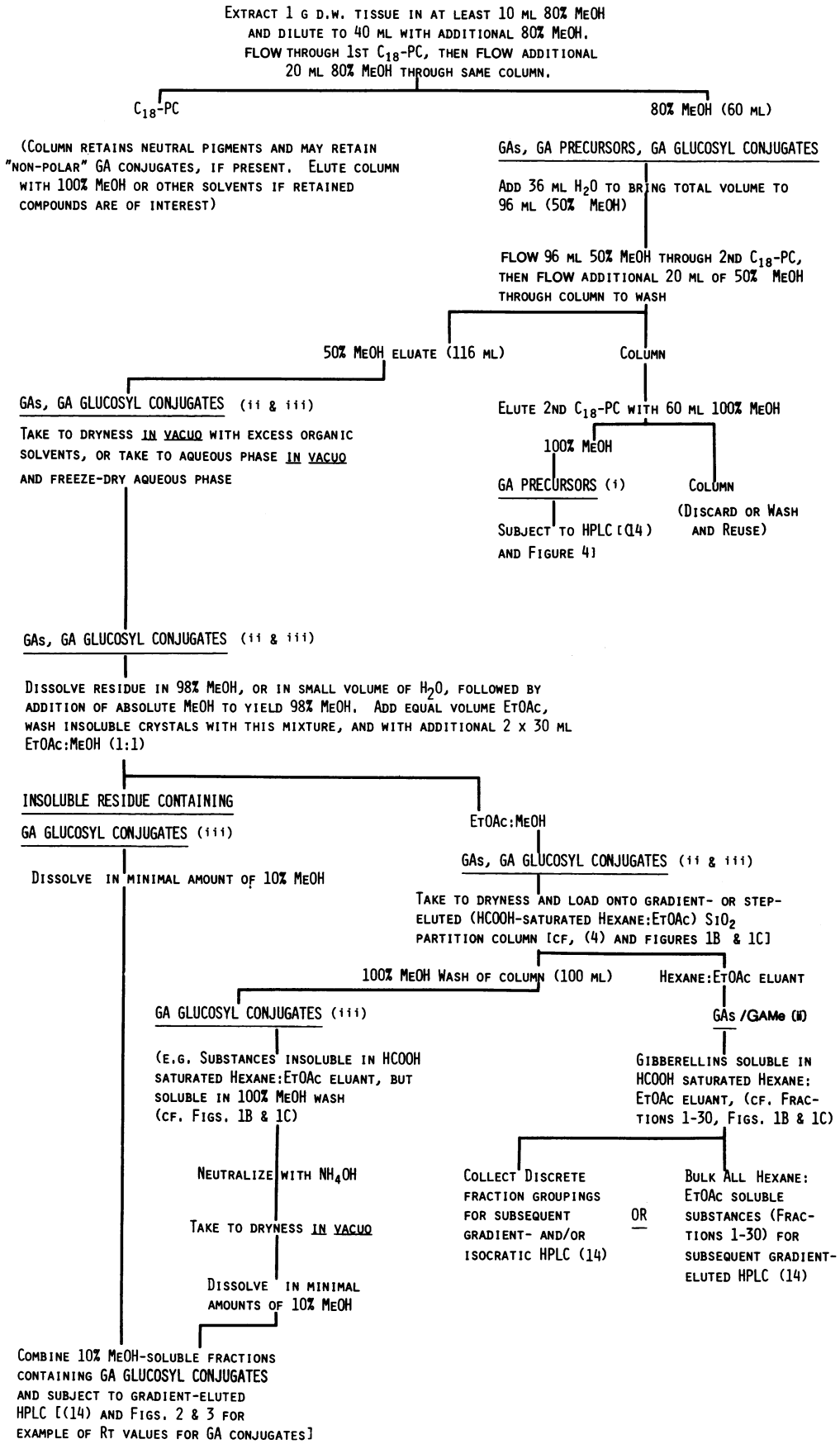


FIG. 5. A flow chart of the recommended procedure for use of the C_{18}-PC method for purification of (i) GA precursors, (ii) GA/GA-Me, and (iii) GA glucosyl conjugates from a 1-g d.w. sample of plant tissue. Increased amounts of tissue will require a proportional scaling up of amounts of C_{18}-reverse phase Bondapak B, and at least a partial scaling up of extraction and C_{18}-PC elution solvents. The pH of the MeOH:H_2O solutions should be maintained at 6.5 (see Table II). This procedure, however, splits GA_{12} aldehyde into fractions (i) and (ii). Use of 40% MeOH (116 ml) to elute the second C_{18}-PC column will allow GA_{12} aldehyde to be subsequently eluted quantitatively in fraction (i) by 60 ml of 100% MeOH. But, use of 40% MeOH then splits GA_9-Me between fractions (i) and (ii), so the user must decide which procedure is most appropriate. Gradient and/or isocratic C_{18} HPLC (1, 12, 14, 32) is recommended for subsequent separation of components within (i, ii, and iii), with detection and quantitation by appropriate methods (*e.g.* radioassay [6–8, 14, 15], definitive bioassay [18, 19], GC-MS [4, 11], or GC-MS-SIM [12]).

procedure) step has not been accomplished.

A convenient procedure (see "Materials and Methods" for details and comments on avoiding occlusion effects) for tissue amounts of about 1 g (or less) involves loading the 50% MeOH eluate residue (about 130 mg for a 1 g d.w. tissue sample) from the second C_{18}-PC (*e.g.* GA and GA glucosyl conjugates) directly on to the SiO_2 partition column, free GA being eluted in the hexane:EtOAc gradient, the GA glucosyl conjugates being washed from the SiO_2 column with excess absolute MeOH.

Where chromatography of free GA at the SiO_2 partition column stage is not required (*e.g.* the free GA and GA conjugate fractions are to be chromatographed subsequently on C_{18} HPLC *in toto*), we have found that short SiO_2 partition columns (5 g SiO_2; column diameter 15 mm i.d.) eluted isocratically with HCOOH-saturated hexane:EtOAc are a rapid and efficient way to effect separation of free GA from GA glucosyl conjugates. Elution of such a column with 70 ml of 5:95 (v/v) hexane:EtOAc yields >95% of the highly polar free [^{3}H]GA_8, and <5% of most nonpolar of GA conjugates, [^{3}H]GA_9 glucosyl ester (Pharis, Koshioka, Janzen, unpublished). Subsequent elution of the column with MeOH (as described in the "Materials and Methods" and "Results") yields almost all of the GA glucosyl conjugates. This procedure would be useful for nonradioactive extracts where one wanted to ensure that free GA would be quantitatively separated from GA glucosyl conjugates. When using radioactive GA, large amounts of residual precursor [^{3}H]GA can be removed from free GA metabolites by step-eluting the SiO_2 partition column sequentially with two isocratic mixtures (*e.g.* 120 ml 45:55 [v/v] hexane:EtOAc to elute the precursor GA, [^{3}H]GA_4, or [^{3}H]GA_{20}, followed by 60 ml of 5:95 [v/v] hexane:EtOAc to elute the free [^{3}H]GA metabolites), GA glucosyl conjugates being eluted subsequently with MeOH (25).

Reuse of the C_{18}-PC material is possible after extensive washing with methanol, chloroform, EtOAc, dimethyl sulfoxide, and H_2O. Washed C_{18} material should of course be checked to ensure that proper elution characteristics are retained (*e.g.* to make certain the C_{18} material is not coated with contaminants that might alter the normal elution pattern) and also for the presence of GA contaminants by an appropriate assay (*e.g.* ^{3}H oxidation; bioassay of MeOH eluate) prior to reuse. Even so, we recommend that only new C_{18} material be used for extracts known to be low in biological activity, or for radioactive extracts where very low levels of radioactivity are expected in metabolites.

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