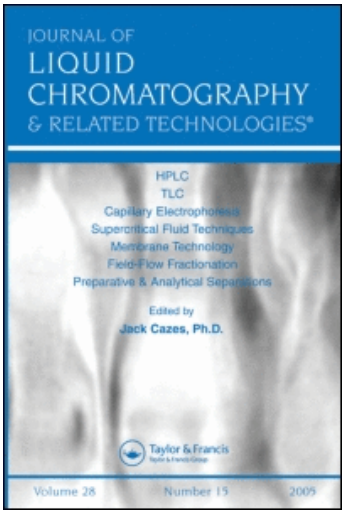

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CLASS FRACTIONATION OF ACIDIC GLYCOLIPIDS AND FURTHER SEPARATION OF GANGLIOSIDES BY OPTLC

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ABSTRACT

The use of the OPTLC method has been extended to the separation of the acidic fraction of the total lipid extract derived from a given blood element. This newly developed method is suitable for the interclass separation of sulphatides and gangliosides and further intraclass separation of gangliosides on the same TLC plate with step gradient development. The elutions can be performed on 10 x 10 cm (or larger) HPTLC plates with 13 parallels on each one. The chromatograms were stained either with orcinol-H_2SO_4 to show class separation (in this case only a single isocratic elution was performed) or with resorcinol-HCl reagent to visualize the ganglioside intraclass

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separation. The chromatograms were evaluated by spectrodensitometric scanning and the reproducibility of the separation was determined.

INTRODUCTION

Separations of lipids generally and especially of glycosphingolipids are usually carried out by various chromatographic methods. For preparative work classical column and layer chromatographic techniques are used. For analytical purposes HPLC (1) and mainly HPTLC (2) procedures are employed. HPTLC plates have been used for the analysis of various, prepurified ganglioside mixtures using one (3,4,5) and two (6,7) dimensional development with various solvent mixtures.

A new layer chromatographic technique, Overpressured Thin Layer Chromatography has been developed in Hungary (8). This technique was introduced into lipid analysis in our previous paper (9), where the basic theoretical and application aspects of OPTLC were cited and discussed.

The present paper describes the application of OPTLC to the separation of acidic glycosphingolipids.

The method was developed on mixtures of pure ganglioside and sulphatide standards and then applied to the separation of the purified acidic fraction of the total lipid extract (TLE) derived from blood elements (e.g. plasma, lymphocytes, granulocytes of normal and leukemic individuals).

Class separation of acidic glycolipids namely sulphatides and gangliosides can be performed by a single isocratic run with chloroform - methanol (C : M = 70 : 30) mixture. The intraclass separation of gangliosides, in the presence of sulphatides, is accomplished by a step gradient run. The first

segment of the gradient profile is the class separation (see above) followed by a consecutive isocratic development with chloroform - methanol - 0.25% KCl in water (C : M : KCl/W = 55 : 36 : 9) mixture.

EXPERIMENTAL

Reagents and materials

All the solvents used were HPLC grade purchased from E. Merck, Darmstadt, F.R.G., the water used was prepared according to (10), from water double distilled from glass and sterilized. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Staining reagents were prepared from analytical grade chemicals, the HPTLC plates used were purchased from E. Merck, Darmstadt, F.R.G. TLE was prepared from human blood elements. Sulphatides (bovine) and GT1b were purchased from Supelco Inc., Supelco Park, Bellefonte, PA, USA. Other ganglioside standards (GM3, GM2, GM1, GD1a, GD1b) were the kind gift of Professor Shimon Gatt, Hadassah Medical School, Dept. of Biochem., Jerusalem, Israel.

Apparatus

Centrifuge: Janetzki K70 MLW, Leipzig, G.D.R.
Incubator: LP 507/1 Labor MIM, Esztergom, Hungary.
Sample applicators: special 1 microliter volume syringe, MTA KKKI, Budapest, Hungary and CAMAG Nanoapplicator, CAMAG, Muttenz, Switzerland.
OPTLC system: Chrompres 10 Overpressured Layer Chromatograph, Labor MIM, Esztergom, Hungary. Instead of its eluent pump a

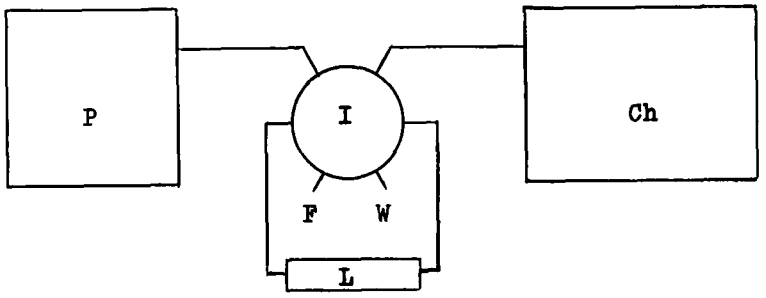


Figure 1. Block diagram of the OPTLC system used.

Ch = OPTLC Chamber

P = Pump

I = Injection valve

L = Loop

F = Filling port

W = Waste

HPLC pump; Beckman 112 SDM, Beckman Instruments Inc., Berkeley Calif., USA, an injector; Altex 210 with a 3ml loop, Beckman Instruments Inc., Berkeley, Calif., USA were used. The block diagram of the system can be seen on Figure 1.

Spectrophotometric densitometer: Opton KM3, Opton Feintechnik GmbH., Oberkochen, F.R.G.

Data system: HP 3354 Lab. Automat. System, Hewlett Packard, Avondale, Calif., USA.

Methods

Sample preparation

Standard glycolipid sample; a mixture was prepared from bovine sulphatides and pure individual gangliosides (GM3, GM2, GM1, GD1a, GD1b, GT1b).

Glycolipid samples from blood elements; the preparation of which has been described elsewhere (11).

Plate pretreatment

All plates used were precleaned by running them in methanol and dried. The OPTLC technique requires that the edges of the plates should be sealed, preferably after scraping off a strip (2-4 mm) of sorbent, by impregnating with IMPRES 1. 3 sides of the plates were sealed and solvent dispersion troughs were scraped into the sorbent layer 14, 17 and 20 mm from the lower edge.

OPTLC and densitometry

Sample application was 200 nl/spot in the case of the standard mixture, with the CAMAG nanoapplicator. Usually 13 samples were applied to a plate at 30 mm from the lower edge and at least 20 mm from the perpendicular edges of the plate. An indicator solution was applied onto the first and last sample spots to help us control the run, also to serve as a reference in calculating migration quotients (relative retention; see later). In the case of glycolipid samples of blood elements 1 microliter was spotted onto the plate. Appropriate sample volumes, for 1 microliter application, were adjusted on the basis of preliminary OPTLC runs.

Development (step gradient) conditions were;

segments	No1	No2
eluents	C:M=7:3	C:M:KCl/W=55:36:9
eluent flow (ml/min)	0.1	0.1
start pressure (bar)	20	20
pillow pressure (bar)	14	14
development distance (cm)	7	14 (overrun)

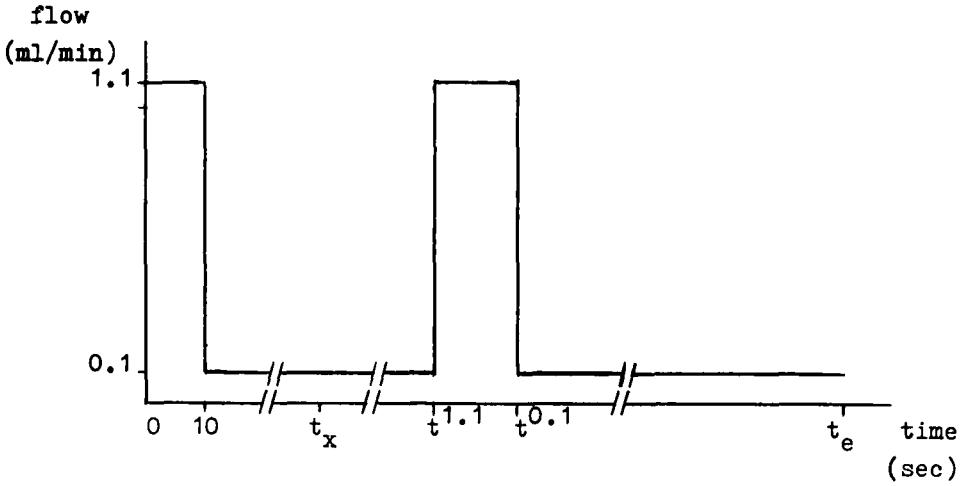


Figure 2. The flow profile employed.

t_x = time of the valve switching

$t^{1.1}$ = t_x + 3'45''

$t^{0.1}$ = t_x + 4'

t_e = time of run end

The step gradient used was the sharp changeover from isocratic segment No1 to No2. The first eluent was pumped by the SDM, the 3 ml volume loop of the injector was filled with the second, No2. eluent. The changeover was accomplished by switching the valve from load to inject. The volume of the connecting tubing from the valve to the plate was taken into calculation.
To achieve ideal dispersion of the eluents, a flow program was used (Figure 2.). The first eluent was pumped until the indicator spots migrated 1.5 cm on the plate, at this moment (t_x) the valve was switched to inject. 3'45'' after switching the

flow was increased to 1.1 ml/min and decreased to 0.1 ml/min 15'' later. The flowrate was maintained at 0.1 ml/min, till the end of the run, when the indicator had migrated 7.0 cm. Staining for class separation, was by immersion of the plate into orcinol-H_2SO_4 reagent and heating at 100°C for 2-3 minutes, for ganglioside separation, by spraying with resorcinol-HCl reagent and heating at 100°C for 15 minutes.

Densitometric conditions in remission mode were:

wavelength:	525 nm
slit:	3.5 x 0.1 mm
scanning speed:	10 mm/min
A/D frequency:	2 Hz

RESULTS AND DISCUSSION

Summarizing the experimental results the OPTLC technique is suitable for the separation of lipid samples. We can now separate the constituent classes of either fraction (neutral and acidic) of the total lipid extract.

In our present paper we described the new separation of the acidic fraction of TLE. The class separation of sulphatides and gangliosides can be achieved by a single isocratic run within 7 minutes, as shown in Figure 3.

As can be seen the resolution is very high between the two classes and the migration of the gangliosides is very small.

Our main purpose was the intraclass separation of the gangliosides in the presence of interfering sulphatides. As we were not interested in the sulphatides at all, the overrun capability of the OPTLC system could be exploited. The

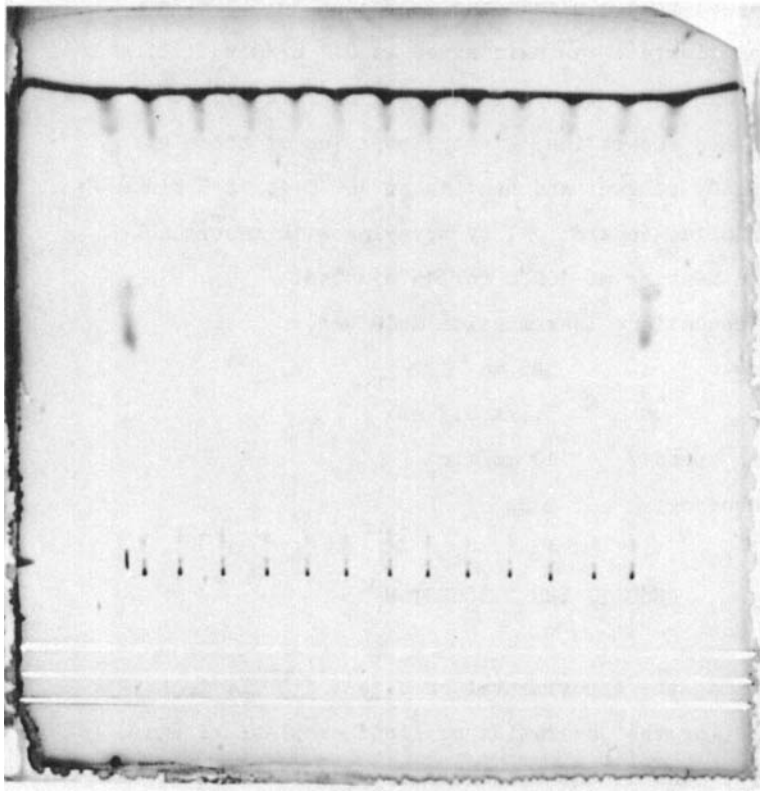


Figure 3. OPTLC separation of acidic glycolipid classes. Plate; Merck HPTLC Si 60. Eluent; C : M = 7 : 3 . Development 7 cm. Staining reagent; orcinol-H_2SO_4.
Samples: 1 & 13; 0.2 µl standard mixture + 0.2 µl indicator solution,
2 - 12; 0.2 µl standard mixture.

sulphatides migrate immediately behind the β front of the first eluent and are eluted from the plate before being reached by the second eluent, which gives excellent separation of the individual gangliosides (Figure 4.). As in such cases, overrun and double development, the conventional R_f has no meaning, it was necessary to find a source of reference for

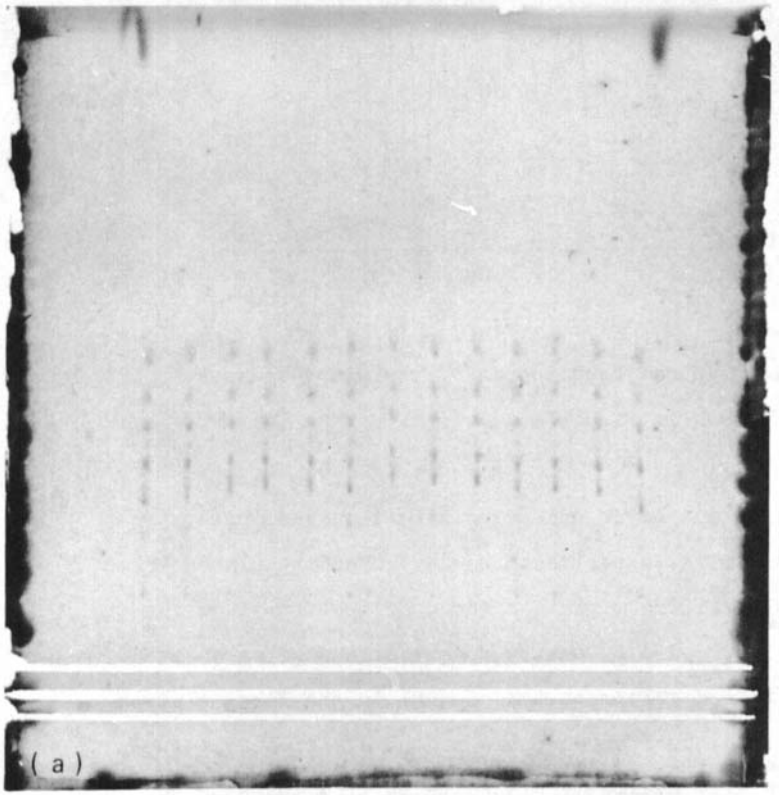
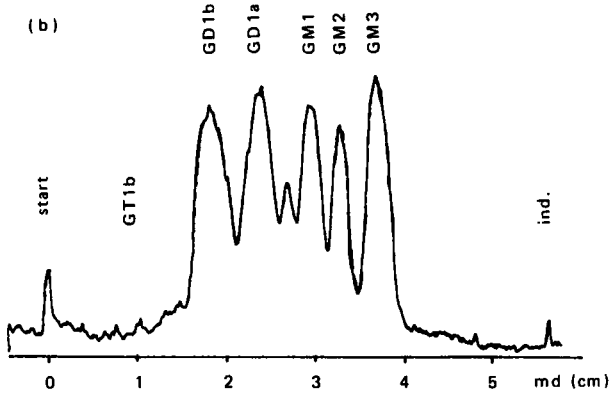


Figure 4. OPTLC separation of acidic glycolipid standards.

4.a. Photograph of the chromatographic plate. Plate; Merck Si 60, staining with resorcinol-HCl. Sulphatides were overrun, see development conditions in text. The samples are the same as for Figure 3.



4.b. A typical densitogram of the plate (lane No7) is shown in Figure 4.a.

standardizing and later characterizing the development process. In our first experiments we developed the plates based on elution time. The migration speed of the front, during the first elution, could be easily measured, and we calculated an approximate development time (equal to the first elution) for the second, overrun. Because of the differences between the commercially available plates (even from the same box), this time based development was not reproducible, so we adapted a different procedure. We selected an indicator, methyl-red, which exhibited very low mobility in C:M=7:3 mixture (R_f=0.2) and has a mobility somewhat higher then all the gangliosides in C:M:KCl/W=55:36:9 mixture.

For the characterization of the separation process we defined the migration quotient (mq) as:

$$mq = \frac{md_G}{md_I}$$

where: md is the migration distance measured for the compound denoted by the subscript,
G subscript stands for gangliosides and
I subscript stands for the indicator.

The quality of the separation method is demonstrated by the numerical data; migration quotients (mq), their averages ($\overline{mq}$), standard deviations (SD) and relative SD (rSD), calculated from the densitograms.

The intraplate reproducibility of the method is demonstrated in Table 1. by the data derived from the densitograms of the plate shown in Figure 4. As the standard deviations and relative standard deviations of the migration quotients show the method is highly reproducible. The relatively high SD-s for GT1b are caused by the small migration distances (the average of which is 8.4 mm), which are influenced more significantly by the variations of sample application.

The interplate reproducibility is shown in Table 2., for nine plates. During the mapping of ganglioside patterns the standard mixture was applied in three places, onto lane 1 and 13 with indicator and to lane 7 without. The rest of the lanes were used for real samples. For the interplate reproducibility studies the center lanes (No7) were used. The migration distance of the indicator was calculated from the averages of the 1. and 13. lanes. Using our standardization method for the development adequate reproducibility can be achieved between plates, but it is advisable to put standards on every plate.

As the results show, the method is highly accurate, reproducible and selective. The method is being used for mapping of ganglioside patterns of plasma, lymphocytes and gra-

Table 1. Intra plate reproducibility of the OPTLC separation of gangliosides

Lane No.	MIGRATION QUOTIENTS (mq)						
	GT1b	GD1b	GD1a	GM1	GM2	GM3	Ind.
1	.16	.19	.23	.3	.35	.42	1
2	.13	.19	.23	.3	.35	.43	1
3	.14	.2	.24	.31	.37	.44	1
4	.14	.2	.25	.32	.37	.45	1
5	.14	.2	.24	.3	.36	.43	1
6	.12	.19	.24	.31	.36	.44	1
7	.12	.21	.26	.32	.37	.44	1
8	.12	.21	.25	.31	.37	.43	1
9	.12	.2	.25	.31	.36	.44	1
10	.12	.19	.23	.31	.37	.43	1
11	.12	.19	.23	.31	.37	.44	1
12	.12	.18	.22	.3	.36	.43	1
13	.08	.15	.23	.3	.37	.45	1
$\overline{mq}$	.13	.19	.24	.31	.36	.44	1
SD.	.02	.01	.01	.01	.01	.01	0
rSD (%)	14.69	7.63	4.15	2.24	1.98	2.02	0

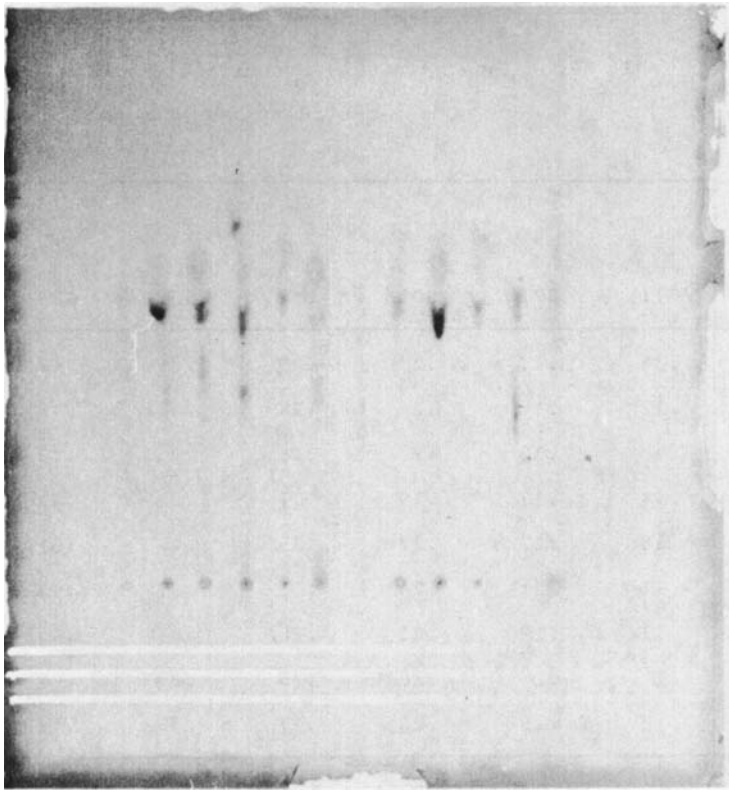


Figure 5. OPTLC separation of gangliosides of acidic glycolipids derived from blood elements.

OPTLC conditions are the same as for Figure 4.

Samples: 1, 7, 13; standard mixture + indicator,
2; MCL plasma,
6; AML plasma (in blast crisis),
3; AMMoL lymphocyte,
AMMoL = Acute Myeloid Monoblastoid L.
CLL = Chronic Lymphoid Leukemia
ALL = Acute Lymphoid Leukemia
4, 5, 9, 10; CLL lymphocyte,
8; ALL lymphocyte,
11; normal granulocyte,
12; blank.

Symbols used: MCL = Mast Cell Leukemia
AML = Acute Myeloid Leukemia

Table 2. Inter plate reproducibility of the OPTLC separation of gangliosides

Lane No.	MIGRATION QUOTIENTS (mq)						
	GT1b	GD1b	GD1a	GM1	GM2	GM3	Ind.
1	.15	.17	.2	.29	.35	.42	1
2	.12	.18	.2	.28	.34	.43	1
3	.1	.18	.2	.28	.35	.43	1
4	.14	.17	.19	.27	.34	.42	1
5	.15	.22	.27	.35	.42	.51	1
6	.11	.21	.25	.31	.37	.46	1
7	.12	.19	.21	.28	.37	.43	1
8	.1	.2	.26	.35	.39	.47	1
9	.12	.19	.23	.27	.36	.44	1
$\overline{mq}$	.12	.19	.22	.3	.37	.45	1
SD.	.02	.02	.03	.03	.03	.03	0
rSD (%)	13.96	9.7	13.69	11.01	7.17	6.22	0

nulocytes of patients with different leukemias. The publication of the results of these experiments is in progress. Here we present one chromatographic plate of selected samples, to show real life application (Figure 5).

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REFERENCES

(1) S. Handa, Y. Kushi, Adv. Exp. Med. Biol., 152, 23 (1982).

(2) R. W. Ledeen, R. K. Yu, Methods in Enzymology, 83, 139 (1982).

(3) S. Ando, N-C. Chang, R. K. Yu, Anal. Biochem., 89, 437 (1978).

(4) J. P. Zanetta, F. Vitiello, G. Vincendon, Lipids, 15, 1055 (1980).

(5) J. Pick, J. H. Breuer, G. Folly, S. R. Hollan, 4th Danube Symp. on Chromatography, Siofok, Hungary, 1981.

(6) G. D. Hunter, G. M. Wiegant, A. J. Dunn, J. Neurochem., 37, 1025 (1981).

(7) V. Chigorno, S. Sonnino, R. Ghidoni, G. Tettamanti, Neurochem. Int., 4, 397 (1982).

(8) E. Tyihak, E. Mincsovics, H. Kalasz, J. Chromatography, 174, 75 (1979).

(9) J. Pick, J. Vajda, L. Leisztner, J. Liquid Chromatogr., in press.

(10) M. Gurkin, J. Ripphahn, Int. Lab., May/June, 63 (1980).

(11) J. Pick, J. Vajda, L. Leisztner, J. Liquid Chromatogr., 6, 2647 (1983).