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Ultrasound-assisted hydrolysis and chemical derivatization combined to lab-on-valve solid-phase extraction for the determination of sialic acids in human biofluids by μ-liquid chromatography-laser induced fluorescence

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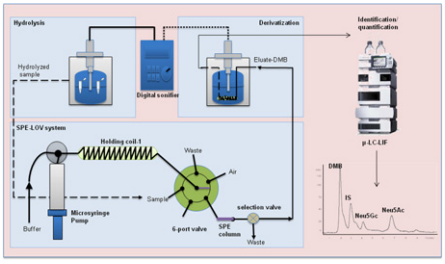
HIGHLIGHTS

- Semiautomated approach for determination of sialic acids in different biofluids.
- Ultrasound-enhanced hydrolysis and derivatization to shorten the analysis time.
- Lab-on-valve approach for automated solid-phase extraction with high concentration and cleanup efficiency.
- Validation of the method by application to biological samples with different characteristics.

GRAPHICAL ABSTRACT



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ABSTRACT

The determination of sialic acids (SIAs) has recently gained interest because of their potential role as markers of inflammatory disorders or chronic diseases. Hydrolysis of conjugated derivatives, solid-phase extraction (SPE) and derivatization steps constitute sample preparation prior to insertion of the analytical sample into a μ-liquid chromatograph-laser induced fluorescence (μ-LC-LIF) detector in the present method for the determination of two representative SIAs of human metabolism. Ultrasound-accelerated hydrolysis released free SIAs, which were efficiently concentrated in a dynamic manner using a lab-on-valve (LOV) module that allows automation of SPE for preconcentration and cleanup. This step was on-line connected with DMB-labeling of SIAs (derivatization), which was shortened from 180 min required with the conventional heating method to 20 min with ultrasound assistance. Individual separation of the target analytes was achieved within 20 min by μ-LC, while LIF detection endowed the overall method with high sensitivity. The LODs and LOQs provided by the method ranged 0.1–0.8 ng mL^{-1} and 0.4–1.0 ng mL^{-1} (between 0.1–0.8 pg and 0.4–1.0 pg expressed as on-column amount), respectively. High efficiency for interferents removal by SPE enabled the application of the method to four different biofluids—serum, urine, saliva and breast milk—for the determination of the target metabolites.

1. Introduction

Sialic acids (SIAs) comprise a family of 43 naturally occurring derivatives of the nine-carbon sugar neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galactononulsonic acid). One branch of the SIAs family is N-acetylated to form *N*-acetylneuraminic acids

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(Neu5Ac), which are the most widespread forms of SIAs and almost the only found in humans. The other branch is based on *N*-glycolylneuraminic acids (Neu5Gc), which are not found in humans, except for individuals affected by certain types of cancers such as colon and breast cancers [1–3].

While a small portion of SIAs occurs as free metabolites in human biofluids, the major part of them is bound to glycosidic forms. Thus, SIAs are commonly present as components of oligosaccharide chains of mucins, glycoproteins, and glycolipids [4]. Cells from higher animals and various microorganisms produce SIAs in a long pathway starting from glucose. An outline of the multiple reactions involving SIAs is shown in Supplementary Fig. 1. Sialic acids behave very different in model animals; in young rats the levels of SIAs in the liver are initially low, rising to a maximum 14–21 days after birth [5]; in mice and guinea pigs SIAs concentration is correlated with age and developmental stage [6]. It has been postulated that human infant liver may also have a limited capacity for synthesizing SIAs during early postnatal life [7]. Human milk also contains a high concentration of SIAs attached to the terminal end of free oligosaccharides, but their metabolic fate and biological role are practically unknown. High concentrations of SIAs in serum have been reported in patients with some inflammatory disorders or chronic diseases, but the range is lowered in healthy humans [8,9].

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The discovery of the role of SIAs as potential biomarkers for different pathologies has increased their clinical interest. Methods for determination of SIAs in biological samples involve two major steps: first, release from individual monosaccharides is achieved by acid or enzymatic hydrolysis; then, the resulting mixtures of monosaccharides can be analyzed by different methods: fractionation, characterization, and quantitation by high-performance liquid chromatography using anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [10], conventional HPLC coupled to fluorescence detection [11] or mass spectrometry detection [12,13], or by gas chromatography with flame ionization detection (GC-FID) [14–16]. Concerning sample preparation, the implementation of a solid-phase extraction (SPE) step to attain higher sensitivity and selectivity for quantitative analysis of SIAs in biofluids is frequent [17]. This is usually carried out by non-polar sorbents such as C18 and C8 to take benefit from non-polar interactions with SIAs [18,19]. Nevertheless, no single cleanup procedure based on polar interactions between the hydroxyl group of the target analytes and a polar sorbent has so far been described.

In this research, an ultrasound-assisted hydrolysis step was planned to precede a cleanup/preconcentration step based on a lab-on-valve (LOV) system. The latter was coupled on-line to an ultrasound-assisted derivatization step with the aim of proposing a semi-automatic method for sample preparation of SIAs in biological samples with easy implementation in the clinical area. The resulting treated sample was inserted into a μ-LC-LIF approach for individual separation and quantitation of *N*-acetylneuraminic and *N*-glycolylneuraminic acids.

2. Materials and methods

2.1. Instruments and apparatus

Ultrasonication was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium–alloy probe (12.70 mm diameter), which was immersed into a lab-made stainless-steel container with eight compartments in a circular arrangement to place test tubes equidistant from the center where the probe was located.

Fig. 1 illustrates the experimental setup used for sample preparation based on three steps: ultrasound-assisted hydrolysis step (A), automated SPE (B) and derivatization of SIAs (C). SPE was carried out with a sequential injection LOV microfluidic system. The LOV system consists of a single-channel high precision bidirectional syringe pump of 1 mL, a holding coil and a multiposition valve. The pump allows aspirating and dispensing micro-volumes of sample and reagents; the valve, a six-port valve made of Plexiglass, has the ports interconnected and also with the syringe pump by a central port. The connections are as follows: port 1 – waste, port 2 – buffer, port 3 – sample, port 4 – not used, port 5 – eluent flow-through to SPE, and port 6 – air. The holding coil (holding coil 1 in Fig. 1—1 mL, 0.3 mm i.d.) is located in between the syringe pump and the six-port valve. A two-position selection valve allows wasting all solutions different from the analytes fraction, which is led to holding coil 2 for derivatization. Therefore, SPE and derivatization were on-line coupled for automated development. Polyetheretherketone (PEEK) tubing (0.5 mm × 0.8 mm i.d.) from Análisis Vínicos (Ciudad Real, Spain) was used to connect the LOV and additional components. Polytetrafluorethylene (PTFE) tube (1.5 mm i.d.) from Análisis Vínicos was used to construct the SPE mini-column, which was packed with aminopropyl (40 μm homogeneous particle size) from Varian (Madrid, Spain). Other tested sorbent material was Chromabond C18 (size 40 μm particle diameter) from Macherey–Nagel (Durem, Germany). The two-position selection valve (VICI, Valco, Houston, USA), connected to the microcolumn channel, enabled to lead the eluate after the SPE step to holding coil 2 (500 μL, 0.5 mm i.d.) in which the eluate was subjected to ultrasound, thus favoring derivatization. The LOV and the selection valve were fully automated and controlled by the FIAlab for Windows version 5.0 software.

Separation of SIAs was performed by an Agilent (Palo Alto, CA, USA) 1100 micro-liquid chromatograph (μ-LC) equipped with a binary capillary pump and an automatic injection valve (1 μL sample loop). The analytical column was a reversed-phase Zorbax SB-C18 (150 mm × 0.5 mm i.d., 5 μm) from Agilent. The overall system was mounted with capillary tubes 75 μm i.d. × 375 μm outer diameter from Teknokroma (Barcelona, Spain). After chromatographic separation, the derivatized analytes were detected by a ZETALIF 2000 325 nm/CE LIF detector from Picometrics (Toulouse, France). The μ-LC was connected to the LIF detector by capillary tubing in which a detection window of 5 mm length was made for collecting the emitted light. Signal acquisition from the LIF detector was monitored and then integrated by the Agilent Chemstation software.

2.2. Chemicals and stock solutions

N-Acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), *N*-acetylneuraminic acid methyl ester (Neu5Ac-methyl ester) and 1,2-diamino-4,5-methylene dioxybenzene (DMB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock standard solutions at 1000 μg mL^{-1} of Neu5Ac and Neu5Gc were prepared in deionized water (DI water) (18 MΩ cm^{-1}) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA). Neu5Ac-methyl ester, used as internal standard, was dissolved at 500 μg mL^{-1} in LC–MS grade methanol from Panreac (Barcelona, Spain). Multistandard solutions were prepared by diluting the stock solutions in water. All the above solutions were stored at −20 °C in glass vials and kept in the dark until use.

LC grade methanol and acetonitrile from Scharlab (Barcelona, Spain) were used to prepare the chromatographic mobile phases. The solution for conditioning the SPE cartridge was 1 mol L^{-1} sodium phosphate solution at pH 3. The activation solution was a