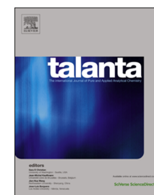
Talanta 118 (2014) 137–146

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



Ultra high performance liquid chromatography–tandem mass spectrometry method for the determination of soluble milk glycans in rat serum

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ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form
27 September 2013
Accepted 5 October 2013
Available online 12 October 2013

Keywords:
UHPLC–ESI–MS/MS
Soluble milk glycans (SMGs)
Carbohydrates
Serum analysis

ABSTRACT

The main objective of the present work was to develop and validate a multicompound method to measure soluble milk glycans (SMGs) in biological fluids such as serum. An ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the identification and quantification of the following SMGs and their precursors 2′-fucosyllactose, 3′-sialyllactose, 6′-sialyllactose, lacto-N-neotetraose, N-acetylneuraminic acid, fucose, lactose and glucose in rat serum samples was set up. These analytes were separated in an Acquity UPLC BEH Amide column using acetonitrile–water gradient with ammonia as additive, in a 10 min run, and were detected and quantified using a triple quadrupole (QqQ) mass spectrometer. The mass spectrometric conditions in negative electrospray ionization mode (ESI−) were individually optimized for each analyte to obtain maximum sensitivity in the Selected Reaction Monitoring (SRM) mode. Selection of two specific fragmentation reactions for each compound allowed simultaneous quantification and identification in one run, ensuring a high specificity of the method. The limits of detection (LODs) ranged from 5 to 70 ng mL^{-1} and the limits of quantification (LOQs) from 20 to 200 ng mL^{-1}. The inter- and intra-day variability was lower than 15% and the recoveries ranged from 85% to 115%. A biological application of the method was also described, specifically the time-course of SMGs in rat serum after an oral challenge.

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1. Introduction

Human milk is a complex biological fluid composed mainly of lipids, proteins, and lactose. Oligosaccharides are the third most abundant component in human milk, after lactose and lipids. They are present in large amounts, ranging from 5 to 23 g L^{-1}. The monosaccharides used for the biosynthesis of human milk glycans are glucose, galactose, N-acetylglucosamine, fucose and N-acetylneuraminic acid (SA, acronym of sialic acid). Despite these basic structures, the possible combinations of monosaccharides and plausible linkages contribute to the overall diversity and complexity of SMG structures to the point that more than 150 structurally distinct SMGs have been identified so far [1].

SMGs exert important biological effects. Namely, they have a prebiotic effect, selectively serving as a source of energy and nutrients for bacteria to colonize the infant intestine [2]. Beyond the prebiotic effects, a large body of evidence suggests that SMGs may protect against infections by mimicking the attachment sites for certain pathogens [2], influence various stages of gut maturation in vitro [3], and prevent necrotizing enterocolitis in neonatal rats [4]. SMGs were previously considered indigestible. However, it is currently known that their structure changes before reaching the colon and that they can be absorbed as they appeared in plasma of neonatal rats [5] and in urine of infants [6]. SMGs can also exert systemic effects. For instance, it has been shown that: 1) fucosylated and sialylated SMGs reduce selectin-mediated leukocyte rolling, adhesion, and activation [7,8]; 2) oligosaccharides are often found as a component of glycoproteins or glycolipids and there is evidence of a role of glycoproteins in adaptive functions of neuronal membrane components determining the efficiency of interneuronal connections [8]; and 3) sialoconjugates have been also shown to participate in the establishment of synaptic pathways, calcium transportation, binding of neurotransmitters, cell-to-cell interactions and axon regeneration [9,10].

An important tool for initiating any study on SMGs is a quantitative method to measure different SMGs in biological matrices. The separation of SMGs has been traditionally a challenge; oligosaccharides are

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http://dx.doi.org/10.1016/j.talanta.2013.10.013

polar compounds that are not well resolved by traditional reversed-phase chromatography. In addition, oligosaccharides do not generally contain strong intrinsic chromophores, resulting in low specificity and sensitivity in optical absorbance detection. Nonetheless, some studies have been published in the scientific literature for the determination of these compounds such as gel permeation chromatography [11], reverse-phase high performance liquid chromatography [12], high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [13,14], capillary electrophoresis [15], precolumn derivation HPLC with different detectors (ultraviolet, photodiode array ultraviolet–visible, differential refractive index detector) [16,17], and also Nuclear Magnetic Resonance Spectroscopy [18].

All of the previously published methods based on LC are limited by cumbersome protocols requiring derivatization precolumns and long-run times, which do not fully resolve the major oligosaccharides to baseline, and have low sensitivities. HPAEC–ECD improves detection limits, but requires a long analysis time. Currently, mass spectrometry offers several advantages such as greater selectivity, specificity and sensitivity. Some of these techniques involve matrix-assisted laser desorption (MALDI) or time-of-flight mass spectrometry (TOF/MS) [19–26], microfluidic chips and mass spectrometry technology (HPLC-Chip/TOF–MS) [27], and negative ion mode electrospray mass spectrometry (ESI–MS) [28,29].

Herein, we report an UHPLC–MS/MS method with negative electrospray ionization using ammonium hydroxide as additive for the separation and determination of specific carbohydrates and metabolites in serum of rats to support pharmacokinetic studies of SMGs carried out to confirm the appearance of these metabolites in serum of rats when they were administered orally. The method focused on four main SMGs: 2′-fucosyllactose (2′-FL), 3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), lacto-N-neo-tetraose (LNnT) and their parental mono- and disaccharides: sialic acid (SA), fucose (Fuc), lactose (Lact) and glucose (Glu). The chemical structures are shown in Fig. 1. To our knowledge, no multicomponent methods for the quantitative determination in serum samples of all the proposed compounds in a single analysis have been previously described in the literature.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC–MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). SA, Fuc, Lact and Glu were supplied by Sigma-Aldrich (Madrid, Spain); 2′-FL, 3′-SL and 6′-SL were all derived from bacterial synthesis; and LNnT was synthesized from a yeast fermentation system and purified by crystallization [30]. The purity and content of each SMG was measured by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD). Acetonitrile (MeCN) LC–MS grade, ethanol (EtOH) HPLC grade and ammonia solution 25% (v/v) eluent additive for LC–MS were from Scharlab (Barcelona, Spain).

A stock solution was prepared by weighing 0.002 g of each compound into a 10 mL flask, except for Lact and Glu, for which 0.005 g were weighed. Then, water was added up to the final volume. The solution remained stable for at least one month at 4 °C. After that, six work standard solutions were prepared for calibration purposes. The first one (WS6) was obtained by dilution to 1 mL of 100 μL of the stock solution. Then, 500 μL, 100 μL, 25 μL and 5 μL of WS6 solution were diluted to a final volume of 1 mL to obtain standards WS5, WS4, WS3 and WS2, respectively. Finally 150 μL of WS2 were diluted to 1 mL to prepare WS1. In all cases, an aqueous solution of ammonia 0.1% (v/v) was used for standard preparation. The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS4) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment. The stock solution was stable for 1 month at 4 °C. The working standard solutions were prepared from the stock solution for each experiment.

2.2. Apparatus and software

Detection and quantification of the studied compounds were performed using an UPLC Acquity® system from Waters (Milford, MA, USA) equipped with a binary pump, a vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector. The chromatograph was connected online to a triple quadrupole mass spectrometer detector (TQD) with an electrospray ionization (ESI) interface. Three different polarity chromatographic columns were tested in order to achieve a good resolution: Acquity UPLC BEH C18 (2.1 mm × 100 mm i.d., 1.7 μm particle size), Acquity UPLC BEH HILIC (2.1 mm × 150 mm i.d., 1.7 μm particle size), and Acquity UPLC BEH Amide (2.1 mm × 100 mm i.d., 1.7 μm particle size) from Waters. MassLynx software version 4.1 was used for instrument control and for data acquisition and analysis.

Analytical balance with a precision of 0.1 mg, vortex-mixer, maximum recovery LC vials and screw caps from Waters, eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom), and speed vac evaporator from Heraeus Instrument Thermo Scientific (Madrid, Spain) were also used.

2.3. Animal manipulation

Thirty Sprague Dawley female rats (~300 g body weight) (Charles River Laboratories, France) were used. The animals were kept in pairs in standard cages at constant room temperature (22 ± 2 °C) and 45–55% humidity under a regular 12 h light/dark schedule. Food and water were freely available. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national laws and EC policies for the Care and Use of Laboratory Animals (RD 2101-2005, 86/609/CEE).

Animals were assigned to five experimental groups ($n=6$). Group 1 received a single dose of 2′-FL (1.75×10^{-3} mol kg^{-1}); group 2, 6′-SL (1.75×10^{-3} mol kg^{-1}); group 3, 3′-SL (1.75×10^{-3} mol kg^{-1}); group 4, LNnT (1.75×10^{-3} mol kg^{-1}) and group 5, a mix of 2′-FL, 6′-SL, 3′-SL, and LNnT (4.37×10^{-4} mol of each SMGs kg^{-1}, representing 1.75×10^{-3} mol kg^{-1} in total).

All the solutions were prepared in water and were administered by intragastric gavage. The animals were fasted for 12 h and a blood sample was taken from the caudal veins (time 0). After the gavage, serial blood samples were collected at 30, 60, 90, 120, 150, 210 and 240 min. Blood samples were allowed to clot and then, centrifuged at 1800 × *g* for 10 min. Serum was stored at −20 °C.

2.4. Sample preparation

The initial extraction protocol was based on the procedures previously proposed in the scientific literature [18,27]. The protocol was then modified and optimized in order to improve the extraction of the analytes from serum samples. An aliquot (80 μL) of serum sample was placed into a 10 mL glass tube and extracted with a solution containing 2.0 mL of a 2:1 (v/v) mixture of chloroform and methanol, and 0.4 mL of deionized water. After shaking for 2 min in a vortex-mixer, samples were centrifuged at 2400 × *g* at 4 °C for 30 min. The lower chloroform layer was

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