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LIQUID CHROMATOGRAPHIC SEPARATION OF PURINES, PYRIMIDINES AND THEIR NUCLEOSIDES ON SILICA GEL COLUMNS

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SUMMARY

The high-performance liquid chromatography of pyrimidine and purine derivatives on plain microparticulate silica gel was studied. Mixtures of dichloromethane, methanol and aqueous ammonium formate–formic acid solutions were used as the mobile phase. Retention data are reported for more than 50 compounds, including the biochemically important nucleobases and nucleosides. By varying the composition of the eluent mixture the chromatographic system provides elution sequences and selectivities that differ markedly from those characteristic of reversed-phase liquid chromatography.

INTRODUCTION

Reversed-phase liquid chromatography (RPLC) on microparticulate octadecylsilica has become the method of choice for the separation of nucleosides and nucleic acid bases in recent years[1,2]. Nevertheless, in some instances plain silica gel was used successfully for the liquid chromatographic separation of these and related substances. Thus, some oxypurines were chromatographed[3] on a Bio-Sil A column with a solvent system consisting of diethyl ether, *n*-propanol and dilute acetic acid. Theobromine, theophylline and caffeine were separated on LiChrosorb SI 60 using dichloromethane–ethanol–water mixtures[4] and also on Partisil 10 with dichloromethane–methanol–aqueous ammonia eluents[5]. Further examples include the separation of five uracil derivatives on LiChrosorb SI 100 using a mixture of water-saturated dichloromethane and isopropanol as the eluent[6] and the chromatography of adenine, adenosine and several cytokinins on a Hypersil column with an ammoniacal chloroform–methanol–water system[7]. The most interesting example in this series is the paper of Evans *et al.*[8] on the separation of urinary pyrimidine bases and nucleosides on LiChrosorb SI 100 with mobile phases composed of dichloromethane, methanol and ammonium formate buffer.

All of the mobile phase systems mentioned above consist of a relatively nonpolar, water-immiscible organic solvent, an alcohol and a small amount of either pure water or an aqueous solution of an ionizable substance. No matter what the exact

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retention mechanism on silica gel with such hydro-organic eluents may be (true liquid–solid adsorption or partition mechanism into a more polar, water-rich liquid stationary phase, developed from the eluent *in situ* in the column), it should differ essentially from RPLC with alkylsilica packings so that different and specific separations could be possible. Nevertheless, a very limited number of purines and pyrimidines have been chromatographed with systems of this type so far. In order to explore better the potentialities of such systems, the present work was undertaken and the dichloromethane–methanol–ammonium formate eluent of Evans *et al.*[8] was modified.

In our studies of nucleic acid chemistry during more than 20 years[9–11] we have often encountered difficulties in the separation and isolation of reaction products and particularly in the identification of nucleic acid components and their analogues in biological materials. These difficulties led us to the utilization of high-performance liquid chromatography (HPLC). The suitability of this method for the separation of closely related compounds was demonstrated in the separation of (*E*)- and (*Z*)-isomers of methyl glyoxylate semicarbazones and in the determination of the *E*/*Z* ratio in their mixtures[12], which are otherwise very difficult to separate[13]. Similarly, HPLC was used successfully in the separation and determination of single epimers of 2′,3′-cyclic sulphites of nucleosides at the rare isomerism located on the sulphur atom[14]. Further, HPLC was used for the quantitative determination of nucleosides and their metabolites in biological materials and the time course of the blood level of arabinosylcytosine and the course of its deamination in rats were determined[15]. The rate of deamination of arabinosylcytosine and its derivatives by deaminase was followed quantitatively by HPLC[16], and this method was similarly used for the quantitative determination of 5-fluorouracil derivatives in connection with their inhibition of *Escherichia coli*[17]. In order to obtain chromatographic retention data for our future work in nucleic acid chemistry, a systematic study of the separation and identification of a series of pyrimidine and purine derivatives was undertaken.

EXPERIMENTAL

The liquid chromatograph was assembled in this laboratory and consisted of a Milton Roy Model 396-57 minipump, a Model 709 pulse damper, a Model 1203 UV III monitor (all from Laboratory Data Control, Riviera Beach, FL, U.S.A.), a home-made septum injector and a Model EZ 13 electronic recorder (Laboratorní Přístroje, Prague, Czechoslovakia). The absorbance of the effluent was monitored at 254 nm.

The columns were made from 6.3 mm O.D., 4.2 mm I.D. LiChroma stainless-steel tubing, 25 cm long, purchased from Applied Science Labs. (State College, PA, U.S.A.). The solvent reservoir was maintained at 20°C and the columns were thermally insulated. Silica gel was packed into the columns with a home-made slurry-packing apparatus; chloroform served as the dispersing medium, the maximum pressure applied during packing being 50 MPa. Three types of silica gel were tried in the preliminary experiments: LiChrospher SI 100, particle size $d_p = 10$ μm; LiChrosorb SI 60, $d_p = 10$ μm; and LiChrosorb SI 100, $d_p = 5$ μm (all from E. Merck, Darmstadt, G.F.R.). Only minor differences were observed in their chromatographic performances. Under comparable conditions, LiChrosorb SI 60 (the material with the largest surface area) displayed the most pronounced tendency to give tailing peaks.

The quantitative retention data given further were obtained with the most efficient 5-μm LiChrosorb SI 100 column.

Mobile phase mixtures were made up by volume from dichloromethane, methanol (both of spectroscopic grade) and ammonium formate/formic acid solutions in deionized water. The relative proportions of the principal constituents of the eluent systems investigated are given in Table I.

TABLE I

COMPOSITIONS OF ELUENT SYSTEMS

System	*Content (parts by volume)*		
	Dichloromethane	*Methanol*	*Aqueous solution*
A1	75	22	3
A2	70	27	3
B1	80	18	2
B2	73	25	2

In addition to the influence of the overall composition (dichloromethane:methanol:water ratio), the influence of the concentrations of ammonium formate and formic acid was also studied. Initial tests revealed that varying the salt concentration from 0.5 to 2.0 *M* had no significant effect on retention; hence, a standard concentration of 0.5 *M* ammonium formate was chosen for the aqueous part of the eluent. On the other hand, the concentration of formic acid, determining the pH of the aqueous solution, proved to be important. Detailed experiments were conducted for three pH levels of the aqueous part, *viz.* pH 4.2 (resulting from the addition of 0.20 mole/l formic acid to the 0.5 *M* ammonium formate solution), pH 3.0 (1.22 *M* formic acid, 0.5 *M* ammonium formate) and pH 2.5 (2.65 *M* formic acid, 0.5 *M* ammonium formate).

Commercially available nucleobases and nucleosides were purchased from Lachema (Brno, Czechoslovakia). The other pyrimidine and purine derivatives were synthesized in this Institute. Standard solutions were prepared in methanol–water mixtures (*ca.* 1:1) so as to contain the solutes at concentrations of roughly 50–200 μg/ml and were injected into the chromatograph in 0.5–2.0-μl volumes, using SGE Type B syringes (Scientific Glass Engineering, Melbourne, Australia).

The emergence time of a peak (or baseline disturbance) obtained by injecting pure methanol into the column was taken as the elution time of a non-retained solute, t_M (ref. 18), and the retention (capacity) factors were calculated in the usual way from $k = (t_R - t_M)/t_M$, t_R being the retention time of the considered compound. Each k value was the mean of at least three measurements.

RESULTS AND DISCUSSION

Tables II and III give the capacity factors (k) of pyrimidine and purine derivatives on silica gel for the different mobile phase compositions used.

As could be expected, mobile phase systems with a higher proportion of the

TABLE II

RETENTION FACTORS (k) FOR PYRIMIDINE DERIVATIVES ON SILICA GEL

Column: packing, LiChrosorb SI 100, 5 μm; length, 25 cm. Mobile phase systems: see Table I.

	Mobile phase							
	A1			*B1*			*A2*	*B2*
pH of the aqueous solution	2.5	3.0	4.2	2.5	3.0	4.2	3.0	3.0
Formic acid concentration (M)	2.65	1.22	0.20	2.65	1.22	0.20	1.22	1.22
2-Hydroxypyrimidine	1.18	1.38	1.58	1.59	1.79	1.91	—	0.87
2-Hydroxy-5-methylpyrimidine	0.84	0.91	1.03	1.06	1.17	1.29	0.47	0.66
Uracil (Ura)	0.75	0.93	1.15	1.04	1.16	1.27	0.37	0.43
Uridine (Urd)	1.66	2.15	2.90	2.92	3.13	3.85	0.60	0.80
2′-Deoxyuridine (dUrd)	1.07	1.33	1.48	1.64	1.87	1.99	0.44	0.57
1-β-D-Arabinofuranosyluracil	1.53	1.92	2.61	2.59	2.97	3.29	0.55	0.70
Thymine (Thy)	0.53	0.66	0.79	0.72	0.75	0.85	0.28	0.31
5-Methyluridine	1.30	1.64	2.17	2.18	2.39	2.82	0.51	0.66
Thymidine (dThd)	0.82	1.01	1.27	1.28	1.36	1.51	0.38	0.47
6-Methyluracil	0.55	0.69	0.82	0.76	0.83	0.94	0.31	0.34
6-Methyluridine	1.12	1.40	1.82	1.80	1.99	2.47	0.47	0.63
6-Methyl-2′-deoxyuridine	0.87	1.00	1.19	1.18	1.28	1.37	0.40	0.52

1,5-Dimethyluracil	0.25	0.26	0.28	0.25	0.26	0.31	0.15	0.20
5,6-Dimethyluridine	0.87	1.03	1.30	1.25	1.38	1.56	0.38	0.49
5-Hydroxymethyluracil	1.47	1.86	2.41	2.34	2.69	2.96	0.57	0.75
Orotic acid	4.85	6.15	8.33	10.34	11.70	—	1.46	2.74
Orotidine	10.73	—	—	—	—	—	2.44	5.11
4,6-Dihydroxypyrimidine	1.67	3.19	7.91	3.00	4.22	—	0.75	1.36
4,6-Dihydroxy-5-methylpyrimidine	1.06	1.29	2.81	1.63	1.94	3.43	—	0.70
4,6-Dihydroxy-5-methyl-1-β-D-Rbf-pyrimidine*	1.56	2.01	4.35	2.77	3.32	5.39	—	0.87
Barbituric acid (B.A.)	0.88	1.12	1.70	1.35	1.61	2.02	0.33	0.59
Cytosine (Cyt)	3.20	3.64	4.00	5.44	5.56	5.46	1.43	2.22
Cytidine (Cyd)	4.57	5.75	7.12	9.20	9.52	11.10	1.58	2.63
2'-Deoxycytidine (dCyd)	3.49	4.20	4.82	6.52	6.84	7.22	1.28	2.23
1-β-D-Arabinofuranosylcytosine	4.20	5.31	6.75	8.66	9.04	10.25	1.44	2.48
1-Methylcytosine (m^1Cyt)	1.74	1.76	1.71	2.36	2.35	2.13	—	1.31
6-Methylcytosine (m^6Cyt)	2.44	2.66	2.66	3.90	3.86	3.56	0.97	1.78
5-Methylcytidine (m^5Cyd)	3.75	4.50	5.38	7.05	7.32	8.19	1.48	2.32
Isocytosine	1.37	1.64	1.90	2.09	2.26	2.34	0.69	0.99
Isocytidine	4.17	5.38	8.34	8.60	9.51	10.56	1.67	3.08
4-Amino-6-hydroxypyrimidine	1.41	1.65	1.99	1.99	2.21	1.96	0.74	0.99
4-Amino-6-hydroxy-1-β-D-Rbf-pyrimidine*	2.18	2.67	3.52	3.61	3.91	—	0.89	1.19

* Rbf = Ribofuranosyl.

TABLE III

RETENTION FACTORS (K) FOR PURINE DERIVATIVES ON SILICA GEL

Column: packing, LiChrosorb SI 100, 5 μm; length, 25 cm. Mobile phase systems: see Table I.

	Mobile phase							
	A1			*B1*			*A2*	*B2*
pH of the aqueous solution	2.5	3.0	4.2	2.5	3.0	4.2	3.0	3.0
Formic acid concentration (M)	2.65	1.22	0.20	2.65	1.22	0.20	1.22	1.22
2-Hydroxypurine	2.15	2.66	3.20	3.57	3.83	4.30	1.59	1.54
Hypoxanthine (Hyp)	1.39	1.68	2.06	2.21	2.38	2.62	0.68	0.92
Inosine (Ino)	2.50	3.21	4.35	4.78	5.17	6.13	1.20	1.40
1-Methylinosine (m^1Ino)	1.40	1.63	1.99	2.23	2.30	2.55	—	0.92
Xanthine (Xan)	1.11	1.40	1.96	1.74	1.88	2.24	0.55	0.61
Xanthosine (Xao)	2.61	3.58	8.09	5.06	6.23	—	0.97	1.47
Theophylline	0.15	0.16	0.14	0.17	0.17	0.20	0.11	0.16
Caffeine	0.06	0.08	0.07	0.08	0.08	0.10	0.08	0.10
Uric acid (U.A.)	2.33	3.36	—	4.52	5.84	7.10	0.84	1.28
Adenine (Ade)	0.92	1.05	1.15	1.27	1.29	1.33	0.62	0.72
Adenosine (Ado)	1.21	1.46	1.77	1.88	2.06	2.27	0.65	0.89
2′-Deoxyadenosine (dAdo)	0.90	1.00	1.07	1.30	1.38	1.45	0.55	0.72
1-Methyladenosine (m^1Ado)	12.50	—	—	—	—	—	5.15	—
N^6-Methyladenine	0.56	0.61	0.64	0.69	0.73	0.75	0.41	0.49
N^6-Methyladenosine	0.74	0.86	0.98	1.06	1.11	1.13	0.45	0.56
N^6,N^6-Dimethyladenine	0.25	0.28	0.27	0.27	0.32	0.33	0.22	0.25
N^6,N^6-Dimethyladenosine	0.41	0.44	0.48	0.55	0.59	0.64	0.30	0.32
Guanine (Gua)	2.41	3.05	3.80	4.43	4.82	4.87	1.50	1.66
Guanosine (Guo)	3.50	4.56	5.94	7.05	7.97	9.05	1.24	1.93
2′-Deoxyguanosine (dGuo)	2.48	3.18	3.86	4.68	4.90	5.58	0.97	1.50
1-Methylguanosine (m^1Guo)	2.37	2.90	3.65	4.25	4.59	—	0.93	1.46
Isoguanosine	4.39	5.68	7.56	9.25	10.40	—	1.65	2.86

less polar component (dichloromethane) act as weaker eluents, and an increase in the methanol and/or water content leads to shorter retention times. With systems A1 and B1 (Table I) the k values lie in the optimal chromatographic range (*ca.* $1 < k < 10$) for most of the substances investigated. Systems A2 and B2 would be useful for the separation of the more retained compounds, such as cytosine derivatives and carboxylated pyrimidines, but for most of the compounds they are rather fast. Also, the influence of pH was less pronounced with these two systems than with either A1 or B1, so that data are given only for a single pH value (pH 3).

With systems A1 and B1, however, changes in the pH (formic acid concentration) of the aqueous part caused considerable effects on both the retention and selectivity of separation. This is demonstrated graphically in Fig. 1, where experimental k values are plotted against formic acid concentration for several selected substances displaying typical behaviour in this respect (data for system B1 were used in Fig. 1; for A1 the dependences, shifted to the lower k region, are similar.) It can be seen that several compound pairs co-eluting under certain conditions can be well resolved when the concentration of the acid has been changed, and in some instances even reversals

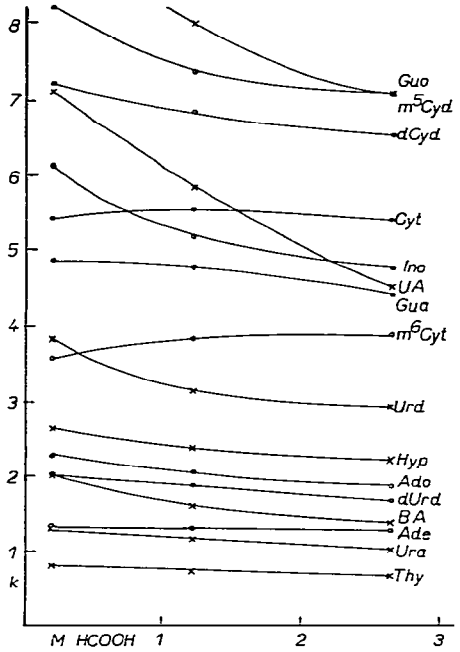

Fig. 1. Dependence of k values on the molarity of formic acid in the aqueous part of the mobile phase system B1.

of the elution order are effected. Obviously, the overall composition of the mobile phase cannot be manipulated at will, as there is a miscibility gap in the underlying ternary solvent system dichloromethane–methanol–water, and additions of formate and formic acid influence the mutual solubilities. Nevertheless, it is evident that the variations attainable in this way provide enough flexibility to affect the retention and selectivity parameters in chromatographic separations of the pyrimidines and purines concerned.

Remarks on structure–retention relationships

Because of the complexity of the interactions necessarily involved, it would hardly be possible to explain the retention behaviour of the main simple pyrimidines and purines in terms of their structures and physico-chemical properties. Nevertheless, some generalizations can be made for similarly substituted compounds, as follows.

(1) In logical contrast to RPLC, methyl groups generally decrease the retention parameters, in proportion to their number. This statement, however, can apply only when the alkyl substituent does not alter the primary structure. Among the compounds investigated, such a notable exception is m^1Ado, which elutes with an extremely high k value after Ado, whereas m^1Ino and m^1Guo emerge before Ino and Guo, respectively, and behave in the normal way. The anomaly must obviously be related to the fact that m^1Ado exists predominantly as the imino tautomer[19], whereas the other adenine derivatives are in the usual amino form. It may be assumed that the two tautomeric structures undergo different retention interactions.

(2) Ribosides possess systematically higher retention factors than their parent bases, which seems to be a logical consequence of the presence of a hydroxylated sugar moiety and their more polar nature. Curiously, in RPLC the order of elution of a certain ribonucleoside–base pair is just the same and by no means 'reversed'; it has been proposed recently[20] that this striking phenomenon may be explained by stacking interactions. Another general observation that can be derived from the data collected here is that the selectivity of separation for ribosides and corresponding bases (*i.e.*, the ratio $k_{nucleoside}/k_{base}$) increases significantly when the formic acid concentration is lowered under otherwise comparable conditions.

(3) The 2′-deoxyribonucleosides elute systematically before the corresponding ribonucleosides, in this instance contrary to their behaviour in RPLC[21,22]. The situation is more complicated, however, when the retention of deoxynucleosides is compared with that of parent bases, and cannot be explained in simple terms of 'polarity'; whereas dUrd and dThd always have distinctly higher k values than Ura and Thy, respectively, the differences are small for Ade, Gua and Cyt derivatives and in some instances the deoxyriboside elutes before the base.

Influence of formic acid

As stated above and illustrated in Fig. 1, variations in the concentration of the formic acid additive (all other conditions remaining unchanged) generally have a marked influence on retention parameters. Some characteristic features can be pointed out in this respect.

For most of the substances investigated, the k values increase when the acid content is decreased. The dependence is strong with structures bearing true hydroxyl groups, such as 5-hydroxymethyluracil, uric acid, barbituric acid and, of course, all the nucleosides and deoxynucleosides. For oxygenated compounds that exist predominantly in the lactam tautomeric form (Ura, Hyp, Xan, etc.), this dependence is weaker but still pronounced. On the other hand, Cyt, Ade, Gua and other purines and pyrimidines containing an amino group (apart from their nucleosides, where the effect of the hydroxyls apparently prevails) are less affected and with the B1 mobile phase system their retention is almost insensitive to changes in the acid concentration; in some instances, their k values even fall in the less acidic region. Obviously, these findings reflect the complex character of the chromatographic retention mechanism. The acidity of the aqueous solution was varied in the pH range 2.5–4.2, but these pH values cannot be taken as a quantitative measure of the entire aqueous–organic eluent mixture. Nevertheless, it is clear that just those compounds which exhibit the greatest retention–acidity dependence must exist as neutral, non-ionized molecules over the whole range of mobile phase compositions used. Thus, the apparent pH influence cannot be attributed to changes in the ionic state of such solutes and must be related to variations in the chromatographic mobile phase–stationary phase system. On the other hand, all of the aminopyrimidines and aminopurines possess basic pK_a constants around 3–4 and should become protonated in a more acidic environment. The relative pH insensitivity of their retention factors could then be explained by assuming that two retention-governing factors cancel out: (a) the chromatographic system as a whole becomes less retentive with the increase in acidity, but (b) the amino compounds change gradually from the neutral to the cationic form and the latter is retained more strongly.

Practical chromatographic aspects

The chromatographic systems described here possess some features that compare favourably with the possibilities offered by RPLC for the separation of nucleic acid components. The biochemically important nucleic acid bases elute in the order Thy < Ura < Ade < Xan < Hyp < Gua < Cyt. In contrast, on octadecylsilica, with weakly acidic aqueous eluents, the typical sequence is Cyt < Ura < Hyp < Gua < Xan < Thy < Ade[2,21], with adenine eluting far from from the other bases. Because of the separate position of Ade and its derivatives in RPLC, it is necessary to use gradient elution in order to separate the whole range of compounds in an acceptable time[21,23]. With plain silica gel and the hydro-organic eluents used in this work, separations of this type are easy to achieve under isocratic conditions. An example is given in Fig. 2, which shows the chromatogram of an artificial mixture of thirteen bases and nucleosides. The excellent resolution of Xan, Hyp, Gua and Urd may also be noted, as these substances come very close together in RPLC systems[23,24].

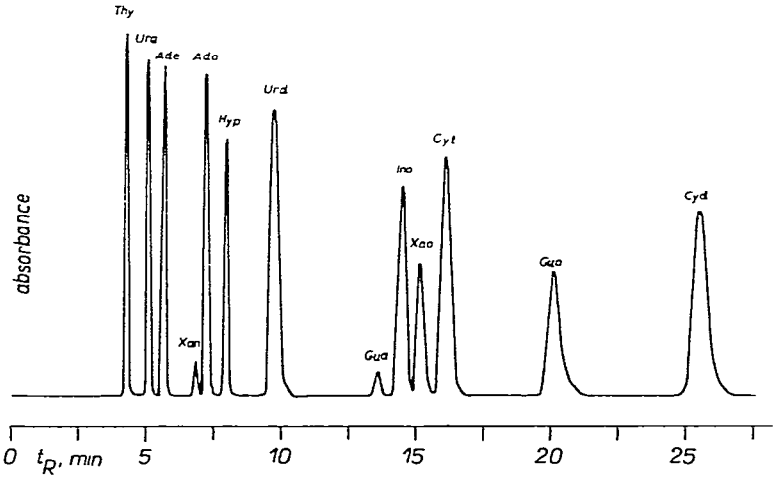

Fig. 2. Separation of bases and nucleosides on silica gel. Column: LiChrosorb SI 100, 5 μm, length 25 cm. Mobile phase system B1 with 2.65 *M* formic acid solution (pH 2.5). Flow-rate, 1.05 ml/min; pressure drop, 7.6 MPa; temperature, 20°C; detection, 254 nm; 0.032 a.u.f.s.; sample size, *ca.* 100 ng of each solute (except for Xan and Gua).

Both column efficiencies and peak shapes were very satisfactory. Plate numbers in the range 10,000–14,000 were typically obtained at flow-rates of 0.7–0.8 ml/min for a 25 cm × 4.2 mm I.D. column packed with 5-μm particles. An interesting finding, however, is that under most conditions the ribonucleosides displayed systematically lower plate numbers (roughly 6000–8000) than the other compound types. Peak profiles were evaluated in terms of the asymmetry factor, $F_t = 100a/b$, where a is the distance between the front peak boundary and the perpendicular drawn from the peak maximum to the baseline, measured at 10% of the peak height, and b is the corresponding distance for the rear boundary. The quantitative values were $F_t = 80$–90 in the most favourable instances, and never dropped below 60.

A certain disadvantage, for scaling-up applications, may be the relatively low sample loadability of the chromatographic systems. On increasing the sample size above 5–10 μg (for a single solute), a limit is reached where individual substances, especially early eluting ones, tend to emerge as sharply shaped peak doublets or even triplets. This phenomenon must be related to their low solubility in the dichloromethane-based hydro-organic eluent, probably causing an overloading effect on the column entrance. On the other hand, no difficulties were encountered upon decreasing the sample size and detection limits in the range 1–2 ng were obtained. Thus, the systems used are suitable for trace analyses.

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