Analysis of *N*-Acetylneuraminic Acid Oligomers by High-Performance Capillary Electrophoresis

Kazuaki KAKEHI, Atsuko HIROSE, Toshiaki TAMAI, Atsushi TAGA
and Susumu HONDA[†]

Faculty of Pharmaceutical Sciences, Kinki University,
Kowakae, Higashi-osaka 577, Japan

A few colominic acid preparations and *N*-acetylneuraminic acid (NANA) oligomers derived thereof were analyzed by capillary zone electrophoresis with low-wavelength UV detection. Colominic acid homologues having degrees of polymerization (d.p.s) up to *ca.* 30 were separated to the baseline level, and homologues having a d.p.s of 30–40 were discriminated from each other using a 100 cm capillary (50 μ m i.d.) and a 25 mM phosphate buffer containing SDS to a concentration of 100 mM as the carrier. A colominic acid preparation was hydrolyzed by a dilute acid (10 mM HCl), an acidic buffer (50 mM acetate buffer, pH 4.8) and neuraminidase from *Arthrobacter ureafaciens*; the products were then analyzed under the established conditions. The information from the obtained results will be useful for estimating NANA oligomers and polymers having a specified d.p.s, and for investigating the biosynthesis and metabolism of glycoconjugates containing these homologous oligosaccharides.

Keywords Capillary electrophoresis, colominic acid, *N*-acetylneuraminic acid, neuraminidase

Poly(sialic acid)-containing glycoconjugates are widely distributed in bacterial capsules¹, fish eggs², nerve tissues³, and human tumors.⁴ They play regulatory roles in cell growth and differentiation, fertilization and neuronal pathogenicity.⁵ It is also known that *E. Coli* produces homologically $\alpha 2 \rightarrow 8$ linked polymers of *N*-acetylneuraminic acid (NANA) (Fig. 1); such a homologous series of NANA polymers is classified as colominic acid. Colominic acid preparations are usually mixtures of NANA polymers having a wide-ranging d.p.s (degrees of polymerization). Colominic acid preparations and their hydrolyzates are often used as standards for studies of the carbohydrate moieties of NANA-containing

glycoconjugates.

Sialic acid oligomers and polymers can be characterized by paper chromatography⁶ as well as thin-layer chromatography.⁷ In liquid chromatography, however, this series of compounds cannot be sensitively detected by absorption in the ordinary UV and VIS regions, even though they can be separated by certain modes. Although a method based on anion-exchange separation and pulsed amperometric detection seems to be promising, but a strong alkali is required for elution. The concentration of the alkali needed to separate larger homologues would be much higher than those hitherto employed to separate neutral carbohydrates.

Fig. 1 Structures of colominic acid homologues.

[†] To whom correspondence should be addressed.

Although high-performance capillary electrophoresis (HPCE) is an analytical method that is noted for its high-resolution separation, it has the same problem concerning the detection of sialic acid oligomers and polymers as that in liquid chromatography. Recent progress in detection technology, however, has enabled monitoring in the low-wavelength UV region. This paper describes the result of an analysis of a few colominic acid preparations and NANA oligomers thereof by HPCE with this detection method.

Experimental

Materials

NANA and neuraminidase from *Arthrobacter urea-faciens* were obtained from Nacalai Tesque (Nakagyo-ku, Kyoto). Samples of colominic acid preparations (average molecular weight: A, 14800; B, 45000) were kindly donated by Drs. Tsukada and Ohta of Marukin Shoyu (Uji, Kyoto). All other reagents for HPCE were of the highest grade commercially available. Deionized water was distilled in a glass vessel. Carriers for HPCE were degassed before use.

HPCE

Optimization of the separation conditions was carried out at ambient temperature using a JASCO (Hachioji, Tokyo) CE-800 apparatus equipped with a UV detector of the deuterium lamp (grating type). Detection was carried out at 200 nm. Analyses of NANA oligomers and polymers under the optimized conditions were performed at $30 \pm 1^\circ\text{C}$ using a Waters (Shinagawa, Tokyo) Quanta 4000 apparatus equipped with a UV detector of the multiple lamp (interference filter type). Detection was carried out at 185 nm using a zinc lamp. A roll of a capillary of fused silica (50 μm i.d.) was obtained from Polymicro Technologies (Phoenix, AZ). A 58-cm portion of the roll was used throughout the work, unless specified. The detection window was made at the 8-cm position from the cathodic end. In all of the analyses, samples were introduced hydrodynamically for 10 s at a 10-cm height.

*Partial hydrolysis of colominic acid in 10 mM hydrochloric acid*⁶

A sample of colominic acid preparation A (500 μg) was dissolved in 10 mM hydrochloric acid (500 μl) in a polypropylene tube; the solution was kept at 80°C in a heating block. After specified intervals, 10- μl portions of the solution were neutralized with 10 mM sodium hydroxide (10 μl) and analyzed by HPCE.

*Partial hydrolysis of colominic acid in an acetate buffer (pH 4.8)*⁷

A sample of colominic acid preparation A (300 μg) was dissolved in 50 mM acetate buffer, pH 4.8 (300 μl); the solution was then incubated at 37°C . After specified intervals, 10 μl portions of the solution were removed and

immediately analyzed by HPCE.

Enzymic digestion of colominic acid by neuraminidase

A sample of colominic acid preparation A (500 μg) was dissolved in 50 mM acetate buffer, pH 4.8 (250 μl). After neuraminidase (10 mU in 50 μl of the same buffer) was added to the solution, the mixed solution was incubated at 37°C . After specified intervals, 20- μl aliquots were removed and immediately analyzed by HPCE. The removed aliquots were not heated for terminating the enzymic action prior to an analysis, because non-enzymic hydrolysis was not negligible under such conditions.

Results and Discussion

Each NANA residue in colominic acid has a carboxyl group, which is dissociated to form an anion in alkaline media. Therefore, colominic acid behaves as polyanions and is attracted to the anode by an electrostatic force. On the other hand, when HPCE is performed in a capillary of fused silica, rapid electroosmotic flow is generated, which flows toward the opposite direction. Consequently, colominic acid is moved to the cathode by the combined effects of electrophoresis and electroosmosis.

If colominic acid has a structure in which each NANA residue is completely independent of other NANA residues, and behaves merely as a multiple form of NANA, the ratio of the electric charge to the molecular mass will be the same; hence, no zone electrophoretic separation occurs. In reality, however, the colominic acid homologues were well separated from each other, as observed for preparation A (average molecular weight, 14800) in Fig. 2a.

Although a few examples of the zone electrophoretic separation of homologous oligosaccharide derivatives [e.g. 2-aminopyridine (AP) derivatives⁸, 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) derivatives⁹] were reported, the charge-to-mass ratio was varied among the d.p.s in these cases, because the molecular mass increased with the d.p., whereas the electric charge remained constant (one AP or one ANTS group was introduced to each oligosaccharide). Since the separation mode in the present work was obviously zone electrophoresis, the reason why the charge-to-mass ratio could vary among homologues was speculated to be as follows. Since all of the carboxyl groups in the NANA residues are considered to be completely dissociated under the conditions employed (pH, ca. 9.4), the electric charge should be proportional to the d.p. with all homologues. On the other hand, the molecular weight is not exactly proportional to the d.p., because one water molecule is removed in each step of chain elongation. The closed circular plot in Fig. 3 shows the change in the $\text{MW}_{\text{NANA}} \cdot \text{d.p.} / \text{MW}$ ratio with the d.p., where MW and MW_{NANA} are the molecular weight of each homologue and that of NANA, respectively.

Fig. 2 Effect of the SDS concentration on the separation of colomonic acid preparation A in 25 mM disodium hydrogenphosphate. Apparatus, JASCO CE-800 without temperature control; capillary, fused silica (58 cm, 50 μ m i.d.); carrier, 25 mM disodium hydrogenphosphate (pH 9.4) without SDS (a), with 50 mM (b) and 100 mM SDS (c); applied voltage, 25 kV; detection, UV absorption at 200 nm. An aqueous sample solution (1 mg/ml) was introduced by gravity (10-cm height, 10 s) from the anodic end of the tube. The numbers indicate d.p.s.

Fig. 3 Curves showing that the non-linear relationship between the molecular weight and the d.p. was not the sole reason for the separation of NANA oligomers. (●) $MW_{NANA} \cdot d.p./MW$ vs. d.p.; (■) RM vs. d.p.

On the other hand the closed rectangular plot in Fig. 3 shows the change in the observed relative mobility (RM) to NANA with the d.p., calculated as $t_{NANA}/t\{(t-t_0)/(t_{NANA}-t_0)\}$, where t , t_{NANA} and t_0 are the migration times of the NANA homologue to be examined, NANA and a neutral marker, respectively. It is apparent that the RM curve was above the $MW_{NANA} \cdot d.p./MW$ ratio curve, and that these two curves did not overlap each other. If the water elimination in chain elongation is the main reason for the observed separation, both curves should be superimposed on each other, because the relative mobility is equal to the $MW_{NANA} \cdot d.p./MW$ ratio for each d.p. value. This discordance suggests another reason for the variation in the charge-to-mass ratio. One possible explanation is the variation of the molecular shape in the carrier. The electrophoretic mobility (μ_{ep}) is affected by the molecular shape, as expressed by Henry's correction function in the basic equation of electrophoresis,

$$\mu_{ep} = k \times \frac{\varepsilon \times \xi \times f(\chi)}{\eta} E$$

where ε , η , and E are the permittivity of carrier, the viscosity of carrier, and the electric-field strength, respectively. All of them are constant for every component. The term ξ is the zeta potential between the analyte molecule and the carrier, and is considered to be varied with the $MW_{NANA} \cdot d.p./MW$ ratio. The symbol $f(\chi)$ designates Henry's function regarding the correction for the molecular shape of the component.¹⁰ Although no positive evidence for a molecular-shape variation is available, it is the most probable that hydrodynamic volume alteration, which corresponds to the alteration of $\xi \cdot f(\chi)$ induced by this molecular shape variation, is the main cause for the diversity of the μ_{eq} values among the homologues.

The separation of NANA oligomers was poorer at low pH's. Various additives were examined in order to improve the separation, keeping the pH at 9.4; it was found that the addition of sodium dodecyl sulfate (SDS) to high concentrations (Fig. 2b and 2c) gave a slight improvement, presumably due to the contribution of micellar electrokinetic chromatography, though only to a slight extent, because NANA oligomers were highly hydrophilic under the conditions employed due to almost complete dissociation. At an SDS concentration of 100 mM (Fig. 2c) homologues having d.p.s up to ca. 15 were discriminated from each other, when analyzed in a 58-cm capillary using a JASCO apparatus without any temperature control. The reproducibility in migration times was excellent. The relative standard deviations in the migration times for di-, tri-, penta-, deca- and pentadecamers were 1.3%, 1.4%, 1.6%, 2.0% and 2.3%, respectively.

The separation was greatly improved in this carrier, when an analysis was performed in a longer (100 cm) capillary using a Waters apparatus with a temperature control at 30°C (Fig. 4). The sensitivity was greatly enhanced (the lower limit, 10^{-5} M) by detection at

Fig. 4 Analysis of the colominic acid preparation A. Apparatus, Waters Quanta 4000 with temperature control at 30°C; capillary, fused silica (108 cm, inlet-detection portion 100 cm, 50 μ m i.d.); carrier, 25 mM disodium hydrogen-phosphate (pH 9.4) containing SDS (100 mM), applied voltage, 15 kV; detection, absorption at 185 nm. NANA (peak 1) was artificially added as an internal marker.

Fig. 5 Relationship between the logarithmic mobility and logarithmic molecular weight.

185 nm. The sensitivity at this wavelength was *ca.* 10-times higher than that at 200 nm, measured with a JASCO apparatus. Under the conditions utilizing a 100-cm capillary, a sample of colominic acid preparation A showed the presence of a number of homologues having d.p.s up to 40. Those homologues having d.p.s less than 30 were separated from each other almost to the baseline level within 70 min. The analysis time (*ca.* 70 min) was rather longer than that in Fig. 2 (<20 min). This was mainly due to difference in applied voltage (15 kV for Fig. 4, 25 kV for Fig. 2). The difference in capillary temperature (30°C for Fig. 4, *ca.* 20°C for Fig. 2) should also be taken into consideration. There was no linear relation between $\log \mu_{ep}$ and $\log MW$

Fig. 6 Analysis of two different colominic acid preparations. Capillary, fused silica (58 cm, 50 μ m i.d.). The other conditions as in Fig. 4. Sample: preparation A (MW 14800), preparation B (MW 45000).

Fig. 7 Course of hydrolysis of colominic acid preparation A in 10 mM HCl at 80°C. Hydrolysis time: a, 10 min; b, 30 min; c, 50 min; d, 180 min. The analytical conditions as in Fig. 6.

(Fig. 5); accordingly, μ_{ep} was not proportional to $(MW)^n$, unlike in ordinary cases of homologous oligosaccharides (for example, PA derivatives of homologous oligosaccharides⁸). This is also evidence suggesting a variation in molecular shape with the d.p.

Figure 6 compares the HPCE profiles of two different colominic acid preparations. A shorter capillary (58-cm) was used in these analyses for economy of the analysis time. Preparation A showed a broad fused peak at about 23 min along with peaks due to small oligomers. Preparation B, having an average molecular weight of 45000, gave a broad peak at *ca.* 26 min, but almost no peaks of small oligomers. Thus, the difference in the molecular distribution is clearly indicated by HPCE under these analytical conditions.

We applied the established analytical procedure for

Fig. 8 Course of hydrolysis of colominic acid preparation A in 50 mM acetate buffer (pH 4.8) at 37°C. Hydrolysis time: a, 12 h; b, 24 h; c, 48 h; d, 72 h. The analytical conditions as in Fig. 6.

observing the reaction course of the depolymerization of colominic acid. Figure 7 shows the course of the acid hydrolysis of preparation A in 10 mM hydrochloric acid at 80°C. When this preparation, having an average molecular weight of 14800, was used, the maximum response was observed at around 23 min for hydrolysis times of up to 50 min, but it shifted to *ca.* 17 min (the peak position of the d.p. 4 oligomer) after hydrolysis for 180 min (Fig. 7d). Hydrolysis for longer periods did not considerably increase the intensity of the monomer peak, but facilitated the formation of small oligomers. This means that small oligomers are relatively stable under such acidic conditions.

Figure 8 shows the course of hydrolysis in a 50 mM acetate buffer (pH 4.8) at 37°C. These reaction conditions are the mildest ones hitherto employed to obtain standard sialic acid oligomers.⁷ The reaction proceeded much more slowly, only a small peak of the monomer being observed after 24 h. With respect to 72-h incubation, the peak of the dimer was the largest. Under these conditions the reaction solution was colored brown, suggesting an accompanying non-hydrolytic degradation.

Figure 9 shows the course of the enzymic digestion by neuraminidase from *Arthrobacter ureafaciens*. The hydrolysis was rapid, and small oligosaccharides were formed already after only 30 min (Fig. 9b). The oligosaccharide profile was not greatly changed until 180 min, while the monomer peak which appeared at 60 min gradually increased. Considering the profile change, this enzyme is considered to have cleaved the glycosidic linkages in larger homologues more easily than those in smaller ones.

The neuraminidase from *Arthrobacter ureafaciens* is known to be an *exo*-type enzyme catalyzing the cleavage of the outermost glycosidic linkage of NANA oligomers

Fig. 9 Course of hydrolysis of colominic acid preparation A by neuraminidase at 37°C. Incubation time: a, 10 min; b, 30 min; c, 60 min; d, 180 min. The analytical conditions as in Fig. 6.

and polymers.¹² If this is the case, the NANA monomer could have been accumulated in the digestion mixture as the incubation time increased. However, the obtained result was inconsistent with this expectation, even though it is taken into consideration that the molar absorptivity of the NANA monomer would be the smallest of all homologues, because it has the smallest number (1) of carbonyl groups. As far as the result shows, this enzyme seems to have difficulty to cleave the outermost NANA residue in smaller homologues. Another possibility is that the enzyme also cleaved interior glycosidic linkages. However, further studies will be necessary to claim this mode of action contradictory to the established mechanism.¹²

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