*Journal of Chromatography*, 605 (1992) 221–225
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 152

# Determination of gangliosides by high-performance liquid chromatography with photodiode-array detection

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(First received December 17th, 1991; revised manuscript received March 3rd, 1992)

ABSTRACT

Mixtures of gangliosides were separated by high-performance liquid chromatography (HPLC) on amino-silica columns according to their negative charge and the length of the carbohydrate portion. The use of an on-line variable wave-length diode-array detector allows the identification of gangliosides on the basis of their UV spectra with maximum absorbance at 196 nm. Accurate analytical data acquired with the diode-array detector allow the qualitative and quantitative determination of gangliosides and therefore eliminate the need for thin-layer chromatography after HPLC separation.

## INTRODUCTION

Gangliosides [1], sialic acid-containing glycosphingolipids, are components of the plasma membrane and act as binding sites on the cell surface. In addition, gangliosides can be linked to cytosol- or plasma-specific carrier proteins; however, their possible regulatory functions have only been hypothesized. The hydrophilic part of the ganglioside [2] is represented by a sialo-oligosaccharide, and a sphingosine and a fatty acid are components of the hydrophobic portion (ceramide). The number of sialic acid residues determines the negative charge of the molecule and therefore regulates the physiological capability of gangliosides to react with toxins, growth factors, antibodies and viruses [3–5].

The growing interest in these molecules has improved the methodologies used for their separation and identification. The most common separation by high-performance liquid chromatography (HPLC) generally employs amino-silica columns and a linear gradient at increasing salt concentrations in the eluent [6]. The low selectivity of conventional UV

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techniques below 200 nm, together with an unfavourable signal-to-noise ratio in the far UV, causes the difficult identification of gangliosides, which are characterized by a lack of chromophores in their molecules. Therefore the identification of gangliosides after separation by HPLC is generally obtained by thin-layer chromatography (TLC) of the UV peaks and subsequent resorcinol staining, which is specific for sialic acid-containing components [7].

The advent of photodiode-array UV detectors in HPLC has provided a valuable tool for solving the analytical problems by greatly improving peak identification, peak purity assessment and quantitation.

In the work reported here anion-exchange chromatography was combined with diode-array detection to separate and identify individual gangliosides. Gangliosides in this paper are named according to the Svennerholm nomenclature [1].

## EXPERIMENTAL

### *Chemicals*

Pre-coated high-performance thin-layer chromatography (HPTLC) plates with silica gel 60 and a LiChrosorb $NH_2$ HPLC column (250 × 4 mm I.D., 5 $\mu$m particle size) were obtained from Merck (Darmstadt, Germany). Ganglioside standards GM3, GM1, GD1a, GD1b and GT1b were purchased from Sigma (St. Louis, MO, USA); GM2 was the kind gift of Fidia Research Labs. (Abano Terme, Italy). Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and asialo GM1 were purchased from Sigma; lactosylceramide was purchased from BioCarb (Lund, Sweden). Water and acetonitrile were of HPLC grade (Merck). All other chemicals were of analytical-reagent grade.

### *Equipment*

A Perkin-Elmer (Norwalk, CT, USA) Series 410 LC pump equipped with a SEC-4 solvent environmental control and a Rheodyne sampling valve with a 50-$\mu$l sample loop were used as the chromatographic system. The detector was a Perkin-Elmer LC-95 variable wavelength UV–visible spectrophotometer interfaced to an Epson PCAX2e computer for system control, data acquisition and report generation; the installed hardware and software were PE OMEGA. The HPLC system included an on-line Perkin-Elmer LC-235 photodiode-array detector equipped with a GP-100 graphics printer.

A Perkin-Elmer Lambda 4B UV–visible spectrophotometer was used to determine the UV spectra of all the gangliosides studied.

### *Chromatographic conditions*

*HPTLC.* A standard ganglioside mixture and ganglioside-containing fractions collected during HPLC separation were assayed on HPTLC plates developed with chloroform–methanol–0.25% aqueous KCl (5:4:1, v/v/v). Gangliosides were detected with the resorcinol spray reagent [7].

*HPLC.* Gangliosides were chromatographed according to the method of Gazzotti *et al.* [8]. The eluent consisted of (A) acetonitrile–5 m*M* disodium phosphate buffered at pH 5.6 by phosphoric acid (83:17, v/v) and (B) acetonitrile–20 m*M* disodium phosphate buffered at pH 5.6 by phosphoric acid (1:1, v/v). The gradient elution programme used was: 7 min with A, 53 min linear gradient from A to A–B (66:34, v/v), 20 min linear gradient from A–B (66:34, v/v) to A–B (36:64, v/v). The flow-rate was 1 ml/min. All the eluents were filtered before use and degassed with helium during chromatography. A 5-$\mu$g amount of each ganglioside or 10 $\mu$g of a ganglioside mixture from bovine brain (GM1 21%, GD1a 40%, GD1b 16% and GT1b 19%) with the addition of 2 $\mu$g of GM3 and GM2 was dissolved in 50 $\mu$l of solvent A and injected. The detection wavelength was fixed at 215 nm.

*Diode-array detector parameters.* Using a pilot signal at 200 nm, spectra were acquired in the range 195–370 nm on the apex and on the ascending and descending part of each peak and were overlapped to determine the peak purity. In parallel, the elution profiles were acquired at 215 nm. The detection limit of the system was determined by injecting scalar concentrations of each ganglioside in the range 2.5–0.078 $\mu$g. The working *y*-axis value was fixed at 0.05 a.u.f.s.

*Spectrophotometric analysis.* The HPLC peaks were collected and their UV spectra recorded in the range 190–220 nm by a PE Lambda 4B UV–visible spectrophotometer. Subsequently the solvents were evaporated under nitrogen and the residues chro-

matographed on HPTLC plates as described previously.

## RESULTS AND DISCUSSION

*Chromatographic analysis of gangliosides*

GM3, GM2, GM1, GD1a, GD1b and GT1b were injected separately and mixed to determine the retention times. In our system, gangliosides were separated according to their negative charge (mono-sialogangliosides precede di- and tri-sialogangliosides) and the length of the carbohydrate portion. The presence of a single ganglioside in each detected UV peak was confirmed by HPTLC. The resorcinol stained the bands containing the ganglioside blue–purple (Fig. 1).

*Diode-array detector analysis*

HPLC of the standard gangliosides was carried out with the on-line photodiode-array detector. All the gangliosides detected showed the same UV spectrum with maximum absorbance at 196 nm. Data on the peak purity of the standard gangliosides used were obtained by comparing the spectra in the ascending, apex and descending portion of each ganglioside containing peak. For standard gangliosides the three spectra were superimposable, indicating the absence of impurities and that the corresponding purity index was 1.0 (Fig. 2). Other experiments carried out on glycolipid extracts obtained from tissues (data not shown) suggested that the peak purity is acceptable if the purity index is between 1.0 and 1.5. In our system, ganglioside identification was addressed by taking into account the retention times and absorbance spectra. With regard to the detection limit, it was found to be as low as 78.125 ng. Linearity was observed up to 2.5 μg (correlation coefficient $r$ = 0.9866).

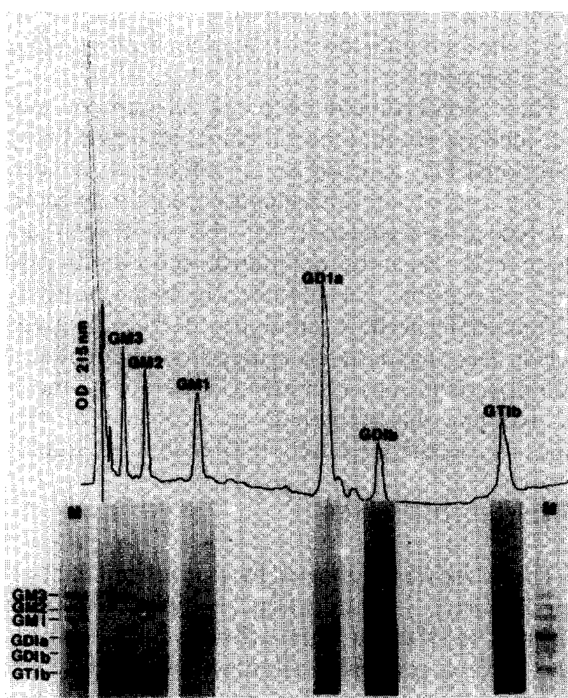


Fig. 1. HPLC and TLC analysis of a standard ganglioside mixture (GM3, 2 μg; GM2, 2 μg; GM1, 2.1 μg; GD1a, 4 μg; GD1b, 1.6 μg; GT1b, 1.9 μg). HPLC separation of standard ganglioside mixture obtained using a LiChrosorb $NH_2$ column; the elution profile was monitored by UV absorbance at 215 nm. Each UV peak was collected, chromatographed on silica gel 60 HPTLC plates and visualized by resorcinol staining. M = markers.

*Spectrophotometric analysis*

The maximum absorbance characteristic of gan-

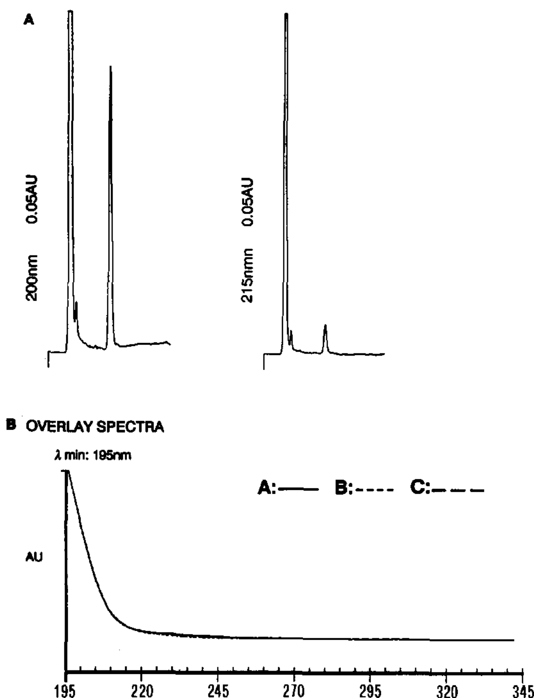


Fig. 2. (A) Chromatograms of standard GM3 detected by diode-array detector at 200 and 215 nm. (B) Peak purity of standard GM3 determined by diode-array detector. UV spectra acquired in the ascending (A), apex (B) and descending (C) portion of the peak were superimposable, indicating the absence of impurity.

gliosides corresponded in our diode-array detector system to the start of the UV acquisition data range (195–370 nm). Therefore it was planned to confirm the diode-array detector results using the Lambda 4B spectrophotometer adjusted in the range 190–220 nm. UV absorbance spectra of all the standard gangliosides, dissolved in the corresponding HPLC eluent fractions, were determined before HPLC analysis. All of them showed a characteristic spectrum with maximum absorbance at 195.8 nm (Fig. 3A). Furthermore, during HPLC separation, all the UV peaks eluting from diode-array detector system were collected. These fractions, when analysed using the spectrophotometer, confirmed the maximum absorbance at 195.8 nm obtained with diode-array detector system, thus suggesting that our HPLC conditions do not interfere with the spectrophotometric properties of ganglioside molecules. The same fractions, when dried and chromatographed on HPTLC plates, gave positive results on resorcinol staining, confirming the presence of gangliosides.

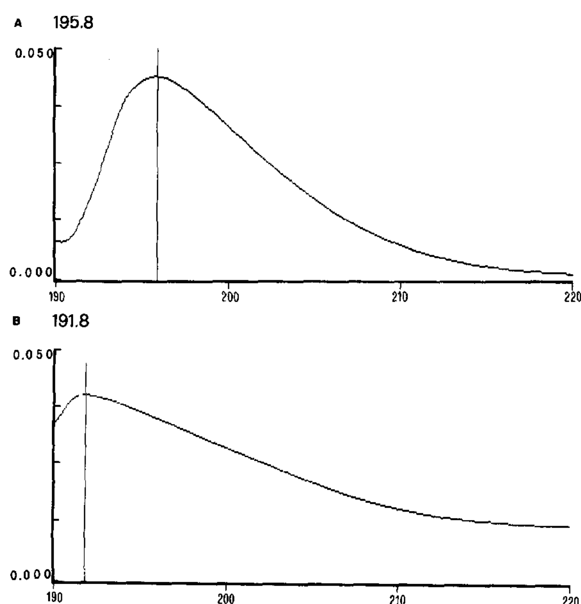


Fig. 3. Absorbance spectrum in the range 190–220 nm of (A) standard GM3 and (B) a collected unretained peak (*i.e.* phosphatidylethanolamine). *x*-Axis, wavelength expressed in nm; *y*-axis, UV absorbance expressed in a.u.f.s. The maximum absorbance is at the top of the *y*-axis.

*HPLC analysis of non-ganglioside glycolipids*

As highly polar lipids can be extracted with gangliosides and can co-elute with gangliosides during HPLC analysis, the retention times of non-sialic acid-containing glycolipids and phospholipids were determined. Under these chromatographic conditions, non-sialic acid-containing glycolipids (asialo GM1 and lactosylceramide) and phospholipids (phosphatidylcholine and phosphatidylethanolamine) were not retained, whereas phosphatidylserine and phosphatidylinositol had retention times of 3.5 and 3.9 min, respectively. All phospholipids tested had a maximum absorbance at 191.8 nm (Fig. 3B). In addition, a chromatographic separation of a mixture of phosphatydilinositol and GM3 (the last phospholipid and the first ganglioside to be eluted) showed two very distinct peaks, the first with the expected retention time (3.9 min) of phosphatydilinositol, the second with expected retention time (6.9 min) of GM3 (data not shown). These data show that under these chromatographic conditions phospholipids do not interfere with ganglioside seperation.

These data on the diode-array detection of gangliosides suggest that the difficulties associated with traditional UV detection at 215 nm can be avoided by the use of the variable-wavelength detector. Analytical data acquired with the diode-array detector make accurate qualitative and quantitative analyses possible. Furthermore, its high sensitivity eliminates the need for subsequent HPTLC analysis (which has until now been used for the identification of gangliosides after HPLC separation), thereby avoiding the accompanying problems of that analysis and the large sample sizes that it requires.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Italian Ministry of Education, National Research Council and Centro Internazionale Studi Diabete. The authors gratefully acknowledge the excellent technical assistance of P. Zoppitelli and the editorial assistance of C. Caputi and S. Salvati.

## REFERENCES

1 L. Svennerholm, *J. Neurochem.*, 10 (1963) 613.
2 R. W. Leeden and R. K. Yu, *Methods Enzymol.*, 83 (1982) 139.

3 W. van Heyningen, *J. Gen. Microbiol.*, 20 (1959) 291.
4 S. Hakomori, *Proc. Natl. Acad. Sci. U.S.A.*, 67 (1970) 1741.
5 S. Spiegel and P. Fishman, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 141.
6 R. Kannagi, K. Watanabe and S. Hakomori, *Methods Enzymol.*, 138 (1987) 3.
7 G. W. Jourdian, L. Dean and S. Roseman, *J. Biol. Chem.*, 246 (1971) 430.
8 G. Gazzotti, S. Sonnino and R. Ghidoni, *J. Chromatogr.*, 348 (1985) 371.