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CHROMATOGRAPHIC BEHAVIOUR OF NUCLEIC ACID CONSTITUENTS AND OF PHENOLS ON CHITOSAN THIN LAYERS

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SUMMARY

The chromatographic characteristics of several nucleic acid constituents and of 36 phenols have been investigated on mixed layers of powdered chitosan and microcrystalline cellulose, with water, water–methanol mixtures and aqueous salt solutions at different pH values as mobile phases. The behaviour of the phenols was strongly correlated with the form in which these compounds were present in solution and therefore with the pH of the eluent. Chitosan was more effective than PEI- and DEAE-cellulose as adsorbent in separating phenols. Analytical applications of chitosan layers are reported.

INTRODUCTION

Chitosan is a β-1,4-linked D-glucosamine polymer prepared by deacetylating the natural polysacharide chitin; it was widely employed by Muzzarelli[1] in studies on inorganic ions by column chromatography. Recently, however, chitosan has been used for the separation of nucleic acid constituents by thin-layer chromatography[2,3], layers of microcrystalline cellulose impregnated with chitosan formate being used.

In this paper, layers of powdered chitosan directly mixed with microcrystalline cellulose have been used, and, in order to compare the characteristics of these layers with those of microcrystalline cellulose (alone or impregnated with chitosan formate), we investigated the behaviour of some nucleic acid constituents; the work was then extended to phenols.

EXPERIMENTAL

The layers of chitosan (300 μm thick) were prepared by mixing 4 g of finely powdered chitosan (100–200 mesh), prepared from chitin according to the deacetylation procedures of Broussignac[4], with 8 g of microcrystalline cellulose in 50 ml of water. Layers of microcrystalline cellulose alone were prepared from 9 g of cellulose in

50 ml of water, and those of microcrystalline cellulose impregnated with chitosan formate were prepared as described by Nagasawa *et al.*[2], using a 0.8% (w/v) solution of chitosan in 0.5% (w/v) formic acid.

The solutions of the test compounds were prepared in water or in water–organic solvent mixtures; such solutions, if required, were neutralized with ammonia in order to prevent gelation of the powdered chitosan layers, which occurs in acid solution. For this reason, acid solutions were not used as mobile phases; indeed, with such mobile phases, layers of microcrystalline cellulose impregnated with chitosan formate exhibit poor reproducibility and irregularities at the solvent front.

The chromatograms were developed by the ascending technique at 25°, and the migration distance was 11 cm unless otherwise stated. The nucleic acid constituents were detected by exposure to UV radiation; phenols were detected by the Boute reaction[5].

RESULTS AND DISCUSSION

Nucleic acid constituents

Table I shows the R_F values of many nucleic acid constituents on development with 0.25 *M* ammonia buffer and with water–methanol (1:1, v/v) on layers of (a) microcrystalline cellulose, (b) microcrystalline cellulose impregnated with chitosan formate, and (c) microcrystalline cellulose mixed with powdered chitosan. With the ammoniacal mobile phase, there is no important difference in the behaviour of the compounds on the three layers; this demonstrates that the addition of chitosan or chitosan formate does not appreciably affect the retention properties of microcrystalline cellulose. Under such conditions of development, the chromatographic data of Nagasawa *et al.*[2] for nucleic acid constituents must be ascribed to the presence on the layers of microcrystalline cellulose and not to chitosan formate.

During development with water–methanol, the nucleosides and nucleic acid bases behave similarly on the three layers, except for xanthine and xanthosine, which are retained to a greater extent on the layers containing chitosan. The behaviour of these two compounds is ascribed to the fact that their acid character is more marked than that of the parent compounds, as evidenced by the pK_a values shown in Table I.

With this developing solvent, the behaviour of the nucleotides on layers containing chitosan (R_F between 0.00 and 0.06) is very different from that on microcrystalline cellulose alone (R_F 0.81 to 0.85). Such behaviour is ascribed to the presence in nucleotide molecules of one or more phosphate groups, which react (probably through an ion-exchange mechanism) with the functional groups of the exchanger. The strong retention of these compounds on chitosan layers can be used analytically for their separation from nucleosides and nucleic acid bases.

The behaviour of xanthine and xanthosine on layers containing chitosan (with water–methanol as mobile phase) shows that layers with powdered chitosan exhibit stronger retention than those impregnated with chitosan formate. This different retention can be ascribed to the different form of the anion exchanger and to the higher content of chitosan in the mixed layer than in the impregnated one.

Phenols

Aqueous eluents. Table II shows the R_F values of 36 phenols on layers of micro-

TABLE I

R_F VALUES FOR NUCLEOSIDES, NUCLEOTIDES AND NUCLEIC ACID BASES ON LAYERS OF MICROCRYSTALLINE CELLULOSE (a) ALONE, (b) IMPREGNATED WITH CHITOSAN FORMATE AND (c) MIXED WITH POWDERED CHITOSAN

Compound	*Mobile phase*						*pK_a of enol form*
	NH_4Cl (0.25 M) + NH_3 (0.25 M)			*H_2O–methanol (1:1, v/v)*			
	a	*b*	*c*	*a*	*b*	*c*	
Xanthine	0.54	0.58	0.56	0.46	0.51	0.35	7.4
Xanthosine	0.74	0.72	0.71	0.82	0.33	0.04	5.75
Xanthosine monophosphate	0.92	0.90	0.96	0.81	0.00	0.00	—
Xanthosine diphosphate	0.93	0.90	0.96	0.85	0.00	0.00	—
Xanthosine triphosphate	0.93	0.83	0.96	0.82	0.00	0.00	—
Thymine	0.76	0.78	0.78	0.75	0.70	0.77	9.8
Thymidine	0.90	0.86	0.86	0.82	0.77	0.83	—
Thymidine monophosphate	0.97	0.93	0.96	0.85	0.04	0.06	—
Guanine	0.39	0.39	0.40	0.41	0.44	0.44	9.2
Guanosine	0.64	0.60	0.60	0.57	0.53	0.52	9.16
Guanosine monophosphate	0.85	0.84	0.91	0.82	0.00	0.00	—
Hypoxanthine	0.57	0.55	0.58	0.57	0.57	0.57	8.9
Inosine	0.78	0.75	0.77	0.64	0.65	0.63	8.75
Inosine monophosphate	0.93	0.90	0.96	0.85	0.00	0.00	—
Adenine	0.34	0.35	0.37	0.50	0.56	0.57	9.8
Adenosine	0.53	0.53	0.52	0.52	0.56	0.59	—
Adenosine monophosphate	0.79	0.81	0.85	0.84	0.00	0.00	—
Adenosine diphosphate	0.79	0.83	0.87	0.85	0.00	0.00	—
Adenosine triphosphate	0.85	0.76	0.93	0.85	0.00	0.00	—
Cytosine	0.75	0.73	0.75	0.65	0.67	0.73	12.2
Cytidine	0.82	0.82	0.80	0.70	0.65	0.72	—
Uracil	0.76	0.74	0.75	0.73	0.73	0.73	9.5
Uridine	0.90	0.84	0.88	0.78	0.72	0.73	9.17

crystalline cellulose and on cellulose–powdered chitosan layers, with 0.1 *M* ammonium acetate or 0.1 *M* sodium bicarbonate as mobile phase. The results indicate that chitosan exhibits a higher retention power and a better selectivity towards the phenols.

On both layers, development with 0.1 *M* ammonium acetate leads to diffuse spots for many compounds, (including phenol), so that their detection is impossible. As the pH of the mobile phase is increased, the quality of the spots improves, and only phenol and *m*-cresol cannot be detected. On development with sodium bicarbonate, however, the number of polyhydric phenols giving rise to elongated spots increases, probably because of the easier oxidizability of such compounds as the pH on the layer is increased. As regards the chromatographic behaviour of the phenols on chitosan layers with the two mobile phases, a general increase in R_F value is observed on changing from ammonium acetate to sodium bicarbonate, except for those phenols of less marked acid character ($pK_a \geqslant 8.7$), which exhibit similar R_F values with the two developing solvents. The chromatographic behaviour of the phenols on the cellulose–powdered chitosan layers seems to depend on the pH value of the mobile phase and on the acid–base character of the compounds. For the dichlorophenols, with ammonium acetate (pH = 7.1) as mobile phase, only 2,6-dichlorophenol ($pK_a = 6.79$) ex-

TABLE II

R_F VALUES FOR PHENOLS ON LAYERS OF MICROCRYSTALLINE CELLULOSE (a) ALONE AND (b) MIXED WITH POWDERED CHITOSAN

n.d. = Not determined; e.s. = elongated spot.

Compound	*Mobile phase*						pK_a*
	CH_3COONH_4 (0.1 M)		$NaHCO_3$ (0.1 M)	H_2O–methanol (v/v)			
				4:1		1:1	
	a	*b*	*b*	*a*	*b*	*b*	
Phenol	n.d.	n.d.	n.d.	0.83	0.78	0.93	9.99
Catechol	0.79	e.s.	0.62	0.82	e.s.	e.s.	9.85
Resorcinol	0.79	0.58	0.61	0.82	0.60	0.77	9.81
Hydroquinone	0.80	0.62	e.s.	0.81	0.62	0.79	10.35
Pyrogallol	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	9.01
Phloroglucinol	0.65	0.60	e.s.	0.75	e.s.	0.65	8.45
Gallic acid	0.74	n.d.	e.s.	0.87	0.01	0.03	4.41
Pyrocatechuic acid	0.80	0.32	0.66	0.88	0.01	0.03	—
m-Cresol	n.d.	n.d.	n.d.	n.d.	n.d.	0.90	10.09
p-Nitrophenol	0.64	0.36	0.61	0.75	0.14	0.42	7.16
o-Nitrophenol	n.d.	0.49	0.71	n.d.	n.d.	0.52	7.23
m-Nitrophenol	0.71	0.50	0.63	0.76	0.50	0.76	8.40
2,4-Dinitrophenol	0.62	0.25	0.53	0.93	0.02	0.04	4.09
2,6-Dinitrophenol	0.75	0.34	0.64	0.94	0.02	0.03	3.71
2,5-Dinitrophenol	0.64	0.30	0.58	0.90	0.02	0.03	5.22
Picric acid	0.68	0.21	0.50	0.90	0.01	0.02	0.60
5-Aminosalicylic acid	0.79	0.49	0.72	0.93	0.03	0.04	2.74
4-Aminosalicylic acid	0.79	0.31	0.65	0.92	0.03	0.03	1.7
2-Amino-5-nitrophenol	0.43	0.28	0.36	0.52	0.31	0.61	—
2-Amino-4-nitrophenol	0.52	0.28	0.49	0.62	0.11	0.37	—
4-Amino-2-nitrophenol	0.60	0.46	0.61	0.66	0.43	0.68	—
2-Amino-4,6-dinitrophenol	0.49	0.14	0.34	0.88	0.00	0.01	—
o-Chlorophenol	n.d.	n.d.	0.63	n.d.	n.d.	0.83	8.48
p-Chlorophenol	0.72	0.55	0.52	0.79	0.62	0.83	9.38
m-Chlorophenol	0.70	0.55	0.55	0.76	0.61	0.83	9.02
p-Bromophenol	0.65	0.48	0.47	0.77	0.56	0.81	8.87
o-Bromophenol	n.d.	n.d.	0.60	n.d.	n.d.	0.81	8.42
2,5-Dichlorophenol	n.d.	0.35	0.58	n.d.	0.28	0.67	7.35
3,4-Dichlorophenol	0.57	0.34	0.45	0.69	0.45	0.79	8.39
2,4-Dichlorophenol	n.d.	n.d.	0.56	0.69	0.37	0.77	7.75
2,3-Dichlorophenol	0.58	0.36	0.57	0.70	0.31	0.71	7.45
2,6-Dichlorophenol	n.d.	0.40	0.65	n.d.	0.14	0.49	6.79
3,5-Dichlorophenol	0.58	0.34	0.50	0.69	0.39	0.77	7.93
1-Naphthol	0.50	0.26	0.27	0.62	0.35	0.76	9.34
2-Naphthol	0.46	0.25	0.25	0.58	0.37	0.78	9.51
Naphthalene-1,5-diol	0.35	0.16	0.16	0.51	0.20	0.58	—

* See ref. 6.

hibits an R_F value higher than those of the other isomers, whereas with sodium bicarbonate (pH = 8.5) the R_F values of the isomers increase with increasing pK_a value in accordance with the differing degrees of deprotonation of these compounds[6].

As regards the mechanism governing the retention of the phenols, and keep-

ing in mind that chitosan is a weak base[1] (and certainly the free-base form predominates at the pH of sodium bicarbonate solution), it follows that interactions between the primary amino group of chitosan and the phenolic hydroxyl groups are the parameters determining the retention.

Aqueous-organic eluents. Table II also shows the R_F values of phenols on layers of microcrystalline cellulose with water–methanol (4:1, v/v) as mobile phase and on layers of cellulose–powdered chitosan with similar mixtures for development.

As has already been pointed out for nucleic acid constituents, the use of aqueous-organic eluents increases the retention power of the chitosan layers. On microcrystalline cellulose, the phenols exhibit high R_F values, independent of their pK_a values and of substituents in the ring. On chitosan layers, however, the phenols are strongly retained and their chromatographic behaviour is correlated with both their pK_a values and the type and number of substituents in the ring. As regards the influence of the substituents in the ring on the chromatographic behaviour of the phenols, it should be noted that polyhydric phenols are retained more than phenol itself, despite their pK_a values, which in some instances (resorcinol and hydroquinone) are similar to, and in others (phloroglucinol) lower than, that of phenol. These compounds can give rise to hydrogen bonds with two or more of the functional groups of chitosan and therefore may be expected to be retained more than phenol.

The introduction into the ring of substituents that markedly increase the acid character of a phenol (*e.g.*, nitro, chloro or bromo groups), produces a decrease in R_F value.

Increase in the methanol concentration in the mobile phase leads to a general increase in R_F values and the production of more compact spots for polyhydric

TABLE III

SEPARATIONS ON LAYERS OF MICROCRYSTALLINE CELLULOSE MIXED WITH POWDERED CHITOSAN

Migration distance = 12.5 cm.

Mixture	*Mobile phase*	R_F
4-Amino-2-nitrophenol	water–methanol (4:1, v/v)	0.41
2-Amino-5-nitrophenol		0.30
2-Amino-4-nitrophenol		0.11
2-Amino-4,6-dinitrophenol		0.00
m-Nitrophenol	water–methanol (1:1, v/v)	0.72
o-Nitrophenol		0.50
p-Nitrophenol		0.41
5-Aminosalicylic acid	CH_3COONH_4 (0.1 *M*)	0.47
4-Aminosalicylic acid		0.30
o-Chlorophenol	$NaHCO_3$ (0.1 *M*)	0.60
p-Chlorophenol		0.50
o-Bromophenol	$NaHCO_3$ (0.1 *M*)	0.58
p-Bromophenol		0.46
3,4-Dichlorophenol	water–methanol (4:1, v/v)	0.44
2,4-Dichlorophenol		0.36
2,5-Dichlorophenol		0.28
2,6-Dichlorophenol		0.14
1- or 2-Naphthol	water–methanol (4:1, v/v)	0.34–0.36
Naphthalene-1,5-diol		0.19

phenols; indeed, some compounds impossible to detect with aqueous salt solutions as mobile phase can be detected in this system.

Comparison of results on chitosan layers with those obtained on cellulose-based anion exchangers (PEI- and DEAE-cellulose)[7] shows that, generally, chitosan exhibits a higher retention power and a better selectivity towards the phenols than do the exchangers cited. The R_F values on chitosan layers with water–methanol (4:1) as mobile phase are appreciably lower than those on PEI-cellulose with water as developer[7]. Further, on chitosan, mono-, di- and trihydric phenols and the three nitrophenols can be separated (see Table II); such separations cannot be achieved on PEI-cellulose, DEAE-cellulose or microcrystalline cellulose.

ANALYTICAL APPLICATIONS

Many separations of phenols can be achieved on the basis of their R_F values with aqueous and alcoholic–aqueous eluents; some are reported in Table III. Also, separations of nitro and polynitrophenols, and chloro and dichlorophenols, are possible.

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