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2-Amino-3-phenylpyrazine, a sensitive fluorescence prelabeling reagent for the chromatographic or electrophoretic determination of saccharides

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Abstract

2-Amino-3-phenylpyrazine is found to be a sensitive fluorescence labeling reagent for saccharides with a reducing end. The labeled monosaccharides show strong fluorescence under various pH conditions, and could be analyzed by both HPLC and HPCE techniques. Laser induced fluorescence detection is also applicable. Following derivatization with 2-amino-3-phenylpyrazine, six monosaccharides are separated by an HPCE system within 23 min in the calibration range of 5 or 10 fmol to 5 pmol (injection amount). The within-day and day-to-day precisions of the monosaccharide determinations are 3.83–4.86% (RSD) and 3.37–4.56% (RSD), respectively. This method was successfully applied to the determination of component monosaccharides in a glycoprotein, bovine serum fetuin.

Keywords: Derivatization, electrophoresis; Aminophenylpyrazine; Saccharides

1. Introduction

Carbohydrate analysis of glycoconjugates is an important technique for the identification of sugar chains, and for the determination of their component monosaccharides. The chemical structures of sugar chains are closely related to the biological activity of the glycoconjugates, especially as regards signal transmission and molecular recognition [1]. In addition, it is well known that a sugar chain has some variety in the same glycoconjugate, leading to differences in the biological activity between these glycoconjugate variants [2,3]. Therefore, precise saccharide analysis is essential for biochemical research into glycoconjugates, and for creating new drugs having sugar moiety such as recombinant glycoproteins.

So far, various analytical methods including MS, GC, LC and CE have been developed for the determination of saccharides [4–11]. Because carbohydrates do not have strong absorbance or fluorescence, precolumn [7] or postcolumn [8] labeling with some reagents to form chromophores or fluorophores is often used for the sensitive determination of saccharides. The aldehyde group at the reducing end or the hydroxyl group are used for the derivatization, and a variety of methods have been reported [6,7,12–14]. Among these methods, reductive amination, which introduces an aromatic amine to the aldehyde group at the reducing end of saccharide before HPLC [15–20] or HPCE [21–25] separation is widely used, and various reagents such as 2-aminopyridine [15,16], aminopyrazine [18–20], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [23] and 9-amino-

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pyrene-1,4,6-trisulfonate (APTS) [24] have been reported. Compared to the methods using hydroxyl groups, reductive amination has the advantage of forming only one product by the derivatization, resulting in precise and convenient quantification. In previous research, we reported aminopyrazine as one of the sensitive fluorescence labeling reagents for monosaccharides and oligosaccharides with a reducing end [18–20]. Although the sensitivity of this reagent is thought to be sufficient, the saccharide analysis needs high accuracy in addition to sensitivity. Thus a reagent which is sufficiently applicable to the multi-dimensional method using various separation techniques under various pH conditions, is required because of the complicated sugar structures as well as the confusion of biological matrices. In this context, the widely used reagents as well as aminopyrazine have some disadvantages: the fluorescence intensities change with pH, and most of the reagents are only applicable to either LC or CE separation.

Therefore, in the present research, we investigated the fluorescence properties of 22 heterocyclic aromatic amines including aminopyridines, aminopyrimidines and aminopyrazines, searching for a novel fluorescence labeling reagent, and found that 2-amino-3-phenylpyrazine (3-APP) shows strong fluorescence under various pH conditions. The precolumn fluorescence labeling conditions of monosaccharides with 3-APP was optimized, and the sensitivity of the determination of monosaccharide labeled with 3-APP was compared to those labeled with 2-aminopyridine and aminopyrazine using both HPLC and HPCE. As an application, component analysis of monosaccharides in a glycoprotein was also performed.

2. Experimental

2.1. Materials

2-Aminopyridine and aminopyrazine was purchased from Nacalai Tesque (Kyoto, Japan). Dimethylamine-borane and all monosaccharides (galactose (Gal), mannose (Man), xylose (Xyl), ribose (Rib), fucose (Fuc), *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc)) were obtained from Wako (Osaka, Japan). Bovine serum fetuin was a product of Sigma (St. Louis, MO, USA). 3-APP and 2-amino-5-phenylpyrazine were synthesized in our laboratory as described by Nakamura et al. [26] and Sugiura et al. [27], and the authenticity and the purity were checked by ^{1}H-NMR, fast atom bombardment (FAB) MS and elemental analyses. Water was purified using a Milli-Q II system (Millipore, Bedford, MA, USA). All other reagents and solvents were of reagent grade and used without further purification.

2.2. Measurement of fluorescence spectra

The heterocyclic aromatic amines were dissolved in 100 m*M* of sodium phosphate buffer (pH values 4.0, 5.0, 6.0 and 7.0) or in 100 m*M* of sodium borate buffer (pH values 8.0, 9.0 and 10.0) to obtain 10 μ*M* solutions. The fluorescence excitation and emission spectra were then measured using an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) with a 1×1-cm quartz cell.

2.3. Precolumn (precapillary) fluorescence labeling of monosaccharides

Derivatization of the monosaccharides using 2-aminopyridine and aminopyrazine was carried out as described by Suzuki et al. [15] and Kiguchi et al. [19], respectively. Briefly, 2-aminopyridine was added to the lyophilized monosaccharide samples, and the mixture was heated at 90 °C for 15 min. The reaction mixture was evaporated, and dimethylamine-borane was added to the residue and heated at 90 °C for 30 min. The resulting solution was dried and applied to the HPCE and RP-HPLC systems described in Sections 2.4 and 2.5.

For the derivatization of the monosaccharides with aminopyrazine, the lyophilized monosaccharides were dissolved in acetic acid containing aminopyrazine and heated at 90 °C for 30 min. Dimethylamine-borane in acetic acid was then added and heated at 90 °C for 5 min. A portion of the reaction mixture was dried and applied to HPCE and HPLC analysis.

Derivatization of the monosaccharides with 3-APP was carried out as follows. The lyophilized monosaccharides (100 nmol) were placed in a glass vial, and dissolved in 10 μl of 0.5 *M* 3-APP in acetic acid.

The vial was tightly capped and heated at 90 °C for 30 min. Then 10 μl of 0.7 *M* dimethylamine-borane in acetic acid was added to the reaction mixture, and the mixture was heated at 90 °C for 15 min. The entire quantity of the reaction mixture was evaporated to dryness using a Speedvac at 40 °C, and the residue was dissolved in 1 ml (or dissolved in 250 μl for the HPCE separation of six monosaccharides, and to determine component monosaccharides of fetuin) of the HPCE running buffer (100 m*M* sodium borate buffer, pH 9.0 or 300 m*M* sodium borate buffer, pH 10.5), and used for HPCE analysis. A part of this solution was diluted 2000 times with water and analyzed using the RP-HPLC system.

2.4. HPCE separation of Gal and GalNAc derivatives

Capillary electrophoresis was performed using an 890-CE system (Jasco, Tokyo, Japan) equipped with a FP-1520 fluorescence detector (Jasco). The electrophoretic separations were carried out using an uncoated fused-silica capillary (Supelco, Bellefonte, PA, USA) of 90 cm (effective length 50 cm×50 μm I.D.). Samples were loaded by hydrodynamic injection (10 cm, 30 s, injection volume 5 nl). Electrophoresis was performed at 25-kV constant voltage in the 100 m*M* sodium borate buffer (pH 9.0). Fluorescence detection of the 2-aminopyridine derivative and the aminopyrazine derivative was carried out at 390 nm (excitation at 320 nm) and 410 nm (excitation at 245 nm) as described in Refs. [15,19], respectively. The 3-APP derivative was detected at 440 nm with excitation at 340 nm. The fluorescence excitation and emission wavelengths used for the determination of 3-APP derivatized monosaccharides were chosen according to the fluorescence excitation and emission spectra of 3-APP derivatives of Gal and GalNAc. Laser-induced fluorescence (LIF) detection of the 3-APP derivative was performed using a He–Cd laser as the excitation (325 nm) light source (ZETALIF detector, Picometrics, Ramonville, France).

2.5. HPLC separation of Gal and GalNAc derivatives

The HPLC system consisted of a PU-980 pump (Jasco), a 7725i injector (Rheodyne, Cotati, CA, USA), an FP-920 fluorescence detector (Jasco) and an 807-IT integrator (Jasco). The analytical column used was a Mightysil RP-18 GP (150×4.6 mm I.D., Kanto Chemical, Tokyo, Japan) kept at 40 °C in a CO-965 column oven (Jasco). The mobile phase was 50 m*M* sodium phosphate buffer (pH 5.0)–acetonitrile (99.2:0.8, v/v) for the separation of the 2-aminopyridine and the aminopyrazine derivatives, and 50 m*M* sodium phosphate buffer (pH 5.0)–acetonitrile (85:15, v/v) for separation of the 3-APP derivatives. The flow rate was 1.0 ml/min. Fluorescence detection of the 2-aminopyridine derivative was performed at 400 nm with excitation at 320 nm, and aminopyrazine and the 3-APP derivatives were detected as described in Section 2.4.

2.6. Hydrolysis of bovine serum fetuin

The hydrolysis and re-*N*-acetylation of fetuin was performed as described by Fu and O'Neill [28]. Briefly, fetuin (50 μg) was dissolved in 100 μl of water, and 100 μl of 8 *M* trifluoroacetic acid (TFA) was added and the mixture was heated at 120 °C for 2 h. The reaction mixture was then dried, and 100 μl of 2-propanol and 1 μg of D-Rib (dissolved in 10 μl of water) were added to the residue. The solution was dried again, and 200 μl of MeOH–pyridine–water (30:15:10, v/v) and 10 μl of acetic anhydride were added to the residue and allowed to stand at room temperature for 30 min. The resulting mixture was dried and derivatized with 3-APP as described in Section 2.3, then analyzed by the HPCE system. An aqueous solution (100 μl) containing Gal, Man, Xyl, Fuc, GlcNAc and GalNAc (1 μg each) was hydrolyzed and analyzed in the same way, and corrected the destruction during acid hydrolysis and yield of derivatization. The HPCE separation of the component monosaccharides of fetuin was performed at a 20-kV constant voltage in 300 m*M* sodium borate buffer (pH 10.5). The other conditions were the same as described in Section 2.4.

3. Results and discussion

3.1. Fluorescence properties of heterocyclic aromatic amines

The fluorescence characteristics of 22 heterocyclic

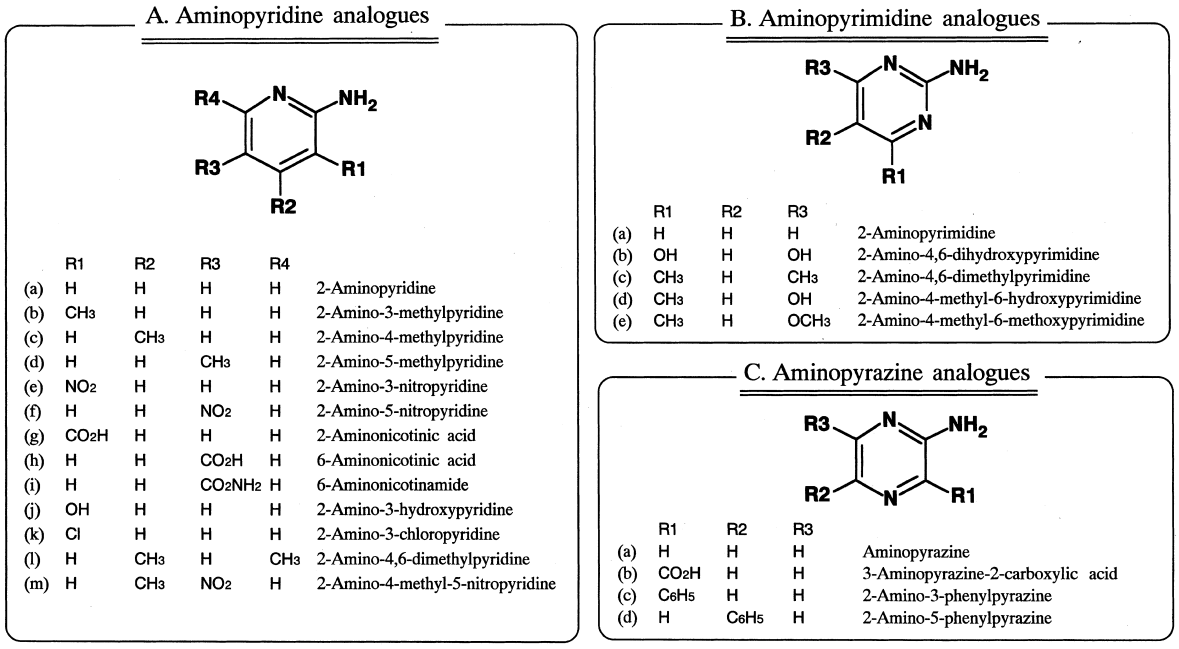

Fig. 1. Structures of the tested (A) aminopyridine analogues, (B) aminopyrimidine analogues and (C) aminopyrazine analogues.

aromatic amines including 13 aminopyridines, five aminopyrimidines and four aminopyrazines were investigated. The tested compounds are shown in Fig. 1. The fluorescence intensities, and the excitation and emission wavelengths of these compounds were investigated in various buffer solutions of pH 4.0–10.0. As shown in Fig. 2, strong fluorescence was observed for 13 of the heterocyclic aromatic amines. The wavelengths of the fluorescence excitation and emission maxima of the heterocyclic aromatic amines change with varying pH; the wavelengths listed in Fig. 2 are those at which the highest fluorescences were observed. The fluorescence intensities of 2-aminopyridine, 2-amino-3-methylpyridine, 2-amino-4-methylpyridine, 2-amino-5-methylpyridine and 2-amino-3-hydroxypyridine are high under acidic conditions, and low under alkaline conditions, while the fluorescence intensities of 2-amino-4,6-dimethylpyrimidine and aminopyrazine are higher under alkaline conditions than under acidic conditions. On the other hand, the fluorescence intensities of 6-aminonicotinic acid, 6-aminonicotinamide, 2-amino-4,6-dimethylpyridine, 2-aminopyrimidine, 2-amino-3-phenylpyrazine and 2-amino-5-phenylpyrazine did not significantly change under the various pH conditions; 2-amino-3-phenylpyrazine (3-APP) and 2-amino-5-phenylpyrazine have especially strong fluorescences. In addition, the fluorescence excitation maximum wavelength of 3-APP is 331 nm, and therefore this reagent is expected to be suitable for use with one of the laser beams (He–Cd laser, 325 nm) as an excitation light source in addition to the conventional fluorescence detector. These results suggest that 3-APP is a useful fluorescence labeling reagent for sensitive determination under various pH conditions, and is suitable for monosaccharide analysis using various separation techniques.

3.2. Precolumn (precapillary) fluorescence derivatization of monosaccharides with 2-amino-3-phenylpyrazine

The sensitivity of monosaccharide determination using 3-APP was compared with those obtained using 2-aminopyridine, a widely used reagent, and aminopyrazine, a reagent we previously reported, under both acidic and alkaline conditions. Gal and

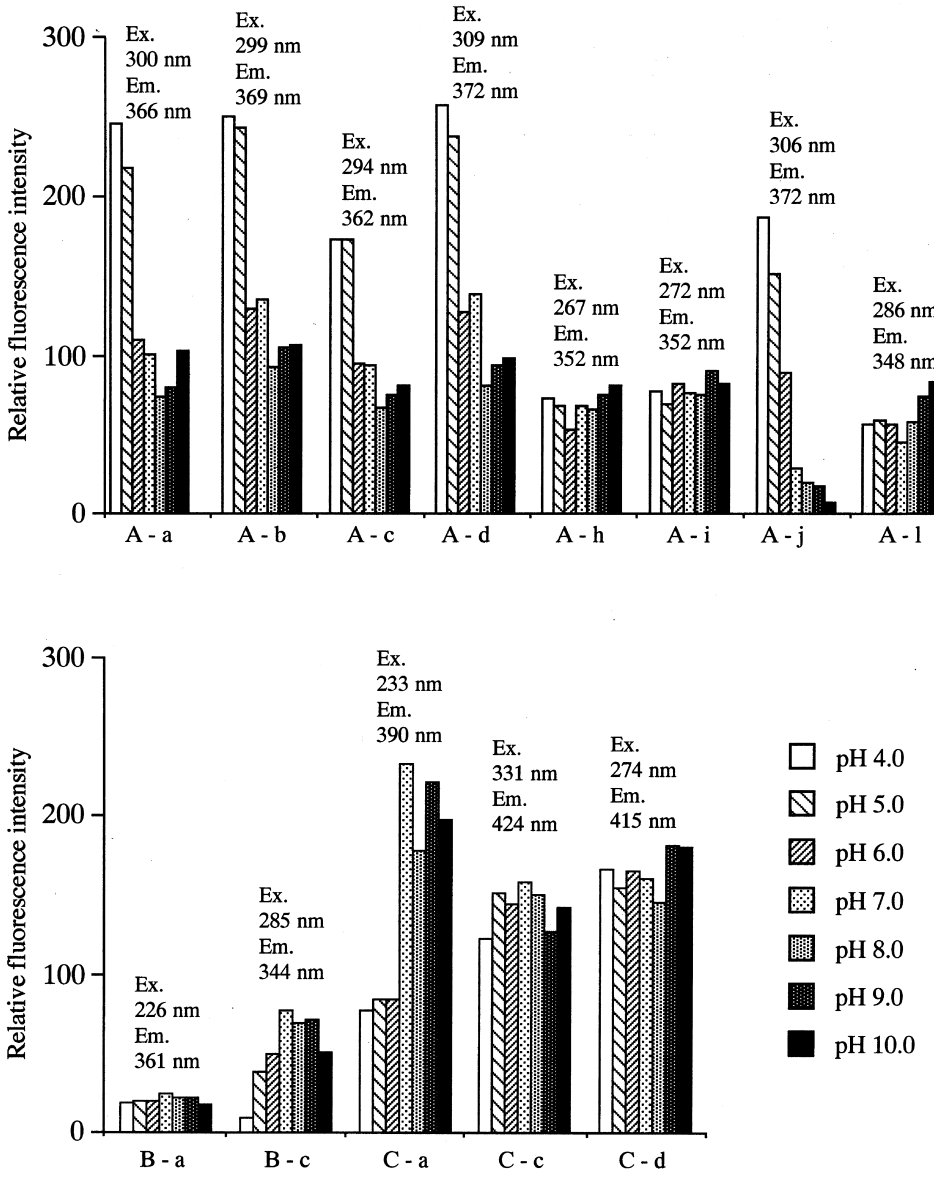

Fig. 2. Relative fluorescence intensities of the aminopyridine, aminopyrimidine and aminopyrazine analogues under various pH conditions. The fluorescence intensity of aminopyridine at pH 7.0 is taken as 100.

GalNAc were used as the monosaccharides. Since this is the first time 3-APP was applied as a fluorescence derivatizing reagent for saccharides, the derivatization conditions were optimized by changing the amount of the added 3-APP, the reaction temperature and the reaction time. The amounts of the labeled monosaccharides formed were monitored by an HPCE system with a fluorescence detector. The peak heights of the labeled monosaccharides increased with increase in the 3-APP concentration up to 0.5 *M*, but did not change much thereafter. The peak heights also increased with increasing reaction temperature, and maximum values were obtained at 90 °C. Under these conditions, the peak heights

reached a plateau after a 30-min reaction. As a result, the monosaccharides were labeled with 0.5 *M* 3-APP at 90 °C for 30 min. Dimethylamine-borane (0.7 *M*) was then added and heated at 90 °C for 15 min to reduce the labeled monosaccharides.

Derivatization of Gal and GalNAc with 2-aminopyridine and aminopyrazine was carried out as previously described [15,19], and the monosaccharides derivatized with three heterocyclic aromatic amines were determined by an RP-HPLC system and an HPCE system with fluorescence detection. The acidic mobile phase of the 50 m*M* sodium phosphate buffer (pH 5.0) in acetonitrile was used for the RP-HPLC and 500 fmol of Gal and GalNAc derivatized with 2-aminopyridine, aminopyrazine and 3-APP were determined. The ratios of the peak heights to the baseline noise (*S*/*N* values) for the 3-APP derivatives were 389 for Gal and 809 for GalNAc, which were higher than those obtained for the 2-aminopyridine derivatives (318 for Gal, and 284 for GalNAc) and aminopyrazine derivatives (17 for Gal, and 72 for GalNAc). The alkaline running buffer of 100 m*M* sodium borate buffer (pH 9.0) was used for the HPCE and the derivatives for 500 fmol of Gal and GalNAc were separated. Under alkaline conditions, the *S*/*N* values for the 3-APP derivatives were 271 for Gal and 374 for GalNAc, which were higher than those obtained for the 2-aminopyridine derivatives (149 for Gal, and 135 for GalNAc) and aminopyrazine derivatives (6 for Gal, and 78 for GalNAc). These results indicate that 3-APP was suitable as a sensitive fluorescence derivatizing reagent for the monosaccharides under both acidic and alkaline conditions. In addition, the 3-APP reagent was successfully applied to LIF detection (He–Cd laser). Fig. 3 shows the electropherogram separating the derivatives for 15 amol of GalNAc and Gal. The sensitivity is about 100 times higher than that obtained by a conventional fluorescence detector. This result also indicates the usefulness of the 3-APP reagent.

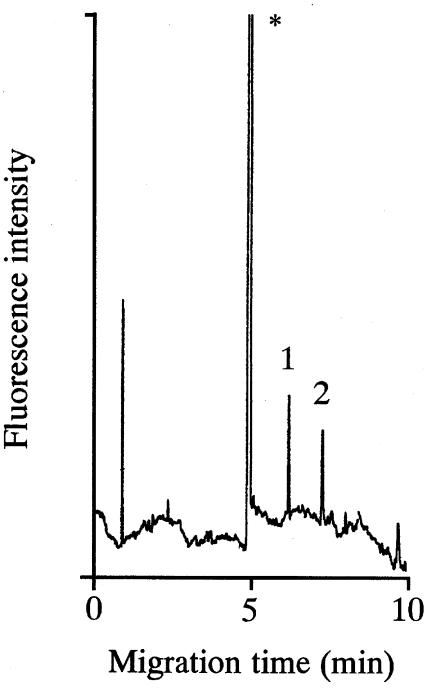

Fig. 3. Electropherogram of Gal and GalNAc derivatized with 2-amino-3-phenylpyrazine obtained by laser-induced fluorescence detection. The amounts of the monosaccharides used are 15 amol for both Gal and GalNAc. Peaks: (1) GalNAc derivative; (2) Gal derivative. Reagent peaks are denoted by asterisks. HPCE conditions are described in the text.

3.3. HPCE separation of monosaccharides derivatized with 2-amino-3-phenylpyrazine

The HPCE separation of six monosaccharides (Gal, Man, Xyl, Fuc, GlcNAc and GalNAc) derivatized with 3-APP was investigated by changing the pH (8.0–11.0) and the concentration (100–300 m*M*) of the running buffer solution used for electrophoresis. These monosaccharides are the neutral and *N*-acetylated monosaccharides widely observed in mammalian glycoproteins [6,12,13,20,28–30]. Higher resolution for the 3-APP labeled monosaccharides was obtained with increased pH of the running buffer solution. However, the separation lacks reproducibility at pH 11.0 because of the high electrical current. Therefore, the buffer solution of pH 10.5 was selected, and the concentration of the borate buffer was then examined. As a result, satisfactory separation of the monosaccharides was obtained using the 300 m*M* sodium borate buffer (pH 10.5). Under these conditions, six monosaccharides derivatized with 3-APP were well separated in about 20 min (Fig. 4). The peak height for GalNAc was about twice as high as those for the other monosaccharides.

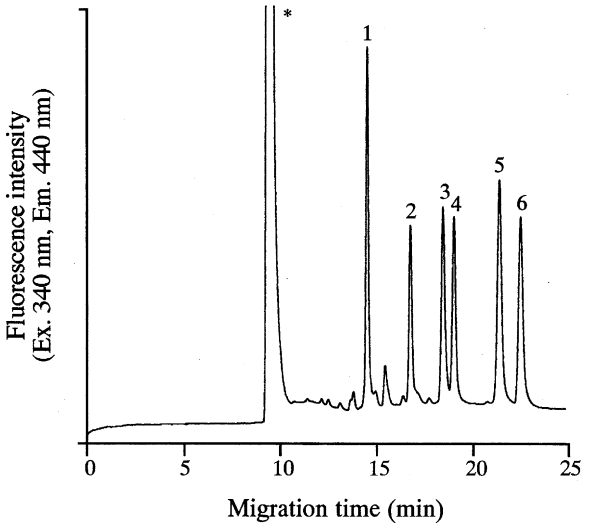

Fig. 4. HPCE separation of six monosaccharides derivatized with 2-amino-3-phenylpyrazine. Peaks: (1) GalNAc derivative; (2) Xyl derivative; (3) GlcNAc derivative; (4) Man derivative; (5) Fuc derivative; (6) Gal derivative. Reagent peak is denoted by an asterisk. HPCE conditions are described in the text.

Under these conditions, glucose, which often appears as a contaminant, eluted between GalNAc and Man as the 3-APP derivatives. The calibration ranges, correlation coefficients, detection limits, within-day and day-to-day precisions are summarized in Table 1. The calibration curves of all the tested monosaccharides showed good linearity in the range of 5 or 10 fmol to 5 pmol (injection amount) with correlation coefficients greater than 0.999. The within-day and day-to-day precisions were less than 5% in all cases. The detection limits of the monosaccharides labeled with other reagents are 10–800 fmol using a conventional fluorescence detector [19,20,30]. It is also reported that the detection limits are about 100 amol using HPCE–LIF detection [23,31]. In the present investigation, the detection limits of the 3-APP labeled monosaccharides are 5–10 fmol using an HPCE system with a conventional fluorescence detector. In addition, the 3-APP labeled monosaccharides could be determined by an LIF detector, and the sensitivity was 100 times higher than that using a conventional fluorescence detector. These results indicate that 3-APP is a suitable fluorescence labeling reagent for the sensitive and accurate determination of monosaccharides.

For application of 3-APP with biological samples, a component analysis of the monosaccharides in a glycoprotein is performed. As a glycoprotein, bovine serum fetuin is used and the neutral and *N*-acetylated monosaccharides are determined. Fetuin was hydrolyzed using 4 *M* TFA and re-*N*-acetylated, then derivatized with 2-amino-3-phenylpyrazine. Ribose was used as an internal standard, and the reaction mixture was analyzed by the HPCE system described above. The electropherogram is shown in Fig. 5. In the reaction mixture of the bovine serum fetuin, GalNAc, GlcNAc, Man and Gal were observed, and the obtained values are as follows: GalNAc, 7.9; GlcNAc, 44.7; Man, 27.5; Gal, 39.9 μg/mg protein. The component monosaccharides of the same preparation of fetuin were determined by our previously reported method using aminopyrazine [20]. The obtained values (GalNAc, 7.4; GlcNAc, 31.4; Man, 33.8; Gal, 40.7 μg/mg protein) are almost the same as those obtained using 3-APP. These values are also consistent with those already reported (GalNAc, 5.4–7.0; GlcNAc, 26.5–56.0; Man, 23.0–30.5; Gal, 34.9–45.9 μg/mg protein) [6,12,13,20,28–30].

In the present investigation, 3-APP was found to

Table 1
Validation of monosaccharide determination using 2-amino-3-phenylpyrazine

Monosaccharide	Linearity range (fmol)	Correlation coefficient (r)	Detection limit (fmol, $S/N=3$)	Precision, RSD (%)	
				Within-day	Day-to-day
GalNAc	5–5000	0.999	5	3.97	4.22
Xyl	10–5000	0.999	10	3.83	4.17
GlcNAc	10–5000	0.999	10	4.85	3.64
Man	10–5000	0.999	10	4.69	3.37
Fuc	10–5000	0.999	10	4.72	4.56
Gal	10–5000	0.999	10	4.86	4.28

The within-day and day-to-day values present the RSD of five analyses using 200 fmol (injection amount) of the monosaccharides.

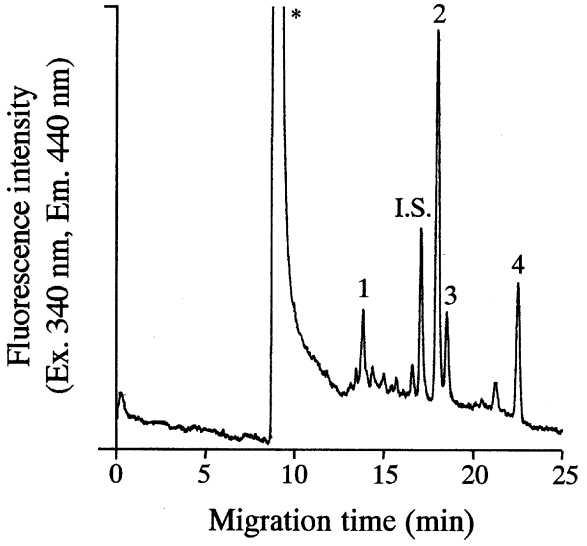

Fig. 5. Electropherogram of the component monosaccharides of fetuin derivatized with 2-amino-3-phenylpyrazine. Peaks: (1) GalNAc derivative; (2) GlcNAc derivative; (3) Man derivative; (4) Gal derivative. Ribose is used as an internal standard (I.S.). Reagent peak is denoted by an asterisk. Other conditions are the same as described in Fig. 4.

be a useful fluorescence labeling reagent for monosaccharides, having both sufficient sensitivity and accuracy. 3-APP labeled monosaccharides have strong fluorescence under various pH conditions, which is thought to be suitable for sensitive analysis using various separation modes. In addition, the labeled monosaccharides could also be detected by an LIF detector, and this 3-APP reagent was successfully applied to the component monosaccharide analysis of glycoproteins. These results indicate that the 3-APP reagent is a powerful tool for the monosaccharide analysis of various biological samples.

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