notes on methodology

Quantitative microanalysis of perbenzoylated neutral glycosphingolipids by high-performance liquid chromatography with detection at 230 nm

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Summary A method is described which permits the quantitative microanalysis of glycosphingolipids in the picomole range by HPLC of their perbenzoylated derivatives with detection at 230 nm. A linear gradient of 1 to 20% dioxane in hexane is used to elute from a Zipax column derivatives that contain up to four sugar residues. Residual absorption due to dioxane is negated by directing the solvent gradient through a pre-column, pre-injector high-pressure reference cell. This path generates a horizontal baseline with a negative and a positive deflection at the beginning and end of the gradient, respectively. Neutral glycosphingolipids can be quantitated either with *N*-acetylpsychosine as internal standard or by comparison of peak areas to those of external standards.

Supplementary key words cerebrosides · glucosylceramide · lactosylceramide · globotriaosylceramide · globotetraosylceramide · globoside

Recently we reported a procedure for the quantitative HPLC analysis of perbenzoylated glycosphingolipids (GSL) (1). That method utilized gradient elution with ethyl acetate in hexane and detection at 280 nm, which is above the ultraviolet (UV) cutoff of ethyl acetate. The method is theoretically 14 times more sensitive if the derivatives are detected at their absorption maximum of 230 nm, but the lack of suitably transparent solvents has to this time prevented use of that wavelength. Dioxane has a UV cutoff below 230 nm and an eluotropic value similar to ethyl acetate, but residual UV absorption by dioxane at 230 nm creates a severe off-scale baseline drift during gradient elution. This problem has now been circumvented by the use of a detector with high-pressure cells (1500 psi) which are joined to the HPLC system so that the eluting solvent passes through the reference cell before entering the injector and column. The perbenzoylated derivatives can be quantitated with *N*-acetylpsychosine as an internal standard or by comparison of peak areas to the response of external standards.

Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NFA, nonhydroxy fatty acid; HFA, hydroxy fatty acid; GlcCer, Glc(β1 → 1)Cer; GalCer, Gal (β1 → 1)Cer; LacCer, Gal(β1 → 4)Glc(β1 → 1)Cer; globotriaosylceramide, $GbOse_3Cer$, Gal(α1 → 4)Gal(β1 → 4)Glc(β1 → 1)Cer; globotetraosylceramide, $GbOse_4Cer$, GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)Glc(β1 → 1)-Cer; AUFS, absorbance units full scale.

Materials and Methods

UV-grade hexane and dioxane (Burdick and Jackson Laboratories, Muskegon, MI) were used for HPLC. UV-grade solvents and chemicals (Fisher Chemical Co., Fairlawn, NJ) were used for tissue extraction and for the isolation of perbenzoylated glycolipids. GlcCer (Supelco, Bellefonte, PA) and LacCer (Miles Laboratories, Inc., Elkhart, IN) standards were purchased and $GbOse_3Cer$ and $GbOse_4Cer$ were prepared from human erythrocyte ghosts. *N*-Acetylpsychosine was synthesized from psychosine (Supelco) and 10% acetic anhydride in pyridine.

HPLC gradients were generated with reciprocating pumps and a solvent programmer (Waters Assoc., Milford, MA, Models 6000 and 660, respectively). Samples were injected with the aid of a universal liquid chromatograph injector (Waters Assoc. Model U6K) with a 100-μl injection loop. Perbenzoylated glycolipids were detected with a variable wavelength UV detector equipped with high-pressure (1500 psi) stainless steel cells (Schoeffel Instruments Corp., Westwood, NJ). The UV detector was well insulated with styrofoam from changes in ambient temperature. In addition, both the sample and reference cells were equipped with heat exchangers to assure that the temperatures of the two cells were the same. The heat exchanger for the reference cell was a pulse dampener we extricated from one of the high-pressure pumps. It was inserted just before the reference cell and rested on top of the lamp housing. Since the housing was well insulated with styrofoam, the approximately 1.5-ml volume of the dampener allowed more than adequate time for the mixing and temperature equilibration of the solvent. We assume that a much smaller volume could be used, but the pulse dampener was the most readily available high-pressure block. The sample cell heat exchanger consisted of a small-volume (approximately 30 μl) block (Schoeffel SFA 226). This exchanger was located inside the lamp housing. Detector output was directed in series to a single-channel computing integrator (Autolabs System I,

Spectra-Physics, Santa Clara, CA) and a strip chart recorder.

The components of the chromatographic system were arranged so that the solvent from the pumping system passed initially through the heat exchanger, then through the reference cell, and next through the injector to the column, heat exchanger, and sample cell of the detector (**Fig. 1**). Plasma glycolipids were isolated by solvent partition and Unisil chromatography as described by Vance and Sweeley (2) and modified by Ullman and McCluer (1). Liver glycolipids were isolated by the same procedure except that a ratio of 4.5 mg Unisil per mg of tissue wet weight was used.

Samples containing more than 200 ng each of mono-, di-, tri-, and tetra-glycosylceramides and, if desired, an appropriate amount of *N*-acetylpsychosine as an internal standard were benzoylated in 500 μl of 10% benzoyl chloride in pyridine for 16 hr at 37°C as described by Ullman and McCluer (1). For samples containing less than 200 ng each of the four types of glycolipids, 50 μl of 10% benzoyl chloride was used according to Jungalwala, Hayes, and McCluer (3). The products were isolated from excess reagents and reaction side products by solvent distribution (1, 3). The benzoylated glycolipids were dissolved in carbon tetrachloride, injected onto a pellicular silica gel (Zipax, E. I. DuPont de Nemours, Inc., Wilmington, DE part #820975001) column (2.1 mm × 50 cm), and eluted with a 13-min linear gradient of 1–20% dioxane in hexane with a flow rate of 2 ml/min. Absorption at 230 nm was recorded.

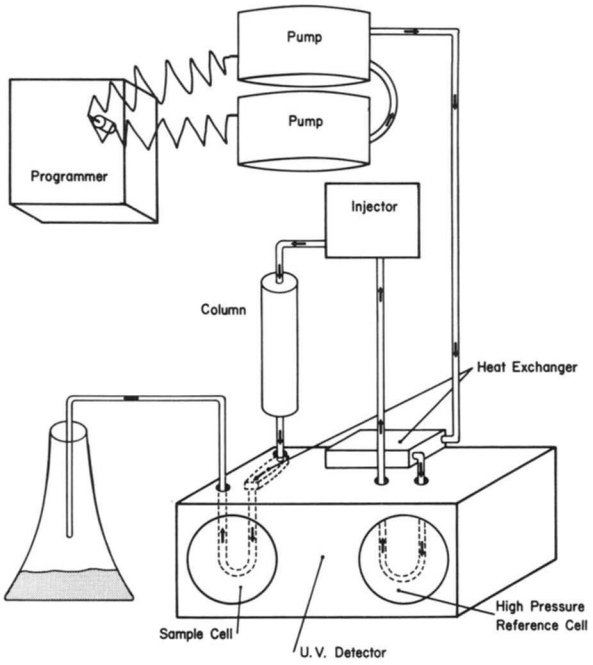

Fig. 1. Diagram of chromatographic system with solvent flow through pre-column reference cell.

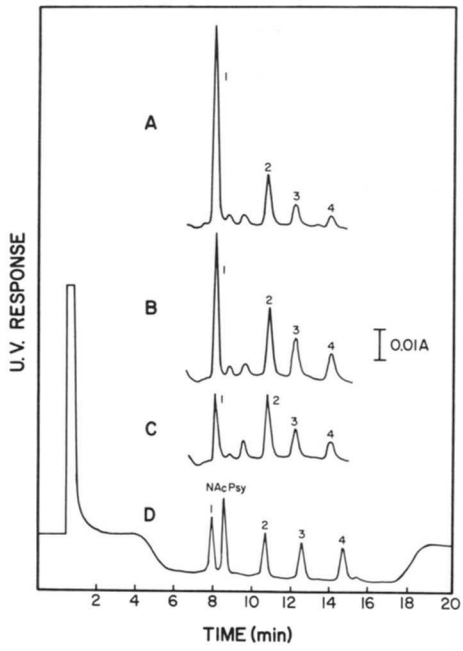

Fig. 2. Chromatograms of perbenzoylated plasma glycosphingolipids and standards. *A*, adult Gaucher's; *B*, juvenile Gaucher's; *C*, normal; *D*, glycolipid standards and *N*-acetylpsychosine with complete chromatogram shown to reveal the negative and positive deflections produced at the beginning and end of the gradients. Peaks are designated as; *1*, glucosylceramide; *2*, lactosylceramide; *3*, globotriaosylceramide; *4*, globotetraosylceramide, NAPsy, *N*-acetylpsychosine.

Results

The baseline generated by directing the solvent gradient through the pre-column reference cell contains a negative and a positive deflection at the beginning and end, respectively (**Fig. 2**). The magnitudes of the deflections are dependent on the volume between the reference and sample cells and on the rate of change in concentration of the absorbing solvent. The quantitative analysis of tissue neutral glycosphingolipids was performed by one of two procedures. Peak areas obtained from perbenzoylated glycolipids were compared to external standard curves as described previously or, for tissues where there were little or no interfering substances, *N*-acetylpsychosine was used as an internal standard. The retention time of the internal standard is slightly greater than that of GlcCer and less than that of LacCer. Chromatograms obtained with mixed standards and samples of glycolipids obtained from normal and Gaucher's disease plasma are shown in Fig. 2. If internal standard is added directly to plasma or tissue samples and processed through the entire isolation procedure rather than added to the isolated glycolipid fractions,

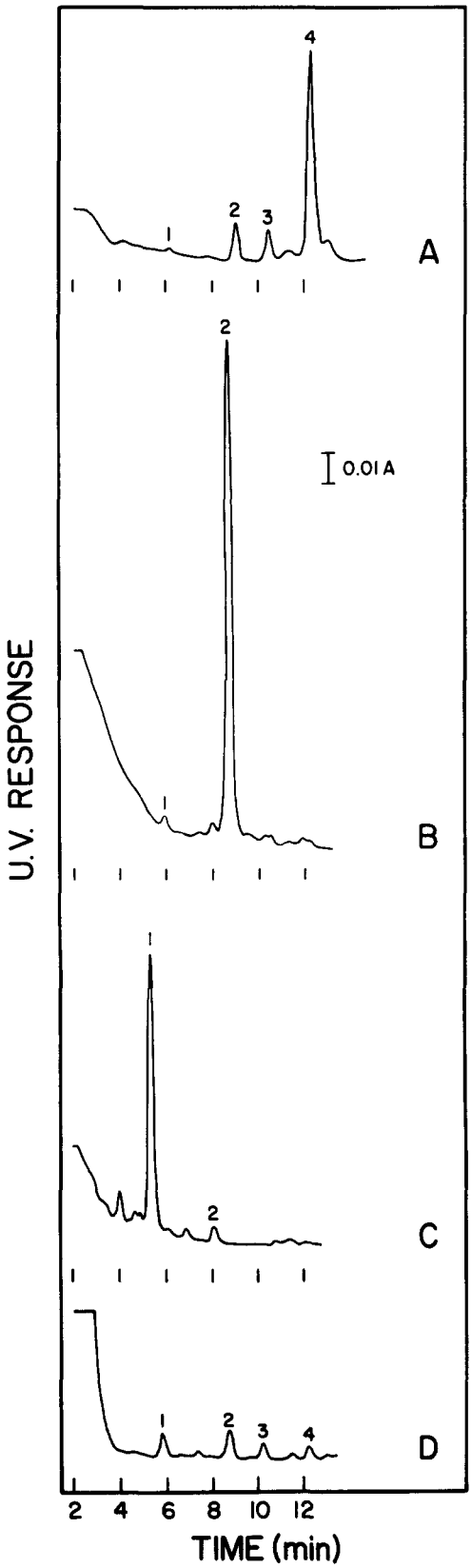

Fig. 3. Chromatograms of neutral glycosphingolipids from various tissues. *A*, human erythrocytes; *B*, human leukocytes; *C*, fetal mouse brain; *D*, human liver. Peaks are designated as in Fig. 3.

losses during the isolation procedure must be taken into account. Recovery of internal standard processed through the glycolipid isolation as compared to directly benzoylated *N*-acetylpsychosine was found to be 80%. We have continued to report glycolipid values that do not reflect losses incurred during the isolation as has been customary (1, 2). The glycolipid values obtained previously with the hexane–ethylacetate and 280 nm detection are the same as those obtained with hexane–dioxane and 230 nm detection, provided identical standards and procedures are utilized. The 230 nm response was shown to be linear for amounts up to at least 1 μg for each neutral glycosphingolipid. The response for glucosylceramide at 230 nm in dioxane was found to be 12 times more sensitive than that at 280 nm in ethyl acetate–hexane. With these chromatographic conditions the lower limit of detection (approximately twice baseline noise) varies from about 2 pmol for GlcCer to about 20 pmol for $GbOse_4Cer$. The difference in sensitivity results primarily from variation in peak geometry. Greater sensitivity for $GbOse_4Cer$ can be obtained if its elution time is decreased by the use of increased concentrations of dioxane in hexane.

Glycolipids obtained from human erythrocytes, leukocytes, and liver and from fetal mouse brain have also been satisfactorily quantitated (**Fig. 3**). The normal erythrocytes show the expected peaks (1) with $GbOse_4Cer$ as the major component. Glycolipids from liver contain major components characteristic of those from plasma and some additional unidentified minor compounds. Lactosylceramide is seen as the major glycolipid of leukocytes and the higher glycolipids $GbOse_3Cer$ and $GbOse_4Cer$ can be observed in fetal mouse brain samples. All tissues examined contained minor constituents which we have not definitely identified.

Discussion

This report describes chromatographic conditions that allow detection of perbenzoylated glycolipids at 230 nm and provides a 12-fold increase in sensitivity over our original procedure which involved detection at 280 nm (1). By directing a linear gradient of 1–20% dioxane in hexane through a pre-column high-pressure reference cell, the UV absorption by dioxane is negated. The total volume between the reference and samples cells and the change in concentration of the absorbing solvent is minimized. The differential change in concentration of the UV-absorbing solvent in the reference and samples cells is reflected by the presence and size of the negative and positive baseline deflections at the beginning and end of the gradient. Once a linear gradient is established, the baseline is

essentially horizontal since the rate of change of the absorbing solvent in the two cells is approximately the same. We have used this procedure with the injector placed both before and after the reference cell. If it is placed before the reference cell, the expulsion of sample from the injector is conveniently seen as a negative response, with certain samples, but this arrangement also creates some artifacts in the baseline. Therefore, we now use the injector placed after the reference cell. To decrease the volume between the reference cell and sample cell, we substituted a 100-μl injection loop for the standard 2-ml loop.

Our results indicate that this procedure is applicable to a variety of tissues; however, chromatographic components observed from each new source must be subjected to characterization in order to definitively establish the glycolipid nature and structure of the observed peaks. Chromatograms from liver and brain show unidentified peaks. It has been shown that these peaks are not artifacts of the perbenzoylation (1). Studies to characterize these unidentified components are currently in progress in this laboratory. Methods for the quantitation of tissue glycolipids require tissue extraction and chromatography on Unisil (1, 2). The recovery of glycolipids from Unisil is not quantitative and varies from 94% for GlcCer to 71% for $GbOse_4Cer$. We have data showing that this recovery is dependent on the amount of Unisil used in the isolation procedure. The sensitivity limitations of the procedure reported here are presently dependent upon this chromatographic step. Efforts to improve this aspect of the procedure are currently underway. Careful attention to the amount of Unisil and eluting solvent used provides reproducible results. In attempts to exploit the high sensitivity of this procedure for the analysis of smaller quantities of tissue, we have encountered additional problems from contaminants introduced during the isolation of the glycolipid fraction. We have found it advantageous to miniaturize the previously used isolation method and to assure that all glassware is scrupulously clean. High-purity HPLC solvents are utilized for glycolipid isolation steps and control samples, without tissue, are routinely processed through the entire procedure in order to detect the introduction of any contaminants.

The use of a pre-column reference cell can surely be applied to other solvent systems. Although detection at 280 nm (1) is adequate for tissues with abundant glycolipids and is preferred for laboratories without access to a variable wave-length detector, detection at 230 nm is by far superior where high sensitivity is required.

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