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Note

Separation of ganglioside molecular species, with homogeneous long-chain base composition, by reversed-phase thin-layer chromatography

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Gangliosides are glycosphingolipids which contain one or more sialic acid residues. They are normal components of the plasma membranes of mammalian cells and are particularly abundant in neuronal membranes[1,2]. They are constituted by a hydrophobic part, the ceramide, which is inserted in the lipid layer of the membrane and by a hydrophilic part, the oligosaccharide chain, which is oriented towards the extracellular medium. The ceramide moiety of the most common gangliosides from the nervous system has a rather homogeneous fatty acid composition, with prevalence of stearic acid, but differs in the long-chain base content. The most abundant long-chain bases are C_{18} and C_{20} compounds, with a double bond at the C_4–C_5 position. Small amounts of the corresponding saturated long-chain bases are also present.

Several thin-layer chromatographic (TLC) procedures, suitable for the separation of ganglioside mixtures into individual entities on the basis of their different oligosaccharide parts, are available[3–12], but a general TLC method which is able to resolve the species having identical oligosaccharide parts but different ceramide parts has not been reported.

Recently an analytical reversed-phase high-performance liquid chromatographic (HPLC) procedure has been described for the separation of ganglioside molecular species homogeneous in the oligosaccharide and long-chain base moieties[13–15].

The aim of the present work is to extend the potential of reversed-phase chromatography to thin-layer analysis of gangliosides. Reversed-phase TLC has been already applied successfully to the separation of several amphiphilic and apolar compounds[16–19].

In this paper we describe a method for the TLC resolution of gangliosides into the molecular species having homogeneous long-chain base content in a simple and rapid way. This method can also be applied to determine the long-chain base content of gangliosides.

The investigation was carried out on six mammalian brain gangliosides, GM2, GM1, Fuc-GM1, GD1a, GD1b and GT1b, which are homogeneous in the oligosaccharide chain for more than 99%.

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EXPERIMENTAL

Materials

All chemicals were reagent grade quality. Solvents were redistilled before use. Water was freshly redistilled in a glass apparatus. Reversed-phase high-performance thin-layer chromatographic plates (HPTLC) (RP-18, F 254 S, 10 × 10 cm) were purchased from Merck (Darmstadt, F.R.G.).

Preparation of gangliosides

Gangliosides GM2, GM1, Fuc-GM1, GD1a, GD1b and GT1b were prepared and structurally identified as previously described[20,21]. GM2 was obtained from a Tay-Sachs brain, all other gangliosides from pig brain. During column chromatography purification, the central fractions of the corresponding eluted peaks, which contained the ganglioside species having the highest percentage of stearic acid were collected. The final purity of all ganglioside preparations with respect to the oligosaccharide part, was assessed according to Ghidoni *et al.*[21] and found to be greater than 99%.

Each standard ganglioside was also separated in the corresponding molecular species, homogeneous in the long-chain base moiety, by reversed phase HPLC, as described by Sonnino and co-workers[13–15]. Ganglioside molecular species containing saturated long-chain bases were obtained from the corresponding unsaturated gangliosides by catalytic hydrogenation [in the presence of platinum(IV) oxide] and characterized as previously reported[13].

Gangliosides were assayed as lipid-bound sialic acid by the resorcinol method[22,23].

Reversed-phase HPTLC

Up to 50 nmol of ganglioside, as bound sialic acid (NeuAc), dissolved in a few microliters of chloroform–methanol (2:1, v/v) were spotted on a 8-mm lane, 1 cm from the lower edge of the plate. The plate was previously activated in an oven at 120°C for 20 min. Prewashing of the plate was unnecessary. The elution solvent system was methanol–acetonitrile–water (19:5:1, v/v). The analyses were performed at 34°C in a ventilated thermostat, using tanks which were preconditioned at the same temperature. Development was stopped when the solvent front reached the upper edge of the plate. Generally the analysis time under the described conditions was less than 20 min.

When densitometric quantification was required, 5 nmol of the GM1 ganglioside molecular species containing *erythro*-C_{18} sphingosine, GM1 (e 18:1), were spotted on the same plate as internal standard.

Densitometric scanning

Gangliosides were rendered visible on the plate by spraying with a fine mist of *p*-dimethylaminobenzaldehyde reagent (Ehrlich reagent)[24]. The plates were covered with a clean glass plate held in place by metal clamps, and heated in an oven at 120°C for 20 min. The chromatograms, after cooling at room temperature and removal of the cover plates, were scanned using a Camag TLC densitometer equipped with a 3390 A Hewlett-Packard integrator. A 620-nm beam, 6 mm wide, was used to scan

the plate from the bottom to the top. The linearity of the relationship between the densitometric response and ganglioside content (as bound NeuAc) was determined by spotting increasing amounts (from 0.25 to 50 nmol) of each ganglioside molecular species on the same plate and referring the densitometric responses to that of GM1 (e 18:1) used as internal standard.

RESULTS AND DISCUSSION

As shown in Fig. 1, each ganglioside homogeneous in the oligosaccharide part, was resolved in four components. For all the examined gangliosides, two main components covering in all cases, together, more than 95% of total sialic acid, and two minor spots, covering in all cases, together, not more than 5% of total sialic acid, were detected. When co-chromatographed with the molecular species of the corresponding ganglioside having a homogeneous long-chain base composition purified by reversed-phase HPLC[16], the two main components for each ganglioside were found to be the molecular species containing the unsaturated long-chain bases C_{18} sphingosine (higher R_F) and C_{20} sphingosine (lower R_F). The two minor spots were found to be the molecular species containing the saturated long-chain bases C_{18} sphinganine (higher R_F) and C_{20} sphinganine (lower R_F). The R_F values of these two minor components were slightly lower than those of the corresponding unsaturated compounds. An example of the comparison between natural GM1 ganglioside and the corresponding ganglioside molecular species, prepared by reversed-phase HPLC, is reported in Fig. 2.

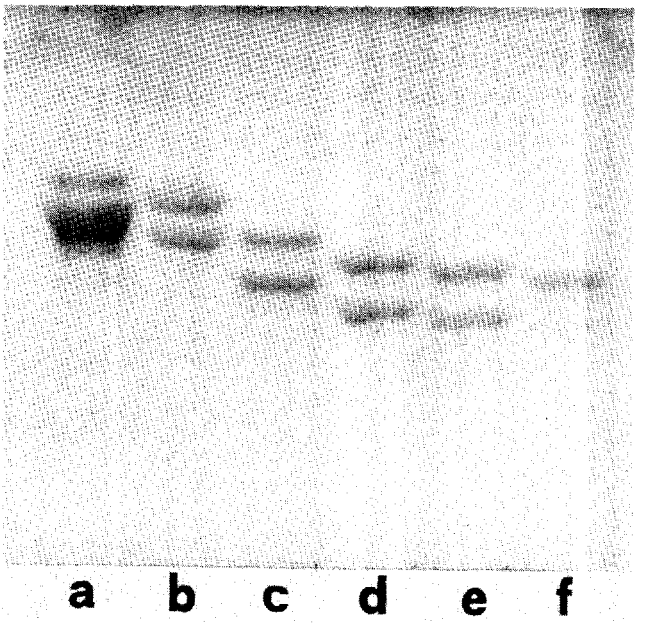

Fig. 1. Separation by reversed-phase HPTLC of gangliosides on the basis of the long-chain base content. (a) GT1b; (b) GD1a; (c) GD1b; (d) Fuc-GM1; (e) GM1; (f) GM2. Solvent system: methanol–acetonitrile–water (19:5:1, v/v); temperature: 34°C.

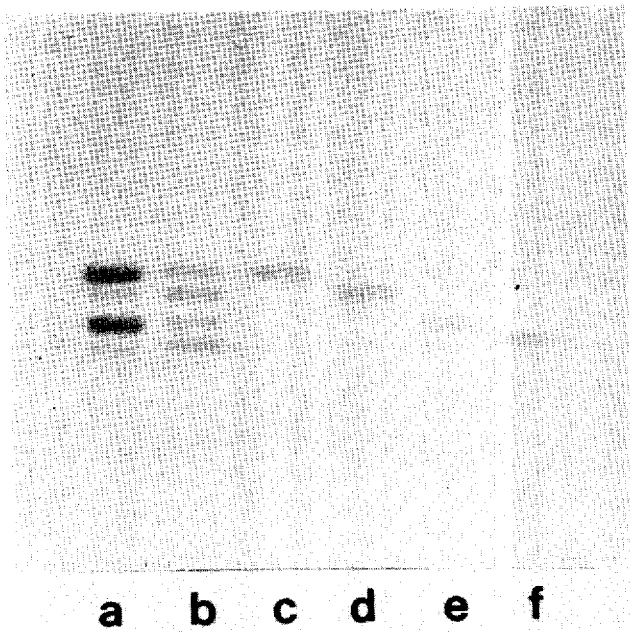

Fig. 2. Separation by reversed-phase HPTLC of GM1 ganglioside molecular species, homogeneous in the long-chain base moiety. (a) natural GM1; (b) mixture of the GM1 molecular species containing C_{18} sphingosine, C_{18} sphinganine, C_{20} sphingosine and C_{20} sphinganine, from the top to the bottom respectively; (c) C_{18} sphingosine containing GM1; (d) C_{18} sphinganine containing GM1; (e) C_{20} sphingosine containing GM1; (f) C_{20} sphinganine containing GM1. Chromatographic conditions as in Fig. 1.

It is worth mentioning that the relative mobilities in reversed-phase chromatography of the various gangliosides are practically opposite to those obtained with conventional silica gel chromatography. Consequently, more sialylated gangliosides and, for the same ganglioside, the molecular species containing the more polar long-chain base (C_{18} sphingosine) behave, in reversed-phase TLC, as fast moving components.

The densitometric responses of all the examined molecular species increased linearly with the ganglioside content up to 40 nmol (as bound NeuAc). The standard deviation was lower than 10% of the mean value for ganglioside amounts between 5 and 40 nmol (as bound NeuAc). They were still acceptably low (*ca.* 15% of the mean values) in the range 1–5 nmol, while they became much higher for a ganglioside content lower than 1 nmol. Therefore precise quantigication was allowed in the range 1–40 nmol of ganglioside (as NeuAc) in the spot. Therefore reversed-phase TLC, followed by densitometric scanning of the plate, can be applied to quantification of the molecular species of each ganglioside.

In order to satisfy the requirements of linearity and acceptable low standard deviation, the maximum difference, in relative concentration, of each molecular species of a ganglioside, should be 1:40.

Siouffi *et al.*[16] reported that some detection problems arise in reversed-phase HPTLC, due to the high content of organic material present on the plate. In the present case, using Ehrlich reagent which consists of an acidic water–ethanol solution,

complete wetting of the plate may be achieved by prolonging the spraying time, with the consequence of a light yellowing, after heating, of the bonded phase. However, this did not influence the densitometric responses, which were in good agreement with those obtained in a previous work[11] using silica gel HPTLC plates.

The quantitative pattern of the molecular species, homogeneous in the long-chain base moiety, from each of the examined gangliosides, is reported in Table I. The reported data clearly indicate the predominance in the nervous system of the ganglioside molecular species that contain unsaturated long-chain bases.

TABLE I

LONG-CHAIN BASE CONTENT OF THE SIX EXAMINED GANGLIOSIDES, DETERMINED BY REVERSED-PHASE HPTLC SEPARATION AND DENSITOMETRIC QUANTIFICATION

Ganglioside	*Long-chain base (%)*			
	C_{18} sphingosine	*C_{18} sphinganine*	*C_{20} sphingosine*	*C_{20} sphinganine*
GM2	69.4	3.1	25.2	2.3
GM1	49.7	3.0	45.7	1.6
Fuc-GM1	36.3	2.0	57.6	4.1
GD1b	42.2	2.2	52.1	3.5
GD1a	38.9	2.8	55.3	3.0
GT1b	20.8	1.8	71.0	6.4

Concluding, this method can be easily applied to check the purity of isolated molecular species of gangliosides and could represent a rapid and simple tool to evaluate the long-chain base composition of single gangiosides.

To date, this procedure has been applied to gangliosides purified from the nervous system of mammals, in which stearic acid is the main fatty acid.

Using gangliosides homogeneous both in the long-chain base and fatty acid moieties, recently prepared in our laboratory, we can preliminarily state that under the described conditions the heterogeinity in the fatty acid moiety does not effect the chromatographic behaviour.

Therefore the method can also be applied easily to gangliosides prepared from extranervous tissues, known to be frequently heterogeneous in the fatty acid moiety. Studies in this direction are already in progress in our laboratory.

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