

Nonchromatographic method for acid sphingomyelinase in WBC lysate using modified Dole solvent

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Purpose: To use a modified fluorometric method to measure white blood cell acid sphingomyelinase activity.

Methods: White blood cell lysates were prepared and used as a source of the enzyme. Two tubes were used for each assay, the second as a blank. In each, N-(NBD-Aminolauroyl)sphingomyelin dissolved in chloroform-methanol (2:1) was added and the organic solvent was removed by nitrogen gas. Acetate buffer, 1% Triton™ X100 solution, and sonicated protein (the reaction tube only) were added to the residue. The mixture in each was then incubated at 37°C for 2 hours, which was followed by the addition of buffer, Dole solvent, heptane, and sodium chloride solution. The sonicated protein was added to the blank tube and NBD-ceramide was extracted by vortex for 5 minutes and brief centrifugation at 3000 rpm. The intensity of fluorescence of the upper phase was determined in a fluorometer at an excitation wavelength of 465 nm and emission wavelength at 530 nm.

Results: In 20 normal patients, the range of enzyme activity was 305–1008 pmol with a mean of 570 pmol. In a proven case of Niemann–Pick disease type A by molecular gene analysis, enzyme activity was undetectable.

Conclusion: The modified method is convenient in a biomedical genetics laboratory where a request for sphingomyelinase is very infrequent.

Keywords: acid sphingomyelinase, Niemann–Pick disease, fluorescent analysis, white blood cells

Introduction

Sphingomyelinases are a group of enzymes that hydrolyze sphingomyelin into phosphorylcholine and biologically-active ceramide and are intimately involved in sphingomyelin-ceramide signaling pathway.¹ At least five different sphingomyelinases have been described that differ in intracellular localization, pH optima, and divalent cation requirement.^{2–6} The lysosomal acidic sphingomyelinase (EC 3.1.4.12) is deficient in types A and B Niemann–Pick disease and therefore the diagnosis of this lipid-storage disorder is confirmed by measuring the activity of this enzyme in cell lysates obtained from patients.^{7,8}

Methods used for sphingomyelinase determination include radioactivity⁹ and colorimetry,¹⁰ but more recent fluorometric methods – by virtue of their sensitivity, high precision, and ease of performance – have gained popularity over others. Since both the substrate and the product of the reaction are fluorescent, they are separated by solvent extraction,¹¹ thin-layer chromatography,¹² or high-performance liquid chromatography.¹³ The unavailability of the pure and commercial resource

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of 11-(9-anthroyloxy) undecanoic acid derivative of sphingomyelin as a substrate, described in detail by Gatt et al,¹¹ prompted the author to use other fluorescent derivatives, namely N-(NBD-Aminolauroyl)sphingomyelin as a substrate and N-(NBD-ceramide) as a product, both available at >98% purity. These two compounds were obtained from Sigma-Aldrich Corporation (St Louis, MO).

Methods

Preliminary experiments showed that NBD-ceramide is not extracted from the acidic buffer solution by Dole solvent (isopropyl alcohol/heptane/sulfuric acid, 0.5 mol/L; 40/10/1 by volume). Increasing the ionic strength of the buffer with sodium chloride enabled perfect separation of the substrate and product. Using equimolar amounts of both resulted in <1% of the substrate displaced in the organic phase, while more than 95% of the product recovered in that phase.

White blood cell lysates were prepared according to Alexander et al¹⁴ and used as a source of the enzyme. Two tubes were used for each assay, the second as a blank. In each, 2500 pmol N-(NBD-Aminolauroyl)sphingomyelin dissolved in chloroform-methanol (2:1) was added and the organic solvent was removed by nitrogen gas. To the residue, 100 μ L acetate buffer (250 mM sodium acetate and 1 mM ethylenediaminetetraacetic acid; pH 5.0), 25 μ L 1% Triton[™] X-100 solution, and 50 μ g sonicated protein (the reaction tube only) were added, and made up to 200 μ L with acetate buffer. The mixture in each was then incubated at 37°C for 2 hours, which was followed by the addition of 300 μ L buffer, 2.5 mL Dole solvent, 2 mL heptane, and 1.5 mL sodium chloride solution (100 mg/mL). The sonicated protein was added to the blank tube and NBD-ceramide was extracted by vortex for 5 minutes and brief centrifugation at 3000 rpm. The intensity of fluorescence of the upper phase (2.5 mL) was determined in a fluorometer at an excitation wavelength of 465 nm and emission wavelength at 530 nm.

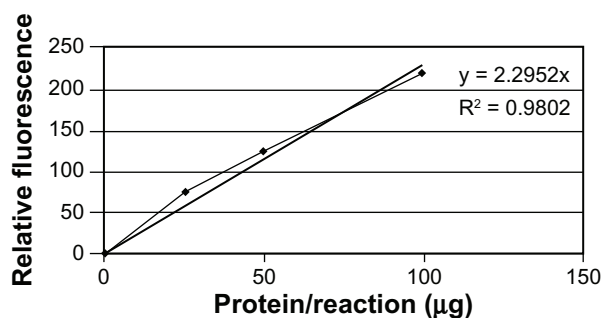


Figure 1 Effect of protein concentration on white blood cell enzyme activity.

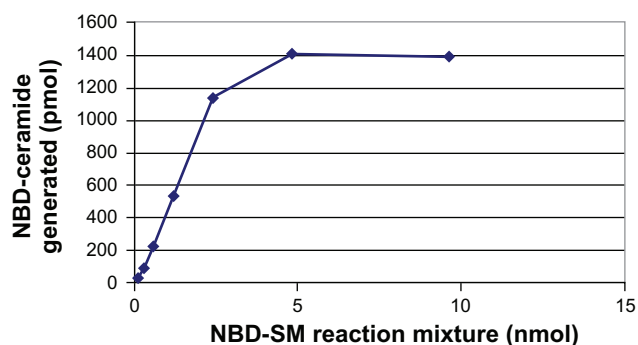


Figure 2 Effect of substrate concentration on white blood cell enzyme activity. Abbreviation: NBD-SM, N-(NBD-Aminolauroyl)sphingomyelin.

Results

Enzyme activity was calculated as NBD-ceramide/mg protein/hour (pmol) using a standard curve generated by adding defined amounts of NBD-ceramide to an identical assay mixture. The correlation coefficient (r^2) was 0.9996 and lower limit of detection was 4.06 ± 0.27 pmol.

The effect of protein concentration was studied by increasing the amounts of white blood cell sonicated protein. A linear relationship was obtained with 25–100 μ g protein ($r^2 = 0.9802$; Figure 1).

The effect of substrate concentration was studied by increasing the amounts of N-(NBD-Aminolauroyl)sphingomyelin using 100 μ g sonicated protein. A plot of NBD-ceramide (pmol) generated is shown in Figure 2.

In 20 normal patients, the range of enzyme activity was 305–1008 pmol with a mean of 570 pmol. In a proven case of Niemann–Pick disease type A by molecular gene analysis, enzyme activity was undetectable.

Conclusion

The modified method is convenient in a biomedical genetics laboratory where a request for sphingomyelinase is very infrequent, for example, in the author's laboratory.¹⁵

Although other sphingomyelinases are not involved in metabolic disorders as in the case of sphingomyelinase, it is tempting to speculate that those under right reaction conditions could also be detected and analyzed. This work is under investigation in the author's laboratory.

Disclosure

The author reports no conflicts of interest in this work.

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