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DETECTION OF SIALURIA BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and selective method for the detection of sialuria by high-performance liquid chromatography is described. The urine sample (2 ml) is purified using a C_{18} cartridge or ion-exchange chromatography, and free N-acetylneuraminic acid is separated on an Aminex HPX-87 cation-exchange column using 3 m*M* sulphuric acid as the mobile phase. The retention time of N-acetylneuraminic acid is ca. 8 min and the detection limit ca. 1 μmol/l. The within-day coefficient of variation is less than 4.9% and the day-to-day coefficient of variation is less than 5.6%. The method was tested on twenty normal individuals and four sialuria patients.

INTRODUCTION

Sialuria is a heterogenous group of diseases characterized by elevated excretion of free N-acetylneuraminic acid in the urine. Four different types of sialuria have been described [1—4]. Mental retardation, dysmorphic features and lysosomal storage in different organs often accompany the disease, but the disease may also have a benign clinical course [4].

The diagnosis is based on the demonstration of elevated excretion of free N-acetylneuraminic acid in urine. In sialuria, the excretion may range from ca. 160 μmol to over 20 mmol per day [1—4], and in control adults it has been reported to be 14—50 μmol per day [5] and 30—80 μg/mg of creatinine at the age of 5—10 years [3]. Free sialic acid can be detected colorimetrically [6]. However, since urine contains a number of substances that may interfere with the thiobarbituric reaction, the detection method must be modified [7, 8]. Thin-layer chromatographic (TLC) analysis allows qualitative detection of N-

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acetylneuraminic acid in the concentration range 30—50 μmol/l [9]. Sensitive and specific methods using gas chromatography (GC) [10] and combined gas chromatography—mass spectrometry (GC—MS) [11, 12] have also been published, but tedious sample preparation makes them too laborious for screening methods in clinical laboratories.

High-performance liquid chromatography (HPLC) has proved to be of value for the detection of different genetic disorders [13, 14] because of simple sample preparation, selectivity and short analysis times. The applicability of HPLC to the quantitative analysis of N-acetylneuraminic acid liberated from glycoconjugates by acid catalysis [15] or enzymatically [16] has been demonstrated. This report describes a simple and selective HPLC method for quantitative analysis of free N-acetylneuraminic acid in urine of both sialuria patients and control subjects.

EXPERIMENTAL

Reagents, reference compounds and general methods

N-Acetylneuraminic acid was purchased from Sigma (St. Louis, MO, U.S.A.). Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Mississauga, Canada), HPTLC plates from E. Merck (Darmstadt, F.R.G.), and Dowex 50W-X8 (H^+) (200—400 mesh) and Dowex 2-X8 (Cl^-) (200—400 mesh) were purchased from Fluka (Buchs, Switzerland). Methyl ester methyl glycoside of N-acetylneuraminic acid was analysed as its O-trimethylsilyl ether derivative [12] using a Hewlett-Packard gas chromatograph Model 5730A equipped with a flame ionization detector and an SE-30 column (25 m × 0.2 mm I.D.). The mass spectra were recorded by a Hewlett-Packard 5992 spectrometer (at 70 eV) under the conditions reported previously [17].

Urine samples

Four patients with diagnosed sialuria (Salla disease) [3], 10, 15, 36 and 38 years of age, as well as fifteen normal adults from 26 to 59 years and five children from 6 to 9 years of age have participated in this study: 24-h urine samples were collected without dietary restrictions and stored frozen if not analysed immediately.

High-performance liquid chromatography

A Perkin-Elmer Series 4 liquid chromatograph was used. It was equipped with a variable-wavelength UV—Vis detector, an ISS-100 autosampler, LCI-100 computing integrator or an R-100A pen plotter, all from Perkin-Elmer (Norwalk, CT, U.S.A.). The quantitative analyses were performed by external calibration. A cation-exchange column (Aminex HPX-87H, 300 × 7.8 mm I.D.) with a cationic micro-guard column purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.) was used. The mobile phase was 3 m*M* sulphuric acid and the flow-rate was 0.6 ml/min. The detection wavelength was 202 nm and the sensitivity was 0.05 absorbance units full scale.

Sample preparation

A 2-ml aliquot of a 24-h urine sample was passed through a Sep-Pak C_{18}

cartridge, pre-eluted with water and washed with 2 ml of water. The eluate and the washing water were collected and 10—30 μl samples were injected into the HPLC column. When the urine samples were purified by ion-exchange chromatography, 2-ml aliquots were passed through columns (2 ml) of Dowex 50W-X8 and Dowex 1-X8, and the eluate was collected. The Dowex 1-X8 column was further eluted with 50 m*M* pyridine acetate buffer, pH 5.2, and the eluate was combined with the previous one for the HPLC analysis.

Characterization of N-acetylneuraminic acid in urine

The elevated amount of N-acetylneuraminic acid in the urine of the sialuria patients was demonstrated by TLC. Similarly, an undiagnosed sialuria was excluded in the case of control subjects. An aliquot of urine corresponding to 10 μg (88 nmol) of creatinine was spotted on the high-performance thin-layer chromatography (HPTLC) plate, the plates were developed in *n*-butanol—acetic acid—water (2:1:1) and N-acetylneuraminic acid was visualized with resorcinol—hydrochloric acid spray [18]. The resorcinol-stainable material was isolated from 50 ml of urine of a sialuria patient with ion-exchange chromatography on Dowex 50W-X8 and Dowex 1-X8 (10 ml). The material not retained on the columns was collected. The Dowex 1-X8 column was eluted with 50 m*M* pyridine acetate buffer, pH 5.2, and the eluate was collected. The eluates were combined, lyophilized, dissolved in water and injected onto the HPLC column. The compounds with retention times similar to that of reference N-acetylneuraminic acid were collected, treated with methanolic hydrochloric acid, converted into the corresponding O-trimethylsilyl ether derivatives and analysed by GC or GC—MS [12].

Testing of the method

The linearity of the detector response for N-acetylneuraminic acid in water was tested over the concentration range 0—4 mmol/l. The linearity for the urine samples was tested as follows: 500 nmol of N-acetylneuraminic acid were added to 1 ml of urine containing originally 34 nmol/ml free N-acetylneuraminic acid. An aliquot was taken for the HPLC analysis, and the rest of the sample was diluted with the original urine (1:1). This procedure was repeated seven times. The line fittings for the calibration curve were made by the linear regression technique. The recovery and reproducibility tests were performed with a solution of 0.3 mmol/l N-acetylneuraminic acid made up in water.

RESULTS

The chromatograms of N-acetylneuraminic acid in water and urine from a normal subject and a sialuria patient are shown in Fig. 1. A compound with the retention time of N-acetylneuraminic acid was detected in both urine samples. A small amount of the compound was isolated from the urine of a sialuria patient and was characterized as N-acetylneuraminic acid on the basis of its retention time in GC and its mass spectrum [12].

The calibration curve for the quantitative estimation of N-acetylneuraminic acid in water, ranging from 3 μmol/l to 4 mmol/l was linear ($r = 0.999$, $n = 13$).

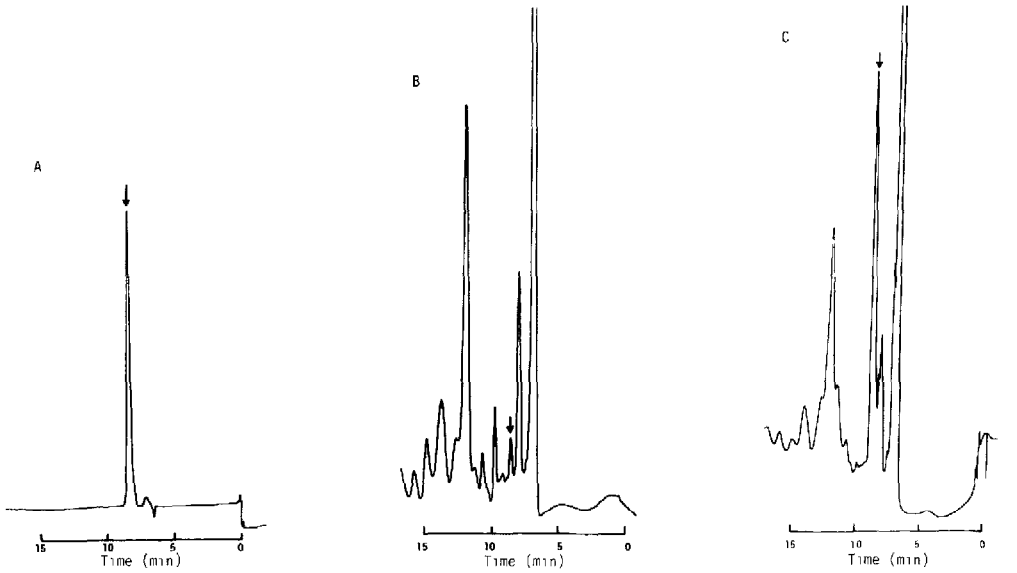

Fig. 1. HPLC separation of free N-acetylneuraminic acid. (A) Standard N-acetylneuraminic acid; (B) urine sample from a control subject; (C) urine sample from a patient with sialuria. The concentration of N-acetylneuraminic acid was 34 μmol/l in the control's urine and 390 μmol/l in the patient's urine.

The line fitted through the data points expressed as detector response (peak height, mm) (y-axis) and the concentration of free N-acetylneuraminic acid (μmol/l) (x-axis) intercepted the y-axis slightly above the origin ($y = 63.1x + 9.85$). The effect of the urine matrix was tested by diluting a urine sample containing a large amount (534 nmol/ml) of N-acetylneuraminic acid with urine containing a small amount (34 nmol/ml) of the compound. The detector response was linear ($r = 0.998$, $n = 13$) over the range 34—534 μmol/l N-acetylneuraminic acid. The regression line intercepted the y-axis slightly below the origin ($y = 63.8x - 8.42$). The slope values were very similar in both cases indicating that the urine matrix did not cause any significant interference with the quantitative estimation of N-acetylneuraminic acid by the present method. The detection limit for the standard N-acetylneuraminic acid in water was 1 μmol/l. In the case of urine samples it was ca. 3 μmol/l. The recovery of the standard N-acetylneuraminic acid after the purification process was 98.3% (range 95.2—100.2%, $n = 6$). The within-day and day-to-day coefficients of variation were $< 4.9\%$ ($n = 10$) and $< 5.6\%$ ($n = 10$), respectively.

The excretion of N-acetylneuraminic acid in control adults was 42—135 μmol per day (mean 74.6 μmol; $n = 15$) (Table I). In children, the corresponding excretion was lower (22—48 μmol per day; mean 33.7 μmol; $n = 5$). When correlated to urinary creatinine content, the values were higher in children than in adults. The excretion in the sialuria patients was 280—1050 μl per day or 93—509 μg/mg of creatinine being ten to twenty times higher than in control subjects.

When ca. 200 Sep-Pak-purified urine samples were analysed in cases suspected of sialuria, the majority of the chromatograms were practically free from interfering peaks. In the minority of cases, containing either a small

TABLE I

URINARY EXCRETION OF FREE N-ACETYLNEURAMINIC ACID IN CONTROL SUBJECTS AND SIALURIA PATIENTS

Urine source	*n*	Excretion of free N-acetylneuraminic acid		
		μmol per 24 h	μmol per mmol of creatinine	μg per mg of creatinine
Controls (26—59 years)	15			
Mean		74.6	5.12	14.0
Range		42—132	2.3—9.7	6.3—27
Controls (6—9 years)	5			
Mean		33.7	10.5	28.7
Range		22.4—48	7.8—15	21—41
Sialuria (10—38 years)	4			
Mean		593	89.7	246
Range		280—1050	34—186	93—509

amount of N-acetylneuraminic acid or interfering peaks in the chromatograms, the urine samples were purified by means of more tedious ion-exchange chromatography. This procedure usually resulted in more effective purification of the samples and allowed a better quantitative estimation of smaller amounts of N-acetylneuraminic acid than the Sep-Pak-purified samples. The quantitative results obtained after different purification procedures were very similar, but ion-exchange chromatography was more tedious and lengthy to perform. All sialuria cases could be diagnosed from the Sep-Pak-purified urine samples, and no further purification was indicated for routine screening purposes of sialuria. When the HPLC analysis time was set to 75 min, at least 50 samples could be analysed in series without interferences.

DISCUSSION

The described HPLC method allows a quantitative analysis of free N-acetylneuraminic acid in the urine of both sialuria patients and control subjects. Because of the simple sample preparation and selectivity, the method was found useful in the diagnosis of sialuria in the clinical laboratory. Although HPLC methods for quantitative estimation of N-acetylneuraminic acid liberated from proteins by acid catalysis [15] or enzymatically [16] have been reported, they could not be applied directly to urine samples.

The daily excretion values of N-acetylneuraminic acid in sialuria patients and control children are of the same order of magnitude as those measured colorimetrically after ion-exchange chromatography [3]. The corresponding values for the adults are according to the present results about three times higher than the earlier ones obtained after the purification of urine by gel chromatography [5]. This difference is probably due to the losses of free N-acetylneuraminic acid during the latter purification processes.

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