Journal of Chromatography A, 1323 (2014) 82–86

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Borate-aided anion exchange high-performance liquid chromatography of uridine diphosphate-sugars in brain, heart, adipose and liver tissues

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ARTICLE INFO

Article history:
Received 6 September 2013
Received in revised form 29 October 2013
Accepted 1 November 2013
Available online 11 November 2013

Keywords:
UDP-sugar
Anion exchange HPLC
Tissue extraction
Boric acid
Ion-pair solid-phase column

ABSTRACT

In this paper we describe a method optimized for the purification of uridine diphosphate (UDP)-sugars from liver, adipose tissue, brain, and heart, with highly reproducible up to 85% recoveries. Rapid tissue homogenization in cold ethanol, lipid removal by butanol extraction, and purification with a graphitized carbon column resulted in isolation of picomolar quantities of the UDP-sugars from 10 to 30 mg of tissue. The UDP-sugars were baseline separated from each other, and from all major nucleotides using a CarboPac PA1 anion exchange column eluted with a gradient of acetate and borate buffers. The extraction and purification protocol produced samples with few unidentified peaks. UDP-N-acetylglucosamine was a dominant UDP-sugar in all the rat tissues studied. However, brain and adipose tissue showed high UDP-glucose levels, equal to that of UDP-N-acetylglucosamine. The UDP-N-acetylglucosamine showed 2.3–2.7 times higher levels than UDP-N-acetylgalactosamine in all tissues, and about the same ratio was found between UDP-glucose and UDP-galactose in adipose tissue and brain (2.6 and 2.8, respectively). Interestingly, the UDP-glucose/UDP-galactose ratio was markedly lower in liver (1.1) and heart (1.7). The UDP-N-acetylglucosamine/UDP-glucuronic acid ratio was also constant, between 9.7 and 7.7, except in liver with the ratio as low as 1.8. The distinct UDP-glucose/galactose ratio, and the abundance of UDP-glucuronic acid may reflect the specific role of liver in glycogen synthesis, and metabolism of hormones and xenobiotics, respectively, using these UDP-sugars as substrates.

1. Introduction

Nucleotide sugars are important substrates for the construction of various extracellular and cell surface glycans and glycoconjugates. One of the nucleotide sugars, uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), also serves an important role as a substrate and regulator of hyaluronan and O-linked N-acetylglucosamine (O-GlcNAc) modification of cytosolic and nuclear proteins [1–3]. Substrate availability has a major effect on the activity of some of the glycosyltransferase enzymes. Nucleotide sugars are derived from glycolysis intermediates which are increased by the aerobic glycolysis of cancer cells, and probably contribute to their changed cell surface glycosylation profiles, increased hyaluronan, and O-GlcNAc signalling [4–9]. Besides cancer, nucleotide sugars are important also in other diseases associated with changed glucose metabolism, like type 2 diabetes.

Despite their importance, relatively little information exists on nucleotide sugar levels in cultured cells, let alone in animal and human tissues *in vivo*. The few previous reports on their tissue levels in animals and humans [10–13] required hundreds of milligrams of starting material, often an unfeasible request in human studies. None of the reported methods for tissue nucleotide sugar isolation has utilized ion-pair solid-phase extraction, a purification step which has turned out to be valuable in the assays from cultured cells. An anion-exchange column has also been used for purification, and is the only procedure reported for the analysis of milligram scale samples from human tissues [10]. However, recoveries were not taken in account in this report.

Many of the previous publications show unsatisfactory separation of either or both of UDP-Glucose/UDP-Galactose (UDP-Glc/UDP-Gal) and UDP-N-Acetylglucosamine/UDP-N-Acetylgalactosamine (UDP-GlcNAc/UDP-GalNAc) pairs. In addition, the possible interference by co-extracted nucleotides is often neglected. Separation and identification of nucleotide sugars have previously been done both by anion-exchange and reversed-phase

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http://dx.doi.org/10.1016/j.chroma.2013.11.004

high-performance liquid chromatography [14–16]. There are also reports on the use of mass spectrometry [17–19] and 1H nuclear magnetic resonance to identify and measure nucleotide sugars from cells [20], but these require equipment and expertise not readily available in many laboratories. In addition, mass spectrometry-assisted identification suffers from the fact that it cannot be combined with all separation methods [19]. Separation of nucleotides and nucleotide sugars has turned out to be difficult but important, as both enter in the same fraction in the isolation processes. In addition, the lack of UDP-Glc separation from UDP-Gal and UDP-GlcNAc from UDP-GalNAc has been problematic in both chromatography and mass spectrometry. A recent ion-pair, reversed-phase high-performance liquid chromatography (HPLC) method has shown thus far the best separation of both UDP-hexoses and UDP-hexosamines in the presence of common nucleotides, but still the UDP-hexose/hexosamine pairs were not baseline separated [15].

In this paper we report a procedure in which the tissue isolation of UDP-sugars has been optimized for recovery and purity, and combined with a novel HPLC analysis that separates the previously difficult UDP-sugar isomers from each other, and from common nucleotides, allowing reproducible quantification from tissue samples as small as a few milligrams. Marked differences were seen in the relative contents of the UDP-sugars in rat liver, adipose tissue, brain, and heart, probably reflecting their distinct needs for the different sugars.

2. Materials and methods

2.1. Reagents

All nucleotide sugar (UDP-Glc, UDP-Gal, UDP-glucuronic acid (UDP-GlcUA), UDP-GlcNAc and UDP-GalNAc) and nucleotide (CMP, UMP, AMP, CDP, GMP, UDP, ADP, CTP, GDP-Mannose (GDP-Man), UTP, ATP, GDP and GTP) standards except UDP-arabinose (UDP-Arab) which was from CarboSource Services, The University of Georgia, Athens, GA, USA) were purchased from Sigma (St. Louis, MO, USA). Sodium acetate, triethylammonium acetate (TEAA), trifluoroacetic acid and phenol, pH 7.9, were from Sigma, butan 1-ol from Merck (Darmstadt, Germany), acetonitrile from BDH Prolabo (VWR, Radnor, PA, USA) and boric acid from Fisher Chemicals (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Tissues

Tissue samples from adult male Wistar rats (Lab animal centre of University of Eastern Finland) were used. Permission for the use of animals was approved by Lab animal centre of University of Eastern Finland. Animals were euthanized using carbon dioxide, combined with cervical dislocation. Liver, abdominal adipose-, brain and heart tissues were collected as quickly as possible, snap frozen in liquid nitrogen, and stored in −70 °C before homogenization and extraction.

2.3. Sugar nucleotide extraction

Nucleotide sugars from the tissues were extracted with a protocol modified from Turnock et al., 2007 [18]. Approximately 10–30 mg of tissues were homogenized in ice cold 80% ethanol using Lysing Matrix D tubes with a FastPrep homogenizer (MP Biomedicals, Santa Ana, CA). In order to measure UDP-sugar loss during the procedure 10 μl of 0.1 mM dilutions of UDP-GlcNAc, UDP-Glc and UDP-GlcUA were added to a part of the samples. Also a sample containing 10 μl of 0.1 mM solution of UDP-GlcNAc, UDP-Glc and UDP-GlcUA standards were diluted directly with 80% ethanol and analyzed to estimate the recoveries. The tissue lysates were centrifuged at 8000 × *g* for 20 min at 4 °C to remove insoluble material. Supernatant was transferred to clean tubes and evaporated in a vacuum centrifuge. The ethanol-insoluble material was suspended in phosphate buffered saline (PBS) and used for the DNA isolation with a phenol extraction and ethanol precipitation. DNA concentration of each sample was measured with nanodrop (Thermo Fisher Scientific) photometer based on A260/280 ratio. The evaporated samples were dissolved in 9% butan 1-ol and lipids removed by three extractions with 90% butan 1-ol. The water soluble fractions were evaporated under vacuum and resuspended in PBS for purification by EnviCarb graphitized carbon columns (Supelco, Sigma) as previously described [21]. In short, EnviCarb columns were activated by 80% acetonitrile and washed with 0.1% trifluoroacetic acid and water. Samples were added and washed with water, 25% acetonitrile and 50 mM TEAA buffer, and eluted with 50 mM TEAA in 25% acetonitrile. The eluates were evaporated in a vacuum centrifuge and stored at −20 °C.

2.4. Anion exchange HPLC

A Dionex (Thermo Fisher Scientific) HPLC system consisting of an ICS-3000 dual pump unit, ICS series variable wavelength detector and Gilson 234 auto injector (Gilson, Middleton, WI, USA) under the control of Chromeleon 6.80 program (Dionex) was used. A CarboPac™ PA1 column (4 × 250 mm; with 4 × 50 mm precolumn, Dionex) was used with a flow rate of 1 ml/min in a 54 °C water bath. The program had a gradient made of the following solutions: (A) ultrapure H_2O, (B) 1.3 M boric acid, pH 7.5, (C) 1 M sodium acetate, pH 7.0, and (D) 1.5 M boric acid, pH 7.5. The column was equilibrated with 45%:55%:0%:0% (v/v/v/v) of solutions A–D. For injections, standards and samples were diluted with ultrapure H_2O. Elution was performed with the following program: T0 = 45%/55%/0%/0%, T23 = 31%/43%/21%/5%, T34 = 21%/37%/32%/10%, T40 = 9%/34%/37/20%, T60 = 5%/23%/56%/16%, T68 = 17%/19%/63/1%, T72 = 15%/17%/67%/1%, T73 = 45%/55%/0%/0% and T78 = 45%/55%/0%/0%. The effluent was monitored at 254 nm. The column was washed weekly with 0.2 M NaOH for about 1 h which was followed at least twice a year with ultrapure H_2O and 1 M HCl. Each day, prior to use, the column was equilibrated with the above gradient without sample.

3. Results and discussion

3.1. Nucleotide sugar isolation from tissues

HPLC analysis of nucleotide sugars from tissues requires disruption of cells and removal of DNA, proteins and lipids from the samples. For that the tissues were first efficiently homogenized and the debris precipitated with ethanol which leaves UDP-sugars in the supernatant. These were further purified by a protocol combining parts of two previously published methods [18,21], including lipid removal by butanol extractions and ion-pair solid-phase extraction with EnviCarb graphitized carbon columns. The samples obtained by the procedure showed very few unidentified peaks, indicating successful purification (Fig. 1B). The unidentified peaks were relatively constant between different tissues and were typically found prior to UDP-sugars (retention time around 17 min) and between GMP and GDP-mannose samples (retention time 43–52 min). The unidentified peaks did not interfere with the identification of UDP-sugars (Fig. 1B). The unidentified peaks originate from the tissue samples as procedure blank gave no peaks above the baseline.

The genomic DNA that remained in the ethanol precipitate was extracted for normalization of the UDP-sugar contents in tissues.