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Chromatography of Indole Derivatives on Sephadex G-15

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The chromatographic properties of indole derivatives, mainly biogenetically related to the plant growth hormone, indole-3-acetic acid, were studied on Sephadex G-15. The eluents used were 70% and 30% aqueous methanol, volatile ethylenediamine-acetate buffers of pH 4.2 and 7.4, and distilled water adjusted to pH 7.4. The adsorption of indole derivatives to Sephadex could be preferentially ascribed to the heterocyclic nucleus which is bound by π complex formation, as well as by hydrogen and hydrophobic bonding. The presence of a side chain, if subject to the same type of binding forces, results not only in stronger sorption but also in some exclusion in accordance with molecular weight. Furthermore, because of the carboxyl groups contained in Sephadex gels, positively charged compounds are bound by ion exchange, whereas negatively charged ones are acted upon by Donnan ion exclusion forces.

INTRODUCTION

Numerous investigations[1–12] of the chromatographic properties of aromatic and heterocyclic compounds have established that they are adsorbed to Sephadex by one or more of the following mechanisms: complex formation, hydrogen bonding, and hydrophobic interaction. The elution of acids and bases is also influenced by the small number of carboxyl groups present in cross-linked dextran gels.

Sephadex chromatography has already been utilized for the separation of indolic compounds[13–20], but the methods used so far, have taken advantage only of some of these binding and exclusion forces. It seemed to us therefore that, by evaluating the multiple interaction of an indole derivative with Sephadex, more efficient procedures for the separation of complex mixtures of these compounds might be designed. For this reason in the present paper the chromatographic properties of a number of indoles, which are, mainly, related to the plant growth hormone, indole-3-acetic acid, are investigated on Sephadex G-15 in several solvents.

EXPERIMENTAL

Materials

Sephadex G-15 (∅ 40—120 μm) and the column (SR 25/45; diameter 25 mm) used in this work were supplied by Pharmacia AB, Uppsala, Sweden. Throughout this work, the same gel bed was used. Its dimensions and characteristic volumes — the total volume (V_t), the void volume (V_o), and the internal volume (V_i) — which are dependent on the eluent applied are given in Tables I and II where the results of the particular experiments are presented.

Indolic compounds were mainly obtained from commercial sources. Methyl esters were prepared from the acids with diazomethane, in the standard way, and 1-O--(indole-3-ethyl)-β-D-glucopyranose (tryptophol glucoside) was also synthesized in this laboratory[21]. Each compound used in this work was previously checked for chromatographic homogenity and, if necessary, submitted to further purification. The ethylenediamine used in buffers was redistilled in vacuo.

The composition of the buffers applied as eluents was (quantities given for 1 litre of buffer):

pH 4.2	ethylenediamine	1.15 ml
	acetic acid	6.00 ml
pH 7.4	ethylenediamine	4.65 ml
	acetic acid	6.00 ml

»Water pH 7.4« was prepared by adding ammonia to distilled water. Methanol concentrations were given as the volume of pure methanol per volume of methanol water mixture.

Chromatography

Since the majority of the investigated compounds is of limited solubility, the sample applied to the column (1—2 mg) was dissolved in 10 ml of the solvent used for chromatography. The following eluents were utilized:

1. aqueous methanol, 70%
2. aqueous methanol, 30%
3. water, pH 7.4
4. ethylenediamine-acetate buffer, pH 7.4
5. ethylenediamine-acetate buffer, pH 4.2

The column and the auxiliary equipment (LKB Ultrorac fraction collector and Uvicord III differential absorptiometer in connection with an automatic recorder) were placed in a cold-room at a temperature of 2—4 °C. The effluent was monitored for indolic compounds by absorbance at 256 and 280 nm. Determinations of elution volumes (V_e) were made at least in duplicate and the arithmetic mean was calculated. The differences between individual determinations were usually less than 1%. In Tables I and II, the peaks are characterized by the positions of their maximum (V_e) and the lengths of their ascending (—) and descending (+) parts. The effluents were also checked by thin-layer chromatography, using the solvent systems and spray reagents recommended by Stahl[22]. For this purpose, aliquots were evaporated in vacuo or, if containing a buffer, they were lyophilized and the residue was warmed up to 40 °C, at about 10^{-3} Torr,* for 2 hours, in order to remove ethylenediamine acetate.

Distribution coefficients (K_D) were calculated by the expression $K_D = (V_e - V_o)/V_i$.

RESULTS AND DISCUSSION

Choice of Experimental Conditions

Preliminary experiments were performed with special regard to the results of several previous workers[3,4,7,12]. Sephadex G-15 was found to be most suitable for the screening of indolic compounds widely different in structure. Eluents were selected so as to cast some light on the kind of interaction of Sephadex and indole derivatives: The chromatographic properties of non-ionogenic compounds were investigated in 70% methanol, where there should be very little, if any, hydrophobic bonding, as well as in 30% methanol and in an aqueous buffer, where the strength of hydrophobic interaction should progressively increase in magnitude[12]. The indolic acids were chromatographed at pH 7.4, at which they are fully dissociated, and at pH 4.2, when only the strongest ones are present in their ionized forms. Comparison of their elution volumes in the ethylenediamine-acetate buffer, pH 7.4, and in distilled water adjusted to the same pH value, should give some insight into the influence of solvent ionic

* 1 Torr = 133.322 Pa

strength on their chromatographic properties. Ethylenediamine-acetate buffers were used in this work, because they may be removed from the effluent by lyophilization.

*Chromatographic Properties of Non-Ionogenic Indole Derivatives***

The elution volumes of 18 non-ionogenic indole derivatives in 70% and 30% methanol and in an aqueous buffer are presented in Table I. Their sorption in 70% methanol, which should be most easily intelligible was studied more thoroughly.

TABLE I

Elution Volumes of Non-Ionogenic Indolic Compounds

Compounds	Elution volumes (ml) in the solvents		
	70% methanol	30% methanol	ethylenediamine-acetate, pH 4.2
Blue dextran (V_o)	70^{+15}_{-10}	82 ± 8	87 ± 15
acetone ($V_o + V_i$)	122 ± 15	149 ± 20	154 ± 15
1. 3-indolyl ethoxymethyl ketone	222 ± 20	—	—
2. 1-methylindole-2-carboxylic acid methyl ester	254 ± 30	—	—
3. indole-3-glyoxylic acid ethyl ester	264 ± 30	—	—
4. 3-indolyl methyl ketone	265 ± 40	—	—
5. 1,2-dimethylindole	266^{+40}_{-30}	—	—
6. indole-3-acetaldehyde	266^{+150}_{-40}	836^{+270}_{-90}	—
7. indole-3-butyric acid methyl ester	267 ± 35	—	—
8. indole-3-aldehyde	277 ± 35	1188^{+140}_{-100}	1893^{+250}_{-300}
9. indole-3-propionic acid methyl ester	278 ± 45	—	—
10. indole-3-acetic acid methyl ester	285 ± 40	1124^{+150}_{-100}	1240 ± 180
11. tryptophol glucoside	296 ± 45	692^{+110}_{-80}	663 ± 65
12. tryptophol	304 ± 45	1038^{+150}_{-110}	1206 ± 150
13. indole-2-carboxylic acid methyl ester	315 ± 35	—	—
14. indole-3-carboxylic acid methyl ester	318 ± 35	1926^{+200}_{-170}	—
15. indole-3-acetonitrile	334 ± 35	1792^{+190}_{-120}	—
16. indole-3-acetamide	337 ± 45	934^{+120}_{-80}	1162 ± 170
17. 2-methylindole	339 ± 30	—	—
18. indole	342^{+45}_{-30}	1356 ± 180	—

The positions of the maxima and the lengths of the ascending (—) and of the descending (+) parts of individual peaks are given. The total volume of the gel bed and the approximate flow rate were 174 ml and 10 ml/h, respectively, for 70% methanol as the eluent, 187 ml and 15 ml/h, respectively, for 30% methanol, and 206 ml and 25 ml/h for the aqueous buffer.

** The nomenclature of indole derivatives is not strictly that recommended by IUPAC but the one employed most frequently.

Sorption in 70% Methanol

The elution volumes of the compounds investigated ranged from 222 ml ($V_o + 2.9\ V_i$) to 342 ml ($V_o + 5.2\ V_i$). However, regardless of individual differences, there remains a common sorption effect which is to be ascribed to the presence of the heterocyclic nucleus. The affinity of the indole ring to dextran gels is, most likely, due to π complex formation and to hydrogen bonding involving the imino proton[7]. The contribution of hydrogen bonding is illustrated by a comparison of the elution volumes of 1,2-dimethylindole and 1-methylindole-2-carboxylic acid methyl ester and their analogues unsubstituted in position 1 of the indole nucleus (Table I).

Whereas the influence of the heterocyclic ring system on the binding of indolic compounds to Sephadex is relatively clear, the contribution of the substituents is more difficult to explain. Their π and hydrogen bonding abilities, which should be the only sorption forces to be considered in 70% methanol cannot be unequivocally deduced from the existing literature. The only firm facts are that the additional π complex given by the cyano group of indole-3--acetonitrile[8] and the additional hydrogen bond involving the rather acidic amido protons of indole-3-acetamide should be outstandingly strong. Actually, the elution volumes of these compounds are found among the largest values included in Table I. However, indole itself has a larger elution volume of even these, most strongly bound, derivatives. It was therefore suspected that the elution of the investigated compounds is influenced not only by their interaction with the gel matrix, but also by molecular sieving. Such a composite separation mechanism, though already postulated by Determann[23], has so far been utilized only sporadically[8].

If molecular sieving has a significant influence on the chromatographical properties of indole derivatives in 70% methanol, their relative elution volumes (V_e/V_o) and the logarithms of their molecular weights (MW) should be correlated linearly. This was indeed observed. If three compounds are left out from the calculations — the nitrile (Compound 15) and the amide (compound 16) with their outstanding binding capacities, and the glucoside (compound 11) with an expected molecular weight to molecular volume ratio different from the other indole derivatives investigated — the significance level is 99.9% for the product moment, and even higher for the rank correlation coefficient.

In spite of the high significance levels obtained for the correlation coefficients, the least-square regression line ($V_e/V_o = 13.101 - 4.119$ log MW) is interesting only from a theoretical point of view: The affinity of a particular indole derivative to the gel matrix may be tentatively correlated to the difference between its observed elution volume and the value predicted by the regression equation. It is, however, just this scattering about the regression line which makes it rather inconvenient for analytical application. We attempted therefore to split up the sample of the investigated indole derivatives (excl. compds. 11, 15, 16) into two groups: One of them comprises the 1-methylated compounds lacking the hydrogen bonding site of the indole nucleus, and those bearing a —(C : O)R moiety (R=H, CH —, CH_2) adjacent to the heterocyclic nucleus which influences the acidity of the *N*-1 hydrogen[24,25]. The remaining 10 compounds represent the other group. The two regression lines obtained by this grouping are practically parallel to each other (Figure 1). The scattering of experimental points about these lines is reduced to such an extent that they

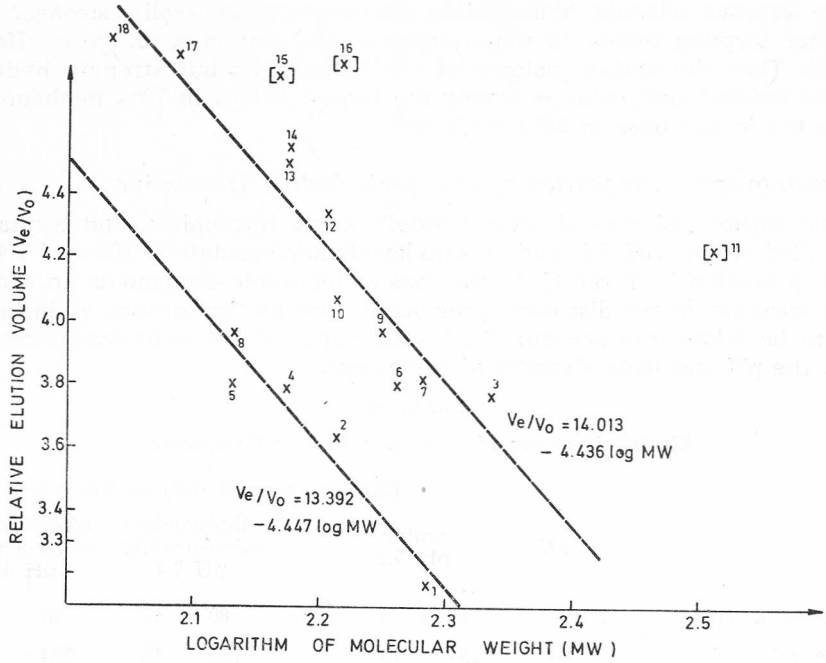

Figure 1. The dependence of relative elution volumes on the logarithms of molecular weights of non-ionogenic indole derivatives.

Compounds: 1, 3-indolyl ethoxymethyl ketone; 2, 1-methylindole-2-carboxylic acid methyl ester; 3, indole-3-glyoxylic acid ethyl ester, 4, 3-indolyl methyl ketone; 5. 1,2-dimethylindole, 6, indole-3-acetaldehyde; 7, indole-3-butyric acid methyl ester; 8, indole-3-aldehyde; 9, indole-3-propionic acid methyl ester; 10, indole-3-acetic acid methyl ester; 11, tryptophol glucoside; 12, tryptophol; 13, indole-2-carboxylic acid methyl ester; 14, indole-3-carboxylic acid methyl ester; 15, indole-3-acetonitril; 16, indole-3-acetamide; 17, 2-methylindole; 18, indole.

To indole-3-acetaldehyde (compound 6) the molecular weight of its methyl hemiacetal was attributed, to which it should be mainly converted in aqueous methanol. For the other carbonyl compounds included in this study, an analogous reaction with methanol is not to be expected[29].

might possibly prove useful for the prediction of relative elution volumes (on Sephadex G-15 in 70% methanol) of similar non-ionogenic compounds not incorporated into this study.

Sorption in 30% Methanol and the Aqueous Buffer

When the concentration of water in the eluent is enhanced, the elution volumes of non-ionogenic compounds increase appreciably (Table I). Since methanol and water are solvents of similar polarity, hydrogen bonding and π bonding are not significantly affected by this change in eluent composition. Neither is gel filtration, because the characteristic volumes of the column remain comparable in magnitude. Therefore, the hydrophobic interaction remains the only mechanism to account for the observed additional sorption. Thus, for example, tryptophol and indole-3-carboxylic acid methyl ester have similar elution volumes in 70% methanol; however, when the methanol concentration decreases to 30%, the elution volume of the relatively hydrophilic alcohol increases about three times, whereas the elution volume of the lipophilic ester increases about six times. This example also indicates that, in prepond-

erantly aqueous solvents, hydrophobic interaction is, generally, stronger, than the other sorption forces. In some instances, the elution sequence is affected, as well. Thus the elution volume of the hydrophilic but strongly hydrogen bonding indole-3-acetamide is among the largest values in 70% methanol and among the lowest ones in 30% methanol.

Chromatographic Properties of Ionogenic Indole Derivatives

The elution volumes of several indolic acids, tryptophan, and tryptamine in distilled water, pH 7.4, and in ethylenediamine-acetate buffers of 7.4 and 4.2 are presented in Table II. In the case of ionogenic compounds, in addition to the sorption forces discussed previously, two further groups of influences have to be taken into account: 1. the pK value of the individual compound and 2. the pH and ionic strength of the eluent.

TABLE II

Elution Volumes of Ionogenic Indolic Compounds

		Elution volumes (ml) in the solvents		
	pK[a]	water pH 7.4	ethylenediamine-acetate pH 7.4	ethylenediamine-acetate pH 4.2
Blue dextran (V_o)	—	80 ± 15	80 ± 15	87 ± 15
acetone ($V_o + V_i$)	—	154 ± 15	154 ± 15	154 ± 15
indole-3-glyoxylic acid	3—4	210 — 240 $^{+\ 20}_{-\ 80}$	600 ± 65	593 ± 65
indole-3-lactic acid	3—4	160 — 190 $^{+\ 10}_{-\ 50}$	402 ± 35	583 ± 60
indole-3-acetic acid	4—5	140 — 175 $^{+\ 30}_{-\ 60}$	369 ± 35	1159 ± 100
indole-3-propionic acid	4—5	215 — 235 $^{+\ 50}_{-\ 70}$	607 $^{+\ 70}_{-\ 50}$	2020 $^{+\ 250}_{-\ 150}$
indole-3-butyric acid	4—5	240 — 255 ± 50	707 $^{+\ 90}_{-\ 60}$	2513 $^{+\ 270}_{-\ 180}$
indole-3-carboxylic acid	7	190 — 230 $^{+\ 30}_{-\ 35}$	549 ± 50	4380 $^{+\ 450}_{-\ 280}$
indole-3-acrylic acid	7	360 — 380 ± 100	2218 $^{+\ 350}_{-\ 125}$	too large for exact determination
tryptophan	2.4/9.4	476 ± 60	490 ± 70	494 ± 65
tryptamine	10.5	not eluted	593 $^{+\ 120}_{-\ 80}$	635 ± 65

[a] The pK values of tryptophan, indolecarboxylic acid (7.00) and indoleacetic acid (4.6) are given according to Perrin[30], Melzer[31], and Cohen et al.[32]. The other values were estimated from the dissociation constants of structurally related compounds[33].

The positions of the maxima and the lengths of the ascending (—) and the descending (+) parts of individual peaks are given. The total volume of the gel bed was 206 ml, the flow rate was 25 ml/h.

As long as the forces arising from the ionogenic nature of these compounds are of the same order of magnitude, their chromatographic properties may be explained in terms of the sorption forces discussed for non-ionogenic compounds. Thus, the elution volumes of the indole-3-alkanoic acids in the three solvents used — except indole-3-carboxylic acid with its significantly larger pK value — are correlated to the number of carbon atoms in the side chain which determines hydrophobicity.

According to Neddermeyer and Rogers[26], the chromatographic properties of anionogenic compounds on Sephadex gels are governed by Donnan equilibria. However, in the case of indolic compounds, their affinity to the gel matrix has to be included as an additional term. Thus, whereas inorganic anions chromatographed in deionized water are completely excluded from the Sephadex beads[26], indole-3-acetic acid, fully dissociated in »water pH 7.4«, retains a K_D-value in the range from 0.81 to 1.28 in this solvent. The other acids investigated have even larger partition coefficients.

In »Water pH 7.4«, the elution volumes of indolic acids are extremely concentration dependent. In some instances, their peaks are skewed, ascending gently and descending steeply, as described for inorganic ions[26], or they are even split. When the solvent is changed from »water pH 7.4« to an ethylenediamine-acetate buffer, the indolic acids emerge in symmetrical or slightly tailed peaks, and the position of the maximum is no longer concentration dependent.

In the neutral buffer, the elution sequence of the acids is the same as in »water« of the same pH. However, due to the presence of a background electrolyte, anion exclusion is partially suppressed, and the K_D-values are larger, generally by a factor of 3.6 ± 0.2. Only for indole-3-acrylic acid, this factor is 7.4. In the case of this extremely insoluble acid, the ionic strength of the neutral buffer is most likely sufficient to give rise to an additional salting-out effect[27,28].

When the pH of the eluent is decreased to 4.2, the majority of the acids investigated ceases to be fully dissociated. This leads to some perturbations in the elution sequence, because an undissociated acid molecule is not subject to Donnan equilibria and freely enters the Sephadex beads. Furthermore, the undissociated carboxyl group is likely to form an additional hydrogen bond to the gel matrix and is less solvated, which makes the undissociated acids more liable to saltingout effects. Thus, indole-3-propionic and indole-3-glyoxylic acids, for example, have almost identical chromatographic properties at pH 7.4, where the two acids are fully dissociated, however, at pH 4.2 indole-3-glyoxylic acid is dissociated to a larger extent and is eluted before the elution of indole-3-propionic acid.

The relative invariance of the elution volume of tryptophan in the eluents used is probably due to the fact that at pH 4.2 this amino acid is mainly in its zwitterion form, and its chromatographic properties are not affected by Donnan equilibria. On the other hand, because of the presence of two charged groups, the molecule of tryptophan is appreciably solvated and hydrophilic, and is therefore eluted before the elution of any of the non-ionogenic compounds investigated in this study.

Tryptamine, as a base, is bound by the carboxyl groups of Sephadex and cannot be eluted by distilled water[20], but only by salt solutions, the cations of which compete for the ion exchange capacities of the gel. The neutral buffer with its larger ionic strength is somewhat more efficient in this respect than the acidic one. Except by ion exchange, tryptamine seems to be bound also by hydrogen bonding involving the primary amino group. This may be concluded from the results of Demetriou et al.[3] obtained under similar working conditions (NaCl—HCl, 50 mM, pH 4.0) according to which *N,N*-dimethyltryptamine — although a stronger base — has a significantly smaller elution volume than tryptamine.

CONCLUSION

Although the results obtained are by no means sufficient for an exact description of the gel chromatographic behaviour of indole derivatives in general, they should at least permit a tentative prediction of the elution sequence of compounds structurally related to those investigated in the present study, and they can be utilized as a guide to practical separation procedures. For instance, the separation of a mixture of acidic, neutral, and basic indole derivatives on Sephadex G-15 should probably be initiated by a sequence of buffers. A proper choice of pH values and ionic strengths will bring about the separation of individual acids and bases which can be washed through the column before non-ionogenic compounds begin to appear in the effluent. These compounds may then be eluted by mixtures of water with methanol or other organic solvents. A method for separating a complex mixture of indolic compounds from plant extracts, based on this general concept, is in progress.

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SAŽETAK

Kromatografija indolskih derivata na Sephadex-G15

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Proučavana su kromatografska svojstva na Sephadex-G15 više derivata indola, koji se mogu dovesti u vezu s biogenezom i metabolizmom indol-3-octene kiseline, biljnog hormona rasta. Upotrebljeni su ovi eluenti: 70% i 30% metanol, hlapivi etilendiamin-acetatni pufer pH = 4,2 i 7,4, te destilirana voda kojoj je pH ugođen na 7,4. Istraživani indolski derivati vežu se na Sephadex raznim silama čiji odnos ovisi o svojstvima molekule i o vrsti eluenta. Vezanju najviše doprinosi heterociklička jezgra, koja s matricom sorbensa može tvoriti π-komplekse, te vodikove i hidrofobne veze. Postrani lanac, ako ima slične mogućnosti, doprinijet će sorpciji, ali će donekle i smanjiti zbog povećanja težine (veličine) molekula. Ionogeni derivati, osim toga, reagiraju s karboksilnim skupinama na Sephadex-u, što dovodi do jačeg vezanja pozitivno nabijenih derivata ionskom izmjenom, odnosno do isključenja negativno nabijenih zbog Donnanovih sila.

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