The analysis of the isoforms of the alpha-1 acid glycoprotein as a biomarker

Thesis of doctoral (Ph.D.) dissertation

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**Introduction**

Alpha-1 acid glycoprotein (AGP) - found in human plasma - belongs to positive acute phase proteins. AGP possesses five N-glycoylation sites and its oligosaccharide content exceeds the 40% of the molecular mass of the glycoprotein. Structural variability is an important feature of AGP. The isoforms of AGP can be derived from the variance of amino acid sequence and from the various structures of the oligosaccharides.

Differences in the sequence of amino acids result in the genetic variants. The structural heterogeneities of oligosaccharides can be originated in the alterations of the monosaccharide content, in the different sequence of the monosaccharides, and in the stereochemistry of the monosaccharides.

The analysis of the isoforms needs special techniques with great sensitivity and selectivity - at the same time. The high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) gives detailed information about the structural heterogeneity of isoforms.
Objectives

The analysis of the genetic variants of AGP and method development for the analysis of the isoforms of oligosaccharides were in the focus of my Ph.D. research.

The aim of my work was to describe the expression rate of the genetic variants applying HPLC-MS method.

The different ratio of the genetic variants of AGP in the healthy and cancerous populations characterize the biomarker feature of AGP under pathologic conditions. However, in the literature contradictory data were published about the acute phase feature of the genetic variants of AGP. I intended to determine the expression rate of the genetic variants in pathological conditions.

Characterization of structural heterogeneities of the oligosaccharide-isoforms is a difficult task. My objective was to work out an accurate HPLC-MS method which makes possible the identification of oligosaccharides and gives information about the isoforms.
Methods

For the analysis of the genetic variants of AGP a developed HPLC-MS method was applied. The peptides cleaved by trypsin were separated on reversed phase nanocolumn. The analysis of peptides was carried out with Q-Tof mass spectrometer. The separated peptides were detected in positive ionisation mode and identified by tandem MS.

The analysis of ORM1 and ORM2 genetic variants were carried out on the basis of selected peptide pairs. The peptide $^{139}$NWGLS$^{144}$YADKPETTK$^{153}$ is characteristic for the expression of ORM1 variants, while the $^{139}$NWGLS$^{144}$FYADKPETTK$^{153}$ for ORM2 variant.

The sample preparation protocol for the analysis of oligosaccharide isoforms was worked out during my Ph.D. work. Oligosaccharides were cleaved enzymatically from the glycoprotein after the denaturation of AGP. For the cleavage Protein-N glycosidase enzyme (PNGase F) was used. The cleaved oligosaccharides were purified with solid phase extraction (SPE). For the separation of the oligosaccharides graphitized carbon column was applied and HPLC gradient method was developed for the optimal separation. The oligosaccharides and fragments were analysed by Q-Tof mass spectrometer in negative ionisation mode.
Results - theses

1. The ratio of AGP genetic variants were determined in samples of healthy individuals and patients suffering from different type of cancer (lymphoma, ovary tumor, melanoma). The applied HPLC-MS method made possible the determination of the expression rate of genetic variants (ORM1 and ORM2). Difference was found in AGP plasma level between the healthy and patients groups. I determined that the AGP levels were 4-fold elevated in the cancerous groups in comparison to the healthy group [1].

2. By variance analysis significant difference was observed in the ratio of ORM2 between the healthy and cancerous groups. However, no significant difference was found between the cancerous groups in the ratio of the genetic variants [Figure 1, 5-8].
Figure 1.: The proportion of ORM2 in the healthy and in the cancerous groups

3. The increase of expression rate of ORM1 and ORM2 can not be characterized by the same rate. The expression of ORM1 was increased at a greater extent in pathological conditions than that of ORM2. Accordig to the results the expression of ORM1 variants was increased 4.5-fold in the cancerous groups, while the increase of ORM2 variant was 2-fold [1].
4. A new sample preparation method was used and optimized for the cleavage of complex, N-glycans of AGP. Solid phase extraction method was worked out for the purification of the oligosaccharides using graphitized carbon columns [2, 15-16].

5. For the characterisation of glycosylation pattern HPLC-MS method was developed. It was found that the graphitized carbon column coupled to mass spectrometry method provides more structural information about the oligosaccharides than the reversed phase column chromatography coupled to mass spectrometry [2, 4].

6. With tandem mass spectrometric measurements I determined the fragmentation of the oligosaccharides. In the spectra the different intensity values of the fragments correlated to the molecule ions indicate the different stereochemistry of the isoforms [12-14].

7. 18 oligosaccharides with different molecular mass and with different monosaccharide content were detected by mass spectrometry. Furthermore optimal liquid chromatography gradient was developed with the graphitized carbon column for the oligosaccharides. The isoforms of oligosaccharides were also separated using the HPLC-MS method [Table 1, 4].
Table 1.: The proportion of the oligosaccharides in the digest of AGP and the number of the isomers identified

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>The proportion of the oligosaccharides (%)</th>
<th>Number of the isomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BiS2</td>
<td>16.8</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>BiS2F1</td>
<td>1.3</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>TriS2</td>
<td>8.0</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>TriS2F1</td>
<td>3.6</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>TriS3</td>
<td>29.2</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>TriS3F1</td>
<td>10.1</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>TriS3F2</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>TetraS2</td>
<td>4.2</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>TetraS2F1</td>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>TetraS3</td>
<td>8.0</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>TetraS3F1</td>
<td>3.3</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>TetraS4</td>
<td>5.9</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td>TetraS4F1</td>
<td>4.2</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>TetraS4F2</td>
<td>0.8</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>PentaS3</td>
<td>0.9</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>PentaS4</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>17</td>
<td>PentaS4F1</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>HexaS3</td>
<td>1.0</td>
<td>4</td>
</tr>
</tbody>
</table>

In the Table 1. the abbreviations of oligosaccharides sign the number of the antenna of the oligosaccharide. „S” refers to the content of sialic acid and the number behind „S” is equal with the number of sialic acids. „F” refers to the content of fucose and the number behind the „F” is equal with the number of fucoses.
Conclusions

The results show that primarily the ORM1, and not the ORM2 variant is responsible for the acute phase response and for the increased concentration of AGP in pathological conditions.

The developed method can be used for the characterization of complex, N-glycans cleaved from glycoproteins and for separation of isoforms. The determination of heterogeneities of glycosylation pattern in pathologic conditions can be a valuable diagnostic tool indicating altered biologic functions. With the use of the presented LC-MS method, detailed characterization of glycosylation pattern in biological samples became possible, by characterizing the major and minor microheterogeneities of corresponding oligosaccharides.
Publications related to the thesis

Articles

   Analytical and Bioanalytical Chemistry
   IF: 3.328

   European Journal of Mass Spectrometry
   14 (2008) 419-422.
   IF: 1.167

Oral presentations

3. Károly Vékey, Olivér Ozohanics, **Lívia Budai**, László Drahos:
   Glycosylation, mass spectrometry and informatics
   8. Igler MS Tage organized by the Institute of Organic Chemistry, University of Innsbruck

4. **Budai Lívia**:
   Az alfa-1 savas glikoprotein oligoszacharid-struktúráinak tömegspektrometriás analízise
   IX. Clauder Ottó Emlékverseny - Különdíj


**Poster presentations**


13. **Lívia Budai,** Ferenc Pollreisz, Oliver Ozohanics, Krisztina Ludányi, László Drahos, Károly Vékey: Glycosylation pattern analysis with mass spectrometry OBEKON symposium Budapest, 1-3 October 2009


**Publications**


