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NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NON-DERIVATIZED GANGLIOSIDE MIXTURES

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SUMMARY

A new analytical and semi-preparative high-performance liquid chromatographic method for the separation of a brain ganglioside mixture into individual components is described. Gangliosides were applied to a LiChrosorb-NH_2 column and eluted with the solvent system acetonitrile–phosphate buffer at different volume ratios and ionic strengths. The elution profile was monitored by flow-through detection of UV absorbance at 215 nm. The separation of mono- to polysialogangliosides was performed in one step in a total elution time lower than 90 min and with high reproducibility.

INTRODUCTION

Gangliosides, glycosphingolipids containing sialic acid, which are normally present in the cell plasma membranes[1,2], are extracted from tissues as mixtures of many species that differ in their oligosaccharide and/or ceramide portions. In recent years, high-performance liquid chromatography (HPLC) has been introduced as a methodological tool for the separation and quantification of glycosphingolipids[3–18]. The described procedures, with analytical aims, generally make use of a pre-column derivatization[3–6,9,12,13,15–18]. However, although these methods have the advantage of being acceptably sensitive, they do not seem to be easily applicable to preparative purposes. Besides, they generally require prewashing before injection to eliminate unspecific by-products that may interfere with gangliosides during UV detection. Recently, non-derivatized gangliosides have been separated on normal-phase silica gel columns with solvent systems containing *n*-hexane[7,14]. Under these conditions direct UV detection, in the wavelength range of ganglioside absorption (190–230 nm), is not convenient owing to solvent cut-off; therefore, tedious control thin-layer chromatographic (TLC) analyses are required.

Gangliosides have also been separated by reversed-phase HPLC, in solvent systems that permit direct UV detection[8,10,11]. In these cases, the analytical and preparative methods allow ganglioside molecular species to be separated because of both the oligosaccharide and ceramide portions, but when it is applied to the resolution of complex ganglioside mixtures, some overlap occurs[10].

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In the present paper, we report a HPLC methodology, that surmounts above the disadvantages and can separate and quantify non-derivatized ganglioside mixtures by direct flow-through reading at 215 nm. The system, which can also be used for preparative purposes, utilizes a LiChrosorb-NH_2 column and a gradient of acetonitrile–phosphate buffer as solvent system. The method has been standardized by the use of nine pure gangliosides and has been applied to the purification of a ganglioside mixture extracted from calf brain.

EXPERIMENTAL

Materials

Silica gel precoated thin-layer plates (HPTLC, Kieselgel 60, 250 μm thick, 20 × 10 cm) were purchased from Merck (Darmstadt, F.R.G.); the total ganglioside mixture was extracted from calf brain and partially purified according to Tettamanti *et al.*[19]. Traces of contaminants were eliminated from the ganglioside lipid extract as follows. The lyophilized crude ganglioside mixture was washed three times with cold acetone (1 ml per 5 mg crude mixture). The organic phase containing less than 0.4% of total ganglioside mixture, as sialic acid, was discarded and the ganglioside mixture was dissolved in doubly distilled water at a concentration of 200 mg of crude mixture per millilitre, sonicated for 2 min and centrifuged for 5 min at 8500 *g*, the clear supernatant containing gangliosides was carefully removed and lyophilized. TLC separation followed by densitometric quantitation[20] of the ganglioside mixture, carried out before and after the described purification steps, did not show any significant differences.

Standard gangliosides were extracted according to Tettamanti *et al.*[19]. GM1, GD1a, GD1b, GT1b and GQ1b were prepared from calf brain, Fuc-GM1 and Fuc-GD1b from pig brain, GM2 from a Tay-Sachs brain and GM3 from human spleen (the nomenclature of Svennerholm[21], for ganglioside designation, is used). All

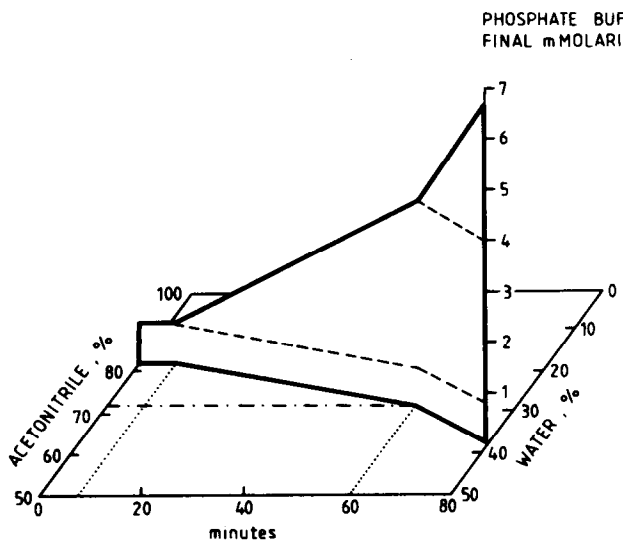

Fig. 1. Graphical representation of the elution gradient used in the HPLC separation of gangliosides.

gangliosides were more than 99.5% pure, as assessed by HPLC analysis following the suggestion of Gazzotti *et al.*[10].

HPLC separation of gangliosides

A 0.1–20-nmol portion of pure GM3, GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b, GQ1b gangliosides, or a 1–50 nmol portion of calf brain ganglioside mixture, as lipid-bound sialic acid, was dissolved in 5–10 μl of redistilled water in a microtube and introduced into a syringe-loading sample injector (Model 7125 Rheodyne) equipped with a 20-μl loop. The microtube was washed with 5–10 μl of redistilled water and the washing was added to the previous sample in order to minimized loss of material. Gangliosides were then chromatographed on a LiChrosorb-NH_2 column, 250 × 4 mm I.D., 7 μm average particle diameter (Merck, Darmstadt, F.R.G.) with a Gilson HPLC apparatus, equipped with an Apple II computer system for the selection of elution gradient.

The separation was carried out at 20°C with a gradient of the following solvent mixtures: solvent A, acetonitrile–5 m*M* phosphate buffer, pH 5.6 (83:17); solvent B, acetonitrile–20 m*M* phosphate buffer, pH 5.6 (1:1). The gradient elution programme was as follows: 7 min with solvent A; 53 min with a linear gradient from solvent A to solvent A–solvent B (66:34); 20 min with a linear gradient from solvent A–solvent B (66:34) to solvent A–solvent B (36:64). The resulting gradient, expressed as acetonitrile and water percentages, and the final phosphate buffer molarity as a function of elution time, is represented in Fig. 1.

A complete analysis took 80 min. The flow-rate was 1 ml/min and the elution profile was monitored by flow-through detection of UV absorbance at 215 nm (Gilson UV detector model Holochrome).

Before a new analysis cycle, the column was washed with solvent B for 10 min and then equilibrated with solvent A for 15 min, in order to eliminate highly polar contaminants.

Preparative HPLC of the ganglioside mixture from calf brain was achieved using preparative LiChrosorb-NH_2, 250 × 25 mm I.D. (Merck), according to the above gradient programme. A 1–5-mg portion of ganglioside mixture (as sialic acid), was dissolved in 100 μl of redistilled water in a microtube, and introduced into the syringe-loading sample injector, equipped with a 200-μl loop. The microtube was washed with 100 μl of redistilled water and the washing was added to the previous sample. Gangliosides were chromatographed at a flow-rate of 39 ml/min. The elution profile was monitored by flow-through detection, as in the analytical procedure, and by TLC (see below). For this purpose 20-ml fractions were automatically collected and 0.1 ml of each fraction was dried, solubilized in 20 μl of chloroform–methanol (2:1) and spotted on a HPTLC plate. After separation, the combined fractions corresponding to each purified ganglioside were dried, dialysed, lyophilized and submitted to analytical HPLC as described.

Thin-layer chromatography (TLC)

TLC of gangliosides was carried out on HPTLC precoated plates under the following conditions: temperature, 18–20°C; solvent system, chloroform–methanol–0.2% aqueous calcium chloride (50:42:11); detection of the spots by spraying with an Erlich reagent and heating at 120°C[20].

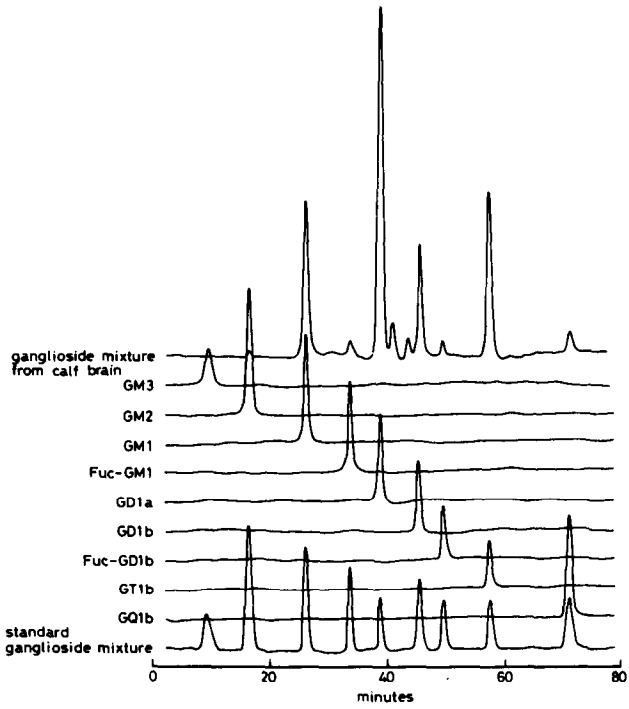

Fig. 2. Application of the HPLC analytical method to the separation of gangliosides present in a calf brain ganglioside mixture. The HPLC profile is compared with that of individual standard gangliosides and with that of a standard ganglioside mixture.

TABLE I

RELATIVE RETENTION TIMES (RRT) AND RELATIVE MOLAR RESPONSES (RMR) OF STANDARD GANGLIOSIDES

GD1a is taken as 1.00.

Ganglioside	*RRT**	*RMR ± S.D. (n = 6)*
GM3	0.202	0.536 ± 0.010
GM2	0.421	0.561 ± 0.006
GM1	0.656	0.607 ± 0.041
Fuc-GM1	0.900	0.621 ± 0.023
GD1a	1.000	1.000 ± 0.014
GD1b	1.184	1.045 ± 0.024
Fuc-GD1b	1.350	1.055 ± 0.041
GT1b	1.503	1.498 ± 0.088
GQ1b	1.869	1.901 ± 0.091

* $RRT = (RT_x - RT_m)/(RT_{GD1a} - RT_m)$, where RT_x is the retention time of the tested ganglioside, RT_{GD1a} is the retention time of GD1a, and RT_m is the dead retention time. S.D. (on six determinations) of RT_{GD1a} and of all RT_X values were lower than 1.2%.

Colorimetric methods

Ganglioside bound sialic acid was determined by the method of Warren[22] after acid hydrolysis of the sample in 0.05 *M* sulphuric acid (1 h at 80°C) and purification of liberated sialic acid by ion-exchange chromatography on a Dowex 2-X8 (CH_3COO^-) column[23]. Pure N-acetylneuraminic acid was used as the standard.

RESULTS AND DISCUSSION

Fig. 2 shows the separation of GM3, GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b and GQ1b standard gangliosides, obtained by analytical HPLC, according to the elution gradient programme in Fig. 1. Fig. 2 also illustrates the chromatographic behaviour of each individual ganglioside injected alone. Gangliosides were eluted from the column depending from their degree of polarity. GM3 and GQ1b gangliosides, which were the first and last to be eluted, displayed retention times of 572 and 4318 s (mean values), respectively. The relative retention times of each ganglioside, referred to GD1a, are listed in Table I.

Although gangliosides show an UV maximum absorption at 195 nm[10], column elution was followed at 215 nm, where the ganglioside absorption corresponded to 60% of that recorded at 195 nm. This was necessary in order to avoid zero-line variation caused by variations in the solvent absorption at 195 nm during the gradient

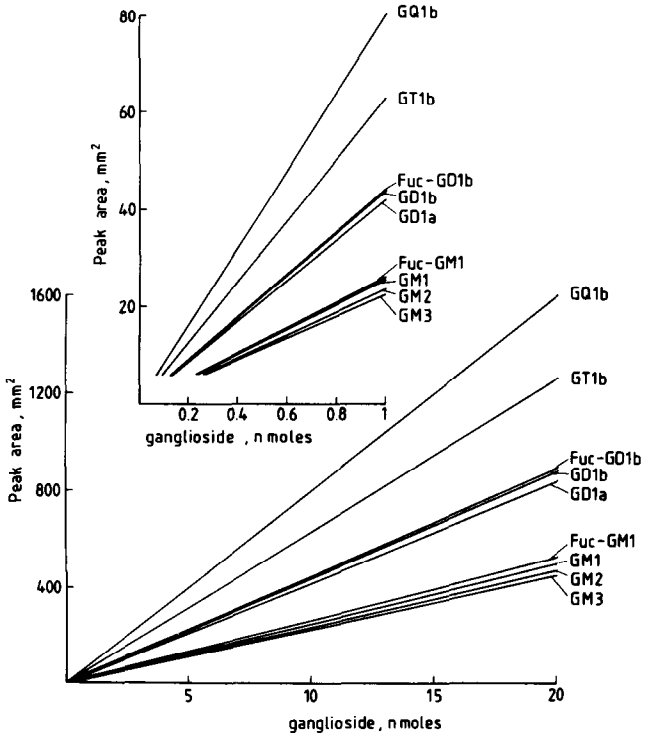

Fig. 3. Application of the HPLC analytical method to ganglioside quantification: relationship between peak area (mm^2 at 0.025 a.u.f.s.) and amount of injected gangliosides (nmol).

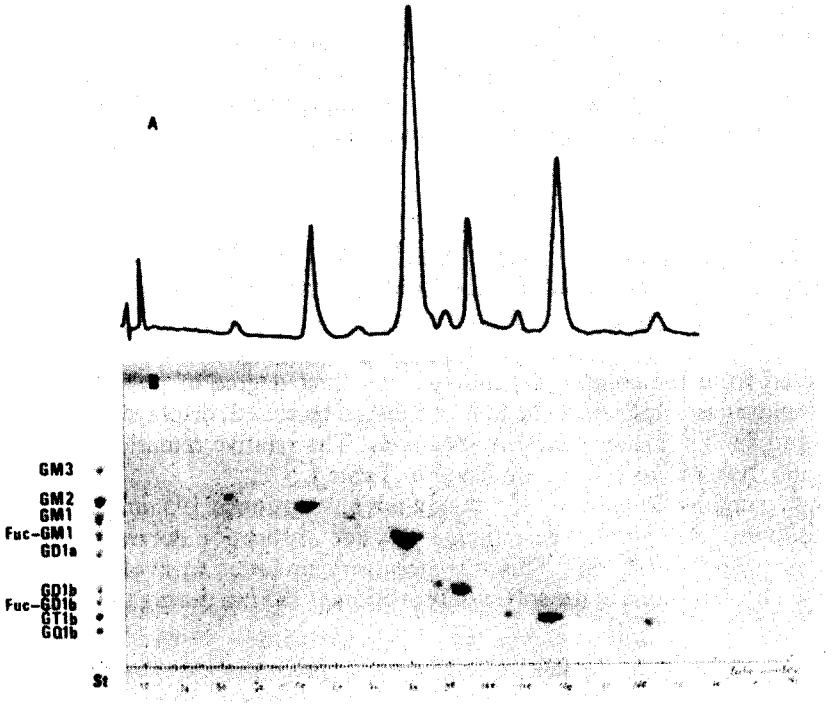

Fig. 4. Application of the HPLC preparative method to the isolation of individual gangliosides from a calf brain ganglioside mixture. (A) HPLC elution profile, recorded by UV absorbance at 215 nm. (B) HPLC elution profile monitored by HPTLC; 20-ml fractions were collected, and 0.1-ml aliquots were spotted after drying and redissolving in 20 μl of chloroform–methanol (2:1). St = Standard ganglioside mixture.

programme. The UV absorbance responses of equimolar amounts of different gangliosides increased with increasing sugar content, and in particular with an increase in the number of sialic acid residues. The response was linear with ganglioside content up to 20 nmol (Fig. 3): at this value the resolving power of the column is still adequate. The lowest amount of each ganglioside considered suitable for quantification is deemed to be that corresponding to an electrical impulse at the recorder of six-fold over the instrumental noise at 0.005 a.u.f.s.; this yields a peak area of *ca.* 30 mm^2. As indicated in Fig. 3, this limit corresponds to 0.26 nmol for GM3, 0.08 nmol for GQ1b ganglioside, and intermediate values for the other gangliosides used. Standard deviation values were less than 1% for ganglioside amounts between 1 and 20 nmol, less than 3% between 0.5 and 1 nmol, and still acceptably low ($\pm$ 5% of mean value) for ganglioside amounts less than 0.5 nmol. The relative molar responses (RMR) of the gangliosides analysed, referred to GD1a as reference standard, are listed in Table I.

The calf brain ganglioside mixture, known to contain four major gangliosides, GD1a, GT1b, GM1 and GD1b, which contain 38%, 16%, 13.5% and 9.4%, respectively[20], of the total brain sialic acid, and a number of minor species, was submitted to analytical (Fig. 2) and preparative (Fig. 4) HPLC separation, according to

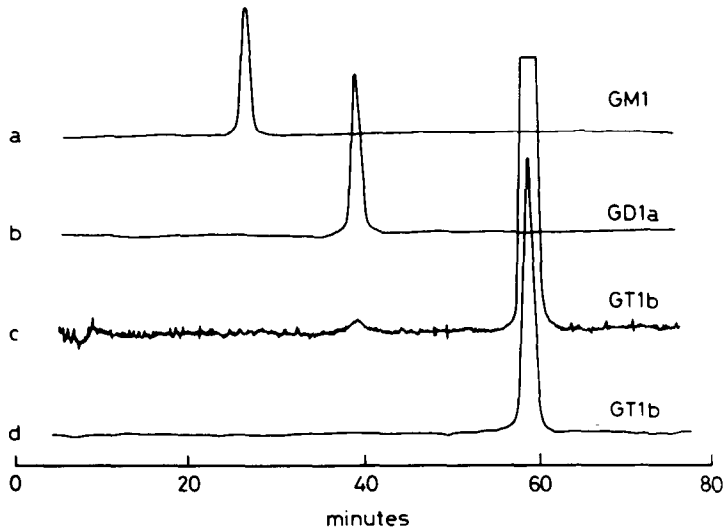

Fig. 5. Application of the HPLC analytical method to verification of the homogeneity of GT1b ganglioside purified by preparative HPLC, from a calf brain ganglioside mixture: (a) 10 nmol of standard GM1, range 0.025 a.u.f.s.; (b) 10 nmol of standard GD1a, range 0.025 a.u.f.s.; (c) 20 nmol of purified GT1b, range 0.05 a.u.f.s.; (d) 20 nmol of purified GT1b, range 0.005 a.u.f.s.

the presented method. Under preparative conditions, up to 5 mg of ganglioside mixture (as sialic acid) could be injected, producing an excellent peak resolution, with practically no overlap, as demonstrated by the TLC column monitoring (Fig. 4). If the injected amount was larger, the resolving power of the column decreased and some cross-contamination occurred.

The purity of each ganglioside separated by the analytical HPLC procedure described was at least 99%, with respect to other contaminant gangliosides. Fig. 5 shows the HPLC analysis of GT1b purified from the calf brain ganglioside mixture: 20 nmol of GT1b were injected and the elution was recorded at two different a.u.f.s. values, in order to detect very minor contaminants. In this particular case, the GT1b ganglioside was more than 99.5% pure.

CONCLUSION

The described procedure fulfills all the requirements of a method suitable for both quantification and preparation of pure ganglioside, starting from complex mixtures. It gives highly reproducible results (1–2% S.D. in retention times), even after hundreds of injections, because the solvent gradient programme is accurately computer-controlled.

The linearity range of the UV responses, and the low calculated standard deviations for RMR values, make this method reliable for accurate quantification of single gangliosides; in addition, the sensitivity of this method is much higher than that provided by conventional colorimetric procedures.

Finally, the ability to detect and quantify, in a precise way, ganglioside contaminants as minor as 0.1% of the total amount injected, makes this procedure very

useful in purity analysis. Adaptations of the method to the resolution of complex ganglioside mixtures extracted from extranervous tissues and body fluids, and from brains of non-mammalian species, are already in progress in our laboratory.

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