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Note

Sialic acid quantitation by analytical isotachophoresis

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The discovery of sialic acids and the investigations which established their importance in glycoprotein chemistry[1], biology and clinical chemistry[2–5] have led to the development of methods of estimating free and bound sialic acid. Apart from the enzymatic assay[6], coloured or UV-absorbing derivatives of sialic acids have been estimated by photometry[7–10]. Preliminary transformations of sialic acid into derivatives are time-consuming and may lead to quantitation errors due to inadequate transformations or to interfering substances. Thus, a specific method of estimating sialic acids which avoids such potential problems is desirable. We wish to report the use of analytical isotachophoresis (ITP) for sialic acid estimation.

MATERIALS AND METHODS

Chemicals were from the following suppliers: N-acetylneuraminic acid (NANA, sialic acid, 5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosaminic acid), β-alanine and caproic acid, all p.a., from Serva (Heidelberg, G.F.R.); 1 *M* HCl, Titrisol, from E. Merck (Darmstadt, G.F.R.); fetuin step IV from Sigma (St. Louis, MO, U.S.A.); and hydroxypropylmethylcellulose (HPMC) from Dow Chemical (Midland, MI, U.S.A.).

Buffer system

Leading electrolyte: 5 m*M* HCl in 0.3% HPMC–β-alanine, pH 3.80 (20°C); conductivity, 0.430 mS. Terminating electrolyte: 10 m*M* caproic acid, pH 3.31 (20°C); conductivity, 0.084 mS.

Experimental conditions

Capillary length, 61 cm; detection wavelength, 254 nm; operating temperature,

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5°C; UV gain, 1.70; thermo gain, 2.00; current, 80 μA; starting voltage, 5.5 $\pm$ 0.1 kV; final voltage, 27.3 $\pm$ 0.1 kV; chart speed, 5 mm/sec; signal input, (a) UV 100 mV, (b) thermo, 100 mV; thermoheight of NANA in % of terminator, 66%.

Calibration line

NANA (200 μg) was dissolved in 1 ml of double-distilled water, and a dilution series in the concentration range 1–200 ng/μl was prepared from this standard. Fig. 1 shows the plot of NANA content *versus* the corresponding zone length in the UV diagram.

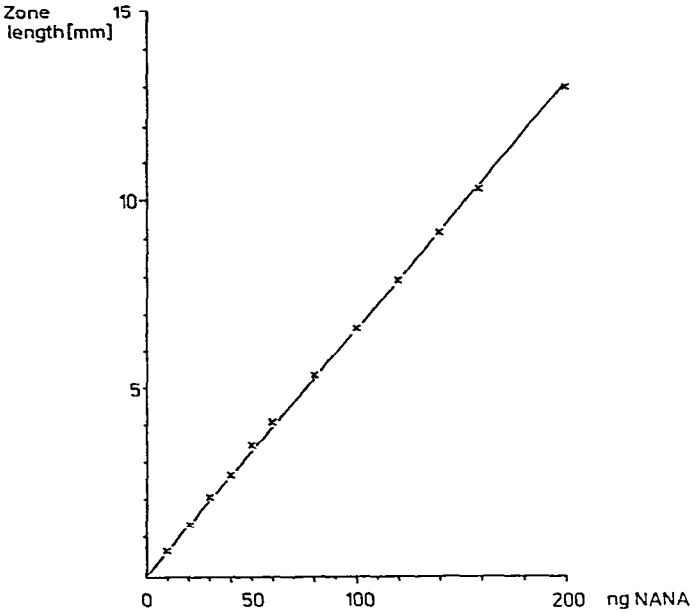

Fig. 1. Calibration line calculated from twelve values, each of which is the mean of three estimates. The accuracy of the zone length estimation is $\pm$0.1 mm, leading to an error of 0.15 ng of NANA. The correlation coefficient r_{xy} = 0.989.

NANA estimation

Under the conditions applied, NANA appears as a trough between two UV-absorbing impurity components of the buffer. Nucleotides may serve as markers too. The trough length is directly proportional to the amount of NANA injected into the system. Trough lengths are estimated by measuring peak–peak distance and half-height widths of the two adjacent peaks. The peak–peak distance, diminished by 50% of the total of both half-height widths, marks the real zone length of NANA.

As the zone length is highly dependent on buffer concentrations and pH, the correlation between the amount of NANA and the zone length was controlled by injection of standards when buffers were newly prepared. The values obtained from the "rule of three" calculation* of sample contents were compared with the values calculated from the calibration line.

* Amount unknown = $\frac{\text{NANA amount of standard} \times \text{trough length measured}}{\text{trough length of standard amount}}$

Desialylation of fetuin

Fetuin (5.81 mg) was dissolved in 1 ml of 80 m*M* HCl at room temperature. Then 100-μl portions of this solution were transferred to eight stoppered vials and incubated in a water bath at 80°C. After 5, 10, 20, 30, 40, 60, 80 and 100 min one vial was removed from the bath, rapidly cooled with ice water, and diluted with double-distilled water, thus producing a 5 m*M* HCl solution. The pH was adjusted to 3.80 with β-alanine. The same procedure was used for glycophorin identification during fractionation. The total NANA content of fetuin was estimated by the thiobarbituric acid assay[7].

RESULTS

Analytical ITP was shown to be a valuable method for NANA estimation. A linear calibration curve could be produced in the range 1–200 ng of NANA, reflecting the high sensitivity and reproducibility of this method. With three estimations per point on the calibration curve, a correlation coefficient of 0.989 was obtained. By using the "rule of three" calculation, together with the evaluation on the calibration curve, an accuracy better than 2% is found even when only small amounts of NANA are being estimated. The contamination of NANA with substances which interfere with photometric assays is not a problem, as unloaded substances like 2-deoxyribose do not migrate whilst components like unsaturated lipids do not migrate under these conditions. Hence, time-consuming preliminary purification and additional corrections of data are unnecessary.

The quantitation of total NANA content by thiobarbituric acid assay resulted in 6.19% ± 0.03% (w/w) of NANA. The NANA content given by the manufacturer's analysis was 6.29% (w/w). Under the dilution conditions described under *Desialylation of fetuin*, a final NANA concentration of 22.5–22.8 ng/μl sample had to be reached.

TABLE I

QUANTITATION OF FREE NANA AFTER INCUBATION OF FETUIN

Incubation with 80 m*M* HCl at 80°C for different times. Zone lengths are means of three estimates. An 8-μl volume of sample was injected. Zone length values are calculated for a sample volume of 1 μl.

Time (min)	*% of total*	*NANA (ng)*	*Zone length (mm)*
5	26.5	6.1	0.40
10	51.0	11.5	0.76
20	70.0	15.8	1.05
30	77.0	17.3	1.16
40	80.0	18.0	1.20
60	83.0	18.7	1.24
80	85.0	19.1	1.27
100	86.0	19.4	1.29

The desialylation curve of fetuin was recorded by relating NANA contents measured by analytical ITP to total NANA content, estimated by the thiobarbituric acid assay (Table I). Fig. 2 shows the corresponding graph.

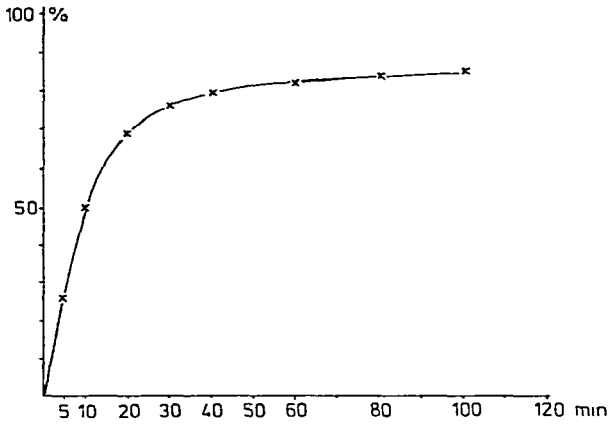

Fig. 2. The desialylation curve of fetuin. Values are given as percentages of total NANA amount present in the fetuin used and estimated by the thiobarbituric acid assay. Within the period of hydrolysis investigated, only 86% of sialic acid was set free. Extrapolation of this curve shows that at 290 min, 100% is reached.

DISCUSSION

Compared with other methods established for NANA quantitation, analytical ITP has many advantages. The small volumes of sample prevent substantial loss of material. The possible variation of injection volume in the range 1–20 μl permits the quantitation of even small amounts of NANA. The injection of large volumes diminishes systematic errors, depending on the inaccuracy of the volumes injected. Chemical transformations prior to quantitation are not necessary, thus considerable time-saving is possible. Moreover, substances such as 2-deoxyribose or unsaturated lipids, which interfere with photometric methods[7,8], do not interfere in ITP because they do not migrate under these conditions.

As NANA readily undergoes decarboxylation when treated with hot mineral acids, the influence of this decarboxylation on NANA concentration during the desialylation experiment was investigated. No significant deviation was found between the NANA content of a sample kept for 80 min in cold 80 m*M* HCl and the same sample kept for 80 min in 80 m*M* HCl at 80°C.

Analytical ITP has been developed as a quantitation method for NANA. Because of its sensitivity, accuracy and rapidity, analytical ITP may become the method of choice for free NANA quantitation.

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