Application of "High-Performance" Liquid Chromatography to the Study of Sphingolipidoses

M. David Ullman, Reed E. Pyeritz, Hugh W. Moser, David A. Wenger, and Edwin H. Kolodny

Quantitative high-performance liquid chromatographic analysis of perbenzoylated sphingolipids has been used to study the correlations of body chemistry to clinical phenomena. Plasma sphingolipids were isolated from 32 Gaucher (β-glucosidase deficiency) and six Fabry (α-galactosidase deficiency) patients by solvent partition and chromatographic separation on silicic acid columns. Plasma sphingolipids from a patient undergoing plasma-exchange were separated from interfering lipids with reversed-phase columns. Liquid-chromatographic analysis of sphingolipids provides useful supportive information for diagnoses because affected individuals are shown to possess increased circulating concentrations of the pathognomonic sphingolipid. We also used this technique to monitor sphingolipid concentrations in plasma and urine sediment during plasma exchange of a patient with Fabry's disease. Regular plasma exchanges produced and maintained decreased concentrations of sphingolipids in plasma, but near pre-exchange concentrations were observed within days after the therapy was terminated.

Additional Keyphrases: diseases of enzyme deficiencies - Gaucher's disease - Fabry's disease - sphingolipids - inherited anomalies - analysis of urine sediment

The contribution of "high-performance" liquid chromatography (HPLC) to the understanding of the sphingolipidoses is becoming increasingly important. Our report will demonstrate some potential areas in which the clinical biochemistry laboratory can utilize analytical HPLC of perbenzoylated sphingolipids to obtain clinical information on these diseases.

The sphingolipidoses are caused by lysosomal enzyme deficiencies, and clinical symptoms manifest themselves through the resulting accumulation of unmetabolized sphingolipids. The discovery of lysosomal enzyme deficiencies has led to enzyme assays that can be used for the pre- and postnatal diagnosis of the specific diseases (1). Although the enzyme assays provide reasonably reliable results, the clinician would be greatly assisted by supportive biochemical information on the accumulation and distribution of the disease-specific sphingolipids and their precursors when "borderline" activities are measured. Early quantitative HPLC analysis of sphingolipids could also help the physician decide what enzyme assays should be performed. Further, because detection of an enzyme deficiency does not necessarily predict the clinical course of the disorder, especially when various subtypes (e.g., infantile, juvenile, and adult) exist, biochemical information relative to the probable clinical course of a disorder would be valuable to the clinician and the family counselor in their respective health care roles. Finally, the clinical evaluation of the efficacy of therapeutic attempts in the sphingolipidoses is difficult because clinical symptoms, which can take years to become manifest, may not show an immediate clinical response to therapeutically altered body chemistry. The HPLC analysis of tissue and body fluid sphingolipid fluctuations during therapeutic attempts will allow the correlation of those fluctuations to the short- and long-term clinical response to therapy.

Before the development of HPLC, quantitative analysis of the sphingolipids required preparative thin-layer chromatography and subsequent measurement of hexose by destructive colorimetric or gas-liquid-chromatographic techniques (2, 3). Insufficient sensitivity and laborious work-ups for those procedures, combined with the low concentrations of sphingolipids in most tissues and body fluids, made large-scale processing of specimens impractical; this encumbered research into sphingolipid biochemistry and correlations to clinical symptomatology in sphingolipidoses. The relatively convenient and sensitive quantitative HPLC analysis for perbenzoylated sphingolipids, with ultraviolet detection at 230 nm, will help the clinical biochemistry laboratory apply fundamental sphingolipid biochemistry to diagnosis, prognosis, and therapeutic monitoring of sphingolipidoses.

Materials and Methods

Specimens

Blood specimens from Gaucher's disease [β-glucosidase (EC 3.2.1.21) deficiency] and Fabry's disease [α-galactosidase (EC 3.2.1.22) deficiency] were collected in green-top heparin-containing tubes. Plasma was isolated by centrifuging the specimens at 1000 × g for 10 min.

Plasma and urine sediment obtained during plasma exchanges were from a 29-year-old man with Fabry's disease. Plasma-exchange therapy was initiated in an attempt to lower circulating amounts of galactosylactosylceramide (Gal-Lac-Cer). After collection of baseline data (4) the patient was subjected to four sessions of plasma exchange over the course of 12 months, each session consisting of three 2-L exchanges spaced two days apart. Data from the second plasma-exchange session are presented here to illustrate the ability of HPLC to monitor fluctuations in circulating sphingolipid. The third session was a sham exchange to test the clinical efficacy of the procedure.

Isolation and Derivatization of Neutral Sphingolipids from Plasma and Urine Sediment

Plasma sphingolipids from Gaucher and Fabry patients were isolated as described by Ullman and McCluer (5). Sphingolipids from plasma samples obtained during plasma exchanges were isolated from total lipid extracts with the use of reversed-phase, rapid sample-preparation columns (C18-Sep-Pak; Waters Associates, Milford, MA 01757). The lipid extract from 0.25 mL of plasma was dissolved in 1 mL of methanol/acetone (95/5 by vol) and placed onto a rapid
The culture tube was capped and shaken vigorously with a mechanical shaker for 15 min. The contents of the tube were filtered through a sintered-glass funnel fitted with a disc of No. 1 filter paper and into a 100-mL filter flask. The culture tube was rinsed with 5 mL of chloroform/methanol (1/1), and the rinse was filtered and collected with the previous fraction.

The contents of the filter flask were transferred to another 20 × 150 mm screw-cap culture tube, and the solvent was removed at room temperature under a stream of nitrogen. The filter funnel residue was returned to the original 20 × 150 mm screw-cap culture tube and reextracted as described above, but with chloroform/methanol (2/1). The extract was combined with the first residue and the solvent removed as before.

After the extract was redisolved in 24 mL of chloroform/methanol (2/1), 6 mL of 0.8 g/L KCl was added. The contents were mixed and centrifuged to clarify the biphasic layers. The upper phase was removed and discarded, and the lower phase was washed with 12 mL of methanol/water (1/1), mixed, and centrifuged as before. The upper phase was again withdrawn and discarded. The lower phase was dried at room temperature under a stream of nitrogen and redisolved in 30 mL of chloroform. We analyzed 0.5-mL aliquots of this solution after each had been subjected to mild alkaline hydrolysis (2). The urine-sediment sphingolipids were derivatized by perbenzoylation (5) and quantitated by HPLC.

**HPLC Procedure**

Quantitative HPLC analysis was performed by a slight modification of the method described by Ullman and McCluer (6). Perbenzoylated sphingolipids were dissolved in carbon tetrachloride, injected onto a pellicular silica gel (Zipax; E. I. Dupont de Nemours, Inc., Wilmington, DE 19899) column (2.1 mm × 50 cm), and eluted with a 10-min linear gradient of 2/98 to 17/83 (by vol) dioxane/hexane solution with a flow rate of 3 mL/min. Absorbance at 240 nm was recorded. The variable-wavelength ultraviolet detector (Model 970A; Tracor Instruments, Austin, TX 78767) used for the analyses was equipped with a high-pressure, flow-through reference cell to balance the residual absorbance of dioxane in the gradient (6).

**Results**

The average of two experiments indicated that about 96% of the [1-14C]-N-stearoyl-glucosylophosphoglycerine was recovered from the reversed-phase rapid sample-preparation column. The reproducibility of the analysis was comparable with that reported previously (5).

Patients with adult Gaucher's disease (Figure 1) frequently possess high concentrations of glucocerebrosides (Glc-Cer) in plasma (7). HPLC analysis of plasma Glc-Cer concentrations from 32 adult Gaucher patients, however, ranged from high normal (4.41 μmol/L) to a sixfold increase (32.71 μmol/L) over the range of control values (2.53 to 6.15 μmol/L). Although the range of plasma Glc-Cer concentrations in Gaucher's disease overlapped with control values, the ratio of Glc-Cer to dihexosylceramide (Lac-Cer) was invariably higher (1.81 to 5.78) than control values (≤1.20). Plasmas from three of the adult Gaucher patients were also examined after splenectomy and were shown to have no significant change in their sphingolipid concentrations. The analysis of plasma sphingolipids from Fabry patients demonstrated the anticipated (8) increase in Gal-Lac-Cer in all cases (Table 1).

We also used HPLC to follow alterations of sphingolipid concentrations in plasma and urine sediment during the course of plasma exchanges in a patient with Fabry's disease. The patient's plasma Gal-Lac-Cer exceeded that in controls, but other sphingolipids were in the normal range (Figure 2).
Table 1. Plasma Sphingolipids in Six Patients with Fabry’s Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Glc-Cer μmol/L</th>
<th>Lac-Cer μmol/L</th>
<th>Gal-Lac-Cer μmol/L</th>
<th>Globoside μmol/L</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>4.80</td>
<td>4.30</td>
<td>5.52</td>
<td>1.45</td>
</tr>
<tr>
<td>2</td>
<td>6.01</td>
<td>5.85</td>
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<td>3</td>
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<td>4</td>
<td>5.50</td>
<td>4.00</td>
<td>5.78</td>
<td>1.46</td>
</tr>
<tr>
<td>5</td>
<td>3.08</td>
<td>2.47</td>
<td>3.34</td>
<td>1.36</td>
</tr>
<tr>
<td>6</td>
<td>4.50</td>
<td>4.35</td>
<td>3.31</td>
<td>0.88</td>
</tr>
<tr>
<td>Control (n = 6)*</td>
<td>5.1 ± 1.5</td>
<td>6.4 ± 1.5</td>
<td>1.7 ± 0.3</td>
<td>1.7 ± 0.6</td>
</tr>
</tbody>
</table>

* Values are mean ± SD.

Regular plasma exchange produced and maintained lowered plasma Gal-Lac-Cer concentrations, but near pre-exchange concentrations were observed five days after the therapy was terminated (Figure 3a). HPLC analysis clearly demonstrated that the sham exchange did not affect circulating concentrations of Gal-Lac-Cer (Figure 3b). From 22 to 29 mg of Gal-Lac-Cer was removed in each plasma-exchange session.

HPLC assay of urine-sediment sphingolipids from the Fabry patient revealed a marked increase in Gal-Lac-Cer (100–700 nmol/12 h) over control values (7 nmol/12 h) (Figure 4).

Quantitation of urine-sediment sphingolipids from the first session of plasma exchange showed that urine-sediment Gal-Lac-Cer excretion increased after each exchange, and that the increase approximated the increased urine volume accompanying each plasma exchange.

Discussion

Past procedures for the quantitative analysis of sphingolipids frequently involved a chromatographic step, usually on a silicic acid column (3, 5), to isolate a sphingolipid fraction from a total plasma lipid extract. Substantial amounts of sphingolipids were lost, however, because of their irreversible binding to the silicic acid; moreover, silicic-acid fractionation of sphingolipids from plasma samples that had been obtained during plasma exchange yielded highly variable recoveries, for as yet unknown causes. Substituting a C18 reversed-phase rapid sample-preparation column for the silicic acid column

Fig. 2. Chromatogram of perbenzoylated plasma sphingolipids, illustrating the increase in Fabry disease Gal-Lac-Cer

Fig. 3. Alterations in plasma Gal-Lac-Cer (a) during second session of plasma-exchange therapy and (b) during sham plasma-exchange therapy.
to isolate crude sphingolipids from plasma total lipid extracts eliminated major losses and provided reproducible recoveries. The sphingolipids were essentially not retained by the reversed-phase column, which provided minimal opportunity for the compounds to be irreversibly bound to the packing material. We had selected radiolabeled Glc-Cer for the recovery study because the sphingolipid theoretically had the greatest retention on the column and, therefore, had the greatest opportunity to become irreversibly bound. More extensive recovery studies of all the major plasma sphingolipids will be reported later.

The procedure is probably not acceptable for tissues or body fluids that contain significant quantities of sphingomyelin, because sphingomyelin (a) is collected with the sphingolipid fraction; (b) is stable to alkaline hydrolysis; (c) does not yield only one peak when derivatized by the method used for these studies; and (d) is eluted with other sphingolipids in the chromatographic system. The very low concentration of plasma sphingomyelin permitted the isolation of sphingolipids by reversed-phase columns.

The quantitation of urine-sediment sphingolipids is a relatively simple and reproducible technique, requiring no column fractionation.

The assumption that increased circulating Glc-Cer was in some part responsible for the transport and deposition of accumulated lipid in the sphingolipidoses suggested that valuable clinical information would be provided by quantitative HPLC analysis of plasma sphingolipids. Quantitative analysis revealed that absolute adult Gaucher plasma Glc-Cer concentrations overlapped control values. Because the analytical procedure permitted the quantitation of other circulating sphingolipids, however, we noted that the ratio of Glc-Cer to Lac-Cer produced no overlap between patients and controls. The increased Glc-Cer to Lac-Cer ratio has been found to occur in some forms of Niemann-Pick disease (9), and so does not constitute a unique diagnostic tool; it is useful, however, as a preliminary test for suggested enzyme analysis or confirmatory information. The consistent relative increase of Glc-Cer in the plasma of adult Gaucher patients and the absolute increase of Gal-Lac-Cer in the plasma of Fabry patients suggest that analytical HPLC may become a very useful supplement to established diagnostic procedures for the sphingolipidoses. Whether analytical HPLC of sphingolipids will help identify the heterozygous states is yet to be determined.

The unaltered sphingolipid concentrations after splenectomy in adult Gaucher's disease is information that can help predict the anticipated postoperative clinical course of the disorder. Therefore, analytical HPLC will contribute to more accurate prognosis of Gaucher's disease and other sphingolipidoses when the pathogenic mechanisms of the accumulated sphingolipids are understood.

HPLC has been successfully utilized to monitor the decrease and gradual re-equilibration of plasma sphingolipids during plasma exchanges. Should this or other potential therapeutic modalities that regulate circulating amounts of sphingolipids prove to be of clinical benefit, quantitative HPLC analysis of sphingolipids could become a useful clinical assay to monitor the maintenance of sphingolipid concentrations in the most therapeutically advantageous range.

The convenient analysis of urine-sediment sphingolipids has also been demonstrated. Because urine sediment contains a large constituency of renal epithelial cells in certain disorders, it can be envisaged as an "indirect" noninvasive biopsy of the renal system. Measurement of urine-sediment sphingolipids during the course of chronic therapy could reveal alterations in intracellular content of the pathognomonic lipids. Difficulties in urine-sediment analyses arise from the variation of urine content and volume with daily events. More useful information on intracellular sphingolipid content in the sphingolipidoses will be gained when a quantifiable marker for urine-sediment cellular composition (e.g., renal epithelial cell-specific protein) is found.

The relevance of HPLC to the clinical biochemistry laboratory in the future holds great potential. Recent improvements in the derivitization of sphingolipids (10) may some day make them amenable to automated analysis. A procedure for the preparative HPLC isolation of sphingolipids (11) may lead to development of uniform standards and substrates for clinical biochemistry laboratories that conduct sphingolipidoses-related assays. Our understanding of sphingolipid biochemistry and its correlation to clinical phenomena will determine the extent to which HPLC can be of value in the clinical biochemistry laboratory.

We gratefully acknowledge the expert technical assistance of Mr. Ricky Akins. D.A.W. is supported in part by NIH grants HD 08315, HD 10494, and NS 10698, and by a Research Career Development
Award (NS00108); H.W.M. is supported in part by grants MS-13513 and HD-10981 from the Public Health Service.

References