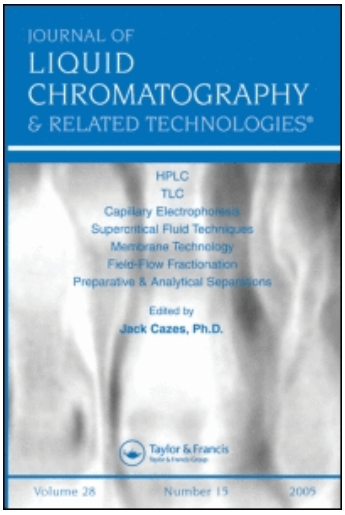

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GANGLIOSIDE PATTERN MAPPING BY GRADIENT OVERPRESSURED LAYER CHROMATOGRAPHY

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ABSTRACT

During the last decade overpressured layer chromatography, a forced flow planar liquid chromatographic technique has undergone significant development since its introduction in 1977. It is a high performance layer chromatographic method in its own right with varied application fields including as a substitute or a pilot technique for modern column liquid chromatography. A great advantage can be its use with gradient elution in the separation of complex biological samples shortening the laborious sample pretreatment process. The analysis of minor components is possible without overloading the chromatographic plate by the suitable selection of the polarity/solvent strength of the consecutive elution steps, overruning the major components (eluting them off the plate) before starting the final development step.

Gangliosides represent a minor group of plasma membrane lipids having very important biological functions such as leukaemia and cancer antigeneity. The specific aim of the study was to perform chromatographic separation and chemical analysis of membrane gangliosides extracted from leukaemic and their normal

equivalent cells, with the objective of determining and comparing the alterations in their compositions. Our step-gradient OPLC method for determining ganglioside patterns of several blood cell membranes, makes available a very effective sample pretreatment, the shortest sample preparation. By the results and the conclusions drawn the leukaemic and normal cells can be distinguished on the basis of GD3 expression and/or the vigorous increase in the ratio of nonpolar to polar gangliosides.

INTRODUCTION

Since its introduction, in 1977 (1), the over-pressured layer chromatographic (OPLC) technique has achieved remarkable results in methodological developments and widespread applications.

The chromatographic separation is carried out in a totally closed, pressurized chamber wherein the vapour phase of the eluent above the sorbent layer is practically inexistant. This eliminates the evaporation and readsorption of the eluent. The constant optimum flow rate of the mobile phase is insured by a solvent pump (2). Because of this forced flow, while in classical layer chromatography the eluent migrates by capillary forces through the sorbent, the efficiency of the separation is improved, the development distance can be increased even on adsorbent layers with very fine particle size distributions.

Both theoretical and practical aspects of the OPLC technique have been extensively investigated by now, i.e. the characteristics of solvent migration, the

spreading of spots, the factors influencing theoretical plate height, the optima of eluent flow rate, migration distance and resolution, the layer thickness, the membrane pressure (3,4,5,6,7,8) etc. The methodological developments were published continuously underlining the flexibility and the advantages (9,10) of the technique and the set up (11). New layers (12), new separations (13) and chromatogram development possibilities (14,15) have also been introduced. OPLC has found widespread applications in the analysis of alkaloids (16), amino acids (17), doping agents (18) and a number of other compounds (19,20), furthermore it can play a very important role in HPLC method development as a pilot technique (21,22).

The OPLC method was introduced to lipid analysis in our previous contributions (23,24). Isolation, identification and quantitative analysis of complex biological samples such as blood cell membrane lipids is a highly complex work. One of the basic problems in analysing complex materials derived from biological origin is the sample preparation/pretreatment. We found the OPLC method to be powerful and versatile, with the help of which we could achieve a short cut in sample pretreatment. Disposing of several prepurification steps, the material and time requirements, also the possibility of incomplete lipid recovery decreased.

With our methodological development (15) the possibility of employing different coupled development steps by changing the mobile phase during the run was introduced. In other words, any kind of gradient forced flow planar chromatographic elution (exponential/linear with or without isocratic steps) can be performed. For ganglioside analysis from the total lipid extract of blood cells an overrun gradient separation composed from three consecutive isocratic steps was selected.

In the present paper the ganglioside pattern mapping of several normal and leukaemic blood cells are summarized. The ganglioside profiles of different cell samples give interesting results of clinical importance. These gangliosides investigated represent one group of glycolipids.

Glycosphingolipids are composed of carbohydrates, sphingosine derivatives and fatty acids. The sphingosine - fatty acid portion, called ceramide, constitutes the hydrophobic part of the glycolipid molecules. The oligosaccharide moiety or substituted monosaccharide and sugaramine units are attached to the primary hydroxyl group of the long-chain base. The glycolipids present in mammalian cells can be divided into three subgroups, namely: neutral glycolipids, sulphatides and gangliosides. Gangliosides are sialic acid conta-

ining glycosphingolipids located almost exclusively on the outer leaflet of the plasma membrane, with their ceramide part embedded into the membrane lipid double layer and the oligosaccharide moiety oriented towards the external fluid. Plasma membrane glycolipids have been found to be influenced by growth and malignant transformations. An enormous progress in the methodology available for isolation and structural characterization of these compounds makes their investigations feasible (25). Changes in the ganglioside profile in human leukaemia have not been studied adequately. The data reported so far is not limited but rather varied and even conflicting. Further, there has been no data on glycolipids in some rare forms of haematological diseases. It is clear that leukaemia and cancer research projects are nowdays in focus, so the importance of a rapid, handy and reproducible method for monitoring leukaemia and cancer antigenes is indispensable for routine clinical laboratory practice.

The specific aim of the study was to perform chromatographic separation and chemical analysis of membrane gangliosides extracted from leukaemic and their normal equivalent cells, with the objective of determining and comparing the alterations in their compositions.

EXPERIMENTAL

Samples, reagents, materials

The samples were taken by leukapheresis, plasmapheresis or as native or anticoagulated blood. From normal controls, single individual samples were collected separately and normal plasma was also used. The samples were processed immediately after collection or kept at -180 ^{0}C in liquid nitrogen or lyophilized, depending on the time and type of the experiments being performed. Cytomorphological, cytochemical and immunocytochemical features were used to classify cases of leukaemia. In the procedure, separating white blood cells (WBC) the contaminating red blood cells (RBC) were eliminated by dextran gravity sedimentation and osmotic lysis, while platelets (P) were removed by differential centrifugation as described in detail previously (26,27). The purified cell preparations had a WBC/RBC ratio of 50:1 and a WBC/P ratio of 5:1. Viability of the cells was greater than 90%.

Pure lipid samples, served as models for method development and standards in ganglioside mapping, were prepared by mixing individual lipids in concentrations corresponding to the cell membrane composition, determined experimentally, obtained from Supelco Inc., Supelco Park, Bellefonte, PA USA.

Reagents for staining and other solutions were prepared from analytical grade chemicals, all solvents used were HPLC grade and chromatographic plates were HPTLC grade silica, purchased from E. Merck, Darmstadt West Germany. The water used was prepared according to (28) from water double distilled from glass and sterilized.

Apparatus

The OPLC instrument was equipped with a Chrompres 10 chamber with water pump, Labor MIM, Esztergom Hungary, a Beckman 112 SDM, Beckman Instr. Inc. Berkeley, CA USA and a Valco C 10 V N 60 ten port valve, VICI AG, Schenkon Switzerland, with two 3 ml loops. Instrument set up and other possible arrangements were detailed elsewhere (15).

Methods

Before the chromatographic separation the total lipid content of the cells examined was extracted. The resulted total lipid extract (TLE) was afterwords incubated in alkalic media and purified on a short reversed phase cartridge to eliminate all the alkali labile acidic lipids interfering with glycosphingolipid separation. The lipid samples derived were separated into main and subclasses and individual gangliosides by the segments of the three-step gradient OPLC pro-

cess. After gradient development the chromatographic plates were stained and evaluated by spectrodensitometry. All details of sample preparation, plate pretreatment, sample application, overpressured layer chromatography and spectrodensitometry were given earlier (24).

RESULTS AND DISCUSSION

Multistep gradient overpressured layer chromatography

The possibility of using gradient elution in conjunction with overrun capability makes possible the direct separation of complex biological materials, and opens a new short cut in the sample pretreatment. As the highly efficient sample preparation became one of the break-throughs of methodologies analysing materials from biological sources, we can state that the gradient OPLC technique gives a convenient method for this purpose. In the given example the sample prefractionation is performed on the same adsorbent layer where the final analytical separation is carried out. In our elution system, in the fractionation of crude lipid extract, the first solvent is the weakest and the later ones become strongers. In this case it is possible to find an eluent series with the aid of which all the uninteresting, interfering components/component groups can be run off the chromatographic plate.

It is also very important from the point of view of the adsorbent capacity, if minor components have to be analysed, as it is the case in glycolipid determination, where the whole ganglioside class amounts altogether max. one percent of the total lipid content. The gangliosides, in our step-gradient development process, migrate only in the last eluent, they are in the mobile phase during a short period of time, resulting in compact spots on the layer.

We have developed the step-gradient OPLC method on standard lipid mixtures, defined a retention factor (Rf) analogue, gave an explanation and an explicit equation for the migration distance (29). With the help of this equation the final migration of a given component after step-gradient development can be calculated in advance from individual isocratic data, measured or given in literature, so the separation can be planned. The confirmation of the equation was also done, the correlation of the calculated and experimentally measured data was examined. Briefly the parameters of the regression curve (Figure 1.) are; slope 1.009, intercept 1.109, s(residual) 2.449 and regression coefficient 0.998, which in themselves summarize the results supporting our hypothesis, the acceptability and validity of our model.

$$m_j = \sum_{i=1}^{n-1} \frac{Rf_{ij} z_i}{1-Rf_{ij}} + Rf_{nj}\left(\sum_{i=1}^{n} z_i - \sum_{i=1}^{n-1} \frac{z_i}{1-Rf_{ij}}\right)$$

m : migration distance
z : elution distance
i : i-th eluent
j : j-th component

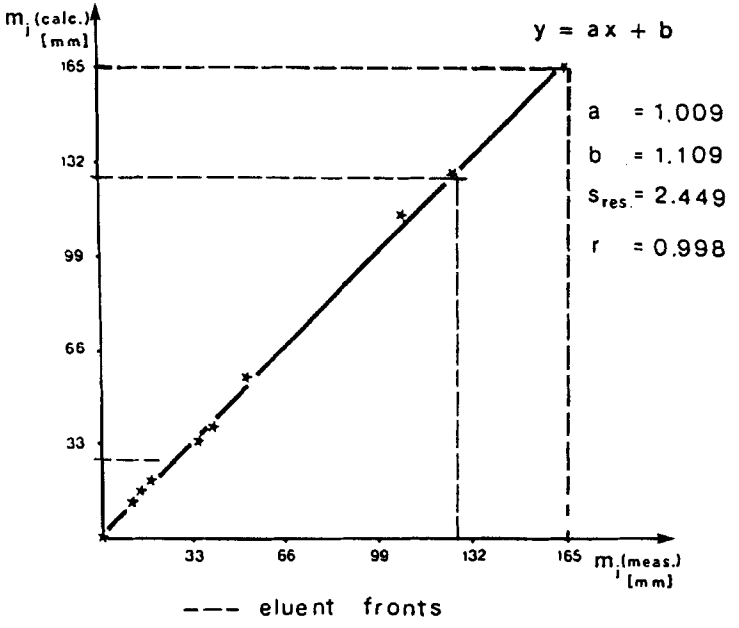

Figure 1. Correlation of measured and calculated migrations in step-gradient OPLC development

Ganglioside pattern mapping

The previously described method was applied for ganglioside pattern mapping of leukaemic blood cells.

The gangliosides of the following samples were analysed;
- normal and leukaemic plasma,
- normal lymphocytes and granulocytes,
- acut myeloblastic leukaemia cells (AML), acut myelomonocytic (AMMoL) and acut monocytic (AMoL) leukaemia cells,
- acut lymphoblastic leukaemia cells (ALL),
- chronic lymphocytic leukaemia cells (CLL),
- mast cell leukaemia cells (MCL),
- Non-Hodgkin's lymphoma cells (NHL) and
- chronic myeloid leukaemia cells (CML).

The ganglioside nomenclature in the text, figures and tables is according to the Svennerholm's system (30), thus:

GM3 II^3NeuAc-LacCer,

GM2 II^3NeuAc-GgOse$_3$Cer,

GM1 II^3NeuAc-GgOse$_4$Cer,

GD3 $II^3(NeuAc)_2$-LacCer,

GD1a II^3NeuAc-IV^4NeuAc-GgOse$_4$Cer,

GD1b $II^3(NeuAc)_2$-GgOse$_4$Cer,

GT1a III^3NeuAc-$IV^3(NeuAc)_2$-GgOSe$_4$Cer,

GT1b II $NeuAc_2$-IV^3NeuAc-GgOse$_4$Cer.

Some representative densitograms of the gangliosides are shown on the next figures (Figure 2. and 3.).

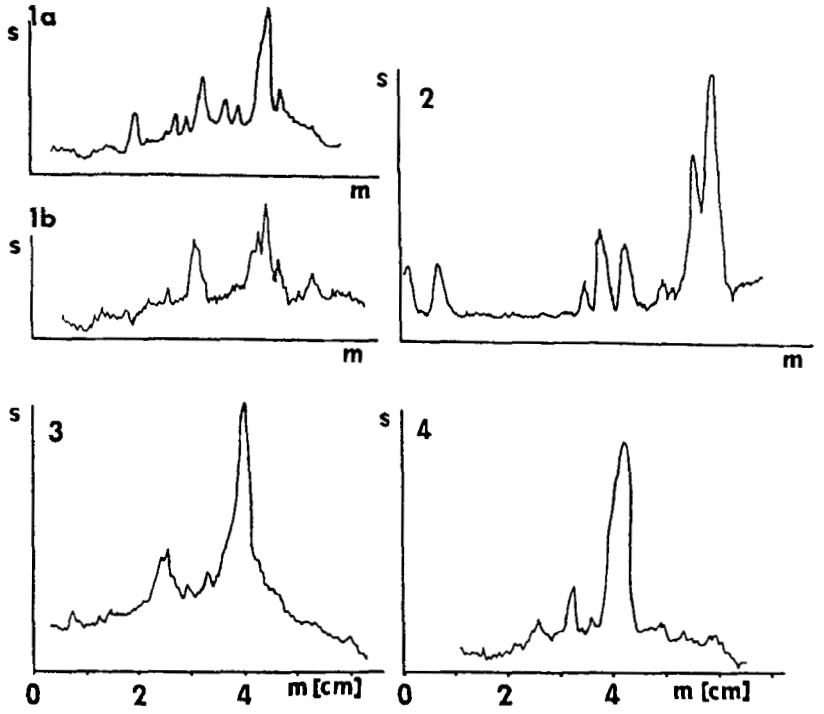

Figure 2. Densitograms of the gangliosides separated from normal lymphocytes and granulocytes (1a and 1b) and leukaemic cells, CLL (2), MCL (3), ALL (4).

The results of ganglioside composition measurements are summarized and compared to the normal equivalent in the following tables (Table 1. and 2.). The qualitative results of the analysis of normal and leukaemic human plasma can be seen in Table 1., while the quantitative ganglioside composition of normal and leukaemic cells are shown in Table 2.

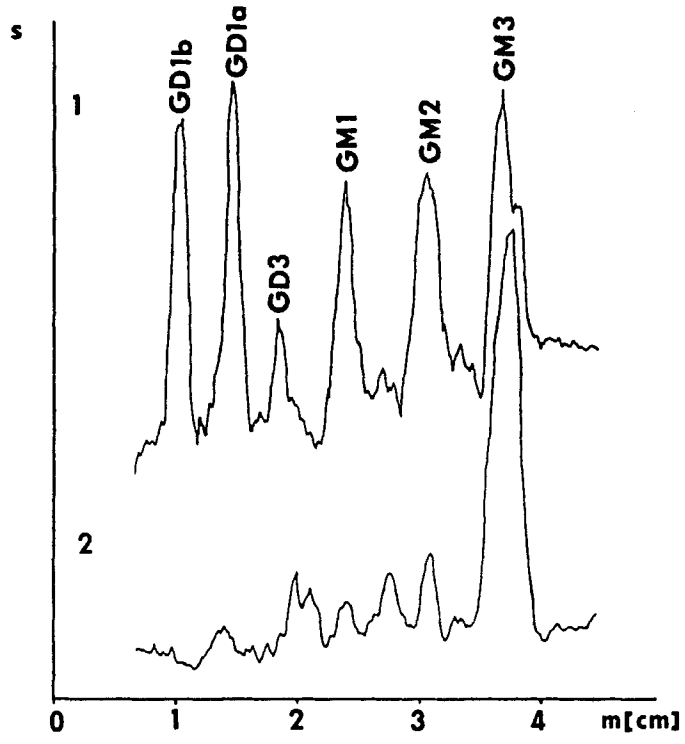

Figure 3. Ganglioside densitograms of standard mixture (1) and pre-B lymphoma cells (2).

In the experiments purified cell preparations from individual patients with four major groups of leukaemia (AML, ALL, CML and CLL) and two of rare haemopoietic malignances, mast cell leukaemia and lymphoblastoid pre-B lymphoma, were analysed for the ganglioside composition by the newly-developed step-gradient OPLC technique. The leukaemic cell populations of the various cell types studied were found to have ganglioside

TABLE 1.

GANGLIOSIDE SPECIES IDENTIFIED IN LEUKAEMIC VS. NORMAL PLASMA

Type of leukaemia	Ganglioside species							
	GM3	GM2	GM1	GD3	GD1a	GD1b	GM1-GD1[x]	GT
AML	+	+	+	+	+	+	4 speaks	+
	+	-	+	+	+	-	2 spaks	-
AML	+	-	+	+	+	+	1 peak	-
ALL	+	-	+	+	+	-	1 peak	+
B-CLL	+	-	+	+	+	-	2 peaks	-
	+	-	+	+	+	-	2 peaks	-
MCL	+	+	+	+	+	-	2 peaks	-
Normal plasma	+	(+)	+	+	+	-	4 peaks	(+)

x Unidentified compounds with TLC mobilities between GM1 and GD1

TABLE 2.

GANGLIOSIDE COMPOSITION OF HUMAN LEUKAEMIA CELLS
VS. NORMAL GRANULOCYTES AND LYMPHOCYTES

Cell type	Ganglioside composition, % of the total							
	GM3	GM2	GM1	GD3	GD1a	GD1b	GM1-GD1[x]	GT
CML	70.3	0.1	0.1	0	4.3	5.7	13.0	6.7
AMoL	51.3	xx	13.4	7.8	4.7	6.0	0	0
AMMoL	47.2	0.1	31.8	0	7.5	0	13.5	0
Granulocytes	8.3	0.1	8.6	0	13.5	13.0	51.2	5.5
NHL xxx	50.8	11.2	7.8	2.3	1.1	2.2	7.4 + 5.5	1.6
MCL	60.8	5.4	5.1	21.5	3.4	2.9	0	0.8
CLL	81.5	0.1	7.6	5.6	1.9	2.2	0	0
ALL	65.7	6.0	24.0	0.1	0.1	0.1	0.1	0
Lymphocyte	37.7	6.1	7.5	0	9.2	10.4	29.2	0

x Unidentified compounds with chromatographic mobilities between GM1 and GD1
xx Unidentified compound (mean: 16.8%) migrating between GM2 and GM1
xxx Unidentified compound (10.3%) migrating between GM2-GM1

profiles characterized by two common features. One is the expression of a ganglioside, GD3, which is not present in normal leucocytes and the other is the predominance of less polar compounds, particularly the GM3, the hematoside, in all types of leukaemia examined.

In summary, leukaemic cells of all cell types studied AML, ALL, CML, MCL, NHL contained abundant amounts of hematoside, low levels of several other components having more complex sugar moieties and/or higher sialic acid content. The GD3 could not be detected in extracts from CML cells, normal lymphocytes and granulocytes. Unlike leucocytes, plasma from patients with different types of leukaemia showed no consistent different in the ganglioside composition.

By the results and the conclusions drawn, the leukaemic and normal cells are distinguishable on the basis of GD3 expression and/or the vigorous increase in the ratio of nonpolar to polar gangliosides. In other words; normal lymphocytes, granulocytes and CML cells can be distinguished from AML, ALL, CLL and pre-B lymphoma cells and leukaemic mastocytes on the basis of GD3 expression. However leukaemic cells can be differentiated from normal leucocytes by the increased proportion of less polar species.

These findings may be of potential clinical importance. It can be stated, that our step-gradient

OPLC method for determining ganglioside patterns of several blood cell membranes, makes available a very effective sample pretreatment, the shortest sample preparation. The techniqual set up, the system configuration is simple, it is easy to operate.

Due to the differential expression of the GD3 ganglioside, the production and application of specific antibodies against this particular ganglioside for diagnosis and treatment of human leukaemia seem to be promising, although the OPLC method is suitable to detect the absence or existance of any individual ganglioside, further extensive studies are still needed.

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