Organic modifiers in the anion-exchange chromatographic separation of sialic acids

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Abstract

The combined effects of the organic modifiers and the ionic strength in the eluent on the separation of sialic acids were investigated on an anion-exchange Mono Q HR5/5 column. A log–log plot of capacity factors of sialic acids vs. eluent anion concentration demonstrates good linearity. The major retention mechanism is explained as anion exchange. Moreover, the plot of capacity factors of sialic acids vs. reciprocal of eluent anion concentration indicates that other retention mechanisms exist in addition to anion exchange. The organic modifiers (methanol and acetonitrile) in the mobile phase have significant influence on the retention time and resolution. The eluent anion concentration and the fraction of organic modifier produce a very flexible system that can be optimized for the separation of sialic acids. Five standard sialic acid derivatives have been separated by choosing a suitable eluent anion concentration and the fraction of organic modifier. The optimized conditions have been applied to separate sialic acids released from bovine submandibular mucin. 5,9-Diacetylneuraminic acid (Neu5,9Ac$_2$) can be separated from N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) but is overlapped with other peaks. Neu5Ac and Neu5Gc are completely separated.

1. Introduction

Sialic acids are components of glycoproteins and glycolipids. They constitute a family of neuraminic acid (5-amino-3,5-dideoxy-D-nonulosonic acid) derivatives [1]. The most common sialic acids are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) (pK values about 2) [2]. Other naturally occurring forms are from O-substitution of one or more of the hydroxyl groups of Neu5Ac or Neu5Gc with acetyl, methyl, lactyl or sulphate groups. Unsaturated and dehydro forms of sialic acids have also been reported in nature [3]. These modifications show tissue specificities and

are known to affect a wide spectrum of biological phenomena. In order to further explore the biological functions of sialic acids, it is necessary to release and separate these compounds from biological materials.

Because sialic acids from biological sources have a low concentration and a high diversity, the separation must be performed by the following multistep procedure: (1) separation from other water insoluble constituents; (2) separation from neutral sugars and other water soluble materials; (3) separation of individual sialic acid derivatives. The first two steps are not difficult and have been established. The present work is concerned with the separation of individual component sialic acids.

Sialic acids are not volatile enough to be

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separated directly by gas chromatography. Liquid chromatography, especially ion-exchange chromatography, because it takes advantage of the ionic character of the carboxyl groups, can be a good choice for separation of sialic acids from neutral sugars. The reported cation [4] and anion [5–8] exchange chromatographies have not yet achieved complete separation of Neu5Ac, Neu5Gc and 5,9-diacetylneuraminic acid (Neu5,9Ac$_2$) (resolution < 1.5). In 1990, Manzi et al. [9] evaluated anion-exchange chromatography of sialic acids on Aminex A-28 and A-29 columns with sodium sulphate as the eluent without organic modifiers, and reported that the overlapping of peaks in complex mixtures is too high and elution times are very close. Moreover, it is easy to miss the presence of a minor component.

In order to further improve the separation of sialic acids, it is necessary to investigate the effect of organic modifier content of the mobile phase on the separation of sialic acids in anion-exchange liquid chromatography. However, until now, according to our knowledge, there is no report on using organic modifiers in this type of separation. In this paper, the effect of methanol and acetonitrile concentrations of the mobile phase on retention and selectivity of five sialic acid derivatives was studied. The optimized conditions can be applied to separate sialic acids released from bovine submandibular mucin (BSM).

Neu5,9Ac$_2$ can be separated from Neu5Ac and Neu5Gc but is overlapped with other peaks. Neu5Ac and Neu5Gc are completely separated. The organic modifiers may be used in other ionic chromatographic separation of charged species.

2. Experimental

2.1. Chemicals

BSM, Neu5Ac, Neu5Gc, cytidine 5'-mono-phospho-N-acetylneuraminic acid (CMP-Neu5Ac), 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Neu2en5Ac) and histamine were purchased from Sigma (St. Louis, MO, USA).

Neu5,9Ac$_2$ was kindly provided by Dr. Chi-Huey Wong (Scripps Research Institute, La Jolla, CA, USA).

2.2. Hydrolysis of BSM [4]

BSM (3 mg) was dissolved in 5 ml 0.01 M hydrochloric acid in a sealed glass tube, the tube was heated for 1 h at 80°C in a water bath incubator with shaking. The reaction mixture was then cooled immediately to room temperature and centrifuged at 50 000 g, the supernatant was filtered through a PVDF syringe filter (0.45 µm), the sample solution was lyophilized and then dissolved in 0.5 ml water. The sample solution was injected onto the HPLC column.

2.3. HPLC

HPLC analysis was performed on a Beckman liquid chromatography apparatus, equipped with a liquid chromatography controller 421A, a programmable detector 166, and a 114M pump. Separations were achieved at room temperature on an analytical pre-packed Mono Q HR 5/5 column (50 × 5 mm) (Pharmacia). Mono Q was a strong anion exchanger based on a beaded hydrophilic resin consisting of monodispersed 10-µm particles with $-CH_2N^+(CH_3)_3$ charged groups. The ionic capacity was 0.27–0.37 mmol/column.

All the standard samples (0.8–2.0 mg) were dissolved in HPLC water. Samples were applied to the column with 20-µl sample loop. The eluent flow-rate was 0.5 ml/min and monitored by the UV detector at 205 nm. Histamine and tetraethylammonium bromide were monitored at 260 nm.

3. Results and discussion

Anion-exchange chromatography is a form of adsorption chromatography in which ionic solutes display reversible electrostatic interactions with a charged stationary phase. A basic equation (Eq. 1) has been published for ion exchange in terms of the capacity factor (k') and the concentration of the eluent anion [E]. It predicts

Table 1
The retention times (min) of histamine and tetraethylammonium bromide at different NaH_2PO_4 concentrations (pH was 4.50 at 7.50 mM)

	NaH_2PO_4 (mM)						
	4.97	7.50	9.94	20.0	40.0	60.0	80.0
$Et_4N^+Br^-$	1.77	1.76	1.77	1.76	1.82	1.73	1.87
Histamine	1.59	1.59	1.59	1.64	1.68	1.70	1.70

a linear dependence of log k' on the logarithm of eluent concentration if the resin capacity (C) is constant [10].

$$\log k' = -(a/b)\log[E] + (a/b)\log C + \text{constant} \tag{1}$$

where a and b are the charges on the sample ion and the eluent ion, respectively.

According to Eq. 1, a plot of log k' *vs.* log [E] will yield a straight line with a negative slope of a/b. The slope varies only with the valence of the solute (a) or the valence of the counterion (b). The capacity factor k' was determined from the retention time of the component (t_r) and that of an unretained compound (t_0): $k' = (t_r - t_0)/t_0$. Histamine [11] and tetraethylammonium bromide were chosen as unretained tracers. The void time determined by histamine was less than that determined by tetraethylammonium bromide, as shown in Table 1. Moreover, histamine has a higher UV absorbance coefficient at 260 nm. Therefore, histamine was preferred for the
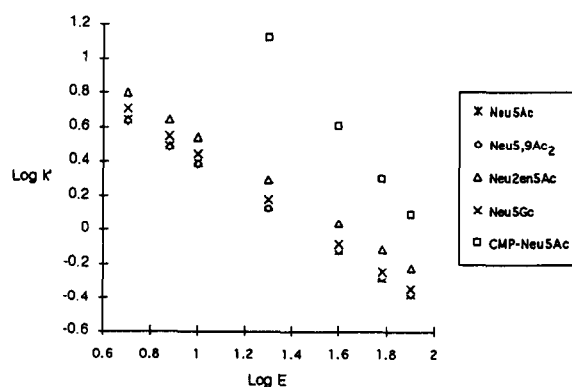

Fig. 1. The log–log plot of the capacity factors of sialic acids and NaH_2PO_4 concentration (mM).

determination of t_0. It should be noted, however, that the accurate determination of k' is still a main problem in liquid chromatography [12].

The effect of the mobile counter anion on the anion exchanger was studied in more detail. The influence of the anion concentration was studied for NaH_2PO_4. The results are shown in Table 2.

It can be seen that CMP-Neu5Ac can be separated by changing the counter anion concentration. The counter anion concentration has significant influence on the retention time of the remaining sialic acid derivatives, but has less influence on the resolution. A plot of log k' *vs.* log of counter anion concentration shows good linear relationship (Fig. 1). The slope of each component is summarized in Table 3. If the resin capacity is constant, this plot should yield lines with a slope of -1 for the first four sialic acids and -2 for the last one in Table 3. The result is

Table 2
The retention times (min) of sialic acids at different NaH_2PO_4 concentrations (pH was 4.50 at 7.50 mM)

	NaH_2PO_4 (mM)						
	4.97	7.50	9.94	20.0	40.0	60.0	80.0
Neu5Ac	8.53	6.41	5.56	3.92	2.95	2.59	2.39
Neu5,9Ac$_2$	8.38	6.36	5.48	3.88	2.94	2.59	2.41
Neu5Gc	9.54	7.12	6.10	4.19	3.07	2.68	2.45
Neu2en5Ac	11.4	8.55	7.29	4.92	3.52	3.00	2.71
CMP-Neu5Ac	nd[a]	nd	nd	23.2	8.29	5.09	3.80

[a] nd = Not determined.

Table 3
Linear regression results of Eq. 1 for the analytes with
NaH$_2$PO$_4$ eluent.

	a/b (calculated)	a/b (predicted)	R^2 [a]
Neu5Ac	−0.86	−1	0.999533
Neu5,9Ac$_2$	−0.85	−1	0.999709
Neu5Gc	−0.88	−1	0.999557
Neu2en5Ac	−0.85	−1	0.995870
CMP-Neu5Ac	−1.71	−2	0.999937

[a] R^2 is a statistical measurement of the validity of the model.
It ranges up to 1, with 1 being optimal.

close to the predicted values from Eq. 1. There-
fore, the primary mechanism for the chromatog-
raphy is anion exchange.

In order to further explore the retention
mechanism, we use the following model to
describe anion-exchange chromatography:

$$R^+E^- + S^- \rightleftharpoons R^+S^- + E^-$$
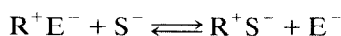
The anion-exchange constant is:

$$K_e = [RS][E^-]/([RE][S^-]) \tag{2}$$

where $R^+ = -CH_2-N^+(CH_3)_3$; $E^- = H_2PO_4^-$;
$S^- = $ Neu5Ac or Neu5Gc anion (R–COO$^-$)

$$HS + H_2O \rightleftharpoons H_3O^+ + S^-$$
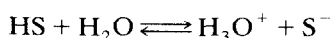
The acid ionization constant is:

$$K_a = [H_3O^+][S^-]/[HS] \tag{3}$$

the capacity factor:

$$k' = n_s/n_m = (C_s/C_m)V_s/V_m \tag{4}$$

where n_s and n_m are moles of solute in the
stationary phase and mobile phase; C_s and C_m
concentration of solute in stationary phase and
mobile phase; and V_s and V_m volume of station-
ary phase and mobile phase, respectively.
The distribution coefficient is:

$$D = C_s/C_m = [RS]/([S^-] + [HS]) \tag{5}$$

$$k' = K_eV_s\{[RE]/[V_m(1 + [H_3O^+]/K_a)]\}(1/[E^-]) \tag{6}$$

where [RE] is determined by the ionic capacity
of the resin.

According to Eq. 6, at infinite anion con-
centration, the plot of capacity factors of
Neu5Ac and Neu5Gc *vs.* the reciprocal of
NaH$_2$PO$_4$ concentration should be linear and
pass through origin. However, in Fig. 2 the
y-intercepts are not zero. This means that some
other mechanisms exist in addition to anion
exchange.

Generally, normal-phase or reversed-phase
separation mechanism exists in anion-exchange
chromatography. Both of these mechanisms
could be affected by organic modifiers. We chose
methanol and acetonitrile as the organic modi-
fiers added into the 7.50 mM NaH$_2$PO$_4$ solution.
The influences of the mobile phases on the
separation capacity factors (k') was investigated
by varying the amount of organic additives. The
results are plotted in Figs. 3 and 4.

It is clear that without organic modifiers, the
system has insufficient selectivity to separate the
sialic acids. However, the selectivity increases
considerably with increasing organic modifier
concentration.

The capacity factors (k') of Neu5Ac and
Neu5Gc increase with increasing organic frac-
tion. We reason that the organic modifiers
change the interaction between solute anion and
stationary phase cation according to Coulomb's
law:

$$F = q_1q_2/(r^2\epsilon)$$

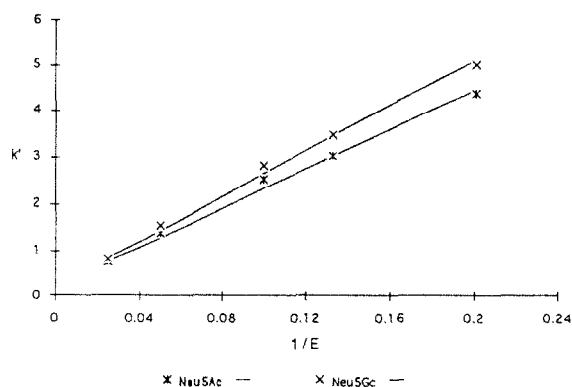where ϵ is the dielectric constant of the medium

Fig. 2. Relationship between the capacity factors of sialic
acids and the reciprocal of NaH$_2$PO$_4$ concentration (mM^{-1}).
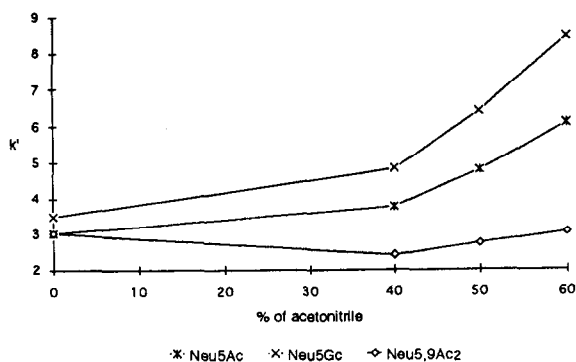
Fig. 3. Relationship between the capacity factors of sialic acids and the fraction of acetonitrile into 7.50 m*M* NaH$_2$PO$_4$ solution.

[13]. $\epsilon(H_2O) = 78.5$ (25°C); $\epsilon(CH_3OH) = 32.7$ (25°C) and $\epsilon(CH_3CN) = 37.5$ (20°C)

When the organic modifier is added, ϵ becomes smaller, the electrostatic attraction force (F) between solute anion and stationary phase cation becomes larger, and the capacity factor (k') becomes larger. The organic additives also affect all the ionic equilibria in the mobile phase.

Very interestingly, at the same organic fraction, the effects of methanol and acetonitrile on k' of Neu5Ac and Neu5Gc are different. At lower organic fractions, the organic solvent influences on the hydrophobic interaction between solute and stationary phase, the secondary mechanism behaves as reversed phase, with the k' value with acetonitrile being less than with
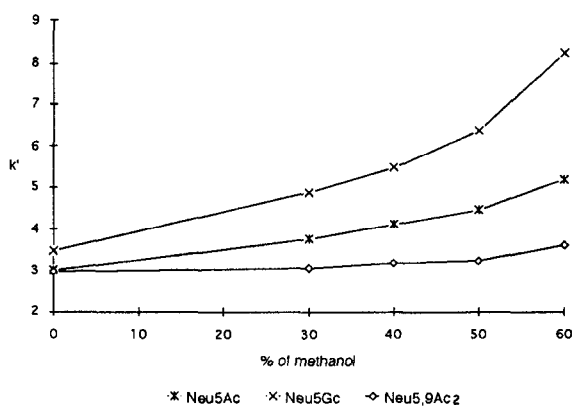
methanol. The organic solvent affecting the hydrophobic interactions is even more obvious with Neu5,9Ac$_2$ in Fig. 3. However, at higher organic fractions, the k' value with methanol is smaller than with acetonitrile. Because methanol instead of acetonitrile can form hydrogen bonds with Neu5Ac or Neu5Gc hydoxyl groups, when more methanol is added in the eluent, it reduces the induced electrostatic interactions between the hydroxyl groups of sialic acids and the positively charged layer on the stationary phase. In this case, at the same fractions of methanol and acetonitrile, the solute has a smaller k' value in methanol than in acetonitrile. Therefore, the secondary mechanism of the anion chromatography involves both hydrophobic and induced electrostatic interactions.

Until now, the retention behavior of sialic acids can be explained at least qualitatively. The complex functions of organic modifiers provide wide flexibility in optimizing the separation of sialic acid derivatives. We found that methanol–7.50 m*M* NaH$_2$PO$_4$ (60:40, v/v) was the best eluent for separation of the sialic acid derivatives.

As a practical application of this study, we separated sialic acids released from BSM. Neu5Ac and Neu5Gc could be completely separated. The results are shown in Figs. 5 and 6.
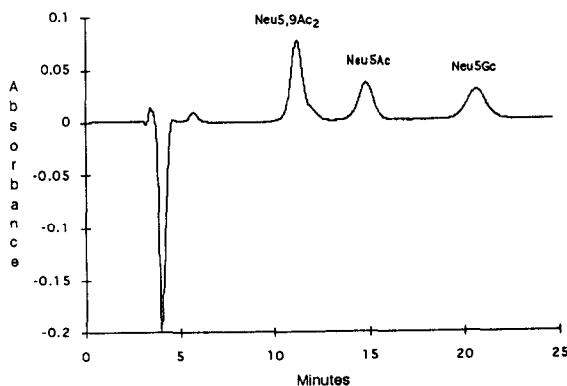

Fig. 5. Chromatogram of a mixture of Neu5,9Ac$_2$, Neu5Ac and Neu5Gc in aqueous solution. The separation was achieved on a Mono Q HR 5/5 column with a HRLC MA7Q anion-exchange column (BioRAD) (50 × 7.8 mm) as guard column. The eluent was methanol–7.50 m*M* NaH$_2$PO$_4$ (aq.) (60:40) solution. UV detector at 205 nm.

Fig. 4. Relationship between the capacity factor of sialic acids and the fraction of methanol into 7.50 m*M* NaH$_2$PO$_4$ solution.
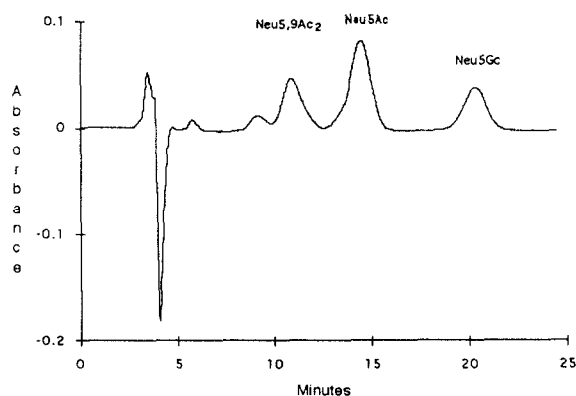
Fig. 6. Chromatogram of the hydrolysate of BSM. BSM (3 mg) was hydrolyzed in 5 ml of 0.01 M HCl for 1 h at 80°C (for sample preparation refer to experimental). The separation was achieved on a Mono Q HR 5/5 column with a HRLC MA7Q anion-exchange column (BioRAD) (50 × 7.8 mm) as guard column. The eluent was methanol–7.50 mM NaH_2PO_4 (aq.) (60:40) solution. UV detector at 205 nm.

The peak just prior to Neu5,9Ac$_2$ in Fig. 6 probably is Neu5,8,9Ac$_3$ or other derivatives with more than one OAc group.

Acknowledgements

References

[1] A.P. Corfield and R. Schauer, in R. Schauer (Editor), *Sialic Acids, Chemistry, Metabolism and Function*, Vol. 10, Springer, NY, 1982, p. 5.
[2] R. Schauer, *Trends Biochem. Sci.*, 10 (1985) 357.
[3] R. Schauer, *Adv. Carbohydr. Chem. Biochem.*, 40 (1982) 131.
[4] H. K. Ogawa, Y. Takeuchi, H. Uchibori, I. Matsumoto and N. Seno, *J. Chromatogr.*, 612 (1993) 145.
[5] A.K. Shukla and R. Schauer, *Anal. Biochem.*, 158 (1986) 158.
[6] A. K. Shukla, N. Scholz, E.H. Reimerdes and R. Schauer, *Anal. Biochem.*, 123 (1982) 78.
[7] G. Herrler, R. Rott, H.D. Klenk, H.P. Müller, A.K. Shukla and R. Schauer, *EMBO J.*, 4 (1985) 1503.
[8] R. Schauer, *Adv. Exp. Med. Biol.*, 174 (1984) 75.
[9] A. E. Manzi, S. Diaz and A. Varki, *Anal. Biochem.*, 188 (1990) 20.
[10] D.T. Gjerde and J.S. Fritz, *Ion Chromatography*, Hüthig, Heidelberg, 2nd ed., 1987, p. 73.
[11] Sj. van der Wal and J.F.K. Huber, *J. Chromatogr.*, 102 (1974) 353.
[12] N. Volpe, *Chromatographia*, 34 (1992) 216.
[13] T.H. Lowry, *Mechanism and Theory of Organic Chemistry*, 3rd ed., 1987, p. 177.