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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PLANT HORMONES

I. SEPARATION OF PLANT HORMONES OF THE INDOLE TYPE

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SUMMARY

A high-performance liquid chromatographic method was developed that permits a very good separation and isolation of substances of the indole type which occur in the metabolic pathways of the naturally encountered phytohormone, indole-3-acetic acid. The method makes use of an anlytical and a preparative column. The stationary phase is silica gel modified by octadecylsilane (reversed phase). Both the isocratic and the gradient elution are performed with a mixture of ethanol and 1% acetic acid as the mobile phase. Plant hormones are detected by UV-detector. The lowest determined amount of the indole derivatives is 5–20 ng. The method was used for analysis of the supernatant from a culture of the bacterium *Pseudomonas putida*, strain K_2.

INTRODUCTION

The biological and physiological significance of phytohormones, important and interesting products of plant and microbial metabolism, has been studied by many physiologists and soil microbiologists. The use of modern high-performance chromatographic methods makes possible the separation, isolation, identification and determination of phytohormones of the indole type found in the natural materials in very low concentrations.

Gas chromatography (GC) has been used for the analysis of methyl-[1–9], trimethylsilyl-[10] and fluorinated derivatives[11] of plant hormones of the indole type either separately or in combination with paper chromatography[9], thin-layer chromatography[8] or column chromatography[4,8], UV spectroscopy[8] and spectrofluorometry[3,4,8]. Preparation of an indole extract from plants by means of gas and column chromatography was described by Powell[4,12]. The analysis of mixtures of the above compounds has been increasingly performed with the use of high-performance liquid chromatography (HPLC). The separation of indole derivatives in biological material by means of HPLC on ion exchangers complemented by UV spectrometric and spectrofluorometric detection was described by Chilcote and Mrochek[13] and Scott *et al.*[14]; Carnes *et al.*[15] separated and identified plant hormones by HPLC on different

types of ion exchangers. The identification was carried out using a UV detector. Reeve and co-workers[16–18] separated and isolated gibberellins, abscissic acid and an artificially prepared mixture of indole derivatives on a high-performance liquid chromatograph of their own construction with a preparative column and an analytical column which was packed with Partisil 10 impregnated with 40% of 0.5 *M* formic acid. The detection of indole derivatives was performed using a UV detector. Sweetser and Swartzfager[19,20] used HPLC to determine nano- to picogram amounts of indole-3-acetic acid in plant tissues; the column was packed with ion exchanger and the detection made use of an electrochemical carbon-paste amperometric detector and/or fluorescence detector. Stationary reversed phase and fluorometric detection was recommended by Berg[21] for separation of substances of the indole type. Durley *et al.*[22] also described an analysis of abscissins and indole-3-acetic acid by HPLC on a reversed phase with the use of a UV detector, combined with GC.

The objective of our study of separation of indole derivatives by HPLC is a rapid and efficient analysis and praparation of substances of the indole type from partially purified extracts of microbial and plant origin.

EXPERIMENTAL

Separation of substances of the indole type was performed on mobile phases including ethanol (for UV spectroscopy), reagent-grade acetic acid (Lachema, Brno, Czechoslovakia) and double-distilled water.

The reference samples of indole derivatives were of the highest available purity and included: indole-3-aminopropionic acid (tryptophan), -3-ethylamine (tryptamine), -3-trimethylamine, -3-glyoxalic acid, -3-acetic acid, -3-carboxylic acid, -2-carboxylic acid, -5-carboxylic acid, -3-ethanol (tryptophol), -3-aldehyde, -3-pyruvic acid (Sigma, St. Louis, Mo., U.S.A.); -3-acetylaspartic acid, -3-glycolic acid, -3-ethyl acetate (Calbiochem, San Diego, Calif., U.S.A.); -3-lactic acid, -3-acrylic acid, indole (Fluka, Buchs, Switzerland); indole-3-acetamide, -3-acetaldehyde, 5-hydroxyindole-3-acetic acid (Koch-Light, Colnbrook, Great Britain); indole-3-methanol (Aldrich Europe, Beerse, Belgium); -3-acetonitrile (Loba Chemie, Vienna, Austria); -3-propionitrile (Schuchardt, München, G.F.R.); N-acetylindole-3-aminopropionic acid, indole-3-propionic acid, -3-butyric acid (Lachema); -3-butyramide (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia).

Bacteria (*Pseudomonas putida*, strain K_2) were cultivated in a mineral medium with the addition of 0.5% glucose and 0.01% L-tryptophan. After 72 h the bacteria were centrifuged, the supernatant was supplemented with sodium diethyldithiocarbamate (Lachema) to prevent the oxidation of indole-3-acetic acid and filtered through a bacterial filter. The volume was reduced to half on a vacuum evaporator at 35° and the proteins were precipitated by saturating the solution with ammonium sulphate. The solution was acidified to pH 2.5 and indole derivatives were extracted five times with 0.4 volume aliquote of methylene chloride which were then evaporated to dryness and the sample was dissolved in ethanol.

Solutions of reference substances and experimental samples, both in ethanol, were injected by means of a 10-μl syringe into the analytical column (amounts 1–5 μl) or by a 50-μl syringe into the preparative column (amounts 10–50 μl).

Analyses of a mixture of substances of the indole type were performed on a Varian 8500 liquid chromatograph with optional programming of the concentration gradient of the mobile phase. The apparatus was equipped with a Variscan LC UV detector permitting a continuous change of the wavelength, with an A 25 line recorder, the CDS 111 chromatography data system and fraction collector (Varian Aerograph, Walnut Creek, Calif., U.S.A.). Chromatographic columns were 25 cm × 2 mm I.D. (A, B) or 50 cm × 8 mm I.D. (C). The stationary phase was silica gel surface-modified by octadecylsilane (reversed phase). Two columns were purchased from Varian: MicroPak CH, particle size 10 μm, with the number of theoretical plates for indole-3-acetic acid, $N = 92$ (A); MicroPak CH, particle size 10 μm, $N = 305$ (C). The third column (B) was packed in our laboratory with LiChrosorb RP-18, particle size 5 μm (E. Merck, Darmstadt, G.F.R.), $N = 361$ (mobile phase 30:70), $N = 324$ (mobile phase 15:85, *cf.*, below).

Column temperature was 25°; the pressure in column A corresponded to 20.5 MPa, in column B to 59.5 and 45.0 MPa and in column C to 23.0 MPa. The mobile phase was in all three cases a mixture of ethanol and 1 % acetic acid: 20:80 (A) 30:70 and 15:85 (B), 20:80 (C). The phase flow-rate during isocratic elution was 40 ml/h (A), 20 ml/h (B) and 180 ml/h (C).

Gradient elution was carried out with the same solvent system as the isocratic one. The sample was injected on column B through which the ethanol–1 % acetic acid mixture (15:85) was allowed to flow. The ethanol content was increased from the beginning of the analysis at a rate of 1.5%/min. After 10 min, when the ratio of the two solvent components had reached 30:70, the elution was continued in an isocratic manner. The flow-rate was 20 ml/h and the pressure of 45 MPa increased gradually to 59.5 MPa.

Qualitative analysis of substances of the indole type was done by comparing their retention volumes with thouse of reference substances on the above columns.

The sensitivity limit for indole derivatives was determined with ethanol solutions of reference substances: $5 \cdot 10^{-5}$ *M* indole-3-acetic acid and indole-3-acetonitrile and $5 \cdot 10^{-6}$ *M* indole-3-aminopropionic acid using UV-detector at 280 nm.

RESULTS AND DISCUSSION

The reversed phase used for the analysis of substances of the indole type yielded satisfactory results. The separation and isolation of the compounds was carried out on two analytical columns (A, B) and one preparative column (C). Among the polar solvents testes, the best results were obtained with the mixture ethanol–1 % acetic acid used as the mobile phase. The isocratic elution of indole derivatives with this mobile phase on a stationary reversed phase also gave satisfactory results.

Satisfactory separation on column A, with the use of the solvent system ethanol–1 % acetic acid (20:80), was achieved in particular with:

(a) Substances of one homologous series, *e.g.*, indole-3-acetic, -propionic, -butyric, -glyoxalic and -pyruvic acid, etc.;

(b) Some derivatives of indole-3-acetic acid which have different functional groups, *e.g.*, 5-hydroxyindole-3-acetic acid, indole-3-acetamide, -acetonitrile, -ethyl acetate, -glycolic acid and -glyoxalic acid; some derivatives of indole-3-propionic acid, *viz.*, indole-3-aminopropionic acid, -propionitrile, -lactic acid; some derivatives of indole-3-butyric acid, *viz.*, indole-3-butyramide;

(c) Some substances with the same functional group in different positions on the indole ring, *viz.*, indole-5-, indole-3- and indole-2-carboxylic acid. The retention volume of the isomer with the carboxy group in position 2 was substantially higher compared with the 3- and 5-isomers. The difference is probably due to the hydrogen bond between the carboxy group in position 2 and imino group in position 1. The 2-isomer is less polar and is thus more strongly retained in the stationary reverse phase (Table I).

Column B exhibited a four-fold higher efficiency for the separation of indole-3-acetic acid; the separation of the derivatives was performed with the above solvent system (ratio of components 30:70) and the sequence was similar to that obtained

TABLE I

RELATIVE RETENTIONS OF INDOLE DERIVATIVES

Columns: MicroPak CH, particle size 10 μm, 25 cm × 2.0 mm I.D. (A); LiChrosorb RP-18, particle size 5 μm, 25 cm × 2.0 mm I.D. (B); MicroPak CH, particle size 10 μm, 50 cm × 8.0 mm I.D. (C). Flow-rates: 40 ml/h (A); 20 ml/h (B); 180 ml/h (C). Pressures: 20.5 MPa (A); 59.5 and 45.0 MPa (B); 23.0 MPa (C). Detection: UV detector Variscan LC, wavelength 280 nm.

Derivatives of indols (I)	*Solvent system, ethanol–1% acetic acid*							
	20:80 (A)		*30:70 (B)*		*15:85 (B)*		*20:80 (C)*	
	$r_{I,s}$	k	$r_{I,s}$	k	$r_{I,s}$	k	$r_{I,s}$	k
5-Hydroxy-I-3-acetic acid	0.26	0.25	0.38	1.00	0.21	0.80	0.26	0.45
I-3-aminopropionic acid	0.32	0.50	0.68	2.57	0.39	2.39	2.30	0.80
I-3-trimethylamine	0.37	0.75	1.51	7.00				
I-3-ethylamine	0.39	0.87	1.46	6.71	0.81	5.80	0.66	2.75
I-3-acetylaspartic acid	0.42	1.00	0.43	1.29	0.30	1.50	0.37	1.10
I-3-acetamide	0.47	1.25	0.51	1.71	0.40	2.40	0.59	2.35
N-acetyl-3-aminopropionic acid	0.58	1.75	0.65	2.43	0.56	3.70	0.66	2.75
I-3-glycolic acid	0.58	1.75	0.62	2.28	0.59	4.00		
I-3-lactic acid	0.66	2.12	0.68	2.57	0.68	4.70	0.76	3.30
I-3-glyoxalic acid	0.74	2.50	0.92	3.86				
I-5-carboxylic acid	0.84	3.00	0.79	3.18				
I-3-carboxylic acid	1.00	3.75	0.97	4.14	0.92	6.70	1.10	5.25
I-3-acetic acid	1.00	3.75	1.00	4.29	1.00	7.40	1.00	4.56
I-3-acetaldehyde	1.00	3.75						
I-3-ethanol	1.21	4.75	1.00	4.29	0.93	6.80	1.51	7.55
I-3-aldehyde	1.53	6.75	1.00	4.29	1.01	7.50	1.65	8.30
I-3-methanol	1.53	6.75	0.92	3.86				
I-3-butyramide	1.53	6.75	1.03	4.43				
I-3-acetonitrile	2.11	9.00	1.32	6.00	1.62	12.6	2.03	10.5
I-3-pyruvic acid	2.16	9.25	1.70	8.00	2.62	21.0	2.12	11.0
I-3-propionic acid	2.21	9.50	1.57	7.29	2.20	17.5	2.36	12.2
I-3-acrylic acid	2.53	11.0	1.76	8.29				
I-3-propionitrile	3.05	13.5	1.65	7.71				
I-2-carboxylic acid	3.15	14.0	2.31	11.2				
Indole	3.31	15.0	1.92	9.14			3.29	17.8
I-3-ethyl acetate	3.89	17.5	2.00	9.57				
I-3-butyric acid	4.37	19.7	2.81	13.9			5.40	29.5
Retention volume of I-3-acetic acid (ml)	2.53		2.46		5.54		67.8	

with column A *i.e.*, 5-hydroxy-indole-3-acetic, indole-3-lactic, -acetic, N-acetylindole-3-aminopropionic, indole-3-propionic, -2-carboxylic, -3-carboxylic, -3-butyric acid, etc. On column B, a number of substances exhibited slightly different retention volumes compared with column A. Their separation was more satisfactory, especially with some biologically interesting substances such as indole-3-aminopropionic acid, -ethylamine, -trimethylamine, -acetonitrile, -pyruvic acid and -propionic acid. On the other hand, the separation of some other substances, *viz.*, indole-3-acetic acid, -butyramide, -glyoxalic acid, -aldehyde, -ethanol, -methanol, became less satisfactory (Table I, Fig. 1a).

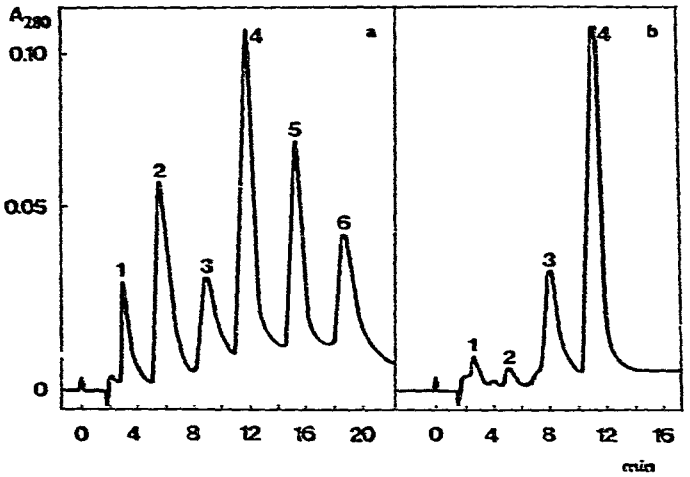

Fig. 1. Gradient elution of an artificially prepared mixture of substances of the indole type (a) and an extract of culture of *Pseudomonas putida* strain K_2 (b). Column, LiChrosorb RP-18 (B). Mobile phase, ethanol–1% acetic acid. Gradient elution: ethanol–1% acetic acid (15:85), 10 min with increasing ethanol concentration at 1.5%/min, 15 min with ethanol–1% acetic acid (30:70). Flow-rate of the mobile phase, 20 ml/h. Pressure, 45–59.5 MPa. Detection, Variscan UV at 280 nm. Peaks: a, 1 = 5-hydroxyindole-3-acetic acid; 2 = indole-3-aminopropionic acid; 3 = indole-3-lactic acid; 4 = indole-3-acetic acid; 5 = indole-3-acetonitrile; 6 = indole-3-pyruvic acid; b, 1 = probably 5-hydroxyindole-3-acetic acid; 2 = indole-3-aminopropionic acid and -acetamide; 3 = indole-3-lactic acid; 4 = indole-3-acetic acid and -aldehyde.

Separation on column B was carried out at a high pressure (59.5 MPa) while the mobile phase flow-rate was half that in column A. A reduction in the proportion of ethanol in the mobile phase (ratios 15:85 or 20:80) resulted in a lowering of the column pressure which imporoved the separation of some substances but led to an increased time of analysis. Column B with the solvent system ethanol–1% acetic acid (15:85) was then used only for measuring the retention volumes of some substances of biological interest (Table I). A gradient elution of the mobile phase during the analysis on column B improved the flow-rate/pressure relationship. A change in ethanol concentration in the mobile phase from the original ratio of 15:85 to 30:70 within 10 min yielded more satisfactory results than those obtained with the isocratic elution (Figs. 2 and 3).

Preparative column (C) which had more than a four-fold higher flow-rate (180 ml/h) gave, with the 20:80 mobile phase, a satisfactory separation of substances;

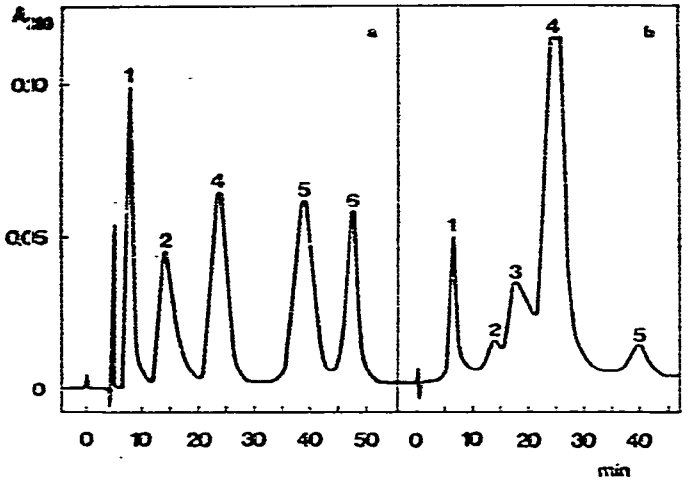

Fig. 2. Preparative HPLC of an artificially prepared mixture of substances of the indole type (a) and an extract of culture of *Pseudomonas putida* strain K_2 (b). Column, MicroPak CH (C). Mobile phase, ethanol–1 % acetic acid (20:80). Flow-rate of the mobile phase, 180 ml/h. Pressure, 23 MPa. Detector, Variscan UV at 280 nm. Peaks: 1 = indole-3-aminopropionic acid; 2 = indole-3-acetamide; 3 = indole-3-lactic acid; 4 = indole-3-acetic acid; 5 = indole-3-aldehyde; 6 = indole-3-acetonitrile.

its efficiency for the separation of indole-3-acetic acid was again three times higher than that of column A.

A lower limit of determination of some biologically significant substances was tested on column A at a wavelength of the UV-detector equal to 280 nm. The value for indole-3-acetic acid was 17.5 ng, for indole-3-acetonitrile 15.6 ng and for indole-3-aminopropionic acid 5.0 ng.

The extract from the supernatant obtained from the culture of *Pseudomonas putida* strain K_2, isolated from wheat rhizosphere, was analyzed on both analytical columns. Fig. 3 shows the chromatogram illustrating the separation of the extract on column A. Comparison of retention volumes of individual components of the extract with appropriate volumes of reference substances showed that peak 1 corresponded to indole-3-aminopropionic acid, peak 3 to indole-3-lactic acid, peak 4 to indole-3-acetic acid and peak 5 to indole-3-aldehyde.

Gradient elution on column B under the above conditions led to a better separation of individual components of the analyzed sample. Peak 1 in Fig. 1b probably represents 5-hydroxy-indole-3-acetic acid, peak 2 corresponds to indole-3-aminopropionic acid and -acetamide, peak 3 to indole-3-lactic acid and peak 4 to indole-3-acetic acid and -aldehyde.

The preparative column C separated the extract into five fractions (Fig. 2b) which were collected separately. The excess of mobile phase was evaporated off and the fractions were analyzed on both analytical columns. Fraction 1 contained indole-3-aminopropionic acid, fraction 2 corresponded to indole-3-acetamide, fraction 3 to indole-3-lactic acid, fraction 4 to indole-3-acetic acid and fraction 5 to indole-3-aldehyde. The results of the qualitative chromatographic analysis were confirmed by biological tests and mass spectrometry.

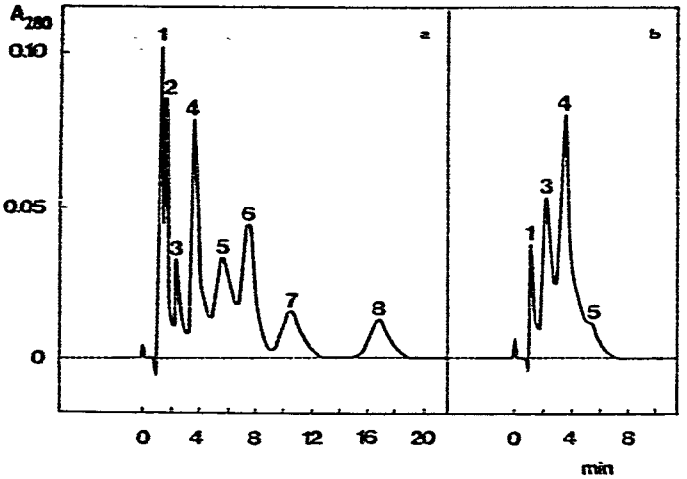

Fig. 3. Chromatogram of an artificially prepared mixture of substances of the indole type (a) and extract of culture of *Pseudomonas putida* strain K_2 (b). Column, MicroPak CH (A) Mobile phase, ethanol–1% acetic acid (20:80). Flow-rate of the mobile phase, 40 ml/h. Pressure, 20.5 MPa. Detector, Variscan UV at 280 nm. Peaks: 1 = indole-3-aminopropionic acid; 2 =indole-3-ethylamine; 3 = indole-3-lactic acid; 4 = indole-3-acetic acid; 5 = indole-3-aldehyde; 6 =indole-3-acetonitrile; 7 = indole-3-propionitrile; 8 = indole-3-butyric acid.

Our experience with the HPLC analysis of compounds of the indole type extracted from bacterial material shows, in keeping with literature data, that separation of these compounds requires the use of a combination of preparative and analytical columns. The identification of the above substances requires the combination of chromatographic and spectroscopic methods such as UV spectrometry, spectrofluorometry and mass spectrometry. The detection is again best with a combination of detectors, *e.g.*, UV detector and spectrofluorometric detector.

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