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# High-performance liquid chromatographic analysis of hexosamines, hexosaminitols, N-acetylhexosamines and N-acetylhexosaminitols by ultraviolet and fluorescence detection at picomole levels

Zhidan Zhang[a], Zhang Renen[b], Guoquan Liu[a,*]

[a]*Institute of Chemistry, Academia Sinica, Beijing 100080, China*

[b]*Glyco-Reagent Laboratory, KEHUA Chemical Corporation, Institute of Chemistry, Academia Sinica, Beijing 100080, China*

**Abstract**

A simple and rapid method for the preparation of 9-fluorenylmethoxycarbonyl (Fmoc) derivatives of hexosamines (glucosamine, galactosamine), hexosaminitols (glucosaminitol, galactosaminitol), N-acetylhexosamines (N-acetylglucosamine, N-acetylgalactosamine) and N-acetylhexosaminitols (N-acetylglucosaminitol, N-acetylgalactosaminitol), is described. The derivatization was performed at room temperature for 5–10 min under neutral and slightly alkaline conditions. The separation of Fmoc-amino sugar and amino sugar alcohol derivatives on various reversed-phase columns (Cosmosil $C_{18}$, Sinopak-S $C_8$ and Develosil $C_{18}$) with isocratic elution was examined. The determination limits of hexosaminitol, N-acetylhexosamine, and N-acetylhexosaminitol derivatives were 0.4, 1.4 and 1.6 pmol with fluorescence detection and 4.6, 16 and 20 pmol with ultraviolet detection, respectively.

*Keywords:* Derivatization, LC; Detection, LC; Hexosamines; Hexosaminitols; Oligosaccharides; Amino sugars; Amino sugar alcohols

## 1. Introduction

The structural information on O-linked oligosaccharides of mucins (mucous glycoproteins) and other glycoproteins containing the GalNAc–Ser (Thr) linkage has been obtained by cleaving the Ser(Thr)–GalNAc linkage, usually through $\beta$-elimination and reduction under mildly alkaline conditions, which leads to the recovery of oligosaccharides as the alditol derivatives [1]. The occurrence of GlcNAc in O-glycosidic linkages has been reported [2]. This precludes further labelling and derivatization at the reducing terminus for determination of the composition and monosaccharide sequence of O-linked oligosaccharides. Therefore, a sensitive and convenient method for the separation and determination of hexosamines and N-acetylhexosamines and the corresponding alditols is essential for obtaining structural information on O-linked oligosaccharides released by alkali-catalysed $\beta$-elimination with reduction.

The use of N-acetylhexosaminitols labelled with sodium [$^3$H]borohydride has been reported

* Corresponding author.

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[3]. High-performance anion-exchange chromatography (HPAEC)–pulsed amperometric detection (PAD) is ineffective for hexosaminitol determination owing to the lack of an acidic anomeric hydroxyl group [4]. However, cation-exchange chromatography–PAD was used for the separation of hexosaminitols recently [5]. Labelling agents for amino acids, phenyl isothiocyanate (PITC) [6] and *o*-phthalaldehyde (OPA) [7], have been used for derivatizing hexosamines and hexosaminitols. Our laboratory has previously used 9-fluorenylmethyl chloroformate (Fmoc-Cl) to label hexosamines [8]. In this work, the use of Fmoc-Cl was extended for labelling hexosaminitols, N-acetylhexosamines and N-acetylhexosaminitols. The separation and determination of these derivatives were performed by RP-HPLC with ultraviolet and fluorescence detection at picomole and low-picomole levels.

## 2. Experimental

### 2.1. Chemical and samples

9-Fluorenylmethyl chloroformate (Fmoc-Cl), glucosaminine·HCl (GlcN), galactosamine·HCl (GalN), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), N-acetylglucosaminitol (GlcNAc-ol) and N-acetylgalactosaminitol (GalNAc-ol) were obtained from Sigma. Galactosaminitol was a gift from Professor V.P. Bhavanandan (Department of Biochemistry, Pennsylvania State University). $NaBH_4$ was obtained from Aldrich.

### 2.2. Preparation of hexosaminitols and N-acetylhexosaminitols

Glucosamine, galactosamine, N-acetylglucosamine and N-acetylgalactosamine (ca. 10 nmol, 10 $\mu$l) were reduced with different molecular ratios of $NaBH_4$ to amino sugar alcohols in 100 $\mu$l of 0.05 *M* NaOH at room temperature for 16 h. Excess $NaBH_4$ was discharged with 100 $\mu$l of acetic acid at 40°C for 4 h, 1 *M* HCl at 40°C for 30 min or 1 *M* trifluoroacetic acid at 40°C for 4 h and dried with nitrogen. The dried sample was repeatedly treated with methanolic HCl (0.1%) to remove boric acid as methyl borate, then the residue was dissolved in water and the pH of the aqueous solution of the sample was near 6.

### 2.3. Derivatization of hexosaminitols, N-acetylhexosamines and N-acetylhexosaminitols with Fmoc-Cl

Derivatization was performed in a 1.5-ml Teflon tube equipped with a screw-cap. To 10 $\mu$l of aqueous solution containing 1–10 nmol of test or control sample were added 100 $\mu$l of 0.2 *M* borate buffer (pH 7.0) for hexosamines, hexosaminitols and N-acetylhexosaminitols, the pH was adjusted to 10 for N-acetylhexosamines, 100 $\mu$l of Fmoc-Cl of different concentrations in acetonitrile were added and the mixture was allowed to react at room temperature for 5–10 min and then injected directly on to the column. Excess Fmoc-Cl and its hydrolysate, Fmoc-OH, can be removed by extracting with pentane 3–4 times if it is necessary.

### 2.4. RP-HPLC of Fmoc-amino sugar derivatives

The HPLC system consisted of an Altex Scientific Model 100 pump, a Hitachi Model 635-A 10-$\mu$l sample valve, a UV detector (254 nm) and a Hitachi F-10 LC spectrofluorimeter. Fmoc-amino sugar derivatives were separated on Cosmosil 5 $C_{18}$ (150 × 4.6 mm I.D.), Sinopak-S 5 $C_8$ (250 × 5 mm I.D.) (Institute of Chemistry, Academia Sinica) and Develosil $C_{18}$ (150 × 4.6 mm I.D.) columns at room temperature using isocratic elution with either 60% aqueous methanol, 30% aqueous acetonitrile or 30% aqueous acetonitrile containing 0.1 *M* acetic acid. The sugar derivatives were detected by fluorescence with excitation and emission at 270 and 320 nm, respectively, or by ultraviolet absorbance measurement at 254 nm.

## 3. Results and discussion

### 3.1. Preparation of amino sugar alcohols

Hexosaminitols and N-acetylhexosaminitols were prepared by reduction of the corresponding

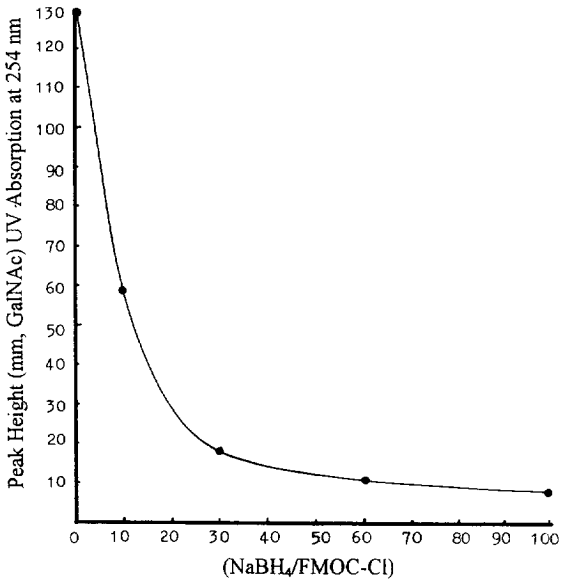



Fig. 1. Effect of $NaBH_4$ on the derivatization of GalNAc with Fmoc-Cl. Derivatization was performed at pH 10.

amino sugars in a large excess of $NaBH_4$. Excess $NaBH_4$ can inhibit the derivatization of the amino sugar alcohols in the next step (Fig. 1). This result shows that unreacted $NaBH_4$ must be decomposed and removed completely before the derivatization of the acetylated amino sugar alcohols with Fmoc-Cl. The decomposition of GlcNAc during the $NaBH_4$ removal using acetic acid at 40°C for 4 h, 1 *M* HCl at 40°C for 0.5 h and 1 *M* trifluoroacetic acid (TFA) at 40°C for 4 h to decompose excess $NaBH_4$ is shown in Fig. 2. These results indicated partial de-N-acetylation to different extents, so that 8.7, 3.6 and 0.4% of GlcN-ol were released from GlcNA-ol in HCl, TFA and acetic acid, respectively. Therefore, acetic acid is both effective in destroying excess of $NaBH_4$ with only minor de-N-acetylation from GlcNAc-ol. When acetic acid was used at room temperature (20°C) overnight, the destruction of $NaBH_4$ was sufficient for following derivatization without any de-N-acetylation.

Fmoc-Cl as a derivatization reagent can react with primary and secondary amino acids [9]. The reaction of Fmoc-Cl with amino sugars take place as shown by the example of N-acetylgalactosamine in Scheme 1.

Fmoc-Cl also reacts spontaneously with water

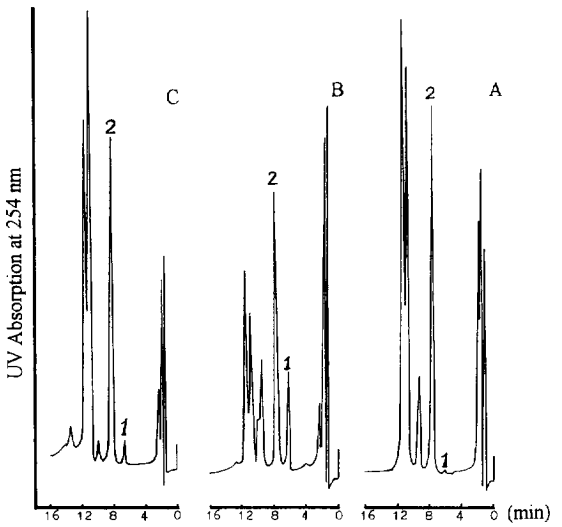
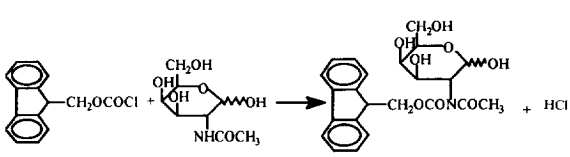



Fig. 2. Chromatogram of GlcNAc-ol obtained from reduction of GLcNAc and various methods for the decomposition of $NaBH_4$. Decomposition conditions: (A) acetic acid at 40°C for 4 h; (B) 1 *M* hydrochloric acid at 40°C for 30 min; (C) 1 *M* trifluoroacetic acid at 40°C for 4 h. Chromatographic conditions: column, Cosmosil 5 $C_{18}$ (150 × 4.6 mm I.D.); mobile phase, 60% (v/v) aqueous methanol. Peaks: 1 = GlcN-ol; 2 = GlcNAc-ol.

to yield the corresponding alcohol as a hydrolysis product. According to the reaction, the pH of the reaction medium is an important factor in controlling both the derivatization and the hydrolysis. The rate of derivatization increases with increasing pH, but is paralleled by an increased rate of hydrolysis of Fmoc-Cl. Optimum conditions are those under which the reactive amino groups most effectively compete for the limited amount of reagent available. The effect of pH on the derivatization of N-acetylhexosamines and N-acetylhexosaminitols has been studied as shown in Figs. 3 and 4. These results suggest that the optimum pH values for derivatization of N-acetylhexosamine and N-acetylhexosaminitol were 10 and 7, respectively. The results of the effect of the molar ratio of Fmoc-Cl to amino




Scheme 1.

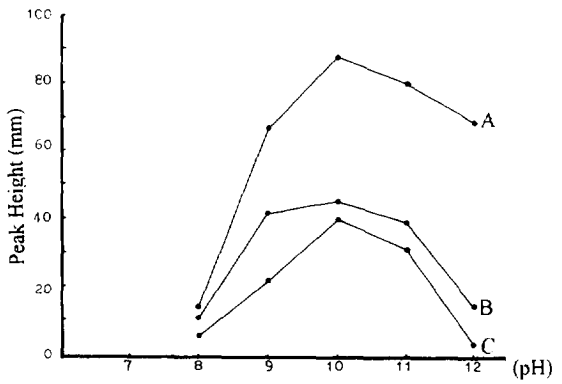



Fig. 3. Effect of pH and molar ratio of GlcNAc to Fmoc-Cl on derivatization. Molar ratio: (A) 1:50; (B) 1:30; (C) 1:20.

sugars on the derivatization indicate that a higher molar ratio is desirable for quantifying the amino sugars and the corresponding alcohols. Fmoc-amino sugar and its alcohol derivatives were stable in the derivatization solution (pH 7) at room temperature for at least 1 week.

### *3.2. RP-HPLC conditions for the separation of Fmoc-amino sugars and their alcohol derivatives*

Various $C_{18}$ and $C_8$ columns have been used to separate Fmoc-hexosamines, N-acetylhexosamines and corresponding alcohol derivatives. The capacity factors ($k'$) and selectivity ($\alpha$) are given in Table 1. According to the equation for the resolution ($R$) [10] and the values of $\alpha$ in Table 1, we can roughly estimate a column having the required resolution power, i.e., the plate number ($N$) for realizing the resolution of a pair of amino sugars.

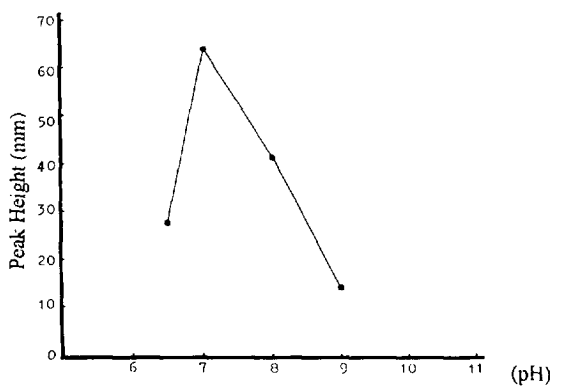



Fig. 4. Effect of pH on derivatization of GlcNAc-ol with Fmoc-Cl.

GlcN and GalN gave two peaks for each amino sugar on all columns used, which presumably represent the anomers. The values of $\alpha$ for GalN-1–GlcN-1 (in system 4) and GalN-2–GlcN-2 (in system 6) are identical, i.e., $\alpha = 1.06$, assuming $R = 1.0$, i.e., the resolution is sufficient for quantifying the amino sugars, and the required resolving power of the column is about $N = 9000$. In the six separation systems, only system 3 provided partial separation of hexosamines (Fig. 5), in which the $\alpha$ for GalN-2–GlcN-2 is 1.07. However, in other systems the values of $\alpha$ for GalN-2–GlcN-2 are 1.02–1.06. In system 1, GalNAc and GlcNAc, and GalNAc-ol and GlcNAc-ol were separated. In system 4, GalN-1, GalN-ol, GalNAc and GalNAc-ol were separated, with GalN-ol being eluted together with GalN-2 (Fig. 6). GlcN, GlcN-ol, GlcNAc and GlcNAc-ol were separated in the system 4 (Fig. 7).

These results showed that amino sugars, N-acetylamino sugars and the corresponding alcohols can be sufficiently resolved from neighbouring or related sugars for identification and quantitative measurement using the six chromatographic systems under the optimum conditions. The retention mechanism was not studied. However, from the elution order and the selectivity of Fmoc-saccharide derivatives on various reversed-phase columns, it is reasonable to assume that the hydrophobic interaction between sugar derivatives and stationary phases is the primary mechanism. However, secondary retention mechanisms, such as hydrogen bonding due to interactions between hydroxyl groups on the sugars and unreacted silanol groups on the silica gel, may play a more important role for improving the selectivity ($\alpha$) of some Fmoc-sugar derivatives. In system 4, the capacity factors of all derivatives studied are lower than on the other two $C_{18}$ columns owing to the column's lower hydrophobicity. However, the $\alpha$ values for GalNAc-ol–GalNAc and GlcNAc-ol–GlcNAc

Table 1
Capacity factors ($k'$) and selectivities ($\alpha$) of amino and acetylamino sugars and their alcohols on various reversed-phase columns

| Sugar | Cosmosil 5 $C_{18}$ | | | | Sinopak-S 5 $C_8$ | | | | | | Develosil $C_{18}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | System 1 (60% MeOH) | | System 2 (30% $CH_3CN$) | | System 3 (50% MeOH) | | System 4 (30% $CH_3CN$) | | System 5 (100 m*M* HOAc)[a] | | System 6 (30% $CH_3CN$) | |
| | $k'$ | $\alpha$ | $k'$ | $\alpha$ | $k'$ | $\alpha$ | $k'$ | $\alpha$ | $k'$ | $\alpha$ | $k'$ | $\alpha$ |
| GalN-1 | 2.07 | | 4.01 | | 1.63 | | 2.02 | | 1.85 | | 2.55 | |
| | | 1.05 | | 1.05 | | 1.12 | | 1.06 | | 1.08 | | 1.03 |
| GlcN-1 | 2.18 | | 4.23 | | 1.82 | | 2.15 | | 2.0 | | 2.65 | |
| | | 1.23 | | 1.30 | | 1.12 | | 1.22 | | 1.15 | | 1.36 |
| GalN-2 | 2.68 | | 5.48 | | 2.04 | | 2.62 | | 2.30 | | 3.61 | |
| | | 1.02 | | 1.05 | | 1.07 | | 1.04 | | 1.02 | | 1.06 |
| GlcN-2 | 2.64 | | 5.24 | | 2.19 | | 2.52 | | 2.34 | | 3.40 | |
| | | 1.07 | | 1.08 | | 1.02 | | 1.08 | | 1.02 | | 1.08 |
| GalN-01 | 2.83 | | 4.85 | | 2.24 | | 2.73 | | 2.38 | | 3.16 | |
| | | 1.02 | | 1.14 | | 1.03 | | 1.11 | | 1.17 | | 1.1 |
| GlcN-01 | 2.77 | | 4.24 | | 2.17 | | 3.02 | | 2.03 | | 2.86 | |
| | | 1.09 | | 1.39 | | 1.16 | | 1.34 | | 1.42 | | 1.52 |
| GalNAc-01 | 3.10 | | 7.59 | | 2.59 | | 4.05 | | 3.38 | | 5.48 | |
| | | 1.02 | | 1.03 | | 1.01 | | 1.09 | | 1.05 | | 1.05 |
| GalNAc | 3.17 | | 7.78 | | 2.62 | | 3.73 | | 3.23 | | 5.21 | |
| | | 1.08 | | 1.02 | | 1.04 | | 1.01 | | 1.01 | | 1.03 |
| GlcNAc | 3.42 | | 7.93 | | 2.85 | | 3.71 | | 3.35 | | 5.32 | |
| | | 1.03 | | 1.02 | | 1.04 | | 1.21 | | 1.11 | | 1.03 |
| GlcNAc-01 | 3.52 | | 8.12 | | 2.72 | | 4.50 | | 3.73 | | 5.50 | |

$k' = (t_r - t_0)/t_0$, where $t_r$ is the retention time of the Fmoc-amino sugars and $t_0$ is the retention time of an unretained peak determined using $KNO_3$ at 254 nm; $\alpha = k'_2/k'_1$, where $k'_1$ and $k'_2$ are the capacity factors of the neighbouring amino sugars.

[a] 30% aqueous acetonitrile containing 100 m*M* HOAc.

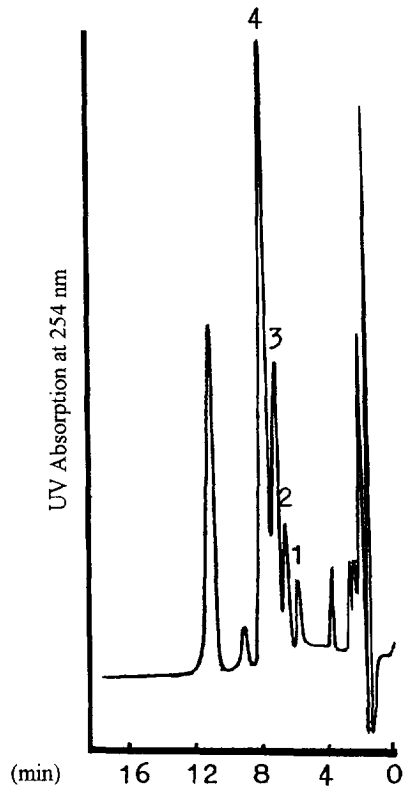



Fig. 5. HPLC of Fmoc-hexosamines. Separation was performed with system 3 in Table 1. Peaks: 1 = GalN-1; 2 = GlcN-1; 3 = GalN-2; 4 = GlcN-2.

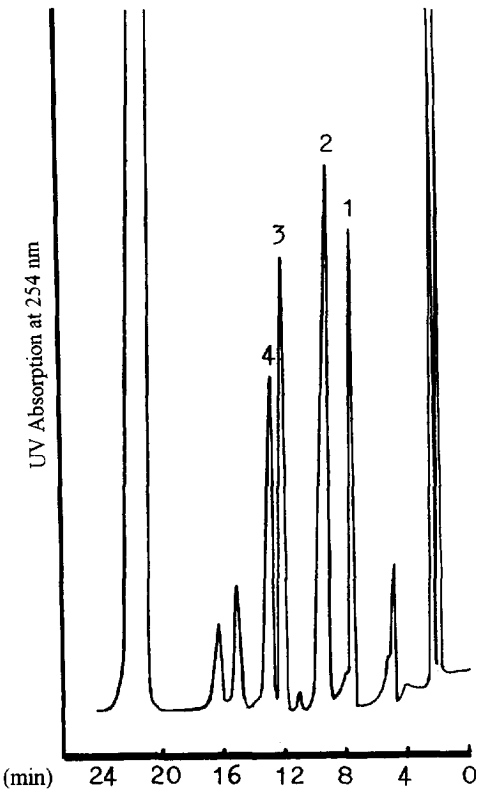



Fig. 6. HPLC of Fmoc derivatizatives of GalN, GalN-ol, GalNAc and GalNAc-ol. Separation was performed with system 4 in Table 1. Peaks: 1 = GalN-1; 2 = GalN-2 + GalN-ol; 3 = GalNAc; 4 = GalNAc-ol.

are obviously higher than in systems 2 and 6 under identical isocratic elution conditions. Therefore, these higher selectivities may be caused by the higher acidity of unreacted silanol groups on the silica gel of Sinopak-1, since it contains more heavy metals. Heavy metals may act to enhance the acidity of silanol groups in their immediate vicinity [11].

### *3.3. Stability of the Fmoc-sugar derivatives*

These studies were performed in order to examine whether the stability of Fmoc-amino sugars is suitable for elucidating the structure of the digestion product by exoglycosidases [12]. Fmoc-GlcN and -GlcNAc derivatives were incubated at pH 4.0, 6.0 and 8.0 for 50 h (Fig. 8). These results show that the stability of GlcN is higher than that of GlcNAc and decreased with increasing pH of the buffer. The recoveries of Fmoc-GlcN and Fmoc-GlcNAc at pH 4.0, 6.0 and 8.0 for 22 h were 88 and 69, 97 and 68 and 85 and 18%, respectively. These results indicated that Fmoc-GlcN is relatively stable in the pH range 4.0–8.0. However, the stability of Fmoc-GlcNAc seemed to be dependent on pH.

### *3.4. Detection limits*

Fmoc-amino sugars possess intense fluorescence and ultraviolet absorption. The optimum fluorescence wavelengths were 270 nm for excitation and 320 nm for emission, and 263 nm for ultraviolet absorption. The detection limits of Fmoc-hexosaminitols and Fmoc-N-acetylhexosamines and the corresponding alcohol derivatives were determined with a signal-to-noise ratio

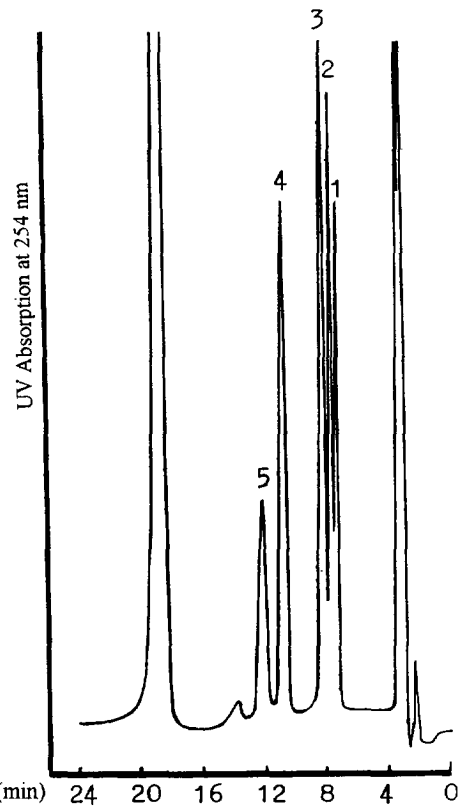



Fig. 7. HPLC of Fmoc derivatives of GLCN, GlcN-ol, GlcNAc and GlcNAc-ol. Separation was performed with system 4 in Table 1. Peaks: 1 = GlcN-1; 2 = GlcN-2; 3 = GlcN-OH; 4 = GlcNAc; 5 = GlcNAc-ol.

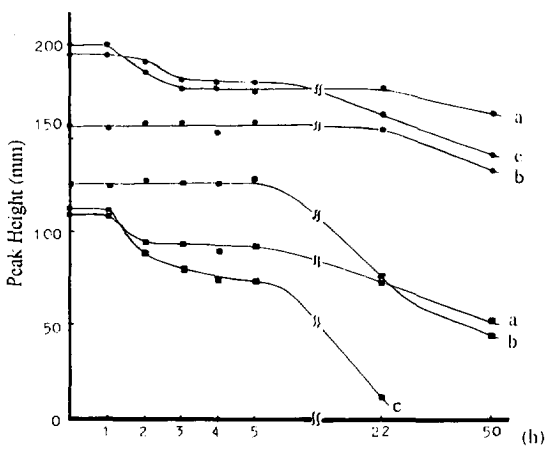



Fig. 8. Stability of Fmoc derivatives of GlcN and GlcNAc. Incubation in (a) 0.1 *M* citrate–phosphate buffer (pH 4.0), (b) 0.1 *M* phosphate buffer (pH 6.0) and (c) 0.1 *M* Tris–HCl buffer (pH 8.0) at 37°C. ● = GlcN; ■ = GlcNAc.

Table 2
Determination limits of hexosaminitols, acetylhexosamines and acetylhexosaminitols

| Sugar | Determination limit (pmol) | |
|---|---|---|
| | Fluorescence detection (ex. 270 nm, em. 320 nm) | Ultraviolet detection (254 nm) |
| GalN-ol | 0.40 | 4.6 |
| GlcN-ol | 0.43 | 4.5 |
| GalNAc | 1.4 | 17 |
| GlcNAc | 1.6 | 16 |
| GalNAc-ol | 3.8 | 20 |
| GlcNAc-ol | 1.6 | 20 |

of 3 and the results are given in Table 2. The results indicate that these derivatives can be determined in the picomole and low-picomole range with ultraviolet and fluorescence detection, respectively.

## Acknowledgements

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