BIOCHEMISTRY OF SPHINGOLIPIDS

IV. SOME NEW PAPER CHROMATOGRAPHIC SYSTEMS FOR THE CHARACTERIZATION OF SPHINGOLIPIDS

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(Received October 4th, 1965)

INTRODUCTION

In the last few years many papers have been published concerning the application of various chromatographic techniques to studies in sphingolipid biochemistry. Chromatography on thin layers of adsorbents, especially, has been widely used for the fractionation of cerebrosides, sulfatides, gangliosides and sphingomyelins. However, only a few publications on the paper chromatography of these substances have appeared[1–6].

For the past few years in our laboratory, we have been investigating the possibility of using paper chromatography for the topographic analysis of sphingolipids in human organs under normal and pathological conditions. This article presents our results on this subject.

EXPERIMENTAL

Preparation of purified sphingolipids

For the preparation of sphingolipids the organs were extracted by the method of FOLCH[7] *et al.* After mild alkaline hydrolysis the isolated mixture of sphingolipids was fractionated on Florisil and DEAE-cellulose. Authentic samples of cerebrosides, gangliosides and sphingomyelins (from bovine brain) were obtained from Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England).

Impregnation of paper with silica gel

Two types of paper impregnated with silica gel were used:

(A) Commercial silica gel paper, Schleicher and Schüll No. 289 containing 35–40 % of silica gel.

(B) Whatman No. 3 paper impregnated with silica gel[8].

Solvent systems

The following solvent systems were used:

System 1: chloroform–methanol–water (12:1.6:0.1);

System 2: tetrahydrofuran–diisobutyl ketone–water (45:9:4);

System 3: tetrahydrofuran–diisobutyl ketone–water (45:5:6);

System 4: chloroform–acetone–glacial acetic acid (9.5:5.7:0.4);
System 5: chloroform–acetone–propionic acid (6.2:7.2:1.0);
System 6: chloroform–methanol–12.5 % ammonium hydroxide (12:7:1.6);
System 7: chloroform–acetone–glacial acetic acid–water (12:1.6:0.4:0.05).

Detection

(*a*) *Rhodamine B.* The dry chromatogram is immersed in a 0.001 % solution of Rhodamine B in 0.25 *M* K_2HPO_4 for approximately 60 min. The excess dye is removed by washing with tap water. Sphingolipids show up as red spots. In ultraviolet light they fluoresce bright yellow or orange.

(*b*) *Chlorine–benzidine–KI.* The dry chromatogram is wetted with an ethanol–acetone mixture (1:1) and exposed to chlorine (0.1 % $KMnO_4$–10 % HCl, 1:1) for 5–10 min. Then it is immediately washed 10 min in running water. When nearly dry the chromatogram is drawn through a 0.5 % benzidine solution in ethanol–water (1:1) with the addition of few crystals of KI and allowed to dry in air. The blue spots are stable for several hours.

(*c*) *Cresyl violet.* The chromatogram is immersed in 0.02 % cresyl violet in 1 % acetic acid for 10 min at 60° or 30 min at room temperature and then washed well in 2 % acetic acid for several hours.

(*d*) *Pinacryptol yellow.* The chromatogram is drawn through an 0.05 % solution of pinacryptol yellow in water and subsequently observed in ultraviolet light. Counterstaining with cresyl violet permits precise identification of sulfatides.

(*e*) *Acriflavine–p-dimethylaminobenzaldehyde.* The dry chromatogram is dipped in 0.002 % acriflavine in 0.1 *M* citrate–HCl buffer solution, pH 2.5, for 10 min. It then is immersed in a 2 % *p*-dimethylaminobenzaldehyde solution in 20 % HCl diluted with *n*-propanol or isopropanol in the proportion (30:70, v/v) before use. As soon as the chromatogram is completely orange in colour (approximately 1–2 min) it is washed several times with water until the background is yellow. The sulfatides appear as bright orange spots. The color is unstable and disappeared after an hour.

(*f*) *Acid fuchsin–uranyl nitrate.* The dry chromatogram is dipped in an 0.02 % solution of acid fuchsin in 0.01 *N* HCl containing 0.2 % of uranyl nitrate for 30 min. Then it is washed three times with an 0.2 % solution of uranyl nitrate in 0.01 *N* HCl. After drying at laboratory temperature, sphingomyelins appeared as red spots on a white background.

The color reactions of individual sphingolipids are summarized in Table I.

TABLE I

COLORATION OF INDIVIDUAL SPHINGOLIPIDS IN SOME DETECTION REACTIONS

Substance	*a*	*b*	*c*	*d*	*e*	*f*
CMH	red-orange	blue	pale blue	—	—	—
CDH	red-orange	blue	pale blue	—	—	—
CTH	red-orange	blue	pale blue	—	—	—
AmGl	red-orange	blue	red-blue	—	—	—
GG	red-orange	weak blue	red-blue	—	—	—
CMHS	red-orange	blue	red-brown	bright yellow	brick-red	—
CDHS	red-orange	blue	red-brown	bright yellow	brick-red	—
SPH	red-orange	blue	blue	—	—	red-violet

CHROMATOGRAPHIC RESULTS

System 1

This solvent system is very suitable for the separation of CMH, CDH, CTH and AmGl*. Each fraction of these substances is further subfractionated into cerebrosides with nonhydroxy and hydroxy fatty acids, the latter having a slower mobility. CMH and CDH, especially, are very well separated. Commercial Schleicher and Schüll No. 289 silica gel paper proved to be superior to Whatman No. 3 silica gel paper, showing minimal streaking and well defined spots. Chromatograms were run on short sheets of paper (solvent front 15 cm) in most experiments. In several cases long chromatograms (solvent front 25 cm) developed by a horizontal technique resulted in a better separation of the spots (Fig. 1).

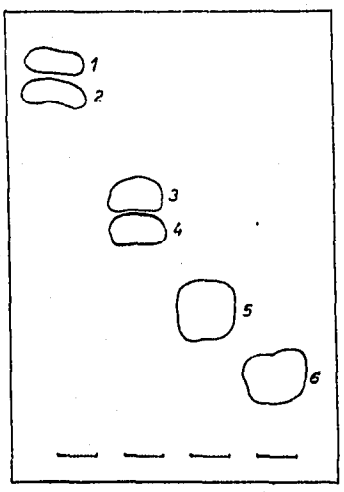


Fig. 1. Separation of human kidney cerebrosides on Schleicher and Schüll No. 289 paper impregnated with silica gel. System 1. Detection: Rhodamine B. 1 = CMH-N; 2 = CMH-OH; 3 = CDH-N; 4 = CDH-OH; 5 = CTH; 6 = AmGl.

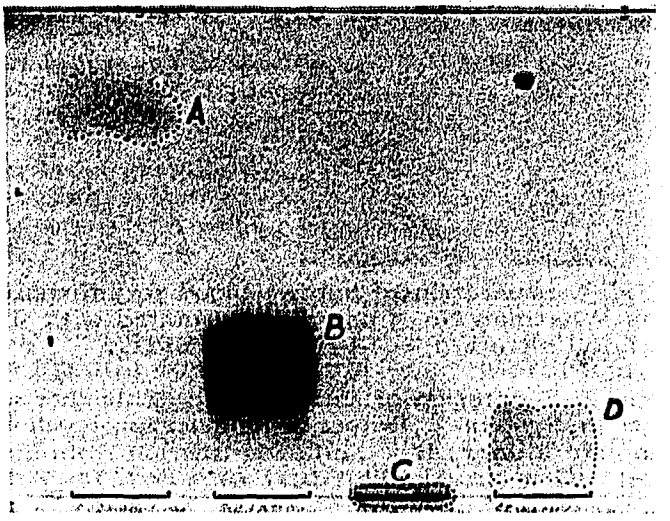


Fig. 2. Separation of human brain sphingolipids on Whatman No. 3 paper impregnated with silica gel in chloroform–methanol–water (12:2.0:0.1). Detection: cresyl violet. A = cerebrosides; B = sulfatides; C = gangliosides; D = sphingomyelins.

If the proportions of methanol or water are increased (*e.g.* chloroform–methanol–water, 12:2.0:0.1 or 12:2.5:0.2) it is possible to differentiate CTH and AmGl (high mobility) on Schleicher and Schüll No. 289 paper. In these systems CMH and CDH run close to the solvent front.

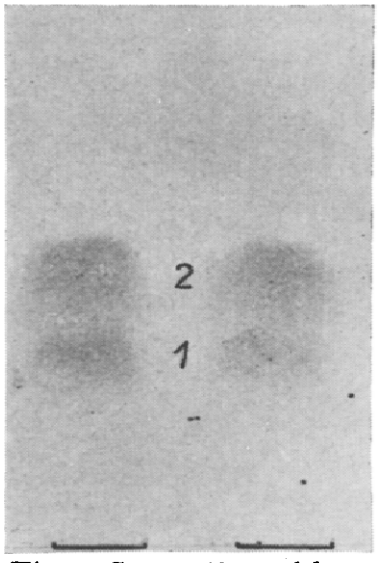
On the other hand an ascending technique using Whatman No. 3 silica gel paper developed for long periods (24 h) led to excellent separation of SPH_{18} and SPH_{24} (Fig. 3).

Sulfatides, gangliosides and sphingomyelins form compact spots without need for further subfractionation on chromatograms developed on short sheets (Fig. 2).

* Abbreviations used: CMH = ceramide-monohexosides; CDH = ceramide-dihexosides; CTH = ceramide-trihexosides; AmGl = aminoglycolipids; GG = gangliosides (G_0–G_6 = symbols for individual ganglioside fractions as used by SUZUKI[9]; CMHS = ceramide-monohexoside-sulfatide; CDHS = ceramide-dihexoside-sulfatide; SPH_{18} = more-polar sphingomyelins containing fatty acids below C_{20}; SPH_{24} = less-polar sphingomyelins containing fatty acids above C_{20}; N = fraction with nonhydroxy fatty acids; OH = fraction with hydroxy fatty acids.

System 2

This system gives a very clear separation of CMHS and CDHS and is very suitable in cases where the sulfatide fraction was purified from other sphingolipids by column chromatography.



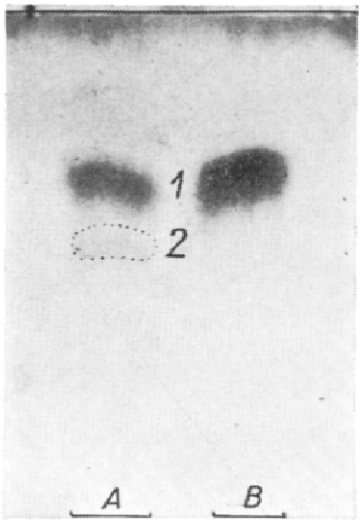


Fig. 3. Separation of human blood serum sphingomyelins by continuous paper chromatography on Whatman No. 3 paper impregnated with silica gel in System 1 visualized with acid fuchsin–uranyl nitrate. 1 = SPH_{18}; 2 = SPH_{24}.

Fig. 4. Separation of sulfatides on Schleicher and Schüll No. 289 paper impregnated with silica gel in System 3. Detection: pinacryptol yellow and cresyl violet. A = sulfatide fraction from human kidney; B = sulfatide fraction from human brain. 1 = CMHS; 2 = CDHS.

System 3

This offers the same resolution as System 2 for CMHS and CDHS but the mobilities are higher. CMH, CDH and CTH are also separated but without further subfractionation. If this system was used two-dimensionally with System 1 an excellent separation of cerebroside fractions was obtained. The spots on silica gel impregnated paper are more compact than on untreated paper as described by SVENNERHOLM[5].

System 4

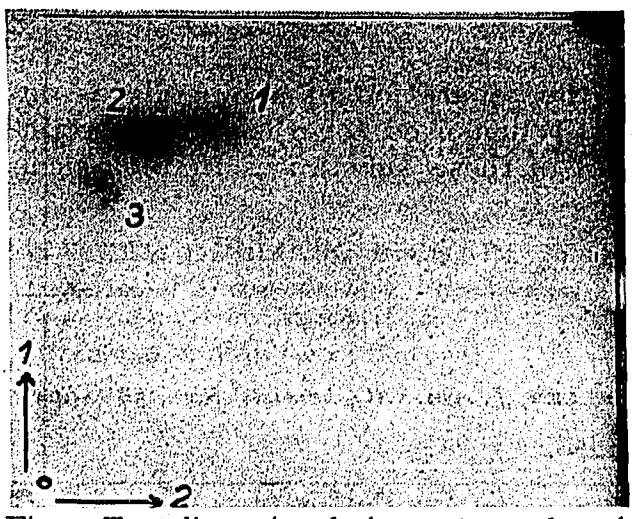
This is advantageous for the differentiation nonhydroxy and hydroxy fatty acid fractions in the CMH fraction. In this system the mobilities of these two fractions are very different. Other sphingolipids remained near or on the start line.

System 5

The system which consists of chloroform and acetone with the addition of propionic acid permits satisfactory separation of CMHS fractions with nonhydroxy and hydroxy acids. A two-dimensional technique using System 3 for the first dimension and System 5 for the second makes a complete separation of all sulfatide fractions possible (Fig. 5).

System 6

The characterization of individual ganglioside fractions is possible with this



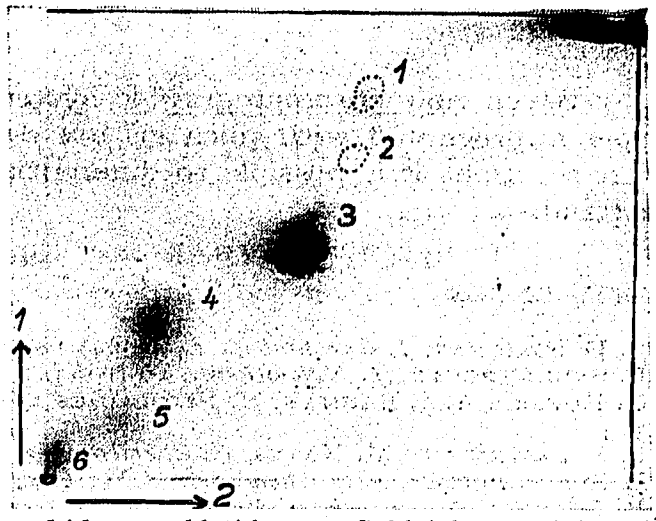


Fig. 5. Two-dimensional chromatography of human kidney sulfatides on Schleicher and Schüll No. 289 paper impregnated with silica gel. First dimension: system 3; second dimension: system 5. Detection: pinacryptol yellow and cresyl violet. 1 = CMHS-N; 2 = CMHS-OH; 3 = CDHS.

Fig. 6. Two-dimensional chromatography of human brain gangliosides on Schleicher and Schüll No. 289 paper impregnated with silica gel. First dimension: system 3; second dimension: system 6. Detection: cresyl violet. 1 = G_0?; 2 = G_5; 3 = G_4; 4 = G_3; 5 = G_2; 6 = G_1.

system. All fractions below G_4 are well separated but have high mobilities. Disialo- and trisialogangliosides eventually aminoglycolipids are only partially resolved. An increase of methanol and ammonium hydroxide concentration (*e.g.* chloroform–methanol–12.5 % ammonium hydroxide, 12:8:2.0) leads to the separation of the trisialogangliosides into 3 fractions and the disialogangliosides into 2 fractions. Ganglioside fractions below G_4 remained unseparated and run near to or with the solvent front as the other sphingolipids. The solvent mixture chloroform–methanol–12.5 % NH_4OH (10:3:0.4) is especially useful for monosialogangliosides, which it separates into 3 fractions. Other gangliosides remain at the start.

The two-dimensional combination (first dimension: system 3; second dimension: chloroform–methanol–12.5 % NH_4OH, 12:7:1.6) gives a good resolution of the whole spectrum of gangliosides (Fig. 6).

System 7

This system is useful for the identification of CMH-N and CMH-OH which are well separated near the starting line. It also appears possible to use this mixture for the separation of ceramides.

DISCUSSION

The chromatographic systems described in this paper show good potentiality for the identification of various types of sphingolipids. Excellent results were obtained especially with the purified fractions obtained after column chromatography on Florisil, DEAE cellulose, silica gel etc. By the combination with certain color reactions it is possible to characterize with good precision the whole range of sphingolipid fractions. The methods described here have a wide application in the analysis of these substances in biological material.

SUMMARY

Seven new chromatographic systems for the separation of sphingolipids on paper impregnated with silica gel are described. When used together with certain color reactions it is possible to differentiate most of these substances in biological material.

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