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Abstract

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Thin-Layer Chromatography of Cerebrosides

The separation, purification, and individualization of cerebrosides have until now remained a difficult task. Alongside the classical methods of fractionation, chromatographic methods have recently begun to be used for this purpose: separation of cerebrosides from other sphingolipids on a column with aluminum oxide ([1]), or passage through a mixture of florisil with ion exchangers ([2]). Weiss, who used chromatography on silica gel with gradient elution by a chloroform–methanol mixture for the separation of sphingolipids ([3]), was able in this way to isolate individual kerasin and cerebron, along with a substance that is not a cerebroside but has a structure close to them, not yet definitively established.

The weakest point of all investigations on the separation of cerebrosides, and to a considerable extent of other sphingolipids as well, is the absence of clear methods for monitoring fractionation, which not infrequently completely devalues the separation methods. The use of paper chromatography for this purpose ([4], [5]), including on impregnated paper ([6]), does not give the proper result, as may also be confirmed by our data. In all cases, owing to their high and similar hydrophobicity for individual representatives, cerebrosides travel as a single diffuse spot with an Rf exceeding 0.85–0.90.

We have established that chromatography in a thin layer, not previously used, is a quite suitable method for monitoring the separation of cerebrosides. By the time the present investigation was completed, a paper by Jatzkewitz and Mehl ([7]) had appeared, in which data are given on thin-layer chromatography on silica gel of various lipids, including the identification of two cerebrosides (kerasin and cerebron).

We carried out thin-layer chromatography of the cerebroside fraction of sphingolipids from bovine brain, preliminarily purified from sphingomyelins, sulfatides, and gangliosides by passing a pyridine solution through a column with basic aluminum oxide (activity 2 according to Brockmann) ([1]); the cerebroside fraction contained no phosphorus or sulfur. The plates used for chromatography were prepared as follows: onto a thoroughly cleaned glass plate (13 × 18) a homogeneous paste of 6–7 g of KSK silica gel (150/200 mesh), 0.35 g of ignited gypsum, and 19 ml of water was applied; the plate was dried for 2–3 hours in air and for 2 hours at 105°. The plates were stored in a desiccator over ignited calcium chloride. As the mobile phase, in contrast to the authors mentioned

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(7), anhydrous systems were used. The most suitable proved to be chloroform-methanol mixtures containing from 10 to 20% methanol.

A cerebroside mixture in an amount of 40 γ in 20 μl of chloroform-methanol mixture (2 : 1) was applied at a distance of 1.5 cm from the edge onto a plate, which was placed in a chamber with solvent for 1 hour; the solvent front usually rose by 10–12 cm. After drying in air,

the chromatogram was sprayed with concentrated sulfuric acid; after heating for 10–15 minutes under a lamp, the cerebrosides appeared as black spots. In our preliminary experiments, the cerebroside fraction, purified as indicated above, gave, when the chloroform-methanol system (80:20) was used, four spots: two intense ones with R_f 0.41 and 0.51, and two weak ones with R_f 0.75 and 0.85.

The method developed was then used by us for monitoring the preparative separation of cerebrosides by distribution chromatography on a silica-gel column. As mentioned, Weiss (3), when separating sphingolipids by this method, isolated only kerasin and cerebron; their individuality, however, remains unclear, since the author did not use any chromatographic control of their homogeneity. In the separation, Weiss used only small amounts of lipids (70–200 mg), citing the need to proceed from a calculation of no more than 4 mg of lipids per 1 g of silica gel. We showed that quite distinct separation also occurs at a loading of 8 mg of the cerebroside fraction per 1 g of silica gel; however, instead of the gradient elution used by Weiss (insufficiently effective because of the closeness of the properties of the individual cerebrosides), elution should be carried out much more slowly than Weiss did, who used a rate of 40–45 ml/hour.

Fig. 1. Chromatographic separation of 400 mg of a mixture of cerebrosides on a silica-gel column (in γ of galactose)

We carried out chromatographic separation of 400 mg of the cerebroside fraction (containing no phosphorus or sulfur, containing 21.3% galactose; calculated for cerebron, 21.7% galactose) on a 20 × 400 mm column with 50 g of silica gel (KSK 75/100 mesh). The cerebrosides were dissolved in 42 ml of a chloroform-methanol mixture (20:1) and applied to a column previously washed with chloroform-methanol (2:1) and pure chloroform. Elution was performed with the following solvents (in order): 230 ml of chloroform, 720 ml of a chloroform-methanol mixture (98:2), and 1150 ml of a chloroform-methanol mixture (95:5). The elution rate was 10–11 ml per hour; the eluate was collected on a collector in 5.5-ml fractions. From each fraction, 0.5 ml of eluate was taken, in which the galactose content was determined by the anthrone method, modified by Roe (8). The elution curve of the cerebrosides from the column, constructed on the basis

Fig. 2. Thin-layer chromatograms on silica gel of cerebrosides in the chloroform–methanol system (85:15).

Figure 2: Fig. 2. Thin-layer chromatograms on silica gel of cerebrosides in the chloroform–methanol system (85:15).

of the galactose content in the individual fractions, is presented in Fig. 1. The fractions obtained upon elution of the column were combined into four groups, A, B, C, D, as shown in Fig. 1. Thin-layer chromatography of all four groups of cerebrosides by the procedure described above, in the chloroform–methanol system (80:20), showed that fraction groups B, C, and D are characterized by a well-formed round spot and contain individual cerebrosides. Fraction group A gave two spots, of which one corresponded in R_f value to the substance of group B, while the other was insufficiently distinct. Repeated thin-layer chromatography of all fraction groups in the less polar chloroform–methanol system (85:15) gave clearer results, showing the presence of five sharply outlined separate spots in group A (Fig. 2, *a*) and confirming the individuality of the cerebrosides of groups B, C, and D (Fig. 2, *b*).

Table 1

Fraction numbers	Group	R_f in chloroform–methanol system, 80:20	R_f in chloroform–methanol system, 85:15
150–182	A	0.70	0.50
150–182	A	0.75–0.85	0.56
150–182	A	–	0.62
150–182	A	–	0.68
150–182	A	–	0.74
183–200	B	0.70	0.50
200–250	C	0.60	0.21
285–385	D	0.55	0.16

The R_f values obtained by us in thin-layer chromatography of groups A, B, C, and D in two systems are given in Table 1.

This result, obtained through the use of thin-layer chromatography, despite its preliminary character, is of considerable interest. Up to the present time the existence

Fig. 2. Thin-layer chromatograms on silica gel of cerebrosides in the chloroform–methanol system (85:15). **a:** *I* –10γ of cerebrosides of group A ($R_f = 0.50; 0.56; 0.62; 0.74$); *II* –mixture of 10$\gamma$ of cerebrosides of group A and 10γ of cerebroside of group B; *III* –10γ of cerebroside of group B ($R_f = 0.50$). **b:** *I* –20γ of cerebroside of group B ($R_f = 0.50$); *II* –10γ of cerebroside of group

C ($R_f = 0.21$); *III* -20γ of cerebroside of group D ($R_f = 0.16$); *IV* -40γ of a mixture of cerebrosides before separation on a silica-gel column.

of four individual cerebrosides has been precisely established: cerebrone (phrenosine), kerasin, nervone, and oxynervone, the last of which has not yet been isolated in pure form. At the same time, in the earlier literature there are repeated indications of the isolation from hydrolysates of cerebroside fractions of higher fatty acids that are not constituents of the four listed

cerebrosides (9, 10); the appearance of these acids was thought to be the result of hydrolysis of impurities of other sphingolipids in insufficiently purified cerebroside fractions. In addition, the existence is well known, alongside galactocerebrosides, of glucocerebrosides, which were isolated from spleen (11) and have not yet been detected in the brain (11, 12).

If one takes into account that, unlike earlier investigators, we used a cerebroside fraction carefully purified chromatographically from impurities of other sphingolipids, then the assignment of the components of group A to impurities of a non-cerebroside nature seems poorly substantiated. The chromatographic analysis of the group of fractions A should rather be regarded as a fairly definite indication of the presence in the bovine brain cerebroside fraction studied by us of as yet unknown cerebrosides, most likely differing in their fatty acids. The data we obtained allow us to suppose that the cerebrosides of groups B, C, and D correspond to kerasine, oxynervone, and cerebrone. Naturally, a more definite conclusion about the nature of the components of groups A, B, C, and D can be made after study of the hydrolysis that we are currently conducting.

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Note: Figure translations are in progress. See original paper for figures.