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Isolation and Analysis of Human Urinary Marker Protein uAGP by Liquid Chromatographic Methods

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1. Introduction

Acid alpha-1-glycoprotein is one of the most glycosylated marker proteins of human blood and urine containing highly structured, antennary oligosaccharide chains. The blood of healthy individuals contains 57 ± 15 mg/dL AGP in average varied slightly by various physiological factors (age, sex, pregnancy). The total protein content of urine in healthy individuals is only 2-8 mg/dL including 2-5% of urinary AGP (uAGP) that depends on many other factors (fluid intake, physical exertion, diseases). According to bioanalytical studies increase in the serum and urinary AGP levels proved to be relevant marker in several (malignant) diseases. [1] In spite of the extensive analysis of serum AGP the glycan structure of uAGP and the mechanism of urinary excretion is not yet clarified. The very low concentration of AGP in urine and large volumes of samples to be applied made necessary to work out novel sample preparation techniques for the isolation and analysis of uAGP. The present study was aimed to develop biocompatible methods for the isolation and purification of uAGP and its glycan units with the systematic application of various liquid chromatographic techniques.

2. Materials and methods

Urine sampling

First morning urine samples from healthy human volunteers of usually 250-500 mL were collected and centrifuged. Before other procedures routine laboratory diagnostic parameters of urine specimen (pH, proteins, blood, etc.) were tested by EL-U Test10 strips. After controlling urine samples were frozen (-25°C) and concentrated by freeze-drying in a proportion of 50 to 1 (v/v).

Preparation of urinary AGP

Desalting and separation of the macromolecular fraction of concentrated urine samples were performed with a preparative Sephadex G-25 (15x2,5 cm) liquid chromatographic column eluted with distilled water. Human urinary total protein content including uAGP eluted first with in the void volume (V_0) of the column was collected and lyophilized then dissolved in 25 mM bis–tris–propane (buffer A, pH 7.5) and applied to a Fractogel EMD TMAE-650 column (Pharmacia HR 5, 5x0,5 cm) using a simultaneous pH–NaCl gradient elution with 25 mM bis–tris-propane − 350 mM NaCl (buffer B, pH 9.5). The uAGP was isolated by preparative ion exchange chromatography as described earlier [2]. Fractions containing uAGP were collected, desalted and lyophilized. Further purification of uAGP was performed by hydrophobic interaction liquid chromatography (HIC). IEX samples of uAGP were dissolved in 0.1 M potassium-dihydrogen-phosphate – 1.0 M $(NH_4)_2SO_4$ buffer (pH 7.0) and applied to a Phenyl Sepharose CL 6B column (5x0,5 cm) using a negative salt gradient elution of $(NH_4)_2SO_4$ concentration. [3] Eluates containing uAGP were desalted and lyophilized. During the purification process the uAGP content in the eluted fractions was controlled by SDS polyacrylamide gel electrophoresis.

Enzymatic release and labeling of uAGP glycans

PNGase F digestion of uAGP and fluorescent labeling of the sugar constituents was performed ac-

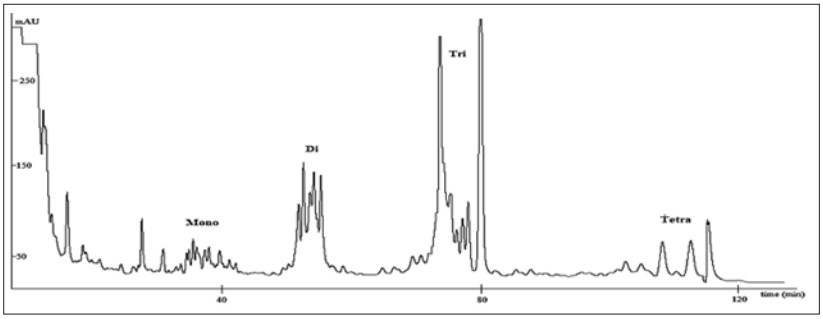

Figure 1 *HILIC separation of mono-, di-, tri and tetraantennary glycan structures of uAGP*

cording to Kremmer et al. [2] The labelled oligosaccharides were freeze-dried under vacuum.

HILIC fractionation of uAGP sugar constituents

The antennary glycan constituents of uAGP were fractionated by hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection at 360 and 425 nm. Labelled uAGP oligosaccharides were dissolved in 200 mM ammonium formate buffer, pH 3.5 – acetonitrile 30:70 (v/v) and applied to a Phenomenex Luna Amino column (15 x 0.2 cm). HILIC separation was driven with a linear gradient elution program where the composition of buffer ratio changed to 95:5 (v/v) in 120 minutes.

3. Results

In order to isolate and purify the total protein content of human urine an optimal combination of freeze-drying and preparative gel chromatographic desalting techniques was developed and applied. To obtain the uAGP content total urinary proteins were fractionated by anion exchange chromatography using a complex elution gradient program. For the further purification of uAGP hydrophobic interaction liquid chromatographic method was developed and successfully applied. Furthermore, sample preparation procedures consisting of the release of N-glycans from uAGP by PNGase-F enzyme hydrolysis and fluorescence labeling with anthranilic acid were performed. Labeled glycan structures of uAGP were analyzed by hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection. ***Figure 1*** demonstrates the characteristic separation and distribution of mono-, di-, tri- and tetraantennary glycan structures of uAGP similarly to patterns of human serum AGP observed earlier. [4]

4. Conclusions

Results presented here show that the examination of the glycan structure of uAGP may provide either valuable information on the applicability of uAGP as a marker or knowledge about the protein excretion processes in kidney. Various preparative and analytical liquid chromatographic techniques was developed and successfully applied for the fast and biocompatible separation and purification of human urinary AGP of adequate purity for structural analysis. Investigating the glycan structure of uAGP HILIC spectra demonstrated various similarities and differences with normal human AGP glycan content.

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