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Note

Analysis of indole derivatives by reversed-phase high-performance liquid chromatography

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The analysis of indole derivatives is of interest both in clinical chemistry and plant physiology. The measurement of indoles present in the brain and spinal fluid is of obvious importance in neurochemistry[1] and modified levels of indole derivatives and their metabolites have been found in patients with cancer of the breast[2] and cancer of the bladder[3]. One of the main groups of plant hormones, auxins, are also indole derivatives. It is generally believed that indole-3-acetic acid (IAA) is the auxin with the most important growth regulatory role, although other indoles do exhibit biological activity[4].

The physiological importance of auxins as plant growth regulators has stimulated the development of an array of methods for the analysis of endogenous IAA and other indole derivatives. Quantification by measuring the fluorescence of IAA after its conversion into indole-α-pyrone has been described[5]. This method is sensitive and is claimed to be very selective so that it can be used to analyse IAA in impure samples. However, in some instances extracts have been found to contain contaminants which interfere with the reaction and lead to the production of inaccurate data[7]. Analysis of indoles by gas chromatography (GC) with alkali flame ionisation[8] and electron-capture detectors[9] has been described. GC of IAA requires derivatization, and sample recovery is not without its problems. Other methods currently in use include combined GC–mass spectrometry (MS)[10]. Although GC–MS is widely regarded as the method of choice, the price and high running costs of MS instrumentation place GC–MS well beyond the reach of many investigators for routine use.

High-performance liquid chromatography (HPLC) has several advantages over other chromatographic procedures in the analysis of indoles: high efficiency coupled with high sample capacity; rapid speed of analysis; simplicity of sample recovery; ability to analyse non-derivatized samples; and relatively low running costs. In addition, as most indoles exhibit strong native fluorescence, the use of a fluorimetric detector can facilitate analysis at the picogram level.

Indoles have been analysed by reversed-phase[1,10], normal-phase[12], ion-pair[13] and silica gel adsorption[14] HPLC. Because of its simplicity the reversed-phase mode is the most commonly employed procedure. There is, however, no information in the literature on the relative abilities of solvents based on methanol, ethanol or acetonitrile to separate indoles by reversed-phase HPLC. This publication reports a study of reversed-phase HPLC of indoles in which attention was directed towards the nature

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of the organic modifier in the mobile phase and the effects of both pH and stationary phase alkyl chain length.

EXPERIMENTAL

The liquid chromatograph consisted of a M 45 and a M 6000 A pump, a U6K injector, a 660 solvent programmer (Waters Assoc.), a UV-III absorbance monitor (LDC) and a 3390 A printer/plotter (Hewlett-Packard). The HPLC columns employed were a 5-μm 100 × 5 mm I.D. Rad-Pak C_{18}, a 10-μm 100 × 8 mm I.D. Rad-Pak C_8 (Waters Assoc.) and a 5-μm 250 × 4.6 mm I.D. Hypersil ODS (Shandon Southern). Mobile phases containing acetic acid and ammonium acetate (Merck) were made up in doubly distilled water. HPLC grade methanol and acetonitrile were supplied by Rathburn (Walkerburn, Great Britain), and ethanol was obtained from A/S Vinmonopolet (Tromsø, Norway). IAA, 5-hydroxy IAA, indole acetamide, indole-2-carboxylic acid, indole-3-carboxylic acid, tryptophol, indole pyruvic acid, indole acetonitrile, indole propionic acid, indole acrylic acid and indole butyric acid (Sigma) were dissolved in methanol at a concentration of 5 $\mu g\ \mu l^{-1}$ and stored in dark at −20°C. Fresh solutions were prepared on a regular basis because of the limited stability of some of the indoles.

RESULTS AND DISCUSSION

Effects of pH

In order to examine the effect of pH, samples were analysed using 10 m*M* pH 7.0 ammonium acetate and 10 m*M* pH 3.5 acetic acid as the aqueous mobile phase and methanol as the organic modifier. The capacity factor values (k') obtained with the various indoles are presented in Tables I and II, which show that acids such as IAA elute much more rapidly at pH 7.0 than at pH 3.5. This is to be expected because at pH 7.0 the carboxyl groups are ionised, and in the reversed-phase mode charged molecules are distributed preferentially into the more polar aqueous mobile phase.

TABLE I

CAPACITY FACTORS (k') OF INDOLES

Column, Rad-Pak C_{18}; solvent A, 10 m*M* ammonium acetate in water; solvent B, 10 m*M* ammonium acetate in methanol.

Name	*k' as function of %B*					
	40%	*30%*	*20%*	*10%*	*5%*	*1%*
5-Hydroxy IAA	–	–	–	0.08	0.15	0.23
Indole acetic acid	–	0.08	0.15	0.62	0.77	1.78
Indole-2-carboxylic acid	0.08	0.15	0.38	0.77	1.15	1.69
Indole-3-carboxylic acid	0.23	0.28	0.62	1.00	1.54	2.15
Indole propionic acid	0.23	0.54	1.00	2.08	3.46	5.85
Indole acrylic acid	0.38	0.92	1.77	3.77	6.31	10.92
Indole butyric acid	0.84	1.35	2.54	5.69	9.62	–
Indole acetamide	1.15	2.38	5.08	11.30	–	–
Tryptophol	2.46	5.08	10.76	–	–	–
Indole pyruvic acid	2.62	5.62	12.0	–	–	–
Indole acetonitrile	3.23	7.46	–	–	–	–

TABLE II

CAPACITY FACTORS (k') OF INDOLES

Column, Rad-Pak C_{18}; solvent A, 10 mM acetic acid in water; solvent B, 10 mM acetic acid in methanol.

Name	*k' as function of %B*					
	60%	*50%*	*40%*	*30%*	*20%*	*10%*
5-Hydroxy IAA	0.15	0.31	0.54	1.15	2.38	6.31
Indole acetamide	0.23	0.31	1.08	2.54	4.92	11.31
Indole lactic acid	0.54	0.92	1.85	4.00	7.85	–
Indole-3-carboxylic acid	0.62	1.15	2.31	5.54	10.77	–
Tryptophol	0.69	1.31	2.69	5.31	10.85	–
Indole acetic acid	0.72	1.31	2.62	5.54	11.15	–
Indole pyruvic acid	0.77	1.46	2.85	5.62	11.69	–
Indole acetonitrile	0.85	1.77	3.69	8.15	–	–
Indole-2-carboxylic acid	1.08	2.38	4.92	10.85	–	–
Indole propionic acid	1.00	2.15	4.62	10.54	–	–
Indole acrylic acid	1.08	2.62	6.31	–	–	–
Indole butyric acid	2.08	3.38	8.23	–	–	–

Owing to the presence of the α-carbonyl group, the side-chain of indole pyruvic acid is a very strong acid. This explains why the retention properties of indole pyruvic acid are similar at pH 3.5 and pH 7.0.

Fig. 1 shows that all the molecules except indole lactic acid can be analysed by gradient elution with methanol and a 10 mM pH 7.0 ammonium acetate buffer. In this system indole lactic acid produced late eluting, broad asymmetric peaks. In addition, 5-hydroxy IAA, indole-2-carboxylic acid, and IAA all eluted rapidly with much lower effective k' values than would be advisable when analysing complex multicomponent samples in which the compounds of interest are trace constituents.

Effect of organic modifier

In order to examine the effect of the organic compound in the mobile phase on the chromatographic properties of indoles, samples were analysed on a Rad-Pak C_{18} column using various ratios of 10 mM acetic acid in methanol, ethanol or acetonitrile.

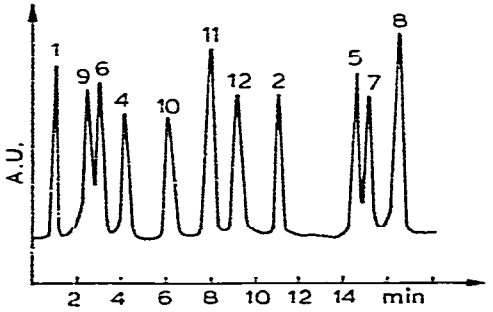

Fig. 1. Separation of indoles. Column, Rad-Pak C_{18}; solvent A, 10 mM ammonium acetate in water; solvent B, 10 mM ammonium acetate in methanol; gradient, 1% B to 50% B in 20 min; detection, UV at 254 nm; flow-rate, 2.0 ml min^{-1}. Peaks: 1 = 5-hydroxy IAA; 2 = indole acetamide; 3 = indole lactic acid; 4 = indole-3-carboxylic acid; 5 = tryptophol; 6 = indole acetic acid; 7 = indole pyruvic acid; 8 = indole acetonitrile; 9 = indole-2-carboxylic acid; 10 = indole propionic acid; 11 = indole acrylic acid; 12 = indole butyric acid.

The k' values obtained are presented in Tables II–IV, and the elution profiles from gradient analysis are illustrated in Fig. 2.

The data in Tables II–IV show that the indoles elute more rapidly with ethanol and acetonitrile than with methanol. Thus smaller volumes of ethanol and acetonitrile are used and this can be an important cost factor. In addition, with these solvents, there is a smaller change in background absorbance during gradient elution.

A comparison of Tables II and III and Figs. 2I and 2II shows that a similar elution order is obtained with methanol- and ethanol-based solvents. However, in both instances the separation of indole-3-carboxylic acid, IAA, indole pyruvic acid and tryptophol is inadequate. Furthermore, indole-2-carboxylic acid and indole propionic acid co-chromatograph in methanol, although they can be partially resolved when ethanol is used as the organic modifier.

TABLE III

CAPACITY FACTORS (k') OF INDOLES

Column, Rad-Pak C_{18}; solvent A, 10 mM acetic acid in water; solvent B, 10 mM acetic acid in ethanol.

Name	*k' as function of %B*					
	60%	*50%*	*40%*	*30%*	*20%*	*10%*
5-Hydroxy IAA	–	–	0.23	0.46	1.00	3.00
Indole acetamide	–	–	0.62	1.31	2.62	6.38
Indole lactic acid	–	0.77	1.08	2.23	4.23	10.31
Indole acetic acid	–	0.69	1.15	2.77	6.00	–
Tryptophol	–	0.69	1.23	2.85	6.00	–
Indole-3-carboxylic acid	–	0.54	1.08	2.92	6.53	–
Indole pyruvic acid	–	0.69	1.23	3.00	6.53	–
Indole acetonitrile	–	0.92	1.77	4.38	10.23	–
Indole propionic acid	–	1.00	1.92	5.23	–	–
Indole-2-carboxylic acid	–	1.23	2.23	6.00	–	–
Indole-3-acrylic acid	–	0.92	2.0	6.62	–	–
Indole-3-butyric acid	0.62	1.62	3.08	9.38	–	–

TABLE IV

CAPACITY FACTORS (k') OF INDOLES

Column, Rad-Pak C_{18}; solvent A, 10 mM acetic acid in water; solvent B, 10 mM acetic acid in acetonitrile.

Name	*k' as function of %B*					
	60%	*50%*	*40%*	*30%*	*20%*	*10%*
5-Hydroxy IAA	–	–	–	0.46	1.00	3.77
Indole acetamide	–	–	–	1.00	2.54	8.62
Indole lactic acid	–	–	0.92	1.77	4.23	13.46
Indole-3-carboxylic acid	–	–	0.69	1.62	4.62	–
Indole pyruvic acid	–	–	1.00	2.08	5.38	–
Indole acetic acid	–	–	1.00	2.15	5.77	–
Tryptophol	–	0.62	1.15	2.23	6.15	–
Indole-2-carboxylic acid	–	0.69	1.38	3.38	10.15	–
Indole propionic acid	–	0.69	1.46	3.85	12.23	–
Indole acrylic acid	–	0.62	1.23	3.31	13.08	–
Indole acetonitrile	0.62	1.23	2.54	5.54	15.54	–
Indole butyric acid	0.60	1.00	2.23	6.08	–	–

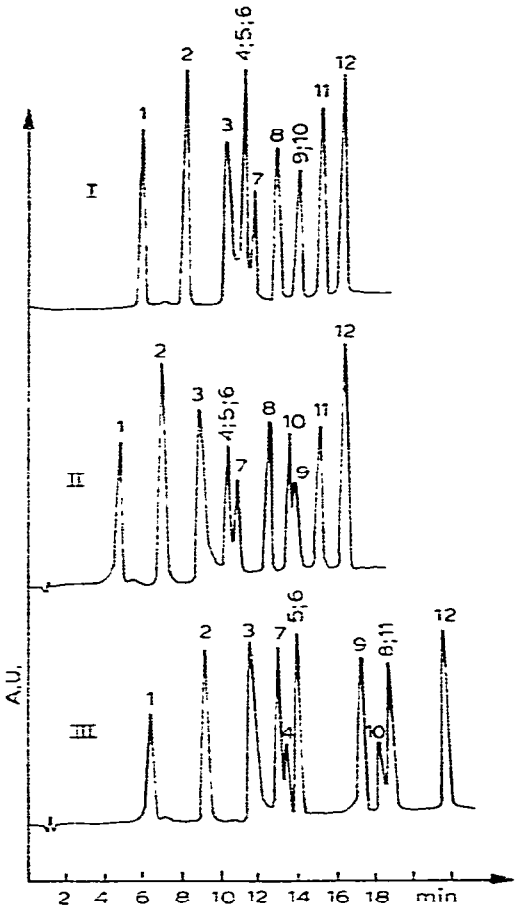

Fig. 2. Separation of indoles. Column, Rad-Pak C_{18}; solvent A, 10 m*M* acetic acid in water; detection, UV at 254 nm; flow-rates, 2 ml min^{-1}. I: Solvent B, 10 m*M* acetic acid in methanol; gradients, 10% B to 70% B in 20 min. II: Solvent B, 10 m*M* acetic acid in ethanol; gradient, 7% B to 50% B in 20 min. III: Solvent B, 10 m*M* acetic acid in acetonitrile; gradient, 5% B to 30% B in 20 min. Peaks as in Fig. 1.

It is a reasonable assumption that indole-3-carboxylic acid behaves in a similar manner to IAA in most of the procedures that are traditionally employed to purify plant extracts. The inability of the Rad-Pak C_{18} column to separate these compounds when a mobile phase modified with either methanol or ethanol is used is, therefore, a potential source of inaccuracy when endogenous constituents are to be analysed. However, the problem can be avoided because an acetonitrile-based solvent provides baseline separation of indole-3-carboxylic acid and IAA (Table IV and Fig. 2III)]. The use of acetonitrile has a further advantage in that its low viscosity reduces the column back-pressures experienced with 3-μm reversed-phase supports.

Choice of column

A comparison of the traces in Figs. 2(I) and 3 and the data in Tables II and V shows that, as far as the analysis of indoles is concerned, there are no significant differences in the selectivity of the Rad-Pak C_{18} and Hypersil ODS columns. The theoretical plate height (H) of the two columns was similar (Rad-Pak C_{18}, $H = 0.026$ mm; Hypersil ODS, $H = 0.033$ mm; both for IAA, $k' = 4.0$). However, as the length

of Rad-Pak columns is restricted to 100 mm, greater efficiences can be generated on a 250-mm Hypersil ODS column. Thus in circumstances where high resolution is required the Hypersil ODS column should be preferred.

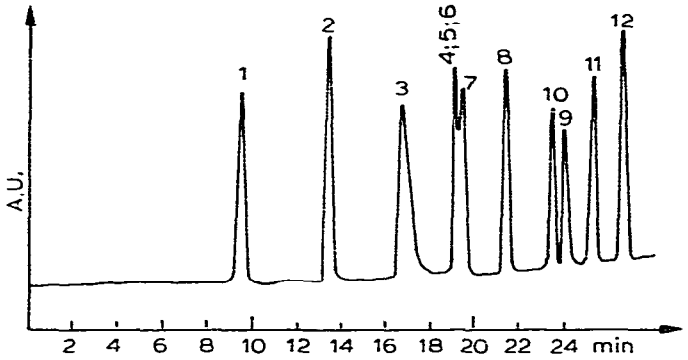

Fig. 3. Separation of indoles. Column, Hypersil ODS; solvent A, 10 m*M* acetic acid in water; solvent B, 10 m*M* acetic acid in methanol; gradient, 25% B to 70% B in 25 min. Peaks as in Fig. 1.

TABLE V

CAPACITY FACTORS (k') OF INDOLES

Column, Hypersil ODS; solvent A, 10 m*M* acetic acid in water; solvent B, 10 m*M* acetic acid in methanol.

Name	*k' as function of %B*					
	60%	*50%*	*40%*	*30%*	*20%*	*10%*
5-Hydroxy IAA	–	–	–	1.09	3.0	9.25
Indole acetamide	–	–	1.34	2.81	4.91	–
Indole lactic acid	–	1.00	2.22	5.43	–	–
Indole-3-carboxylic acid	–	1.22	2.88	7.19	–	–
Indole acetic acid	–	1.28	3.16	6.63	–	–
Indole pyruvic acid	–	1.31	3.00	7.00	–	–
Tryptophol	–	1.38	3.03	7.22	–	–
Indole acetonitrile	–	1.78	4.53	–	–	–
Indole propionic acid	1.03	2.53	6.53	–	–	–
Indole-2-carboxylic acid	1.19	2.97	6.97	–	–	–
Indole acrylic acid	1.16	2.91	8.63	–	–	–
Indole butyric acid	1.59	4.16	–	–	–	–

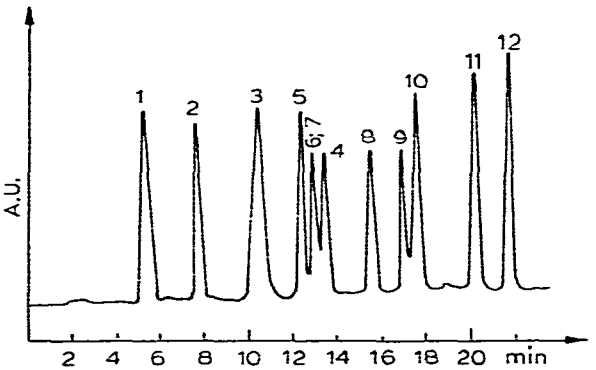

Fig. 4. Separation of indoles. Column, Rad-Pak C_8; solvent A, 10 m*M* acetic acid in water; solvent B, 10 m*M* acetic acid in methanol; gradient, 12% B to 60% B in 30 min. Peaks as in Fig. 1.

In order to assess the effect of alkyl chain length, a Rad-Pak C_8 column was compared with its C_{18} equivalent using a methanol–10 m*M* acetic acid mobile phase. Fig. 4 and Table VI indicate that the more polar C_8 column possesses some distinctive properties, because indole-3-carboxylic and tryptophol separate from IAA, and indole-2-carboxylic acid and indole propionic acid are also resolved. Although indole pyruvic acid co-chromatographs with IAA this is not an insurmountable problem, at least as far as the analysis of IAA is concerned, as IAA exhibits strong native fluorescence whereas indole pyruvic acid does not.

TABLE VI

CAPACITY FACTORS (k') OF INDOLES

Column, Rad-Pak C_8; solvent A, 10 m*M* acetic acid in water; solvent B, 10 m*M* acetic acid in methanol.

Name	*k' as function of %B*					
	60%	*50%*	*40%*	*30%*	*20%*	*10%*
5-Hydroxy IAA	–	0.54	0.92	1.92	3.69	10.15
Indole acetamide	–	1.08	2.00	3.85	7.23	–
Indole lactic acid	–	1.69	3.00	5.69	11.54	–
Tryptophol	–	2.23	4.31	8.23	–	–
Indole acetic acid	–	2.23	4.46	9.00	–	–
Indole pyruvic acid	–	2.38	4.76	9.07	–	–
Indole-3-carboxylic acid	–	2.30	4.69	11.38	–	–
Indole acetonitrile	1.23	3.00	6.46	13.85	–	–
Indole-2-carboxylic acid	1.46	3.85	8.46	–	–	–
Indole propionic acid	1.46	4.15	8.69	–	–	–
Indole acrylic acid	1.62	4.69	11.76	–	–	–
Indole butyric acid	2.08	6.08	15.08	–	–	–

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