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GEL FILTRATION CHROMATOGRAPHY OF FLUORESCENT PHENOLIC AND HETEROCYCLIC COMPOUNDS*

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SUMMARY

A systematic study has been made of the chromatographic behavior of individual phenolic and heterocyclic compounds on Sephadex G-15. The partition coefficients of these compounds with distilled water or an acid–salt solution as eluants were determined.

Compounds with a carboxylic acid substituent on the ring structure were found to be excluded from the gel beads with distilled water as the eluant. However, these compounds were adsorbed on columns developed with the acid–salt solution.

Phenolic and indolylic compounds with an amine group in the aliphatic side chain were bound to the gel beads by the cationic properties of the gel matrix. Development of the column with the acid–salt eluant afforded the fractionation of the phenolic and indolylic compounds.

Hydroxy, methoxy and dihydroxy substituents on the ring structure resulted in increased adsorption when the columns were developed under conditions in which ion-exclusion effects were not operative or suppressed.

The degree of adsorption by the gel matrix was found to be affected by the functional groups in the aliphatic side chain of the compounds. For an acid–salt column, the order of elution for either phenolic or indolylic compounds was in the following sequence: amine, glycol, and finally carboxylic acid derivatives.

A method is presented for the separation of carboxylic acid derivatives from amino acid or glycol derivatives with the distilled water eluant, and subsequently, for the fractionation of phenolic and indolylic compounds with the acid–salt eluant.

INTRODUCTION

Gel filtration chromatography affords a method for the fractionation and quantitative recovery of small and large molecules[1,2]. The primary applications of

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cross-linked dextran gel have been the separation of large molecules by virtue of the molecular dimensions or the removal of small molecules from larger ones. GELOTTE's report demonstrated the applicability of dextran Sephadex for the fractionation of small molecules[2]. Since then, systematic studies of the chromatographic behavior of small molecules on the gel material have been relatively few[3–5]. The recent review of adsorption phenomena on Sephadex by JANSEN has attempted to explain the interactions and anomalous behavior of small molecules on cross-linked dextran[6].

In the course of attempting to fractionate certain fluorescent aromatic and heterocyclic compounds on dextran gel Sephadex G-15, it became apparent that further studies were necessary to properly utilize the ion-exclusion, ion-exchange, and adsorption properties of the gel matrix. Our findings agree with other investigators on the chromatographic behavior of aromatic and heterocyclic compounds. However, examination of analogues with different functional groups has provided several new facets on the interactions of solutes with the gel matrix.

This paper deals with the chromatographic migration of aromatic and heterocyclic hormones, their metabolites, and certain structurally related analogues on columns of Sephadex G-15. In addition, a novel method is presented that permits the separation of phenolic or indolylic acids from analogous glycols, and the fractionation of phenolic and indolylic amines on a single chromatographic column.

METHODS AND MATERIALS

Column preparation and operation

The cross-linked Dextran, Sephadex G-15 (Pharmacia AB), used in the study had a particle size of 40–120 microns and a water regain of 1.5 g/g. The dry powder was mixed with glass distilled deionized water, and the fines were removed by repetitive mixing, sedimentation and decantation.

Glass columns with O-ring joints (Kontes Glass Co.) and a stopcock with a Luer joint delivery tip were employed. The column dimensions were 1.2 cm in diameter and 50 cm in length. A coarse frittered glass disc at the bottom of the column supported the gel column.

The chromatography columns were prepared by addition of the slurry of gel beads to a height of 42 cm of packed gel. With the aid of an extension tube clamped on to the top of the column, the packed gel was uniformly raised to an approximate height of 40 cm by applying pressure with a column of water from an elevated reservoir. With the stopcock closed, the gel beads were allowed to settle. Firm tapping of the column promoted further settling of the gel beads. Final compression of the gel column was accomplished by percolating distilled water from an elevated reservoir through the column for several hours. This procedure provided uniformly packed columns that exhibited no layering or banding of the gel when viewed with a strong light. A flow rate of 35 $\pm$ 4 ml/h was used for all the chromatographic runs.

All compounds were obtained from commercial sources and were used without further purification. Each compound was dissolved in 0.05 *M* phosphate buffer pH 6.1, and the amount applied to the gel column was varied according to the relative fluorescence of the compound.

A 0.2 ml volume of each compound was applied directly onto the gel surface and allowed to percolate into the gel beads. Three additions of 0.1 ml of eluant were

made to insure the complete introduction of the sample into the gel surface. The space above the surface was filled with 10 ml of the eluant, and the column was connected to the reservoir with a plastic tube.

The two eluants used for development of the gel columns were (a) deionized distilled water, and (b) 50 millimolar sodium chloride solution adjusted to pH 4.0 with dilute hydrochloric acid. The latter eluant will be referred to as the acid–salt solution in the text. The chromatographic behavior of the compounds was first studied with the distilled water equilibrated columns and subsequently with the acid–salt equilibrated columns.

Repetitive use of gel columns resulted in a compaction of the gel surface and a resultant decrease in flow rate of the eluant. This effect was counteracted by periodically stirring the top 2 cm of the gel beads and then allowing them to settle.

Fluorometric monitoring of columns

A fluorometer (Turner—Model 111) with a flow-cell attachment was used to continuously monitor the fluorescent content of the column effluent solution. A potentiometric recorder (Bausch & Lomb—VOM-5), set at a speed of 3 inches/hour, provided a tracing of the elution of each compound. The fluorometer lamp (Westinghouse F4T5/UV 31) had a continuous emission from 270 to 340 nanometers (nm) and a peak emission at 306 nm. The light filter system consisted of a primary filter (Baird-Atomics Interference Type) with peak transmission at 280 nm, and a secondary filter (Corning CS No. 7-60) with peak transmission at 360 nm. The aperture openings used were at the 1 × or 3 × setting. All of the compounds, with the exception of xanthurenic acid, gave a fluorescence response with the described lamp and light filter combination. The latter compound gave an absorption response.

RESULTS

Presentation of data

The elution volume (V_e) for each compound represents the volume of eluant required for elution from a 40 cm column of Sephadex G-15 with a total bed volume (V_t) of 50 cc. The V_e measurement was made from the time of introduction of the sample onto the gel surface until the point of maximum fluorescence on the recorder tracing. The fluorescence elution tracings obtained for most of the compounds were essentially quasi-symmetrical peaks.

The partition coefficient (K_{av}) for each compound was calculated from the equation,

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_0 is the void volume of the column and V_t is the total volume of the gel beads. The V_0, determined with Blue Dextran 2000 (Pharmacia AB), was found to be 20 ml.

Reproducibility of elution volumes

The consistency of column operation was evaluated by repetitive chromatography of individual compounds using distilled water as the eluant. A mixture of three compounds was used to test the reproducibility of elution volumes on an acid–

TABLE I

REPRODUCIBILITY OF ELUTION VOLUMES (V_e) ON SEPHADEX G-15

	V_e (ml) for each run			
	No. 1	*No. 2*	*No. 3*	*No. 4*
Individual Compounds: Water Eluant				
5-Hydroxy-indoleacetic acid	29	30	32	—
3-Methoxy-4-hydroxy-phenethylglycol	71	70	—	—
Mixture: Acid–Salt Eluant				
Metanephrine	51	52	53	53
Tryptamine	113	117	121	123
Serotonin	144	150	157	162
	Ratios			
Separation Factors				
Tryptamine–Metanephrine	2.24	2.24	2.31	2.34
Serotonin–Metanephrine	2.86	2.86	2.90	3.09
Serotonin–Tryptamine	1.28	1.28	1.29	1.32

salt column. The results are shown in Table I. Although there were shifts in the elution volume of individual compounds, calculation of separation ratios showed that the relative fractionation of the compounds remained fairly consistent.

Distilled water elution study

Carboxylic acid, amino acid, or glycol derivatives of heterocyclic or phenolic compounds were found to be readily eluted by distilled water (Table II). Those compounds with a carboxy group were eluted with relatively small volumes of eluant, regardless of the structure of the parent compound. The small elution volumes for compounds with a prominent carboxy substituent is due to the ion-exclusion properties of the gel matrix.

TABLE II

WATER ELUTION OF AROMATIC AND HETEROCYCLIC ACIDS AND GLYCOLS

Compound	*Quantity* (μg)	V_e(ml)	K_{av}
Kynurenic acid	5	29	0.30
5-Hydroxy-indoleacetic acid	10	29	0.30
4-Hydroxy-phenyllactic acid	65	30	0.33
3-Methoxy-4-hydroxy-mandelic acid	65	31	0.37
3-Methoxy-4-hydroxy-phenylacetic acid	65	31	0.37
3,4-Dihydroxy-phenylacetic acid	65	31	0.37
Anthranilic acid	1	32	0.40
Indole-3-acetic acid	10	32	0.40
Xanthurenic acid	20	32	0.40
Vanillic acid	10	35	0.50
Tyrosine	100	51	1.03
3,4-Dihydroxyphenylalanine	100	58	1.27
3-Methoxy-4-hydroxy-phenethylglycol	100	71	1.70
3,4-Dihydroxy-phenethylglycol	100	91	2.37

The two phenolic amino acids that were tested showed slightly larger elution volumes. This effect could be attributed to an interaction of the alpha amino group with the carboxylic group. Interestingly, the alpha hydroxy group or the ortho amino group on some of the carboxylic acid compounds had no effect on the exclusion of the acidic analogues.

Both derivatives of phenethylglycol also required large volumes of water for elution. These results suggest that the phenolic amino acid and glycol derivatives are adsorbed by the gel matrix.

The effects of ring substitutions, such as methoxy, hydroxy or dihydroxy groups, on the relative elution volumes were apparent only for those compounds that were adsorbed. Furthermore, the two dihydroxy derivatives were adsorbed to a greater degree than tyrosine or 3-methoxy-4-hydroxy-phenethylglycol.

Acid–salt elution study

Compounds with an aliphatic amine group, applied to distilled water equilibrated columns, could not be eluted with distilled water. Elution of amines could be effected only after incorporation of small amounts of electrolytes or dilute acid into the eluant. This binding of amines by the gel is due to weak cationic properties of the gel. The elution volumes and K_{av} values for a series of phenolic and indolylic amines obtained with the acid–salt eluant are shown in Table III.

The most prominent feature in Table III is the larger volumes of eluant required for the elution of the indolylic amines when compared with the phenolic amines. Apparently, the indoles are adsorbed to a greater degree by the gel matrix than the phenolic amines.

Although the acid–salt eluant tends to neutralize the ion-exchange properties of the gel, there is still some evidence of a residual ionic interaction with some of the derivatives. Evidence for this effect is shown by smaller elution volumes for mono- and dimethyl- derivatives of the primary amines. The following pairs of compounds

TABLE III

ACID–SALT ELUTION OF AROMATIC AND HETEROCYCLIC AMINES

Compound	*Quantity* (μg)	$V_e(ml)$	K_{av}
Tyramine	100	55	1.17
DL-Metanephrine	100	55	1.17
DL-Octopamine	100	58	1.27
3-Methoxy-4-hydroxy-phenethylamine	100	60	1.33
DL-Normetanephrine	100	62	1.40
L-Epinephrine	100	62	1.40
DL-Norepinephrine	100	69	1.67
3,4-Dihydroxy-phenethylamine	100	72	1.73
N,N-Dimethyl-tryptamine	40	87	2.23
N,N-Dimethyl-5-hydroxy-tryptamine	4	113	3.10
Tryptamine	35	115	3.17
N-Acetyl-5-methoxy-tryptamine	20	130	3.67
5-Methoxy-tryptamine	40	148	4.27
Serotonin (5-hydroxy-tryptamine)	40	157	4.57
N-Acetyl-5-hydroxy-tryptamine	10	249	7.63
Indole	100	328	10.27

illustrating this effect are: metanephrine–normetanephrine; epinephrine–norepinephrine; N,N-dimethyltryptamine–tryptamine and N,N-dimethyl-5-hydroxy-tryptamine–serotonin.

From the results for methylated derivatives, a similar reduction in elution volume with the two N-acetylated compounds was anticipated. However, both an increase and a decrease in elution volumes was noted. The N-acetyl derivative of 5-methoxy-tryptamine showed a decrease in elution volume of 18 ml, whereas the volume required for the elution of N-acetyl-5-hydroxy-tryptamine was 92 ml greater than for the parent compound.

The addition of a functional group to an aromatic or heterocyclic "nucleus" had a definite effect on the elution volumes with the acid–salt column. Changing from a 4-hydroxy to a 3-methoxy-4-hydroxy or to a 3,4-dihydroxy derivative resulted in a progressively larger K_{av} value. This effect was observed for both the phenethylamine and phenethanolamine type compounds. The elution volumes for tryptamine, 3-methoxy-tryptamine and serotonin showed a similar relationship for the indolylic amines.

Since ion-exchange effects are suppressed by electrolyte-containing eluants, the chromatographic behavior of some of the indolylic acids, phenolic acids, and glycols was reexamined with the acid–salt elution system. Table IV shows the elution volumes of these compounds in each of the elution systems. The findings from this comparative study are:

(a) The two compounds, dihydroxyphenylalanine and 3-methoxy-4-hydroxy-phenethylglycol that were adsorbed on the distilled water equilibrated column, behaved identically on the acid–salt column.

(b) The four compounds that were excluded by distilled water were adsorbed by the gel with the acid–salt eluant.

(c) The phenolic acids tended to be well separated from the indolylic acids.

Furthermore, it was noted that the acidic analogues of certain compounds were adsorbed to a greater degree than the corresponding amines on the acid–salt column. The elution volumes shown in Table V for four pairs of acid and amine analogues clearly illustrate this point.

Two stage elution chromatograms

The results obtained from the study of single compounds with the two eluant systems were then applied to effect the separation of a selected mixture of compounds. The mixture was applied to a distilled water equilibrated column and distilled water

TABLE IV

COMPARISON OF ELUTION VOLUMES OF COMPOUNDS ON DISTILLED WATER OR ACID–SALT COLUMNS

Compound	*Water (ml)*	*Acid–salt (ml)*
5-Hydroxy-indoleacetic acid	29	250 (T)*
3-Methoxy-4-hydroxy-mandelic acid	31	65
3-Methoxy-4-hydroxy-phenylacetic acid	31	88 (T)
Indole-3-acetic acid	32	203 (T)
3,4-Dihydroxyphenylalanine	58	59
3-Methoxy-4-hydroxy-phenethylglycol	71	68

* T = Tailing of peaks.

was percolated through the column for 3 h. The eluant was then changed to the acid–salt solution and allowed to run for 5 h. The fractionation of the mixture of phenolic and indolylic compounds using this two stage elution system is shown in Fig. 1.

In the fractionation of the mixture of compounds, the three properties of the gel matrix, ion-exclusion, ion-exchange and adsorption were utilized.

TABLE V

ELUTION VOLUMES OF AMINE AND ACID ANALOGUES WITH THE ACID–SALT ELUANT

Compound	V_e
Metanephrine	55
3-Methoxy-4-hydroxy-mandelic acid	65
3-Methoxy-4-hydroxy-phenethylamine	60
3-Methoxy-4-hydroxy-phenethylacetic acid	88
Tryptamine	115
Indole-3-acetic acid	203
Serotonin	157
5-Hydroxy-indoleacetic acid	250

DISCUSSION AND CONCLUSIONS

Our investigation of the chromatographic behavior of aromatic and heterocyclic compounds on Sephadex has uncovered new relationships on the fractionation of these classes of compounds by the gel material. The adsorption of aromatic and heterocyclic compounds noted by FLODIN[1] and the ion-exclusion and ion-exchange properties of the gel reported by GELOTTE[2] were also observed in the present study. However, the systematic examination of phenolic and indolylic acids, amines, amino acids, glycols, ring structure and aliphatic substitutions of this study now provides a greater insight for the application of Sephadex for the fractionation of complex mixtures of small molecules.

The exclusion and adsorption effects that are operative on distilled water

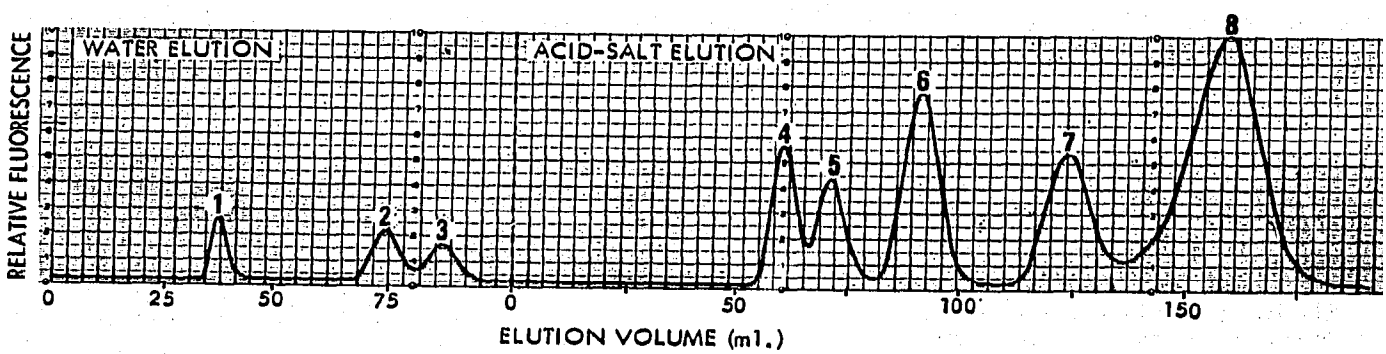

Fig. 1. Fractionation of phenolic and indolylic compounds on Sephadex G-15. Distilled water was used for the first phase and 0.05 *M* sodium chloride, pH 4.0 solution for the second phase of column development. Column size: 1.2 × 40 cm. 1 = Vanillic acid (1 μg); 2 = 3-methoxy-4-hydroxy-phenethylglycol (100 μg); 3 = 3,4-dihydroxy-phenethylglycol (100 μg); 4 = normetanephrine (200 μg); 5 = 3,4-dihydroxy-phenethylamine (200 μg); 6 = N,N-dimethyltryptamine (40 μg); 7 = tryptamine (10 μg); 8 = serotonin (10 μg).

columns afford a method for the separation of compounds with carboxy groups from the amino acid and glycol derivatives. From the data in Table II it is apparent that the nature of the ring structure or the nuclear substitutions have but a minimal effect on the chromatographic behavior of the monocarboxylic acids. However, the introduction of an alpha amino group tends to suppress the exclusion effect as was noted with the two phenolic amino acids. The exclusion of monocarboxylic acids, nucleotides and benzoic acid derivatives has been reported by GELOTTE[2] and WOOF AND PIERCE[5].

The phenolic amino acids and glycols that were adsorbed on the distilled water columns also showed the rather marked effects of nuclear substitutions. In both cases, the dihydroxy compounds had larger elution volumes than the corresponding 4-hydroxy or 3-methoxy-4-hydroxy derivatives. Similar results for a large number of phenols have been reported[5].

The ion-exchange properties, due to the negatively charged groups in the gel matrix, result in the binding of basic compounds on the distilled water column. Neutralization of the electrostatic interactions of solute and gel matrix by the incorporation of an electrolyte or acid into the eluant results in the elution of the basic compounds. The chromatographic behavior of basic compounds on an electrolyte-equilibrated column depends primarily on the degree of adsorption, and secondarily on the residual electrostatic charges on the gel beads. Both effects have been observed with the acid–salt column. Our conclusion for the existence of residual electrostatic charges (weak ion-exchange effects) is based on the larger partition coefficients obtained with the primary amines in comparison to the corresponding methylated or acetylated derivatives.

The planarity of a molecule markedly influences the degree of adsorption by the gel. Biphenyl and naphthyl derivatives have been reported to have larger elution volumes than simple phenols[5]. Our study with phenolic and indolylic compounds has shown a greater adsorption by the gel for the latter class of compounds.

Substitutions on the aromatic nucleus that extend the planarity of the molecule may result in either ion-exclusion or an increase in adsorption. WOOF AND PIERCE have shown that carboxy and nitro groups decrease adsorption, whereas methoxy and hydroxy groups increase the adsorption of compounds by the gel matrix[5]. These effects are attributed to the electron-donating properties of the substituent groups. Similar effects of methoxy and hydroxy groups on phenolic and indolylic compounds have been noted in our study.

The effect of the chemical nature of the aliphatic portion of an aromatic or heterocyclic compound on the chromatographic behavior has not been previously reported. Our finding of the stronger adsorption of carboxy derivatives in comparison to amine analogues on the acid–salt column led to an examination of the sequence of elution of compounds with different functional groups. The sequence found was in the following order: amines, alcohols and carboxylic acids. The examples illustrative of this elution sequence are 3-methoxy-4-hydroxy-phenethylamine, glycol and acetic acid derivatives with elution volumes of 60, 68 and 88 ml, respectively. This sequence of elution has been found to be primarily dependent on the pH of the eluant[7].

Finally, the two stage elution system used for the separation of a mixture of phenolic and indolylic compounds demonstrates the applicability of gel filtration chromatography for the facile separation of compounds. By using a distilled water

column, the exclusion of the strongly acidic compounds and the separation of amino acid and alcoholic compounds can be accomplished. A subsequent change to an electrolyte-containing eluant permits the elution and separation of phenolic and indolylic amines. Although the acidic compounds are not fractionated on the distilled water column, it is possible to collect this fraction and effect a separation of these types of compounds by rechromatography on an acid–salt column.

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