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Determination of cytidine 5′-monophospho-N-acetylneuraminic acid pool size in cell culture scale using high-performance anion-exchange chromatography with pulsed amperometric detection

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Abstract

A simultaneous determination of cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) and its metabolic products, cytidine, CMP and Neu5Ac by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) using a Carbo-Pac PA1 column is described. Preparation of the samples involved a single purification step of the crude cell extract on DEAE-Sepharose. The method is adequate to quantify the amount of CMP-Neu5Ac produced by one culture dish; equivalent to $6 \cdot 10^6$ cells. In addition, a method for desalting and recovery of the separated material was developed to determine the cellular concentration of CMP-Neu5Ac in Madin Darby canine kidney (MDCK) cells. The addition of 5 m*M* N-acetylmannosamine to the culture medium gave rise to a 6.4-fold elevation of this value.

Keywords: Cytidine 5′-monophospho-N-acetylneuraminic acid; N-Acetylneuraminic acid; Galactono-1,4β-lactone; Glucuronate; Saccharides; Nucleotides

1. Introduction

Cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) is the activated form of N-acetylneuraminic acid (Neu5Ac) which serves as a direct precursor for the synthesis of sialylated oligosaccharides in glycoproteins and glycolipids. Although interest in the metabolism of sialic acids, and especially the regulation of sialyl transferases, has grown over the last years, there are but few reports on actual measurements of the cellular concentration of CMP-Neu5Ac (for review see Ref. [1]), e.g. the pool size of this sugar nucleotide available for biochemical reactions. Earlier methods employing paper chromatography and thiobarbituric acid [2,3] were found to be complicated and time consuming. A previous HPLC separation of CMP-Neu5Ac on an anion-exchange column has been reported [4], but the use of an acidic eluent resulted in a rapid hydrolysis of the sugar nucleotide. High-performance anion-exchange chromatography using an alkaline elution protocol combined with pulsed amperometric detection (HPAEC–PAD) has proven to be a method well suited for the separation of neutral [5] and acidic sugars [6] as well as the analysis of activated

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UDP-sugars [7]. Its widespread application is due to a sensitive determination without previous derivatisation. In the present paper we report a method for the simultaneous analysis of CMP-Neu5Ac and Neu5Ac using HPAEC–PAD. Employing this procedure, the cellular concentration of CMP-Neu5Ac in Madine Darby canine kidney (MDCK) cells was determined. Furthermore, it was shown that treatment of cells with N-acetylmannosamine (ManNAc), a direct precursor for CMP-Neu5Ac, resulted in a manifold increase in the concentration of this sugar nucleotide.

2. Experimental

2.1. Materials

Monosaccharides for standards were purchased from Sigma (Deisenhofen, Germany). The 50% (w/w) NaOH solution was from Baker (Deventer, Netherlands). Ammonia solution (32%), ammonium acetate, sodium acetate and acetic acid (96%, Suprapur) were from Merck (Darmstadt, Germany) and ammonium hydrogencarbonate was from Fluka (Buchs, Switzerland). CMP-[4,5,6,7,8,9-^{14}C]Neu5Ac ($0.65 \cdot 10^5$ dpm/nmol) was obtained from Amersham (Braunschweig, Germany).

2.2. Cell culture

MDCK cells were cultured in 100-mm plastic culture dishes with Dulbecco's modified Eagles medium (DMEM) containing 10% heat-inactivated fetal calf serum (Eurobio, Les Ulis, France), 0.58 g/l L-glutamine (Merck) and 2 ml penicillin-streptomycin (500×) (Boehringer, Mannheim, Germany). Cells were tested and found to be free from mycoplasma contamination. For all experiments, cells were plated in a density of 10^5 cells/cm^2 and were allowed to adhere for 6 h. For subsequent treatment, cells were maintained in medium with or without ManNAc for 24 h. Cellular protein was determined by the BCA (biocimchomimic acid assay [8] using bovine serum albumin (Sigma) as a standard.

2.3. Cytotoxicity assay

Cytotoxicity of different concentrations of ManNAc to MDCK cells was determined according to Culvenor et al. [9] by measurement of the alkaline phosphatase activity of the cells. By means of this assay, toxic effects of the ManNAc concentrations used were excluded. A concentration of 5 m*M* ManNAc was the highest concentration usable without toxic effects after 24 h of incubation.

2.4. Isolation and purification of CMP-Neu5Ac from cells

All steps were carried out at 4°C. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped into 3 ml 0.05 *M* NH_4HCO_3/NH_3 buffer, pH 8.0, using a rubber policeman. For pool determination, 5550 dpm CMP-^{14}C-Neu5Ac were added to each sample as a tracer. The suspension was sonicated with the micro-tip of a W-375 sonicator (Heat Systems-Ultrasonics, Planview, USA) using 50% duty cycle and 40% power output for 2 min. By staining with Giemsa's solution (Merck), this procedure was shown to be sufficient to disrupt the cell nuclei. The samples were centrifuged at 100 000 *g* for 45 min to precipitate the cell membranes. Ice-cold ethanol was added to the supernatant to a final concentration of 60%. Insoluble material was removed by a second centrifugation step at 100 000 *g* for 45 min. The samples were concentrated to a final volume of 1 ml by evaporation. A DEAE-Sepharose column (Pharmacia, Uppsala, Sweden) with 0.5-ml bed volume, acetate form, was prepared. To this end, 0.5 ml of resin, which should be sufficient to bind 25 mmol of a bivalent ion like CMP-Neu5Ac, was poured into a column with an inner diameter of 5 mm and converted to acetate form with 2.0 *M* aqueous ammonium acetate (NH_4OAc) as described [10]. The probe was loaded on the column and subsequently rinsed with 10 bed volumes distilled water and 10 bed volumes 0.02 *M* NH_4OAc. CMP-Neu5Ac was eluted with 10 bed volumes 0.07 *M* NH_4OAc and the volatile salt was removed by freeze-drying. For separation on HPAEC, galactono-1,4β-lactone and glucuronate, 2 nmol each, were added as internal standards. Due to the alkaline elution conditions, galactono-1,4β-lactone was measured as galactonic acid by HPAEC–PAD.

2.5. Chromatographic conditions

For separation of the acidic monosaccharides by anion-exchange chromatography, a Dionex DX-300 gradient chromatography system and a Dionex PAD II detector (Dionex, Idstein, Germany) were employed. Chromatographic data were recorded using the Spectra Physics Model 4270 integrator (San Jose, CA, USA) and evaluated with Spectra Physics software.

Acidic monosaccharides were separated using the following conditions. Eluent 1 was 140 m*M* NaOH, and eluent 2 was 140 m*M* NaOH containing 600 m*M* sodium acetate. Separation was performed on a CarboPac PA 1 column with a CarboPac PA guard column (both from Dionex) at a flow-rate of 1 ml/min. The elution program started with 4% eluent 2 for 1 min, followed by a linear gradient from 4 to 46% of eluent 2 for 44 min. The column was washed with 100% eluent 2 for 10 min and subsequently equilibrated for 10 min with 4% eluent 2. Using this program, injections could be made every 65 min. Acidic monosaccharides were detected with PAD pulse settings as follows: $E_1=0.05$ V ($t_1=420$ ms); $E_2=0.75$ V ($t_2=180$ ms); $E_3=-0.2$ V ($t_3=360$ ms).

2.6. Desalting of pooled peaks from HPAEC separation

Desalting of the potential CMP-Neu5Ac peak from analysed samples was necessary for subsequent acid hydrolysis to confirm the identity of the material with authentic CMP-Neu5Ac. To this end, a strong anion-exchange resin, Dowex AG 50W-X12 (BioRad, Munich, Germany), was converted from H^+ to NH_4^+ form. For this, 2 ml of resin were packed in a column and rinsed twice with distilled water. Subsequently, the column was rinsed with 1 *M* aqueous ammonia solution until the pH value of the eluent changed from acidic to basic. The pooled material from HPAEC was applied to the resin (2 ml of resin for 1 ml eluate), and the column was rinsed with 8 ml distilled water. Using this procedure, sodium in the probe was exchanged for ammonium, resulting in ammonium acetate, a volatile salt. The probe was freeze-dried twice to remove the ammonium acetate completely.

3. Results

3.1. Separation of monosaccharide standards by HPAEC–PAD

HPAEC separation of Neu5Ac, Neu5Gc and CMP-Neu5Ac is shown in Fig. 1A. The cleavage products, cytidine and CMP, resulting from acid hydrolysis of CMP-Neu5Ac were included to the standard. Fig. 2 shows the structures of the analytes and the reactions involved in their interconversions. For identification of unknown peaks galactono-1,4β-lactone and glucuronate were added as internal standards. These monosaccharides were chosen because they had not been found within mammalian cells. Using the chromatographic conditions described, baseline separation of the compounds could be achieved. The chromatographic parameters, retention times and relative response factor referring to the internal standards, are presented in Table 1.

Stability of CMP-Neu5Ac during separation was assessed by incubating 2 nmol in elution buffer (140 m*M* NaOH, 250 m*M* NaOAc) at 35°C and quantifying the remaining amount of CMP-Neu5Ac at different times. Logarithmic fitting of the data gave a hydrolysis rate of 14% per 40 min, i.e. during the retention time of CMP-Neu5Ac.

3.2. Purification of CMP-Neu5Ac on DEAE-Sepharose column

To achieve an enrichment of CMP-Neu5Ac from crude cytosolic extracts, a method employing batch elution on DEAE-Sepharose was established. Neu5Ac, Neu5Gc and CMP-Neu5Ac, 1 nmol each, were placed on a DEAE-Sepharose column, 0.5 ml bed volume, acetate form, in 1 ml distilled water. The column was washed once with 5 ml of distilled water and eluted with 5 ml of a given concentration of ammonium acetate. Galactono-1,4β-lactone and glucuronate were added as internal standards. The eluate was freeze-dried twice to remove the ammonium acetate and the amount of neuraminic acid recovered was analysed by HPAEC–PAD. The results are shown in Fig. 3. Both Neu5Ac and Neu5Gc were poorly bound to the column and eluted at a concentration of 10 m*M* ammonium acetate. CMP-Neu5Ac first appeared at 40 m*M* and was eluted

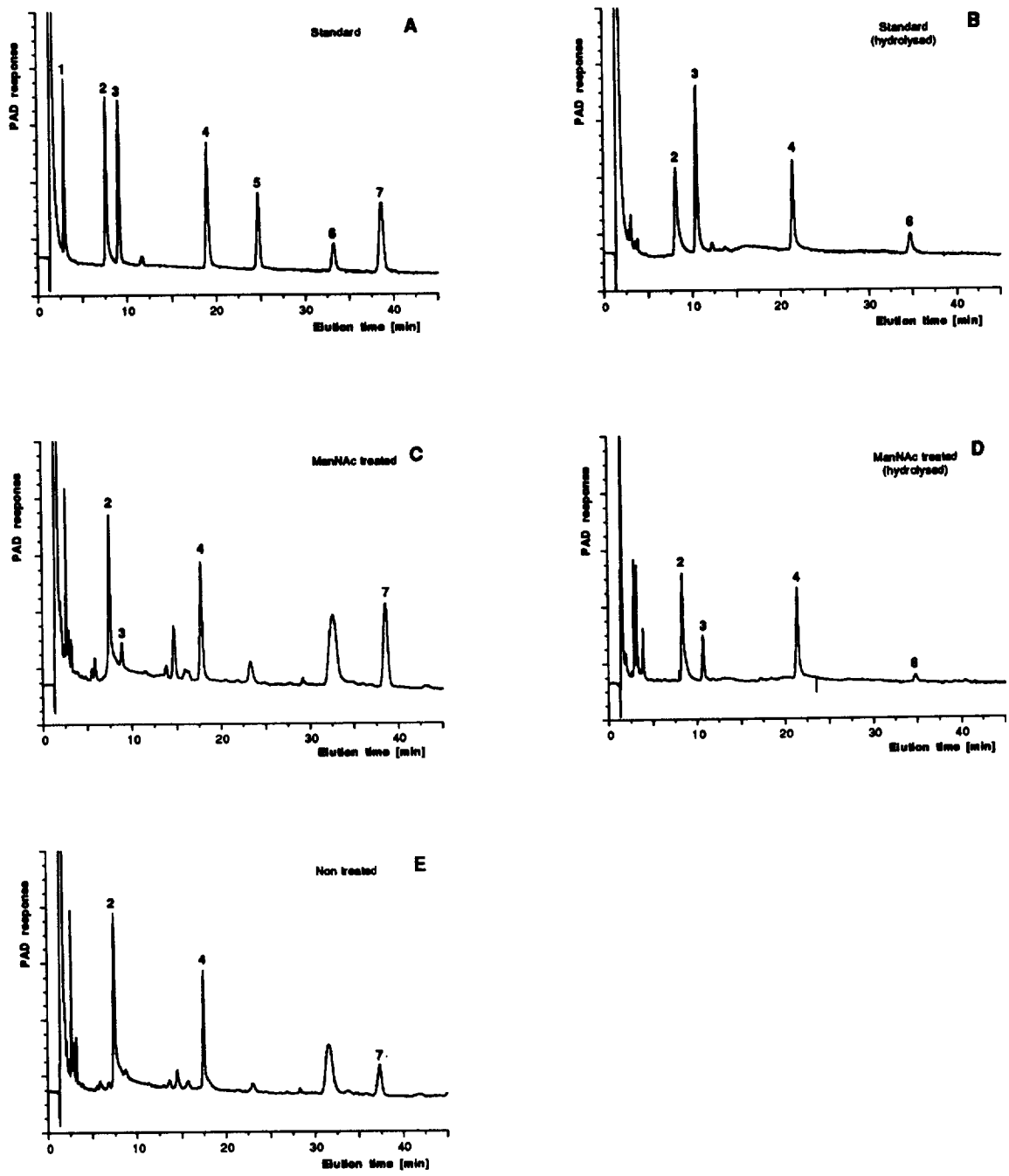

Fig. 1. Determination of CMP-Neu5Ac by HPAEC–PAD. Standard CMP-Neu5Ac and CMP-Neu5Ac derived from MDCK cells grown in the presence or absence of ManNAc were separated by HPAEC–PAD as described in the Experimental. Peaks: 1=cytidine, 3=Neu5Ac, 5=Neu5Gc, 6=CMP and 7=CMP-Neu5Ac. Galactono-1,4β-lactone (2) and glucuronate (4), 2 nmol each, were added as internal standards for the indentification of compounds from their relative retention time. Detection is by PAD at 300 nA full scale for all chromatograms. (A) Separation of standards, 1 nmol each. (B) CMP-Neu5Ac (2 nmol) was hydrolysed and the cleavage products were separated as described. (C) CMP-Neu5Ac prepared from MDCK cells treated with 0.5 m*M* ManNAc for 24 h. (D) The putative CMP-Neu5Ac peak from (C) was collected, desalted and hydrolysed as described in the text. (E) Analysis of CMP-Neu5Ac from MDCK cells grown in the absence of ManNAc.

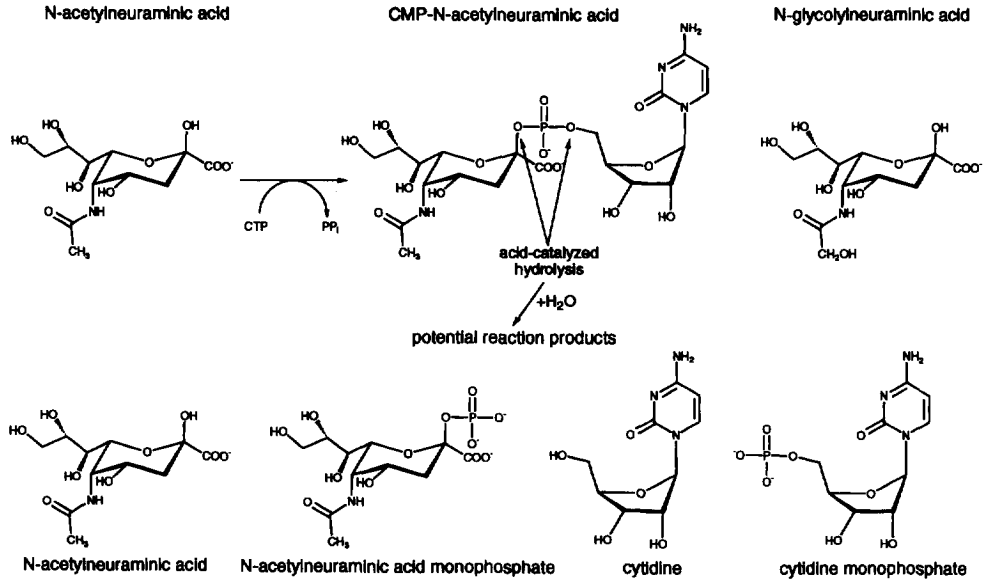

Fig. 2. Structures and interconversions of the analytes measured. N-Acetylneuraminic acid is converted to its activated form, CMP-N-acetylneuraminic acid, by the activity of the CMP-acylneuraminate synthase (EC 2.7.7.43) in the cell nucleus [11]. The conversion of N-acetylneuraminic acid in a free or bound form to N-glycolylneuraminic acid is catalysed by the N-acetylneuraminate monooxygenase (EC 1.14.99.18). Hydrolysis of CMP-Neu5Ac led to the four potential products shown and phosphate.

completely with 70 m*M* ammonium acetate. For the purification of CMP-Neu5Ac from crude extract, the column was therefore washed with 5 ml of distilled water and 5 ml of 20 m*M* ammonium acetate to remove weekly charged contaminations. Subsequently CMP-Neu5Ac was eluted using 5 ml of 70 m*M* ammonium acetate.

3.3. Determination of CMP-Neu5Ac in MDCK cells

The isolation of CMP-Neu5Ac from MDCK cells was carried out essentially as described [2,3]. Because CMP-Neu5Ac is very acid labile, a basic (pH 8.0), volatile buffer system was employed. Addition-

Table 1
Retention times and response factors of the compounds separated

	Retention time (min)	Relative retention time (min)	Molar response factor relative to	
			Galactono-1,4β-lactone	Glucuronate
Cytidine	2.92±0.04	0.60±0.02	1.55±0.08	1.68±0.05
Neu5Ac	8.79±0.13	1.13±0.01	2.30±0.08	2.50±0.04
Neu5Gc	23.95±0.05	2.50±0.01	1.60±0.08	1.73±0.03
CMP	32.31±0.02	3.26±0.01	0.71±0.04	0.77±0.03
CMP-Neu5Ac	37.53±0.04	3.73±0.02	1.04±0.05	1.13±0.02

The compounds were separated using the conditions described in Experimental. Data are the average of 5 injections±S.D. For determination of the relative retention time, the retention times of the internal standards galactono-1,4β-lactone (7.39±0.17 min) and glucuronate (18.41±0.04 min) were defined as 1 and 2, respectively.

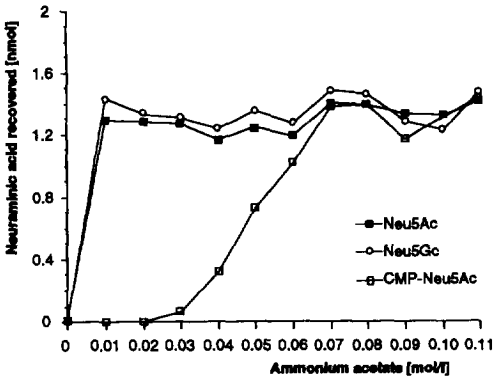

Fig. 3. Separation of CMP-Neu5Ac, Neu5Ac and Neu5Gc on DEAE-Sepharose. CMP-Neu5Ac, Neu5Ac and Neu5Gc, 2 nmol each, were applied to a DEAE-Sepharose column and eluted with 5 ml of a given concentration of ammonium acetate. The amount of neuraminic acids recovered was determined by HPAEC–PAD. For experimental details see text. Each data point represents a single determination. The experiment was repeated once and similar results were obtained.

ally, one purification step on DEAE-Sepharose was performed as described above to remove interfering substances for the separation on HPAEC. Fig. 1E shows a HPAEC separation of a sample of untreated MDCK cells, i.e. cells grown in the absence of ManNAc, prepared as described. Using three different concentrations of NaOH, 70, 100, and 140 m*M* a peak eluting at the same relative retention time as authentic CMP-Neu5Ac was identified (data not shown). To provide further evidence for the identity of this substance to CMP-Neu5Ac, the peak was collected and desalted as described in Experimental. In the desalting procedure the conversion of the resin from H^+ to NH_4^+ form was done since in preliminary experiments the H^+-form of the resin led to a considerable hydrolysis of CMP-Neu5Ac. The desalted material was hydrolysed under mild acidic conditions employing 0.2 *M* acetic acid at 80°C for 1 h. The dried sample was resolved together with internal standards on HPAEC. These experiments were performed using MDCK cells stimulated with ManNAc, since more of the nucleotide sugar was produced. Results are shown in Fig. 1C and D. After hydrolysis and re-chromatography, the putative CMP-Neu5Ac peak vanished. Instead, two peaks showing the same relative retention time as authentic Neu5Ac and CMP appeared, the former possessing the entire radioactivity of the tracer CMP-^{14}C-Neu5Ac added to the samples. Normalizing on the base of the added radioactivity, the yield of the method was calculated to be approximately 10%. The pool size of CMP-Neu5Ac in MDCK cells was determined to 3.39±0.04 nmol/mg protein in four independent experiments, corresponding to a precision of the method of about 1%.

3.4. Accumulation of CMP-Neu5Ac in MDCK cells in response to ManNAc treatment

The effect of ManNAc, a direct precursor for Neu5Ac synthesis, on the size of the CMP-Neu5Ac pool in MDCK cells was determined by means of the method described above. As shown in Table 2 the pool size of CMP-Neu5Ac is increased by ManNAc concentrations higher than 0.5 m*M*. Addition of 5 m*M* ManNAc to the cell medium for 24 h led to an approximately 6-fold elevation of the concentration of CMP-Neu5Ac as compared with control.

4. Discussion

For a wide variety of mono- and oligosaccharides the analysis and separation by HPAEC–PAD has been shown to be a sensitive method which is suitable to detect and quantify amounts of monosaccharides as little as 25 pmol [5]. We adapted this method for the separation and quantification of CMP-Neu5Ac, which is the activated Neu5Ac substrate for sialyltransferases. Former analytical methods employed paper chromatography for separation and the thiobarbituric acid test for quantification of the neuraminic acids [2,3,12]. This experimental protocol involves many steps and is time consuming. A report of Rump et al. [4] describes the HPLC separation of CMP and CMP-Neu5Ac on a Partisil-10 SAX anion-exchange column at pH 4 combined with UV detection at 262 nm. Unfortunately, no lower detection limit was reported. The drawback of their experimental procedure is the rapid hydrolysis of CMP-Neu5Ac at pH 4. Furthermore, the UV detection was limited to nucleotides and nucleotide sugars whereas the method presented allows the

Table 2
ManNAc increases CMP-Neu5Ac pool size in MDCK cells

ManNAc (m*M*)	CMP-Neu5Ac determined[a] (nmol)	dpm	CMP-Neu5Ac computed[b] (nmol)	Protein (mg)	nmol CMP-Neu5Ac/ mg protein
0	0.95	395	8.99	2.63	3.42
0.005	0.84	336	9.30	2.43	3.38
0.05	1.10	406	10.11	2.56	3.95
0.5	1.96	437	16.75	2.64	6.34
5	5.69	347	61.37	2.80	21.92

MDCK cells were prepared as described in Experimental. ManNAc was added to the cell culture dishes in the concentrations indicated in column 1 and the cells were incubated for 24 h. Cells were harvested with the addition of CMP-^{14}C-Neu5Ac as a tracer. The amount of internal standard added was 3750 dpm, as counted after separation by HPAEC–PAD. CMP-Neu5Ac was separated by HPAEC–PAD as described and the pool size was determined in consideration of the recovery rate evaluated by the radioactivity added. Each data point represents a single experiment.

[a] For recovery and pool size determination, the amount of nmol CMP-Neu5Ac was determined from peak areas only; not by means of the internal sugar standards.

[b] Total amount of CMP-Neu5Ac was computed by the formula: CMP-Neu5Ac (computed)=3750/dpm·CMP-Neu5Ac (determined); where 3750 dpm is the amount of internal standard added as determined by counting the CMP-Neu5Ac peak with liquid scintillation after separation by HPAEC–PAD.

simultaneous identification of CMP-Neu5Ac cleavage products, i.e. cytidine, CMP and Neu5Ac.

The response factors of the phosphorylated compounds CMP-Neu5Ac and CMP relative to the internal standards shown in Table 1 are about half the values for the related compounds Neu5Ac and cytidine, respectively. This is in accordance to findings of Townsend et al. [13] for monophosphorylated/non-phosphorylated oligosaccharides. Hydrolysis of CMP-Neu5Ac during separation was determined to be 14%. This value is of interest only if the material is to be pooled and further utilized. Quantification is not affected, since the standard is hydrolysed to the same degree and the nmoles are computed by the ratio of the peak areas.

Desalting of the material was attempted first by Dionex anion self-regenerating suppressor ASRS-I. This equipment exchanges Na^+ in the eluate for H^+ at the surface of a thin membrane. Unfortunately, it could not exchange completely the high amounts of Na^+ resulting from the eluate composition necessary for elution of CMP-Neu5Ac. Even if a complete removal of Na^+ ions could be achieved, a second disadvantage was that the exchange resulted in high concentrations of acetic acid in the samples leading to a rapid hydrolysis of CMP-Neu5Ac. Therefore, desalting was performed on a Dowex cation-exchange resin. To avoid the formation of acetic acid as well as the known catalysis of acid hydrolysis by Dowex H^+-form resins [14], the resin was converted to the NH_4^+ form. Employing this procedure, one has to keep in mind that the affinity of the resin is higher for NH_4^+ than for Na^+. As a consequence, an excess of resin has to be utilized. Two ml of resin (NH_4^+ form) were found to completely remove the Na^+ present in 1 ml sample.

Concerning the CMP-Neu5Ac pool size in cultured cells there are only a few reports. In chinese hamster ovary cells, Briles et al. [2] found 1.6 nmol CMP-Neu5Ac/mg protein and cultured human skin fibroblasts examined by Thomas et al. [12] contained 2.5 nmol CMP-Neu5Ac/mg protein. These data are in good agreement with the 3.4 nmol we determined in MDCK cells. Thomas et al. [12] also reported a stimulating effect of ManNAc on CMP-Neu5Ac biosynthesis. According to their data, incubating human skin fibroblasts for 72 h in medium containing 10 m*M* ManNAc resulted in an approximately 2-fold elevation of the CMP-Neu5Ac pool, but this does not result in an increased incorporation of Neu5Ac into glycoproteins and glycolipids.

In conclusion, a rapid and sensitive method suitable for the determination of CMP-Neu5Ac in nanomolar scale is presented. Additionally, a desalting procedure is described for the recovery and further analysis of the material following separation.

The method is suitable for the determination of the effect of different substances on the CMP-Neu5Ac pool size in cell culture scale ($6 \cdot 10^6$ cells).

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