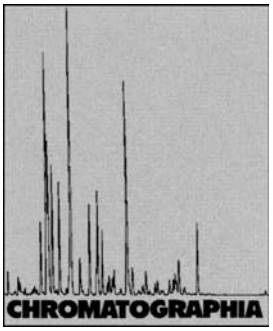
A Novel Method for the Separation and Purification of Human Serum Acid Alpha-1-Glycoprotein. Liquid Chromatographic and Mass Spectrometric Investigation of Tryptic Fragments



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É. Szöllősi[1], T. Kremmer[1,✉], K. Ludányi[2], T. Imre[2], G. Schlosser[2], M. Boldizsár[1], B. Vincze[1], K. Vékey[2]

[1]National Institute of Oncology, Department of Biochemistry, P.O. Box 21, 1122 Budapest, Hungary; E-Mail kremmer@oncol.hu
[2]Chemical Research Center of the Hungarian Academy of Sciences, P.O. Box 17, 1525 Budapest, Hungary

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Abstract

Analytical ion-exchange chromatography resulted in significantly elevated human serum acid alpha-1-glycoprotein (AGP) levels in cancer patients (153–194 $mgdL^{-1}$) and in acute inflammation (161 $mgdL^{-1}$) compared to healthy individuals (58.5 ± 17.4 $mgdL^{-1}$) and non-tumorous diseases (61.6 ± 5.0 $mgdL^{-1}$). A novel method of sample preparation and dye-ligand affinity chromatography was worked out and applied to the fast and biocompatible isolation and purification of AGP. Solvent extraction and precipitation provided nearly 100% recovery of serum AGP, while affinity chromatography using a new generation of dye-ligand phase (Toyopearl AF-Blue) resulted in AGP with a total yield over 85%. Tryptic fragments of AGP were separated by reversed phase liquid chromatography (RP-HPLC) and identified either with on-line ESI-MS, or off-line with PNGase F digestion and NH_2-phase HPLC. Off-line analysis provided further data on the distribution of antennary oligosaccharide structures. Predominance of bi- and triantennary chains was shown in human serum AGP (38 and 30%, respectively), while significant elevation in the relative proportion of tetraantennary compounds (9.6 vs 15.0%) and a 2-3 fold increase in the total amount of fucose containing structures were observed in cancer patients compared to the healthy controls.

Keywords

Column chromatography – mass spectrometry
Human serum acid alpha-1-glycoprotein (AGP)
Trypsin and Endoglycosidase F digestion
Glycopeptides and oligosaccharides

Introduction

Recent reviews on the bioanalytical application of high performance separation techniques indicate clearly that purity and integrity of biologically active natural analytes are the most important prerequisites in the structural and functional studies [1, 2]. Apparently, a significant development and demand of increasing the specificity and biocompatibility of sample preparation and separation methods can be observed [3, 11]. Among the numerous biopolymers investigated carbohydrate-rich complex proteins (cell membrane compartments, antigens, interferons, erythropoietin, etc) attend increasing interest either in bioanalytical, or pathophysiological aspects [4–12]. The sialoglycoprotein character of circulating glycoconjugates including a great majority of conventional serological tumor markers and acute phase serum protein fractions (AGP, alpha-1-antitrypsin, haptoglobin, ceruloplasmin, C-reactive protein, immunoglobulins, etc.) has been recognized [7, 10–18]. From theoretical and practical points of view the most heavily glycosylated serum acid alpha-1-glycoprotein (AGP, orosomucoid) proved to be particularly interesting in the laboratory diagnosis of malignant diseases and in structural investigations as well [5, 17, 19–26]. AGP is an acidic (pI ≅ 2-3) acute-phase protein fraction of human serum with exceptionally high (∼45% *w*/*w*) carbohydrate content and molecular mass of 36–38 kDa [19]. Physico-chemical characteristics, biological functions and pathophysiological role of human serum AGP are recently reviewed [20, 21]. Among various pathological conditions significantly increased serum AGP levels can be measured in cancer compared to healthy individuals [20, 27–29]. Sample preparation, gel-, ion-exchange and affinity chromatographic methods were widely applied to the separation and purification of AGP [20, 25, 30–33]. However, the time-consuming multi-step procedures and low-degree of biocompatibility make dubious in some cases

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the purity and structural integrity of AGP produced. To overcome these disadvantages in the present work a simple, fast and extremely mild sample preparation and affinity chromatographic purification using a new generation of dye-ligand stationary phase were worked out and applied.

It has been shown that the carbohydrate content of glycoconjugates can be formulated by antennary oligosaccharide structures consisting of neutral sugars, hexosamines (N-acetyl glucose and galactose amines) and N-acetyl neuraminic (sialic) acids [5, 9, 19–25]. Recent observations suggest that molecular informations (sugar code) concerning to various physiological and/or pathological events (pregnancy, rheumatic arthritis, cancer, etc.) can be detected in the exceptionally variable composition and structure of glycan chains [5–7, 10, 21]. The carbohydrate content of AGP consisting of five glycan units is formulated mostly by bi-, tri- and tetraantennary oligosaccharide structures with sialic acid at the end of chains and fucose attached to N-acetyl glucosamines [5, 17, 21, 26]. Liquid chromatographic (RP-HPLC), gel and/or capillary electrophoretic and mass spectrometric (MALDI, ESI) techniques including enzymatic (tryptic, PNGase F) digestion are purposefully used in the structural investigation of AGP [11, 12, 34]. HPLC-coupled ESI-MS was favourably applied to oligosaccharides derived from AGP [35]. It has been suggested that malignant diseases may induce detectable changes in the carbohydrate structure of AGP [5–7, 26, 41, 42]. Our preliminary observations indicated that AGP derived from cancer patients contained significantly higher fucose and sialic acid content in comparison to healthy controls [36].

Considering the significance of structural changes in human serum AGP the present study was aimed to develop a new combination of sample preparation and liquid chromatographic methods for the isolation and purification of the biopolymer and its carbohydrate compartments. Furthermore, RP-HPLC and MS techniques were developed and applied to identify and characterize the glycopeptide tryptic fragments of AGP, as well as to reveal the changes in their antennary oligosaccharide structures induced by cancer.

Experimental

Materials

Solvents of HPLC purity ($CHCl_3$, methanol, ethanol, CH_3CN, H_2O, Suprapur, LiChrosolv, gradient grade), reagents (NaCl, Na_2HPO_4, NH_4HCO_3, 2-aminobenzoic (anthranilic) acid) were purchased from Merck (Darmstadt, Germany) and Toyopearl AF-Blue HC-650M from TOSOH Biosciences (Stuttgart, Germany). Desalting was made on Sephadex G-25 (superfine, Pharmacia, Uppsala, Sweden). Trypsin (TPCK-treated), dithiothreitol, and 4-vinylpyridine from Sigma, and trifluoroacetic acid from Aldrich Chemical Co., were obtained. PNGase F (recombinant, glycerol free) was from Roche Diagnostics GmbH (Mannheim).

Serum Sampling

Venous blood was taken without anticoagulants from volunteer healthy individuals (normal controls) as well as from hospitalized patients fasting overnight. After spontaneous clotting at room temperature serum samples were isolated by centrifugation (5000 rpm, 10 min, 0–4 °C) and kept at −20 °C until use. The clinical status of patients, the type and stadium of malignancy in cancer was characterized and verified by conventional laboratory markers, imaging techniques and pathology. Ovarium carcinoma patients with high (over 200 mg dL^{-1}) serum AGP level were selected for structural investigations.

Sample Preparation and Determination of Serum AGP

Sample preparation, either in analytical, or preparative scale was performed by non-miscible phase solvent extraction as follows. One *vol* of human serum was diluted with 3.5 *vol* of ice-cold water and extracted with 15 *vol* of chloroform-methanol mixture (2:1 *v*/*v*) by vigorous shaking at 0 °C for 45 min. Solvent phases were separated by centrifugation (1000 g, 0–4 °C, 20 min) and the methanol-water extract (upper phase) was collected. The protein content of the extract was precipitated with two volumes of ice-cold ethanol, kept for 30 min at 0–4 °C then centrifuged (1000 g, 0–4 °C, 20 min). The precipitate was dissolved in 10 mM phosphate buffer A (pH: 5.8). AGP concentrations of sera and in each step of purification were measured according to our microanalytical ion-exchange chromatographic method [29].

Dye-ligand (Cibacron Blue) Affinity Chromatography of AGP

Cibacron Blue F3GA dye-ligand affinity chromatography was performed on a Toyopearl AF-Blue HC-650M column (5 × 1 cm I.D. 40–90 μm, TOSOH Biosciences) equilibrated with 10 mM phosphate buffer A (pH 5.8) at a flow rate of 2 mL min^{-1}. One ml of sample (ethanol precipitate solved in 10 mM phosphate buffer A) containing 20 mg of proteins (approx. 10 mg AGP) was applied and eluted with 10 mL phosphate buffer A then changed immediately to 10 mM phosphate buffer B (pH: 6.8) containing 2M NaCl. UV-detectable fractions (see Fig. 2) were collected, desalted on a Sephadex G-25 column (15 × 1.6 cm I.D., superfine, Pharmacia, Uppsala, Sweden) and liophylized. Protein content of fractions were measured by ion-exchange chromatography and investigated by SDS-polyacrylamide gel electrophoresis [37] and mass spectrometry carried out in linear mode using a Bruker Biflex™ MALDI-TOF mass spectrometer with a 337 nm nitrogen laser. Accelerating voltage was 19.5 kV. Chromatography was operated using an FPLC LCC-500 system (Pharmacia-LKB, Uppsala, Sweden) consisting of two P-500 pumps, UV-1 detector at 278 nm and equipped with a HP 3392A Integrator.

Treatment of AGP with Trypsin

Glycopeptide fragments of purified AGP were obtained by digestion with trypsin according to Dage et al. [17]. In the sample preparation 5 mg AGP was denatured in 1 mL 100 mM tris-HCl buffer (pH:8.3) containing 6 M Gu.HCl at 50 °C for 30 min, reduced with 0.2 mg dithiothreitol at 37 °C for 3 h and alkylated with 75 μL 4-vinylpyridine at 37 °C for 30 min. Finally, 3 mL distilled water was added to the mixture and centrifuged (10000 rpm, 30 min). Supernatant was

collected and desalted on Sephadex G-25. Fraction eluted with the void volume was freeze-dried. Alkylated AGP was dissolved in 0.5 mL 200 mM NH_4HCO_3 (pH:8.5) and digested with 200 μg TPCK-treated trypsin (Sigma) at 37 °C for 24 h. Prior to RP-HPLC tryptic digest was ultrafiltrated on Microcon centrifugal filter (Amicon, cut-off 10 kDa) and purified on Isolut C18 SPE cartridges (IST) conditioned with 0.1% TFA then eluted with 70% (*v*/*v*) acetonitrile - 0.1% TFA. Eluate was dried under nitrogen stream.

Reversed Phase Liquid Chromatography (RP-HPLC) of Tryptic Fragments

RP-HPLC separation of tryptic fragments was performed on a VYDAC Selectapore 300P wide-pore C18 column (25 × 0.46 cm I.D., 5 μ) equilibrated with 15% (*v*/*v*) acetonitrile-0.1% TFA for 10 min, then eluted using a linear gradient to 50% (*v*/*v*) acetonitrile-0.1% TFA in 80 min. Chromatography was carried out with an ISCO Model 2350 pump, 2360 Gradient Programmer and V^4 Absorbance detector (Lincoln, Nebraska, USA). Flow rate was 0.5 mL min^{-1} and detection at 215 nm. Data were handled with a JASCO-BORWIN (Tokyo, Japan) Ver 1.50 chromatography softwer program. Fractions were collected in time periods marked in Fig. 4 and freeze-dried.

PNGase F Treatment of Tryptic Fragments

To obtain antennary oligosaccharide structures bound to glycopeptides tryptic fragments collected from VYDAC Selectapore 300P wide-pore C18 column were digested with PNGase F (Endoglycosidase F, Boehringer) according to Elliott et al. [25] in 50 mM ammonium hydrocarbonate buffer (pH: 7.5) at 37 °C for 24 hours. Prior to derivatization digests were freeze-dried.

NH_2-HPLC Separation of Antennary Oligosaccharides

Oligosaccharides obtained by PNGase F treatment of tryptic fragments were derivatized with anthranilic acid according to Anumula et al. [38] and separated on apHera^{TM} NH_2 polyamine-bonded polymer based column (250 × 4.6 mm I.D., 5 μm, Astec) using a gradient elution program with solution A (2% acetic acid - 1% tetrahydrofuran in water) and solution B (5% acetic acid – 1% tetrahydrofuran – 3% triethylamine) according to the time schedule: Equilibration was with 30% B for 10 min then linear increase to 95% B in 80 min, washing with 100% B for 10 min. Separations were carried out with flow rate 1 mL min^{-1} at 50 °C (thermostated). HPLC was performed with a Merck-Hitachi LiChroGraph system consisting of an L-6200A Intelligent Pump, AS-2000A Autosampler, JASCO FP-1520 fluorescence detector (λ_{ex}: 360 nm, λ_{em}: 425 nm) and D-2500 Chromato-Integrator with D-7000 Interface Module and D-7000 HPLC System Manager.

On-line RP-HPLC–ESI Mass Spectrometric Analysis of Tryptic Fragments

Tryptic fragments derived from human serum AGP were separated on a VYDAC Selectapore 300P wide-pore C18 narrow-bore column (25 × 0.21 cm I.D., 5 μ) using a Perkin-Elmer HPLC system (Norwalk, USA) consisting of a Series 200 micro pump and autosampler, 785A UV/VIS detector (276 nm) and analysed on line with a Perkin-Elmer Sciex Instruments (Thornhill, Canada) API 2000 triple quadrupol mass spectrometer. Elution program was identical to the RP-HPLC conditions excepted that flow rate was 0.2 mL/min. Injection volume was 20 μL. Absorbance at 276 nm was monitored prior to ESI/MS. The instrument was fitted with an ESI source used in positive mode. ESI source temperature and needle voltage were 200 °C and 5 kV. Nitrogen was used as nebulizer, dryer and curtain gase with 150, 350 and 200 kPa, respectively. Orifice voltage was 150 V. Lens voltages of the mass spectrometer were optimized to give maximum signal to noise ratio. Spectra were obtained over the range of *m*/*z* 160-1800.

Table 1. Human serum AGP levels in healthy controls and in various diseases

Subjects	(*n*)	Serum AGP mg dL^{-1} (mean ± SD)
Healthy individuals	35	58.5 ± 17.4
Acute inflammation* (st. post op.)	31	161 ± 59
Ovary and cervix carcinomas	58	194 ± 70
Lymphomas	30	153 ± 87
Gastrointestinal tumors	35	185 ± 48
Other (non-tumorous) patients	15	61.6 ± 5.0

* In 2-3 days after surgical treatment

Results and Discussion

Measuring of AGP concentration in human serum under various pathophysiological conditions has been reviewed [20]. However, only few comprehensive data are available on the applicability of serum AGP in the malignant diseases [27–29, 39–42]. Our results summarized in Table 1 demonstrated that in average 2-3 times higher serum AGP levels could be measured in cancer patients compared to healthy individuals, but there were no significant differences between the various forms of malignancy. Temporary elevated AGP concentrations could also be measured in non-tumorous patients with acute inflammation. On the other hand, non-tumorous patients with various diseases resulted in serum AGP values practically in the normal range. In a more extensive trial (not presented here) it has been demonstrated that changes in serum AGP proved to be a useful tumor marker in monitoring of the disease.

It has been suggested that malignant diseases may induce molecular changes in the glycan moiety of human serum AGP [5–7, 42]. In order to investigate the oligosaccharide structure of AGP our further studies had been focused on selected cancer patients with serum AGP over 200 mg dL^{-1}. Fig. 1 shows the working scheme of our investigations. To isolate human serum AGP in 10–50 mg scale a new combination of sample preparation and dye-ligand affinity chromatographic methods has been developed. Modifica-

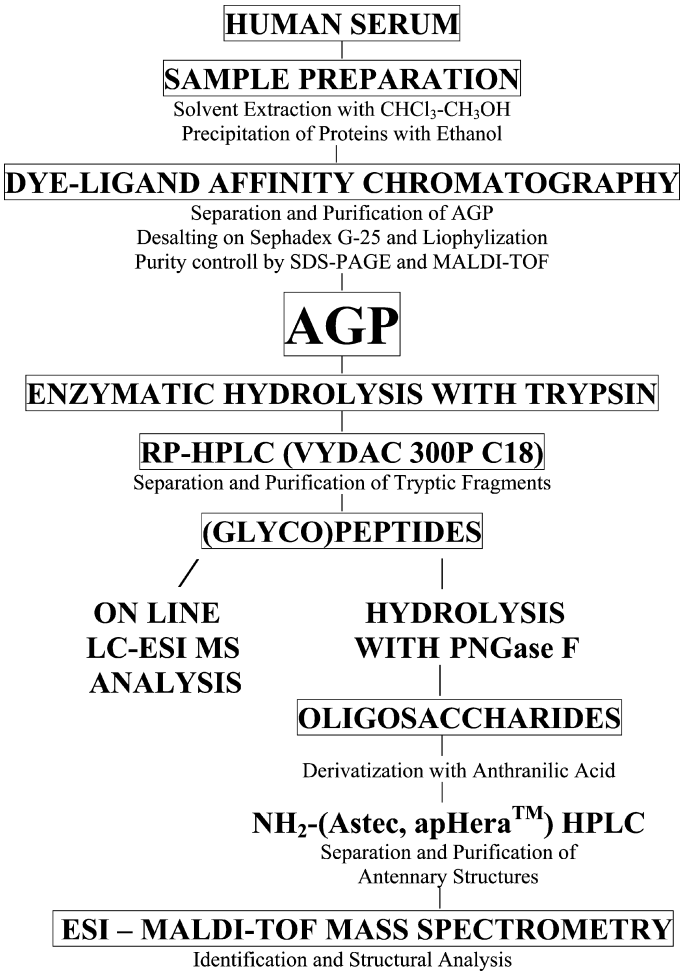

Fig. 1. Working scheme for the isolation and structural investigation of human serum AGP

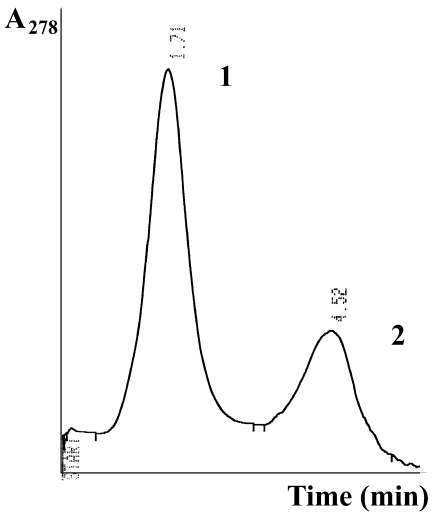

Fig. 2. Dye-ligand (Cibacron Blue) affinity chromatography of human serum AGP. 20 mg of proteins (extracted in sample preparation and precipitated with ethanol) was solved in 1 mL 10 mM phosphate buffer (pH: 5.8) and applied to a Toyopearl AF Blue HC-650M column (5 × 1 cm, I.D.) equilibrated with 10 mM phosphate buffer (pH: 5.8). Flow rate: 2 mL min^{-1}. UV detection: 278 nm

tion of our sample preparation reported previously [24] provided basic advantages over the other procedures. Instead of long-term dialysis (dilution) and/or various denaturing (strongly acidic) treatment of serum, as well as of ion-exchange purification used extensively [30–33] extraction and forthcoming precipitation of extracted proteins with ethanol eliminated either the bulk of serum proteins (mainly albumin), and/or solvents and low molecular contaminants in two single and entirely mild steps. In addition, a concentrated sample solved in any kind of buffer, or eluents could be prepared and applied to affinity chromatography. It has been demonstrated that AGP can be isolated from serum by non-miscible solvent extraction with a recovery over 95% [29]. However, considering the unavoided loss of water-methanol extract in phase separation the total yield of AGP is about 89%. The present investigations showed that 2:1 *vol* ratio of abs. ethanol to water-methanol extract resulted in quantitative precipitation of its protein content. Polyacrylamide gel electrophoresis and ion-exchange chromatography indicated that this sample contains predominantly AGP contaminated with traces of other serum proteins. Fig. 2 demonstrates that the purification of a sample prepared by the above method on a Toyopearl AF Blue affinity column resulted in two distinct fractions eluted either with 10 mM phosphate buffer at pH 5.8 (fractions 1), and with a step-wise change to phosphate buffer at pH 6.8 containing 2M NaCl (fraction 2). Analytical ion exchange chromatography, electrophoresis and MALDI-TOF MS methods verified (not shown here) that fraction 1 represented AGP, while other contaminating serum proteins (mainly albumin) appeared in fraction 2. The total yield of AGP including desalting on Sephadex G-25 column (as a crucial requirement of MS) was over 80%. In spite of its simplicity the advantage of this procedure can be characterized by the use of a new generation of Cibacron Blue-coupled, macroporous (1000 Å), polymeric backbone structured matrix with binding capacity of 15–20 mg protein mL^{-1} and significantly better hydrodynamic properties and chemical stability than that of former dye-ligand affinity phases. However, the capacity of the column in respect to AGP unbound under the conditions of elution is practically indifferent, while important for the elimination of contaminating proteins retarded.

Investigating the glycopeptide structure tryptic fragments of AGP were separated by wide-pore (VYDAC) RP-HPLC and analysed by on-line LC - ESI-MS. Fig. 3 shows the UV (A), total ion (B) and selected ion (*m*/*z* 366) chromatograms (C) of tryptic digests derived from human serum AGP. In general, peptide and glycopeptide fragments of AGP, as well as autolytic fragments of trypsin could be distinguished in mass spectra. Total ion chromatogram was similar to the UV spectrum (Fig. 3A-B), while oligosaccharide content of glycopeptides were detected by a special mass spectrometric technique. Selected ion chromatograms of *m*/*z* 204 (HexNAc) and/or 366 (Hex-HexNAc) corresponding to the most intense glycan related ions were developed [12, 26]. These ions (in this particular case *m*/*z* 366) provided a fragmentation pattern specific only to glycopeptides to

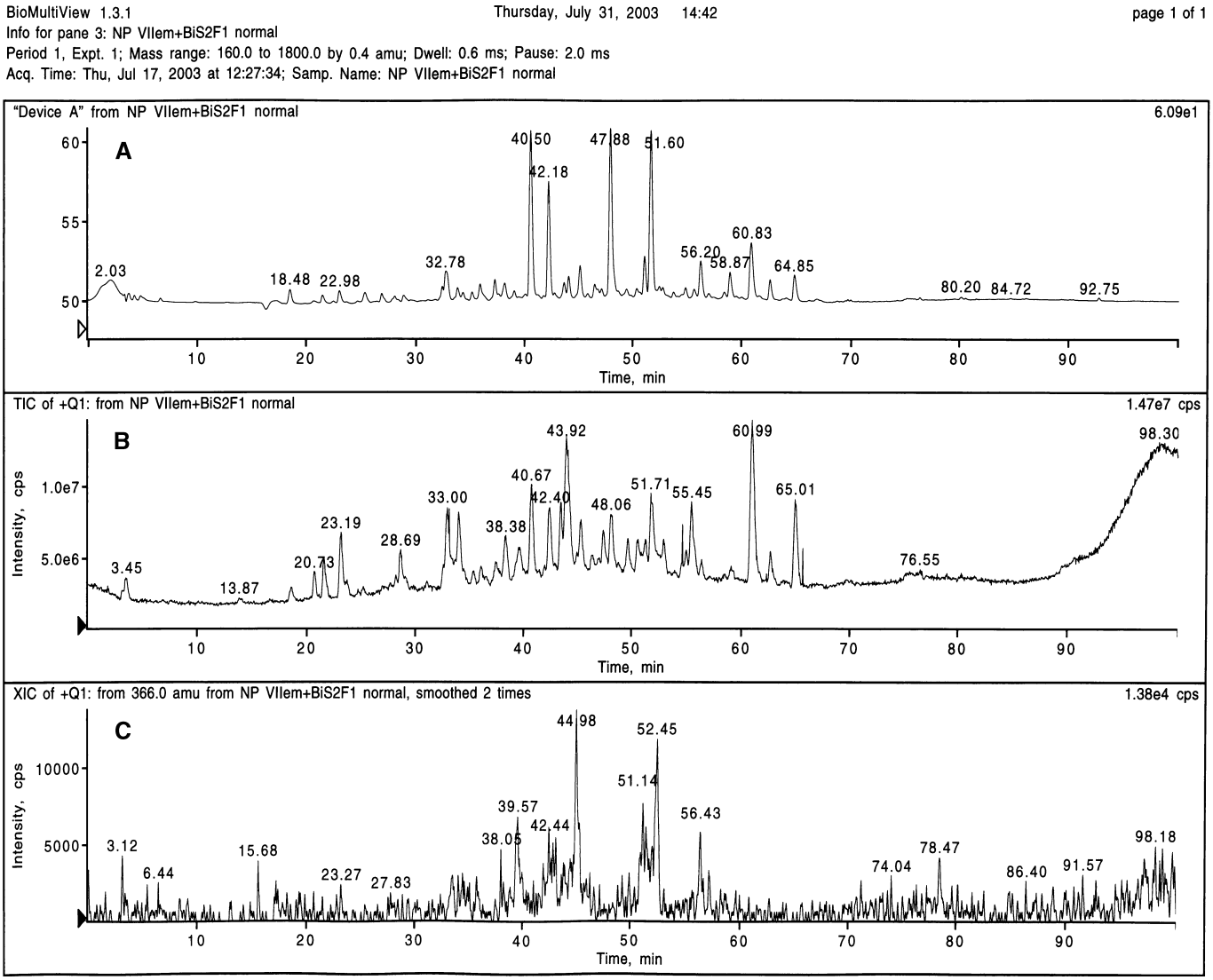

Fig. 3. On-line RP-HPLC – ESI MS analysis of tryptic fragments derived from human serum AGP. A – UV/276 nm, B – Total ion and C – Selected ion (m/z 366) chromatograms. RP-HPLC was carried out on a narrow-bore VYDAC Selectapore 300P C18 (25 × 0.21 cm, I.D., 5 μ) column using linear gradient elution with 15–50% (v/v) acetonitrile- 0.1%TFA in 90 min at ambient temperature. Flow rate: 0.2 mL min^{-1}, UV detection: 276 nm

identify them as they eluted by HPLC (Fig. 3C).

The oligosaccharide content of glycopeptides derived from human serum AGP was simultaneously identified and characterized by NH_2-phase HPLC. Fig. 4 shows the separation of antennary oligosaccharide structures followed by PNGase F enzyme digestion and derivatization of tryptic fragments obtained off-line from the RP-HPLC (Fig. 3A). Similarly to the selected ion chromatogram (Fig. 3C) patterns of Fig. 4 demonstrate that characteristic antennary oligosaccharide structures can be detected in the distinct fractions of tryptic fragments (mainly in the 40–45, 45–50 and 55–60 min segments of RP-HPLC). Both methods provided congruent results on the retention and character of glycopeptides derived from AGP and made possible to localize them in the RP-HPLC spectrum. In addition, NH_2-HPLC separation of anthranilate derivatives of oligosaccharides gave more detailed informations on the distribution of antennary structures in human serum AGP. According to the relative fluorescence intensities at 426 nm it could be concluded that the oligosaccharide content of human serum AGP was predominantly consisted of bi- and triantennary chains in average 38 and 30%, respectively. At the same time significant elevation in the relative proportion of tetraantennary compounds (9.6 *vs* 15.0%) and a 2-3 fold increase in the total amount of fucose containing structures were observed in cancer patients compared to the healthy controls. In accordance with our previous observations [36] RP-HPLC and MS analysis of tryptic fragments derived from serum AGP indicated anomal distribution of tri- and tetraantennary oligosaccharide structures in cancer. Further investigations are in progress to characterize these molecular changes in details.

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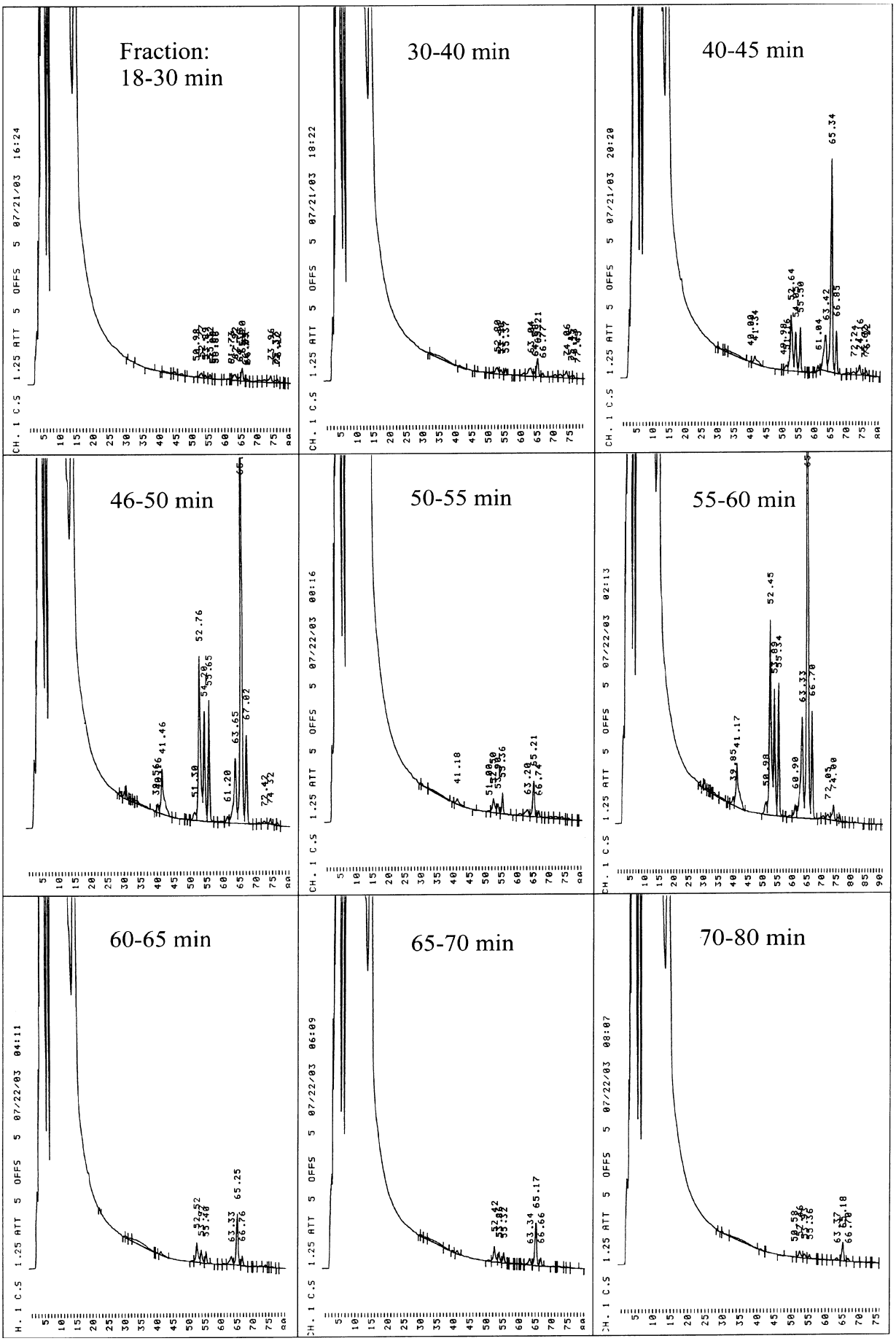
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Fig. 4. NH_2-HPLC separation of anthranilate derivatives of antennary oligosaccharide structures derived from the tryptic fragments of human serum AGP on an apHera^{TM} NH_2 polyamine bonded polymer based column (250×4.6 mm I.D., 5 μm, Astec) using a gradient elution program (see Experimental). Time periods correspond to the off-line collected fractions of RP-HPLC in Fig. 3

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References

1. Brown PR (1973) High Pressure Liquid Chromatograpy, Biochemical and Biomedical Applications. Academic Press, New York
2. Gelpi E (2003) J Chromatogr A 1000:567–581
3. Wang H, Hanash S (2003) J Chromatogr B 787:11–18
4. Hounsell EF (1994) Adv. Carbohydr. Chem Biochem 50:311–350
5. Van Dijk W, Havenaar EC, Brinkman–Van der Linden ECM (1995) Glycoconjugate J 12:227–233
6. Dall'Olio EJ (1996) Clin Pathol Mol Pathol 49:M126–M135
7. Hounsell EF, Young M, Davies MJ (1997) Clin Sci 93:287–293
8. Taylor CM (1998) Tetrahedron 54:11317–11362
9. Drickamer K, Taylor M (1998) TIBS 23:321–324
10. Rüdiger H, Siebert H-C, Solis D, Jiménez-Barbero J, Romero A, von der Lieth CW, Diaz-Maurino, T, Gabius H-J (2000) Current Med Chem 7:389–416
11. Harvey DJ (2001) Proteomics 1:311–328
12. Charlwood J, Bryant D, Skehel JM, Camilleri P (2001) Biomol Engineering 18:229–240
13. Damm JBI, Kamerling JP, van Dedem GWK, Vliegenthart JFG (l987) Glycoconjugate J 4:129–144
14. Hokke CH, Bergwerff AA, van Dedem GWK, Kamerling JP, Vliegenthart FG (1995) Eur J Biochem 228:981–1008
15. Mortz E, Sareneva T, Julkunen I, Roepstorff P J (1996) J Mass Spectrom 31:1109–1118
16. Rahbek-Nielsen H, Roepstorff P, Reischl H, Wozny M, Koll H, Haselbeck AJ (1997). J Mass Spectrom 32:948–958
17. Dage JL, Ackermann BL, Halsall HB (1998) Glycobiology 8:755–760
18. Wu JT, Nakamura RM (1997) Human circulating tumor markers. Current concepts and clinical applications. American Society of Clinical Pathologists Press, Chicago
19. Schmid K (1989) In: Alpha1-acid glycoprotein. Genetics, Biochemistry, Physiological Functions, and Pharmacology, P Baumann, WE Müller, CB Eap, JP Tillement (eds.) Alain R Liss Inc, New York, pp. 7–22
20. Kremer JMH, Wilting J, Janssen LMH (1988) Pharmacol Rev 40:1–47
21. Fournier T, Medjoubi-N N, Porquet D (2000) Biochem Biophys Acta 1482:157–171
22. Kishino S, Nomura A, Sugawara M, Iseki K, Kakinoki S, Kitabatake A, Miyazaki K (1995) J Chromatogr B 672:199–205
23. Tamura K, Shibata Y, Matsuda Y, Ishida N (1981) Cancer Res 41:3244–3252
24. Eggert FM, Jones M (1985) J Chromatogr 333:123–131
25. Elliott MA, Elliott HG, Gallagher K, McGuire J, Field M, Smith KD (1997) J Cromatogr B 688:229–237
26. Treuheit MJ, Costello CE, Halsall HB (1992) Biochem J 283:105–112
27. Patel PS, Adhvaryu SB, Balar DB, Parikh BJ, Shah PM (1994) Anticancer Res 14:747–752
28. Harvey HA, Lipton A, White D, Davidson E (1981) Cancer 47:324–327
29. Kremmer T, Boldizsár M, Kovács J, Paulik E, Bencsik K, Szajáni B (1995) J Liquid Chromatogr 18:1207–1218
30. Birkenmeier G, Kopperschläger G (1982) J Chromatogr 235:237–248
31. Laurent P, Miribel L, Bienvenu J, Vallve C, Arnaud P (1984) FEBS Letters 168:79–83
32. Hervé F, Millot M-C, Eap CB, Duché J-C, Tillement J-P (1996) J Chromatogr B 678:1–14
33. Azzimonti F, Atchley DH, Morrison CA, Dodd S, Boulton DW, DeVane CL, Arnaud P (2003) J Chromatogr B 678:1–14
34. Rudd PM, Dwek RA (1997) Current Opinion Biotech 8:488–497
35. Huang L, Riggin RM (2000) Anal Chem 72:3539–3546
36. Kremmer T, Ludányi K, Szöllősi É. Imre T, Schlosser G, Boldizsár M, Vincze B, Vékey K (2003) Biomed Chromatogr (in press)
37. Végh S, Kremmer T, Boldizsár M, Gesztesi KA, Szajáni B (1991) Clin Chim Acta 203:259–268
38. Anumula KR, Du P (1999) Anal Biochem 275:236–242
39. Turner GA, Skillen AW, Buamah P, Guthrie D, Welsh J, Harrison J, Kowalski A (1985) J Clin Pathol 38:588–592
40. Ganz PA, Ma PY, Wong HJ, Elashoff RM (1987) J Clin Oncol 5:472–479
41. Duché J-C, Urien S, Simon N, Malaurie E, Monnet I, Barré J (2000) Clin Biochem 33:197–202
42. Moule SK, Peak M, Thompson S, Turner GA (1987) Clin Chim Acta 166:177–185
43. Charet J-C, Watine J, Lepretre A, Raimbault C, Charet P (1996) Clin Biochem 29:287–290