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Thin layer chromatography of gangliosides

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Abstract Thin layer chromatography is the easiest way to analyze the total glycosphingolipid mixtures extracted, and, in some cases, partially purified from tissues and cultured cells. Several solvent systems have been introduced to separate the complex mixtures as a function of their composition, presence of contaminants and, in some cases, of their quantity. In addition, colorimetric, enzymatic, immunological and radiochemical detection procedures are available for their recognition. The method does not allow to determine the chemical structure of separated molecules, but gives a very economical and very accessible first information on their possible structure on the basis of their chromatographic mobility in comparison with standards, and of their reactivity to the staining procedures. In this paper we show how to perform mono and two-dimensional thin layer chromatography of the total lipid mixture extracted from mouse brains and, in a few cases, from cells in culture. Table 1 shows the structures of reported lipids.

Keywords Glycosphingolipids · Gangliosides · Sphingolipids · TLC

Introduction

Glycosphingolipids (GSL) are a wide class of lipids sharing the same basic structure represented by ceramide. Ceramide is an hydrophobic molecule constituted by a long chain amino-alcohol, called sphingosine, linked by an amidic bond with a fatty acid.

Gangliosides are a group of complex glycosphingolipids which characteristically contain sialic acid as a component of their carbohydrate chain. They appear to be ubiquitous in vertebrate tissues and have been found in some invertebrates too [1]. However, their concentration in brain (where they are present in substantially higher concentrations in gray matter) far exceeds that in other tissues [2, 3]. Their accumulation and pattern alteration in some neurological diseases and their possible involvement in functions, such as synaptic transmission and memory formation, as well as in several neurological disorders [4], are additional reasons that evoked a special interest for brain gangliosides. On the other hand, sulfatides are associated primarily with myelin and they account for 4–6% of total lipids of brain. The loss of sulfatides is thus a specific marker for demyelinating neurodegenerative diseases.

The functional relevance of GSL is not restricted to the nervous system. For example, they have been implicated in several aspects of malignant transformation as modulators of cell growth, apoptosis, motility and invasiveness [5]. More in general, GSL organized in membrane domains with a peculiar lipid composition and supermolecular organization are able to control and modulate the activity of several membrane proteins involved in signal transduction [6]. Thus, the availability of sensitive and widely applicable analytical method for GSL analysis is of great interest. However, their complexity has always represented a challenge for scientists. It should be recalled that this complexity is not limited to the structure of their carbohydrate moieties: GSL molecular species of gangliosides differ as well in their hydrophobic components, fatty acids and long chain bases [7].

Liquid chromatography (LC) is one of the oldest techniques used for the analysis of complex lipid mixtures

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coming from biological samples. Nowadays the most informative LC methodology is probably the HPLC (high pressure liquid chromatography) used as preparative technique for the further mass spectrometry analysis in a LC-MS system. This kind of approach makes possible the precise characterization of the nature and the quantification of the amount of each lipid species contained in the injected sample, but it is clearly a technique that requires an important capital outlay both for the instrumentation and for the professional training. Similar considerations could be done for the gas chromatography approach, coupled or not to a mass spectrometer.

To overcome these economic and instrumental restrictions, an opportune use of thin layer chromatography (TLC) has proven along the years to have enormous advantages when applied to the study of lipid mixtures deriving from biological samples.

This methodological approach is easily exploitable in almost all the biochemical laboratories because it does not require a sophisticated equipment or a complex instrumental training. The nowadays wide commercial availability of high quality pre-cast TLC plates overcomes the major problem associated with this technique, *i.e.* the preparation of the chromatographic support. TLC is not only cheap and accessible; it is extremely versatile due to the great range of separation conditions that can be achieved by combining different chromatographic supports and different separating solvent mixtures. Moreover, the chromatographic plate can represent a valid support for many different chemical or immunochemical treatments, useful for a more precise characterization of the nature of the separated lipids, and allowing, in some cases, to reach a very high sensitivity and specificity in the final detection step.

In this paper we discuss the application of TLC for the analysis of gangliosides in the total lipid mixtures obtained from cell cultures and brains, paying a particular attention to the lipid extraction procedure, a crucial point to obtain a quantitative isolation of all the classes of lipids present in the original samples. This aspect is particularly relevant for biological samples deriving from neuronal cell cultures or nervous tissues, in which the amount of gangliosides, the more hydrophilic lipids (whose extraction yield is strongly influenced by the extraction conditions and by the presence of water in the sample) is much higher than in other samples of different origin.

Materials

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus.

Trypsin, KCl, reagents for cell cultures were from Sigma Chemical Co. (St Louis, MO, USA). Basal modified Eagle's medium (BME), Eagle minimum essential medium (EMEM) with Earle's Salts and fetal calf serum, were purchased from EuroClone (Leeds, UK).

MF discs mixed cellulose esters, hydrophilic, 0.025 μm for micro dialysis were from Millipore.

Sphingosine was prepared from cerebroside [8]. [1-^{3}H] sphingosine was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro[^{3}H]hydride [9] (radiochemical purity over 98%; specific radioactivity 2 Ci/mmol).

Neuraminidase (*Clostridium perfringens,* type V), horseradish peroxidase-conjugated cholera toxin B subunit, *o*-phenylenediamine tablets and galactosylceramide were from Sigma (St Louis, MO, USA).

High performance silica gel precoated thin layer plates (TLC Kiesegel 60) were purchased from Merck.

Lipids to be used as standard were extracted from rat brain [10], purified by partitioning [11], and structurally characterized [12]. Lactosylceramide, and glucosylceramide were prepared by acid hydrolysis of ganglioside GM1 [13].

Granule cells, obtained from the cerebellum of 8-days-old Sprague-Dawley rats, were prepared and cultured as described [14]. Cells were plated in 100-mm dishes at a density of 9×10^6 cells/dish with BME containing 10% heat-inactivated fetal calf serum and cultured for 8 days to fully differentiated neurons [15]. Normal human skin fibroblasts were obtained by the punch technique, cultured, and propagated as described [16] in 100-mm dishes (≈0.35 mg of protein/dish), using EMEM supplemented with 10% FCS. Confluent fibroblasts were used for the experiments.

Brain tissue was from 7-months-old C57 mice.

Methods

Treatment of cell cultures with [1-^{3}H]sphingosine

Cells were incubated with 3×10^{-8} M [1-^{3}H]sphingosine (5 ml/dish) for 2 h pulse followed by 48 h chase. The pulse of rat cerebellar granule cells (CGC) was performed at the 6th day in culture and the radioactive sphingosine was dissolved in cell-conditioned medium. Human skin fibroblasts were radiolabelled in a pre-confluent stage with radioactive sphingosine dissolved in cell culture medium. After the pulse period, the medium was removed and replaced with cell-conditioned medium or with fresh medium (for neurons and fibroblasts, respectively) without radioactive sphingosine for the chase period. Under these conditions, free radioactive sphingosine was barely detectable in the cells and all cell sphingolipids, including

ceramide, sphingomyelin, neutral glycolipids and gangliosides were metabolically radiolabelled. Tritium-labelled phosphatidylethanolamine was also obtained due to the recycling of radioactive ethanolamine formed in the catabolism of [1-^{3}H]sphingosine [17].

The radioactivity associated with cells was determined by liquid scintillation counting: it corresponded to 145 nCi/mg and to 333 nCi/mg of cellular proteins, respectively for CGC and human fibroblasts.

Lipid extraction from cells and brain tissue

Cell dishes were washed twice with ice cold PBS containing 0.4 mM Na_3VO_4 and then scraped in PBS and collected in centrifuge tubes. The cell pellets were obtained by centrifugation at 4°C at 1,600×*g* for 10 min and 450×*g* for 5 min for CGC and human fibroblasts, respectively, and then resuspended in iced water to be snap frozen and lyophilised. Fresh brains were weighted, minced with a razor blade and homogenized in iced water (500 mg of fresh tissue/ml) and sonicated, maintaining all samples in ice immersion. The homogenized tissues were snap frozen and lyophilised.

Lipids from lyophilized cells (corresponding to about 0.40 mg of proteins for labelled cells and to around 1.35–3.9 mg of proteins for not labelled cells) and lyophilised brain samples (corresponding to about 400 mg of fresh tissue) were extracted with chloroform/methanol/water 2:1:0.1 by vol. [18]. This first extraction was performed by adding 1550 μl and 1860 μl of the solvent system, respectively for cells and for tissue. The total lipid extracts were separated from the protein pellet by centrifugation at 13,400×*g* for 15 min. The cell pellets were subjected to a second lipid extraction by adding 250 μl of chloroform/methanol 2:1. The tissue pellets were subjected to two other extractions, using the same volume of the solvent system respect to the first extraction.

Protein content determination

The protein content was determined on the pellets obtained after the total lipid extraction. The pellets were digested in 1 M NaOH overnight and then diluted to 0.05 M to be used for protein estimation according to Lowry [19], using bovine serum albumin as the reference standard.

Phase partitioning

Aliquots of the total lipid extracts obtained both from cell cultures and from mouse brain were further subjected to a two-phase partitioning [11], resulting in the separation of an aqueous phase containing gangliosides and in an organic phase containing all other lipids. Briefly: an amount of water, corresponding to the 20% of the total volume of lipid extracts, was added to each total lipid extract. The solutions were centrifuged at 2,300×*g* for 15 min, obtaining the separation of two phases. The aqueous phases were transferred in other tubes and a similar volume of methanol/water 1:1 was added to the organic phases. The samples were mixed and the two phases were separated again by centrifugation.

The organic and aqueous phases were then dried under nitrogen flow and then resuspended in a known volume of chloroform/methanol 2:1, whereas the aqueous phases were resuspended in a small volume of water (< of 70 μl) to be subjected to micro dialysis against water for 4–5 h. At the end of the dialysis the samples were lyophilised and then resuspended in a known volume of chloroform/methanol 2:1.

The two phases thus obtained, as the total lipid extracts, were used for the TLC separation and analysis.

Micro dialysis

This procedure allows to efficiently remove salts from aqueous phases, working with micro-volumes. Floating membrane filters replace the dialysis sac. A glass container was filled with water and membrane filters were allowed to float on the water surface. Aqueous phases resuspended in about 50 μl of water were dropped on the centre of the membrane discs. The apparatus was covered to prevent evaporation. During the time of dialysis, the volume of the samples increased and after 4–5 h the dialyzed aqueous phases were recovered and lyophilised.

Alkaline treatment on the organic phases

Alkaline treatment allows to remove glycerophospholipids from the organic phases, breaking their ester bonds, and maintaining unaltered the amide linkage of sphingolipids. This procedure allows to remove several chromatographic interferences. Aliquots of organic phases were dried under nitrogen flow and the residue was resuspended with 100 μl 0.6 M NaOH in methanol and allowed to stand at 37°C for three h and overnight at room temperature. The reaction was blocked by adding 120 μl 0.5 M HCl in methanol. Finally, after phase separation (by adding 1,050 μl of chloroform/methanol/water 70:18:17), the new organic phases were used for TLC analysis.

Mono-dimensional TLC

The lipid residue dissolved in chloroform-methanol, 2:1 by vol. was applied on a 3 mm lane at 1.5 cm from the plate bottom edge. Different samples were applied maintaining a 3–5 mm distance. The plate was immersed into the chromatographic solvent system (1 cm deep) and chromatographed in a closed tank allowing the solvent to reach the

TLC top edge (TLC size, 10×10 cm). Chromatography was carried out at room temperature in the range of 20–25°C.

The aqueous phases were analyzed using solvent systems composed by chloroform/methanol/0.2% aqueous $CaCl_2$ in different reciprocal proportions, such as 40:60:25; 45:55:10; 50:42:11 and 60:35:8 by vol.

The organic phases were separated using the solvent system chloroform/methanol/water in the ratio of 110:40:6 by vol.

Sulfatide in the alkali-treated total lipid extracts was analyzed using the solvent system chloroform/methanol/water 70:25:4 by vol.

Galactosylceramide in the alkali-treated organic phases was analyzed using a TLC plate previously treated by spraying with 4% sodium tetraborate/methanol 1:3 by vol. and maintained at 120°C for 1 h, and the solvent system chloroform/methanol/2M NH_3 70:30:3 by vol.

Two-dimensional TLC

The lipid residue dissolved in chloroform–methanol, 2:1 by vol. was applied at 1.5 cm from the plate edge bottom and left side as a small circle of 1–1.5 mm diameter. The plate was immersed into the first chromatographic solvent system (1 cm deep) and chromatographed in a closed tank allowing the solvent to reach the TLC top edge (TLC size, 10× 10 cm). The plate was dried, rotated 90° left and immersed in the second solvent system (1 cm deep) allowing the solvent to reach the TLC top edge.

For the identification of *O*-acetylated gangliosides, the TLC plate was exposed to ammonia vapours in a closed tank; alkaline treatment removed the *O*-acetyl groups, linked to the sialic acid residues, and caused a differential chromatographic behaviour of these gangliosides in the second chromatographic run. Briefly: after the first chromatographic run, the plate was dried and vertically put on a support inserted into a tank containing 17 M NH_4OH (1–2 cm deep). Tank must be closed and great care is necessary to avoid any contact between the plate and the liquid. After 5 h, the plate is thoroughly dried and submitted to the second chromatographic run.

The aqueous phases were analyzed using the solvent systems composed by chloroform/methanol/0.2% aqueous $CaCl_2$ in the ratio of 50:42:11 by vol. in both the first and second run.

The organic phases were analyzed using the solvent system chloroform/methanol/water in the ratio of 110:40:6 by vol. in both the first and second run.

Colorimetric staining

Gangliosides were recognized using Ehrlich reagent, prepared dissolving 6 g of *p*-dimethylaminobenzaldehyde in a mixture of 200 ml 37% hydrochloric acid and 800 ml ethanol. The plate was moderately sprayed with the reagent, immediately and firmly covered with a 10×10 cm glass pre-warmed at 120°C, and maintained for 10 min at 120°C in oven [20]. Gangliosides reacted as grey spots.

All lipids were recognized using the anisaldehyde reagent, prepared dissolving 0.5 ml 4-methoxybenzaldehyde in a mixture of 50 ml glacial acetic acid and 1 ml 97% sulphuric acid. The TLC plate was thoroughly sprayed and then maintained for 15 min at 120°C in oven [21]. Lipid spots showed different colours: from green to brown.

All glycolipids were recognized using the aniline/diphenylamine reagent prepared dissolving 4 g of diphenylamine and 4 ml aniline in a mixture of 20 ml 85% phosphoric acid and 200 ml acetone. After a moderate spraying the plate was maintained at 120°C for 5–10 min in oven [22].

Sulfatide was recognised using cresyl violate acetate reagent (10 mg in 1% acetic acid) The TLC was soaked for 5 min in this staining solution and then washed several times with 1% acetic acid/methanol, 3:1. Sulfatide acquired red-violet colour against blue-violet of all the other lipids [23]

3–4 nmoles of glycosphingolipids were applied on the TLC plate for the colorimetric staining.

Identification was accomplished by chromatographic comparison with standard glycolipids.

Cholera toxin staining

Gangliosides belonging to the gangliotetraose series were detected directly on the TLC plate by the sialidase-cholera toxin procedure: sialidase converts gangliosides to GM1 and cholera toxin B specifically recognizes the oligosaccharide chain of GM1 ganglioside [24]. Identification of GM1 within the mixture was performed omitting the sialidase treatment. Briefly: after chromatographic running, the TLC plate was well dried and fixed with a polyisobutylmethacrylate solution prepared dissolving 1.3 g of polyisobutylmethacrylate in 10 ml chloroform and diluting 8 ml of this solution with 42 ml hexane. The TLC plate was immersed in this solution three times and then allowed to dry for 1–2 h. The dried TLC was soaked for 30 min in 0.1 M Tris–HCl pH 8.0, 0.14 M NaCl supplemented with 1% bovine serum albumin (BSA). The incubation with *Clostridium perfringens* sialidase was performed overnight at room temperature. The enzyme concentration was 0.12 U/ml in 0.05 M acetate buffer pH 5.4, 4 mM $CaCl_2$. Then the TLC was overlaid with HRP-conjugated cholera toxin subunit B diluted 1:1000 in PBS with 1% BSA for 1 h. After several washings with PBS, the TLC plate was developed with OPD-substrate (1 tablet in 50 ml citrate–phosphate buffer pH 5+20 μl H_2O_2) for 5 min.

0.3–0.4 nmoles of glycosphingolipids were applied on the TLC plate for the colorimetric staining.

Identification was accomplished by chromatographic and behavioural comparison with standard gangliosides.

Radioimaging

Tritium metabolically radiolabelled glycosphingolipids and phosphatidylethanolamine were visualized by digital autoradiography performed with a Beta-Imager 2000 instrument (Biospace, Paris). Total 200–1,000 dpm were applied on the plate and the image was acquired for 24–48 h. Identification was accomplished by chromatographic comparison with standard radiolabelled gangliosides and phosphatidylethanolamine.

Results and discussion

The total lipid extracts and the aqueous and organic phases obtained by partitioning are the sources for analysis of glycosphingolipids by TLC. The use of total lipid extract allows to obtain information simultaneously on all the sphingolipids, namely glycosphingolipids, sphingomyelin and ceramide, at the same time (see Table 1 for nomenclature and structures of reported lipids), but only a small amount of lipids can be applied on the TLC to obtain a good separation, due to the high content of glycerophospholipids in the total mixture. In addition, this approach is very useful to analyse those compounds that partition in both the aqueous and organic phases, like sulfatide. The aqueous phase is the source for ganglioside analysis, while the organic phase gives information on the neutral glycosphingolipids. Figure 1 shows schematically the different analytical pathways for TLC analyses.

Many solvents have been used in the past for TLC separation of glycosphingolipids. We can consider that the system comprising chloroform, methanol and aqueous solutions (or water) is now world wide used. Nevertheless, completely different results are obtained due to small differences in the ratio of the three components, as shown in Fig. 2.

Table 1 The lipids reported in the text

Lipids	Definition
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
Sph	(2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene/eicosene (sphingosine)
Cer	(2*S*,3*R*,4*E*)-2-(acyl)amino-1,3-dihydroxy-octadecene/eicosene (ceramide)
SM	Cholinephosphorylceramide (sphingomyelin)
GalCer	β-Gal-(1–1)-Cer
GlcCer	β-Glc-(1–1)-Cer
SM4s	$^{-}O_3S$-3-β-Gal-(1–1)-Cer
LacCer	β-Gal-(1–4)-β-Glc-(1–1)-Cer
Gg3Cer	β-GalNAc-(1–4)-β-Gal-(1–4)-β-Glc-(1–1)-Cer
Gg4Cer	β-Gal-(1–3)-β-GalNAc-(1–4)-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GM3	α-Neu5Ac-(2–3)-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GD3	α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GM2	β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GD2	β-GalNAc-(1–4)-[α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GM1	β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GD1a	α-Neu5Ac-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
O-Ac-GD1a	α- Neu5,9Ac_2-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GalNAc-GD1a	β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GD1b	β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
O-Ac-GD1b	β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5,9Ac_2-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GT1a	α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
O-Ac-GT1a	Unknown position of the acylating group in GT1a
GT1b	α-Neu5Ac-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
O-Ac-GT1b	α-Neu5Ac-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5,9Ac_2-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer
GQ1b	α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-Cer
O-Ac-GQ1b	α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5,9Ac_2-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-Cer
O-Ac_2-GQ1b	unknown position of the two acylating groups in GQ1b

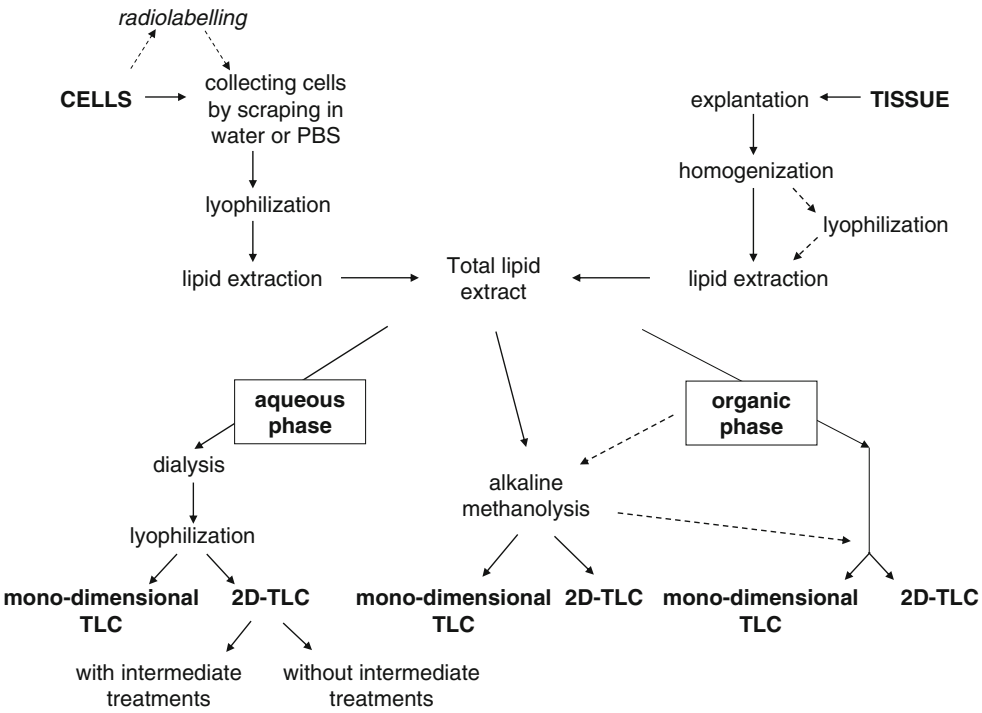


Fig. 1 Schematic representation of the analytical pathways for complex lipids TLC

Figure 3 shows the TLC separation of the total ganglioside mixture prepared from rat cerebellar granule cells, human fibroblasts and mouse brains. The use of chloroform/methanol/0.2% $CaCl_2$ in the volume ratio of 50:42:11, as solvent system and the use of three different staining systems allowed to separate and tentatively recognize, by comparison with ganglioside standards and by specific ganglioside features, up to 12 compounds. Lanes A of Fig. 3 show the ganglioside staining with *p*-dimethylaminobenzaldehyde reagent, considered one of the more specific reagent for aminosugars; lanes B show the ganglioside staining with cholera toxin, known to display a very high affinity for ganglioside GM1 (a few pmoles of GM1 can be recognized) and much less for GD1b; in lanes C, the plate after chromatography was treated with sialidase to hydrolyse gangliosides and then stained with cholera toxin to identify gangliosides that contain the GM1 core structure. Lanes D show the radioimaging of the separation of the ganglioside mixtures from cells in culture metabolically labelled with tritiated sphingosine. Only a very minor

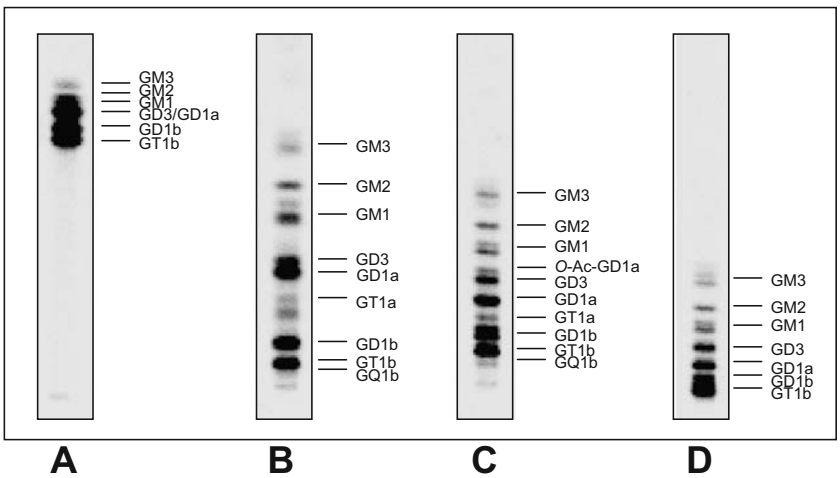


Fig. 2 Examples of TLC separations with different solvent systems. Radioimaging of the TLC separation of the ganglioside mixture (aqueous phase) from [1-^{3}H]sphingosine metabolically labelled rat cerebellar granule cells. Solvent systems were composed by chloroform/methanol/0.2% $CaCl_2$ in the volume ratio of 40:60:25 (*A*), 45:55:10 (*B*), 50:42:11 (*C*) and 60:35:8 (*D*). Acquisition time 30 h; total radioactivity, 500 dpm/lane

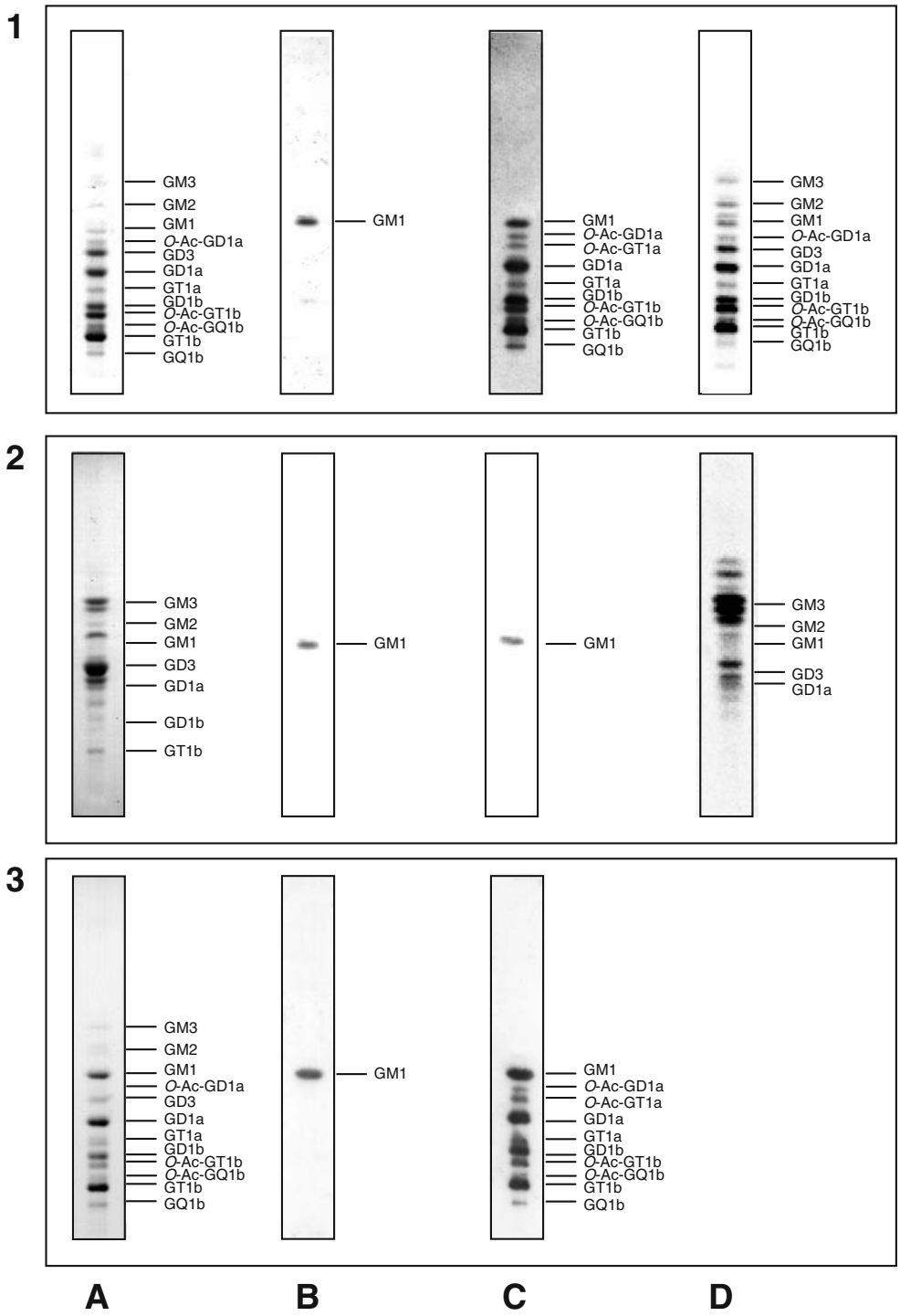


Fig. 3 TLC of gangliosides. TLC separation of the ganglioside mixture (aqueous phase) from rat cerebellar granule cells (*1*), from human fibroblasts (*2*) and from 7 months old mouse brains (*3*) using the solvent system chloroform/methanol/0.2% $CaCl_2$ 50:42:11 by vol. Lanes *A*: staining with *p*-dimethylaminobenzaldehyde reagent; lipids from 350–500 μg of cellular or tissue proteins. Lanes *B*: staining with cholera toxin; lipids from 35–50 μg of cellular or tissue proteins. Lanes *C*: staining with cholera toxin after sialidase treatment; lipids from 35–50 μg of cellular or tissue proteins. Lanes *D*: radioimages of cell metabolically tritium labelled gangliosides; lipids (500 dpm) from 5 μg of cellular proteins; acquisition time, 30 h

quantity of lipids is necessary for this analysis and this allows very high chromatographic resolution. 15–20 dpm per spot are sufficient for recognition and quantification.

Figure 4 shows the separation of lipid mixture present in the organic phase prepared from rat cerebellar granule cells, human fibroblasts and mouse brains. The organic phase prepared from total lipid extract contains glycerophospholipids, sphingomyelin, ceramide, neutral glycosphingolipids and glycerophospholipids as major components. These latter interfere with correct separation, recognition and quantification of sphingolipids (lanes A and C). Characterization of sphingolipids is achieved after alkaline treatment of the total mixture. The process hydrolyses ester linkages but does not affect the amide linkages. Thus, the alkaline treatment followed by partitioning to remove the water soluble compounds formed during the reaction and salts

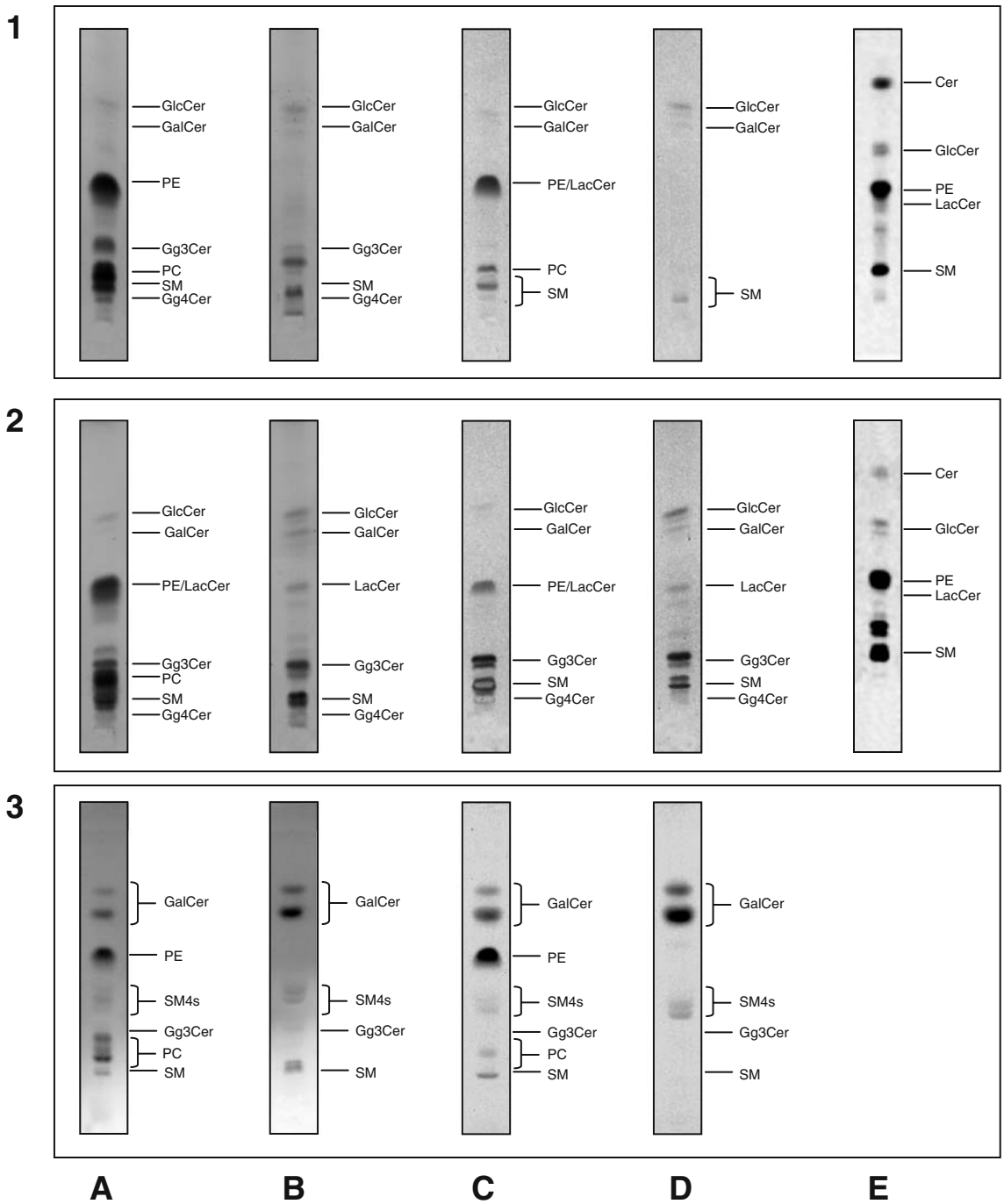


Fig. 4 TLC of neutral glycosphingolids. TLC separation of the lipids of the organic phases obtained from rat cerebellar granule cells (*1*), from human fibroblasts (*2*) and from 7 months old mouse brain (*3*) using the solvent system chloroform/methanol/water 110:40:6 by vol. Lanes *A*: staining with anisaldehyde reagent; lipids from 100–150 μg of cellular or tissue proteins. Lanes *B*: lipids from an alkali-treated organic phase; staining with anisaldehyde reagent; lipids from 200–300 μg of cellular or tissue proteins. Lanes *C*: staining with aniline/diphenylamine reagent; lipids from 200–250 μg of cellular or tissue proteins. Lanes *D*: lipids from an alkali-treated organic phase; staining with aniline/diphenylamine reagent; lipids from 400–500 μg of cellular or tissue proteins. Lanes *E*: radioimages of cell metabolically tritium labelled lipids; lipids (500 dpm) from 1–3 μg of cellular proteins; acquisition time 30 h

(lanes B and D) allow a good separation and identification of sphingolipids. Sphingolipids from cells metabolically tritium labelled with sphingosine do not require to be alkali-treated before analysis. In this case, the small amount of lipids applied on the plate allows to get a good sphingolipid separation even in the presence of glycerophospholipids. In addition, feeding cells with sphingosine that contains tritium at position 1, allows to detect also phosphatidyleth-

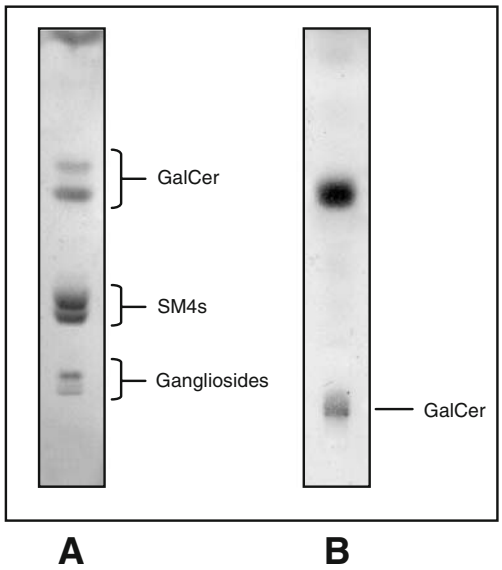
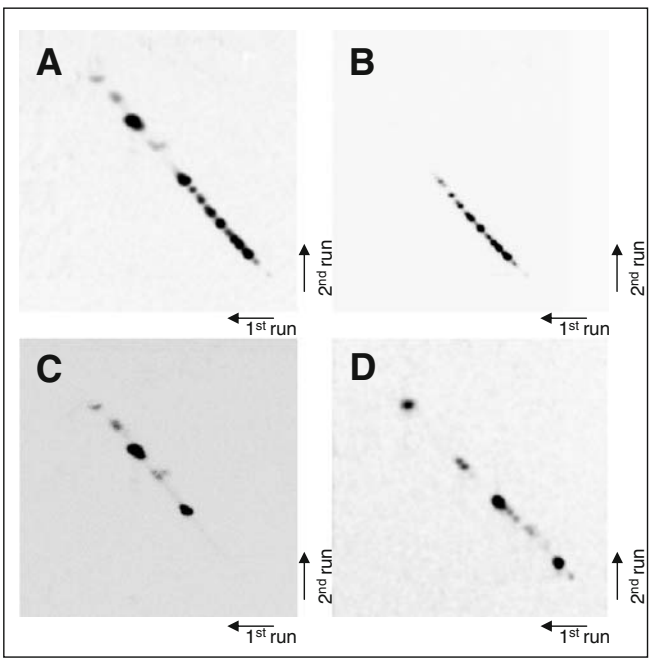


Fig. 5 TLC analysis of sulfatide and galactosylceramide from 7 months old mouse brains. TLC analysis of sulfatide and neutral glycolipids from 7 month old mouse brains. *A*: solvent system, chloroform/methanol/water 70:25:4 by vol.; staining with cresyl violet acetate reagent; alkali-treated total lipid extract from 500 μg of total brain proteins. *B*: TLC plate pre-treated with 4% tetraborate Na^+ / methanol 1:3 by vol. and maintained at 120°C for 1 h; solvent system, chloroform/methanol/2 M ammonia, 70:30:3 by vol.; staining with aniline/diphenylamine reagent; alkali-treated total lipid extract from 400 μg of total brain proteins

anolamine, as radioactive compound, due to the recycling of radioactive ethanolamine.

Figure 5 shows the analysis of sulfatide and galactosylceramide. Sulfatide partitions in both the organic and aqueous phases, thus we analysed it using the total lipid extract after treatment with alkali to remove glycerophospholipids. The cresyl violet reagent, specific for sulfatides, is useful for the identification of this class of compounds. Analysis of galactosylceramide requires great attention to avoid overlapping with glucosylceramide and other contaminants. Pre-treatment of the TLC plate with tetraborate and alkaline chromatographic solvents are useful for its separation and recognition.

Two-dimensional TLC can be performed using the same or different solvent systems for the two runs. Figure 6 shows radioimages of the two-dimensional TLC separations of the total complex lipid mixture, aqueous phase and organic phase from rat cerebellar granule cells metabolically tritium labelled with [1-^{3}H]sphingosine. A, B and C were developed with solvent system chloroform/methanol/0.2% aqueous $CaCl_2$ in the volume ratio of 50:42:11 for both runs. D shows the separation obtained using chloroform/methanol/water in the volume ratio of 110:40:6. About 1000 dpm total lipid extract deriving from 10 μg of cell proteins were applied on the plate, and the image was acquired in 40 hr. Comparison within A, B, and C, and



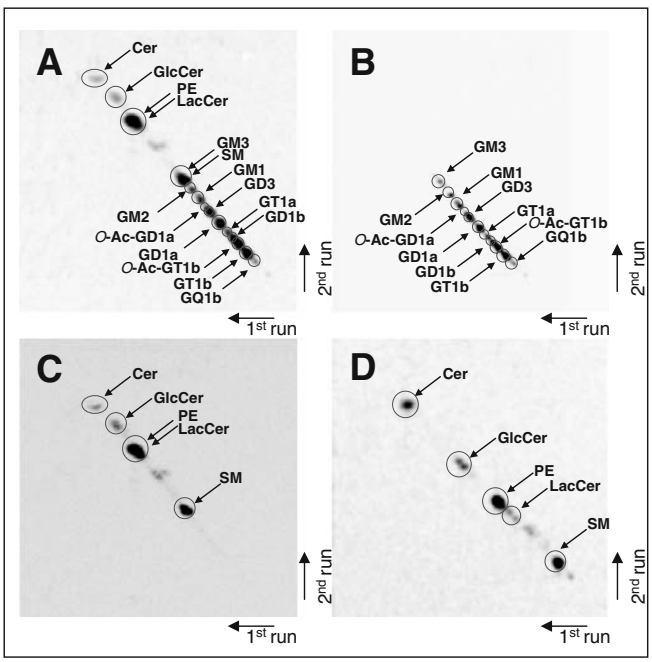


Fig. 6 Two-dimensional TLC of metabolically tritium labelled lipids from rat cerebellar granule cells. Radioimaging of the two-dimensional TLC of the lipids from metabolically tritium labelled rat cerebellar granule cells. Total lipid extract (*A*), aqueous phase (*B*) and organic phase (*C*) were separated using the solvent system chloroform/methanol/0.2% $CaCl_2$ 50:42:11 by vol., for runs. The organic phase was also separated (*D*) using the solvent system chloroform/methanol/water 110:40:6 by vol. for both runs. Lipids (1000 dpm) from 10 μg of cellular proteins; acquisition time, 40 h. In the right side of figure we assigned the names to identified compounds

comparison of C with D allowed to recognize over 20 compounds. Among these, phosphatidylethanolamine and up to 15 sphingolipids were tentatively recognized on the basis of their chromatographic behaviour.

Solvent systems that contain ammonia are often very useful for the second run of bi-dimensional chromatography, allowing very good separation [25]. However, it is necessary to recall that nervous system gangliosides comprise for *O*-acetylated sialic acid-containing compounds [26, 27]. Their identification prevents any alkaline treatment of the total mixture or the use of alkaline chromatographic solvent systems. In the past, we developed a two-dimensional TLC method with intermediate ammonia treatment to specifically detect alkali-labile gangliosides [28]. The plate is developed with the same solvent system in both runs, so that gangliosides remain aligned along a diagonal after chromatography. An intermediate treatment with ammonia vapours releases the acylamide (very often the acetamide) leaving the alkali-stable ganglioside. *O*-acetylated gangliosides are more polar than the corresponding alkali-stable derivatives, and after ammonia treatment and the second chromatographic run, they remain located under the diagonal that comprises for the non modified gangliosides, and horizontally aligned with the corresponding alkali-stable compound. Figure 7 shows the two-dimensional TLC of gangliosides from mouse brains. A shows gangliosides stained with *p*-dimethylaminobenzaldehyde reagent after separation performed without ammonia treatment, where all the gangliosides are aligned along a diagonal; B shows the same separation as in A but with intermediate treatment with ammonia vapours. 6 spots are now located under the diagonal of alkali-stable compounds, indicating that they are alkali-labile. According to their chromatographic behaviour in the second run and to their horizontal alignment with GD1a, GT1a, GD1b, GT1b, and GQ1b, they were tentatively identified as *O*-acetylated derivatives of these gangliosides. Two alkali-labile derivatives of GQ1b were recognised. One was previously characterised as the derivative of GQ1b with the Neu5,9Ac_2 external to the inner disialosyl residue [28]. The second

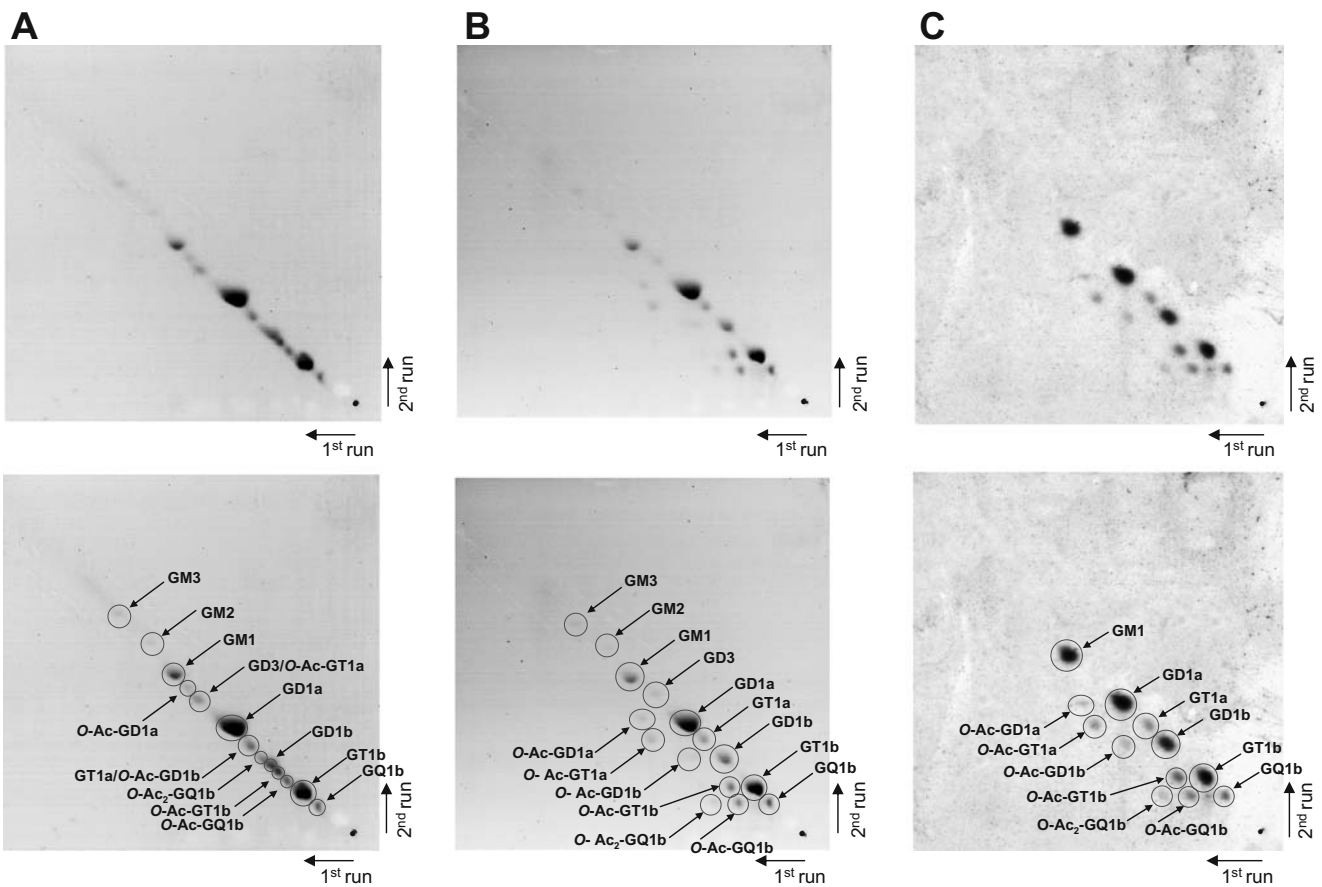


Fig. 7 Two-dimensional TLC of gangliosides from 7 month old mouse brains. Chromatography were carried out using the solvent system chloroform/methanol/0.2% $CaCl_2$ 50:42:11 by vol., for both runs. *A*: staining with *p*-dimethylaminobenzaldehyde reagent; gangliosides from 700 μg of total brain proteins. *B*: two-dimensional TLC with intermediate ammonia treatment; staining with the *p*-dimethylaminobenzaldehyde reagent; gangliosides from 700 μg of total brain proteins. *C*: two-dimensional TLC with intermediate ammonia treatment; staining with cholera toxin after sialidase treatment; gangliosides from 70 μg o f total brain proteins

compound should be a di-*O*-acetylated derivative on the basis of its chromatographic behaviour. The presence of the GM1 core in these alkali-labile gangliosides was then defined by staining with cholera toxin after sialidase treatment, as shown in C of Fig. 7. This is the first report on the combination of the separation of a ganglioside mixture by two-dimensional TLC with the sialidase-cholera toxin staining procedure. This new information suggests that TLC remains a valid analytical procedure that at low cost and in short time allows to collect data that can suggest the structure of glycosphingolipids before to move to expensive and not always accessible procedure, like nuclear magnetic spectroscopy or mass spectrometry. In relationship with this latter point, in the last years direct combinations of TLC and mass spectrometry have been proposed [29, 30]. This requires very expensive instruments and an expertise not always present in all research groups. Nevertheless, TLC separation can be the starting analytical process to prepare samples that, after far western blotting [31] or scraping of the silica gel areas from the TLC plate, elution and eventual column chromatography purification, can be ready to MS analyses. We show in Fig. 8, as example, the MS analysis of the gangliosides from ovarian carcinoma A2780 cells, obtained after TLC separation. After TLC, four main spots were recognised [32]. These were then characterized by MS as the molecular species containing C16/18 and C24:0/:1 of GM3 and GM2.

Glycosphingolipids are a group of compounds comprising for some hundreds species differing in both the oligosaccharide and ceramide moieties. They are extracted from tissues or cells together with many other complex lipids, so that their TLC analysis and characterization are not easy in some cases. Our aim in preparing this manuscript was to describe, on the basis of our own

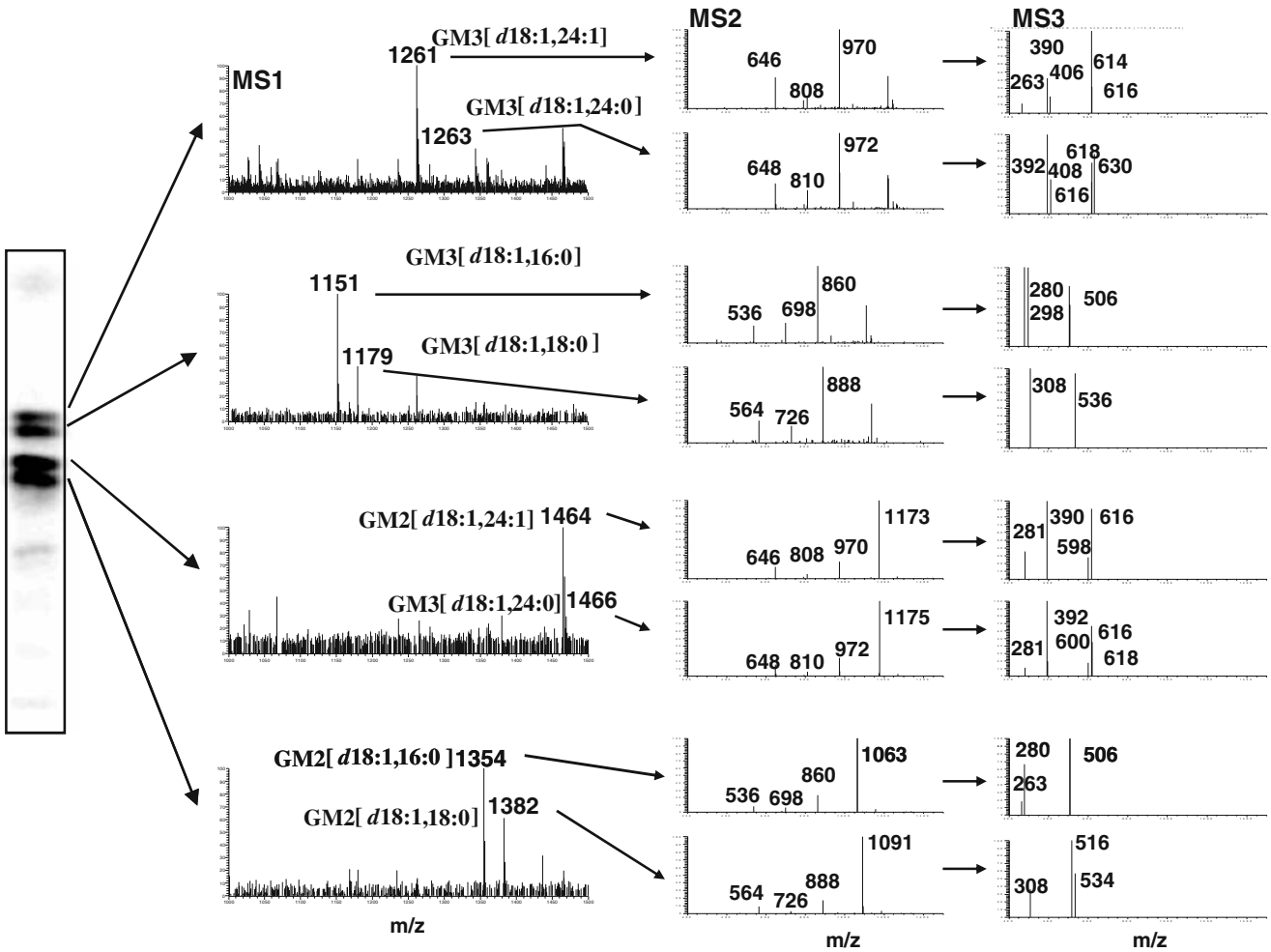


Fig. 8 Identification of the main gangliosides from an ovarian carcinoma A2780 cell line. The ganglioside mixture from A2780 cells was separated by TLC; chromatography was carried out using the solvent system chloroform/methanol/0.2% $CaCl_2$ 50:42:11, by vol. The four main compounds were analysed by mass spectrometry after blotting. MS experimental condition as reported in [32]; figure shows the four MS1 spectra and the corresponding MS2 and MS3 spectra derived from the main ions. MS3, obtained from the ceramide ion, allows to assign the correct structure of both fatty acid and long chain base

experience, some guidelines for practical performance of thin layer chromatography techniques, applied to the basic and first steps of characterization of total lipid mixture obtained after tissue or cell extraction and partially purified by partitioning and alkaline hydrolysis. According to this, we mainly describe the separation of ganglio series gangliosides from the nervous systems and from a few cells in culture, in the presence or after phase separation from the neutral lipids. Nevertheless it is necessary to recall that many other compounds, such as lacto and globoseries glycolipids, complex sulfatides and many other glycosphingolipids can be components of the total lipid mixtures. In these cases, separation of acid glycosphingolipids from neutral compounds by DEAE Sepharose [33] or separation of alkaline glycosphingolipids, such as lyso-glycosphingolipids, plasmalopsychosines and glyceroplasmalopsychosine, from the other lipids by carboxymethyl Sepharose [34], can be taken in account to improve results.

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