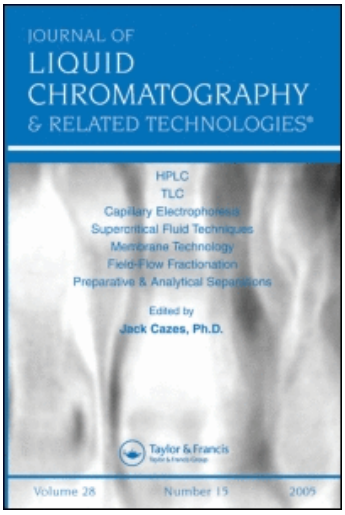

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SEPARATION OF SEVERAL MAIN GLYCOLIPIDS INTO CLASSES AND PARTIALLY INTO SPECIES BY HPLC AND UV-DETECTION

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ABSTRACT

The separation and the estimation of several main glycolipid classes as well as of some of these classes into main subclasses (or species) by reverse phase and normal phase high performance liquid chromatography and UV detection, is described.

Two different gradient elutions onto a C_{18} column and onto a silica column, respectively, and an isocratic elution onto a strong cation exchange column were performed, with detection at 206nm. Separations were achieved within 30min. in the reverse phase and normal phase modes and within 10min. in the cation exchange mode.

The following glycolipids standard classes were tested: gangliosides, sulfate cerebrosides (sulfatides), digalactosyl-diglycerides, galactosyl-cerebroside as well as ceramides (the backbone of sphingoglycolipids), N-palmitoyl-sphingosine (a synthetic ceramide) and a phospholipid, cardiolipin. Some species of digalactosyl-diglycerides and galactosyl-cerebrosides were also separated.

Application of the present method on the separation of glycolipids from animal tissues is represented.

INTRODUCTION

Glycolipids of plant and animal tissues origin were traditionally isolated from neutral lipids and phospholipids by glass column techniques while thin layer chromatography (TLC), high performance TLC (HPTLC) and high performance liquid chromatography (HPLC) have also been used.

Normal phase HPLC (NP-HPLC) on silica based columns of different type has been used for the separation of several individual glycolipid classes.[1-15] Reverse phase HPLC (RP-HPLC) on C_{18} columns has been used for the classification of glycolipid fractions or for the separation of glycolipid subclasses into molecular species[13-22] while anion exchange HPLC (AX-HPLC) for the separation of gangliosides[23-25] and HPLC on Iatrobeads for glycosphingolipids.[26-30]

NP-HPLC was used for separation of cerebrosides (CER),[1,2,3,5] ceramides (CERA),[3,12] sulfatides (SULF),[4] digalactosyl-diglycerides (DGDG),[5] monogalactosyl-diglycerides (MGDG),[5] sterol-glycosides (STGL),[5] glycosphingolipids (GLSP)[9,10,13] heterocyst type of glycolipids benzoylated derivatives[11] and gangliosides (GANG).[6-8,14,15] The simultaneously separation by NP-HPLC of more than one class of glycolipids only in a few cases have been reported as in the case of the separation of MGDG, DGDG, STGL and CER[5] while other cases have been reported for the species separation of glycosphingolipids.[9,10.13]

RP-HPLC has been reported for the separation of some major GANG subclasses into molecular species,[14,15,21] for the separation of sophorolipids subclasses,[19] for the separation of heterocyst-type glycolipids into subclasses,[22] for the resolution of GLSP[13,17,18] and galactosyl-cerebrosides (GALCER).[20]

AX-HPLC has been used for the separation of GANG.[23-25] Iatrobead separations have been used for GLSP,[26,29,30] GANG[27] and SULF.[28]

The detection of the referred HPLC analysis of the glycolipids were usually performed by techniques other than UV because of the low UV detectability of the underivatized glycolipids. Namely, refractive index (RI) detection,[22] light-scattering detection (LSD),[19] flame ionization detection (FID),[5] fluorescence (FL)[16] have been used.

UV detection for underivatized glycolipids have been reported for GANG,[7,15,21,24,25,27,29] for GLSP,[10,18,31] cyanobacterial heterocyst-type glycolipids[22] while UV- detection for derivatized glycolipids have been reported for GANG,[6] GLSP,[9,13] cyanobacterial heterocyst-type glycolipids[11] and galactosyl-ceramides.[20]

As it can be seen from the above review the simultaneous separation of more than one class of underivatized glycolipids has been reported only in a few cases.[5] This separation was achieved by NP-HPLC while RP-HPLC have been used only for the separation of one particulate glycolipid class or subclass each time into molecular species and AX-HPLC for separation of one particular subclass moiety each time into species.

In the present method the simultaneous separation of four major glycolipid standard classes as well as the separation of these classes into subclasses is represented for the first time by using an easy to run one step gradient RP-HPLC method, by UV-detection at 206nm while an alternative separation by NP-HPLC and a strong cation exchange HPLC (SCX-HPLC) fractionation are also introduced. The present RP-HPLC and NP-HPLC methods are applied to the separation of glycolipids isolated from beef brain.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, purchased from Merck (Darmstadt, G). HPLC solvents were purchased from Ruthburn (Walkerburn, Peebleshire, UK). Lipid standards of HPLC grade were obtained from Supelco (Bellefonte, PA, USA). Bovine brain was also used immediately after the sacrifice of the animal.

Table 1

Retention Times of Individual Standards (min)

Peak No	Glycolipids	RP-HPLC	NP-HPLC	SCX-HPLC
SF	----	3.02	3.02	3.02
1	GANG	8.16	28	----
2	SULF	9.06	10.05	4.21
3	SULF	14.15	SF	5.71
4	(NPSP)	11.41	----	----
5	(NPSP)	15.94	----	----
6	(CARD)	12.87	30.02	----
7	DGDG	13.27	24.05	4.18
8	DGDG	14.37	(24.05)	(4.18)
9	DGDG	15.71	(24.05)	(4.18)
10	GALCER	16.87	26.21	4.32
11	GALCER	21.39	(26.21)	(4.32)
12	GALCER	26.05	(26.21)	(4.32)
13	(CERA)	18.12	SF	4.43
14	(CERA)	23.54	6.05	7.94

Abbreviations: SF, solvent front; GANG, gangliosides; SULF, Sulfatides; NPSP, N-palmitoyl-sphingosine; CARD, cardiolipin; DGDG, digalactosyl-diglycerides; GALCER, galactosyl-cerebrosides; CERA, ceramides.

Standard and Sample Preparation

All standards were prepared as 5% solutions in chloroform/methanol (1:1). Total lipids were isolated from bovine brain by extraction and the glycolipids were separated on a silicic acid glass column chromatography as will be described elsewhere.

Chromatography

HPLC was performed on a dual pump Jasko (Tokyo, Japan) model 880-PU HPLC, supplied with a 330μL loop Rheodyne (P/N 7125-047) injector. A Jasko model 875 UV spectrophotometer was used as detector at 206nm (0.4 a.u.f.s.). The spectrophotometer is connected to a Hewlett-Packard (Avondale, PA USA) Model HP - 3396A integrator - plotter. Three different types of

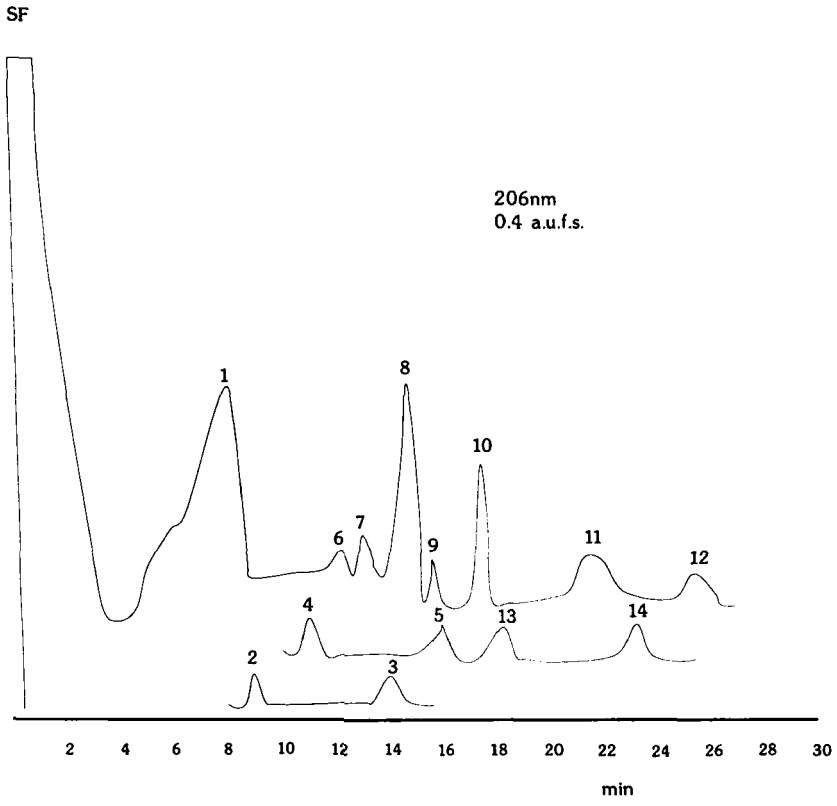

Figure 1. RP-HPLC chromatograms of glycolipid standards. Chromatographic conditions in Results and Discussion section. Peak identification in Table 1.

columns were used: a cation exchange column, SS 10μm Partisil 25cm x 4.6mm I.D., PXS 10/25 SCX from Whatman (Clifton, NJ, USA), an absorption column, Silica 25cm x 4.6mm I.D., from Hichrom H5 (Reading, Berkshire, England) and a nucleosil-300, C_{18} column 7μm, 250 x 4mm I.D. from Analysentechnik (Mainz, G). The flow rate was lmL/min.

RESULTS AND DISCUSSION

Glycolipids analysis was performed by using three different column types, a reverse phase, Fig. 1 and 2, a normal phase, Fig. 3, and a strong cation exchange, Fig. 4.

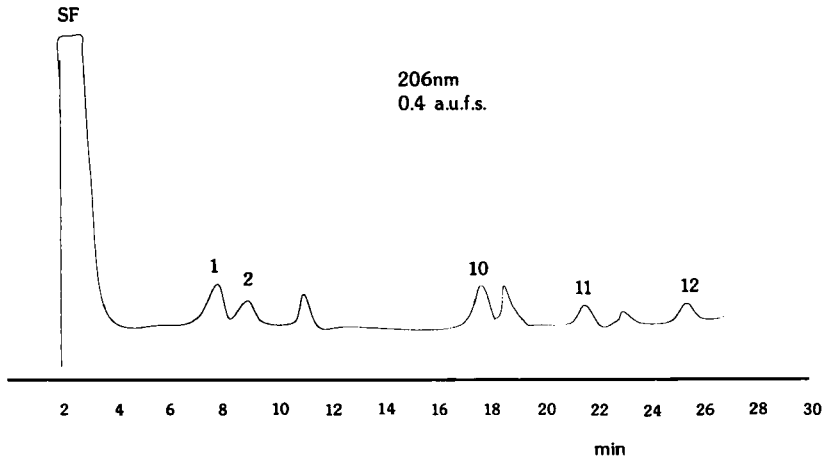

Figure 2. RP-HPLC representative chromatograms of glycolipid fraction from beef brain sample. Chromatographic conditions as in Fig. 1 and in Results and Discussion section. Peak identification in Table 1.

The analysis of standards was performed by using the optimum amounts of each individual substance each time for the UV detection limit of the used instrumentation, as shown in Fig. 1, 3A and 4. Each individual glycolipid standard was injected separately and the resulted peak(s) was collected and co-chromatographed on TLC plates with authentic standards in order to confirm the elution and the retention times (RT's) of the examined glycolipid standard. Similar TLC were also used for the identification of the HPLC peaks from the examined natural sample of beef brain (Fig. 2, 3B).

The mobile phase introduced with the RP-HPLC mode, Fig. 1, was a linear gradient from 100% methanol/water (4:1) to 100% acetonitrile/methanol (7:5) in 10min. and then hold for 15min.. A 25min. elution was sufficient for the separation of four glycolipid classes, as represented in Table 1 with respective RT's. The examined glycolipids were eluted in the following order, GANG as two overlapped species (peak 1), SULF as two species (peaks 2 and 3), DGDG as three species (peaks 7, 8 and 9) and GALCER as three species (peaks 10, 11 and 12). N-palmitoyl-sphingosine (NPSP) was eluted as two species (peaks 4 and 5) and CERA as two species (peaks 13 and 14). Cardiolipin (CARD) was injected in order to compare the RT's of the examined glycolipids with the RT of a relatively non polar phospholipid.

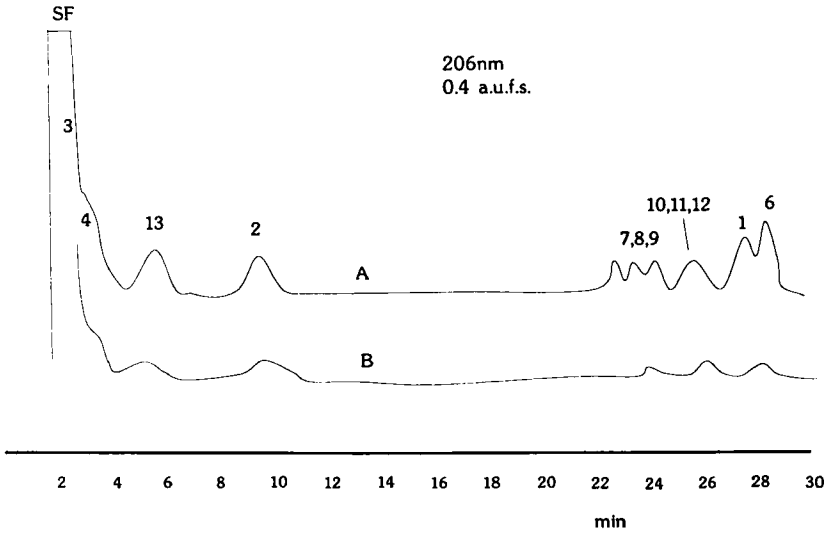

Figure 3. NP-HPLC representative chromatograms (A) of glycolipids standards and (B) of glycolipid fraction from beef brain sample. Chromatographic conditions in Results and Discussion section. Peak identification in Table 1.

Since it has been referred[31] that neutral lipids are also eluted under the above conditions, they should be isolated from glycolipids by other techniques before the analysis performed on HPLC. On the contrary, phospholipids are eluted with the solvent front (SF) (under the above conditions) and they do not interfere in the analysis on the HPLC with the exception of cardiolipin which is eluted among glycolipids.

A glycolipid sample isolated from beef brain was fractionated by this mode as shown in Fig. 2 and revealed the existence of GANG, SULF and GALCER.

A two step isocratic elution was chosen for the separation of glycolipids onto a silica column as shown in Fig. 3. A 15min. elution with 100% acetonitrile followed by a gradient change to 100% methanol within 10min. and hold for 10min., proved to be sufficient for the distinct separation of the relatively low polarity glycolipids from the relatively polar ones, with relatively long intermediate times. Namely, species of SULF (as well as CERA) which were eluted in the first 12min., show a 12 minute difference from DGDG and

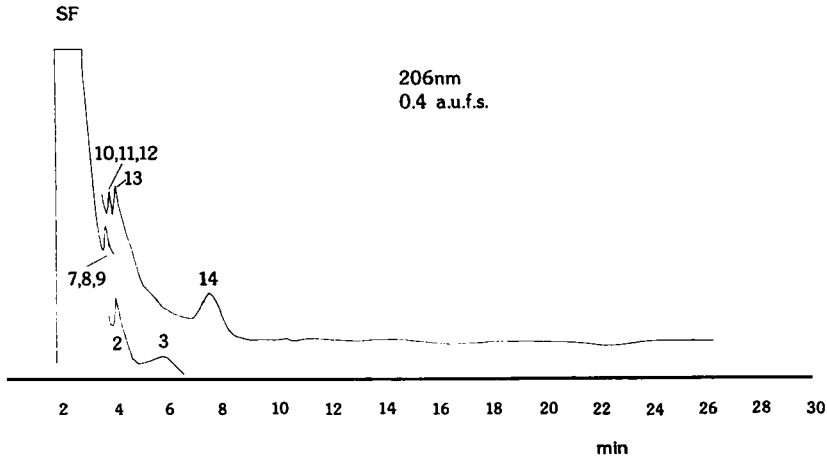

Figure 4. SCX-HPLC representative chromatograms of glycolipid standards. Chromatographic conditions in Results and Discussion section. Peak identification in Table 1.

GANG which were beginning to elute at 23min. The peaks of DGDG and GANG appeared as riders on the "mountain" of the elution solvent change. SULF were elute with solvent front and can be collected and analysed by the RP-HPLC mode. In Fig. 3B is shown the separation of beef brain glycolipids. This method offers an easy collection of the clearly separated glycolipid classes and thus it could be very useful for semi- or preparative purification of individual glycolipid classes.

A strong cation exchange column (Fig. 4) was also used with 100% acetonitrile as elution system. Under these conditions the glycolipids (SULF, DGDG and GALCER) were eluted within 8min. but their separation was insufficient.

The above experimental data show that separation of glycolipids can be achieved with the use of a RP-HPLC as well as with a NP-HPLC but RP-HPLC is superior since it permits also the sufficient separation of glycolipids subclasses and species.

The efficiency of the described method is shown by the analysis of an animal origin sample.

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