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Determination of sialic acids in biological fluids using reversed-phase ion-pair high-performance liquid chromatography

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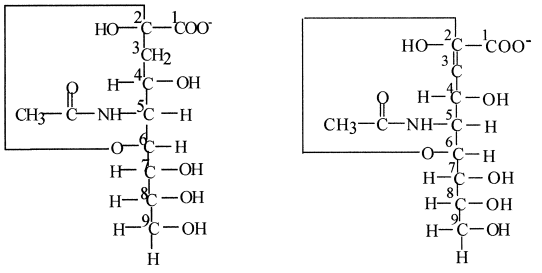
Abstract

A simple, rapid and sensitive reversed-phase ion-pair high-performance liquid chromatographic method for the determination of *N*-acetylneuraminic acid and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid in biological fluids is described. Determination of *N*-acetylneuraminic acid released by acidic hydrolysis, in serum, urine and saliva, and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid in urine, without hydrolysis, was accomplished by injecting the sample without derivatization, into the chromatograph. Measurements were carried out isocratically within 6 min using a C_{18} column and a mobile phase of aqueous solution of triisopropanolamine, as ion-pair reagent, 60 m*M*, pH 3.5 at room temperature with UV absorbance detection. The present method is reported for the first time for the determination of sialic acids in biological fluids. Recoveries in serum, urine and saliva ranged from 90 to 102% and the limits of detection were 60 n*M* and 20 n*M* for the two sialic acids, respectively. The method has been applied to normal and pathological sera from patients with breast, stomach, colon, ovarian and cervix cancers, to normal urine and urine from patient with sialuria and to normal saliva.

Keywords: Sialic acids; *N*-Acetylneuraminic acid; 2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid

1. Introduction

Sialic acids, acylated derivatives of neuraminic acid, mainly occur as nonreducing terminal residues of carbohydrate chains of glycoproteins and gangliosides in biological materials. More than 25 different kinds of sialic acids have now been reported in nature. The most commonly occurring sialic acid is *N*-acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid, Neu5Ac) (Fig. 1a). They can also be *O*-substituted at the 4, 7, 8 and 9 positions, giving rise to a wide



(a) Neu5Ac (b) Neu5Ac2en

Fig. 1. Chemical structures of Neu5Ac (a) and Neu5Ac2en (b).

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variety of compounds and isomers. Unsaturated and dehydro forms of sialic acids have also been reported, like 2-deoxy-2,3-dehydro-*N*-acetylneuraminc acid, Neu5Ac2en (Fig. 1b) [1,2]. There has been great interest in the determination of sialic acids in man. For instance, increased Neu5Ac concentration in serum has been reported in patients with inflammatory disorders, myocardial infarction and chronic glomerulonephritis [3]. Furthermore, serum sialic acids are raised in certain cancers, including malignant melanoma and cancers of the brain, gastrointestinal and gynecologic system, thus they have been used as tumour markers [4–6]. Whereas serum sialic acids are of limited use in the initial diagnosis of malignant disease, serial measurements can be valuable in detecting the development of secondary metastases or in monitoring the tumour bulk in response to treatment [7]. On the other hand, increased urinary levels of free or conjugated Neu5Ac were found in lysosomal storage disorders, such as Salla disease and sialidosis [8,9].

A variety of methods are available for the detection and estimation of sialic acids. Spectrophotometric [10,11], fluorimetric [12] and enzymatic [13] methods have been reported for the determination of sialic acids. Most of these methods have been criticised on the grounds of lack of specificity due to interfering substances and in addition they cannot estimate individual sialic acids in mixtures. Enzymatic methods, supposedly more specific, may underestimate total sialic acids concentrations due to the incomplete hydrolysis of sialoconjugates by neuraminidase [11]. Gas chromatographic methods, alone or in combination with mass spectrometry [14,15], are useful for simultaneous determination of sialic acids but require time consuming derivatization processes prior to analysis.

During the last 15 years, application of liquid chromatographic methods gradually increased compared to other methods, because of the advantages of allowing rapid and direct determination of underivatized or derivatized samples with amperometric [16], spectrophotometric [17] and fluorimetric [18] detection. In addition, many ion-exchange liquid chromatographic methods [19,20] have been used, due to the ionic character of sialic acids. However, to the best of our knowledge, no reports have appeared of the determination of sialic acids in biological fluids using ion-pair liquid chromatography.

Recently, we reported a new reversed-phase ion-pair high-performance liquid chromatographic method with UV absorbance detection, using triisopropanolamine (TIP) as ion-pair reagent, for the separation and determination of five selected sialic acids (Neu5Ac, *N*-glycolylneuraminic acid (Neu5Gc), Neu5Ac2en, cytidine 5′-mono-phospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) and *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2)) with biological importance [21]. This study has led us to the successful application of the above method for the determination of Neu5Ac in serum, urine and saliva and of Neu5Ac2en in urine.

2. Experimental

2.1. Chemicals and materials

Deionized water was passed through a Millipore system (C > 18.2 MΩ cm^{-1} (25°C), TOC<3 ppb). TIP, M_r 191.27, 97% was purchased from Fluka (Sigma-Aldrich Company, Athens, Greece). Neu5Ac, M_r 309.3, 98% and Neu5Ac2en, M_r 291.3, 95% were purchased from Sigma (Sigma-Aldrich Company, Athens, Greece). *Ortho*-phosphoric acid, purity 85%, was supplied from MERCK (Darmstadt, Germany).

Sep-Pak Plus cartridges C_{18}, 12% C, pore size 125 D, particle size 80 μ*M* and HVLP 0.45 μm Millipore filters were purchased from Millipore, Waters Chromatography (Malva, Athens, Greece).

2.2. Biological samples

Ten normal sera and nineteen pathological sera from patients with stomach cancer (four), intestinal cancer (three), ovarian cancer (four), cervix cancer (four) and breast cancer (four) were obtained from male and female subjects. Blood was drawn after an overnight fast by venipuncture and allowed to clot for 30 min at room temperature. After centrifugation for 10 min at 1000 *g*, the serum was pipetted off and stored at −18°C until analysed. Ten 24 h urine samples from five male and five female healthy individuals with normal creatinine clearance and one urine sample from patient with sialuria, were centrifuged at 4000 *g* for 10 min. The supernatants were analyzed immediately or stored at −18°C. Neu5Ac and Neu5Ac2en are stable for as long as 1 month

under these storage conditions. Ten saliva samples from seven male and three female healthy individuals were collected as follows: an individual was asked to rinse out his mouth with water and then chew a piece of rubber from several min. The first mouthful of saliva was discarded; thereafter the saliva was collected into a small glass bottle [22]. Saliva samples were analyzed immediately after collection.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of a model Waters 600 Controller, a solvent delivery system and a syringe-loading sample injector valve (20-μl loop) was used. The separation was performed in a reversed-phase column LiChrospher RP-18 (250×4 mm I.D., particle size, 10 μm, Waters Associates) at ambient temperature. The samples were eluted with aqueous TIP solution 60 m*M*, pH 3.5, adjusted with concentrated H_3PO_4, at a flow-rate of 0.6 ml min^{-1}. Mobile phase degassing was performed automatically, on line, under stream of He. The eluted peaks of Neu5Ac and Neu5Ac2en were recorded at 215 and 240 nm, respectively, using a Waters 486 spectrophotometric detector fitted with a 8-μl flow cell. Data processing was carried out using software Millenium 2.10.

Column can be used for more than 1000 injections with only a small decrease in the theoretical plate number, if it is washed with H_2O: methanol (1:1, v/v) at a flow-rate of 1.0 ml min^{-1} for 30 min every day after analysis has been performed [23].

2.4. Procedure for the determination of Neu5Ac in serum, urine and saliva

To 100 μl of sample, placed in a vial, were added 1.90 ml of 0.050 *M* H_2SO_4. The vial was closed and heated at 80°C for 1 h to hydrolyze the sample [24]. After cooling, 2.0 ml of the hydrolyzed serum sample were passed through a cartridge Sep-Pak plus C_{18}, pre-eluted with 2.0 ml of water, at flow-rate 1 drop per s. In the case of urine or saliva, hydrolyzed sample was passed through a HVLP 0.45 μm Millipore filter. The eluate, in all cases, was collected and 20 μl sample were injected into the chromatograph. Neu5Ac concentration in the samples was calculated by comparing the height of the peak obtained from the samples with that obtained using a standard Neu5Ac aqueous solution in the appropriate concentration. The latter was subjected to the same sample analytical procedure.

2.5. Procedure for the determination of Neu5Ac2en in urine

Aliquot of urine sample was passed through a HVLP 0.45 μm Millipore filter and 20 μl of the sample were injected into the chromatograph. Neu5Ac2en concentration in the samples was calculated by comparing the height of the peak obtained from the samples with that obtained using standard Neu5Ac2en aqueous solutions 60 μ*M*, which was subjected to the same sample analytical procedure.

3. Results and discussion

3.1. Determination of total Neu5Ac in serum

As reported in the literature, Neu5Ac is the major sialic acid derivative found in serum, at a concentration range 2.0–3.0 m*M*, mainly bonded to glycoproteins and glycolipids [25]. Deconjugation is normally achieved by means of acid hydrolysis [24,26] or enzymatic digestion [27,28]. Using the latter procedure the yield of sialic acid is not quantitative, as it is dependent on different factors which are usually difficult to control, such as *O*-acetylation and the conformation of the glycosidic linkage. In the present work, hydrolysis was carried out using sulphuric acid [24].

Conversely, the most common problem in HPLC on injection of a serum sample is the binding of proteins to the column. Therefore, it is best to avoid putting them on the column since removal is difficult and time consuming [23]. For this purpose, cartridges Sep-Pak Plus C_{18} were used. Recovery for standard aqueous solutions of Neu5Ac was found to be 98%.

The peak of Neu5Ac was identified on the basis of the retention time compared with that of the standard compound, after co-chromatography of the standard and sera. Retention time in standard hydrolysed Neu5Ac aqueous solutions is 5.018±0.029 min

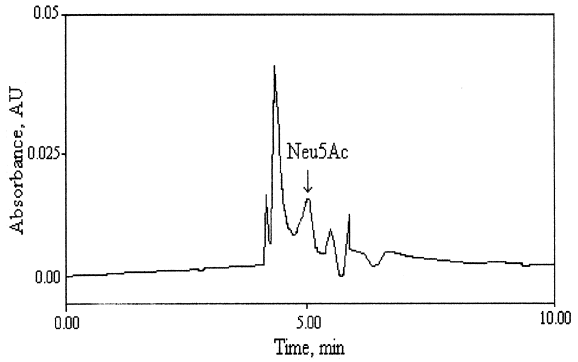

Fig. 2. Typical chromatogram for a hydrolysed standard Neu5Ac aqueous solution, 3.0 m*M*. Mobile phase: TIP 60 m*M*, pH 3.5, flow-rate 0.60 ml min^{-1}, ambient temperature, λ=215 nm.

(n=10) and in sera is 4.984±0.039 min (n=30), therefore retention of Neu5Ac is not affected by the serum matrix. Fig. 2 shows a chromatogram for a hydrolysed standard Neu5Ac aqueous solution under procedure 2.4. Typical chromatograms obtained with sera from a normal subject and from an ovarian cancer patient are shown in Figs. 3 and 4, respectively.

Neu5Ac concentration in the samples was calculated by comparing the height of the peak obtained from the samples with that obtained using a standard Neu5Ac aqueous solution of 3.0 m*M*. Calculations based on peak area have the same measurement reliability. In addition, to compare the results, calibration curve for Neu5Ac in water (range 0.41–4.0 m*M*, N=5×2) showed a linear relation between the concentration of the compound and the detector

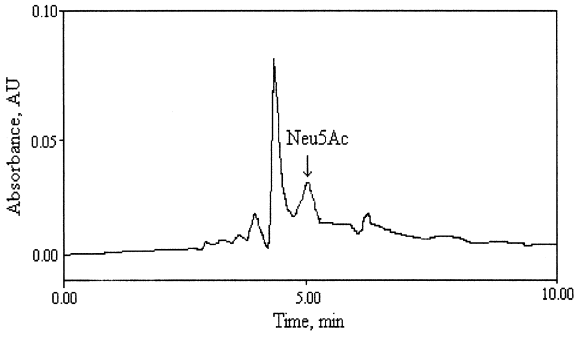

Fig. 3. Typical chromatogram of total Neu5Ac, 2.8 m*M*, in normal serum. Mobile phase: TIP 60 m*M*, pH 3.5, flow-rate 0.60 ml min^{-1}, ambient temperature, λ=215 nm.

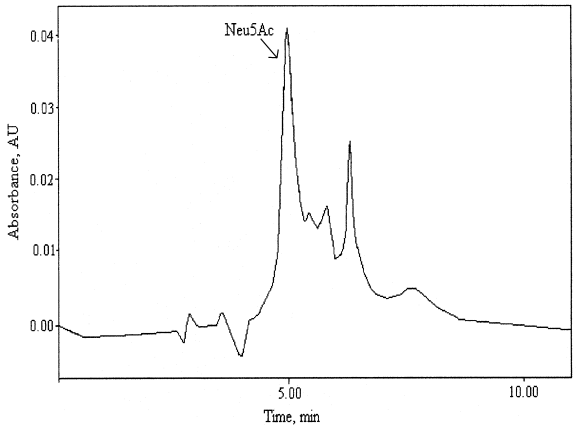

Fig. 4. Typical chromatogram of total Neu5Ac, 3.5 m*M*, in pathological serum from patient with ovarian cancer. Chromatographic conditions as Fig. 3.

response (peak height). The slope of the fitted line was 0.00194±0.00001, the y-intercept was −0.000171±0.000030 (r=0.999) and the mean analytical error was found to be 0.8%. The plot was constant for three months with a deviation of the slope equal to 5.1%. Eventually, reliable results were obtained using quantitation procedure based on one standard aqueous solution.

Repeated determinations (n=4) indicated that coefficient of variation of Neu5Ac was 2.7% at 3.0 m*M* level. Detection limit, which is defined as (3 $s_{y/x}$/slope of calibration plot) and limit of quantification, which is defined as (10 $s_{y/x}$/slope of calibration curve), were found to be 60 n*M* and 200 n*M*, respectively. Recovery, which was examined in the range 1.0–5.5 m*M* by taking a serum sample in which Neu5Ac was present at a concentration of 1.02 m*M* and to aliquots of that adding Neu5Ac at concentrations of 0.64, 1.28, 2.26, 3.40 and 4.60 m*M*, was found to be 98±4% (n=5×2) (Table 1).

The concentrations of total Neu5Ac in human sera from healthy volunteers and from patients with different cancers were determined under the procedure reported in Section 2.4. The same results have also been reported by other methods [29]. As might be expected, the mean value in normal sera (2.3±0.6 m*M*, n=10×2, range 1.36–3.4 m*M*) is significantly different compared to the mean value in pathological sera-including cancer of stomach, colon and ovaries-(3.8±0.7 m*M*, n=15×2, range 2.2–4.8 m*M*) accord-

Table 1
Recovery study of Neu5Ac added to normal serum

Added [Neu5Ac][a] (m*M*)	Found [Neu5Ac][a,b] (m*M*)	Recovery[c] (%)
0	0.051	–
0.032	0.081	94
0.064	0.113	97
0.113	0.167	103
0.170	0.225	102
0.230	0.271	96
		$\bar{x}\pm s=98\pm4$

[a] Concentrations refer to hydrolysed samples, meaning a 20-fold dilution.

[b] The values were calculated using standard Neu5Ac solution 3.0 m*M* diluted 20-fold.

[c] Duplicate runs.

ing to the Mann–Whitney test within a 95% confidence interval [30]. Using the same statistical test, the importance of the differentiation in concentration of Neu5Ac was statistically checked for each kind of cancer. Concentrations of Neu5Ac were found to be significantly increased in sera from stomach cancer patients (3.3 ± 1.1 m*M*, $n=4\times2$), colon cancer patients (3.9 m*M* with $s\ll0.05$ m*M*, $n=3\times2$), cervix cancer patients (4.2 ± 0.7 m*M*, $n=4\times2$) and ovarian cancer patients (4.02 ± 0.04 m*M*, $n=4\times2$). On the other hand, concentrations in sera from breast cancer patients (2.0 ± 0.9 m*M*, $n=4\times2$) were almost identical with those in normal sera.

3.2. Determination of Neu5Ac and Neu5Ac2en in urine

It has been reported that human urine contain free Neu5Ac, ~40 μ*M*, and bound Neu5Ac at the level of 250 μ*M* [31]. Neu5Ac2en was found for the first time in nature from a sialuric patient's urine and its normal urine value is about 50 μ*M* [32,33]. Since Neu5Ac2en is lacking the glycosidic hydroxyl group, it occurs in a free form in biological materials, so that the hydrolysis step is not necessary.

In the present work, chosen detection wavelength for Neu5Ac2en is 240 nm ($\epsilon_{240\ nm}=5600\ M^{-1}\ cm^{-1}$), which differs from the common wavelength for sialic acids, 215 nm, due to their carboxylic group [21]. The reason for choosing 240 nm is to avoid interference from urine substances that co-

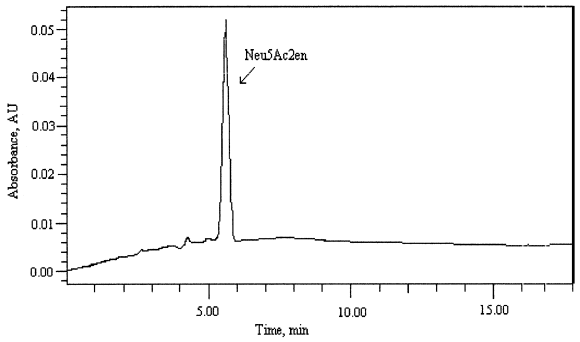

Fig. 5. Typical chromatogram of standard Neu5Ac2en aqueous solution, 80 μ*M*. Mobile phase: TIP 60 m*M*, pH 3.5, flow-rate 0.60 ml min^{-1}, ambient temperature, $\lambda=240$ nm.

elute with Neu5Ac2en, resulting in large positive errors.

The peaks of Neu5Ac and Neu5Ac2en were identified on the basis of the retention time compared with those of the standard compounds, after co-chromatography of the standards and urine. Retention time of Neu5Ac in urine is 4.652 ± 0.021 min ($n=9$). Retention time in standard Neu5Ac2en aqueous solutions (Fig. 5) is 5.583 min with $s\ll0.05$ ($n=8$) and in urine is 5.656 ± 0.098 min ($n=10$). A typical chromatogram obtained for Neu5Ac2en in urine from a normal subject is shown in Fig. 6. Observed peaks with retention time 3.40 and 4.30 min may be attributed to SO_4^{2-} and Cl^-. Identification was carried out after addition of the two substances to urine samples, resulting in the increase of the peak height.

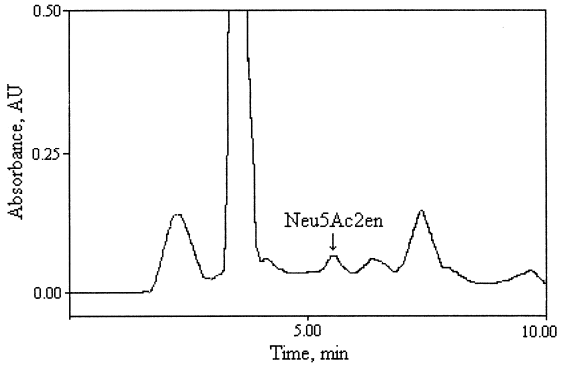

Fig. 6. Typical chromatogram of Neu5Ac2en, 42 μ*M*, in normal urine. Mobile phase: TIP 60 m*M*, pH 3.5, flow-rate 0.60 ml min^{-1}, ambient temperature, $\lambda=240$ nm.

Neu5Ac and Neu5Ac2en concentrations in the samples were calculated by comparing the height of the peaks obtained from the samples with those obtained using standard Neu5Ac and Neu5Ac2en aqueous solutions of 200 and 60 μ*M*, respectively. Calibration plot for Neu5Ac2en in water ranges from 0.040 m*M* to 0.400 m*M* ($n=5\times2$). The slope of the fitted line was 0.555 ± 0.003, the *y*-intercept was 0.00162 ± 0.00070 ($r=0.9999$) and the mean analytical error was found to be 0.90%.

Repeated determinations ($n=5$) indicated that the coefficient of variation of determination of Neu5Ac and Neu5Ac2en in urine were 2.5% at 43 μ*M* and 2.2% at 4.7 μ*M*, respectively. The detection limit and the quantification limit for Neu5Ac2en were found to be 20 n*M* and 66 n*M*, respectively. Recoveries, in the range 130–735 μ*M* for Neu5Ac and 50–135 μ*M* for Neu5Ac2en, were found to be $92\pm2\%$ ($n=5$) and $95\pm3\%$ ($n=4$), respectively (Table 2).

Total Neu5Ac and Neu5Ac2en contents in urine from ten healthy volunteers, five males and five females, could be more conventionally expressed as follows: total Neu5Ac ranges from 43 to 435 μ*M* with median value 303 μ*M*, $n=10\times2$, and Neu5Ac2en ranges from 4.7 to 78 μ*M* with median value 32 μ*M*, $n=10\times2$. These results agree well with those reported previously [31,34]. Furthermore, it is noticed that values range widely for both sialic acids, but seem independent of sex.

In addition, free Neu5Ac was determined in urine sample from patient with sialuria, as illustrated in Fig. 7, and was found to be 304 μ*M*. Therefore free sialic acid excretion was increased about 7-fold compared with the value in a normal urine sample, ~40 μ*M*, as reported in the literature [31]. Concentrations of total Neu5Ac and Neu5Ac2en were found to be 382 and 16 μ*M*, respectively.

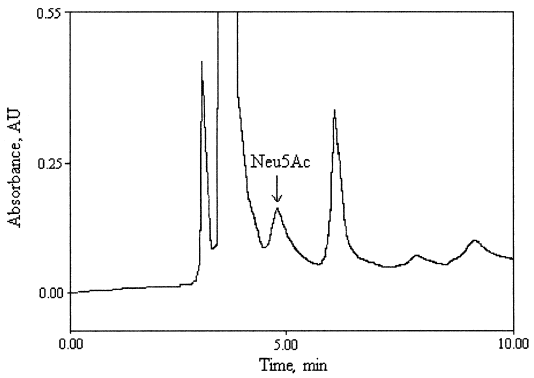

Fig. 7. Typical chromatogram of free Neu5Ac, 304 μ*M*, in urine from patient with sialuria. Chromatographic conditions as Fig. 3.

3.3. Determination of total Neu5Ac in saliva

A typical chromatogram obtained in saliva from a normal subject is shown in Fig. 8. Retention time of Neu5Ac in saliva is 4.761 ± 0.031 min ($n=9$).

Neu5Ac concentration in the samples was calculated by comparing the height of the peak obtained

Table 2
Recovery studies of Neu5Ac and Neu5Ac2en added to normal 24-h urine sample

Added (μ*M*)		Found (μ*M*)		Recovery[d] (%)	
[Neu5Ac][a]	[Neu5Ac2en]	[Neu5Ac][a,b]	[Neu5Ac2en][c]	Neu5Ac	Neu5Ac2en
0	0	6.47	40.5	–	–
2.41	17.1	8.64	56.1	90	91
4.42	42.2	10.4	81.5	89	97
6.14	74.0	12.3	111	95	95
18.9	96	23.7	134	91	97
32.5		36.7		93	
				$\bar{x}\pm s=92\pm2$	$\bar{x}\pm s=95\pm3$

[a] Concentrations refer to hydrolysed samples, meaning a 20-fold dilution.
[b] The values were calculated using standard Neu5Ac solution 202 μ*M* diluted 20-fold.
[c] The values were calculated using standard Neu5Ac2en solution 128 μ*M*.
[d] Duplicate runs.

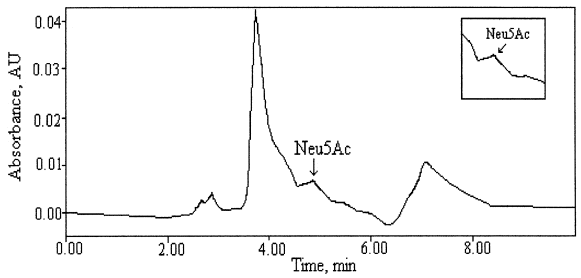

Fig. 8. Typical chromatogram of total Neu5Ac, 456 m*M*, in normal saliva. Chromatographic conditions as Fig. 3.

from the samples with that obtained using a standard Neu5Ac aqueous solution of 601 μ*M*.

Coefficient of variation of the determination of Neu5Ac in saliva was 3.6% at 276 μ*M* level ($n=6$). To saliva sample various amounts of Neu5Ac ($n=3\times2$) were exogenously added to test the analytical recovery for the range 183–861 μ*M*. The experimental results showed that recovery was 96±5%.

Saliva samples from ten healthy adult subjects, seven males and three females, were collected to determine total Neu5Ac. Concentration range is 178–456 μ*M*, with median value 276 μ*M*, $n=10\times2$. Increased Neu5Ac in four saliva samples-Neu5Ac concentrations greater than 375 μ*M*-may be attributed to the fact that these samples were provided by smokers and it is reported that the smoking habit increases Neu5Ac concentration [35].

4. Conclusions

The aim of the present study was to analyze and evaluate quantitatively sialic acids present in biological fluids, using, for the first time, a new reversed-phase ion-pair liquid chromatographic method. This method is rapid, simple and accurate and is successfully applied for the determination of Neu5Ac to normal and pathological sera from patients with different types of cancer, normal urine and urine from patient with sialuria and normal saliva. Neu5Ac2en has also been determined in normal urine. Results are in agreement with the literature.

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References

[1] R. Schauer, Methods Enzymol. 50 (1978) 64.
[2] R. Schauer, Angew. Chem. Int. Edit. 12 (1973) 127.
[3] P.J. Waters, E. Lewry, C.A. Pennock, Ann. Clin. Biochem. 29 (1992) 625.
[4] S. Kakari, G. Michaelidis, S.T. Besbeas, A.S. Ferderigos, M. Tsitsoura, E. Poulaki, Anticancer Res. 4 (1984) 3.
[5] S. Kakari, G. Avgoustatos, A.S. Ferderigos, E. Poulaki, P. Sakka, A. Karamplianis, E. Konstadinidis, G. Constantopoulos, Anticancer Res. 4 (1984) 313.
[6] E. Stringou, K. Chondros, J. Kouvaris J, S. Kakari, K. Papavassiliou, Anticancer Res. 12 (1992) 251.
[7] E. Moran, R. O'Kennedy, R. Douglas Thornes, Biochem. Soc. Trans. 13 (1985) 937.
[8] M. Renlund, M.A. Chester, A. Lundblad, J. Parkkinen, T. Krusius, Eur. J. Biochem. 130 (1983) 39.
[9] J.A. Lowden, J.S. O'Brien, Am. J. Hum. Genet. 31 (1979) 1.
[10] L. Warren, J. Biol. Chem. 234 (1959) 1971.
[11] M.E. Spyridaki, P.A. Siskos, Anal. Chim. Acta 327 (1996) 277.
[12] K.S. Hammond, D.S. Papermaster, Anal. Biochem. 74 (1976) 292.
[13] S. Teshima, K. Tamai, Y. Hayashi, S. Emi, Clin. Chem. 34 (1988) 2291.
[14] J. Casals-Stenzel, H.P. Buscher, R. Schauer, Anal. Biochem. 65 (1975) 507.
[15] Y. Sugawara, M. Iwamori, J. Portoukalian, Y. Nagai, Anal. Biochem. 132 (1983) 147.
[16] A.E. Manzi, S. Diaz, A. Varki, Anal. Biochem. 188 (1990) 20.
[17] H.K. Ogawa, Y. Takeuchi, H. Uchibori, I. Matsumoto, N. Seno, J. Chromatogr. 612 (1993) 145.
[18] K.R. Anumula, Anal. Biochem. 230 (1995) 24.
[19] J. Xia, P.J. Gilmer, J. Chromatogr. A 676 (1994) 203.
[20] M. Fritsch, C.C. Geilen, W. Reutter, J. Chromatogr. A 727 (1996) 223.
[21] M.-E. Spyridaki, P.A. Siskos, J. Chromatogr. A 831 (1999) 179.
[22] D.S. Young, E.W. Bernes Jr., in: C.A. Burtis, E.R. Ashwood (Eds.), Tietz Textbook of Clinical Chemistry, 2nd ed, W.B. Saunders Company, Philadelphia, 1994, Ch. 2, p. 74.

[23] M.C. McMaster, HPLC. A Practical User's Guide, VCH, New York, 1994.
[24] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Biochem. 164 (1987) 138.
[25] R. Schauer, Adv. Carbohydr. Chem. Biochem. 40 (1982) 131.
[26] A. Varki, S. Diaz, Anal. Biochem. 137 (1984) 236.
[27] A.K. Shukla, R. Schauer, J. Chromatogr. 244 (1982) 81.
[28] S. Hara, M. Yamaguchi, Y. Takemori, M. Nakamura, J. Chromatogr. 377 (1986) 111.
[29] M. Crook, Clin. Biochem. 26 (1993) 31.
[30] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, in: Ellis Horwood, New York, 1986, Ch. 5, p. 129.
[31] J. Romppanen, I. Mononen, Clin. Chem. 41 (1995) 544.
[32] J.P. Kamerling, J.F.G. Vliegenthart, R. Schauer, G. Strecker, J. Montreuil, Eur. J. Biochem. 56 (1975) 253.
[33] J. Haverkamp, R. Schauer, M. Wember, J.-P. Farriaux, J.P. Kamerling, C. Versluis, J.F.G. Vliegenthart, Hoppe-Seyler's Z. Physiol. Chem. 357 (1976) 1699.
[34] K. Hayakawa, C. De Felice, T. Watanabe, T. Tanaka, K. Iinuma, K. Nihei, S. Higuchi, T. Ezoe, I. Hibi, K. Kurosawa, J. Chromatogr. 620 (1993) 25.
[35] P.S. Patel, G.N. Raval, R.R. Rawal, G.H. Patel, D.B. Balar, P.M. Shah, D.D. Patel, Cancer Lett. 82 (1994) 129.