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Journal of Chromatography A, 831 (1999) 179–189

JOURNAL OF CHROMATOGRAPHY A

Development of a new direct reversed-phase ion-pair high-performance liquid chromatographic method for the separation and determination of sialic acids

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Received 2 June 1998; received in revised form 5 November 1998; accepted 6 November 1998

Abstract

A new reversed-phase ion-pair high-performance liquid chromatographic method has been developed for the determination of the following five selected sialic acids: *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, cytidine 5′-mono-phospho-*N*-acetylneuraminic acid and *N*-acetyl-9-*O*-acetylneuraminic acid. Three ion-pair reagents, their concentration, the pH of the mobile phase, flow-rate and the presence of organic modifiers were studied, with a C_{18} column, at 215 nm and at room temperature. For the first time, triisopropanolamine, triethanolamine and tetraoctylammonium bromide were used as ion-pair reagents for the separation of sialic acids. A mobile phase of an aqueous solution of triisopropanolamine 60 m*M*, pH 3.5 at a flow-rate 0.60 ml/min, was chosen as optimum using a chromatographic response function. The developed method is direct, precise with mean relative standard deviation of 2.0% and accurate, with mean analytical error of 5.0%, sensitive with detection limit at the n*M* level, specific, time analysis is approximately 20 min and inexpensive, considering that triisopropanolamine is a low cost reagent and organic solvents are not used for the separation.

Keywords: Ion-pairing reagents; Sialic acids; Neuraminic acids; Sugars

1. Introduction

Sialic acids are a family of 9-carbon carboxylated 2-keto sugars usually found as the terminal non-reducing sugars of glycoproteins and gangliosides. 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). One modification is the substitution of one of the hydrogens of *N*-acetyl group by a hydroxyl group, giving rise to *N*-glycolylneuraminic acid, Neu5Gc. Most of other sialic acids arise from *O*-substitution of one or more of the hydroxyl groups of Neu5Ac with acetyl, methyl, lactyl or sulfate groups. Unsaturated and dehydro forms of sialic acids have also been reported, like 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, Neu5Ac2en [1]. 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 different types of cancer [2,3]. On the other hand, increased urinary levels of free or conjugated

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0021-9673/99/$ – see front matter
PII: S0021-9673(98)00948-0

Neu5Ac were found in lysosomal storage disorders [4,5].

Many techniques for determining sialic acids, including spectrophotometry [6], spectrofluorimetry [7], enzymology [8], gas chromatography (GC) [9] and high-performance liquid chromatography (HPLC) with spectrophotometric [10], fluorimetric [11] and amperometric [12] detection have been described. In addition, many ion-exchange liquid chromatographic methods [13,14] have been performed, due to the ionic character of sialic acids. During the last 15 years, application of liquid chromatographic methods gradually increased compared to other methods [15]. However, to the best of our knowledge, no reports have been appeared for ion-pair liquid chromatography (IP-LC) for the separation of sialic acids.

In this report, we have developed a new reversed-phase ion-pair HPLC method for the separation and determination of five selected sialic acids: Neu5Ac, Neu5Gc, Neu5Ac2en, cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA) and *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2) (Fig. 1). A systematic study of the parameters that influence the selectivity and the retention of sialic acids on a C_{18} reversed-phase column at 215 nm and at room temperature, is presented. Optimisation of the method was achieved using the chromatographic response function, CRF. Tertiary amines triisopropanolamine (TIP) and triethanolamine (TEA) and the salt of quaternary amine tetraoctylammonium bromide (TOA·Br) were studied as ion-pair reagents. The use of the above substances as ion-pair reagents has not been reported to date.

2. Experimental

2.1. Materials

Deionized water was passed through a Millipore system (>18.2 MΩ/cm (25°C), TOC<3 ppb). Acetonitrile and methanol were HPLC grade. TIP, M_r 191.27, 97% and TOA·Br, M_r 546.8, 98% were purchased from Fluka and TEA, M_r 149.19, >99% from Ferak. Neu5Ac, M_r 309.3 98%, Neu5Gc, M_r 325.3 90%, Neu5,9Ac_2, M_r 351.3 95%, Neu5Ac2en, M_r 291.3 95% and CMP-NANA, M_r 614.5 93%, were purchased from Sigma. Orthophosphoric acid, purity 85%, was supplied by Merck.

2.2. Instrumentation

Double beam spectrophotometer Hitachi U2000 was used for taking UV spectra.

The HPLC system consisted of a model Waters 600 Controller, a solvent delivery system, a syringe-loading sample injector valve (20-μl loop) and a Waters 486 spectrophotometric detector fitted with a 8-μl flow cell was used. Reversed-phase columns LiChrospher RP-18 (250×4 mm I.D., particle size, 10 or 5 μm) were purchased from Waters Associates. Column temperature was ambient. Data processing was carried out the using software MILLENIUM 2.10.

The dead volume was determined by injection of mobile phase, TIP 120 m*M*, pH 3.5 and was found to be $t_o = 1.200 \pm 0.002$ min ($n = 3$) at a flow-rate of 1.00 ml/min.

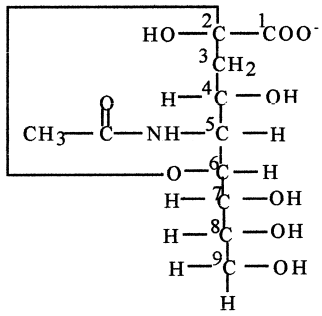
2.3. Procedure

Before measurements, it is imperative to equilibrate the column with the mobile phase, which has been already filtered with Millipore apparatus through HVLP 0.45-μm filters. Mobile phase degassing was performed automatically, on line, under stream of He. Chromatographic settings (composition of mobile phase, flow-rate, purge, wavelength, time analysis) were adjusted using MILLENIUM 2.10. Sialic acids standards aqueous solutions (20-μl) were injected into HPLC system. All standard solutions were filtered through HVLP 0.45-μm Millipore filters. Data acquisition and data analysis were performed using MILLENIUM 2.10.

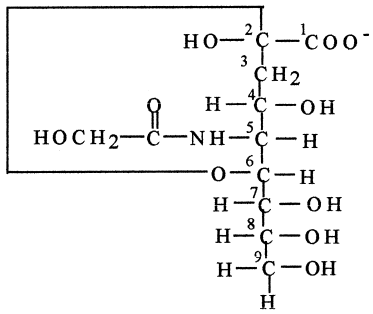
The column can be used for more than 1000 injections with only a small decrease in the theoretical plate number if it is washed with water–methanol (1:1, v/v) at a flow-rate of 1.0 ml/min for 30 min every day after analyses have been performed [16].

3. Results and discussion

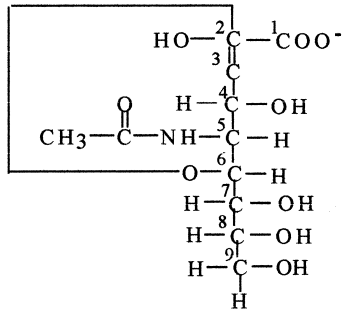
Before considering IP-LC, initial experiments were carried out using reversed-phase liquid chroma-



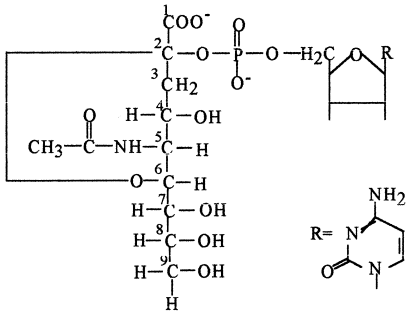
(1) Neu5Ac



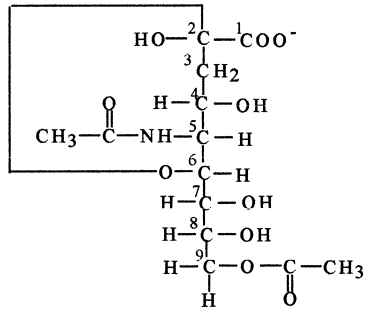
(2) Neu5Gc



(3) Neu5Ac2en



(4) CMP-NANA



(5) Neu5,9Ac$_2$

Fig. 1. Chemical structures of sialic acids. (1) Neu5Ac, (2) Neu5Gc, (3) Neu5Ac2en, (4) CMP-NANA and (5) Neu5,9Ac$_2$.

tography (RPLC) without ion pairing. However, RPLC method development was unable to achieve an adequate separation due to very poor band spacing and it is known that IP-LC provides an additional selectivity option. The use of tertiary and quaternary amines to the successful separation of carboxylic

acids [17] and anionic dyes [18] as well as the fact that sialic acids are strong acids with $pK_a \approx 2.5$ [19], lead to the application of IP-LC for the separation of sialic acids.

3.1. Choice of sialic acids

From a total of about 25 sialic acids that are distributed in nature, Neu5Ac, Neu5Gc, Neu5Ac2en, CMP-NANA and $Neu5,9Ac_2$ were chosen in this study, due to their biological and diagnostic importance in man [20].

Neu5Ac is a physiological component of serum, urine, saliva, milk, amniotic and cerebrospinal fluid and consequently its determination is useful in pathological situations, such as cancer and storage lysosomal disorders. Neu5Gc is not detected normally in biological fluids in man, except in malignant neoplasia, such as melanoma and endometrium cancer, where its concentration is significantly increased. Neu5Ac2en is found in urine and in traces in saliva and serum. CMP-NANA is an important intermediate compound of sialic acids metabolism and has been isolated from liver and brain tissues, but is not found in plasma due to the fact that it does not penetrate cell membrane. Its determination is difficult and a clean-up procedure is necessary before quantitation. $Neu5,9Ac_2$ is detected in tissues and in traces in different biological fluids. It is a specific receptor of virus influenza C, but it prevents binding of viruses influenza A and B [1,20]. The mixture of the above sialic acids has not been successfully separated to date.

3.2. Choice of ion-pair reagent for the separation of sialic acids

The nature of ion-pair reagent is crucial to the efficiency of the separation of different mixtures with IP-LC. Usually, the objective in selecting a particular ion-pair reagent is to be able to achieve a significant column uptake of the reagent or of the ion pairs, for a reasonable reagent concentration [21].

In the case of sialic acids, the ion-pair reagent has to be a rather lipophilic compound positively charged, such as the protonated tertiary or quaternary amines. Firstly, TIP, which has three short carbon chains of three carbon atoms, was used. Every chain possesses a hydroxyl group, which contributes to the nature and number of bonds that influence the retention of the compounds to the column. In addition, TEA, with a methylene group less than TIP, was also tested. Finally, TOA·Br, which is more hydrophobic than TIP and insoluble in water, due to the four carbon chains of eight carbon atoms, was studied. To the best of our knowledge, none of the above compounds has been used as ion-pair reagent.

3.3. Choice of wavelength

All standard sialic acids aqueous solutions absorb in the region 205–215 nm, due to the carboxylic group, and Neu5Ac2en and CMP-NANA absorb, additionally, at 240 and 275 nm.

UV spectra of sialic acids in: (i) TIP solution 55 m*M*, pH 3.5, (ii) TEA solution 0.60 m*M*, pH 5.0 and (iii) TOA·Br 0.72 m*M* pH 6.0 in MeOH 35%, were taken and compared with the spectra in water. The pH was adjusted with concentrated H_3PO_4. The pattern of the spectra was unchanged in all the above cases, so the absorbance of the ion pairs is due to sialic acids. A wavelength of 215 nm was chosen for detection of the mixture of sialic acids, because at lower wavelengths, absorbance tends to increase sharply causing interference in the case of organic solvents, such as methanol.

The apparent molar absorptivities, $\epsilon_{215\sigma nm}$, for sialic acids in aqueous and TIP solutions are given in Table 1. A decrease in ϵ_{215nm} values in TIP solutions compared to aqueous solutions may be attributed to the partial cover of carboxylic group of sialic acids molecules from TIP, which is present in large excess.

Table 1
Molar absorptivities, ϵ_{215nm}, of sialic acids in water solutions and in TIP aqueous solutions, 55 m*M*, pH 3.5

Sialic acids	ϵ_{215nm} (H_2O) (M^{-1} cm^{-1})	ϵ_{215nm} (TIP) (M^{-1} cm^{-1})
Neu5Ac	860	525
Neu5Gc	1390	470
Neu5Ac2en	4200	3300
CMP-NANA	13 625	3545
$Neu5,9Ac_2$	1160	125

Sialic acids in TEA and TOA·Br solutions gave ϵ_{215nm} values of the same magnitude as TIP.

3.4. Study of triisopropanolamine as ion-pair reagent

The ion-pair reagent concentration was usually varied from 5.0 to 100 m*M*. Therefore, an excess of ion-pair reagent over the analyte was used in order to create a larger ion-pairing effect. This approach provides a wide range of separation selectivity, thereby improving chances for a successful separation. At concentrations <5.0 m*M*, small variations in ion-pair reagent concentration may cause large changes to the system efficiency. On the contrary, concentrations over 100 m*M*, usually show solubility problems [22]. The pH of the mobile phase was chosen so that both sialic acids and the counterion were completely ionised. The best results are obtained when pH value differs at least one unit from the pK_a of the compounds. When the pH and the ion-pair reagent concentration are varied simultaneously, considerable control is achievable over both the retention range and the band spacing [23].

Taking the above principles into consideration, a concentration range of 5.0–90 m*M* and a pH value of 2.0–6.0 were studied in TIP aqueous solutions. Phosphate buffer was used for pH adjustment.

Capacity factors, *k*, in different compositions of mobile phase, were found to be 0.5–2.5 for Neu5Ac, Neu5Gc, Neu5Ac2en and CMP-NANA and 4.0–10.0 for Neu5,9Ac_2 (Table 2). The difference in capacity factors for Neu5,9Ac_2 may be attribute to the acetyl group of C-9 in the external carbon chain of the molecule, resulting in further attraction of Neu5,9Ac_2 to the stationary phase.

For each of eight TIP concentrations, a mean increase of 41% in *k* values is noticed, when the pH is increased from 2.0 to 6.0, for the five sialic acids. On the contrary, for each of the five pH values, the mean variation in *k*, when the concentration is increased from 5.0 to 10 m*M*, is +30% and from 10 to 90 m*M* is only +12%. Fig. 2 shows the dependence of capacity factor on the TIP concentration and the pH of the mobile phase. Therefore, we can conclude that: (a) pH is a more significant factor than TIP concentration and (b) according Eq. (1) [17], the relatively small dependence of *k* on the ion-pair reagent concentration, means that either absorption of TIP to stationary phase or formation of ion pairs in mobile phase is negligible. Considering that TIP possesses three small carbon chains, one can suppose that binding of TIP in stationary phase is negligible. Eq. (1) is expressed as follows:

$$k = (k_o + B[\text{TIP}])/(1 + P[\text{TIP}]) \quad (1)$$

where k_o is the capacity factor of sialic acids in absence of ion-pair reagent and *P* is either the stability constant of the complex sialic acid–TIP or the equilibrium constant for the binding of TIP to the stationary phase. The physical meaning of factor *B* depends on the particular mechanism which governs sialic acid retention in the presence of the ion-pair reagent.

3.5. Triethanolamine as ion-pair reagent

Once the separation with TIP had been successfully achieved, a similar approach was used for TEA. In this case, we studied concentration range from 5.0 to 60 m*M* and pH value from 2.5 to 5.5, adjusted with concentrated H_3PO_4. Results can be generalised as follows: considerable variations in *k* values are noticed at 5.0 m*M*, whereas at higher concentrations, *k* is fairly independent of TEA concentration and pH.

3.6. Tetraoctylammonium bromide as ion-pair reagent

TOA·Br solutions were prepared in organic solvent, such as acetonitrile or methanol, because of its low solubility in aqueous solutions. TOA·Br concentration ranged from 0.100 to 25 m*M*. The upper limit of the concentration is limited by solubility problems. Apparent pH value was studied in the region from 3.0 to 7.4, adjusted with concentrated H_3PO_4.

Sialic acids were not separated under any of the above experimental conditions, even replacement of the column with a new one with 5 μm particle diameter, did not improve results. Therefore use of TOA·Br is not recommended for the following reasons: first, addition of organic solvent to the mobile phase (acetonitrile, methanol) causes the

Table 2
Capacity factor, k, values for five sialic acids, for different TIP concentrations and pH values of mobile phase, flow-rate 1.00 ml/min, $t_0 = 1.200 \pm 0.002$ min, $n = 3$

Mobile phase		Capacity factor, k[a]				
[TIP], m*M*	pH	Neu5Ac 3.0 m*M*	Neu5Gc 1.00 m*M*	Neu5Ac2en 0.41 m*M*	CMP-NANA 0.060 m*M*	Neu5,9Ac$_2$ 0.50 m*M*
5.0	2.0	1.11	0.45	1.35	1.68	3.89
	2.5	1.10	0.59	1.38	1.71	4.02
	3.0	1.24	0.63	1.51	1.93	4.23
	3.5	1.34	0.76	1.65	2.07	5.54
	6.0	1.45	0.90	1.74	2.07	5.78
10	2.0	1.14	0.79	1.56	1.94	5.12
	2.5	1.11	0.90	1.65	2.05	5.89
	3.0	1.49	0.99	1.75	2.17	7.01
	3.5	1.53	1.12	1.78	2.11	7.75
	6.0	1.74	1.32	1.87	2.22	9.44
20	2.0	1.18	1.01	1.57	1.95	5.40
	2.5	1.12	1.02	1.68	2.10	6.21
	3.0	1.50	1.26	1.76	2.26	7.30
	3.5	1.56	1.36	1.81	2.14	7.86
	6.0	1.76	1.46	1.90	2.28	9.47
40	2.0	1.22	1.03	1.59	1.96	5.65
	2.5	1.15	1.14	1.70	2.10	6.76
	3.0	1.51	1.29	1.76	2.26	7.63
	3.5	1.58	1.34	1.83	2.21	7.95
	6.0	1.79	1.57	1.93	2.31	9.48
50	2.0	1.24	1.04	1.58	1.97	5.74
	2.5	1.16	1.15	1.71	2.10	6.93
	3.0	1.53	1.28	1.80	2.28	7.74
	3.5	1.59	1.32	1.82	2.24	8.05
	6.0	1.80	1.54	1.94	2.35	9.49
60	2.0	1.25	1.06	1.60	2.02	5.95
	2.5	1.17	1.16	1.70	2.12	7.32
	3.0	1.54	1.31	1.82	2.32	7.88
	3.5	1.62	1.32	1.87	2.24	8.22
	6.0	1.83	1.52	1.96	2.36	9.52
70	2.0	1.26	1.06	1.61	2.10	6.10
	2.5	1.17	1.14	1.72	2.12	7.60
	3.0	1.56	1.29	1.82	2.32	8.17
	3.5	1.65	1.32	1.90	2.25	8.27
	6.0	1.86	1.51	1.98	2.38	9.55
90	2.0	1.28	1.10	1.62	2.12	6.21
	2.5	1.18	1.15	1.74	2.15	7.65
	3.0	1.58	1.32	1.82	2.33	8.25
	3.5	1.66	1.34	1.92	2.25	8.26
	6.0	1.88	1.50	2.01	2.38	9.58

[a] Duplicate runs.

disability of ion pairs to be retained to the column, resulting in small retention times up to 1 min. TOA is a bulk molecule and its strong lipophilic character does not differentiate ion pairs of sialic acids, consequently all ion pairs show the same retention behaviour.

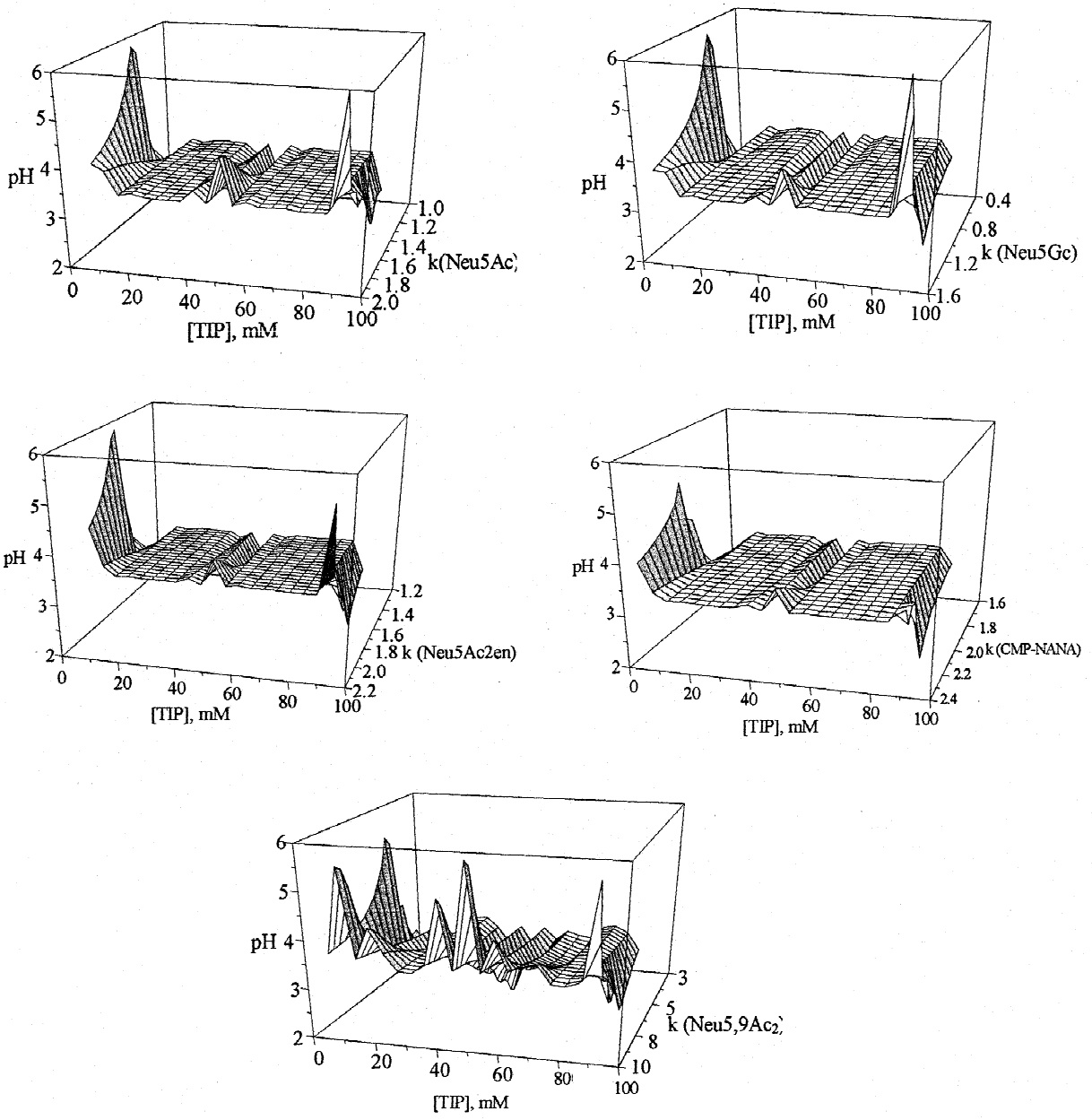


Fig. 2. Three-dimensional charts showing the dependence of capacity factor, *k*, of Neu5Ac, Neu5Gc, Neu5Ac2en, CMP-NANA and Neu5,9Ac_2 on TIP concentration and pH value of mobile phase. Results from Table 2.

3.7. Optimisation of chromatographic separations

Selectivity can be optimised following a wide range of protocols varying from a trial and error approach to automated computer-aided methods [22]. The present study is better suited for the quantitative prediction of the optimisation of experimental conditions, such as ion-pair reagent concentration and pH, with the use of CRF, which takes into account the simultaneous importance of resolution, *R*, total

number of detectable peaks and the separation time [24]. CRF can be expressed as follows:

$$\mathrm{CRF} = \sum_{i-1}^{n-1} R_i + n^a - b|T_A - t_{R,L}| - c(T_0 - t_{R,1}) \quad (2)$$

where n is the number of peaks observed, T_A the maximum desired time analysis, $t_{R,L}$ the observed retention time for the last detected peak, T_0 the minimum desired retention time for the first detected peak, $t_{R,1}$ the observed retention time for the first detected peak and a, b and c are weighting factors that can be adjusted to change the importance of the various contributions to the CRF. In this application $a=1$, $b=0.5$ and $c=1$. In Table 3, results for CRF calculations for TIP are presented. The optimum TIP concentration and pH of the mobile phase were found to be 60 m*M* and 3.5, respectively, where $\mathrm{CRF}_{max}=13.6$.

Fig. 3 presents a typical chromatogram showing the separation of the mixture of five sialic acids under optimum conditions. Elution of sialic acids was achieved in the following order: Neu5Gc> Neu5Ac>Neu5Ac2en>CMP-NANA>Neu5,9Ac$_2$. Resolution values for the adjacent peaks (1), (2), (3) and (4), which correspond to Neu5Gc, Neu5Ac, Neu5Ac2en and CMP-NANA are 1.21, 1.13 and 1.20, respectively. In such a chromatogram, one peak appears for each analyte and a number of additional peaks which are characteristic of the chromatographic system (system peaks), like the first negative peak in Fig. 3. The number of observed system peaks depends on the complexity of the mobile phase and whether each individual component is coupled to the stationary phase equilibria which affects the instantaneous concentration of the monitored ion [22].

Table 3
Calculated CRF values for different TIP concentrations and pH of mobile phase for the optimization of the separation of five sialic acids mixture, flow-rate 1.00 ml/min

[TIP], m*M*	pH	CRF[a]	[TIP], m*M*	pH	CRF[a]
5.0	2.0	3.94	50	2.0	7.68
	2.5	4.45		2.5	8.61
	3.0	7.40		3.0	12.0
	3.5	9.64		3.5	12.9
	6.0	9.86		6.0	13.0
10	2.0	5.76	60	2.0	8.00
	2.5	6.34		2.5	9.28
	3.0	9.23		3.0	11.6
	3.5	12.3		3.5	13.6
	6.0	12.7		6.0	13.0
20	2.0	6.48	70	2.0	8.08
	2.5	7.24		2.5	9.2
	3.0	10.2		3.0	11.8
	3.5	12.8		3.5	13.2
	6.0	12.8		6.0	13.2
40	2.0	7.03	90	2.0	8.18
	2.5	8.24		2.5	9.18
	3.0	11.5		3.0	11.8
	3.5	12.7		3.5	13.2
	6.0	12.4		6.0	13.6

[a] Duplicate runs.

The results of the optimisation for TEA showed that maximum value of CRF was found to be 24.2 and corresponds to 15 m*M* TEA and pH 4.5. But, under these conditions, CMP-NANA gave two peaks and this fact may complicate its quantitation. So, the optimum conditions were chosen with the CRF value <24.2 and in this case all sialic acids peaks are separated and CMP-NANA gives a single peak. On the basis of the above requirements, optimum CRF value is 20.6 and corresponds to 5.0 m*M* TEA and pH 5.5. The elution order does not change compared to TIP.

When the optimum conditions were repeated after 1 month, the results for TIP were unchanged for the separation and quantitation of sialic acids mixture. Conversely, TEA proved to be an unstable, and was not used for quantitation of sialic acids, therefore TIP is the preferred ion-pair reagent.

3.8. Effect of organic modifiers on separation

The presence of non-aqueous solvents in mobile phase, such as acetate 0.6%, methanol 10% or acetonitrile 10%, did not allow for peak separation and negative peaks complicated the chromatogram. Therefore the use of organic solvents is not recommended.

3.9. Effect of flow-rate on separation

Flow-rate in the range 0.60–1.50 ml/min, for TIP optimum conditions, was studied. Lower values were not studied in order to avoid extensive runs. The upper value was determined from the pressure limits

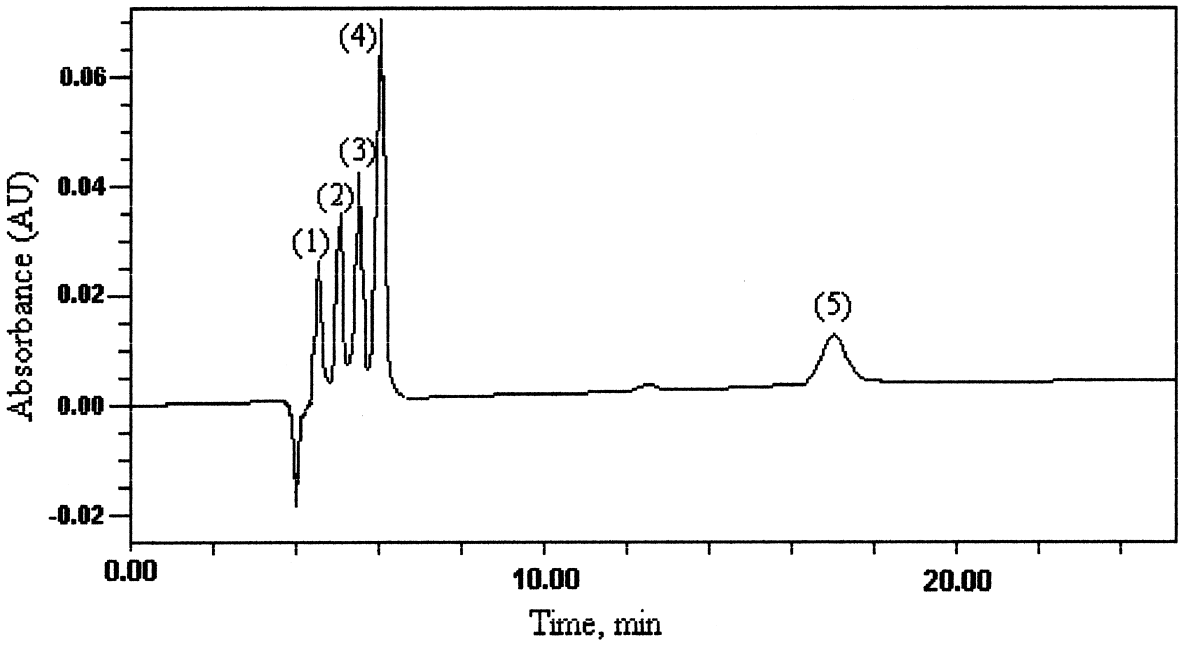


Fig. 3. Typical chromatogram of a mixture of five standard sialic acids under optimum conditions, TIP 60 m*M* and pH 3.5. Peaks: (1) Neu5Gc 0.31 m*M*, t_R 4.533 min, (2) Neu5Ac 0.39 m*M*, t_R 5.050 min, (3) Neu5Ac2en 0.070 m*M*, t_R 5.600 min, (4) CMP-NANA 0.135 m*M*, t_R 6.083 min, (5) Neu5,9Ac_2 0.45 m*M*, t_R 17.233 min.

(3500 p.s.i.) of the column. The optimum value for CRF was found to be 19.5 for flow-rate 0.60 ml/min (Table 4).

3.10. Effect of temperature on retention time

All experiments were carried out at ambient temperature. Measurements of t_R of Neu5Ac, for the period spring to autumn for 20°C temperature variation, showed a deviation of about 1.5% ($t_R \pm s = 5.002 \pm 0.087$ min, $n = 32$) and no significant changes in band spacing. This finding is surprising, because it is rare in IP-LC that a change in temperature does not lead to changes in separation [23]. This behaviour of the system is advantageous for performing measurements at ambient temperature. The explanation can be attributed to the small dependence of equilibrium constants of complexes of ion-pair reagent–sialic acid on temperature.

Table 4
Calculated CRF values for TIP 60 m*M*, pH 3.5 and variable flow-rates of mobile phase for the optimization of the separation of five sialic acids mixture

Flow-rate (ml/min)	CRF[a]
0.60	19.5
0.70	18.4
0.80	18.7
1.00	19.1
1.50	18.1

[a] Duplicate runs.

3.11. Analytical characteristics of the method

Analytical characteristics of sialic acids, in optimised conditions, were determined and summarised in Table 5. Detection limits, which are defined as 3 $s_{y/x}$/slope of calibration curve, are found to be in the nmol range and precision between days, in peak height, does not exceed 4.0%. The within-day retention time of sialic acids shows standard deviation near to zero, due to the great stability of the pump.

The calibration curves for each sialic acid in aqueous solutions are expressed by the following equations:

Table 5
Analytical characteristics for the determination of five standard sialic acids in aqueous solutions in the reversed-phase ion pair chromatographic method, experimental conditions as in Fig. 3

	NGNA	Neu5Ac	Neu5Ac2en	CMP-NANA	Neu5,9Ac$_2$
Mean analytical error (%)	3.0	4.0	0.90	5.0	3.4
Molar absorptivity, ϵ_{215nm} (M^{-1} cm^{-1})	490	525	3300	3550	125
Response factor RF (AU/μmol)	3.95	4.45	30.0	33.5	1.05
Detection limit,[a] (nM)	82	41	12	4.3	130
Quantitation limit,[b] (nM)	273	136	41	14	432
Minimal detectable amount,[c] (pmol)	1.64	0.820	0.240	0.086	2.60
Precision (%)					
(within day, $n=6$)	1.0	1.0	1.0	1.0	2.0
(between days, $n=6$)	2.7	3.2	2.5	3.7	3.7

[a] 3 $s_{y/x}$/slope.
[b] 10 $s_{y/x}$/slope.
[c] Detection limit×20 μl.

Neu5Ac: $y = (0.084 \pm 0.001)x + (0.00050 \pm 0.00007)$,

range: 0.050 − 0.50 mM, $n = 4 \times 2$

Neu5Gc: $y = (0.083 \pm 0.002)x - (0.00031 \pm 0.00002)$,

range: 0.050 − 0.50 mM, $n = 4 \times 2$

Neu5Ac2en: $y = (0.555 \pm 0.004)x + (0.0016 \pm 0.0007)$,

range: 0.040 − 0.40 mM, $n = 4 \times 2$

CMP − NANA: $y = (0.58 \pm 0.01)x + (0.00073 \pm 0.00004)$,

range: 0.0050 − 0.050 mM, $n = 4 \times 2$

Neu5,9Ac$_2$: $y = (0.0202 \pm 0.0001)x + (0.00012 \pm 0.00007)$,

range: 0.100 − 1.00 mM, $n = 4 \times 2$

where y = peak height in absorbance units (AU) and x = sialic acid concentration (mM).

Calibration curves were performed with aqueous standard sialic acids solutions with a mean analytical error of less than 5.0%. Analytical error was the same with sialic acid solutions in mobile phase. Calibration curves based on peak area have the same measurement reliability.

4. Conclusions

A new direct reversed-phase ion-pair chromatographic method has been developed for the successful separation and determination of a mixture of five selected sialic acids, using TIP as ion-pair reagent, with detection at 215 nm and at room temperature.

Present method shows the following advantages: (a) this mixture of sialic acids is separated for the first time, (b) the separation was achieved with a common column C_{18}, (c) there is no time-consuming step, like derivatisation of sialic acids to less polar compounds, (d) detection is performed with a simple spectrophotometer, (e) it is sensitive, minimum detection amount is in the pmol range, (f) it is specific, (g) time analysis is ≈20 min, (h) is inexpensive for not using organic solvents or expensive chemical reagents.

The presented method is currently used for the determination of sialic acids in biological fluids.

Acknowledgements

M-H. E. S. is grateful to the State Scholarships Foundation for financial support.

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