Separation of molecular species of sphingomyelin and ceramide by argentation and reversed-phase HPLC

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Summary A convenient method for the separation of molecular species of complex lipids such as sphingomyelin and ceramide by argentation high performance liquid chromatography (HPLC) is described for the first time. Molecular species separation of these complex lipids was achieved for quantitative analysis and preparative work by the combined use of argentation and reversed-phase HPLC.—**Smith, M., P. Monchamp, and F. B. Jungalwala.** Separation of molecular species of sphingomyelin and ceramide by argentation and reversed-phase HPLC. *J. Lipid Res.* 1981. **22:** 714–719.

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Argentation high performance liquid chromatography has been used for the separation of a variety of compounds such as drugs (1–3), insect sex attractants (4, 5), prostaglandins (6), and fat soluble vitamins (7). A method for the separation of triglyceride mixtures including the positional isomers by argentation HPLC has been described (8). However, its use has not been adequately explored within the lipid field. Here we describe the separation of saturated and unsaturated fatty acid-containing sphingomyelins and ceramides by this technique.

Previously we have reported partial separation of molecular species of sphingomyelin, phosphatidylcholine, cerebrosides, and ceramides by reversed-phase HPLC (9–13). With this method, however, 'critical pairs' were not completely resolved. Complex lipids having one double bond in the fatty acid side chain eluted together with the lipid having a saturated fatty acid with two less carbon atoms, e.g., sphingomyelins with 24:1 and 22:0 fatty acids were eluted together. Here we show that argentation HPLC separated the lipids based upon the number of double bonds independently of the number of carbon atoms.

Abbreviations: HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; NFA, nonhydroxy fatty acids; HFA, α-hydroxy fatty acids; TLC, thin-layer chromatography.

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Thus, by the combined use of argentation and reversed-phase HPLC, different molecular species of complex lipids are almost completely resolved.

EXPERIMENTAL METHODS

Materials

Sphingomyelin from beef brain was purchased from Supelco, Bellefonte, PA. Hydroxy fatty acid-containing ceramide (bovine) was purchased from Applied Science Labs., State College, PA. 3-O-Benzoylated nonhydroxy fatty acid-containing ceramide was prepared by benzoylation of bovine brain sphingomyelin and hydrofluoric acid hydrolysis to remove the phospocholine moiety (14). HPLC solvents were from Burdick and Jackson, Inc. (Muskegan, MI). All other chemicals were of reagent grade from Fischer Scientific Co. (Medford, MA).

Instrumentation

The HPLC analysis was performed with a Waters Associates (Milford, MA) model 6000 solvent delivery system and model U-6K injector. The argentation HPLC was performed with a Chrompak (Whittier, CA) silver column (25 cm × 4.6 mm i.d.). The reversed-phase HPLC was with a Nucleosil-5 μm C_{18} column (30 cm × 4 mm i.d.) or with a Waters Associates Radialpak A Cartridge (10 cm × 8 mm i.d.) as previously described (9, 10). A variable wavelength spectromonitor from Schoeffel Instrument Co. (Westwood, NJ) was used at either 205 nm for underivatized sphingolipids or at 230 nm for benzoylated sphingolipids.

Method

Benzoylation of sphingomyelin. Sphingomyelin (1 mg) was benzoylated with 0.5 ml of 10% benzoic anhydride in tetrahydrofuran at 70°C for 2 hr. After the reaction, the solvent was evaporated under N_2 and the product was dissolved in 1 ml of chloroform. The sample was chromatographed on a silicic acid (500 mg) column with 10 ml each of chloroform, chloroform–methanol 8:1 (v/v), and methanol, respectively. The methanol fraction contained the 3-O-benzoylated sphingomyelin. This fraction was evaporated under N_2 and redissolved in a suitable volume of the HPLC solvent.

Argentation HPLC of benzoylated sphingomyelin was performed on the Chrompak silver column with methanol–isopropanol 8:2 as the solvent pumped isocratically at a flow rate of 1 ml/min.

Reversed-phase HPLC of benzoylated sphingomyelin was performed on the Nucleosil 5 μm C_{18} column with methanol–acetonitrile–potassium phosphate buffer, pH 7.4, 5 mM 100:20:1 (v/v) as the solvent pumped isocratically at a flow rate of 2 ml/min. The molecular species associated with individual peaks were analyzed for sphingosine and fatty acid as described previously (9, 10).

Argentation HPLC of hydroxy fatty acid-containing ceramide and 3-O-benzoylated nonhydroxy fatty acid-containing ceramide. The 3-O-benzoylated NFA-containing ceramide was resolved on the Chrompak silver column with hexane–isopropanol 9:1 (v/v) at a flow rate of 0.5 ml/min. However, for better resolution of the minor peaks, the same solvent in the ratio of 9.5:0.5 was pumped at a flow rate of 0.5 ml/min.

Argentation HPLC of HFA-containing ceramide was performed without derivitization on the Chrompak silver column with hexane–isopropanol 9:1 (v/v) as the solvent pumped isocratically at a flow rate of 1.5 ml/min.

Reversed-phase HPLC of hydroxy fatty acid and 3-O-benzoylated non-hydroxy fatty acid-containing ceramide was performed either on Nucleosil-5 μm C_{18} column or with a Radialpak A cartridge with methanol pumped at a flow rate of 2 ml/min.

Analysis of fatty acid methyl esters by GLC. The fatty acid methyl esters obtained after methanolysis with anhydrous HCl (15) were analyzed by GLC on either 3% OV-1 (on 80–100 mesh Supelcoport) or 10% SP-2340 (on 100–120 mesh Chromosorb W) as described previously (9, 10).

RESULTS

3-O-Benzoylated sphingomyelin

Sphingomyelin, when benzoylated with benzoic anhydride, consistently formed 3-O-benzoylated sphingomyelin with a 90–95% yield. With radioactive sphingomyelin, the benzoylation reaction formed a single product as determined by TLC and HPLC analysis (16). The reversed-phase HPLC of the 3-O-benzoylated sphingomyelin from bovine brain resulted in the separation of several molecular species, **Fig. 1A.** The fatty acid and sphingoid base composition of 3-O-benzoylated sphingomyelin associated with each peak was similar to that of unbenzoylated sphingomyelin published previously (9) and is not reported here. The separation is based upon the chain length and number of double bonds in the fatty acid and the long chain base of the molecule (9). Sphingomyelin with a monounsaturated fatty acid was not

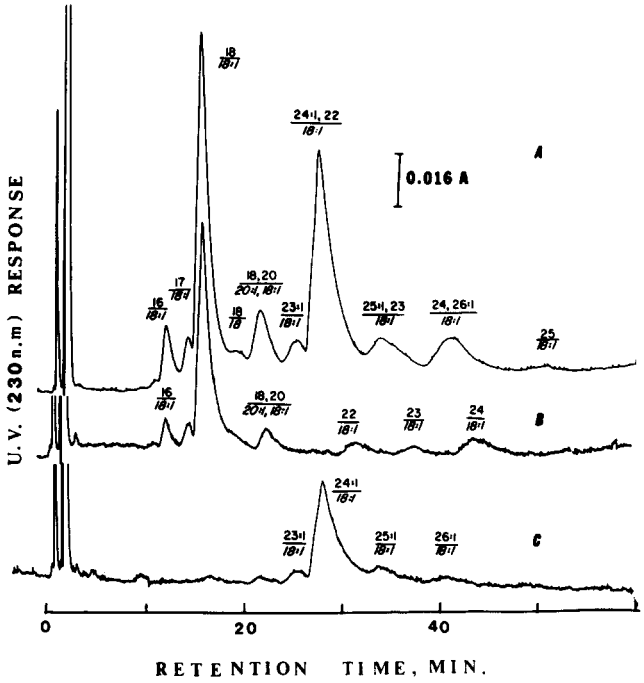

Fig. 1. Reversed-phase HPLC analysis of bovine brain 3-O-benzoylated sphingomyelin on Nucleosil-5-C_{18} column. The solvent was methanol–acetonitrile–phosphate buffer, pH 5.4, 5 mM (100:20:1, by volume) pumped at a flow rate of 2 ml/min. Benzoylated sphingomyelin, 100 μg, (A); benzoylated sphingomyelin in peak 1 (B) and peak 2 (C) collected from the silver column (see Fig. 2). The major fatty acid (upper lettering, "numerator") and the long chain base (lower lettering, "denominator") composition of the sphingomyelin is given near each peak.

completely resolved from sphingomyelin with a saturated fatty acid having 2 less carbon atoms.

The argentation HPLC of the benzoylated sphingomyelin is shown in **Fig. 2.** The benzoylated sphingomyelin was clearly resolved into two separate peaks. These peaks were collected individually and rechromatographed on the reversed phase column

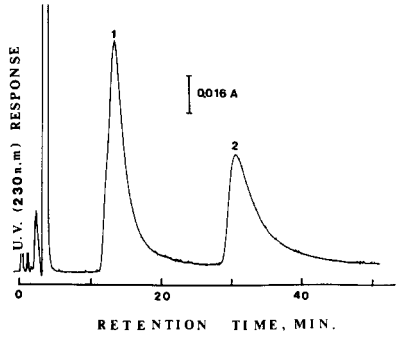

Fig. 2. Argentation HPLC of 3-O-benzoylated sphingomyelin (50 μg) on Chrompak silver column with methanol-2–isopropanol 8:2 (v/v) as the solvent. The flow rate was 1 ml/min. Peaks 1 and 2 were collected and analyzed by reversed phase HPLC (see Fig. 1, B and C) and by GLC (see Fig. 3).

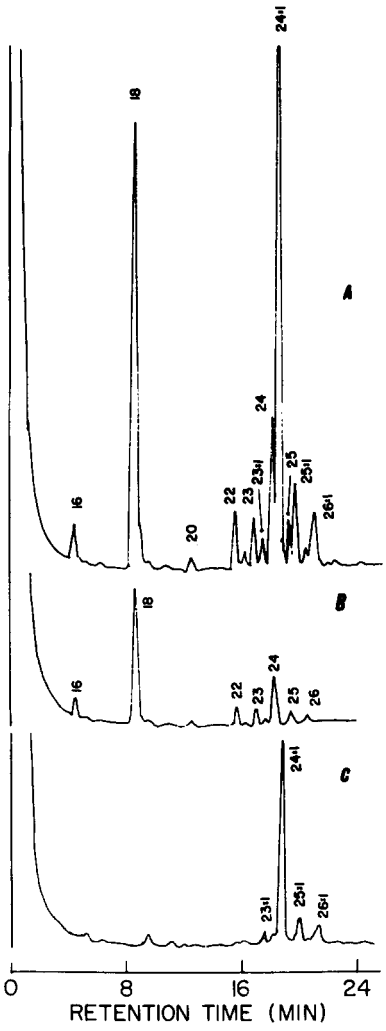

Fig. 3. GLC analysis on SP-2340 column of fatty acid methyl esters of the bovine brain total sphingomyelin (A) and of sphingomyelin in peak 1 (B) and peak 2 (C) collected from the silver column (see Fig. 2).

(Fig. 1B and 1C). GLC analysis of the fatty acids of the total sphingomyelin and of the sphingomyelin collected in peaks 1 and 2 from the silver column is given in **Fig. 3.** The results showed that peak 1 from the silver column was due to 3-O-benzoylated sphingomyelin with only saturated fatty acids, mainly 18:0 with small amounts of 16:0, 22:0, 23:0, and 24:0 (Fig. 3B); whereas, peak 2 contained sphingomyelin with only the monounsaturated fatty acids, mainly 24:1 with small amounts of 23:1, 25:1, and 26:1 (Fig. 3C).

3-O-Benzoylated nonhydroxy fatty acid-containing ceramide

Reversed-phase HPLC of the 3-O-benzoylated NFA-containing ceramide is shown in **Fig. 4A.** The argentation HPLC of the same lipid is given in **Fig.**

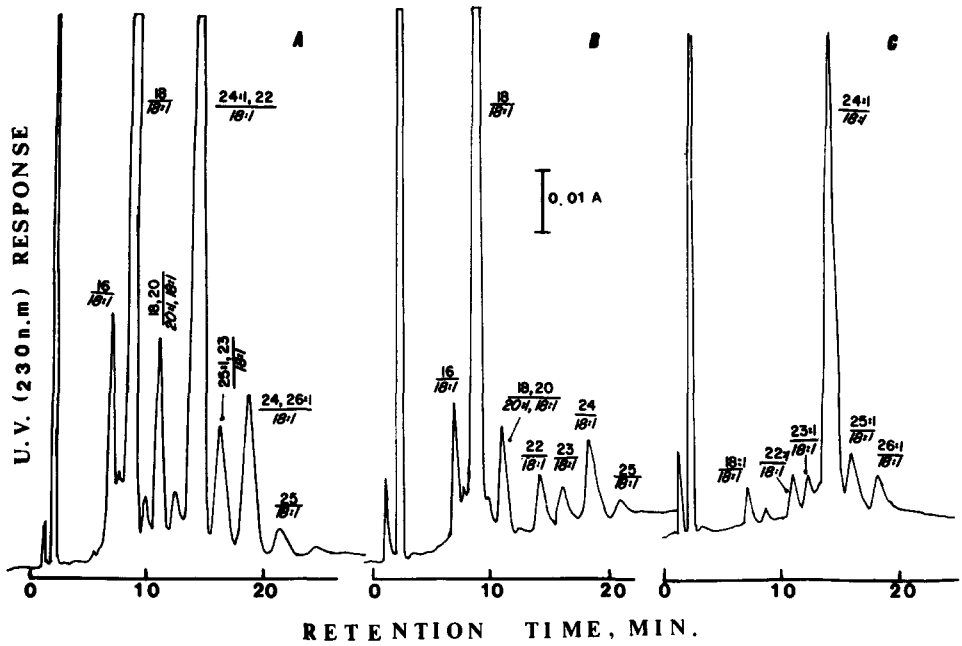

Fig. 4. Reversed-phase HPLC analysis of 3-O-benzoylated NFA-containing ceramide on Nucleosil-5-C_{18} column. The solvent was methanol pumped at a flow rate of 2 ml/min. Total 3-O-benzoylated NFA-containing ceramide, 50 μg (A); benzoylated NFA-containing ceramide in peak 1 (B) and peak 2 (C) collected from the silver column (see Fig. 5A). The major fatty acid (upper lettering) and the long chain base (lower lettering) composition of the NFA-containing ceramide is given near each peak.

5A. The benzoylated NFA-containing ceramide was resolved into four peaks with a shoulder on the front peak. When the same compound was chromatographed with a more nonpolar solvent, the front shoulder was resolved into a separate peak, 1′ in Fig. 5B. A separate peak (2′) also appeared in between the two major peaks, Fig. 5B. However, the time required for the latter resolution was twice as long.

The reversed-phase HPLC of the benzoylated NFA-containing ceramide, which separated as two major peaks (1 and 2) on the silver column (Fig. 5A), is given in Fig. 4B and C. The HPLC (Fig. 4B and C) and GLC (not presented) analyses of the fatty acids of the total ceramide and of the ceramide collected in the peaks from the silver column showed that peak 1 (Fig. 5A) was due to saturated fatty acids while peak 2 was due to monounsaturated fatty acids. The fatty acid and sphingoid analysis of the 3-O-benzoylated ceramides associated with the individual peaks in Fig. 4B and C showed that each peak represented more than 90% of the listed fatty acid and sphingoid base.

The amount of ceramide in peaks 1′,2′,3, and 4 (Fig. 5B) was too small for accurate HPLC and GLC analysis of the fatty acids and long chain bases. However, it is probable that peaks 1′ and 2′ may be due to sphinganine-containing ceramides with saturated and monounsaturated fatty acids, respectively. Peaks 3 and 4 could be due to ceramides with di- and tri-unsaturated fatty acids, respectively. However, accurate assignment of these minor peaks remains to be determined.

HFA-containing ceramide

We have been interested in the preparation of individual molecular species of HFA-containing ceramide

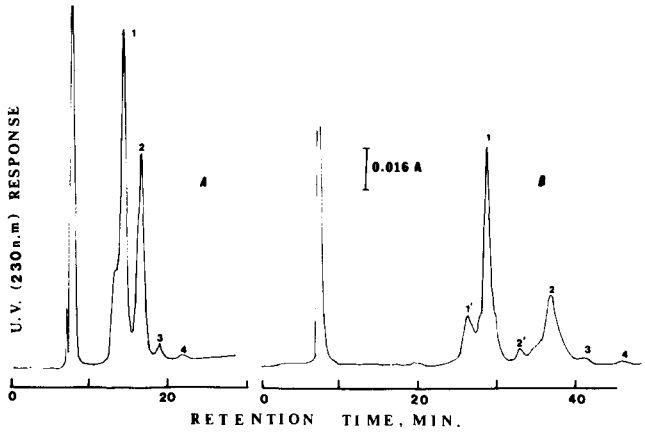

Fig. 5. Argentation HPLC of 3-O-benzoylated NFA-containing ceramide (8 μg) on Chrompak silver column with hexane-2-isopropanol 9:1 (v/v), A or 9.5:0.5 (v/v) B, as the solvent. The flow rate was 0.5 ml/min. Peaks 1 and 2 in A were collected and analyzed by reversed phase HPLC (See Fig. 4, B and C) and by GLC.

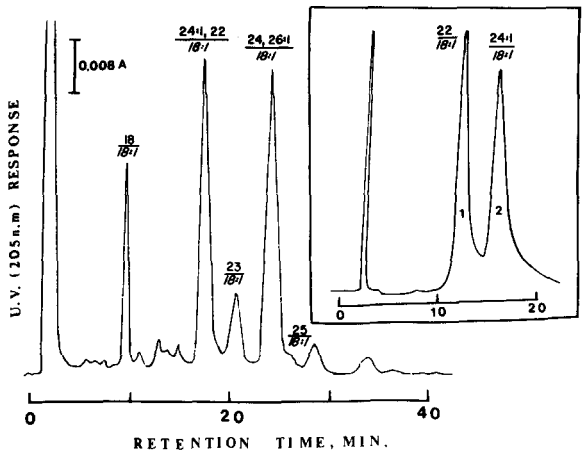

Fig. 6. Reversed-phase HPLC analysis of HFA-containing ceramide (600 μg) on the Waters Radialpak A cartridge. The solvent was methanol pumped at a flow rate of 2 ml/min. Inset: Argentation HPLC of the mixed 24:1, 22:0 HFA-containing ceramide peak collected from the reversed-phase column and rechromatographed on Chrompak silver column. The solvent was hexane–isopropanol 9:1 (v/v) pumped at a flow rate of 1.5 ml/min. Peaks 1 and 2 were collected and analyzed by GLC for fatty acid methyl esters.

for use as the substrate of the enzyme UDP-galactose: HFA-containing ceramide galactosyl transferase, in order to determine its substrate specificity.

The reversed-phase HPLC of the unbenzoylated HFA-containing ceramide is given in **Fig. 6.** The 24:1, 22:0 hydroxy fatty acid-containing ceramide was collected from the HPLC column and reinjected on the silver column. This mixture was resolved into two separate peaks (Fig. 6 inset). The GLC fatty analysis of the two peaks indicated that front peak 1 contained only 22:0 whereas peak 2 contained only 24:1.

DISCUSSION

The biological properties of membranes such as fluidity, enzymic activity, and membrane integrity are controlled by the acyl group composition of lipids in the membrane bilayers (17). There has been special interest in the analysis and preparation of different molecular species of membrane complex lipids. With the advent of reversed-phase HPLC, better separation of molecular species of intact complex lipids has been achieved (9, 10, 18–22) compared to earlier separation techniques (23–25). However, with reversed-phase HPLC some of the critical pairs were not completely resolved (9, 10, 18, 19, 22). With the availability of the commercially prepared silica bonded silver columns, this difficulty can be overcome. Argentation HPLC resolves the lipids based upon the capacity of the Ag^+ ions to complex with the olefinic double bonds in the molecule. Our results show that critical pairs of lipids that were not resolved in the reversed-phase mode were resolved on the silver column. Thus, molecular species separation of complex lipids such as sphingomyelin, NFA- and HFA-containing ceramide was achieved for quantitative analysis and preparative work by the combined use of argentation and reversed-phase HPLC. For quantitative analysis it is necessary to derivatize the lipid; however for preparative work no derivatization is necessary since the lipid elution can be monitored at 205 nm (9). The benzoylated derivatives are stable for months provided they are not exposed to alkali. The Ag^+ in the column is complexed with sulfonic acid which is bonded to 10 μm silica particles and appears to be quite stable. We have continuously used the column for almost 6 months. Occasional loss of resolution could be restored by washing the column at 55°C with polar solvents, such as methanol, to remove impurities.

Sphingomylein and NFA- and HFA-containing ceramides usually have either saturated or *cis*-monoene-containing fatty acids and long chain base with mostly *trans*-monoene double bond (sphingenine) or small amounts of saturated long chain base (sphinganine). Argentation HPLC has the capacity to separate *cis* and *trans*, as well as other geometric positional isomers besides lipids, with different degrees of unsaturation under proper chromatographic conditions. A hint to such separation is given by the chromatogram of 3-O-benzoylated-NFA-containing ceramide (Fig. 5). Combined argentation–reversed phase HPLC has been applied in our laboratory to separate molecular species of phosphatidylcholine and other phospholipids from natural sources that contain more than one or two double bonds.

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