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Determination of free N-acetylneuraminic acid in human body fluids by high-performance liquid chromatography with fluorimetric detection

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ABSTRACT

Determinations of both the free and bound form of N-acetyl-neuraminic acid (NANA) in several human body fluids, such as serum, cerebrospinal fluid (CSF), saliva, urine, amniotic fluid, and milk were carried out by HPLC with fluorimetric detection. The method utilized 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) as a fluorimetric derivatizing reagent. Free-form NANA was obtained from the body fluids after ultrafiltration with Microcon 10 (YM-10 cellulose membrane, filtration limit M_r = 10 000, Amicon). The DMB derivative of NANA was separated isocratically by a Nucleosil $5C_{18}$ column with a mixture of 0.1 *M* sodium phosphate buffer (pH 2.0)–methanol (75:25, v/v). A gradient elution system was used for urine analysis. Analysis times were 10–30 min. Recoveries of free NANA by ultrafiltration were satisfactory: 95.66 ± 1.80% for serum and 97.27 ± 1.55% for CSF, respectively. The high sensitivity and specificity render this method applicable to all the body fluids tested. Although a physiological role for free NANA has not yet been elucidated, the method presented promises to contribute to the basic understanding of the NANA metabolism.

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INTRODUCTION

Over 30 compounds with a variety of biological roles are known as sialic acids [1]. N-acetylneuraminic acid (NANA) has been reported to be the predominant form of sialic acid in mammals [1], whereas a variable, but often minor percentage is represented by N-glycolylneuraminic acid (NGNA). While a small portion of the total sialic acid is free in tissues and body fluids, most is bound by an alpha-glycosidic linkage to glycoconjugates [2]. To date, no definite physiological role has been attributed to unbound NANA. NANA is a constituent of the plasma membrane, present in most acute-phase reactant proteins. In human plasma a large quantity of NANA is found in orosomucoid, alpha-1-anti-trypisin, haptoglobin, ceruloplasmin, fibrinogen, complement proteins, and transferrin. Increase in total serum NANA concentrations has been reported in malignant tumors, and bacterial infections [3]. On the other hand, intralysosomal accumulation of free NANA is the major biochemical feature of Salla's disease [4] and infantile sialic acid storage disease [5].

In human tissues and body fluids, the determination of NANA has been classically carried out by the photometric resorcinol reaction [6] or thiobarbiturate (Warren's) method [7]. More recently, an enzymatic method has also been described [8]. However, low sensitivity hampers all these methods. Moreover, in photometric methods interference from several compounds is likely to occur when they are applied to naturally turbid body-fluid samples, such as milk. Therefore, accurate knowledge of the free NANA concentrations in human body fluids is still surprisingly lacking.

Recently, an HPLC method with fluorimetric detection for the determination of NANA and NGNA through derivatization by 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) has been described by Hara *et al.* [9,10]. The method is highly sensitive (detection limit 57 fmol) and suitable for microassay (0.02 ml sample volume is sufficient for the analysis). In the present study, an already existing pre-column derivatization and HPLC with fluorimetric detection was improved and applied to the determination of free NANA and NGNA in human body fluids and some animal sera after ultrafiltration.

EXPERIMENTAL

Chemicals and reagents

NANA and NGNA were purchased from Sigma (St. Louis, MO, USA). DMB, sodium hydrosulfite, and 2-mercaptoethanol were from Wako Pure Chemical Co., Osaka, Japan. Malic acid, pyruvate, ascorbate, lactic acid, isocitrate and glyceric acid were from Nacalai Tesque, Kyoto, Japan.

Specimens

Fresh sera from eight healthy Japanese school children and two 4-month-old babies were obtained. Fresh sera from three healthy volunteers (all males, age: 26, 30, and 36 years) were also obtained. Fresh human breast milk samples (n = 3) were obtained from the Boshi-Hoken-In Hospital, Tokyo, Japan, and were stored at −20°C. Cerebrospinal fluids (CSF) were provided by the Division of Neurology, National Children's Hospital, Tokyo, Japan, from both ventricular and spinal punctures from patients with subacute sclerosing panencephalitis (SSPE, n = 3), and other neurological diseases such as mitochondrial encephalopathy (n = 1), congenital obstructive hydrocephalus (n = 1), Rett's syndrome (n = 1), and muscular dystonia (n = 1). Saliva samples (n = 3) were taken by the suction method from healthy volunteers. Urine samples from healthy volunteers (n = 3) were collected and filtered through an Ekicrodisk 13 (Gelman Japan Prod., pore size 0.2 μm), and stored at −20°C until the date of assay. Amniotic fluid specimens (n = 5) were provided by the Division of Dismorphology, National Children's Medical Research Center, Tokyo, Japan; the samples were taken at week 16–19 of the gestational age, on a prenatal screening programme for chromosomal aberrations and stored at −20°C. Rat sera (n = 3) were obtained from Sprague–Dawley rats (7 weeks old). Equine (thoroughbred; n = 3), bo-

vine (n = 3), and porcine (n = 3) sera were obtained from Obihiro Zootechnic University, Obihiro, Hokkaido.

Stability of free and total NANA concentrations in serum

In order to simulate sample-handling conditions possibly occurring during collection and storage, 10 ml of fresh blood samples obtained from 3 healthy adult volunteers (all males, age 26, 30, and 36 years, respectively) were aliquoted (5 ml) into polyethylene terephthalate test tubes containing olefin oligomer (Sepaclean-A), 17 mm × 99 mm; + WAX) and into polymethacrylic acid polymer test tubes (Centrifuge tube A, 17 mm × 102 mm; − WAX, Eiken Kizai Co., Tokyo, Japan), and centrifuged at 1050 g for 10 min. The + WAX and − WAX plasmas or sera (one ml) were then aliquoted (0.2 ml) into five glass test tubes (10 mm × 75 mm) and stored at −20°C. After storage at −20°C, specimens were thawed in tap water, and allowed to stand at room temperature (23°C) for 0, 1, 2, 3, and 4 h, respectively, before assay. The + WAX and − WAX samples were then analyzed for free and total NANA contents.

Ultrafiltration of body fluids

A 0.5-ml sample of body fluid was ultrafiltered through an ultrafiltration membrane (Microcon 10, Lot PFA028, Amicon Division, W. R. Grace & Co., Boverly, MA, USA) at 14 000 g for 10–30 min. A 0.02-ml portion of the ultrafiltrate was utilized for the determination of free NANA.

Determination of bound NANA

A 0.025-ml sample of serum, milk, saliva, and urine was added to 0.475 ml of 0.05 M H_2SO_4 and incubated for 1 h at 80°C, according to the procedure of Silver *et al.* [11]. For CSF and amniotic fluid, a 0.05-ml sample volume was added to 0.05 ml of 0.1 M H_2SO_4 and incubated for 1 h at 80°C.

High-performance liquid chromatography

The chromatographic apparatus (Hitachi, Tokyo, Japan) consisted of a 665A-11 liquid chromatograph, a sample injector (Rheodyne Model 7125) with a 0.1-ml loop, and a spectrofluorometric detector (RF-550, Shimadzu, Kyoto, Japan). The analytical column was a Nucleosil 120-5C_{18} (250 mm × 4.6 mm I.D., Macherey-Nagel, Düren, Germany) and was heated at 45°C by a column oven (Hitachi 655A-52). A line filter (GL Sciences, Tokyo, Japan) was inserted between the injector and the column. The flow-rate was 1.0 ml/min with a column-inlet pressure of approximately 100 kg/cm^2. A 1.5 l sodium phosphate buffer (0.1 M NaH_2PO_4, pH 2.0) was mixed with 0.5 l of methanol and utilised as eluent. Fluorimetric detection was carried out at excitation and emission wavelengths of 373 nm and 448 nm, respectively [9]. Standards of NANA and NGNA eluted at retention times (t_R) of 8.05 and 6.45 min, respectively. In this separating system, reacting compounds such as sodium pyruvate (t_R = 21.4 min) and L(+) sodium ascorbate (t_R = 4.50 min) did not interfere with the NANA and NGNA assay. Other alpha-keto groups containing substances such as DL-lactic acid, DL-malic acid, trisodium isocritrate, and calcium salt of glyceric acid gave no fluorescent derivatives under the described conditions.

For urine analysis, a linear gradient from solvent A (0.1 M sodium phosphate buffer, pH 2.0–methanol = 90:10, v/v) to solvent B (methanol) in 30 min was used. The gradient method reduced the analysis time of urine samples to less than 30 min.

Derivatization of NANA

The procedure was carried out essentially according to Hara *et al.* [9,10]. A 0.02-ml amount of ultrafiltrated or acid-hydrolyzed body fluid sample was added to 0.2 ml of DMB reagent (1.7 M acetic acid, 0.75 M 2-mercaptoethanol, 18 mM sodium hydrosulfite, 7 mM DMB). After incubation at 50°C for 2.5 h, a variable (0.005–0.05 ml) portion of the reaction mixture was directly injected into the HPLC system.

Recovery test

Standard NANA was added in the amount of 16.2 nmol to 0.1 ml of CSF, and in the amount of

1.62 nmol to 0.1 ml of serum. NANA analyses were performed as described above.

RESULTS AND DISCUSSION

Human body fluids

Determinations of free NANA concentrations in several human body fluids were carried out by a highly sensitive high-performance liquid chromatographic method with fluorimetric detection. Two neuraminic acid standards (NANA and NGNA) were well separated under the elution conditions described in Experimental, as in Ref. [9]. The method allowed clear detection of as low as 22 pmol of NANA and NGNA (data not shown), thus confirming the high sensitivity of the technique [9].

Recovery tests for free NANA in two representative body fluids (serum and CSF) were carried out, and the results were quantitative; *i.e.* recovery values of 97.3 ± 1.6 (mean ± S.D.) for CSF and 95.7 ± 1.8 for serum were obtained, respectively. The high and constant recoveries for free NANA analysis showed that the new ultrafiltration tool Microcon 10 is satisfactorily applicable to free NANA analyses in body fluids.

Since free NANA may be liberated from sialylated proteins during storage, stability experiments were performed with serum samples. Results are shown in Fig. 1, where the effects of the serum preparation methods are also compared. The results indicated that the free and total NANA concentrations were relatively stable during storage or when left at room temperature up to 4 h. On the other hand, a significant reduction (approximately 40%) in free NANA concentrations was observed in sample preparations in the wax-containing test tubes (+ WAX). It is suggested that absorption of free NANA by the wax material may occur when test tubes containing olefin oligomers are used.

Typical chromatograms obtained for free NANA analyses from different human body fluids are shown in Fig. 2. NGNA was not detected in any of the examined human body fluids. Free NANA analysis was completed within 10 min for serum, milk, saliva, CSF, and amniotic fluid, using simple isocratic conditions. For urine samples, the gradient analysis was more convenient than the isocratic one (Fig. 2, right side bottom panel). Analysis times for urine were less than 30 min.

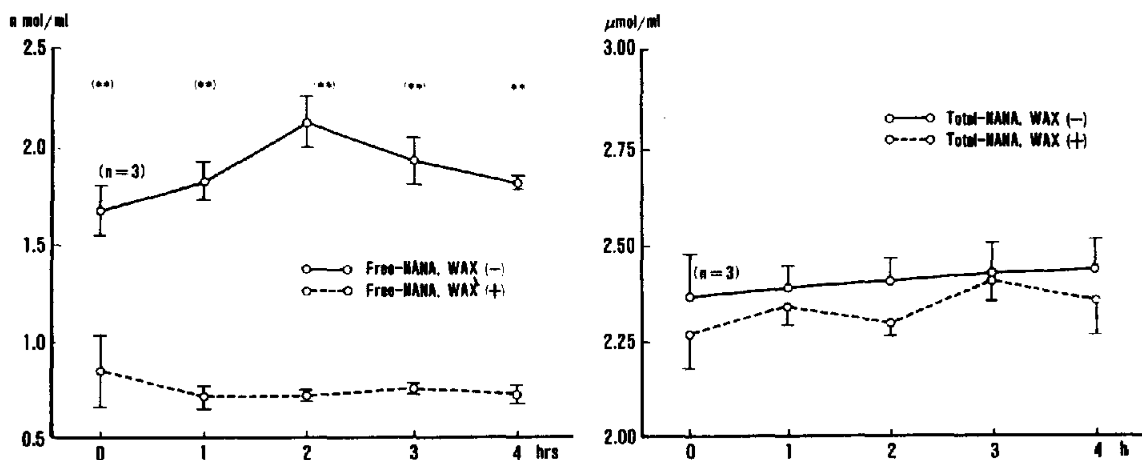

Fig. 1. Changes in free and total NANA concentrations by different serum preparation methods and storage at room temperature. Fresh serum samples from three healthy adults were processed for NANA analysis as described in the Experimental section. Left panel: Free NANA concentrations. Right panel: Total NANA concentrations. Solid lines and dashed lines show serum samples prepared from tubes without and with wax (− WAX and + WAX), respectively. Bars represent S.D. (**) in left panel: significant different means (Wilcoxon test, p less than 0.01).

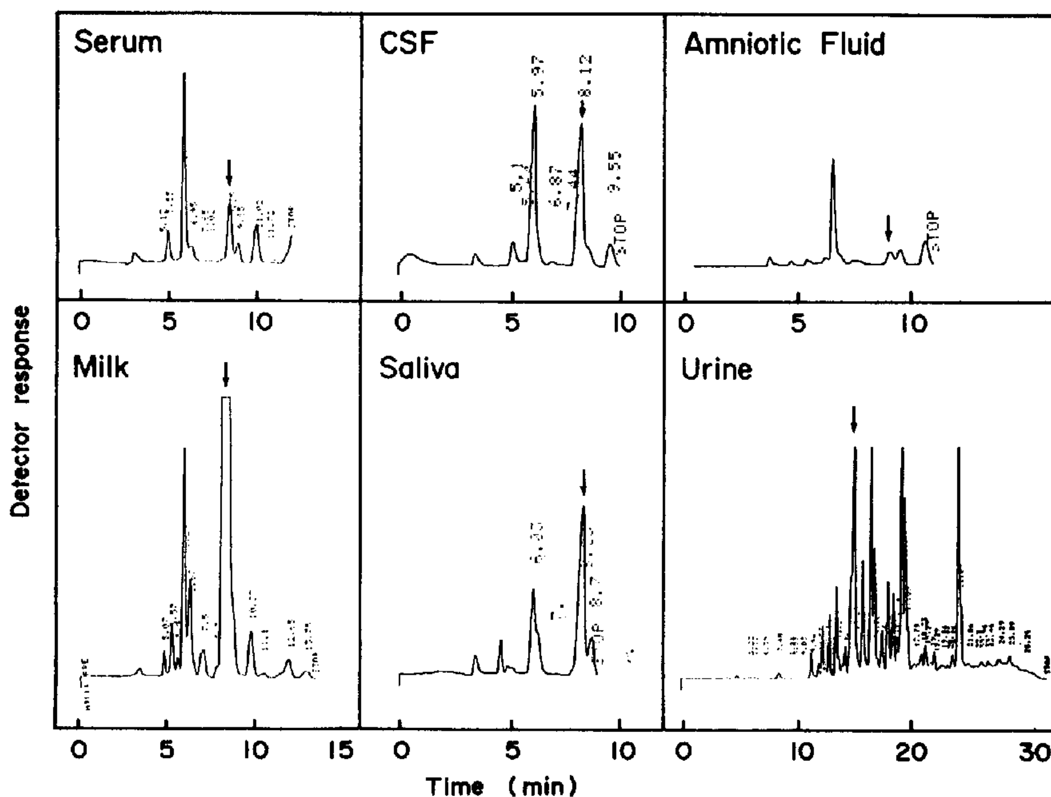

Fig. 2. Typical examples of analyses for free NANA in human body fluids. Ultrafiltrates of body fluids were obtained with Microcon 10, and derivatized as described in the Experimental section. A 0.01-ml portion of the derivatized solution was directly injected into the HPLC system.

The results of the analyses for free NANA concentrations are shown in Tables I and II, where total NANA concentrations and ratios for free/total NANA concentrations are also indicated for reference. Total NANA concentrations of urine and serum were similar to the values reported in Ref. [9] and Refs. [9–11], respectively. Among the examined body fluids, the highest free NANA levels were measurable in milk, while the lowest free NANA concentrations were found in serum. The physiological relevance for the observed one-hundred-fold-difference in the mean extreme free NANA contents is not clear at this time. Furthermore, it is worthy to note that free/total NANA ratios seem body fluid specific, with mean extreme values ranging widely from 0.05% for serum to 29% for CSF.

Free NANA concentrations in CSF showed the largest variability. Serum has a very low free/total NANA ratio. This may be used as a specific body fluid marker. The free NANA contents of the urine samples listed in Table I could be more conventionally expressed as follows: free NANA (mean ± S.D.) = 10.62 ± 2.59 mg/g creatinine, n = 3).

Animal sera

In the system used, simultaneous determination of free and total NGNA and NANA in the sera of three animal species was possible. The results for the free NGNA levels (mean ± S.D.; nmol/ml, n = 3) were as follows: swine, 2.16 ± 0.62; equine, 0.37 ± 0.019; bovine, 2.62 ± 0.76; rat, 0.516 ± 0.059, respectively. The results for the free NANA levels were: swine, 1.6 ± 0.08; equine, 0.324 ± 0.053; bovine, 0.676 ± 0.202;

TABLE I

FREE AND TOTAL NANA CONTENT IN HUMAN "EXTERNAL" BODY FLUIDS

Sample No.	Age (y)	Sex	Free NANA (nmol/ml)	Total NANA (nmol/ml)	Free/total NANA ratio
Milk					
1	28	F	108	4100	0.03
2	24	F	157	3140	0.05
3	34	F	263	3735	0.07
Mean ± S.D.			176 ± 64.7	3658 ± 395	0.05 ± 0.16
Urine					
1	34	F	69.7	275.0	0.25
2	36	M	46.4	215.0	0.22
3	44	M	33.7	124.0	0.27
Mean ± S.D.			49.9 ± 14.9	204.7 ± 62.1	0.24 ± 0.02
Saliva					
1	21	F	34.1	264.0	0.13
2	30	M	4.4	73.2	0.06
3	45	M	27.7	113.0	0.24
Mean ± S.D.			22.1 ± 12.8	150.1 ± 82.2	0.14 ± 0.07
AF[a]					
1		F[b]	1.01	227.0	0.004
2		M[b]	4.84	250.0	0.019
3		M[b]	2.86	257.0	0.011
4		M[b]	3.54	189.0	0.019
5		M	5.02	193.0	0.026
Mean ± S.D.			3.45 ± 1.46	223.2 ± 28.1	0.016 ± 0.007

[a] AF = amniotic fluid.

[b] Gender assessed by chromosomal analysis (QFQ-banding).

rat, 1.76 ± 0.25, respectively. Total NGNA and NANA values in these animal sera (data not shown) were similar to those of Hara *et al.* [10]. High free NGNA levels were detected in swine and bovine sera, and high free NANA levels were found in swine and rat sera. The levels of free NANA and free NGNA in porcine, equine, bovine, and rat sera were demonstrated by this method for the first time.

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TABLE II

FREE AND TOTAL NANA CONTENTS IN HUMAN "INTERNAL" BODY FLUIDS

Sample No.	Age (y)	Sex	Free NANA (nmol/ml)	Total NANA (nmol/ml)	Free/total NANA, ratio
CSF					
1	0.3	M	18.0	63.9	0.28
2	6	M	8.8	36.0	0.24
3	9	F	28.8	71.3	0.40
4	9	F	9.4	22.1	0.43
5	11	F	8.7	37.4	0.23
6	16	F	30.3	103.2	0.29
7	20	M	5.8	28.3	0.21
Mean ± S.D.			15.7 ± 9.4	51.7 ± 26.9	0.29 ± 0.08
Serum					
1	0.3	M	1.6	1580	0.0010
2	0.3	F	0.9	1780	0.0005
3	4	M	1.7	2560	0.0006
4	4	F	1.0	2370	0.0004
5	5	M	0.8	2540	0.0003
6	5	F	0.5	2480	0.0002
7	10	M	1.8	2150	0.0008
8	10	F	1.1	2780	0.0004
9	15	M	0.9	2450	0.0004
10	15	F	1.1	2380	0.0005
Mean ± S.D.			1.40 ± 0.40	2307 ± 351	0.0005 ± 0.0002

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