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HIGHLY SENSITIVE DETERMINATION OF N-ACETYL- AND N-GLYCOLYLNEURAMINIC ACIDS IN HUMAN SERUM AND URINE AND RAT SERUM BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A simple, rapid and highly sensitive high-performance liquid chromatographic method for the determination of N-acetyl- and N-glycolylneuraminic acids in serum and urine is described. The neuraminic acids, released by hydrolysis of serum and urine, are converted in dilute sulphuric acid with 1,2-diamino-4,5-dimethoxybenzene, a fluorogenic reagent for α-keto acids, to highly fluorescent derivatives. The derivatives are separated isocratically within 8 min by reversed-phase chromatography using a Radial-Pak cartridge C_{18} column and detected fluorimetrically. The limit of detection is 40 fmol (12 pg) for both neuraminic acids in 10-μl injection volume [0.3 nmol (90 ng)/ml) of serum or urine]. This sensitivity permits the precise determination of the neuraminic acids in 5 μl of serum or urine. The method was applied to the determination of the neuraminic acids in sera from normal subjects and cancer patients, normal urine and rat serum.

INTRODUCTION

Sialic acids, acylated derivatives of neuraminic acid, mainly occur as non-reducing terminal residues of carbohydrate chains of glycoproteins or glyco-

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lipids in biological materials. Sialic acids in human serum reflect the levels of sialoglycoproteins and sialoglycolipids. Increased serum or urinary levels of these compounds have been observed in inflammatory diseases, cancer or various other diseases [1—4] and congenital metabolic disorders [5, 6]. Sialic acids present in human and experimental animals are mostly N-acetylneuraminic acid (NANA) and/or N-glycolylneuraminic acid (NGNA). Thus, a simultaneous assay for NANA and NGNA in serum and urine may be useful for diagnosis and therapy of these diseases.

Many methods, including spectrophotometric [7, 8] and spectrofluorimetric [9—11] techniques, have been reported for the determination of total sialic acids in biological materials. The spectrophotometric method using thiobarbituric acid [7, 8] has been the most widely used. However, the method is not very selective and sensitive for sialic acids and suffers from interferences with 2-deoxy sugars such as 2-deoxyglucose and 2-deoxyribose. Selective and simultaneous assay of NANA and NGNA has been performed by gas chromatography (GC) [12], gas chromatography—mass spectrometry (GC—MS) [13] and high-performance liquid chromatography (HPLC) with UV detection [14—16]. GC and HPLC with UV detection have limited sensitivities and do not enable the determination of the neuraminic acids at picomole levels. Although the GC—MS method is extremely sensitive, it requires sophisticated instrumentation and a rather tedious sample clean-up procedure. Recently, a fluorimetric HPLC method using 4′-hydrazino-2-stilbazole, a fluorogenic reagent for α-keto acids, has been developed for the determination of NANA and NGNA in human plasma and urine [17]. The method is sensitive enough to determine NANA in human plasma or urine of 10 μl or more. However, the method has not been applied to the determination of NGNA, which occurs in physiological fluids from experimental animals at extremely low concentrations.

Recently, we have found that 1,2-diamino-4,5-dimethoxybenzene (DDB), a fluorogenic reagent for α-keto acids [18, 19], reacts with NANA and NGNA under conditions different from those for α-keto acids to form highly fluorescent compounds. Using the above finding, we developed a sensitive, simple and rapid HPLC method for the determination of NANA and NGNA in low amounts of serum and urine.

EXPERIMENTAL

Reagents and solutions

Unless stated otherwise, all chemicals and solvents were of analytical-reagent grade. Deionized and distilled water was used. NANA and NGNA were purchased from Sigma (St. Louis, MO, U.S.A.). DDB monohydrochloride was prepared as described previously [20]; it is now available from Dojindo Labs. (Kumamoto, Japan).

DDB solution (7.0 m*M*) was prepared by dissolving DDB monohydrochloride in water containing 0.7 *M* β-mercaptoethanol and 4.6 m*M* sodium hydrosulphite. The solution was stored in the dark and used within a day.

Apparatus

Uncorrected fluorescence excitation and emission spectra of the eluate were

measured with a Hitachi 650-60 fluorescence spectrophotometer (Tokyo, Japan) fitted with a 20-μl flow-cell with spectral bandwidths of 5 nm for both the excitation and emission monochromators.

A Hitachi 635A high-performance liquid chromatograph equipped with a high-pressure sample injector (10-μl loop) and a Shimadzu RF-530 fluorescence spectromonitor (Kyoto, Japan) equipped with a 12-μl flow-cell operated at 369 nm excitation and 453 nm emission were used. The column was a Radial-Pak cartridge C_{18} (100 × 8 mm I.D.; particle size 5 μm; Waters Assoc., Milford, MA, U.S.A.). The column could be used for more than 1000 injections with only a small decrease in theoretical plate number if washed with aqueous methanol (1:1) at a flow-rate of 1.2 ml/min for ca. 30 min everyday after analyses.

Serum and urine samples

Human sera were obtained from healthy volunteers in our laboratories and from hospitalized cancer patients (Kyushu Cancer Center Hospital, Fukuoka, Japan). Urines (24 h) from healthy volunteers were collected in the presence of 10 ml of toluene as a preservative [17]. Rat sera were obtained from male Wister rats (13 weeks old).

Procedure

To 5-μl aliquots of serum or urine samples, placed in a screw-capped 1.5-ml vial, were added 200 μl of 25 m*M* sulphuric acid. The vial was tightly closed and heated at 80°C for 1 h to hydrolyse the sample. After cooling, 200 μl of the DDB solution were added to the resulting solution and the mixture was heated at 60°C for 2.5 h in the dark to develop the fluorescence. The reaction mixture was cooled in ice-water to stop the reaction. A 10-μl aliquot of the resulting solution was injected into the chromatograph. For the establishment of calibration curves, a series of standard mixtures of NANA and NGNA [1—3500 nmol (0.3—1100 μg) each per ml] was prepared, and the standards (5 μl each) were carried through the procedure, without the hydrolysis. The peak heights in the chromatogram were used for the quantification of the neuraminic acids.

The mobile phase used for the HPLC separations was a mixture of methanol—acetonitrile—water (15:8:77). The flow-rate was 2.0 ml/min (ca. 69 bar). The column temperature was ambient (ca. 25°C).

RESULTS AND DISCUSSION

Derivatization conditions

Chromatograms obtained by treating a standard mixture of NANA and NGNA at various reaction temperatures are shown in Fig. 1. NANA and NGNA reacted with DDB at low ambient temperatures (0—37°C) to give single fluorescent peaks (Fig. 1A, peaks 2 and 1, respectively). However, at these temperatures, maximum and constant peak heights could not be attained even after 4 h (Fig. 2a and b). Higher reaction efficiencies were obtained at higher temperatures (50—100°C) (Fig. 2). However, at these temperatures, the additional peaks for both NANA and NGNA (Fig. 1B and C, peaks 4 and 3,

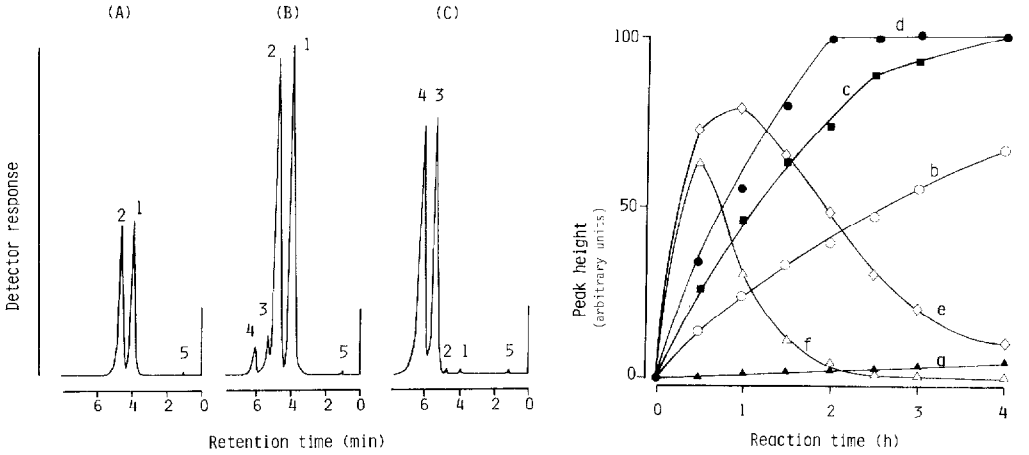

Fig. 1. Chromatograms of the DDB derivatives of NANA and NGNA. Portions (5 μl) of a standard mixture of NANA and NGNA (2 μmol/ml) were treated as in the procedure at various temperatures: A, 37° C; B, 60° C; C, 100° C. Detector sensitivity = 1. Peaks: 1 and 3 = NGNA; 2 and 4 = NANA; 5 = DDB.

Fig. 2. Effect of reaction temperature and time on the fluorescence development for peak 2. Portions (5 μl) of NANA were treated as described in the procedure. Temperatures: a, 0° C; b,37° C; c, 50° C; d, 60° C; e, 80° C; f, 100° C.

respectively) appeared in the chromatogram and increased in height with time. Moreover, at 80—100° C, peaks 2 and 1 decreased in height with time after 1—4 h (Fig. 2); peaks 2 and 1 were employed in the present method. At 60° C, the peak heights reached almost maxima after heating for 2 h. Thus, 2.5-h heating at 60° C was chosen. The fluorescence excitation (maximum, 369 nm) and emission (maximum, 453 nm) spectra of peak 2 (NANA) and peak 1 (NGNA) were virtually identical. The DDB derivatives in the final mixture were stable for at least 3 h in daylight at room temperature.

The derivatization reaction was efficient in dilute sulphuric or hydrochloric acid solutions, but not in neutral and alkaline solutions. Since sulphuric acid was used for the hydrolysis of serum and urine samples in the method, the following DDB reaction with the neuraminic acids was performed in sulphuric acid. The acid concentration in the final solution in the range 12—20 m*M* gave almost maximum and constant peak heights for both neuraminic acids; 12.5 m*M* sulphuric acid was selected, which could be attained easily by adding an equal volume of DDB dissolved in water to the hydrolysed sample. The concentration of DDB in the solution giving the most intense peaks was greater than ca. 6 m*M*; 7.0 m*M* was used as an optimum concentration. β-Mercaptoethanol in the DDB solution stabilized DDB during the reaction. The peak heights for the neuraminic acids reached maximal and constant values at β-mercaptoethanol concentrations greater than 0.5 *M* in the DDB solution; 0.7 *M* was used in the procedure. Sodium sulphite served to stabilize the fluorescent products from NANA and NGNA. The sulphite concentrations in the DDB solution in the range 2.3—9.2 m*M* gave the most intense peaks; 4.6 m*M* was chosen in the procedure. The peak heights were ca. 1.3 times higher than those in the absence of the sulphite. On the other hand, the heights of

peaks 3 and 4 decreased with increasing sulphite concentration (1.0—20.0 m*M* in the DDB solution).

Interferences

Many substances examined gave no fluorescent derivatives under the described conditions, even at a concentration of 5 nmol per 5 μl; i.e. sugars such as D-xylose, D-ribose, 2-deoxy-D-ribose, D-fucose, D-glucose, D-galactose, D-fructose, D-glucosamine, D-galactosamine, glucuronic, mannuronic, iduronic and galacturonic acids, maltose, cellobiose, gentiobiose, lactose, and benzaldehyde, vanillin, *p*-hydroxybenzaldehyde and dehydroascorbic acid.

Chromatography

Since both NANA and NGNA gave two peaks each (Fig. 1B) under the recommended derivatization conditions, these four peaks should be separated for the simultaneous determination of the neuraminic acids. The separation was studied on reversed-phase columns, Radial-Pak cartridges C_{18}, C_8 and CN, using methanol, acetonitrile, water and their mixtures as mobile phases. The best separation was achieved on a Radial-Pak cartridge C_{18} with a mixture of methanol—acetonitrile—water (15:8:77). Under these conditions, the retention times of DDB derivatives of the α-keto acids (2.5 min for α-ketoglutaric acid, 13.7 min for pyruvic acid and 20—60 min for the other α-keto acids) were different from those of NANA and NGNA. Thus, these acids did not interfere with the determination of NANA and NGNA.

The precision of the method was established by ten repeated determinations using standard mixtures of NANA and NGNA [5 nmol (1.5 μg) and 0.2 nmol (60 ng) each per 5 μl]. The coefficients of variation were 2.0 and 4.8% for NGNA and 3.4 and 4.2% for NANA, respectively. The detection limits were 40 fmol (12 pg) for both NANA and NGNA (10-μl injection volume) at a signal-to-noise ratio of 2. The sensitivity is at least 40 times higher than that obtained with the HPLC method using 4′-hydrazino-2-stilbazole.

Determination of NANA and NGNA in human serum and urine

Serum and urine were hydrolysed in the usual manner to release NANA and NGNA from glycoproteins and glycolipids [17]. Both neuraminic acids were stable under the hydrolysation conditions.

Typical chromatograms obtained with sera from a normal subject and from a liver cancer patient with metastases are shown in Fig. 3A and B, respectively. Fig. 4 shows the chromatogram of a urine sample from a healthy volunteer. The peaks of NANA (Figs. 3 and 4, peaks 2) were identified on the basis of the retention time and fluorescence excitation and emission spectra compared with those of the standard compounds, and co-chromatography of the standard and sera with aqueous 50—100% methanol as mobile phase.

Only NANA was detected in human serum and urine. This confirms that NGNA is not present in the human body [17]. Small peaks 6 and 7 in Fig. 4, which could be ascribed to α-ketoglutaric and pyruvic acids, respectively, appeared in the chromatogram obtained with urine, in which the α-keto acids occur in relatively large amounts [19]. However, these peaks were not observed in the chromatograms of human sera, because the α-keto acids are present in trace amounts in human serum [19].

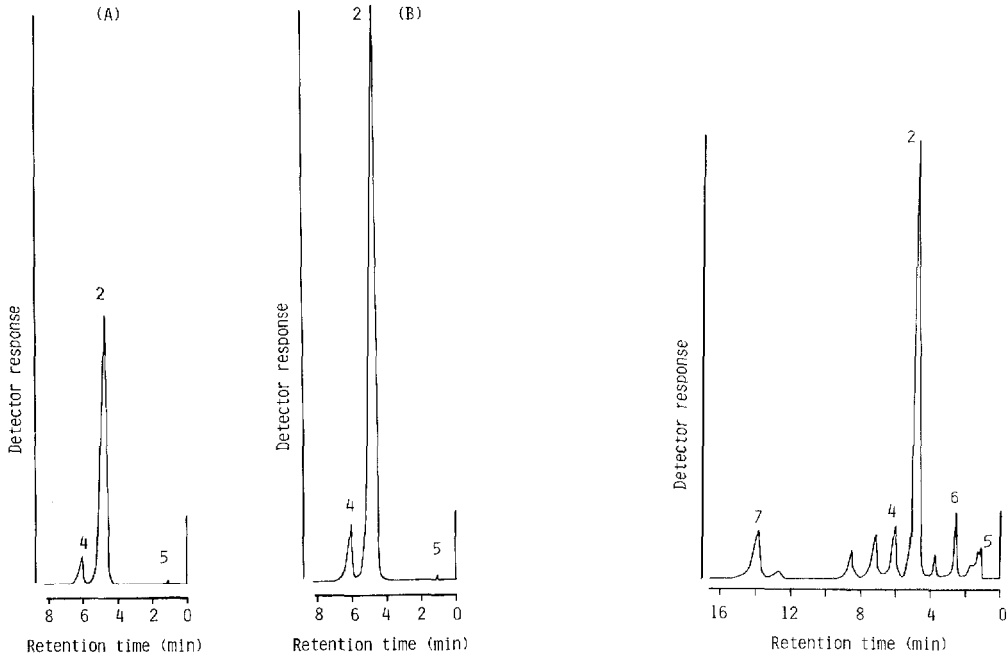

Fig. 3. Chromatograms of the DDB derivatives of NANA in sera from (A) a normal subject and (B) a liver cancer patient with metastases. Portions (5 μl) of normal and cancer patient sera were treated as described in the procedure. Detector sensitivity = 1. Peak assignment is the same as in Fig. 1.

Fig. 4. Chromatogram obtained with normal urine. A portion (5 μl) of normal human urine was treated as described in the procedure. Detector sensitivity = 4. Peaks: 2 and 4 = NANA; 6 = α-ketoglutaric acid; 7 = pyruvic acid.

Linear relationships were observed between the peak heights and the amounts of NANA and NGNA added to normal serum and urine up to at least 18 nmol (5.6 μg) per 5 μl. The recoveries of NANA and NGNA added to 5 μl of normal serum and urine in the amount of 5.0 nmol (1.5 μg) were 100.7 ± 2.2 and 101.0 ± 2.3% for serum, and 99.5 ± 3.2 and 98.7 ± 4.6% for urine (mean ± standard deviation, n = 10 in each case), respectively.

Comparison with the thiobarbituric acid method [7, 8] (requiring 100 μl of serum) using a normal pooled serum spiked with 0—1.8 μmol (0—540 μg) of NANA per ml gave a correlation coefficient of 0.974 (n = 10) and the regression equation for the present HPLC method (x, μg/ml of serum) against the thiobarbituric acid method (y, μg/ml of serum) was $y = 1.05x + 118.8$. This indicates that the present HPLC method gives lower values of NANA than the thiobarbituric acid method. This is due to interference with some endogenous sugars in human serum in the spectrophotometric method.

The concentrations of NANA in human sera from healthy volunteers and from patients with liver cancer were determined by this method (Table I). The levels of NANA in 24-h urines from healthy subjects are given in Table II. As might be expected, the mean values in normal serum and urine were slightly lower than those given by other workers using the thiobarbituric acid method [7, 8]; the same results have also been reported by the other HPLC method

TABLE I

CONCENTRATIONS OF NANA IN SERA FROM CONTROL SUBJECTS AND PATIENTS WITH LIVER CANCER

Age	Sex*	Concentration (μg/ml)	Age	Sex*	Concentration (μg/ml)
Control subject			*Liver cancer patient with metastases*		
21	F	461.3	55	F	828.5
21	F	468.0			
21	F	475.4	*Liver cancer patient without metastases*		
24	F	426.4	50	M	352.4
24	F	520.3	54	M	321.6
22	M	475.2	65	M	358.0
22	M	474.9	72	M	290.9
22	M	505.9	74	M	482.7
27	M	531.1	74	M	531.7
34	M	544.7			
59	M	531.3			
Mean		492.2			
S.D.		35.0			

*M = Male; F = female.

TABLE II

URINARY EXCRETION (24 h) OF NANA IN URINE FROM HEALTHY VOLUNTEERS

Age	Sex*	Urinary excretion (mg per day)
22	F	67.9
22	F	66.5
22	F	77.1
24	F	65.7
25	F	88.9
22	M	99.5
23	M	101.4
23	M	103.3
27	M	145.9
35	M	126.2
Mean		94.2
S.D.		25.4

*M = Male; F = female.

[17]. The concentration of NANA was significantly increased in serum from a liver cancer patient with metastases. On the other hand, the concentration in sera from liver cancer patients without metastases was almost identical with that in normal sera. This is in agreement with the previous findings [21].

Determination of NANA and NGNA in rat serum

Fig. 5 shows a typical chromatogram obtained with rat serum. NANA and

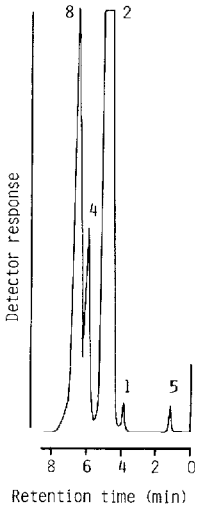

Fig. 5. Chromatogram of a sample of rat serum. A portion (5 μl) of rat serum was treated as described in the procedure. Detector sensitivity = 6. For peaks 1—5, see Fig. 1; peak 8 is unidentified.

TABLE III

CONCENTRATIONS OF NANA AND NGNA IN RAT SERUM

	Concentration (μg/ml)	
	NANA	NGNA
1	522.7	21.4
2	559.8	25.0
3	624.8	20.9
4	528.9	17.3
5	541.3	16.8
6	535.1	20.9
7	615.5	18.3
8	525.8	16.8
9	671.2	20.9
10	640.3	20.5
Mean	576.5	19.9
S.D.	52.8	2.5

NGNA peaks in male Wister rat serum were identified in the same way as for human serum and urine. Unknown peak 8 may be due to the endogenous substance in rat serum, which is not present in human serum. The concentrations of NANA and NGNA in rat serum determined by this method are shown in Table III. The levels of NANA and NGNA in rat serum were first determined by the present HPLC method.

The present HPLC method gives a satisfactory sensitivity in the quantification of NANA and NGNA. In this study, 5 μl of serum and urine were used for precise sampling. However, the sensitivity of the method permits the determination of NANA in only 0.5—1.0 μl of serum. This method is also rapid

and simple to perform and can therefore be applied for routine use in clinical investigations. Furthermore, the method may be very useful for the investigations of glycoproteins and glycolipids.

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