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Original Research Article

Profiling gangliosides from milk products and other biological membranes using LC/MS

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ABSTRACT

Gangliosides are an important class of glycosphingolipids involved in numerous biological processes such as neuronal development, host–pathogen interactions and gastrointestinal health. Due to the highly heterogeneous nature and relatively low abundance of gangliosides, characterization of gangliosides in biological membranes is challenging. Existing methods for ganglioside analysis are quite time consuming and require expensive high resolution mass spectrometers. A rapid method combining reversed phase chromatography and mass spectrometry was developed using a triple-quadrupole mass spectrometer operating in multiple reaction monitoring mode. The ganglioside species were separated with a Poroshell 120 EC-C18 column and analysed under the negative ion mode. This method allows a sensitive, specific, and quantitative assay for profiling gangliosides. The method is developed for analysis of gangliosides in the milk fat globule membrane of whole milk and applied to other biological membranes. Application includes the cellular membrane of prostate cancer cells. In summary, the method allows various biological membranes to be screened for over 600 gangliosides from 12 classes (GM1, GM2, GM3, GM4, GD1, GD2, GD3, GD4, GT1, GT2, GT3, and GT4) in less than three hours. In summary, expressed as % of relative amounts: 1.5% GM3, 80.2% GD3, 14.4% GT3, 1.5% GM1, 2.4% GD1 were observed in whole milk; 2.5% GD1, 88.2% GD3, 2.5% GM1, 2.2% GM3, 0.2% GT2, 4.2% GT3 were observed in buttermilk and 10.6% GD1, 55.6% GD3, 1.6% GM1, 12.2% GM3, 19.2% GT3, 0.9% GT4 were observed in colostrum.

1. Introduction

Gangliosides are complex amphiphilic lipids associated with the plasma membrane of mammalian cells and other biological membranes (Levery, 2005; Skaper et al., 1989; Sonnino et al., 2006; Stoffel, 1971). Gangliosides and all glycosphingolipids are composed of a glycan moiety linked to a ceramide portion (Lopez and Schnaar, 2009). The distinguishing characteristic of gangliosides is the presence of one or more sialic acid residues (Khatun et al., 2013) within the glycan chain. Gangliosides are divided into different classes based on the number of sialic acids present: GM (mono-sialylated), GD (di-sialylated) GT (tri-sialylated) (Svennerholm et al., 1972; Rodden et al., 1991). Further classification is based on oligosaccharide chain length. The ceramide consists of N-acylsphingosine in which the acyl residue is linked by an amide bond to a long-chain fatty acid (Skaper et al., 1989; Rueda, 2007).

The amphiphatic nature of milk gangliosides determines the biological function; the hydrophobic ceramide determines how gangliosides are embedded in biological membranes while the glycan moiety modulates various recognition and adhesion processes (Sonnino et al., 2006; Tettamanti, 2004; Khatun et al., 2013). Cow milk contains about 3–5% of fat in droplets surrounded by a milk fat globule membrane which contains bioactive or nutraceutical glycoproteins and glycolipids (Singh, 2006). In milk, for example, gangliosides are linked to the milk fat globule membrane (MFGM) where the ceramide portion is anchored into

Abbreviations: GM, mono-sialylated gangliosides; GD, di-sialylated gangliosides; GT, tri-sialylated gangliosides; MFGM, milk fat globule membrane; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography; T-LBSA, total lipid bound sialic acid; MS, mass spectrometry; CID, collision induced dissociation; LC, liquid chromatography; SPE, solid phase extraction; MRM, multiple reaction monitoring; ANSA, 8-anilino-1-naphthalene-sulfonic acid; ESI, electrospray ionization; FA, fatty acid; FBS, fetal bovine serum; PBS, phosphate buffered saline; HTST, high temperature, short time; LTLT, low temperature, long time; S, saturated ceramide; M, monounsaturated ceramide; P, polyunsaturated ceramide; CE, collision energy.

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the membrane and the glycan moiety is interacting with the external environment (Keenan, 1974; Keenan et al., 1972). Dietary milk gangliosides may promote intestinal immunity development in the neonate, growth of beneficial microflora such as *Bifidobacteria* and suppress growth of pathogens such as *Escherichia coli* (Rueda, 2007; Rueda et al., 1998a, 1998b; Gil and Rueda, 2002). Gangliosides have been recognized as regulators in signaling pathways (Buccoliero and Futerman, 2003) immunomodulators (Potapenko et al., 2007) and modulators of ion channels (Ledeen and Wu, 2002). A Canadian study determined ganglioside intake in a healthy human population that consumed egg, tuna, beef, milk and other milk products (yogurt and cheese). Minimal amounts of ganglioside intake, around <200 mg/day, were detected in a healthy diet of 2000 kcal/day (Pham et al., 2011). There is no literature to suggest an optimal intake of gangliosides.

Thus gangliosides represent a very interesting, although challenging, class of biomolecules to study. Challenges in ganglioside analysis are due to high levels of variability in ceramide composition (carbon number, degree of saturation) and carbohydrate core (number and nature of saccharides) and also due to the relatively low abundance of gangliosides in biological membranes. Conventional analysis involves the use of thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) (Lacomba et al., 2010) followed by colorimetric methods using-hydrochloric (HCl) acid or orcinol–sulfuric acid reagents which quantify total lipid bound sialic acid (T-LBSA) (Rueda, 2007; Yu and Ariga, 2000). These approaches are very time-consuming and do not provide simultaneous information regarding ceramide or carbohydrate composition.

An emerging tool in ganglioside analysis is mass spectrometry (MS) (Fong et al., 2011; Lee et al., 2011; Sørensen, 2006). An MS based approach offers several key advantages, including sensitivity and selectivity (Lacomba et al., 2010). MS based methods offer direct information on both the ceramide and carbohydrate composition (Fong et al., 2009; Lee et al., 2013). The application of gas-phase dissociation experiments, such as collision induced dissociation (CID), can also provide structural information. Using CID the position and anomeric configuration of the glycosidic linkages can be determined (Lee et al., 2011).

Although these MS-based approaches represent significant improvement in analysis of gangliosides, some limitations remain. For example, the existing MS methodology is still quite time-consuming, with long liquid chromatography (LC) runs prior to MS detection. Existing techniques require the use of expensive high resolution mass instruments, such as Orbitrap or Fourier transform ion/cyclotron resonance mass spectrometers (Lee et al., 2011; Fong et al., 2009). We present a method for rapid profiling of gangliosides from various biological membranes using a low resolution triple-quadrupole MS operating in multiple reaction monitoring mode (MRM). In the MRM mode, the first quadrupole scans multiple masses of gangliosides selected with precursor ion to pass. The precursor ion is one sialic acid (Neu5Ac). In the second quadrupole or collision cell, some fragmented ions are generated (Gillette and Carr, 2013). The third quadrupole is set to allow sialic acid to pass and to collect fragments. Triple quadrupole MS with MRM has been recently used due to the high specificity for simultaneous detection of multiple components in complex samples (Zhang et al., 2012; Lee et al., 2013; Giuffrida et al., 2014; Huang et al., 2014).

This method provides key advantages in terms of sensitivity, specificity and quantitative analysis. Every aspect of the method was optimized for MS detection; this included optimizing the extraction of gangliosides from dairy products and the LC separation prior to MS analysis. This method was optimized using bovine whole milk, colostrum and powdered buttermilk as a source of gangliosides and was applied to evaluate the effect of pasteurization, sample size, ganglioside extraction procedure and on the detection of the gangliosides in these milk products. The application of this method was tested in another biological system to determine the main ganglioside present in prostate cancer cells.

2. Materials and methods

2.1. Materials

Bovine buttermilk powder (7% of fat) was provided by Parmalat (Toronto, Ontario, Canada). Bovine buttermilk powder was reconstituted with Milli-Q water at 25% (w/v). Fresh unpasteurized pooled whole milk (~3.5% of fat) and colostrum (~5.5% of fat) were obtained from the University of Alberta Dairy Research Technology Centre from Holstein cows. Acetic acid, HCl and sodium bicarbonate were obtained from Fisher Scientific Company (Ottawa, Ontario, Canada). Resorcinol, glucose, HEPES, 28% ammonia and 8-anilino-1-naphthalene-sulfonic acid (ANSA) were obtained from Sigma Aldrich (Oakville, Ontario, Canada). Ammonium acetate was obtained from Anachemia (NY, USA). Sodium pyruvate, glutamine, fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gibco (Burlington, Ontario, Canada). Sep-Pak C18 cartridges were obtained from Waters Corporation (Milford, MA, USA). All LC/MS solvents were of LC/MS grade and solvents for ganglioside extraction were ACS grade (Fisher Scientific Company, Ottawa, Ontario, Canada). Whole milk samples were pasteurized in a preheated water bath at 63.7 °C for 30 min for the low temperature, long time (LTLT) pasteurization treatment (62 °C for 30 min), and at 72.7 °C for 15 s for the treatment of high temperature, short time (HTST) pasteurization. Both pasteurization treatments were followed by cooling in a water bath (<5 °C).

2.2. Ganglioside extraction and purification

Samples were extracted according to a Folch method for extracting animal tissue lipids (Folch et al., 1957). This method consisted of chloroform–methanol (2:1, v/v) extraction with a solvent to aqueous ratio of 20:1. The mixture was homogenized and shaken vigorously (20 min). After shaking, $CaCl_2$ (0.025%, w/v) was added to the mixture followed by another 20 min of shaking. The solution was either left overnight at 4 °C or centrifuged to allow the phases to settle. The upper methanolic phase (aqueous phase) was purified by SPE passing through Sep-Pak C18 cartridges. Prior to sample loading, cartridges were prewashed with 5 mL of Milli-Q water, 5 mL of methanol, 5 mL of chloroform–methanol (2:1, v/v), 5 mL of methanol and 5 mL of Milli-Q water. The aqueous phase was passed through a prewashed Sep-Pak C18 cartridge and then washed with 10 mL of Milli-Q water to remove salts. Gangliosides were eluted with 2 mL of methanol and 10 mL of chloroform–methanol (2:1, v/v) (Williams and McCluer, 1980), dried under N_2 gas at 34 °C, and re-dissolved with 500 μL of methanol/water (1:1, v/v). To evaluate the optimal sample size for a MS-based approach, the Folch method was applied to various milk aliquots, from 50 μL to 5 mL. The ratio of all organic solvents to sample size was kept constant at 20:1. The necessity of the solid phase cleanup was also evaluated, as direct injection of the aqueous phase was analysed as well as a reconstituted sample with 500 μL of methanol/water (1:1, v/v) of the entire aqueous phase after drying under N_2 gas at 34 °C.

2.3. Standard ganglioside preparation

Gangliosides were extracted from whole milk as described by Jennemann and Wiegandt (1994). Use of nonhomogenized whole fresh milk for analysis assures that ganglioside structure has not