Quantitative analysis of brain galactosylceramides by high performance liquid chromatography of their perbenzoyl derivatives

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Abstract A high performance liquid chromatographic (HPLC) method for analysis of galactosylceramides as their benzoyl derivatives has been devised. Samples containing 10–150 nmoles of monohexosylceramides are benzoylated by heating for 60 min at 60°C in 0.5 ml of 10% (v/v) benzoyl chloride in pyridine. The products are purified by solvent distribution and analyzed by HPLC. The benzoylated cerebrosides with nonhydroxy fatty acids are separated from those with hydroxy fatty acids on a Zipax column with 7% ethyl acetate in hexane as a solvent and UV absorption at 280 nm is recorded. This isocratic procedure can be applied directly to chloroform–methanol extracts of adult brain with a relative standard deviation of 3.0% for cerebrosides with nonhydroxy fatty acids and 4.0% for cerebrosides with hydroxy fatty acids. Sulfatides do not interfere in the assay and can be converted to cerebrosides after desulfation by mild acid methanolysis. Benzoylated glucosyl- and galactosylceramides can be separated on a MicroPak NH_2 column with 1.5% 2-propanol in cyclopentane as the chromatographic solvent.

Supplementary key words sulfatides · nonhydroxy fatty acid cerebrosides · hydroxy fatty acid cerebrosides · glucosylceramide · galactosylceramide

Monohexosylceramides (cerebrosides) have the general structure 1-*O*-*β*-hexosyl-*N*-acyl sphingosine and are widely distributed in nature. They are found with differing fatty acids, hexoses, and long chain bases (1). Galactosylceramides in brain are major components of the myelin sheath and characteristically contain 2-hydroxy fatty acids (HFA) as well as nonhydroxy fatty acids (NFA) (2). These compounds accumulate actively during myelination and continue to increase at a slower rate to adulthood (3, 4). Galactosylceramides are precursors of sulfatides, which are also present in high concentration in myelin. Glucosylceramides are major components of premyelinated brain and are probably synthesized in neurons (5). Glucosylceramides are the predominant monoglycosylceramides of extraneural organs.

Cerebrosides have generally been measured after solvent extraction, base hydrolysis, and/or column chromatographic purification followed by TLC separation and subsequent measurement of hexose by colorimetric or GLC methods (6). Sulfatides have been similarly measured by purification and hexose analysis or by mild acid hydrolysis and sulfate analysis (7). A method based on the formation of a dye complex is also available for the measurement of total sulfatide (8).

The analysis of cerebrosides by high performance liquid chromatography (HPLC) becomes a practical and convenient procedure if derivatives are prepared that allow the utilization of a sensitive ultraviolet detector. Previous studies on the qualitative HPLC analysis of neutral glycosphingolipids (9), the structure of benzoylated cerebrosides (10), the quantitative HPLC analysis of ceramides (11) and the analysis of sphingolipids as their 3-keto sphingosine derivatives (12) have been reported. This report describes procedures for the separation and measurement of NFA and HFA galactosylceramides by isocratic HPLC of their benzoates. Sulfatides can be similarly measured as cerebrosides after desulfation in acidic methanol. We have also shown that benzoylated NFA glucosyl- and galactosylceramides can be separated on a MicroPak NH_2 column.

MATERIALS AND METHODS

Standards and reagents

Calf brain cerebrosides were kindly supplied by Dr. Y. Kishimoto; ^{14}C-labeled cerebrosides were

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

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prepared by Dr. Lyle Hayes by injection of [U-^{14}C]serine into rat brains and purification of the brain cerebrosides to constant specific activity by repetitive preparative TLC. [^{35}S]Sulfatide was obtained from Dr. J. Dulaney and synthetic *N*-stearoyldihydroglucocerebroside was obtained from Miles Laboratories, Inc., Kankakee, Ill. NFA-cerebrosides and HFA-cerebrosides were prepared from a human brain cerebroside fraction by benzoylation with benzoic anhydride (10), and HPLC separation by repeated injection on a 2 mm ID × 25 cm Zorbax column (E. I. duPont de Nemours and Co., Wilmington, Del.) eluted with 1% acetonitrile in methylene chloride as the mobile phase. The resulting fractions were debenzoylated (1) and the cerebroside concentration was measured by the orcinol assay (13). NFA-cerebroside and HFA-cerebroside standards were also purchased from Supleco, Bellefonte, Pa. All solvents and reagents were reagent grade and used without further purifications.

Apparatus

An HPLC apparatus, constructed from a Waters Associates (Milford, Mass) Model 6000 pump and a Laboratory Data Control (LDC, Rivera Beach, Fla.) Model 1285 UV monitor operated at 280 nm coupled to a strip chart recorder was employed for the standard isocratic procedure. This detector was also operated at 254 nm as indicated. We have also employed the Varian Model 4000 constant pressure gas driven reservoir pump (Varian Associates, Palo Alto, Cal.) in conjunction with the Zipax column. Ultraviolet spectra were recorded with a Unicam SP 1800 spectrometer (Pye Unicam Ltd., Cambridge, England). A 2.1 mm ID × 50 cm stainless steel tube dry packed with Zipax, a controlled surface porosity support, average particle size 27 μm (Instrument Products Division, E.I. DuPont de Nemours & Co., Inc., Wilmington, Del.), was used for the standard isocratic procedure. The surface of Zipax is made of layers of microspheres of silica that are sintered to produce a mechanically stable porous surface of known dimensions. The benzoyl derivatives of glucosyl and galactosylceramides were separated on a prepacked MicroPak NH_2-10 column, 2.0 mm ID × 25 cm (Varian Associates). MicroPak NH_2 is microparticulate silica gel with a polar bonded phase consisting of 3-aminopropyl silane groups.

Standard isocratic procedure

Samples containing 10–150 nmoles of cerebroside are dried over P_2O_5 in 13 × 100 mm Teflon-lined screw cap test tubes and benzoylated with 0.5 ml of 10% (v/v) benzoyl chloride in dry pyridine for 60 min at 60°C. The reaction mixture is dried at 50°C under a stream of nitrogen and mixed with 3.0 ml of 95% alkaline–methanol (95 vol of methanol and 5 vol of 1% aqueous Na_2CO_3). This solution is extracted with 5.0 ml of hexane, the lower phase is discarded and the hexane is washed twice more with the 95% alkaline–methanol and then with 95% methanol. The hexane phase is dried under a stream of nitrogen and the residue is dissolved in 100 μl of carbon tetrachloride. One to 20 μl of the sample is injected onto the HPLC column with an appropriate sensitivity setting on the UV monitor. As an elution solvent, we have routinely used 7% aqueous ethyl acetate in hexane. Separation of the NFA-cerebroside peak from impurities eluting at the solvent front can be improved by elution with a solvent containing less ethyl acetate, but analysis time is increased and sensitivity is decreased. Aqueous ethyl acetate is prepared by mixing dry ethyl acetate and water-saturated ethyl acetate in a ratio of 5:1 by volume. This hexane–ethyl acetate solvent provides satisfactory resolution and reproducible adsorbant activity day to day. Measurement of cerebrosides is performed by comparison of the peak area response of the sample to that of cerebroside standards that have been benzoylated, extracted, and injected in a similar manner. Peak areas were measured by the cut and weigh method.

RESULTS

Chromatography

Chromatograms obtained from the analysis of standard cerebrosides and tissue samples with the ethyl acetate–hexane solvent system are shown in **Fig. 1**. In the early stages of this work (10) we employed 0.13% methanol in pentane as an eluting solvent. Although this solvent has the advantage of being transparent to UV light at 230 nm and 254 nm, it was found difficult to reproduce adsorbant activity from day to day and it was necessary to vary the methanol concentration in order to obtain satisfactory chromatography. The perbenzoylated NFA-cerebrosides, which are diacylamines (10), elute first and are completely separated from the HFA-cerebroside derivatives. The results obtained from the direct benzoylation of lyophilized myelin and a chloroform–methanol extract of brain illustrate the application of the method to crude samples (Fig. 1). Better separation from material at solvent front can be obtained if quantities of sample containing less than 2 μg of cerebroside are injected. The method is

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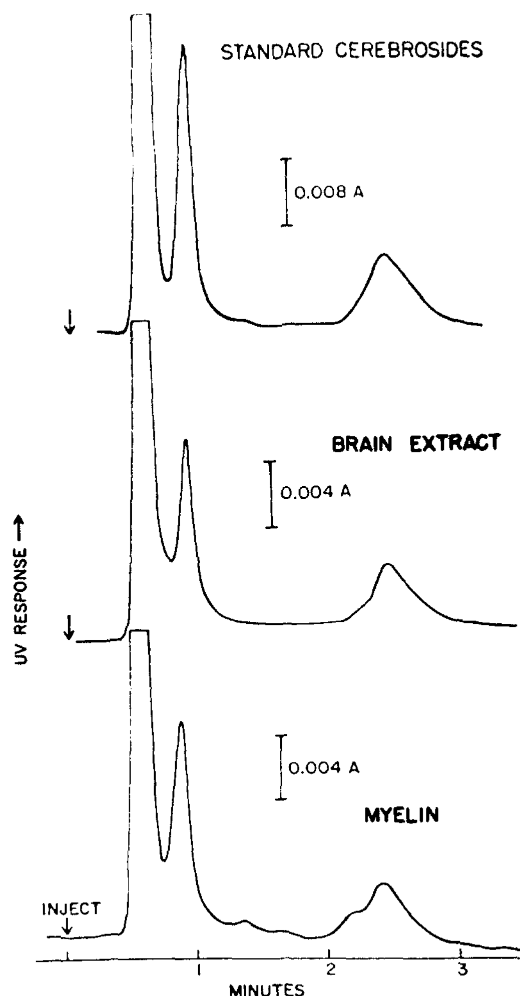

Fig. 1. Standard isocratic HPLC analysis of cerebrosides. Standard cerebrosides: 97.6 μg of calf brain cerebrosides benzoylated and 10% injected. Brain extract: a chloroform–methanol extract of mouse brain equivalent to 5.0 mg wet wt. benzoylated and 10% injected. Myelin: purified lyophilized myelin equivalent to 94.5 μg of myelin protein benzoylated and 5% injected. The peaks resulting from benzoylated NFA-cerebrosides and HFA-cerebrosides have elution times of 0.9 and 2.5 min respectively. The samples were injected onto a 2.1 mm × 50 cm Zipax column and eluted with 7% ethyl acetate in hexane at a flow rate of 1.0 ml per min. UV absorption at 280 nm was recorded.

equally applicable to isolated glycolipid fractions or cerebrosides eluted from TLC plates.

NFA-glucosyl and galactosylceramides are not well resolved with these Zipax chromatographic conditions but can be separated on a MicroPak NH_2 column with 1.2% 2-propanol in cyclopentane as the mobile phase as shown in **Fig. 2**. The NFA-cerebroside peak from the Zipax column can be collected and glucosyl- and galactosylceramides subsequently separated. Direct analysis of benzoylated brain extracts with the MicroPak NH_2 column is not satisfactory and we demonstrated that lactosylceramide is not well resolved from HFA-galactosylceramide under these chromatographic conditions.

Relative response

We have previously reported (10), that benzoylation of NFA-cerebrosides with benzoyl chloride results in amide acylation in addition to normal *O*-acylation, while only *O*-acylation occurs when HFA-cerebrosides are benzoylated. The molar response for the NFA derivative at 254 nm with the LDC monitor was found to be 2.5 times larger than that for the HFA derivative. At 280 nm, on the other hand, the NFA/HFA response ratio was found to be 1.2. In order to substantiate that the difference in response ratio at 254 nm resulted from a difference in their absorption spectra, we chromatographed the benzoylated cerebrosides with the methanol–pentane system, collected the NFA and HFA fractions separately and recorded their UV absorption spectra in the chromatography solvent (**Fig. 3**) with the Pye Unican spectrophotometer. The spectra of the samples showed the expected λ_{max} at 230 nm and gave qualitative verification that the large response ratio at 254 nm measured on the LDC UV monitor was a result of spectral difference of the two derivatives. The shoulder at 205 nm is believed to be an artifact. Both derivatives show similar side band absorption at 280 nm. Response differences at 280 nm noted above are due to some extent to preferential loss of small amounts of the HFA-cerebroside derivative during the solvent distribution. Spectra obtained with samples dissolved in the ethyl acetate–hexane solvent showed similar spectral differences in the region of 254 nm. Thus the routine HPLC analyses were done at 280 nm because the UV responses more accurately reflect relative concentrations and because the ethyl acetate–hexane solvent is more transparent at the higher wavelength and, consequently, the monitor can be operated at higher sensitivity.

Benzoylation

Conditions for the benzoylation of cerebrosides were studied. The time course of the reaction was initially investigated with 20% (v/v) benzoyl chloride in pyridine maintained at 20°C. The amount of HFA-cerebroside product, as measured by HPLC, reached a maximum level in 20 min while the amount of NFA-cerebroside product was still increasing at 130 min. The experiment was repeated at various temperatures. At 60°C the benzoylation of HFA-cerebrosides appeared complete after 15 min and that of NFA-cerebrosides after 30 min. Standard reaction conditions of 60°C for 1 hr were adopted.

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The effect of benzoyl chloride concentration was also studied. Cerebrosides were benzoylated with 5, 10, and 20% benzoyl chloride. The samples benzoylated with 10 or 20% benzoyl chloride gave the same yield of products. With 5% benzoyl chloride the amount of NFA-cerebroside product was decreased by 9.5% while the yield from HFA-cerebroside remained unchanged. We have used 10% benzoyl chloride in pyridine as the standard reaction reagent.

Standard curves

The linear relation between the amount of cerebrosides benzoylated and the recorder response is shown in **Fig. 4**. The response ratio of NFA-glucosylceramide to NFA-galactosylceramide was 1.0, while that of the NFA- to HFA-cerebrosides was 1.2. These response factors are dependent to a small extent on losses during solvent distribution, chromatographic solvent composition, and monitor differences so that standard cerebrosides must be run simultaneously with all samples. We determined the concentration of NFA-cerebroside and HFA-cerebroside in a mixed beef brain cerebroside preparation by comparison with the purified individual NFA and HFA standards and then utilized the mixed preparation as a working standard. The concentrations of the primary standards were established by hexose determinations (orcinol assays) and by reference to the synthetic *N*-stearoyldihydro-glucocerebroside on a weight basis.

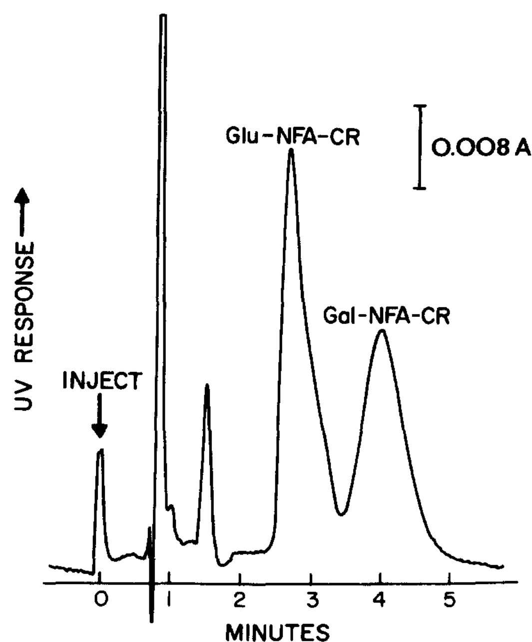

Fig. 2. HPLC separation of glucosyl- and galactosylceramides containing NFA. Approximately 10 μg of each of the benzoylated derivatives was injected onto a 2.0 mm × 25 cm MicroPak NH_2 column that was eluted with 1.2% 2-propanol in cyclopentane at a flow rate of 1.0 ml per min. The peak shown at 1.5 min. is an unidentified random contaminant.

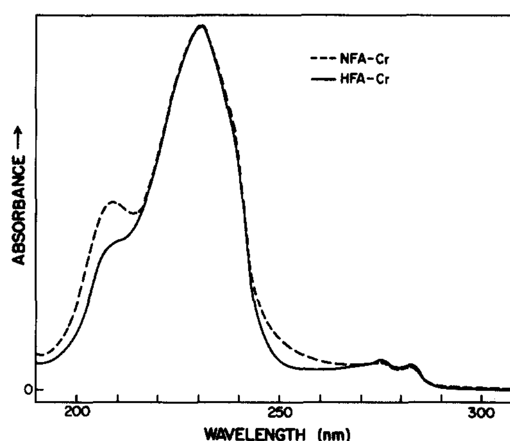

Fig. 3. Ultraviolet absorption spectra of the benzoylated derivatives of NFA and HFA cerebrosides. Equal quantities of the two derivatives (approximately 50 μg) were chromatographed with the methanol–pentane solvent system and peaks collected in equal volumes of solvent and spectra directly recorded. Chromatographic solvent from the column was collected and used as the spectral reference.

Specificity, recovery, and reproducibility

To detect any interference in the measurement of cerebrosides by sulfatides (through desulfation and subsequent benzoylation), 20 nmoles of beef brain sulfatide was benzoylated and processed by the standard isocratic procedure. The amount of cerebrosides detected was 3.4 percent on a molar basis of the sulfatide reacted. This level of interference is low and, since sulfatides are normally present in tissues at a fraction of the level of cerebrosides, this interference can usually be considered insignificant.

The efficacy of the cerebroside benzoylation in the presence of contaminating lipids was tested. Whole mouse brain was extracted with 20 vol of chloroform–methanol 2:1 (v/v), and filtered. An aliquot of the crude extract was removed and the remainder washed according to Folch, Lees, and Sloane Stanley (14). The lower phase was removed, taken to dryness, and subjected to mild alkaline methanolysis according to the method of Vance and Sweeley (15). Equivalent aliquots of the alkali-stable lipids and crude extract were benzoylated and analyzed by HPLC. The amount of benzoylated cerebrosides from the two fractions was the same within the error observed for cerebroside standards, indicating that removal of saponifiable lipids had no effect on the yield of benzoylated cerebrosides.

The recovery of cerebrosides from crude brain

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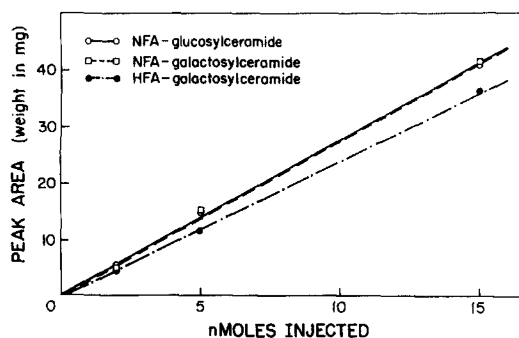

Fig. 4. Relation of UV response at 280 nm (peak wt.) to amount of cerebroside analyzed by the standard assay procedure. Each point represents the result from injection of 10% of the individually benzoylated samples.

extracts was tested. ^{14}C-Labeled galactosylceramides were added to a chloroform–methanol extract of mouse brain and an aliquot processed according to the standard procedure. Seventy percent of the added radioactivity was recovered in the hexane phase. When the hexane phase was examined by HPLC, more than 90% of the radioactivity injected was recovered in the two benzoylated cerebroside peaks.

The hexane phases were also examined by TLC in benzene–ethyl acetate 9:1 (v/v) and all of the detectable radioactivity was associated with the two benzoylated products. When lipid extracts from rat brain, to which had been added ^{14}C-labeled galactosylceramides, were benzoylated and subsequently washed with 80% alkaline methanol (4 vol of methanol and 1 vol of 1% aqueous Na_2CO_3) instead of 95% methanol, the percent recovery of radioactivity in the hexane phase was 96.7% ($\pm 3.9\sigma$). When this hexane phase was examined by TLC, all of the radioactivity was found associated with the two fully benzoylated products and, when examined by HPLC, 94.9% ($\pm 5.1\sigma$) of the radioactivity was recovered in the two benzoylated cerebroside peaks. Thirty-five percent of the radioactivity was associated with the NFA-cerebroside peak and 60% was associated with the HFA-cerebroside peak. These data indicate that the losses resulting in only a 70% recovery in the standard isocratic procedure, which involves washes with 95% methanol, are due to partitioning rather than incomplete benzoylation. Furthermore, since the response ratio of NFA to HFA cerebrosides at 280 nm was found to be 1.1 with the 80% methanol washes and 1.2 with the 95% methanol, any preferential loss of the HFA derivative is small. The washes with 95% methanol are recommended in the standard isocratic procedure because they are more effective in removing excess reagent.

The reproducibility of the standard procedure for the measurement of NFA-cerebroside and HFA-cerebroside in a chloroform–methanol extract from whole mouse brain was determined. Aliquots of extracts equivalent to 5.0 mg of wet tissue were measured into nine tubes, dried under N_2, and analyzed by the standard procedure. The mean NFA-cerebroside concentration was 4.74 nmoles per mg wet weight tissue and the mean HFA-cerebroside concentration was 6.92 nmoles per mg wet weight tissue with relative standard deviations of 3.0% and 4.0%, respectively.

In order to obtain comparison with an independent method, three adult rat brain extracts were analyzed for cerebrosides by the HPLC method and by the TLC–orcinol method of Neskovic et al. (13). The average values (± mean deviation) were 11.8 ± 0.3 and 11.3 ± 2.1 nmoles per mg wet weight tissue for the HPLC and orcinol methods, respectively.

The ratios of NFA-cerebroside to HFA-cerebroside in these samples were also calculated and found to be 0.49, 0.50, and 0.54. Hoshi, Williams, and Kishimoto (16) reported a ratio of 0.64 for adult rat brains as determined by gas–liquid chromatographic analysis of cerebroside fatty acids. The ratios of NFA-cerebrosides to HFA-cerebrosides obtained by HPLC were also directly compared on the same samples to the ratios obtained by the GLC method (16). Extracts of adult rat brain grey matter (cerebral cortex) and white matter (mid-brain) were prepared and aliquots were removed for benzoylation and HPLC analysis. From the remaining portion of the extracts, cerebrosides were isolated and the hydroxy and nonhydroxy fatty acids were analyzed by GLC with the use of internal standards. The NFA/HFA ratios for grey and white matter as measured by GLC were 0.34 and 0.40, respectively, whereas the ratios for grey and white matter obtained by HPLC analysis of the lipid extracts were 0.32 and 0.42, respectively. These data show that the same values are obtained by both methods.

Conversion of sulfatides to cerebrosides

Sulfatides are desulfated by mild acid methanolysis to form cerebrosides (17). Convenient conditions for the quantitative conversion of sulfatides to cerebrosides that can be subsequently assayed by HPLC were sought. [^{35}S]Sulfatide was dissolved in 0.05 N anhydrous methanolic–HCl and incubated at 40°C and at room temperature. At various time intervals aliquots were removed and partitioned according to Folch et al. (14) and radioactivity in the two phases was determined. Seventy percent of the sulfatide was cleaved after 4 hr at room temperature, while sulfate liberation was in excess of 99% after

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80 min at 40°C. To test whether significant cleavage of the glycosidic bond occurred at 40°C after 80 min, calf brain cerebroside was subjected to these hydrolysis conditions and the resulting lower phase lipids benzoylated and examined for benzoyl ceramides (11) by TLC. An aliquot equivalent to 100 μg of cerebrosides was spotted so that if more than 1% hydrolysis occurred benzoylated ceramides would have been detected. There were no detectable ceramides formed from cerebroside under these hydrolysis conditions.

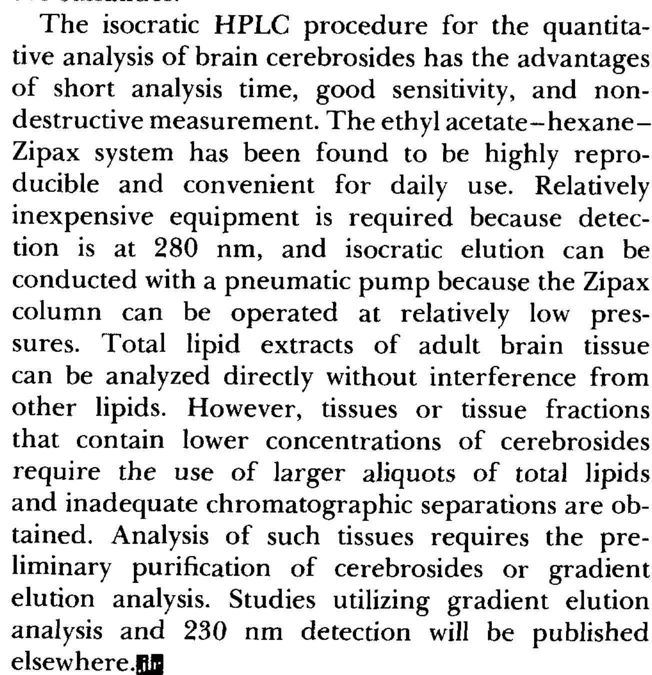
DISCUSSION

Benzoylation of NFA-cerebrosides with benzoyl chloride results in amide acylation in addition to normal *O*-acylation, but HFA-cerebrosides form only *O*-acyl derivatives under these conditions. NFA-cerebrosides cannot be recovered completely because alkaline methanolysis of the perbenzoyl NFA-cerebroside results in the formation of *N*-benzoyl-psychosine as well as the parent cerebroside (10). Benzoylation with benzoic anhydride in pyridine (110°C for 16 hr) forms only *O*-acyl derivatives and cerebrosides can be recovered by alkaline methanolysis but sulfatides are completely converted to benzoylated cerebrosides during the anhydride reaction. Therefore, we have chosen the chloride benzoylation for the analytical procedure because the 1.0 hr reaction time is more convenient and sulfatides do not desulfate and interfere appreciably under these conditions.

The differences in the absorption spectra (Fig. 3) of NFA- and HFA-cerebroside derivatives results in a difference in UV response of greater than two when the LDC monitor is operated at 254 nm. Although the sensitivity of the procedure for the analysis of the NFA-cerebroside is decreased when measurement is made at 280 nm, we prefer to operate at this wavelength where the relative response is close to one. Standard cerebrosides must be run simultaneously to establish UV response for each chromatographic system and validity of analysis of different tissue samples should be verified with recovery studies or internal standards. If a multi-wavelength UV monitor is used at 230 nm the sensitivity is about 14 times higher than at 280 nm but this requires the use of different chromatographic solvents that are transparent at 230 nm.

Several considerations are involved in choosing the proper mobile phase–column combination for the analysis of a sample. When HFA- and NFA-cerebrosides are to be separated and measured, the hexane–ethyl acetate mobile phase with the Zipax column performs the separation adequately and has been found to be more reproducible than the methanol–pentane solvents originally employed (10). If separation is desired between NFA-glucosyl and NFA-galactosylcerebrosides this can be accomplished with the cyclopentane–2-propanol mobile phase on the MicroPak NH_2 column. We have found it convenient to do initial analysis with the hexane–ethyl acetate–Zipax system and to collect the NFA-cerebroside peak for subsequent chromatography on the MicroPak NH_2 column in order to detect any glucosylceramide in the sample.

We have shown that sulfatides do not interfere in the analysis of cerebrosides and that sulfatides can be quantitatively converted to cerebrosides by desulfation in acidic methanol. Although we have not utilized this procedure for tissue sulfatide analysis, it theoretically provides a sensitive method that does not require previous separation from cerebrosides and, in addition, it allows independent measurement of NFA and HFA sulfatide. However, because such values are obtained by difference, the analysis of small quantities of sulfatides in the presence of large amounts of cerebrosides would give rise to considerable error. Sulfatide analysis would best be preceeded by TLC or DEAE-Sephadex isolation of the sulfatides.



The isocratic HPLC procedure for the quantitative analysis of brain cerebrosides has the advantages of short analysis time, good sensitivity, and non-destructive measurement. The ethyl acetate–hexane–Zipax system has been found to be highly reproducible and convenient for daily use. Relatively inexpensive equipment is required because detection is at 280 nm, and isocratic elution can be conducted with a pneumatic pump because the Zipax column can be operated at relatively low pressures. Total lipid extracts of adult brain tissue can be analyzed directly without interference from other lipids. However, tissues or tissue fractions that contain lower concentrations of cerebrosides require the use of larger aliquots of total lipids and inadequate chromatographic separations are obtained. Analysis of such tissues requires the preliminary purification of cerebrosides or gradient elution analysis. Studies utilizing gradient elution analysis and 230 nm detection will be published elsewhere.

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REFERENCES

1. Martensson, E. 1970. Glycosphingolipids of animal tissue. *In* Progress in the Chemistry of Fats and Other Lipids. R. T. Holm, editor. Pergamon Press, New York, N.Y. 365–407.
2. Kishimoto, Y., and N. S. Radin. 1966. Metabolism of brain glycolipid fatty acids. *Lipids.* **1:** 47–61.
3. Kishimoto, Y., and N. S. Radin. 1959. Composition of cerebroside acids as a function of age. *J. Lipid Res.* **1:** 79–82.
4. Kishimoto, Y., W. E. Davies, and N. S. Radin. 1965. Turnover of the fatty acids of rat brain gangliosides, glycerophosphatides, cerebrosides, and sulfatides as a function of age. *J. Lipid Res.* **6:** 532–536.
5. Radin, N. S., A. Brenkart, R. C. Arora, O. Z. Sellinger, and A. L. Flangas. 1972. Glial and neuronal localization of cerebroside metabolizing enzymes. *Brain Res.* **39:** 163–169.
6. Sweeley, C. C., and D. E. Vance. 1967. Gas chromatographic methods for the analysis of carbohydrates in lipids. *In* Lipid Chromatographic Analysis. G. V. Marinetti, editor. Marcel Dekker, Inc. New York, N.Y. 465–495.
7. Kates, M. 1972. Techniques of Lipidology. American Elsevier Publishing Co., New York, N.Y.
8. Kean, E. L. 1968. Rapid, sensitive spectrophotometric method for quantitative determination of sulfatides. *J. Lipid Res.* **9:** 319–327.
9. Evans, J. E., and R. H. McCluer. 1972. High pressure liquid chromatography of neutral glycosphingolipids, *Biochem. Biophys. Acta.* **270:** 565–569.
10. McCluer, R. H., and J. E. Evans. 1973. Preparation and analysis of benzoylated cerebrosides. *J. Lipid Res.* **14:** 611–617.
11. Sugita, M., M. Iwamori, J. E. Evans, R. H. McCluer, J. T. Dulaney, and H. W. Moser. 1974. High performance liquid chromatography of ceramides: application to analysis in human tissues and demonstration of ceramide excess in Farber's disease. *J. Lipid Res.* **15:** 223–226.
12. Iwamori, M., H. W. Moser, R. H. McCluer, and Y. Kishimoto. 1975. 3-Keto-sphingolipids: applicatiion to the determination of sphingolipids which contain 4-sphingenine. *Biochim. Biophys. Acta.* **380:** 308–319.
13. Neskovic, N., L. Sarlieve, J. Nussbaum, D. Kostic, and P. Mandel. 1972. Quantitative thin-layer chromatography of glycolipids in animal tissues. *Clin. Chim. Acta.* **38:** 147–153.
14. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
15. Vance, D. E., and C. C. Sweeley. 1967. Quantitative determination of the neutral glycosylceramides in human blood. *J. Lipid Res.* **8:** 621–630.
16. Hoshi, M., M. Williams, and Y. Kishimoto. 1973. Characterization of brain cerebrosides at early stages of development in the rat. *J. Neurochem.* **21:** 709–712.
17. Stoffyn, P., and A. Stoffyn. 1963. Direct conversion of sulfatides into cerebrosides. *Biochem. Biophys. Acta.* **70:** 107–108.

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