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Short communication

Determination of free *N*-acetylneuraminic acid in urine by high-performance liquid chromatography using 3-[(1-{[4-(5,6-dimethoxy-1-oxoisoindolin-2-yl)-2-methoxyphenyl]-sulfonyl}pyrrolidin-2-yl)carbonylamino]phenylboronic acid as a fluorescent labeling reagent

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Abstract

A highly sensitive high-performance liquid chromatography (HPLC) method for the determination of urinary *N*-acetylneuraminic acid (NeuAc) using 3-[(1-{[4-(5,6-dimethoxy-1-oxoisoindolin-2-yl)-2-methoxyphenyl]sulfonyl}pyrrolidin-2-yl)carbonylamino]phenylboronic acid as a fluorescent labeling reagent was developed. The labeling reaction was carried out at 30 °C for 30 min in the presence of pyridine. The derivative was monitored at Ex 314 nm and Em 388 nm. The detection limit of NeuAc was about 48 fmol per injection. The relative standard deviations of within-day and between-day precisions were 2.6–3.3 and 1.7–3.3%, respectively. Urine diluted 10 times with distilled water was analyzed by employing the standard-addition method. The concentrations were 8–89 nmol/mg creatinine (30±28 nmol/mg creatinine, $n=9$).

Keywords: *N*-Acetylneuraminic acid

1. Introduction

Elevated urinary excretion of free *N*-acetylneuraminic acid (NeuAc) is a condition unique to Salla disease, infantile free sialic storage disease and sialuria [1–4]. The determination of urinary free NeuAc can provide useful information in the diagnosis of these diseases.

For the determination of urinary free NeuAc, spectrophotometric methods [5,6], gas chromatography–mass spectrometry (GC–MS) [2], high-performance liquid chromatography (HPLC)–UV detection [7,8] and HPLC-fluorescence detection [9] have been reported. However, spectrophotometric methods using periodate/resorcinol and thiobarbituric acid are not selective and are affected by interference from other biological compounds. The GC–MS method requires a rather tedious sample clean-up procedure and HPLC–UV detection methods do not provide sufficient sensitivity. The HPLC-fluorescence detection method using 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB)

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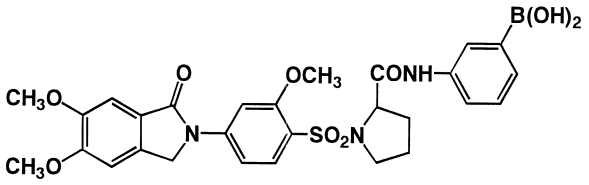

Fig. 1. The structure of DMPB.

is selective and highly sensitive. However, the labeling reaction of NeuAc with DMB requires a long time (2.5 h).

Recently, we reported on a new fluorescent labeling reagent, 3-[(1-{[4-(5,6-dimethoxy-1-oxoisoindolin-2-yl)-2-methoxyphenyl]sulfonyl}pyrrolidin-2-yl)carbonylamino]phenylboronic acid (DMPB, in Fig. 1), having boronic acid that reacts with some diol compounds (1,2-diols or 1,3-diols) to form cyclic boronates [10]. We found that DMPB reacted with NeuAc having a diol structure to form a fluorescent derivative. In this paper, a highly sensitive HPLC method for determining urinary NeuAc using DMPB was developed.

2. Experimental

2.1. Chemicals and solvents

All chemicals were of analytical-reagent grade, unless stated otherwise. DMPB was prepared from 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl) [11] as described previously [10]. NeuAc and acetonitrile (HPLC-grade) were obtained from Wako (Osaka, Japan) and pyridine (dehydrated) was from Kanto Chemicals (Tokyo, Japan). L-Leucine (Leu) and tetra-*n*-butylammonium bromide were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Synthesis and analytical data of internal standard (I.S., DMS derivative of Leu, DMS-Leu)

A reaction mixture of Leu (40 mg, in 30 ml borate buffer) with DMS-Cl (70 mg, in 100 ml acetone) was mixed and stirred at 70 °C for 20 min. The solution was evaporated to remove acetone and then acidified with concentrated hydrochloric acid (ca. 100 μl) to obtain a precipitate. The precipitate was recrystallized from methanol to obtain DMS-Leu as white plates (yield 58 mg, m.p. 271–273 °C). Analysis: calculated for $C_{23}H_{28}N_2O_8S$, C 56.08, H 5.73, N 5.69; found, C 56.04, H 5.71, N 5.68%. EI-MS (*m*/*z*): 447 ($[M\text{-}COOH]^+$). ^1H NMR (δ, ppm, in $CDCl_3$): 0.78–0.83 (7H, m, CH_3–CH–CH_3 of leucine), 1.43 (2H, m, CH_2 of leucine), 3.84 (1H, m, –CH–COO), 3.85, 3.88 and 3.89 (3H each, s each, OCH_3 each), 4.97 (2H, s, CH_2 of isoindoline), 7.25 (2H, s, aromatic-H of isoindoline), 7.44 and 7.70 (1H each, d each, H of benzene ring, J=8.43Hz each), 7.86 (1H, s, H of benzene ring).

2.3. Biological samples

Urine samples were collected from nine healthy volunteers (six male and three female) with normal creatinine clearance. The samples were analyzed immediately or stored at 4 °C until assay.

2.4. Instrumental conditions

The HPLC system consisted of two BIP-I HPLC pumps, a GP-A50 gradient programmer, a 880-51 2-line degasser (Jasco, Tokyo, Japan), a Rheodyne Model 7125 injector (20-μl loop), an FS-8010 fluorescence detector (Tosoh, Tokyo, Japan), a C-R6A recorder (Shimadzu, Kyoto, Japan), an ECS-1 eyela cool (Eyela, Tokyo, Japan) and a Dx-80 thermo minder (Titec, Tokyo, Japan). A TSK-gel ODS-80T_S (4.6×150 mm, I.D., 5 μm, Tosoh, Tokyo, Japan) connected to a TSK-guardgel ODS-80T_S (3.2×15 mm, I.D., Tosoh) as a guard column was used at 10 °C. For determination of free NeuAc in urine, a stepwise elution of (A) phosphate buffer (1 m*M*, pH 7) containing tetra-*n*-butylammonium bromide (1.75 m*M*)–(B) acetonitrile was employed. A stepwise elution program was an isocratic elution of 37% B for 12 min, followed by a stepwise increase to 80% B to wash the column for 10 min and then a stepwise decrease to 37% B to re-equilibrate the column for 15 min. The flow-rate program was 0.7 ml/min for 12 min, followed by 1.0 ml/min for 22 min and then 0.7 ml/min for 3 min. The fluorescence intensities were monitored at exci-

tation (Ex) and emission (Em) wavelengths of 314 and 388 nm, respectively.

2.5. Labeling procedure for NeuAc and HPLC analysis

A mixture of standard solution of NeuAc (20 μl) and NaH_2PO_4 (1 m*M*, 20 μl) was evaporated to dryness in a nitrogen gas stream at 50 °C. The residue was reacted with DMPB (0.5 m*M* in 2% pyridine–acetonitrile, 100 μl) at 30 °C for 30 min. After cooling, I.S. solution (0.2 μ*M* DMS-Leu in methanol, 900 μl) was added to the reaction mixture, and an aliquot (20 μl) of the resulting mixture was subjected to HPLC.

2.6. Procedure for determination of free NeuAc in urine

Urine was diluted 10 times with distilled water and analyzed by employing the standard-addition method. Two aliquots (20 μl each) of the diluted urine sample were taken in test tubes and was water (20 μl) or a standard solution of NeuAc (10 μ*M*, 20 μl) was added, respectively. Then, each solution was evaporated to dryness in a nitrogen gas stream at 50 °C. The residue was reacted with DMPB and analyzed by HPLC as described above.

3. Results and discussion

3.1. Chromatographic separation

DMPB reacted with NeuAc to produce a fluorescent derivative that was separated by reversed-phase HPLC. The derivative was monitored at Ex 314 nm and Em 388 nm, which were the maximum fluorescence wavelengths of derivatives of some diols with DMPB [10]. Typical chromatograms obtained from a standard solution, blank, diluted urine and diluted urine spiked with a standard solution of NeuAc are shown in Fig. 2. The peaks of the derivative of NeuAc and I.S. were eluted at 8.6 and 10.3 min, respectively. To prevent a rise in the baseline and disorder of separation, dilution of the reaction mixture with methanol by adding I.S. solution was required. The effect of the column temperature was examined at 10 and 25 °C. The peak area due to NeuAc decreased to 46% by measurement at 25 °C compared with the measurement at 10 °C. Probably, the derivative of NeuAc is unstable at higher temperatures. The complete separation of peaks due to

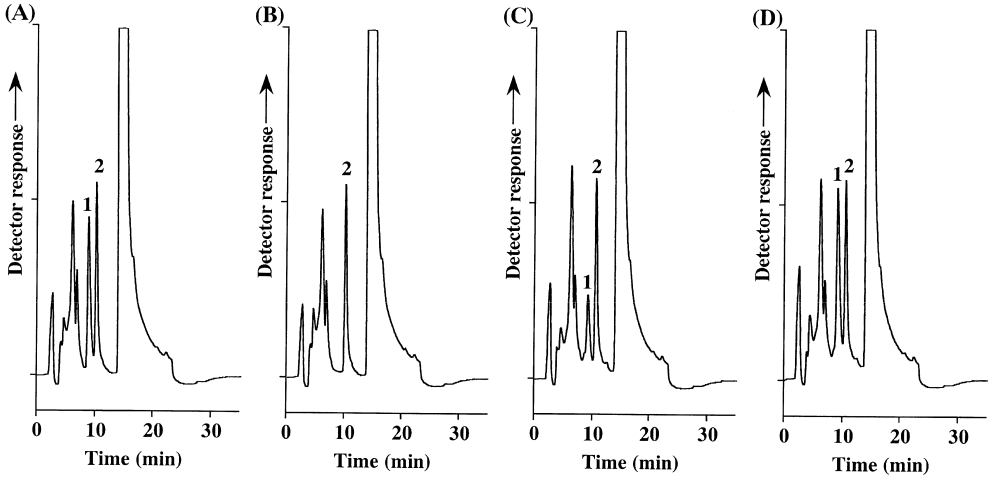

Fig. 2. Chromatograms obtained from (A) a standard solution, (B) a reagent blank, (C) diluted urine and (D) diluted urine spiked with a standard solution according to the procedure described in Section 2. Peaks: (1) NeuAc; (2) I.S. NeuAc concentration: standard solution, 10 μ*M*; diluted urine, 5. 6 μ*M*.

NeuAc and IS was achieved by ion-pair chromatography using tetra-*n*-butylammonium bromide at a concentration of more than 1.5 m*M*. The pH and concentration of phosphate buffer in the mobile phase also affected the separation. The peak due to NeuAc was delayed and broadened at acidic pH (less than pH 6). At higher concentrations of phosphate buffer (more than 5 m*M*), the peaks due to NeuAc and blank were partially overlapped because the peak due to NeuAc was eluted earlier while the peak due to blank (retention time: 7 min) was delayed. Therefore, 1 m*M* phosphate buffer (pH 7) was used. The relative standard deviations ($n=5$) of the retention times of NeuAc and IS were 0.64 and 0.68%, respectively.

3.2. Selection of I.S.

Some derivatives of amino acids (L-hydroxyproline, L-proline, L-alanine, L-isoleucine, L-phenylalanine and Leu, 10 μ*M* each) with DMS-Cl were examined as I.S., as the derivatives show the almost same fluorescence spectra as the derivative of DMPB. The derivative of Leu (10.3 min) was separated completely from the peaks due to NeuAc and blank reagent, while other derivatives (6.2, 7.1, 8.0, 10.1 and 12.6 min, respectively) overlapped with the peaks due to NeuAc or blank reagent. Therefore, the derivative of Leu with DMS-Cl was synthesized and used as I.S.

3.3. Labeling reaction conditions

In general, the formation of cyclic boronate proceeded in the presence of basic catalysts such as pyridine. When the effect of the pyridine concentration on the labeling of NeuAc with DMPB was examined, the maximum peak area was obtained above 1% of pyridine in the DMPB solution.

The effect of reaction time was also tested at various temperatures. As shown in Fig. 3, the labeling reaction proceeded at 30 °C and was completed within 20 min.

The reaction of NeuAc with DMPB was interfered with by water. Therefore, the diluted urine was evaporated before the labeling reaction. As salts also affected the labeling reaction, the effect of salts such as NaH_2PO_4, Na_2HPO_4, NaCl, $NaHCO_3$ and KCl (1 m*M* each, 20 μl) was examined using a standard solution of NeuAc. The results are shown in Table 1. The reaction was improved in the presence of NaH_2PO_4 or Na_2HPO_4 although the other salts interfered with the labeling reaction. When the amount of NaH_2PO_4 was tested, the peak area reached the maximum in the range of 10–120 nmol. On the other hand, when urine was analyzed, addition of salts caused the peak area due to NeuAc to decrease. Therefore, the salt was not added to urine.

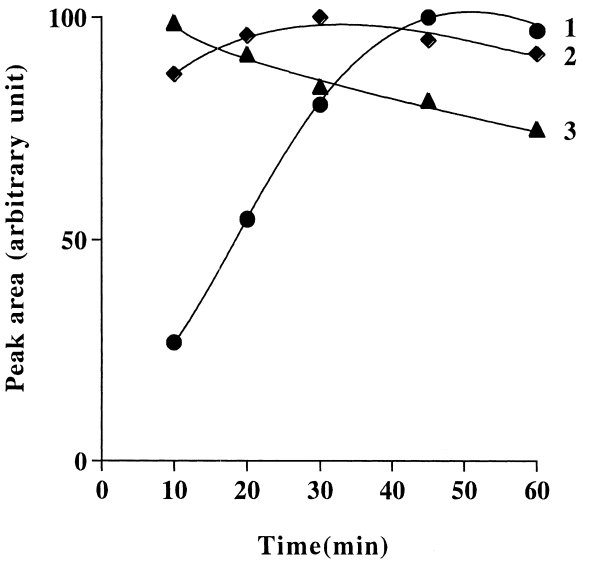

Fig. 3. Effect of reaction time and temperature on the labeling reaction of NeuAc with DMPB. Curves: (1) 10 °C; (2) 30 °C; (3) 50 °C.

Table 1
Effect of salt on the labeling reaction of NeuAc with DMPB

Salt[a]	Relative peak area ratio[b]
NaH_2PO_4	2.3
Na_2HPO_4	2.0
NaCl	0.7
$NaHCO_3$	0.5
KCl	0.4

[a] Amount: 20 nmol.
[b] The peak area ratio of NeuAc/I.S. obtained from the reaction without salt was taken as 1.0.

3.4. Linearity, detection limit, precision and recovery

The relationship between the peak area ratio of NeuAc to I.S. and the concentration of NeuAc (0.5–100 μ*M*) was linear. The detection limit of NeuAc was about 48 fmol per injection. The within-day (seven replicate assays in 1 day) and between-day (five different days) precisions were evaluated using standard solutions (10 and 20 μ*M*). The relative standard deviations of within-day were 3.3 and 2.6%, respectively, and those of between-day precisions were 3.3 and 1.7%, respectively. The recovery tests were examined using diluted urine spiked with standard NeuAc (10 and 20 μ*M*). When the recoveries were obtained from the slope ratios of regression equations with/without urine, they were 66.7–82.1% ($n=4$). As the recoveries were fairly low and fluctuated depending on urine, the standard addition method was employed for determination of urinary NeuAc.

3.5. Influence of other hydroxyl compounds

The influence of other hydroxyl compounds on the determination of NeuAc was examined with urine spiked with D-ribose, D-glucose, D-mannitol, catechol and *N*-glycoylneuiraminic acid (NeuGc). These hydroxyl compounds except for NeuGc did not interfere with the determination of NeuAc. Although the peak due to NeuGc was eluted at 7.2 min and partially overlapped with that of NeuAc, NeuGc is said to be absent in normal urine and exists in only cancer cells at very low concentration [9,12]. Therefore, the influence of NeuGc on the determination of urinary NeuAc is negligible.

3.6. Determination of NeuAc in urine

The concentrations of NeuAc in normal urine were determined by means of the standard addition method. That is, it is the area ratio due to the standard NeuAc to IS the result of subtracting the peak area ratio of the urinary intact NeuAc to IS from the peak area ratio of NeuAc in the urine spiked with standard NeuAc to IS. Therefore, the concentration of NeuAc in diluted urine was obtained from the peak area ratio of the urinary intact NeuAc to IS using the concentration of the spiked standard NeuAc and the area ratio due to the standard NeuAc to I.S. Urinary creatinine was measured with Creatinine Test Wako (Wako). The concentrations were 8–89 nmol/mg creatinine (mean value: 30±28 nmol/mg creatinine, $n=9$). These values were similar to those reported previously [1,9].

The proposed method for determination of urinary NeuAc employing HPLC with fluorescence detection is highly sensitive, reliable and useful in clinical research.

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