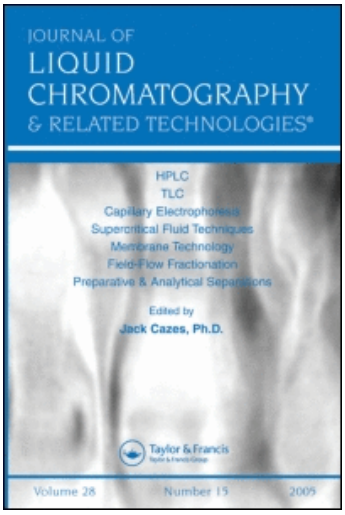


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# A VARIANT FOR ISOLATION OF SERUM GANGLIOSIDES

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## ABSTRACT

The serum gangliosides are of great diagnostic importance. A step-by-step procedure for their isolation from serum is described and it includes the following stages: a) dehydration of the sample; b) total lipids extraction; c) non-polar lipids removal by preparative TLC; d) elimination of the blood sugar by Sep-Pak technique; e) TLC of the ganglioside fractions. Azeotropic distillation of the mixture of serum water/n-propanol was used for sample dehydration. Triple extraction was carried out: with cyclohexane (I); chloroform : methanol = 1 : 1 (v/v) (II) and chloroform : methanol = 1 : 2 (v/v) (III). The extracts were combined and the non-polar lipids were removed. The eluate composition was checked out by HPTLC with a modified mobile phase: chloroform:methanol:0.1 M sodium lactate = 55 : 40 : 10 (v/v/v). Comparative data for the $R_f$ values of the ganglioside fractions as well as recovery data are presented. This method is also applicable for milk samples.

## INTRODUCTION

Gangliosides are glycosphingolipids that consist of sialic acid containing carbohydrate residue linked to ceramide, i.e. a N-fatty acyl derivative of a sphingoid linked carbon chain base.[1] Within vertebrates they occur in virtually all tissues and body fluids. In each species and each tissue their molecular patterns differ and undergo changes during development and under various physiological influences.[2] The majority of gangliosides are situated in the outer leaflet of the cell membrane. However, gangliosides also occur in non-cell associated form in blood plasma and other body fluids.[3] Although the concentrations of gangliosides reported in human serum vary, it is now established that elevated levels occur in some pathological conditions such as cancer, atherosclerosis and multiple sclerosis.[4,5]



In order to study the significance of gangliosides in the pathogenesis of different disorders it is very important to determine the concentration and composition of these lipids in the serum. Due to the low concentrations of gangliosides in serum (700 μg) ganglioside-bound sialic acid/100 mL sera,[5] it is difficult to obtain ganglioside extracts free from contaminants. Several methods for extracting, purifying and analysing gangliosides from serum or plasma have been reported.[6-10] All methods use a total lipid extraction as the first step. Gangliosides are then separated from other lipids by a partition procedure and further purified from contaminants. The separation of the lipids according to their polarity is usually performed by a phase distribution in a system of extraction solvents (mainly chloroform, methanol and water).[7-9] This step is followed by gel filtration and silicic acid column chromatography.[6,10] Most of the techniques used for ganglioside isolation are multistage and time consuming. A variant for isolation of serum gangliosides after suitable express dehydration and three-step extraction of the total lipids with solvents with increasing polarity followed by purification of the ganglioside fractions by selected TLC techniques is described in this article.

## MATERIAL AND METHODS

One mL serum was pipetted in a distillation flask and mixed with 10 mL n-propanol. The flask was mounted to a rotary vacuum evaporator and the serum water was removed by azeotropic distillation at 35-40°C; 15-20 mm Hg for 10-12 min. The rest was suspended in 10 mL of cyclohexane which was vacuum evaporated at the same conditions. The dehydrated sample dissociated mainly in the form of fine granules.

**Extractions**

The granules were transferred in a centrifugation tube containing 1 g pure quartz sand and 0.3 g kieselguhr. The residue stuck to the flask wall was washed with 15 mL of cyclohexane (first extraction) and eventually ultrasonication could be used. The suspension formed was added to the centrifugation tube and the mixture was vigorously stirred for 10 min and centrifuged at 4000 $min^{-1}$ for 10 min. The upper phase was transferred to a vial. The residue in the vacuum flask was washed repeatedly with 15 mL of a mixture of chloroform : methanol = 1 : 1 (v/v).

After sonication the solution was transferred to the tube and the extraction procedure was repeated (second extraction). The third extraction was performed as already described but with a mixture of chloroform : methanol = 1 : 2 (v/v). The combined extracts were evaporated at 35°C under nitrogen until 10-15 mL of solution were left in the vial. For analytical purposes the extracts could be processed separately.



**Non-polar Lipids Removal**

The concentrated extract was applied onto a preparative TLC plate (10x10 cm) and chromatographed with a mobile phase chloroform : methanol : 0.3% $CaCl_2$ as described in.[11] After peripheral marking of the ganglioside carrying zones (orcinol reagent) the sorbent was scrapped, placed in a column and successively eluted with a mixture (8 mL) of chloroform : methanol = 1 : 1 (v/v) and 12 mL of methanol. The eluate was concentrated at 35°C under nitrogen and its composition was checked out by HPTLC (aluminium plates, Merck, Germany). Modified mobile phase: chloroform : methanol : 0.1 M sodium lactate = 55 : 40 : 10 (v/v/v). After finishing the checking procedure the eluate was evaporated to dryness.

**Sep-Pak Procedure**

The Sep-Pak (Sep-Pak Cartridge, Millipore Corporation, Milford, Massachusetts, U.S.A.) extraction was performed according to.[12] The final eluate was concentrated and a sample was spotted onto a HPTLC glass plate (10x10 cm); mobile phase (see above). The spots were visualised by spraying with orcinol reagent (0.5% solution in 20% sulfuric acid) followed by local heating at 110°C. Bovine brain gangliosides could serve as a test mixture for identification.

**Recovery Data**

We have accomplished an additional experiment to determine the overall recovery. A standard mixture containing ganglioside fractions, obtained from bovine brain, was added to the native serum and the sample was processed as already described. The result was calculated according to the following equation:

$$\mu g \text{ sialic acid / mL serum} = \frac{A.F.Q}{a.b.g}$$

where:
A = absorbance at 580 nm
F = sialic acid content (μg) in the standard sample absorbance of the standard sample at 580 nm ratio
Q = whole ganglioside carrying area scrapped area ratio
a = percentage of the total extract applied onto the preparative TLC plate
b = percentage of the concentrated eluate after the Sep-Pak procedure used for sialic acid determination
g = serum sample (mL)

The technique for spectrophotometric determination of sialic acid is described in a previous work.[11]



## RESULTS AND DISCUSSION

The equal amount of the ganglioside mixture added to the serum sample is:

$$A_1F = 0.4 \times 170 = 68.0 \ \mu g$$

where: $A_1$ - absorbance at 580 nm

A sample without addition of test mixture was worked separately:

$$\mu g \text{ sialic acid / mL Serum} = \frac{0.022 \times 170 \times 1.5}{0.8 \times 0.7 \times 2.0} = 10.0$$

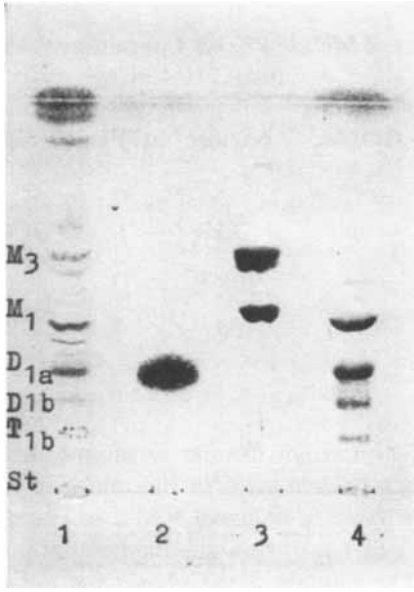


**Figure 1**. Thin-layer chromatogram of: (1) gangliosides after Sep-Pak procedure (test mixture added); (2) blood sugar (glucose); (3) GM1 and GM3 standards; (4) test mixture of bovine brain gangliosides; mobile phase: chloroform : methanol : sodium lactate = 55 : 40 : 10 (v/v/v); visualization by spraying with orcinol reagent.



A serum sample with test mixture was added for recovery:

$$\mu g \text{ sialic acid / mL serum} = \frac{0.164 \times 170 \times 1.5}{0.8 \times 0.7 \times 1.0} = 74.6$$

$$\frac{74.6 - 10}{68} \times 100 = 95\% \text{ recovery}$$

The control chromatogram of the first extract (cyclohexane) shows presence of cholesterol, neutral lipids and phospholipids, some blood sugar (glucose) and traces of gangliosides. Both second and third extracts contain traces of non-polar lipids, the main quantity of serum gangliosides and part of the blood sugar. The last one could be effectively removed by the Sep-Pak method. Control chromatograms illustrating the extraction procedure are given in Figure 1 (see text below).

**Table 1**

**$R_f$ (x 100) Values of Mobile Phases Containing 0.1M Sodium Lactate and 0.2% $CaCl_2$**

| | Sialic Acid | Glucose | Lactose | GT1b | GD1b | GM1 | GM3 |
|---|---|---|---|---|---|---|---|
| 0.1M Na lactate | 11 | 40 | 25 | 16 | 30 | 72 | 82 |
| 0.2% CaCl2 | 15 | 55 | 33 | 8 | 20 | 91 | 97 |



The choice of n-propanol for the azeotropic distillation proved to be successful. According to data cited in the literature the composition of the azeotropic mixture is water : n-propanol = 1 : 2.6. The sample dehydration was carried out at a ratio of 1 : 10 this was the optimal process to be completed within 8-10 min. The addition of cyclohexane at the dehydration stage was meant to remove the excess of n-propanol and to granulate the dry serum residue. Samples from rat serum, cow milk and human milk were dehydrated in a similar manner. Differences in the water, lipid and protein content did not affect the proposed method. The successive extraction of the dry serum residue with solvents with increasing polarity ensured a total drawing out of the sample gangliosides so that a four extraction step was not necessary. The sample solvents ratio in this three-stage procedure was 1 : 40 (v/v). Removing the total serum lipids (cholesterol, triacylglycerols and phospholipids) is necessary for simplifying and making the Sep-Pak procedure that follows more effective. A modified mobile phase consisting of chloroform : methanol : 0.1 M sodium lactate = 55 : 40 : 10 (v/v/v) was used in the analytical TLC and it was compared with a known one[5] consisting of chloroform : methanol : 0.2% $CaCl_2$ = 50 : 45 : 10 (v/v/v). The corresponding $R_f$ (x100) values at 30°C ambient temperature are given in Table 1.

According to the chromatographic conditions (sorbent, mobile phase, ambient temperature), the glucose spot could overlap the GD1a and GD1b fractions and the same is valid for the lactose and GD1b and GT1b fractions respectively. In case this is avoided (as on the chromatogram in Figure 2) the information about the serum gangliosides is sufficient and the Sep-Pak procedure can be refrained.

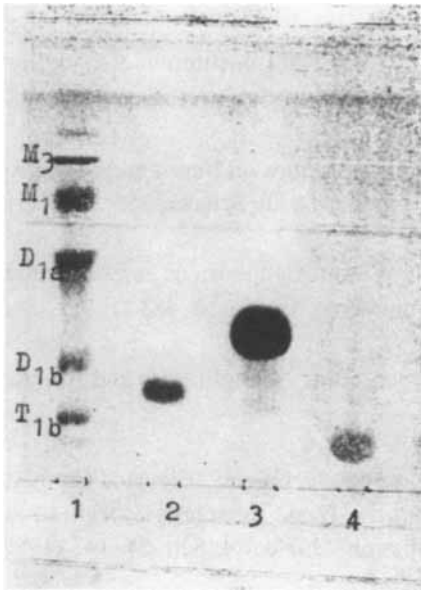


**Figure 2.** Thin-layer chromatogram of: (1) standard mixture; (2) lactose; (3) glucose; (4) sialic acid; mobile phase and visualization as in Fig. 1; for the $R_f$ values see text.



The GM3 fraction forms about 50% of the total gangliosides and it is of great diagnostic importance.[5] According to the data for the $R_f$ values, glucose does not overlap the GM3 spot and the Sep-Pak procedure could also be avoided for this reason. However, it is absolutely necessary when the serum gangliosides are quantified spectrophotometrically by the lipid-bound sialic acid.[11]

This variant for isolation of gangliosides from serum and also, from other biological fluids, is rapid and inexpensive and it is fairly suitable for routine diagnostic studies in the clinical practice.

## ACKNOWLEDGEMENTS

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