Journal of Chromatography, 195 (1980) 257–264
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,801

BRAIN GANGLIOSIDES: AN IMPROVED SIMPLE METHOD FOR THEIR EXTRACTION AND IDENTIFICATION

J. A. J. RANDELL
Department of Science, Bristol Polytechnic, Coldharbour Lane, Bristol (Great Britain)
and
C. A. PENNOCK*
Department of Child Health, University of Bristol, Bristol Maternity Hospital, Bristol (Great Britain)
(First received July 30th, 1979; revised manuscript received February 29th, 1980)

SUMMARY

Total ganglioside extracts prepared from brain tissue were concentrated either by dialysis against Carbowax or by employing Millipore filter cones. Thin-layer chromatography was then carried out using silica gel plates. After location of the various fractions quantitation was effected by direct densitometry.

The methods that have been adopted are rapid and suitable for the study of brain gangliosides in post mortem and biopsy material in a clinical chemistry laboratory.

INTRODUCTION

Gangliosides serve as surface membrane receptors and play a significant role in maintaining the internal environment of the body. They are a class of negatively charged glycosphingolipids the molecules of which contain both hydrophilic and hydrophobic regions. The hydrophilic moiety consists of a carbohydrate portion to which one or more sialic acid residues are attached and the hydrophobic moiety is ceramide. Details of results of structural analysis studies are given in a review by Svennerholm[1] and are summarised in Table I.

The functional role of the gangliosides is still speculative but they have been implicated in several neurological mechanisms[2–5]. Genetically determined attenuation of one or more of the enzymes involved in ganglioside metabolism leads to severe neurological dysfunction in affected patients and is usually accompanied by ganglioside accumulation[6] such that both concentration and pattern of human brain gangliosides differs markedly from the normal.

One of the obstacles in the clinico-chemical study of the gangliosides has been the lack of a simple and reliable method for the isolation of gangliosides from tissue extracts. Difficulties have arisen primarily because of the formation of easily contaminated micelles in aqueous and organic solvents and inefficient extraction and concentration procedures. Most methods are based upon that of Folch *et al.*[7] whereby total

0021-9673/80/0000–0000/$02.25

TABLE I

STRUCTURE AND NOMENCLATURE OF BRAIN GANGLIOSIDES

The structures and nomenclature shown are based on the work of Svennerholm[1]. This nomenclature is used throughout the text with the addition of GQ 1, (a ganglioside similar in structure to GT 3 but having four sialic acid residues) and G7 of unknown structure; the nomenclature adopted by Zanetta *et al.*[12].

Compound	*Structure*	*Symbol*
Monosialoganglioside	Gal 1 → 4 Gluc 1 → Cer 3 ↑ NANA 2	GM3
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 ↑ NANA 2	GM2
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 3 ↑ ↑ Gal 1 NANA 2	GM1
Disialoganglioside	Gal 1 → 4 Gluc → Cer 3 ↑ NANA 2 → 8 NANA 2	GD3
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 ↑ NANA 2 → 8 NANA 2	GD2
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 3 ↑ ↑ Gal 1 NANA 2 → 8 NANA 2	GD1b
	GalNac 1 → 4 Gal 1 → 4 Gluc → Cer 3 3 ↑ ↑ Gal 1 NANA 2 3 ↑ NANA 2	GD1a
Trisialoganglioside	GalNac 1 → 4 Gal 1 → 4 Gluc → Cer 3 3 ↑ ↑ Gal 1 NANA 2 → 8 NANA 2 3 ↑ NANA 2	GT1

lipids are extracted in chloroform–methanol mixture and the gangliosides partitioned into an aqueous phase which is then dialysed. Numerous modifications have been applied to increase total yield or to reduce contamination by non-ganglioside material one of the most successful of which appears to be that of Suzuki[8]. The final dialysis can be avoided using the technique of Carter and Kanfer[9] whereby water insoluble calcium complexes of the gangliosides are formed.

The simplest and most effective procedures for the separation of lipophilic mixtures are systems relying upon adsorption chromatography. As a consequence silica gel has proved to be the chromatographic support of choice and both thin-layer (TLC) and column chromatography have been utilised. Several solvent systems have been formulated including chloroform–methanol–water mixtures[10] and more recently tetrahydrofuran–water[11] and methyl acetate–isopropanol–water[12] which achieve good resolution with rapid separation time. Harth *et al.*[13] have applied total lipid extracts directly to silica gel plates and have obtained ganglioside separation with some purification by using three solvent systems successively, fractionation is however time consuming.

After chromatograms have been developed the gangliosides may be visualised using orcinol[14], resorcinol[15] or Ehrlich's reagent[16] which detect the sialic acid residues. Such procedures afford a means of ready qualitative assessment and quantitative evaluation by direct-scanning densitometry[17,18].

We describe here our own improved method based on selective combination of some of these techniques, for the isolation and concentration of brain gangliosides and their identification.

EXPERIMENTAL

Extraction procedures

All chemicals and reagents (British Drug Houses, Poole, Great Britain) were of analytical grade purity. Solvents were used without further purification. Homogenisation of samples was achieved with a Polytron homogeniser (Northern Media Supply, Brough, Great Britain), and extracted samples were applied to chromatograms with Hamilton syringes. Visking cellophane dialysis tubing (8/32) was obtained from Medicell International (London, Great Britain) and filter cones were obtained from Amicon (High Wycombe, Great Britain).

A representative sample of brain tissue was weighed and chopped into small pieces. Portions of approximately 1 g in weight were homogenised in nineteen volumes of chloroform–methanol (2:1, v/v) for 1 min. The homogenates were combined and rehomogenised for a further 2 min. After centrifugation, the supernatant was removed, four volumes of 0.1 *M* potassium chloride was added to it and the resultant homogenised for 1 min. The upper phase was then removed and the lower phase washed twice with ten volumes of theoretical upper phase containing potassium chloride (*i.e.* chloroform–methanol–0.1 *M* potassium chloride; 3:48:47). All the upper phases were pooled and the residual chloroform driven off under a stream of nitrogen. The final extract was then concentrated by sealing the resultant solution in Visking dialysis tubing and subjecting it to overnight dialysis against 20% Carbowax at 4°C. The concentrate was freeze dried and after weighing, was redissolved in 1 ml of distilled water prior to application to the chromatography plate. Alternatively, the sample was concentrated by centrifugation in a Millipore centriflow membrane cone RF25. About 7 ml of the extract was placed in the cone and concentrated to approximately 1 ml. Further additions were made to the cone and the procedure repeated until the whole aqueous phase had been concentrated. The inside of the cone was washed with this residual fluid to recover any material that might have adhered to the surface. This filtrate was freeze dried and after weighing the residue was dissolved in 1 ml of distilled

water and submitted to TLC. When knowledge of this weight was not required the filtrate was applied directly.

Chromatography procedure

Merck silica gel G 60, a medium porosity gel containing 13% calcium sulphate and having a mean pore diameter of 60 Å, was used for the preparation of thin-layer plates for chromatography. Precoated plates were also purchased from E. Merck (Darmstadt, G.F.R.) both glass backed, 5 × 20 cm, and aluminium foil-backed 20 × 20 cm, each 250 μm thick.

Chromatography was done in Shandon Universal TLC Chromotanks (Shandon, London, Great Britain) and plates were dried with a domestic hairdryer and sprayed with location reagents using Shandon spray apparatus (Shandon). Activation of plates was achieved in a hot-air oven which was also used for complete location of chromatographed material. Scanning of thin-layer plates was achieved on a Chromoscan 200 with a scan 201 (Joyce, Loebl & Co., Burlington, MA, U.S.A.). The weighing of samples of portions of chromatographic scans less than 1 mg in total weight was done on a Sauter five place microbalance.

Samples were applied to the plates as a band (1.8 cm from the lower edge of the plate) thereby making visualization easier and reducing the amount of tailing that might occur during development. The band, normally spread over 1.5 cm, was applied in the form of a series of tiny confluent drops. A hairdryer was used to evaporate off the sample solvent when necessary. The total volume applied to the plate was dependant upon the weight of the lyophilate dissolved. A 200-μg amount of ganglioside in solution was applied to either pre-prepared commercial plates or glass backed plates prepared by us. The solvent mixtures were prepared freshly for each chromatographic run and the chromotography tanks were lined with Whatman 3 MM paper which dipped into the solvent. Plates were chromatographed in either chloroform–methanol–1.4 *M* ammonium hydroxide (55:40:10) or tetrahyfrofuran–0.05 *M* potassium chloride in various combinations. The chromatogram was allowed to develop until the solvent front was within 2 cm of the upper edge of the plate. Excess solvent was removed by the use of a hairdryer in a fume cupboard followed by final complete removal by placing the plate in an oven at 120°C for 3 min. The location reagent consisted of a solution containing 200 mg of resorcinol in 100 ml of 4 *M* hydrochloric acid with 2.5 ml of 0.1 *M* copper sulphate added. (The solution is prepared at least 4 h before use but may be kept in a dark brown bottle at 4°C for about 4 weeks). The dried plates were sprayed lightly and covered carefully with a clean plate before being placed in an oven 120°C for about 20 min. A blue colour against a white background reveals the presence of sialic acid-containing compounds. Quantitation of gangliosides was achieved by placing the plate in a Chromoscan 200 which was adjusted to zero absorbance on an unstained portion of the plate area before scanning the stained gangliosides automatically. A red filter (610 to 750 nm), which was complementary to the blue colour of the localised gangliosides, was used and the 1:4 cam on the instrument was used to scale expand the reading which was recorded simultaneously in the form of a line graph so that for each band a peak was obtained. Peak areas were calculated by cutting out the peak from the paper trace and weighing the paper on a suitable five place microbalance.

RESULTS AND DISCUSSION

Thin-layer chromatographic investigation[19] of several published modifications of the Folch[7] extraction procedure showed that they were incomplete, traces of the smaller-molecular-weight components GM3 and G7 being present in the organic residues after extraction. At the present time this is not important since the relative concentrations of these fractions do not appear to have any primary diagnostic significance. This may change in the future when it will be necessary to modify existing methods.

When a general lipid location reagent was applied to developed chromatograms of the purified extracts it was apparent that the degree of purity achieved was roughly proportional to the sophistication of the extraction method. The compromise technique described here was selected on the basis of degree of contamination and the time involved.

The two modifications for the concentration of ganglioside extracts seem to be successful. The cone method is rapid and would appear to have a place in the hospital laboratory repertoire where a result is required on a very small sample. Routinely the method can also be used prior to lyophylisation. A minor disadvange is that several seedings have to be made to ensure optimal results. Chromatography of the reconstituted filtrate from the cone shows an absence of gangliosides, however the presence of sialic acid in the filtate demonstrated by the method of Warren[20] indicates the possibility of some breakdown during the procedure but if this is the case then such losses are non-selective since the relative percentage of each ganglioside remains unchanged within the tolerances that can be applied. Dialysis against Carbowax produced similar relative proportions of ganglioside fractions (Table II) and was selected for the standard procedure not only on the grounds of cost but because dialysis takes place overnight and therefore no working time is lost.

It was considered that the time taken and the degree of separation achieved using the classical chloroform–methanol–water system for chromatography did not warrant its routine use consequently the solvent of Eberlein and Gercken[11] was examined. Variations in relative proportions of tetrahydrofuran–0.05 *M* potassium chloride significantly altered the separation of the gangliosides (Fig. 1) and it was confirmed that the original solvent system suggested was in fact optimal but that a shorter development time was feasible. If the relative proportion of tetrahydrofuran to aqueous potassium chloride was maintained at 5:1 then concentration changes over the range 0.025–0.1 *M* potassium chloride were without signicance.

A comparison of different types of thin-layer plate showed that the resolution on commercial glass backed plates was better than that achieved on laboratory prepared plates. However foil backed commercial plates showed a lowering in relative R_F values and a tendency for the coating to lift during the location process. Furthermore, the densities of the resultant bands were so reduced that only half the sensitivity was realised. Van den Eijnden[10] observed that poor results were obtained when purified gangliosides were separated on precoated commercial plates, the addition of potassium chloride and calcium chloride to the chromatographic solvent markedly improving resolution. Consequently a number of other compounds were employed in place of 0.05 *M* potassium chloride within the tetrahydrofuran system, these included hydrochloric acid, sodium chloride, acetic acid and sodium hydroxide (all at 0.05 *M*

TABLE II

COMPARISON OF THE RELATIVE PROPORTION OF GANGLIOSIDES FOUND USING TWO DIFFERENT METHODS FOR SAMPLE CONCENTRATION

Gangliosides are identified from left to right in order of increasing mobility in tetrahydrofuran–water with added potassium chloride (see text).

Sample	*Method*	*Relative % of each fraction*									
		GQ1	*GT1*	*GD1b*	*GD2*	*GD1a*	*GD3*	*GM1*	GM_2	GM_3	G_7
1	Cone	1.94	14.62	5.93	—	54.77	4.90	15.50	—	1.72	0.62
	Carbowax	1.52	15.87	6.87	—	53.02	4.51	16.05	—	1.86	0.30
2	Cone	trace	1.08	5.42	—	24.12	7.26	53.87	—	5.12	3.23
	Carbowax	trace	1.23	4.93	—	22.18	6.42	54.72	—	7.12	3.40

concentration) and calcium chloride, calcium acetate, calcium sulphate and sodium sulphate (all at 0.025 *M* concentration). Results indicated that it is the chloride ion only that has a significant affect, other ions and changes in pH appeared to be insignificant. It is probable that the chloride ion is bound by electrostatic attraction to the sialic acid residues of the gangliosides thereby reducing the strong interaction of dissociated sialic acid and the silica residues of the plate. Thus band broadening and other specific interactions between gangliosides are decreased, this being reflected in changes in R_F values and better resolution (Fig. 2).

Attempts at quantitation of the gangliosides using resorcinol location followed by densitometric scanning showed that the scan area of the peaks obtained were linearly proportional to the amount of ganglioside present when varying amounts of between 50 and 350 μg of a total ganglioside extract were seeded. Over the range 100–

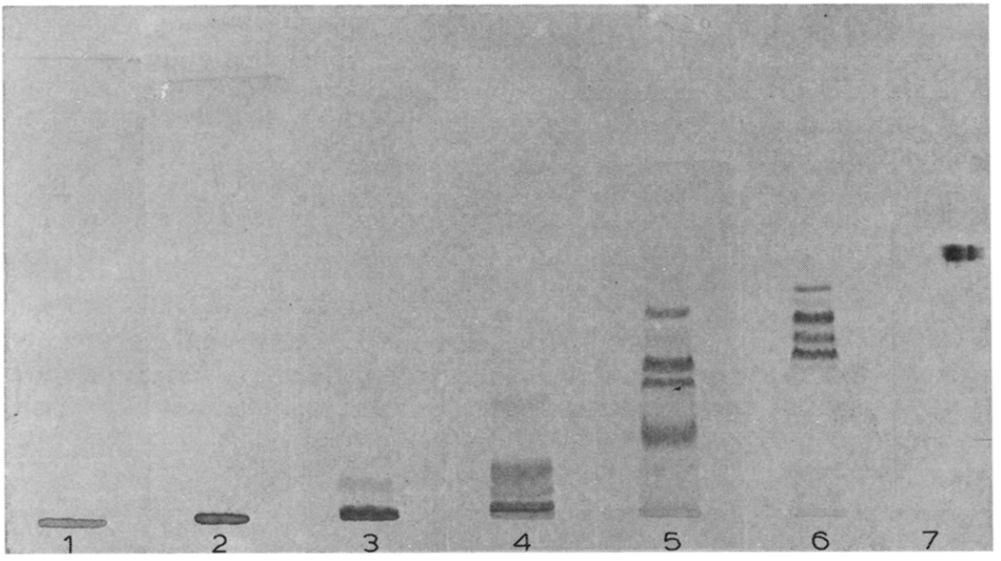

Fig. 1. The effect of alteration of the relative volume of aqueous potassium chloride to tetrahydrofuran on TLC separation of brain gangliosides. The relative volume of potassium chloride to five volumes of tetrahydrofuran was: 1(0), 2(0.4), 3(0.6), 4(0.8), 5(1.0), 6(1.2), 7(2.0).

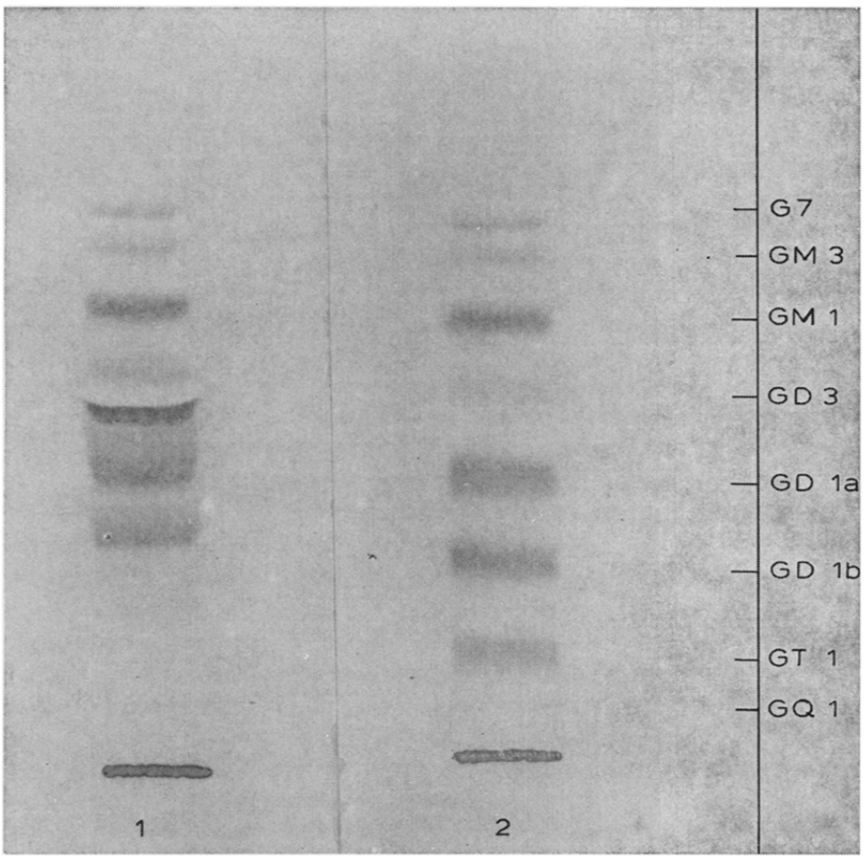

Fig. 2. The effect of chloride ion on the TLC of brain gangliosides. 1 = Tetrahydrofuran with water (5:1); 2 = tetrahydrofuran with 0.05 *M* potassium chloride (5:1).

300 μg there was little difference in calculated relative percentages of each fraction except when this quantity was very low and therefore the scan figure subject to error. A maximum scan error of less than 5% was obtained for all fractions other than GM3 (7%) and G7 (31%), however, both of these constitute extremely small fractions within the total (2% and 0.3% respectively) and the results obtained are diagnostically useful for the major gangliosides. A problem associated with resorcinol location is that the keeping properties are poor and fading occurs within 3 days, the background taking on a pink colouration making rescanning impracticable.

The overall thin-layer procedure using the optimal tetrahydrofuran–potassium chloride solvent appears to be more efficient under the conditions used than at first reported since a further fraction is identified as lying between the seeding line and GT1. Because this band and certain subfractions of other bands appear to be the same as those isolated by Zanetta *et al.*[12] using a different solvent system it was decided to adopt their nomenclature.

We have successfully used the method described for the identification of GM1 gangliosides in human and cat brain affected by GM1 gangliosidosis and the demonstration of GM2 ganglioside in patients with Tay Sachs disease.

We have also found increased amounts of a ganglioside migrating as GD2 in brain from patients with San Filippo syndrome (mucopolysaccharidosis III) and are currently using the technique to evaluate the long term effect of histological fixation.

REFERENCES

1 L. Svennerholm, *J. Lipid Res.*, 5 (1964) 145.
2 H. Dreyfus, P. F. Urban, P. Bosch, S. Edel-Harth, G. Rebel and P. Mandel, *J. Neurochem.*, 22 (1974) 1073.
3 P. H. Fischman and R. O. Brady, *Science*, 194 (1976) 906.
4 H. Rahmann, H. Rosner and H. Breer, *J. Theor. Biol.*, 57 (1976) 231.
5 H. Dreyfus, S. Harth, P. F. Urban and P. Mandel, *Vision Res.*, 16 (1976) 1365.
6 K. Suzuki, in S. M. Aronson and B. W. Volk (Editors), *Inborn Disorders of Sphingolipid Metabolism*, Pergamon, New York 1967, p. 215.
7 J. Folch, M. Lees and G. H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
8 K. Suzuki, *J. Neurochem.*, 12 (1965) 629.
9 T. P. Carter and J. N. Kanfer, *Methods Enzymol.*, 35 (1975) 549.
10 D. H. van den Eijnden, *Hoppe–Seyler's Z. Physiol. Chem.*, 352 (1971) 1601.
11 K. Eberlein and G. Gercken, *J. Chromatogr.*, 106 (1975) 425.
12 J. P. Zanetta, F. Vitiello and J. Robert, *J. Chromatogr.*, 137 (1977) 481.
13 S. Harth, H. Dreyfus, P. F. Urban and P. Mandel, *Anal. Biochem.*, 86 (1978) 543.
14 E. Klenk, U. W. Hendricks and W. Gielen, *Hoppe–Seyler's Z. Physiol. Chem.*, 330 (1962) 140.
15 L. Svennerholm, *J. Neurochem.*, 10 (1963) 613.
16 R. Kuhn and H. Wiegandt, *Chem. Ber.*, 96 (1963) 866.
17 F. Šmíd and J. Reinišová, *J. Chromatogr.*, 86 (1973) 200.
18 K. Sandhoff, K. Harzer and H. Jatzkewitz, *Hoppe–Seyler's Z. Physiol. Chem.*, 349 (1968) 283.
19 J. A. J. Randell, *MSc. Thesis*, University of Bristol, 1977.
20 L. Warren, *J. Biol. Chem.*, 234 (1971) 1959.