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Journal of Chromatography A, 728 (1996) 343–350

JOURNAL OF CHROMATOGRAPHY A

Analysis of the 9-fluorenylmethoxycarbonyl hydrazide labelling of neutral and sialic acid-containing oligosaccharides by reversed-phase high-performance liquid chromatography

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Abstract

Maltoheptaose (M7) and neuraminyllactose (NeuAc-Lac) as models of neutral and sialyl oligosaccharides were derivatized with 9-fluorenylmethoxycarbonyl hydrazide (FMOC hydrazide) at 37°C in acetonitrile–water (50:50) containing 0.5–5% acetic acid without causing any desialylation. The reproducibility of the derivatization of M7 with FMOC-hydrazide was examined and the average relative standard deviation ($n = 9$) was 2.8%. The oligosaccharide FMOC hydrazones were separated on a Cosmosil C_{18} column by isocractic elution. The determination limits of M7 and NeuAc-Lac derivatives are at the picomole level for ultraviolet detection and the sub-picomole level for fluorescence detection.

Keywords: Derivatization, LC; Oligosaccharides; Maltoheptaose; Neuraminyllactose; 9-Fluorenylmethoxycarbonyl hydrazide

1. Introduction

In recent years, the structure and biological activities of glycoproteins have generated intense interest. In order to understand the precise roles that they play in biological processes, isolation and structural determination are often required. However, glycoproteins are usually available in only limited amounts (usually in the range 1–100 μg), which makes it difficult to determine the sequence, position and a nomeric configurations of glycosidic linkages in their carbohydrate chains [1].

Highly sensitive detection methods have been reported for oligosaccharides, such as tritium labelling at the reducing end of sugars by sodium [^{3}H]borohydride reduction [2–4]. HPAEC, using pellicular resins with a pulsed amperometric detector, has been reported for measuring oligosaccharides with high sensitivity (picomole range) without resorting to pre- or postcolumn derivatization [5]. Several methods for precolumn labelling of oligosaccharides of glycoproteins are also available. Of these, the most widely used is the reductive animation method, including the use of 2-aminopyridine [6–10], 7-amino-4-methylcoumarin [11], aniline, *p*-aminoacetophenone, ethyl *p*-aminobenzoate [12,13] and *n*-alkyl *p*-aminobenzoates [14]. However, in some instances some NeuAc residues were re-

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0021-9673/96/$15.00
SSDI 0021-9673(95)00873-X

leased from the sialic acid-containing oligosaccharide during the derivatization. We have previously reported the fluorescence reagent 9-fluorenylmethoxycarbonyl hydrazide (FMOC hydrazide) for labelling monosaccharides [15,16]. In this investigation, this reagent was used for labelling oligosaccharides. The optimization of the conditions for the derivatization of neutral and sialyl oligosaccharides with FMOC hydrazide, the reproducibility of the derivatization reaction and the stability and the detection limits of oligosaccharide FMOC hydrazones were examined.

2. Experimental

2.1. Chemicals and samples

FMOC hydrazide (purity >99%) was obtained from Glyco-Reagent Laboratory, Kehua Chemical, Institute of Chemistry, Academia Sinica (Beijing, China), maltoheptaose (M7) from Hayashibara Biochemical Laboratories and neuraminyllactose (NeuAc-Lac) from Sigma. Isomaltooligosaccharides were obtained by partial hydrolysis of dextran in dilute hydrochloric acid according to the method of Yamashita et al. [17].

2.2. Derivatization of oligosaccharides with FMOC hydrazide

All derivatizations were performed in a 13 × 20 mm I.D. Teflon tube with a screw-cap. To 10 μl of 60% methanol containing 1–100 nmol of M7 or NeuAc-Lac were added 110 μl of acetic acid (0.1–10% of final concentration) and 100 μl of FMOC hydrazide in acetonitrile. The molar ratio of oligosaccharide to FMOC hydrazide was 1:50 to 1:1000. The mixture was reacted at different temperature in a 50% aqueous solution. The derivatives were diluted progressively with the mobile phase used for eluting the column and injected directly on to the column. When a high molar ratio of FMOC hydrazide to oligosaccharides was used in the reaction, the large excess of reagent was removed by extraction with chloroform.

2.3. High-performance liquid chromatography

The apparatus used for HPLC consisted of an MIC Altex Model 100A pump, a Hitachi Model 635A injector with a 10-μl loop, a Hitachi UV 254 monitor, a Hitachi 650-10LC spectrofluorimeter (excitation at 270, emissional 320 nm) and a Bio-Rad Series 800 HPLC system. The separation of oligosaccharide FMOC hydrazones was performed on a Cosmosil 5 C_{18} reversed-phase column (150 × 4.6 mm I.D.) with methanol–water (60:40) as eluent at room temperature.

3. Results and discussion

3.1. Optimization of derivatization conditions

The reaction of FMOC hydrazide with aldehyde groups is a specific acid-catalysed reaction [18]. Therefore, the pH of the reaction medium plays an important role in the derivatization of oligosaccharides with FMOC hydrazide. We have previously reported that acetic acid, as an acidic medium, was used for the derivatization of sugars with FMOC hydrazide and found that the optimum concentration of acetic acid was around 0.25% at 65°C [15]. In this work, we studied further the optimum pH and reaction temperature for labelling neutral and sialyl oligosaccharides. The effect of the concentration of acetic acid with various derivatization temperatures and reaction times on the yield of M7 FMOC hydrazone was observed. The reaction was carried out in 0.1, 0.25, 0.5, 2.5, 5 and 10% acetic acid with a molar ratio of M7 to FMOC hydrazide of 1:50 at 65°C for 1 h (Fig. 1A) and then continued at room temperature (24–30°C) for 48, 144 and 230 h (Fig. 1B, C and D). In Fig. 1A, the results show that the derivatization yields increased with increasing concentration of acetic acid. In Fig. 1B and C, the results show that the derivatization could occur even if at room temperature in 0.1–10% acetic acid and the yields obtained in the range 0.5–2.5% acetic

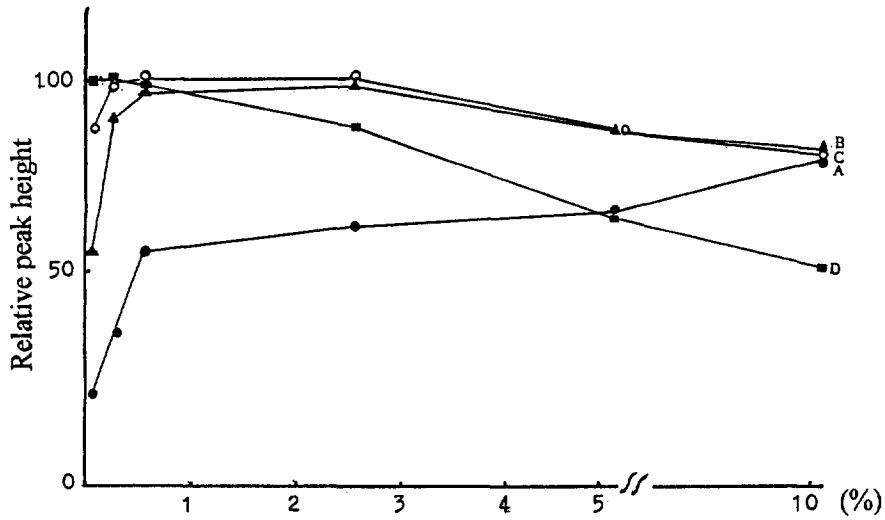

Fig. 1. Effects of the concentration of acetic acid and reaction temperature on derivatization of M7 with FMOC hydrazide. (A) Derivatization performed at 65°C for 1 h; (B), (C) and (D) derivatizations continued at room temperature (24–30°C) for 48, 144 and 230 h, respectively.

acid (corresponding pH 3.23–2.83) almost reached a similar high level. These results indicate that the derivatization is complete and the derivatives are stable under such conditions. However, the speed of reaction in the range 0.1–0.25% acetic acid is too slow for the derivatization of oligosaccharide. Fig. 1D shows the final yields of all the derivatization procedures. The yields obtained with 5 and 10% acetic acid (corresponding pH 2.65 and 2.47) were 60 and 53%, respectively, of the yields obtained in range 0.1–0.5% acetic acid. These results indicate that higher concentrations of acetic acid (<5%) and higher temperatures (65°C) could damage the stability of the derivative and reduce the derivatization yield. Fig. 2 shows the derivatization of M7 with FMOC hydrazide carried out in 0.5% acetic acid at 37°C for 6.5 h. In fact this derivatization could be completed in 2.5% acetic acid in 2.5 h.

Compared with neutral oligosaccharides, sialic acid-containing oligosaccharides partially release sialic acid residues during the derivatization, particularly when precolumn derivatization is performed under extreme reaction conditions such as in reductive amination methods [6]. The derivatization conditions for NeuAc-Lac with FMOC hydrazide were studied with the aim of obtaining the structurally intact form of the sialylated derivative. The reaction of NeuAc-Lac with FMOC hydrazide in 1.0% acetic acid at various temperatures was examined. Fig. 3 shows that sialic acid residues were released when the derivatization was performed at 60°C. However, NeuAc-Lac was stable during the reaction at 37°C (Fig. 4A). Fig. 4B shows that partial release of NeuAc residues occurred dur-

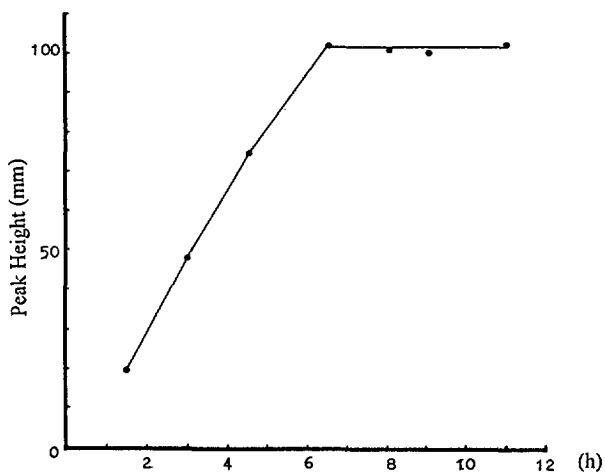

Fig. 2. Effect of reaction time on the derivatization of M7 with FMOC hydrazide at 37°C and 0.5% acetic acid.

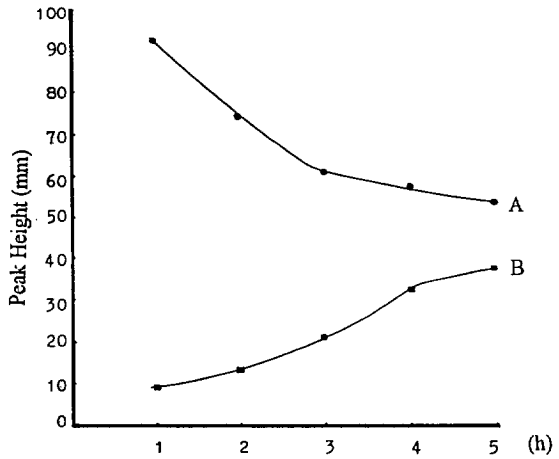

Fig. 3. Instability of N-acetylneuraminyllactose during derivatization with FMOC hydrazide at 60°C. Chromatographic conditions as in Fig. 4. (A) NeuAc-Lac; (B) Lac.

ing the reaction at 60°C for 2 h and estimated that about 18% of NeuAc residues were released from NeuAc-Lac according to the peak height for quantitative injection of lactose (Fig. 4C). Fig. 5 shows the results of the derivatization of NeuAc-Lac with FMOC hydrazide in 0.5, 2.5 and 5% acetic acid at 37°C. The results mentioned above show that the optimum concentration of acetic acid and temperature for the derivatization of neutral and sialyl oligosaccharides with FMOC hydrazide should be 0.5–5% acetic acid at 37°C.

3.2. Effect of molar ratio of FMOC hydrazide to oligosaccharide on the yield of derivatization

The reaction of FMOC hydrazide with aldehyde groups is an addition reaction. The addition product then releases water to form a stable product, FMOC hydrazone. Therefore, increasing the molar ratio of FMOC hydrazide to M7 would be of benefit to improve the yield of M7 FMOC hydrazone. As shown in Fig. 6, the yield of M7 FMOC hydrazone increased linearly with increasing molar ratio. Thus the yield of M7 FMOC hydrazone at a 1:1000 ratio of M7 to reagent is nearly twenty times higher than the yield with a 1:50 ratio. Our experiments showed that the detection limits of M7 and NeuAc

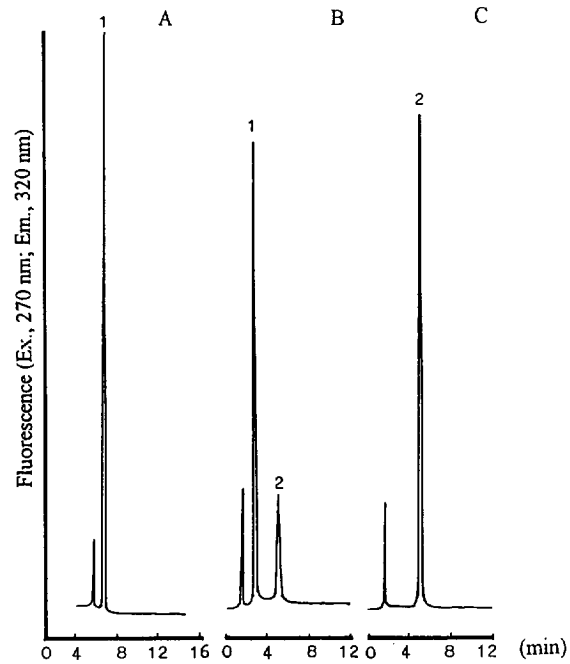

Fig. 4. Chromatograms of derivatization of lactose and N-acetylneuraminyllactose with FMOC hydrazide. Chromatographic conditions: column, Cosmosil 5 C_{18} (150 × 4.6 mm I.D.); mobile phase, methanol–water (60:40, v/v); fluorescence detection. (A) Derivatization of NeuAc-Lac with FMOC hydrazide in 1% acetic acid at 37°C for 4 h; (B) derivatization of NeuAc-Lac with FMOC hydrazide in 1% acetic acid at 60°C for 2 h; (C) derivatization of lactose with FMOC hydrazide in 1% acetic acid at 60°C for 2 h. Peaks: 1 = NeuAc-Lac; 2 = lactose.

derivatives at 1:1000 molar ratio with a signal-to-noise ratio of 3 were 40 and 50 fmol for fluorescence detection and 1 and 2 pmol for ultraviolet detection, respectively.

Although the FMOC hydrazide and breakdown products were eluted after the sugar FMOC hydrazones without interfering with the separation of sugar derivatives, the elution of a large amount of excess reagent is time consuming. Therefore, removing the excess reagent is necessary for shortening the analytical time. The efficiency of removing excess FMOC hydrazide from the derivatization reaction solution was examined by extraction with water-saturated organic solvents. The extraction was performed with 300 μl of organic solvent to extract 150 μl of derivatization solution once. The results

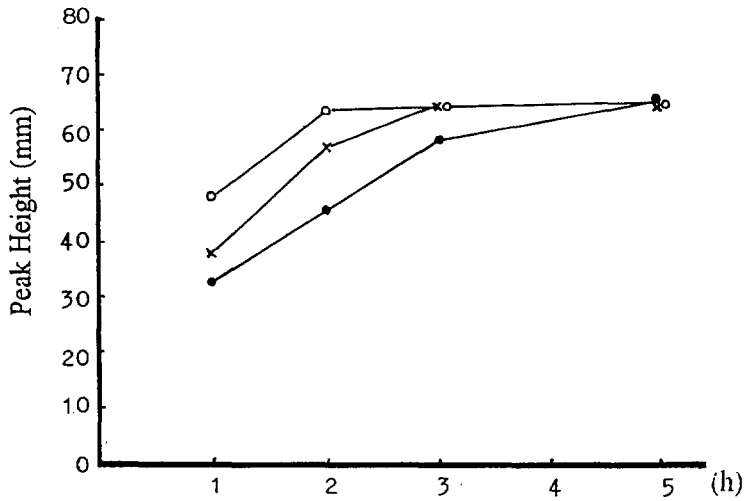

Fig. 5. Effect of concentration of acetic acid on derivatization of N-acetylneuraminyllactose with FMOC hydrazide at 37°C. Chromatographic conditions as in Fig. 4. (●) 0.5%; (×) 2.5%; (□) 5% acetic acid.

(Table 1) showed that higher polar solvents, such as ethyl acetate, cause a loss of 7.6% of M7 FMOC hydrazone and 19% of Lac FMOC hydrazone, whereas with non-polar solvents, such as pentane, the efficiency of the removal of excess reagent is poor and only 43% FMOC hydrazide is removed. Chloroform shows excellent efficiency and 99% FMOC hydrazide was removed in one run without loss of the derivatives. However, this extraction procedure is not suitable for removing excessive FMOC hydrazide from the derivatization of monosaccharides with FMOC hydrazide, since some monosaccharide FMOC hydrazones can be partially extracted into the chloroform phase. In that case, pentane could be used instead of chloroform.

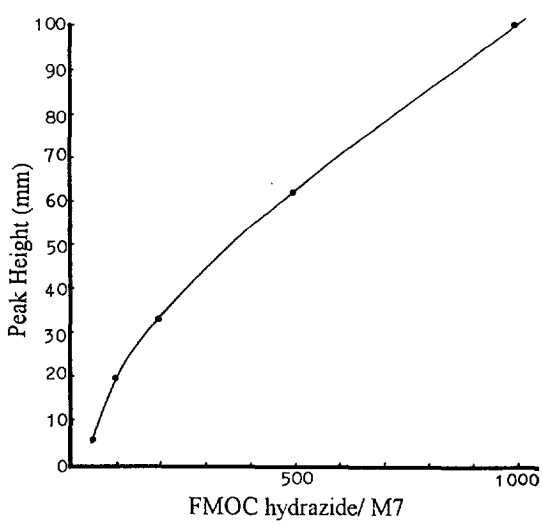

Fig. 6. Effect of molar ratio of M7 to FMOC hydrazide on the derivatization yield. Reaction was performed in 1% acetic acid at 37°C for 5 h.

3.3. Chromatography of partial acid-hydrolysed dextran

Fig. 7 shows the results obtained for analysis of isomaltooligosaccharides, a homologous series of reducing oligosaccharide. Isomaltooligosaccharide FMOC hydrazones up to hexadecasaccharides were separated completely within 40 min by isocratic elution with acetonitrile–water (20:80) on a Cosmosil C_{18} column. Each peak represent a unique oligosaccharide with the degree of polymerization indicated by the numbers above the peaks. This result shows that the high hydrophobicity of oligosaccharide FMOC hydrazones is of benefit to achieve the effective separation of multi-component oligosaccharides by RP-HPLC. The selectivity of neighbouring oligosaccharide of isomaltooligosaccharides was 1.07–1.08 (with a degree of polymerization in the range 9–16). The data indicate that there is a

Table 1
Comparison of efficiencies of removal (%) of excess reagent by solvent extraction[a]

Solvent	FMOC hydrazide in water	Lac FMOC hydrazone in organic solvent	M7 FMOC hydrazone in organic solvent
Chloroform[b]	0.28	–	–
Ethyl acetate[b]	1.2	19	7.6
n-Pentane[b]	57	–	–

[a] 150 μl of derivatization solution were extracted with 300 μl of solvent once.
[b] All of the solvents for extraction were presaturated with water.

certain relationship between the degree of polymerization and capacity factor of oligosaccharide homologues.

3.4. Stability of the M7 FMOC hydrazone

The stability of the M7 hydrazone was examined and the results showed that it can be stored in the derivatized solution containing 1% acetic acid and at 4°C for at least 2 weeks. The stability of M7 hydrazones in buffers of different pH and at 37°C for 20 h was examined further and the results are shown in Fig. 8. The recoveries of the derivative in borate (pH 8.0), phosphate (pH 6.0) and acetate (pH 4.0) buffers were 95, 74 and 56%, respectively. These results show that the stability of M7 FMOC hydrazone

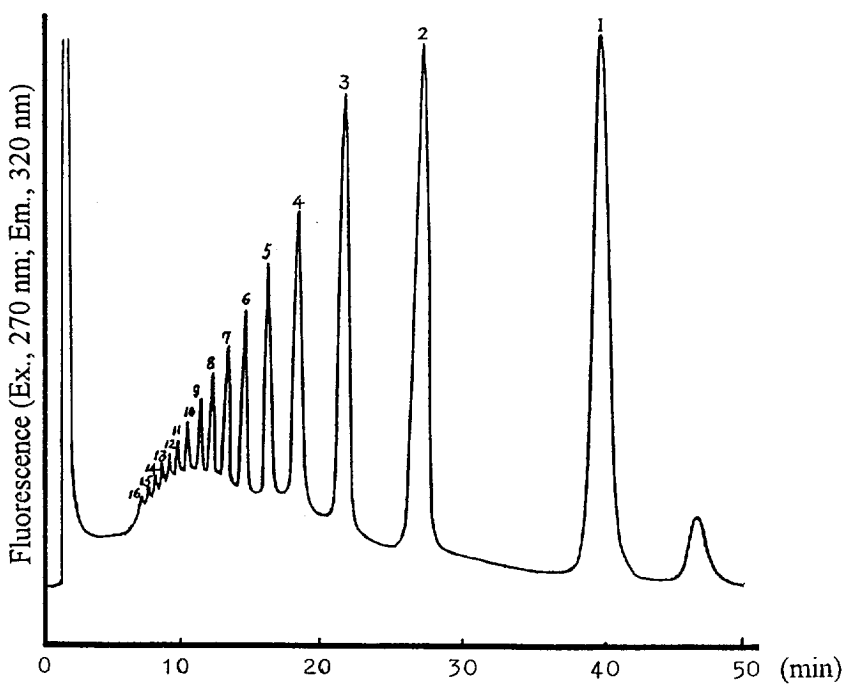

Fig. 7. Separation of partial acid hydrolysates of dextran. Numbers on chromatographic peaks represent degree of polymerization. Mobile phase, acetonitrile–water (20:80, v/v). Other chromatographic conditions as in Fig. 4.

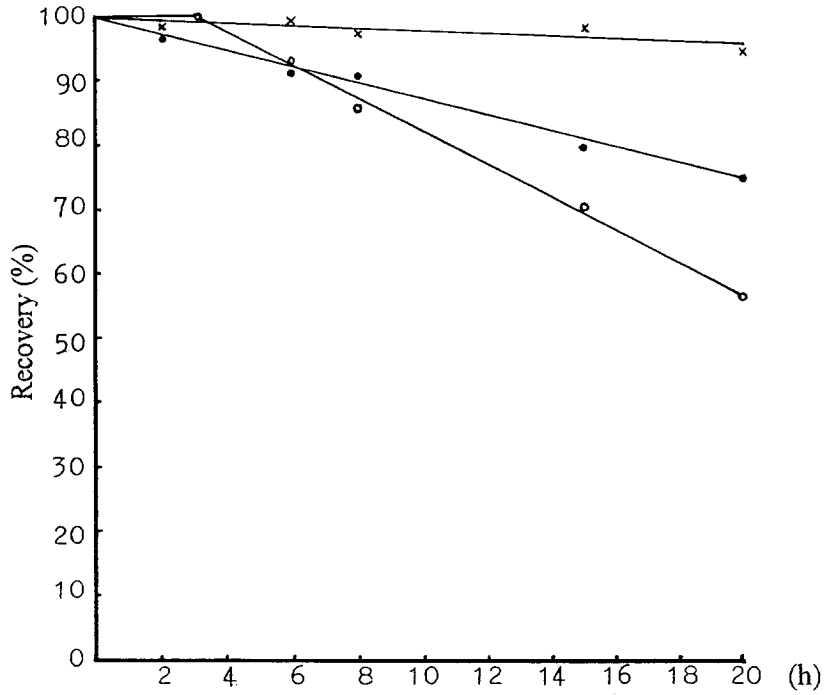

Fig. 8. Stability of M7 FMOC hydrazone. Incubation in (×) 0.1 *M* borate buffer (pH 8.0), (●) 0.1 *M* phosphate buffer (pH 6.0) and (○) 0.1 *M* acetate buffer (pH 4.0) at 37°C. Chromatographic conditions as in Fig. 4.

decreased with decrease in the pH of buffer solution.

3.5. Reproducibility of the derivatization of FMOC hydrazide with M7

The reproducibility of the derivatization of M7 with FMOC hydrazide was studied using 1% acetic acid at 37°C for 6 h. Nine aliquots of M7 were derivatized by the normal procedure described under Experimental. The analysis of each derivatized sample was performed on a C_{18} column with three identical isocratic runs. Peak areas were used to measure the ultraviolet response (263 nm). The average relative standard deviation ($n = 9$) at 100 pmol was 2.8%.

4. Conclusion

We have described a simple, reliable and sensitive method for determining neutral and sialic acid-containing oligosaccharides by means of RP-HPLC. This method has the advantages that the derivatization conditions for oligosaccharides with FMOC hydrazide are mild and can be used to label sialic acid-containing oligosaccharides without causing any desialylation. The derivatives are stable, the derivatization solution could be injected directly without further treatment and separated on a C_{18} column with isocratic elution and ultraviolet and fluorescence detection could be used for determining oligosaccharide derivatives at picomole and sub-picomole levels, respectively.

Acknowledgements

We thank Professor Rou-Li Zhou and Mr. Ye Zhang of the Beijing Medicine University, and Professor Yi-Xian Sha and Mrs. Rui Zhao of the Institute of Chemistry, Academia Simica, for their help during the course of this work. This

work was supported by grants (29475216) from the Chinese National Foundation Committee of Natural Sciences.

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