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Short Communication

Preparative separation of ganglioside GM_3 by high-performance liquid chromatography

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Abstract

A preparative high-performance liquid chromatographic method for the purification of ganglioside GM_3 is described. The method utilizes a Zorbax-NH_2 column and methanol–2-propanol–acetonitrile–phosphate buffer as the eluent. The elution profile was monitored by flow-through detection of UV absorbance at 215 nm. The purification of ganglioside GM_3 was performed in a total elution time of less than 15 min.

1. Introduction

Gangliosides are normal membrane components, located almost exclusively at the outer leaflet of plasma membranes [1]. Dramatic changes in ganglioside composition and metabolism during ontogenesis, differentiation and oncogenic transformation suggest a specific role of gangliosides in the regulation of cell growth and cellular interaction [2]. The observation of effects on growth factor-stimulated receptor phosphorylation was the first evidence of the participation of gangliosides in the molecular mechanism associating cell growth control [3,4]. Confirmation and extension of these results are important. Usually 1– 20 mg of GM_3 of purity not less than 99% are sufficient for experiments with cell cultures. The aim of this work was to develop a convenient and inexpensive HPLC procedure appropriate for the preparative isolation of GM_3 from a mixture containing other gangliosides and non-ganglioside impurities. This paper describes a procedure for the preparative HPLC of gangliosides GM_3-NeuGc and GM_3-NeuAc with on-line UV detection.

2. Experimental

HPLC-grade acetonitrile (LiChrosolv) was purchased from Merck (Darmstadt, Germany). Water was purified with a Milli-Q system (Millipore). All other solvents were redistilled before use. Commercial chemicals were of analytical-reagent grade or the highest grade available.

Precoated high-performance thin-layer chromatographic (HPTLC) plates with Kieselgel 60 were obtained from Merck and DEAE Sephadex A-25 from Pharmacia (Uppsala, Sweden).

Ganglioside GM_3 and N-acetylneuraminic acid from Sigma (St. Louis, MO, USA) and GM_3-

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NeuGc from equine erythrocytes, prepared according to Miyazaki et al. [5], were used as standards.

The procedure for extraction and phase separation was similar to a previously described method [6] for the isolation of total gangliosides from male Wistar rat (3–6 months old) liver (160 g) and equine erthrocytes (5 l). Monosialogangliosides from the upper methanol–water phase were prepared by DEAE-Sephadex A-25 (acetate form) column chromatography (7 × 3 cm I.D. column) with 0.03 *M* ammonium acetate in methanol as eluent, as described [1]. The desalting of gangliosides was carry out on LiChroprep RP-18 column (4 × 3 cm I.D.) similarly to a described method [7]. The methanol solutions were evaporated and lipids were dissolved in water and lyophilized. The yield of monosialogangliosides was 23 mg from rat liver and 420 mg from equine erythrocytes.

HPLC was performed on a Gilson apparatus, equipped with a Rheodyne Model 7125 sample injector.

2.1. Preparative HPLC separation of ganglioside GM_3

Ganglioside GM_3-NeuGc was dissolved in 2-propanol–water (1:2, v/v) to give a 10 mg/ml concentration and 0.1–2.0 ml of this solution was introduced into the sample injector. Ganglioside was then purified on a Zorbax-NH_2 (8 μm) column (250 × 21.4 mm I.D.) (DuPont) with methanol–2-propanol–acetonitrile–30 m*M* sodium phosphate buffer (pH 5.6) (168:84:24:35, v/v) as eluent at a flow-rate of 20 ml/min and UV detection at 215 nm.

Monosialogangliosides from equine erythrocytes were separated under the same conditions as standard GM_3-NeuGc.

Methanol–2-propanol–acetonitrile–30 m*M* sodium phosphate buffer (168:96:23:20, v/v) was used for the separation of GM_3-NeuAc from the monosialoganglioside fraction of the rat liver by injection of up to 1 ml (10 mg/ml) of sample solution.

2.2. Analytical HPLC

A 1 mg/ml solution of ganglioside GM_3 in water was introduced into the injector and then separated on a Diasorb-130-NH_2 (6 μm) column (150 × 4 mm, I.D.) with a Diasorb-130-NH_2 (6 μm) guard column (50 × 4 mm I.D.) (Bio-ChimMak, Russian Federation) with methanol–2-propanol–acetonitrile–30 m*M* sodium phosphate buffer (pH 5.6) (168:96:23:20, v/v) as eluent at a flow-rate of 1.5 ml/min and UV detection at 215 nm.

2.3. Analytical methods

Chloroform–methanol–0.2% $CaCl_2$ (60:35:8, v/v) was used as the mobile phase for HPTLC. Spots were revealed with orcinol–iron(II) chloride (Sigma) and resorcinol–HCl spray reagents [8], ninhydrin and a solution of sulphuric acid in ethanol.

Sialic acid was measured quantitatively with resorcinol–HCl reagent [9]. Pure N-acetylneuraminic acid was used as the standard.

3. Results and discussion

Several methods for the separation of gangliosides by preparative HPLC have been developed [10–13]. 2-Propanol–hexane–water and chloroform–methanol water are used as eluents with HPTLC control of the collected fractions. Unfortunately, on-line monitoring of the separation by short-wavelength UV detection cannot be used in these procedures, because gradient elution and highly absorbing eluents were used. The aim of this study was to establish appropriate conditions for the rapid preparative HPLC separation of ganglioside GM_3 with UV flow-through detection.

Gazzotti et al. [14] presented an HPLC method with an aminopropyl-modified silica gel stationary phase and UV detection at 215 nm. However, the eluent used (acetonitrile–phosphate buffer) is not suitable for preparative purposes because of the low solubility of the

gangliosides. Replacement of acetonitrile by methanol, in which gangliosides show better solubility, resulted in a significant decrease in the capacity factor (k'). That is why we introduced a component with a smaller elution strength than methanol–2-propanol. The proportions of the components in the eluent were optimized to achieve the maximum and rapid yield of ganglioside with 99% purity. An example of the purification of 1, 10 and 20 mg of ganglioside GM_3-NeuGc is shown in Fig. 1. The recovery of ganglioside after HPLC purification and desalting was more then 96% by measurement of the sialic acid content.

Fig. 2 illustrates the preparative separation of the equine erythrocyte monosialoganglioside fraction. A 4.5-mg amount of mixture was separated within 14 min using 250 ml of eluent. The 3.1-mg yield of pure GM_3-NeuGc was homogeneous according to analytical HPLC under the same conditions (Fig. 4) and HPTLC (Fig. 5).

The optimum capacity factor for the preparative isolation of ganglioside GM_3-NeuAc from rat liver was obtained with a small change in the eluent composition. Fig. 3 illustrates the preparative separation of GM_3-NeuAc from a mixture containing non-ganglioside impurities. The collected substance was homogeneous according to analytical HPLC and HPTLC (Figs. 4 and 5).

Both gangliosides obtained by the developed preparative HPLC method did not contain im-

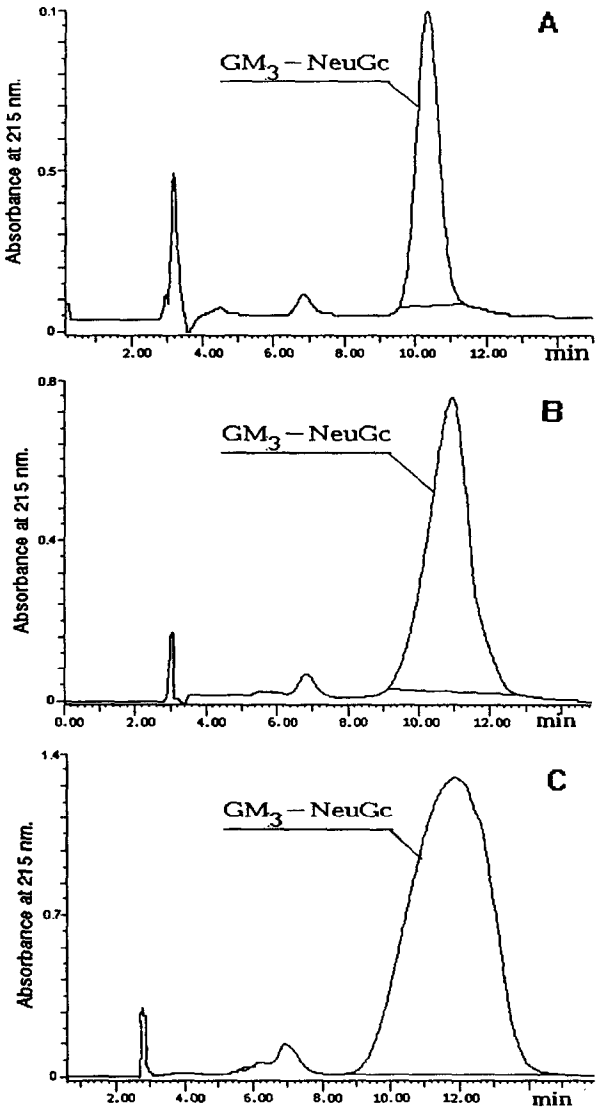

Fig. 1. Application of the preparative HPLC method to the purification of ganglioside GM_3-NeuGc. Amount: (A) 1; (B) 10; (C) 20 mg. The substance was dissolved in 2-propanol–water (1:2, v/v) to give a 10 mg/ml concentration and 0.1, 1.0 and 2.0 ml of sample solution were injected.

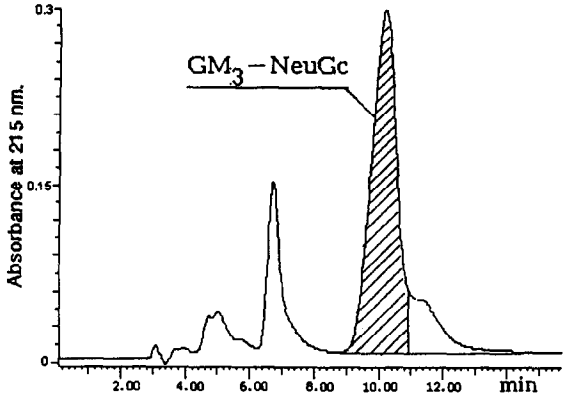

Fig. 2. Application of the preparative HPLC method to the isolation of the ganglioside GM_3-NeuGc from 4.5 mg of monosialoganglioside fraction of equine erythrocytes. The substance was dissolved in 2-propanol–water (1:2, v/v) to give a 10 mg/ml concentration and 0.45 ml of sample solution was injected.

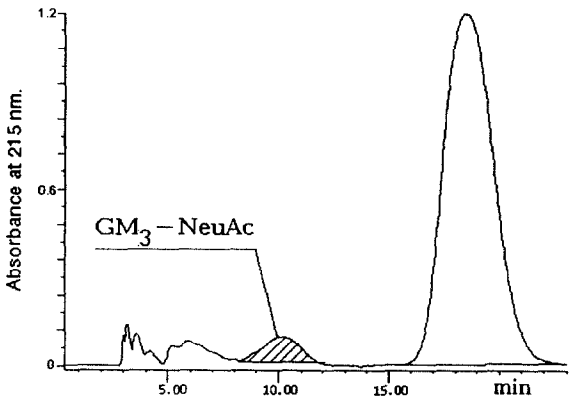

Fig. 3. Application of the preparative HPLC method to the isolation of the ganglioside GM_3-NeuAc from 3 mg of monosialoganglioside fraction of rat liver. The substance was dissolved in 2-propanol–water (1:2, v/v) to give a 10 mg/ml concentration and 0.3 ml of sample solution was injected.

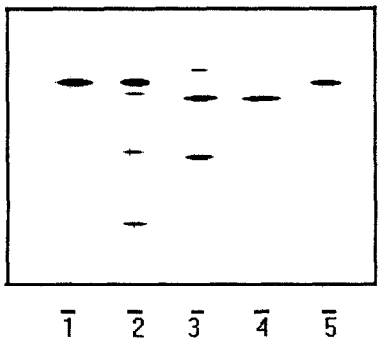

Fig. 5. HPTLC of gangliosides 1 = Standard of GM_3-NeuAc; 2 = monosialogangliosides from rat liver; 3 = monosialogangliosides from equine erythrocytes; 4 = GM_3-NeuGc purified by HPLC; 5 = GM_3-NeuAc purified by HPLC. Spots were revealed using both resorcinol–HCl spray reagent and a solution of sulphuric acid in ethanol.

purities detectable with ninhydrin and sulphuric acid reagents.

In conclusion, we have demonstrated the application of an aminopropyl-modified silica gel column for the preparative separation of ganglioside GM_3 from equine erythrocytes and rat liver with methanol–2-propanol–acetonitrile–30 m*M* sodium phosphate buffer as eluent and UV detection. The same combination of stationary phase and eluent can be used for the analytical HPLC of ganglioside GM_3.

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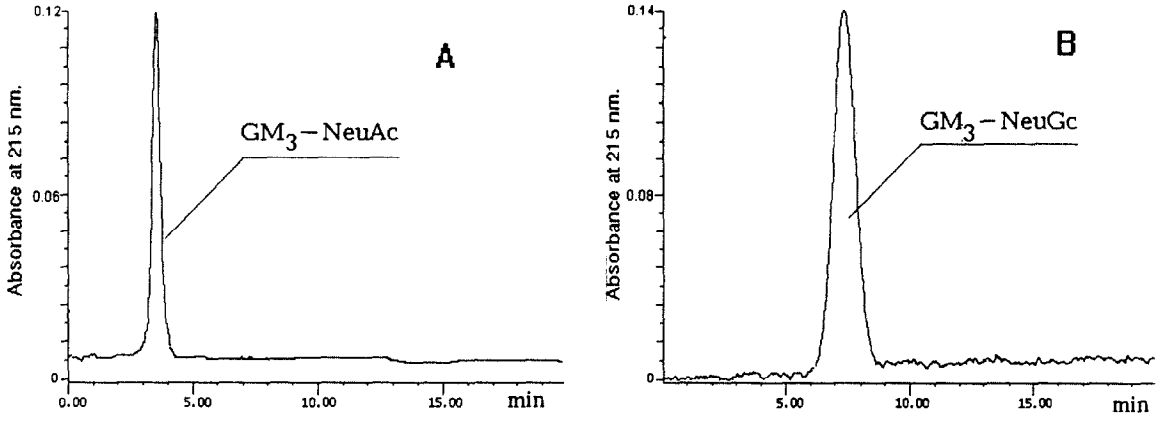

Fig. 4. Application of the analytical HPLC method to verification of ganglioside GM_3 purity after preparative separation. (A) GM_3-NeuAc; (B) GM_3-NeuGc. The substance was dissolved in water to give a 1.0 mg/ml concentration; 20 and 30 μl of sample solution were injected.

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