High Performance Liquid Chromatography for the Detection of Homozygotes and Heterozygotes of Niemann-Pick Disease

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Summary

A method is developed for the detection of homozygotes and heterozygote carriers of Niemann-Pick disease type A by employing a high performance liquid chromatography (HPLC) technique for the measurement of sphingomyelin (SP) as well as for the assay of the enzyme sphingomyelinase (SPase) in skin fibroblasts and amniotic fluid cell cultures. A simple, less than 10-min, HPLC of the lipid extract of fibroblasts (about 2×10^6 cells) and measurement of the ratio of the area of SP peak to that of other phospholipids present in the cell-lipid extract show accumulation of SP in Niemann-Pick type A fibroblasts compared to normal. The SPase assay was performed by HPLC measurement of the substrate SP before and after hydrolysis by the enzyme. By employing this technique, it was shown that cells from patients with Niemann-Pick disease type A have less than 10% of the normal SPase activity, whereas the heterozygote carriers of this disease have about 50% of the normal activity.

Speculation

The HPLC method for the measurement of SP could be used for preliminary screening of a large number of samples suspected of Niemann-Pick disease. The diagnosis could be further confirmed by the actual SPase assay.

Niemann-Pick disease is characterized by SP lipidosis, foam cells in marrow, and hepatosplenomegaly. The accumulation of SP is caused by a deficiency of the enzyme SPase (2). This lysosomal enzyme splits SP into ceramide and phosphorylcholine. Various types of Niemann-Pick disease differ with respect to degree of SP storage in the visceral organs and the nervous system, age of onset and death, and severity of neurologic involvement (6). SPase is markedly reduced in the infantile form, type A. In type B, in which there is no neuronopathic involvement, large amounts of SP storage occurs in the visceral organs and the activity of SPase is about 10% of the normal level. In type C, the total SPase activity has been reported to be within normal limits (3) or about 50% of the normal (7). Some accumulation of SP is found in the visceral organs of type C patients. Callahan *et al.* (3) have detected by isoelectric focusing a specific deficiency of an isoenzyme of SPase in liver and brain from type C patients.

The current method for the assay of SPase requires the use of radiolabeled SP (1, 15). This material, although now commercially available, is expensive. In a recent method, an analog of SP (2-hexadecanoylamine-4-nitrophenylphosphorylcholine) has been employed (7). This material, however, is not a natural substrate for SPase. Other published methods involve quantitative paper chromatography of SP (4) or thin layer chromatographic analysis (TLC) of liberated ceramide and phosphoryl-

choline (8) before and after enzymatic conversion. We have recently described a sensitive method for the separation and measurement of SP by HPLC (9). In this report we describe an application of this method for the assay of SPase, as well as for the measurement of accumulated SP in amniotic fluid cells and cultivated skin fibroblasts for the detection of homozygotes and heterozygotes of Niemann-Pick disease.

MATERIALS AND METHODS

MATERIALS

Bovine sphingomyelin obtained from Supelco, Bellefonte, PA was used as the substrate. The purity of the material was checked by TLC. Acetonitrile and methanol, the solvents used for HPLC, were of ultraviolet spectral grade (Burdick Jackson Labs., Muskegon, MI) and have cut-offs below 200 nm. All other chemicals and reagents were of analytic grade.

CELL CULTURE

Methods for obtaining skin and amniotic fluid samples have been described previously (12, 13). The cells from these materials were cultured according to previously published procedures (13).

ENZYME ASSAY

SPase activity was determined with bovine SP in extracts of cultured skin fibroblasts or amniotic fluid cells as described below. The medium in which the cells were grown was washed away with phosphate-buffered saline, pH 7.4, 10 mM. The cells were treated with 1 ml trypsin, 0.25% in sodium citrate-buffered saline solution, pH 7.8, 10 mM, per dish for 10 min at 37°. The cells were washed with phosphate buffered saline solution and centrifuged at $1000 \times g$ for 10 min. The cells were rewashed as above and the pellet was frozen at -20° .

Approximately $5-15 \times 10^6$ cells were homogenized in 0.5-1 ml 0.1 M KCl, Tris-HCl buffer, 0.05 M, pH 7.0, containing 1.0% Triton X-100 (4). The homogenate was centrifuged for 1 hr at $50,000 \times g$. The supernatant was carefully removed. The incubation mixture contained 0.2 mg SP ($0.266 \mu\text{mol}$), acetate buffer, 0.1 M, pH 5.2, containing 0.25% Triton X-100 and an appropriate amount of the cell supernatant protein in a final volume of 0.2 ml. The incubation mixture before the addition of cell supernatant protein was sonicated in a Bransonic 220 sonic bath for 5 min and incubated with the protein at 37°, with vigorous shaking for 4 hr, unless stated otherwise. After the incubation, 7.5 ml chloroform-methanol 2:1 were added. The blank reaction mixture contained the same amounts of enzyme protein, SP, and buffer, but chloroform-methanol (2:1) was added immediately after the addition of enzyme protein. Each assay, including blanks, was performed either in duplicate

or triplicate. The chloroform-methanol extract was washed with 0.2 vol 0.9% saline and the phases were separated by centrifugation. The lower phase was rewashed three times with a mixture of chloroform-methanol-0.9% (w/v) saline (3:48:47, by volume). The washed extract was then dried under N_2 and the lipids were dissolved in 100 μ l ethanol. A suitable portion of the extract (usually 10–20 μ l) was then injected on a Micropak SI-10 (Varian, Palo Alto, CA) HPLC column, 50 cm $\times$ 2.1 mm (internal diameter), essentially according to our previously published procedure (9). The column was developed with a mixture of acetonitrile-methanol-water (85:21:14, by volume) at a flow rate of 1.5 ml/min. The detection was at 203 nm with a Laboratory Data Control (Riviera Beach, FL) spectromonitor I coupled to a strip-chart recorder.

The amount of SP present in the blanks as well as in the incubated samples was determined by injecting known amounts of SP standard. The peak areas were measured by cutting the paper and weighing or by a Spectra Physics (Santa Clara, CA) computing integrator, Autolab System I. Each sample was injected at least twice and the response was averaged. The quantity of SP before and after the incubation was calculated. The specific activity of the enzyme is reported as nanomoles of SP hydrolyzed per mg protein per hr. The protein was assayed by the procedure of Lowry *et al.* (11).

DETERMINATION OF SPHINGOMYELIN IN LIPID EXTRACTS

The cell ($4\text{--}15 \times 10^6$ cells) pellets were warmed at 60° for 15 min with 5 ml chloroform-methanol (1:1, v/v). The precipitated proteins were centrifuged and reextracted with 2.5 ml chloroform. The combined lipid extracts were washed once with 1.4 ml saline and twice with 1.0 ml theoretic upper phase (5). The extracts were dried and redissolved in 100 μ l ethanol. About 10–20% of the sample was injected onto the HPLC column as described previously. The response due to phosphatidylcholine (PC), phosphatidylethanolamine (PE) + phosphatidylserine (PS), and SP was measured by cutting the area of the corresponding peaks and weighing the paper or by a Spectra-Physics computing integrator Autolab System I. Each sample was injected at least twice and the response averaged.

RESULTS

ASSAY OF SPHINGOMYELINASE

A typical HPLC separation of the lipid extract of the incubation mixture is illustrated in Figure 1. The peak after the solvent front was due to PC which was extracted from the cell supernatants. The peak due to SP after the incubation was smaller than that before the incubation with normal cultured cell extracts. Since equivalent amounts of material were injected, the area of the PC peak remained the same before and after the incubation. This also indicated that PC was not hydrolyzed and that its degree of unsaturation was not altered during the incubation. The SP peak in Figure 1, *a* and *b* represents 53 nmol and 24.1 nmol SP, respectively. The enzyme activity in the precipitates which remained after the extraction of the cells with Triton X-100 was negligible (4). The amount of SP present in the enzyme extracts of cultured cells was negligible as compared to added bovine SP and did not interfere in the assay.

Figure 2 represents the activity of the enzyme measured by varying the protein concentration of fibroblast cell extracts. The activity of the enzyme was linear up to about 350 μ g protein. The activity was linear up to 8 hr with 180 μ g protein (Fig. 3).

Table 1 shows the activity of enzyme measured in the Triton X-100 extracts of skin fibroblasts and amniotic cells from fetuses or patients with Niemann-Pick disease and heterozygous carriers. In a typical experiment the incubation was for 4 hr and the enzyme was extracted from $4\text{--}10 \times 10^6$ cells. The amount of protein used was about 0.1–0.3 mg. The assay was performed in duplicate at two different protein levels. The SPase activity in the enzyme extract of the cells of those with Niemann-Pick

disease, type A was found to be 0–10% of the control values. The control values varied between 108–208 nmol/mg/hr between experiments. However, within the experiment, the variation was less. Such variations in control values of the SPase activity are also reported by others (7). The levels of SPase activity in the fibroblasts of the patient with Niemann-Pick type C was about 55–60% of the control values. Cultured skin fibroblasts obtained from carriers of Niemann-Pick type A had intermediate levels of SPase activity (Table 1, experiment 4). Case 445 in experiment 4 (Table 1) was a possible heterozygous carrier since he was the brother of case 440, a female obligate heterozygote. Case 445 showed about one-half the activity of SPase of the normal control and thus appeared to be a carrier of the gene for Niemann-Pick disease. The skin fibroblasts of the Niemann-Pick type C heterozygotes had SPase activity similar

Fig. 1. The HPLC analysis of lipid extracts of incubation mixtures before (*a*) and after (*b*) incubation. The incubation mixture contained sphingomyelin and Triton X-100 extract of normal cultured fibroblasts. The amount of protein was 250 μ g. Twenty percent of the total sample was injected in each case. Other conditions are described in the text. S.F., solvent front.

Fig. 2. Effect of varying amounts of protein on the hydrolysis of SP. Proteins from cultured skin fibroblasts were extracted and incubated as described in the text.

Fig. 3. Hydrolysis of SP by extracts of cultured skin fibroblasts as a function of time. Each incubation mixture contained 180 μ g protein. Other conditions were the same as described in the text.

Table 1. Specific activity of SPase in enzyme extracts of cultured skin fibroblasts and amniotic fluid cells

Diagnosis	Sphingomyelinase activity, nmol/mg/hr	
	Average	Range
Skin Fibroblasts		
Exp 1		
Normal control (1202) ¹	108 (3) ²	100-118
Niemann-Pick type A (439)	4.8 (2)	0-9.6
Exp 2		
Normal control (1654)	153.5 (2)	144-163
Normal control (1202)	121 (2)	114-128
Niemann-Pick type A (1294)	5.1 (2)	0-10.3
Niemann-Pick type A (609)	4.3 (2)	0-8.6
Exp 3		
Normal control (455)	128 (2)	127-129
Normal control (344)	138 (2)	136-140
Niemann-Pick type C (1001)	75 (2)	72-78
Exp 4		
Normal control (1030)	208 (3)	182-234
Normal control (1654)	180 (3)	166-192
Niemann-Pick obligate type A heterozygote (440)	76 (2)	67-86
Niemann-Pick obligate type A heterozygote (444)	93.5 (2)	85.6-101.4
Niemann-Pick suspected type A heterozygote (445)	101.5 (2)	98.3-104.6
Niemann-Pick heterozygote type C (1560)	187 (2)	177-197
Niemann-Pick heterozygote type C (1561)	175 (2)	173-177
Niemann-Pick heterozygote type C (1562)	177 (2)	172-182
Amniotic fluid cells: Exp 5		
Normal control (1664A)	150 (2)	142-158
Normal control (1664B)	110.5 (2)	110-111
Normal control (1662)	144.6 (2)	137-152.3
Normal control (1658)	168 (1)	
Niemann-Pick type A (1653)	8.1 (3)	2.6-10.9

¹ Represents case number.

² Represents number of assays performed.

to normal control subjects. The cultured fibroblasts from the Niemann-Pick cases, the heterozygotes, and some of the control subjects were also assayed for SPase using radioactive substrate (5). The specific activity measured by this method was within % of the values reported in Table 1.

DETERMINATION OF SPHINGOMYELIN IN LIPID EXTRACTS

A typical HPLC separation of PE, PS, PC, and SP in the lipid extracts of fibroblasts from a normal control subject and that of a patient with Niemann-Pick type A is shown in Figure 4. PE and PS were eluted together in this system. It has been shown previously that the response measured at 203 nm for the phospholipids is primarily due to the double bond present in the molecule and the response varies with the degree of unsaturation (9). Thus, although the amount of PC (measured by lipid phosphorus after TLC separation (10)) was much higher than that of PE (ratio 4:1) in skin fibroblasts, the area of the HPLC peak for PE + PS is about 1.4-2 times larger than that of PC. This is because the amount of unsaturation in PE is greater. This ratio does not alter in the case of Niemann-Pick disease (Table 2).

SP constitutes about 8-10% of the total phospholipids in normal human fibroblasts. The area of the peak due to SP constitutes only about 3% of that of total phospholipids. This suggests that the amount of unsaturation in SP of fibroblasts is lower than that of other phospholipids. The relatively small peak observed for SP of the normal skin fibroblasts could thus be accounted for as due to low content and lower unsaturation. The ratio of area of the peak for sphingomyelin to that of PE + PS in normal skin fibroblasts varied from 0.048-0.058 with an average ratio of 0.054. This ratio was about 2-3 times higher in the case of Niemann-Pick type A fibroblasts (Table 2). This ratio is slightly elevated in the case of Niemann-Pick type C and in other unrelated disease fibroblasts. The average ratio of the area of the peak for SP to that of PC in normal skin fibroblasts was 0.09. However, this ratio was about 2 times higher in the case of Niemann-Pick type A fibroblasts and only slightly higher in the case of Niemann-Pick type C and Niemann-Pick type A and C heterozygotes. This ratio also appeared to be slightly elevated in the case of other unrelated disease fibroblasts. These results suggest an accumulation of SP relative to other phospholipids in the fibroblasts of Niemann-Pick type A as compared to normal fibroblasts and demonstrate that type A cells can be identified with this convenient measure.

DISCUSSION

HPLC has become a useful tool for the rapid and efficient analysis of minute amounts of compounds which are not volatile. The analytic method described here for the detection of homozygotes (including *in utero*) and heterozygote carriers of Niemann-Pick type A is shown to be possible in two different ways. A simple 10- to 12-min HPLC analysis of the lipid extract and measurement of the ratio of the area of the SP peak to that of either the PC or the PE + PS peak shows accumulation of SP in Niemann-Pick type A fibroblasts compared to normal and thus detection of such cases. However, detection of type B, C, or the heterozygotes of Niemann-Pick disease is not possible by this method since the characteristic ratio is not significantly different from control subjects and other possible diseases. It has been reported that the percentage of SP is generally increased in the brains of patients with Tay-Sach's disease, GM₁-gangliosidosis, metachromatic leukodystrophy, and phenylketonuria, besides large increases found in the infantile and juvenile forms of Niemann-Pick disease (14). It is possible that the slightly elevated levels of the SP/PE and SP/PC ratios found in the fibroblasts of the patients with chondrodystrophy and the metachromatic leukodystrophy heterozygote could be due to the tendency of elevation of SP in tissues, which is also reflected in the fibroblasts in culture. The results obtained on the analysis of SP and other phospholipids of fibroblasts of Niemann-Pick type A are in agreement with those of Uhlendorf *et al.* (16). They reported on accumulation of SP 1.5-2 times the normal in the lipid extracts of skin and amniotic cell cultures of Niemann-Pick type A, analyzed by TLC and phosphorus determination. The direct HPLC method for the measurement of SP could be used for preliminary screening of large number of samples. The

Fig. 4. The HPLC analysis of lipid extracts of human skin fibroblasts from (a) normal control and (b) Niemann-Pick type A. The chromatographic solvent was acetonitrile-methanol-H₂O (75:21:14, by volume). Other conditions for HPLC analysis are described in the text. S.F., solvent front. The lower chromatographic pattern in the figure was recorded at 10 times less sensitivity than the upper one.

Table 2. Analysis of SP, PC, and PE + PS in lipid extracts of cultured skin fibroblasts by HPLC

Case no.	Diagnosis	Ratio, ¹ × 100		
		(PE + PS)/PC	SP/(PE + PS)	SP/PC
1202	Normal control	151.5	5.7	8.65
1650	Normal control	159.7	5.4	8.58
1654	Normal control	222.0	4.82	10.7
1028	Normal control	140.0	5.78	8.05
541	Metachromatic leucodystrophy, heterozygote	152	6.91	10.5
456	Chondrodystrophy	137	7.45	10.3
1731	Hypophosphatasia	206	3.92	8.07
439	Niemann-Pick type A	150.5	14.5	20.85
609	Niemann-Pick type A	151.8	14.7	18.4
1653	Niemann-Pick type A	145	9.0	13.1
1294	Niemann-Pick type A or B?	142	6.8	9.42
1001	Niemann-Pick type C	142	7.6	10.8
440	Niemann-Pick heterozygote type A	102	7.2	7.1
444	Niemann-Pick heterozygote type A	160	5.55	8.85
1560	Niemann-Pick heterozygote type C	140	6.3	8.8

¹ Average of two separate determinations.

diagnosis could be further confirmed by the actual SPase assay with Triton X-100 extract of the cells using the HPLC method. It is also possible to detect the carriers of this disease by the SPase assay as performed with the application of HPLC. These diagnoses are possible for type A disease. The detection of type C disease and its heterozygotes is not possible by our method since fibroblasts of patients with type C disease appear to have activity similar to those of type A heterozygotes, whereas type C heterozygotes have normal SPase activity.

The SPase assay is done with bovine SP, which has predominantly C₂₄₌₁ (52%) C₁₈₌₀ (21%) fatty acid. It is not yet known whether the enzyme has preference for particular species of SP and whether there are any differences between normal and deficient cells. Many investigators, however, have reported that

the fatty acid composition of the SP accumulating in various organs of the patients with type A disease is very similar to that of SP in the normal tissue (6). We did observe negligible but consistent, less than 8–10% of the normal, specific activity of SPase in type A disease fibroblasts and amniotic fluid cells, especially at a slightly higher protein concentration. Others have reported much lesser amounts of this activity in type A disease (7). The differences could be due to different substrates used in the method of assay of the enzyme.

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