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Determination of sialic acids in human serum by reversed-phase liquid chromatography with fluorimetric detection

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ABSTRACT

A simple, rapid and highly sensitive reversed-phase liquid chromatographic method has been developed for the determination of sialic acids in human serum. The sialic acids, released by hydrolysis of serum, are converted in borate buffer with malononitrile to highly fluorescent compounds. The reaction mixture is separated isocratically within 5 min using an octadecyl-bonded silica column and a mobile phase of methanol and ammonium acetate buffer (15:85, v/v; pH 5.5). Measurement of the fluorescence intensity of the reaction mixture at 434 nm with irradiation at 357 nm allowed determination of 30–1000 ng/ml of sialic acids with high reproducibility. The limit of detection was 2 ng/ml. Intra-day and inter-day coefficients of variation for assaying 300 ng/ml N-acetylneuraminic acid (NANA) were 1.5% (n = 9) and 2.6% (n = 7), respectively. The recoveries of NANA were 98.5–101.1% for serum. The method has been used for clinical determinations.

INTRODUCTION

Sialic acids, acylated derivatives of neuraminic acids, are important as components of glycoproteins and glycolipids. Sialic acids in human serum reflect the levels of sialoglycoproteins and sialoglycolipids. Increased serum levels of these compounds have been observed in patients with cancer and various other diseases [1–4] and in patients with congenital metabolic disorders [5,6]. Sialic acids present in human serum are mostly N-acetylneuraminic acid (NANA). Thus, an assay for NANA in serum samples may be useful for diagnosis and treatment of these diseases.

Many techniques for determining sialic acids, including spectrophotometric [7,8] and spectrofluorimetric [9] techniques, gas chromatographic methods [10] and high-performance liquid chromatography (HPLC) with ultraviolet [11,12] and amperometric [13] detection, have been described. The most widely used methods are thiobarbituric acid colorimetric and fluorimetric assays using sodium arsenite as a reducing agent. Nevertheless, all inorganic arsenic salts have now been classified as carcinogens and rigid standards for their use have been established [14]. Gas chromatographic methods require a tedious derivatization process before analysis and have limited sensitivities. HPLC with ultraviolet detection does not require derivatization, but the sensitivity is unsatisfactory. An HPLC method with fluorimetric detection using 1,2-diamino-4,5-dimethoxybenzene (DDB), a fluorogenic reagent for α-keto acids, has been developed for the determination of NANA in human serum and urine [15]. The method is sensitive enough to determine NANA in 5 μl of human serum or urine. However, apart from the fact that the reaction of NANA with DDB is time consuming (heated at

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60°C for 2.5 h in the dark), additional peaks also appeared in the chromatogram. This made it inconvenient to estimate the peak-height or -area ratio of the reaction compounds with accuracy.

Honda *et al.* [16] have reported a fluorimetric procedure using malonamide as a fluorogenic reagent for the determination of reducing carbohydrates. Using this finding, we have succesfully developed a sensitive, simple and rapid reversed-phase liquid chromatographic method for the determination of sialic acids in small amounts of serum.

EXPERIMENTAL

Apparatus

The HPLC system used was an HP 1090M liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a Model K501 high-pressure sample injector (20-μl loop; Shanghai Scientific Instruments Factory, Shanghai, China), thermostated column compartment and a 1046A fluorescence detector (Hewlett-Packard) operated at 357 nm excitation and 434 nm emission. Chromatographic separations were carried out on a YWG octadecylsilane (C_{18}) column (200 × 4.6 mm I.D.; particle size 10 μm; Dalian, China) at ambient temperature. Control of the instrument, data storage, evaluation, integration and reporting were performed by an HP Series 300 computer (Hewlett-Packard, Boeblingen, Germany). A Model 071 pH meter (Beckman Instruments, Fullerton, CA, USA) with a pencil combination Beckman electrode was employed for pH measurements in various solutions.

Reagents

HPLC-grade methanol (Linhai Chemicals Factory, Zhejiang, China) and ammonium acetate (Nanjing Chemical Reagent Factory, Jiangsu, China) were used to prepare the mobile phase. NANA (Tokyo Kasai Kogyo, Japan) and malononitrile (Fluka, Buchs, Switzerland) were also used. All chemicals, except where stated otherwise, were of analytical-reagent grade and the water used in this assay was doubly distilled.

Mobile phase

The mobile phase was a methanol–buffer mixture (15:85, v/v). This solution was passed through a 0.45–μm membrane filter (Millipore, Bedford, MA, USA) and was then degassed before use. The buffer was 0.010 *M* ammonium acetate, which was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml of water and the pH was adjusted electrometrically to 5.5 with acetic acid. The mobile phase flow-rate was 1.0 ml/min with a typical back-pressure of 125 bar.

Preparation of solutions

Stock standard solutions (500 μg/ml) were prepared by dissolving 25 mg of NANA in 50 ml of water and storing in a refrigerator. Malononitrile solution (0.8%) was prepared by dissolving 0.8 g of malononitrile in 100 ml of water and storing in a refrigerator. Borate buffer was prepared by dissolving sodium tetraborate in water and the pH was adjusted with hydrochloric acid and sodium hydroxide.

Analytical procedure

A 100-μl aliquot of 0.05 *M* sulphuric acid was added to 3 μl of serum in a 2-ml test-tube and vortex-mixed. The test-tube was tightly closed and heated at 80°C for 30 min to hydrolyse the sample. After cooling rapidly under running water 200 μl of the malononitrile and 1.5 ml of the borate buffer solution (0.15 *M*, pH 9.5) were added to the resulting solution and vortex-mixed again. The mixture was then heated at 80°C for 20 min to develop the fluorescence. After cooling, a 20-μl aliquot of the resulting solution was injected into the chromatograph.

RESULTS AND DISCUSSION

Fluorescence spectra

The fluorescence spectra of the reaction compound of NANA and malononitrile were scanned from 300 to 500 nm. The results showed that the excitation and emission maxima were 357 and 434 nm, respectively.

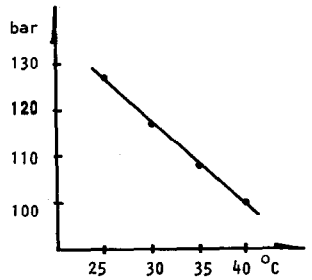

Fig. 1. Dependence of column pressure on oven temperature.

Chromatographic conditions

To achieve the optimum resolution, the separation temperature, the type of mobile phase, its composition, pH, buffer and buffer concentration were all studied systematically. In all experiments dealing with the optimization of liquid chromatographic parameters, the concentration of malononitrile solution was 0.2%. The solution was heated in a boiling water-bath for 15 min.

As shown in Fig. 1, the column pressure depended on the oven temperature, but its effects on the retention and peak area were not obvious.

A 200 × 4.6 mm I.D. octadecylsilane YWG column was chosen, with various proportions of methanol in aqueous ammonium acetate buffer and different pH values as the mobile phase. Fig. 2a shows the dependencies of the retention time of the reaction compound of NANA and malononitrile on the composition and pH of the mobile phase at a given buffer salt concentration, respectively. An increasing methanol concentration reduces the retention, whereas decreasing the pH increases retention. However, the detector response (peak area) changed only slightly with varying acidity or composition of the mobile phase.

A study was made of the effect of the buffer salt concentration in the mobile phase on the chromatographic peaks at a given acidity and composition. The results indicated that the retention time is reduced with a decrease in the buffer salt concentration (Fig. 2b), but no change in peak area was seen. Hence at a given buffer salt concentration the retention of the reaction compound can be easily controlled within a large appropriate range by adjusting the pH or methanol content of the mobile phase.

On the basis of these results the chromatographic parameters recommended together with the 200 × 4.6 mm I.D. YWG column for this procedure were as follows: the concentration of ammonium acetate buffer solution was selected as 0.010 *M* and the pH was adjusted to 5.5 with acetic acid. The ratio of ammonium acetate buffer solution and methanol was selected as 85:15

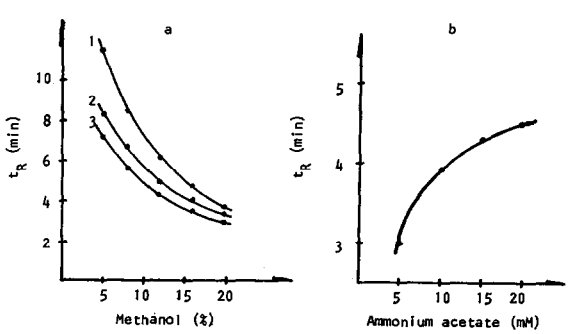

Fig. 2. Optimization of conditions for the mobile phase. (a) Dependence of the retention time of the fluorescent product of NANA on the methanol composition and pH of the mobile phase. 1 = pH 4.5; 2 = pH 5.5; and 3 = pH 6.5. (b) Dependence of the retention time of the fluorescent product on the buffer salt concentration in the mobile phase.

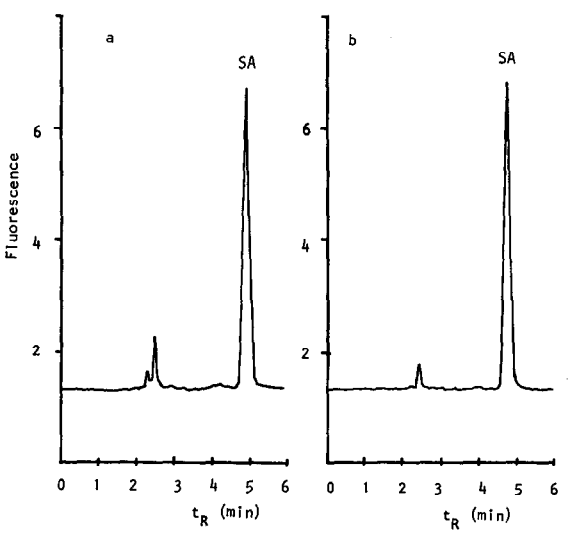

Fig. 3. Chromatograms of the fluorescent product of NANA in serum (a) and a standard solutions containing 0.60 μg/ml NANA (b).

(v/v), delivered at a flow-rate of 1.0 ml/min at ambient temperature. Fig. 3 shows the chromatograms of the standard and serum samples. Under the described chromatographic conditions, the reaction compound of NANA and malononitrile had a retention time of approximately 5 min.

Optimization of reaction of sialic acids with malononitrile

To obtain the optimum conditions for the reaction of sialic acids with malononitrile, the reagent concentration, buffer concentration and pH, reaction time and temperature and acid hydrolysis time have been studied in detail. In all experiments dealing with the optimization of the reaction, the mixed solution was heated in a boiling water-bath for 15 min.

The effect on the peak area of changing the reagent concentration was studied. The results indicated that the peak area increased with increasing reagent concentration, but the responses were unchanged when the reagent concentration was above 0.7%, was shown in Fig. 4. In this work the reagent concentration was selected as 0.8%.

The effects of the pH of the reaction medium and the buffer salt concentration on the peak area at a given reagent concentration are shown in Fig. 5. The change of the pH of the reaction medium affected the fluorescence and the maximum fluoresence intensity was obtained at pH 9.5. The fluoresence intensity of NANA increased with increasing borate buffer concentratin at a given pH value. However, this was not

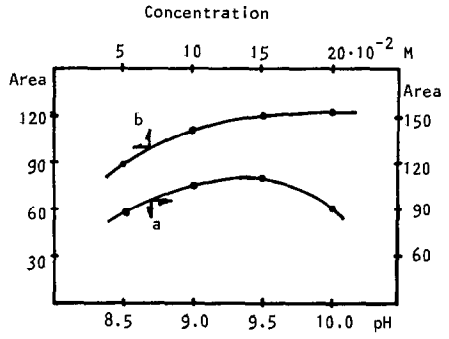

Fig. 5. Optimization of conditions for the reaction medium. (a) Effect of the pH of the reaction medium on the peak area. (b) Effect of the buffer salt concentration on the peak area.

seen when the buffer concentration was greater than 0.15 *M*. Thus a buffer with a pH value of 9.5 and a concentration of 0.15 *M* was used here.

The effects of reaction time on the peak area were investigated at 60, 80 and 100°C. Fig. 6a shows that the reaction time can be shortened by an increase in reaction temperature. However, higher reaction efficiency was obtained at 80°C. At this temperature the peak-area responses were hardly changed, with a reaction time longer than 15 min. The reaction time and temperature were therefore selected at 20 min and 80°C, respectively.

Acid hydrolysis of serum samples will help to release most of the sialic acids in serum. The effect of hydrolysis time on the peak area at 80°C was investigated. Fig. 6b shows that the peak-area responses were unchanged at 80°C with

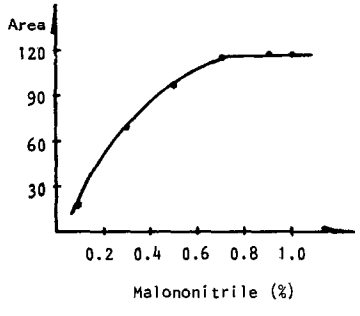

Fig. 4. Effect of changing the reagent concentration on the peak area.

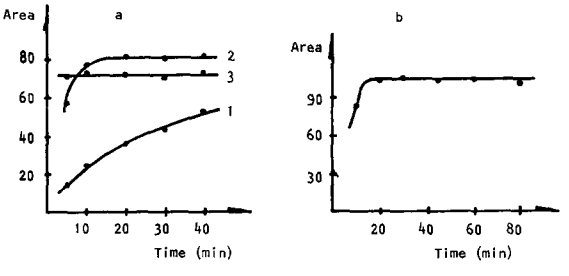

Fig. 6. Effects of time and temperature on fluorescence generation. (a) Effects of reaction time and temperature. Temperature: 1 = 60°C; 2 = 80°C; 3 = 100°C. (b) Effect of acid hydrolysis time.

more than 20 min reaction time. In this method the hydrolysis conditions of 80°C for 30 min were adopted.

Precision

Intra-day reproducibility studies, evaluated by assaying nine solutions containing 300 ng/ml NANA, yielded a coefficient of variation of 1.5%. Inter-day reproducibility studies, evaluated by assaying the same concentration nine times over a seven-day period, was 2.6%.

Linearity and detection limit of method

A series of solutions containing 0.030, 0.250, 0.500, 0.750 and 1.000 μg/ml NANA were prepared to study the relationship between the peak area and the concentrations of NANA under selected conditions. The results showed that the peak area was linearly related to the NANA concentration for the range 0.030–1.000 μg/ml. The linear equation for the concentration *versus* peak area was $y = 923.2x + 0.04$, with a correlation coefficient of 0.9998. The detection limit was 2 ng/ml.

Recovery

To the serum samples in which NANA was determined ($n = 6$) various amounts of NANA were exogenously added to test the analytical recovery. The samples, after being hydrolysed and reacted, were analysed by HPLC. The experimental results showed that the recovery of NANA was 98.5–101.1% for serum samples.

Determination of NANA in normal subjects

Blood samples from twelve healthy adult subjects were collected to determine NANA. The average value was 503.3 ± 36.7 μg/ml. These results agree well with those reported previously [15].

CONCLUSIONS

In this work a reversed-phase HPLC method with fluoresence detection has been developed for the separation and determination of sialic acids. The high sensitivity of this fluorescent reaction using malononitrile, a fluorogenic reagent for sialic acids, gives a low detection limit. The method is also rapid, simple and accurate. It could therefore be used for routine clinical analysis.

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