Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1079 (2005) 354–365
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Application of capillary zone electrophoresis and reversed-phase high-performance liquid chromatography in the biopharmaceutical industry for the quantitative analysis of the monosaccharides released from a highly glycosylated therapeutic protein

K. Račaitytė, S. Kiessig, F. Kálmán *

Unit Analytics, Solvias AG, CH-4002 Basel, Switzerland

Available online 18 April 2005

Abstract

Two assays for the quantitative determination of the neutral and amino-monosaccharides attached to a therapeutic glycoprotein were developed using capillary zone electrophoresis (CZE) and RP-HPLC. These assays meet the strict batch release requirements of the quality control in biopharmaceutical industry. The monosaccharides were released from the glycoprotein by hydrolysis with 2N trifluoroacetic acid. In the CZE assay the monosaccharides were reacetylated prior to derivatization with 8-aminopyrenesulfonic acid (APTS), reacetylation in the glycoprotein matrix was investigated in detail. The RP-HPLC method used pre-column derivatization with anthranilic acid in methanol–acetate–borate reaction medium; reacetylation was not necessary. However, epimerization of the different monosaccharides was observed and studied in detail. For the quantitative assay, separation of the amino-monosaccharide epimers had to be developed. The HPLC assay was validated.

Keywords: Monosaccharides; Glycoproteins; Derivatization; Capillary zone electrophoresis; RP-HPLC; Epimerization; Reacetylation; Pharmaceutical analysis; Batch release

1. Introduction

A substantial part in the manufacturing process of pharmaceuticals is the assessment of product quality as identity, content and purity. Many regulatory guidelines and examples are apparent how to describe the appropriate and specific product characteristics by physico-chemical, microbial and analytical methodologies for conventional small therapeutic molecules, e.g. in testing monographs of the European and Japanese Pharmacopoeia or USP, in guidelines from the International Conference of Harmonization (ICH) [1], by the FDA. The situation is different for the today strongly emerging area of therapeutic biomolecules as proteins, glycoproteins, complex carbohydrates, liposaccharides, DNA therapeutics, virus particles, etc. obtained by biotechnological processes [2–11], which have a highly complex composition and structure. The biological production process itself shows usually a high variability, which introduces high product diversity [2–4,10,11]. Even from production batches only limited amounts of material might be available, requiring sensitive analytical technology. In conclusion, today there is a huge demand for the development of novel, straight forward, efficient and comprehensive analytical methodology, which is able to describe and secure product quality for this diverse class of complex therapeutic biomolecules.

It has been well documented, that the glycan composition and glycan structure of glycoproteins has a strong impact on their biological/therapeutic activity [2,6,12]. The degree of glycosylation as well as the glycosylation pattern of proteins produced in mammalian cells is largely influenced by

Abbreviations: AA, anthranilic acid, 2-aminobenzoic acid; APTS, 8-aminopyrene-1,3,6-trisulfonic acid trisodium; Fuc, fucose; Gal, galactose; GalN, galactosamine; GalNac, *N*-acetylgalactosamine; Glc, glucose; GlcN, glucosamine; GlcNac, *N*-acetylglucosamine; HPAEC-PAD, high-pH anion exchange chromatography with pulsed amperometric detection; LIF, laser induced fluorescence; Man, mannose; ManN, mannosamine; MS, monosaccharide; PBS, phosphate buffered saline; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TOC, total organic carbon

* Corresponding author.
E-mail address: franka.kalman@solvias.com (F. Kálmán).

0021-9673/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.03.080

the actual cell line as well as cell culture conditions used for production [13]. A sensitive measure for the consistency of glycoprotein drug substances/preparations with respect to their glycosylation is given by the determination of the molar ratio of individual monosaccharides with respect to protein. Typically the neutral monosaccharides galactose, mannose and fucose and the amino-monosaccharides *N*-acetylglucosamine and *N*-acetylgalactosamine are found and have to be determined in mammalian glycoproteins. There are no methods available for direct quantitative determination of complex carbohydrates attached to the protein backbone [14]. That means, the carbohydrate moiety needs to be cleaved from the glycoprotein and subsequently hydrolyzed completely in order to obtain the monosaccharide building blocks. Decomposition of the released monosaccharides during the cleavage has to be avoided, since that would adulterate their quantitation. Acidic hydrolysis using 2N trifluoroacetic acid for several hours at about 100 °C is the most common hydrolysis procedure [14–19].

Traditionally, both chromatography [12,16,20,21] and electrophoresis [22] are employed for the analysis of carbohydrates. Sensitive detection of monosaccharides is hampered by the absence of effective chromophores or fluorophores. Detection without derivatization by measurement of the refractive index or absorption in the UV region at 190–210 nm is restricted to the μmol to nmol range, respectively [23]. Quantitative composition analysis of oligosaccharides has relied in the past to a large extent on GC separation and flame ionisation detection of trimethylsilyl or alditol acetate monosaccharide derivatives. GC has good sensitivity, but derivatization chemistry is tedious and it results usually in very complex separation patterns due to stereochemical isomeric reaction products of the monosaccharides [14,24]. One of the most widely used method for sensitive (<1 nmol), quantitative analysis of monosaccharides employs high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [14,19,25,26]. It gives high resolution of all common monosaccharides in less than 30 min [14] and has the advantage of not requiring pre-column derivatization of monosaccharides [26]. However, due to the high pH of the eluent it needs special equipment, what is not commonly available in pharmaceutical quality control laboratories. Alternatively, sensitive, no derivatization requiring, fast (<20 min) carbohydrate analysis by CZE was developed using high alkaline pH (>12) electrolytes to ionize the carbohydrates. Using 2,6-pyridine-dicarboxylic acid as indirect UV agent for Glc a 70 μmol limit of detection was obtained [27]. Amino acids and peptides from glycoprotein hydrolysate do not interfere with the detection of carbohydrates as reported for HPAEC-PAD [25]. However, CZE separation at such high pH makes the baseline noisy and to our experience the CZE system unstable.

Today, sensitive (<1 nmol) methods using RP-HPLC with fluorescence detection or capillary electrophoresis with LIF detection are available for the quantitative determination of monosaccharides (see for HPLC reviews [12,16], for CE review [22]). These methods are based on precolumn derivatization of the monosaccharides by introducing fluorescence tags. This approach allows the analysis of glycoproteins in μg/ml protein concentration range, a typical concentration for pharmaceutical glycoprotein drug products. Among the various methods described for derivatization [28] reductive amination that introduces an aromatic amine to the aldehyde group of the carbohydrate is a widely applied procedure [18,29–36]. A broad set of labels for carbohydrate laser-induced fluorescence (LIF) detection, the most sensitive detection mode in CE, have been described, e.g. 2-aminopyridine [37,38], aminobenzoic acids [15,39], phenylmethylpyrazolone [29–31], 8-aminonapthalene-sulfonic acid [32–35], 8-aminopyrenesulfonic acid (APTS) [18,36,40], 7-amino-4-methylcoumarin [41] and 2-aminoacridone [42,43]. Among them APTS labelling is the most common approach in CE [22]. Detection limits as low as about 1 pmol for monosaccharide standards can be estimated from the literature [18,40]. APTS provides charges to the uncharged monosaccharides what is advantageous for their analysis by CZE [22,40].

RP-HPLC is the most widely used separation technique in today's pharmaceutical industry [44]. Common fluorescent tags used for labelling of the monosaccharides prior to RP-HPLC analysis are: anthranilic acid (AA), 2-aminobenzamide, 2-aminopyridine [12,16], phenyl isothiocyanate [12], 9-fluorenylmethoxycarbonylhydrazine, 7-amino-4-methylcoumarin, 7-amino-1,3-naphthalene-disulphonate [12]. Among the RP-HPLC methods, separation and detection based on AA is reported to provide the highest sensitivity. Detection limits about 5 pmol for hexose standards can be estimated [12,16]. The main advantage of AA is its suitability for the quantitative determination of both amino- and neutral monosaccharides without re-*N*-acetylation of the aminohexoses. Labelling with all other fluorescent tags requires, that the hexosamines have to be *N*-acetylated prior to their fluorescence labelling [16,18].

The present study describes the development of a sensitive quantitative assay for determination of the neutral monosaccharides (galactose, mannose and fucose) and the amino-monosaccharides (glucosamine and galactosamine) attached to a highly glycosylated therapeutic glycoprotein for batch release in the biopharmaceutical industry. The usefulness of the CZE method with APTS-labelling [18,40] and RP-HPLC method with AA derivatization [12,16] is discussed, validation results of the RP-HPLC assay are presented.

2. Experimental

2.1. Reagents and samples

2.1.1. Monosaccharides

D(+)-GlcN·HCl (>99%), D(+)-GalN·HCl (≥99%), D(+)-Gal (≥99.5%), D(+)-Glc (≥99.5%), D(+)-Man

(≥99.5%), D(+)-Fuc (>99%), GlcNac (>99%), GalNac (>98%) were supplied by Fluka (Buchs, Switzerland). D-ManN·HCl (>99%) was supplied by Sigma (Buchs, Switzerland).

2.1.2. Capillary electrophoresis

High purity APTS was obtained from Beckman Coulter (Palo Alto, CA, USA). Sodium cyanoborohydrate solution (1 mol/l) in THF was provided by Aldrich (Cat. No. 29,681-3, Buchs, Switzerland). Citric acid, boric acid, sodium hydrogen carbonate, 50% NaOH solution, 0.1 mol/l HCl and acetic anhydride were obtained in analytical reagent grade from Fluka (Buchs, Switzerland). Water (≥18.0 MΩ cm, TOC <20 ppb) was taken from a Milli-Q system (Millipore, Bedford, MA, USA).

CZE separation buffers were prepared as follows: 240 mM borate buffer pH 9.0: 1.48 g boric acid were dissolved in approximately 80 ml water. The pH of the obtained solution was adjusted to 9.0 using 50% sodium hydroxide solution. The obtained solution was then transferred completely into a 100 ml volumetric flask and adjusted to 100 ml with water. 120 mM borate buffer pH 10.2: 0.74 g boric acid were dissolved in approximately 80 ml water. The pH of the solution obtained was adjusted to 10.2 using 50% NaOH solution. The solution obtained was transferred completely into a 100 ml volumetric flask and adjusted to 100 ml with water.

2.1.3. RP-HPLC

AA (≥98%), sodium acetate anhydrous (≥99%), sodium cyanoborohydride (≥95%), 1-aminobutane (≥99.0%), boric acid (≥99.8%) and trifluoroacetic acid (≥99.5%) were purchased from Fluka (Buchs, Switzerland). Methanol (LC grade), THF (LC grade), phosphoric acid (85%) and acetic acid (glacial) were supplied by Merck, Darmstadt, Germany. Water (≥18.0 MΩ cm, TOC <20 ppb) was taken from a Milli-Q system (Millipore).

2.1.4. Glycoprotein

The glycoprotein under investigation had a concentration of approximately 1 mg/ml and was dissolved in PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate). Protein concentration was determined prior to analysis by measuring absorption at 280 nm and calculating content by using its specific absorption coefficient.

2.2. Sample preparation

2.2.1. Sample preparation for CZE

2.2.1.1. Standard solutions. Sample preparation was performed as described by Chen et al. [18] in an adapted manner. Six single stock solutions of GalN, ManN, GlcN, Man, Glc, Fuc and Gal containing 5 mmol/l of the appropriate monosaccharide in PBS were prepared. The stock solutions were diluted 1:10 using PBS. By mixing the appropriate volumes of the diluted stock solutions three different monosaccharide standard solutions 1–3 were obtained: *CZE-standard solution* 1: GalN (1.5 nmol), GlcN (15 nmol), Man (18 nmol), Glc (8 nmol), Fuc (2 nmol), Gal (18 nmol) per vial. *CZE-standard solution* 2: GalN (2.5 nmol), GlcN (35 nmol), Man (12 nmol), Glc (8 nmol), Fuc (4 nmol), Gal (10 nmol) per vial. *CZE-standard solution* 3: GalN (0.5 nmol), GlcN (25 nmol), Man (6 nmol), Glc (8 nmol), Fuc (6 nmol), Gal (26 nmol) per vial. The monosaccharide CZE-standard solutions 1–3 were mixed with TFA. The final concentration of TFA in each solution was 2N. In a next step the monosaccharide CZE-standard solutions 1–3 containing 2N TFA were heated for 5 h at 100 °C. Aliquots of the "hydrolyzed" standard solutions were prepared containing 1/10 of the original volume each. The aliquots were placed in fresh 500 μl tubes and evaporated to dryness using a vacuum centrifuge concentrator. If not used immediately the dried samples were stored at −18 °C.

2.2.1.2. Protein samples. Approximately 40 μg of the glycoprotein were placed in a screw top vial completed with 8 nmol Glc and hydrolyzed in 2N TFA for 5 h at 100 °C. Aliquots of the hydrolyzed protein sample were prepared containing 1/10 of the original volume each. Each aliquot of the hydrolyzed protein sample was placed in fresh 500 μl tube and evaporated to dryness using a vacuum centrifuge concentrator. If not used immediately the dried samples were stored at −18 °C.

2.2.1.3. Reacetylation. The aliquots of the "hydrolyzed" CZE-standard solutions 1–3 as well as the aliquots of the hydrolyzed protein samples were subject to derivatization with or without reacetylation. If reacetylation was applied, the samples were reconstituted in the 500 μl tube with 5 μl of 25 mM sodium hydrogen carbonate, pH 9.5. In the following 2 μl acetic anhydride were added and the sample was vortexed. Afterwards the samples were incubated at room temperature for 30 min. Finally they were concentrated to dryness using a vacuum centrifuge concentrator.

2.2.1.4. Derivatization. Fifty milligrams of APTS were completely dissolved in 48 μl citric acid aqueous solution (1 mol/l). The solution obtained was mixed in equal parts with 1 mol/l sodium cyanoborohydrate solution in THF. This mixture (derivatization reagent) was prepared immediately before use. To the dried aliquots of the standards or protein samples either after or without reacetylation 5 μl of the derivatization reagent were added. The mixture was vortexed and incubated for 90 min at 55 °C in a water bath. The derivatization reaction was stopped by the addition of 200 μl water. Aliquots of the derivatized samples were stored at −18 °C. For CZE analysis the samples were diluted 10fold with distilled water prior to analysis.

2.2.2. Sample preparation for HPLC

Hydrolysis and derivatization were carried out as described by Anumula [12,15].

2.2.2.1. Standard solutions. Stock solutions were prepared as described in Section 2.2.1. By mixing the appropriate volumes of stock solutions three HPLC-standard solutions were obtained: *HPLC-standard solution* 1: GlcN (12.5 nmol), GalN (0.625 nmol), Gal (7.5 nmol), Man (5.0 nmol) and Fuc (1.25 nmol) per vial. *HPLC-standard solution* 2: GlcN (25.0 nmol), GalN (1.25 nmol), Gal (15.0 nmol), Man (10.0 nmol) and Fuc (3.75 nmol) per vial. *HPLC-standard solution* 3: GlcN (37.5 nmol), GalN (2.5 nmol), Gal (22.5 nmol), Man (15.0 nmol) and Fuc (6.25 nmol) per vial. Additionally two standard solutions with Glc as internal standard were prepared in PBS: *HPLC-standard solution A*: GlcN (22.5 nmol), GalN (15 nmol), Gal (22.5 nmol), Man (22.5 nmol), Fuc (15.0 nmol) and Glc (22.5 nmol) per vial. *HPLC-standard solution B*: GlcN (28.0 nmol), GalN (1.6 nmol), Gal (22.8 nmol), Man (14.0 nmol), Fuc (5.6 nmol) and Glc (10.5 nmol) per vial. All HPLC-standard solutions were mixed with TFA (20% final TFA concentration) and heated for 5 h at 100 °C. Afterwards they were dried in a vacuum centrifuge evaporator without heat. The dried samples were reconstituted in 100 μl of 1% (w/v) sodium acetate solution and derivatized with AA reagent as described below.

2.2.2.2. Protein samples. Thirty to eighty micrograms of glycoprotein were hydrolyzed in 20% trifluoroacetic acid for 5 h at 100 °C. In the following they were dried in a vacuum centrifuge evaporator without heat. The dried samples were reconstituted in 100 μl of 1% (w/v) sodium acetate solution and derivatized with AA reagent as described below.

2.2.2.3. Derivatization. A methanol–acetate–borate solution was prepared by dissolving 2.4 g sodium acetate and 2 g boric acid in 100 ml methanol. AA reagent was prepared by dissolving 30 mg AA and 20 mg sodium cyanoborohydride in 1 ml of the methanol–acetate–borate solution. The reconstituted carbohydrate samples were mixed with 100 μl AA reagent and heated for 1 h at 80 °C. Thereafter, the samples were cooled to ambient temperature and diluted to 1 ml with HPLC eluent A. In order to remove particles from the solution and to protect the system in particular the column from contamination and blockage, after dilution with eluent A sample solutions were filtrated through a Cetrifugal Ultrafree®–MC 0.45 μm (Millipore) filter unit.

2.3. CZE analysis

CZE analysis was performed on a PACE 5010 or a PA 800 from Beckman Coulter (Palo Alto, CA, USA) equipped with a LIF detector and Argon ion laser (excitation at 488 nm). The fluorescence emission at 520 nm (band pass filter) was collected. Fused silica capillaries (Polymicro, Phoenix, AZ, USA), internal diameter 30 μm, total length 27 cm (20 cm effective length to the detector) in case of the P/ACE 5010 or 31 cm (21 cm effective length to the detector) in case of the PA 800 were used, separation buffer: 240 mM borate buffer pH 9.5 or 120 mM borate buffer pH 10.2. A field strength of 741 V/cm resulting in a current of 35 μA or 925 V/cm resulting in a current of 53 μA with normal polarity (anode at autosampler end) was applied, respectively. The capillary temperature was 20 °C. Samples were kept in the autosampler at 5 °C, hydrodynamic injection using a pressure of 3.45 kPa (0.5 p.s.i.) for 5 s was used. A new capillary was constituted by flushing it in sequence with 0.1 N HCl (5 min), water (5 min), 1 M NaOH (10 min), water (5 min) and separation buffer (10 min), applying a pressure of 138 kPa (20 p.s.i.). Between the runs the capillary was flushed with separation buffer for 5 min.

2.4. HPLC analysis

All LC separations were performed on a liquid chromatograph 1100 Series (Agilent Technologies, Waldbronn, Germany) equipped with an integrated degasser, a quaternary pump, an autosampler, a cooling device and a fluorescence detector. For detection the following wavelengths were used: excitation 230 nm, emission 425 nm. Control of the HPLC system and data evaluation was performed with an Agilent ChemStation, version A.09.03 (Agilent Technologies, Waldbronn, Germany). The separation was carried out at a temperature of 25 °C and the samples were stored in the autosampler at 4 °C. *Separation system I*: C_{18} reversed phase column: YMC-Pack ODS-A 150 mm × 4.6 mm I.D., particle size 5 μm, pore size 12 nm (YMC Separation Technologies, YMC Europe, Schermbeck, Germany). Precolumn: Phenomenex C_{18}, 4 mm × 3 mm I.D. (Phenomenex, Aschaffenburg, Germany). Mobile phase: Eluent A contained 0.3% 1-aminobutane, 0.5% phosphoric acid and 1% THF in water, eluent B consisted of equal parts of solvent A and acetonitrile [15]. The following gradient program was used: 0–35 min 6% B isocratic, 35–55 min linear gradient 6–12% B, 55–65 min 100% B isocratic. Re-equilibration of the column was performed with 100% A for 20 min. The flow rate was 0.85 ml/min. *Separation system II:* C_{18} reversed-phase column: Hypersil BDS 150 mm × 4.6 mm, particle size 3 μm (Thermo Hypersil, Kleinostheim, Germany). Precolumn: Phenomenex C_{18}, 4 mm × 3 mm ID (Phenomenex). Mobile phase: sodium acetate–methanol; eluent A contained 50 mM sodium acetate buffer pH 4.1 in water, eluent B contained 20% eluent A in methanol. The following gradient program was used: 0–35 min 3% B isocratic, 35–80 min linear gradient 3–8% B, 80–85 min linear gradient 8–9% B, 85–90 min 9% B isocratic, 90–95 min 100% B linear gradient. In order to ensure the reproducibility from run to run, the column was washed then with 100% B for 20 min and re-equilibrated with 3% B for 24 min. The flow rate was 0.7 ml/min.

3. Results and discussion

3.1. Application of APTS-labelling and CZE for the quantitative determination of monosaccharides released from a highly glycosylated glycoprotein drug substance

Sample preparation and CZE were performed according to Chen et al. [18] in an adapted manner (see Section 2). Two different CZE systems were tested, one used 240 mM borate pH 9.0, the other 120 mM borate pH 10.2. Both separation systems showed similar, reproducible performance for the monosaccharide standards. Peak shape and number of theoretical plates of the monosaccharides were better for the 240 mM system. The advantage of the 120 mM system was, that ManNac was baseline separated from the other amino-monosaccharides and APTS-labelling impurity peaks, not relevant for the present assay development. Visualization of the monosaccharides was performed after APTS labelling by LIF detection, optimization of labelling conditions was reported in detail in several publications [17,18,45]. A high yield of APTS-labeled carbohydrate standards was obtained in a robust and reproducible manner. In the following the monosaccharide standards were subjected to hydrolysis prior to labelling with APTS. During acidic hydrolysis *N*-acetylated-amino-monosaccharides are deacetylated and the reactivity of the amino-monosaccharides with APTS is strongly reduced [18], decreasing drastically their detection sensitivity. Reacetylation of the sample with acetic anhydride can solve this problem [18]. A comparison of the determination of 1.5 nmol GlcN and 0.15 nmol GalN with and without reacetylation is given in Fig. 1. Glucosamine can be detected in the acetylated and non-acetylated form, but the loss in sensitivity for the APTS–GlcN response in comparison to APTS–GlcNac is more than 15-fold. For the reacetylated standard, the electropherogram shows a complete transformation of GlcN into GlcNac. The non-acetylated GalN cannot be detected at the concentration used. The recovery of the other neutral monosaccharides Gal, Man and Fuc is not influenced by reacetylation.

During derivatization with APTS the carbonyl group, available only on the reducing end of the neutral monosaccharides reacts with the primary amine of APTS. That means that APTS is attached to each monosaccharide in a one-to-one stoichiometry. Assuming the same reactivity for all the monosaccharides, all of them should have a similar concentration—fluorescence response dependency. Due to the complexity of the method, well known matrix effects on the labelling reaction [2] as well as small reaction volumes and a varying test sample matrix a three point calibration for each monosaccharide was employed. Linear response–concentration plots for standard solution are summarized in Table 1. Poor correlation was obtained for all monosaccharides. The absolute fluorescence responses were varying by about 50% of the mean value (data not shown). These large fluctuations were probably due to differences in the absolute yield of the derivatization reaction in the different reaction vials. In order to improve the correlation Glc, not apparent in the glycoprotein, was added and the reduced peak area of each monosaccharide was normalized by the reduced peak area of Glc. As expected and shown in Table 1, this normalization procedure improves the reproducibility of the data significantly and supports the hypothesis, that the fluctuations are due to differences in the absolute yield of the derivatization. The normalized linear response–concentration plots for all APTS-monosaccharide derivatives show similar slopes, indicating a similar reactivity of the different monosaccharides.

In a next step, the monosaccharide content of a glycoprotein test samples was analyzed, results are compared to the theoretical values in Table 2, a typical electropherogram is shown in Fig. 2A. In comparison to CZE-standard mixtures (Fig. 1) the electropherogram shows more peaks, which are probably due to side reactions of APTS with constituents of the hydrolyzed and reacetylated glycoprotein matrix. The

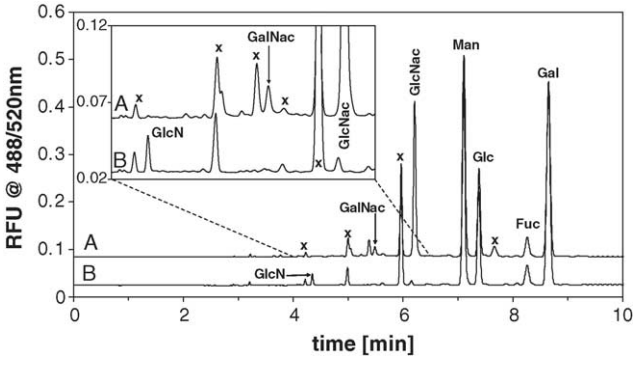

Fig. 1. CZE separation of an APTS-labelled monosaccharide standard mixture (CZE-standard solution 1) treated like glycoprotein drug substance with acidic hydrolysis, followed by reacetylation (A) and without reacetylation (B), CZE separation buffer: 240 mM borate pH 9.0, for other parameters, see Section 2. Peaks caused by labelling solution or by the reacetylation are marked with "x".

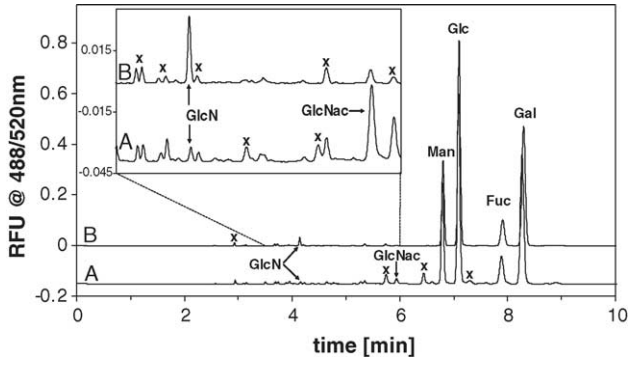

Fig. 2. CZE separation of monosaccharides released from glycoprotein drug substance by acidic hydrolysis, subject to reacetylation (A) and not reacetylated (B), followed by labelling with APTS, CZE separation buffer: 240 mM borate pH 9.0, for other parameters, see Section 2. The sample was supplemented with 25 nmol Glc for peak area normalization before acidic hydrolysis.

Table 1
Results of a linearity plot for the monosaccharides GalN, GlcN, Man, Fuc and Gal

Linearity plot		GalN (0.05–0.25 nmol)	GlcN (1.5–3.5 nmol)	Man (0.6–1.8 nmol)	Fuc (0.2–0.6 nmol)	Gal (1.0–2.6 nmol)
Without normalisation	[*S*]	133462	152738	198952	208852	182690
of reduced peak area	[*I*]	−747	7718	−14756	6448	22874
MS to reduced peak area of glucose	[*r*]	0.970	0.916	0.951	0.957	0.929
With normalisation of	[*S*]	0.817	0.973	1.135	1.383	1.188
reduced peak area	[*I*]	−0.005	−0.026	−0.007	0.007	0.027
MS to reduced peak area of glucose	[*r*]	0.996	0.994	0.999	0.994	0.997

The concentration of each (re)acetylated, APTS-labelled monosaccharide was plotted against its fluorescence intensity and subject to linear regression analysis. CZE-standard solutions 1–3 were used, each solution was prepared three times ($n = 9$). The slope [*S*], intercept [*I*] and the correlation coefficient [*r*] are presented. The results were calculated with and without normalisation of the reduced peak area MS. In case of normalisation the reduced peak area of each monosaccharide was divided by the reduced peak area of glucose. CZE separation buffer: 120 mM borate pH 10.2. For other parameters, see Section 2.

recovery of neutral monosaccharides is within a range of 84–90%. In contradiction, the amino-monosaccharides GalN and GlcN show poor recovery of 12 and 17%, respectively. When analyzing an independently prepared CZE-standard solution 1, treated like a test sample, recoveries for all monosaccharides are between 96 and 102% (Table 2). In Fig. 2, the electropherogram of the reacetylated, APTS-labeled glycoprotein test sample (Fig. 2A) is compared to a test sample not subjected to reacetylation (Fig. 2B). It is clearly seen, that in the reacetylated test sample the amino-monosaccharide GlcN was not fully converted to the GlcNac. *N*-acetyl-GalN, only present in small quantities in the test sample, cannot be detected in the non-acetylated sample and only in small quantities in the reacetylated sample. As discussed before, the sensitivity for non-reacetylated amino-monosaccharides is much less than for the acetylated amino-monosaccharides. It follows, that an incomplete reacetylation in the hydrolyzed glycoprotein matrix might be an explanation for the poor recovery of the amino-monosaccharides. The monosaccharides are released from the glycoprotein by acidic hydrolysis with 2N TFA at 100 °C for 5 h. These are similar conditions as used for protein hydrolysis prior to their quantitative amino acid analysis [46]. During the liberation of the monosaccharides from the protein with 2N TFA one might assume a partial hydrolysis of the protein backbone, resulting in protein fragments. These amino acids and peptides with free amino groups probably compete for acetylation with the amino-monosaccharides, resulting in their incomplete reacetylation. Several efforts were made to increase the recovery of the APTS labeled *N*-acetyl-amino-monosaccharides in the hydrolyzed glycoprotein matrix as optimizing the reacetylation medium, using higher buffer capacity and higher concentrations of acetic anhydride, but all these attempts failed. Probably sample cleanup after hydrolysis or other optimization steps of the reacetylation reaction are needed to get higher yields of the amino-monosaccharides. For economy of time and resources the assay was not developed further.

Table 2
Analysis of monosaccharide content

Content (MS)	GalN	GlcN	Man	Fuc	Gal
(A) Test sample analysed by CE					
Recovery (theory) (%)	12 ± 4	17 ± 4	84 ± 4	90 ± 7	90 ± 7
(B) CE-standard solution 1					
Theory (nmol)	0.15	1.50	1.80	0.20	1.80
Found by CZE (nmol)	0.15	1.53	1.80	0.19	1.73
Recovery (theory) (%)	100	102	100	95	96
(C) Test sample analysed by HPLC					
Recovery (theory) (%)	63 ± 2	80 ± 3	82 ± 2	93 ± 3	86 ± 2

(A) Recovery of monosaccharides released from glycoprotein test sample compared to content calculated from the glycan structure of the glycoprotein. The test sample was prepared (hydrolyzed) three times, from each preparation three aliquots were subject to reacetylation, labelling and analysis ($n = 9$). (B) Recovery of CZE-standard solution 1, prepared once like test sample. Three point external standard calibration was performed with CZE-standard solutions 1–3, each prepared three times. The results were calculated with normalisation of the reduced peak area MS to glucose. CZE separation buffer: 120 mM borate pH 10.2. For other parameters, see Section 2. (C) Recovery of monosaccharides released from glycoprotein test sample. The test sample was prepared six times and each injected once. Three point external standard calibration was performed with HPLC-standard solutions 1–3, each prepared once. For conditions, see Section 2, separation system II.

3.2. Application of AA-labelling and RP-HPLC for the quantitative determination of monosaccharides released from a highly glycosylated glycoprotein drug substance

Sample preparation and chromatographic separation were performed in an adapted manner to Saddic and Anumula [12] (see Section 2). The method is based on quantitative, equimolar pre-column derivatization of monosaccharides with AA. Reductive amination is performed in methanol–acetate–borate reaction medium. Parameters affecting the derivatization, such as temperature, water content, hydrolysis time, reaction time and concentration of AA are described in detail elsewhere [12,15,16]. Prior to derivatization, the individual monosaccharides are released from glycoprotein by acidic hydrolysis with 2N TFA. During method development, it was found that 5–6 h of hydrolysis are optimal for quantitative monosaccharide determination (data not shown).

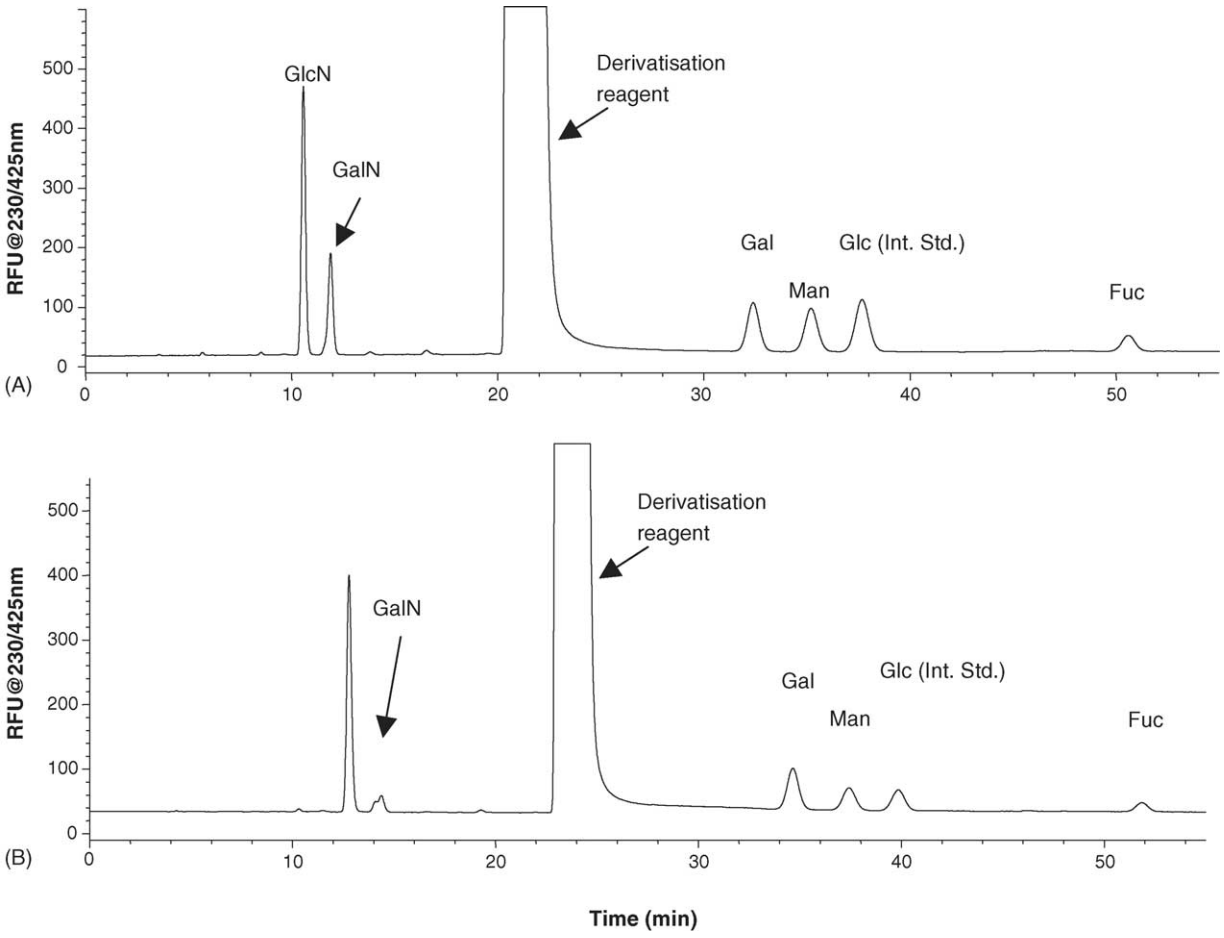

Fig. 3. RP-HPLC separation of AA labelled monosaccharides on a YMC-Pack ODS-A column using 1-aminobutane/phosphoric acid/tetrahydrofuran/water/acetonitrile mobile phase, for other parameters, see Section 2, separation system I. (A) HPLC-standard solution A, (GlcN, GalN, Gal, Man, Fuc and Glc), (B) HPLC-standard solution B (GlcN, GalN, Gal, Man, Fuc and Glc), with monosaccharide concentrations at their expected concentration in the test sample, calculated from the glycan structure of the glycoprotein.

The RP-HPLC separation of the six AA-monosaccharide standards Man, Gal, Fuc, GalN, GlcN and Glc in the nanomolar concentration range is shown in Fig. 3A. The monosaccharides elute in two groups: the amino- and the neutral monosaccharides. All monosaccharides AA-derivatives are well separated from each other and from the excess of reagent. Glc, not present in the glycoprotein under investigation, was tested as internal standard. As discussed in Section 3.1, external three point calibration for each monosaccharide was used. The separation system proved to be very reproducible and the method showed excellent sensitivity. In Fig. 3B, the separation of the six monosaccharide standards at the expected concentration is presented. It is seen that the AA-GalN peak appears at low concentrations as a splitted peak, a reproducible phenomenon. The splitted peak was also observed in glycoprotein samples. Since this splitted peak would hamper the precise quantitation of GalN, various attempts were made to get the splitted AA-GalN peak separated into two baseline resolved peaks by slightly changing the chromatographic system I as altering the gradient, varying the 1-butylamine/phosphoric acid ratio and the amount of THF in the mobile phase. These efforts were not successful.

Attempts were made to identify the origin of the unknown peak co-eluting with AA-GalN. During early phase method development ManN was considered as an internal standard for the quantitation of the amino-monosaccharides, but AA-ManN co-elutes with AA-GalN. At higher concentrations they elute in one peak similar to the AA-GalN peak in Fig. 3A, at the lower concentration they elute as splitted peaks similar to AA-GalN peak in Fig. 3B. It was suspected, that the additional peak co-eluting with AA-GalN is AA-ManN. There was no plausible explanation, why one would find ManN in a standard solution mixture of the five monosaccharides under investigations. In the following the individual AA-labelled amino-monosaccharides standards ManN, GlcN, and GalN were analyzed, the chromatograms are shown in Fig. 4. Indeed, AA-GlcN can be well separated from AA-GalN, but AA-GalN is eluting at approximately the same time as AA-ManN. Surprisingly, it is also seen that every amino-monosaccharide investigated is eluting in two peaks. The ManN standard solution gives an additional peak at the

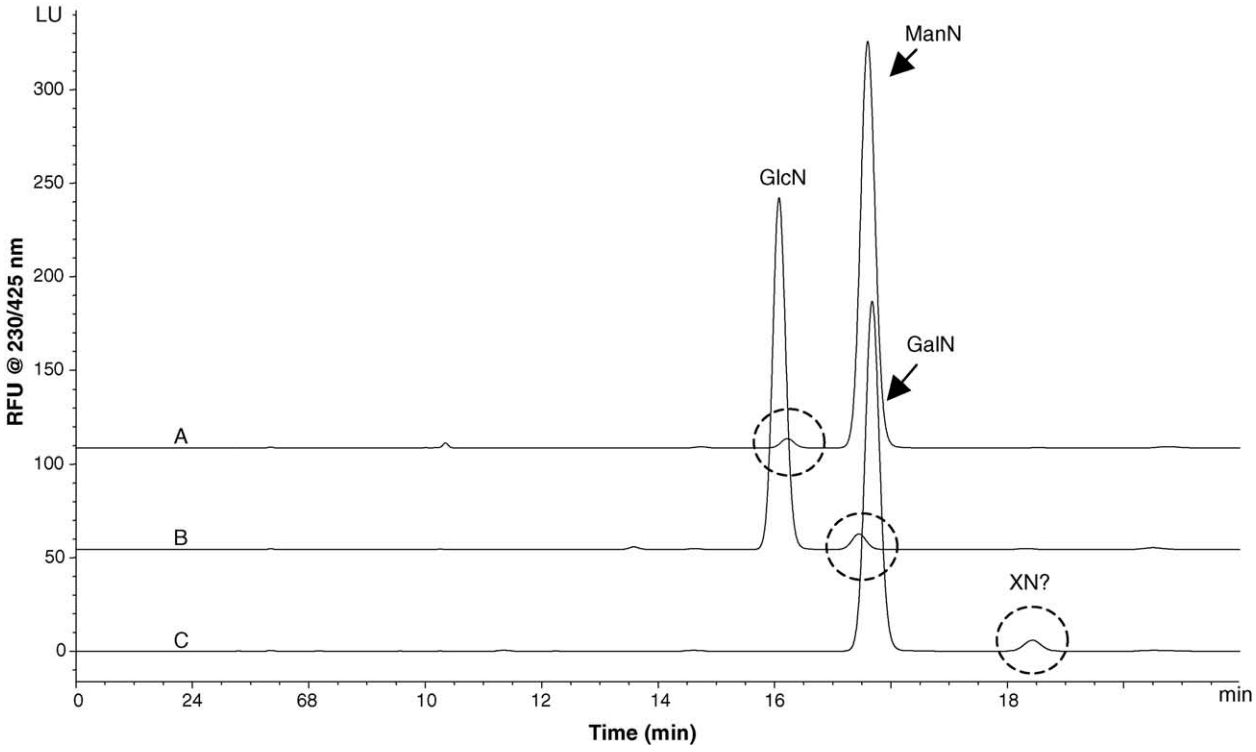

Fig. 4. Separation of individual AA-labelled amino-monosaccharides: (A) ManN, (B) GlcN, (C) GalN, injection: 0.5 nM of each amino-monosaccharide, for other parameters, see Section 2, separation system I.

retention time of AA-GlcN (Fig. 4A), the GlcN standard shows an additional peak with the retention time corresponding to AA-ManN (Fig. 4B), an additional peak (AA-XN?) was also observed for GalN standard solutions (Fig. 4C). The content of the additional peak in the GlcN solutions with the retention time corresponding to ManN was about 5%, in the ManN standard solutions about 2% and in the GalN solutions about 3.7%. These ratios proved to be independent on the amino-monosaccharide concentration (Table 3). However, the purity of the amino-monosaccharide standards was >99%. In the CZE analysis using APTS-labelling (Section 3.1) never impurity peaks in the above amounts were observed in the individual amino-monosaccharide standards. Further, the "impurity" peak ManN found in GlcN-standard appears in similar amounts in the glycoprotein test sample as well (estimated from the splitted GalN peak). From structural investigations it is known that the glycoprotein does not contain ManN, likewise the CZE analysis of the glycoprotein did not show any ManN in the glycoprotein test samples. Analyzing literature data it was found that derivatization of GlcN with AA in methanol–acetate reaction medium is accompanied by epimerization of GlcN to ManN [15], what would explain the presence of the ManN in the GlcN standard as well as in the glycoprotein test sample. Furthermore, based on the review of Samuel et al. [47] one can assume that the epimerization can occur in both directions as the inversion of ManN to GlcN is also possible, explaining the GlcN peak in the ManN standard. Epimerization of the activated stereocenter of hexoseamines is due to protonation/deprotonation of their adjacent aldehyde carbonyl in the acidic reaction medium and can easily be understood considering all equilibria between their different species in solution (hemiacetal, open chain, keto and enol form). Assuming that epimerization of an activated stereocenter in GalN occurs with the same mechanism as in GlcN and ManN, the inversion of GalN to the rare amino-monosaccharide talosamine is suspected [47]. This

Table 3
Ratio of peak area hexosamine standard/peak area respective impurity at two hexosamine standard concentrations

Hexosamin	Hexosamine/respective impurity peak (identification see Fig. 4)	Ratio peak area respective impurity/peak area hexosamine, (%)	
		0.05 nmol[a]	0.5 nmol[a]
GlcN	ManN/GlcN	4.7	4.8
ManN	GlcN/ManN	1.9	2.1
GalN	XN?/GalN	3.6	3.7

For conditions, see Section 2, separation system II.
[a] Amount of hexosamine (per injection).

hypothesis was not proven, since talosamine is not commercially available. Only literature for its synthesis via a novel dihydroxylation reaction was found [48]. As mentioned before, the ratios of AA-XN?/AA-GalN, AA-ManN/AA-GlcN and AA-GlcN/AA-ManN are constant at given derivatization conditions and independent on the hexosamine concentration (Table 3). It follows, that the epimerization itself does not disturb the quantitation of the individual monosaccharides by external calibration. Since GalN is one of the amino-monosaccharides to be determined in the glycoprotein, and AA-GalN is co-eluting with the AA-GlcN epimer AA-ManN, the separation needed to be optimized in order to assure precise quantitation of GalN.

In consequence, a new separation system (separation system II) using acetate–methanol mobile phase and a Hypersil BDS C_{18} stationary phase was developed. Fig. 5 shows the analysis of monosaccharide HPLC-standard solution 2, containing all five monosaccharides to be investigated, in comparison with a glycoprotein test sample. It is seen that all monosaccharides AA-derivatives are well separated from each other and the AA-GalN and AA-ManN peak are baseline separated. It is also evident that the presence of the protein matrix and an excess of labelling reagent in the separation mixture do not disturb the separation. The separation system proved to be very robust. Using separation system II the monosaccharide content of glycoprotein was determined and compared to the theoretical values. For the neutral monosaccharides comparable results with the CZE analysis were obtained (Table 2). However, the recovery for the amino-monosaccharides was much higher, about 63–80%. One hundred percent recovery of the monosaccharides in comparison to theoretically calculated values was not expected, since the glycoprotein drug substance is a heterogenic mixture of differently glycosylated proteins. The full recovery of monosaccharides was proven in model experiments during method validation, as described in the following section.

3.3. Validation of the AA-labelling/RP-HPLC assay for the quantitative determination of monosaccharides of a highly glycosylated glycoprotein

The assay is intended for use in a routine lot release environment. Therefore, the requirements for assay performance are stringent, especially in terms of linearity, precision and accuracy [1]. Acceptance criteria, in particularly percent of relative standard deviation, are recommended by the ICH [1]. The assay was validated for its intended use: to characterize the glycoprotein for its content of neutral monosaccharides and to investigate batch to batch consistency as well as product stability with a required precision of 10% error. The adjustments are justified by the complexity of the assay as given by the many steps in sample preparation and by the complexity and heterogeneity of the test samples. The validation results and validation acceptance criteria are summarized in Table 4.

3.3.1.1. Linearity

The linear range of the assay was validated using five monosaccharide standards. The plots of concentration against response were linear in the tested range of 0.25–37.6 nmol/ml for GlcN, 0.25–2.5 nmol/ml for GalN, 0.6–22.9 nmol/ml for Gal, 0.6–15.0 nmol/ml for Man, and 0.8–6.4 nmol/ml for Fuc, six concentrations per monosaccharide, with double injection were analyzed. In Table 4, it is seen that the monosaccharides show similar slopes, indicating a similar reactivity of the different monosaccharides with AA.

3.3.1.2. Accuracy/recovery

Recovery was established by spiking a representative test sample (glycoprotein drug substance) with different amounts of GlcN, GalN, Gal, Man and Fuc (six concentrations each, covering the linearity range, single injection). Results were obtained by calculating the difference of the respective monosaccharide content found in the non-spiked test sample and the spiked one. Mean recovery values for the accuracy of the assay were obtained between 96.1 and 100.2%.

3.3.1.3. Repeatability/precision

Precision and repeatability of the assay were confirmed by three independent validation tests: precision/repeatability of the whole process, precision/repeatability of injection and intermediate precision. The repeatability of whole process was demonstrated by preparing and analyzing one test sample as well as HPLC-standard solution 2, six times each. The precision of injection was confirmed by injecting six times a test sample preparation. Intermediate precision, proving the transferability of the assay to other release laboratories, was demonstrated by preparing and analyzing the same two test samples by two different operators, with different batches of chemicals and reagents including calibration solutions, on two different HPLC instruments and with two different batches of the RP-HPLC columns (Table 4). One can conclude that the assay performs with high precision/repeatability.

3.3.1.4. Stability of solutions

In order to ensure, that the test samples prepared are not degraded or altered after sample preparation prior to analysis in the autosampler at 4 °C, the stability of solution was investigated analyzing a representative test sample as well as HPLC-standard solution 2 directly after preparation, 33, 47 and 65 h, the change in response (peak area) was monitored. It was found that the labelled, ready to analyse test and reference samples are stable at least 65 h. The change of response of each monosaccharide peak in resulting chromatograms was between 0.2 and 5.2%.

3.3.1.5. Quantitation limit (LOQ)

The LOQ of the assay based on the 10× signal-to-noise ratio (ICH) and relative standard deviation of the response ($S_{rel} \leq 20\%$, $n = 6$) was 5.0 pmol for GlcN and GalN, 12.6 pmol for Gal and Man, and 15.0 pmol for Fuc per injection.

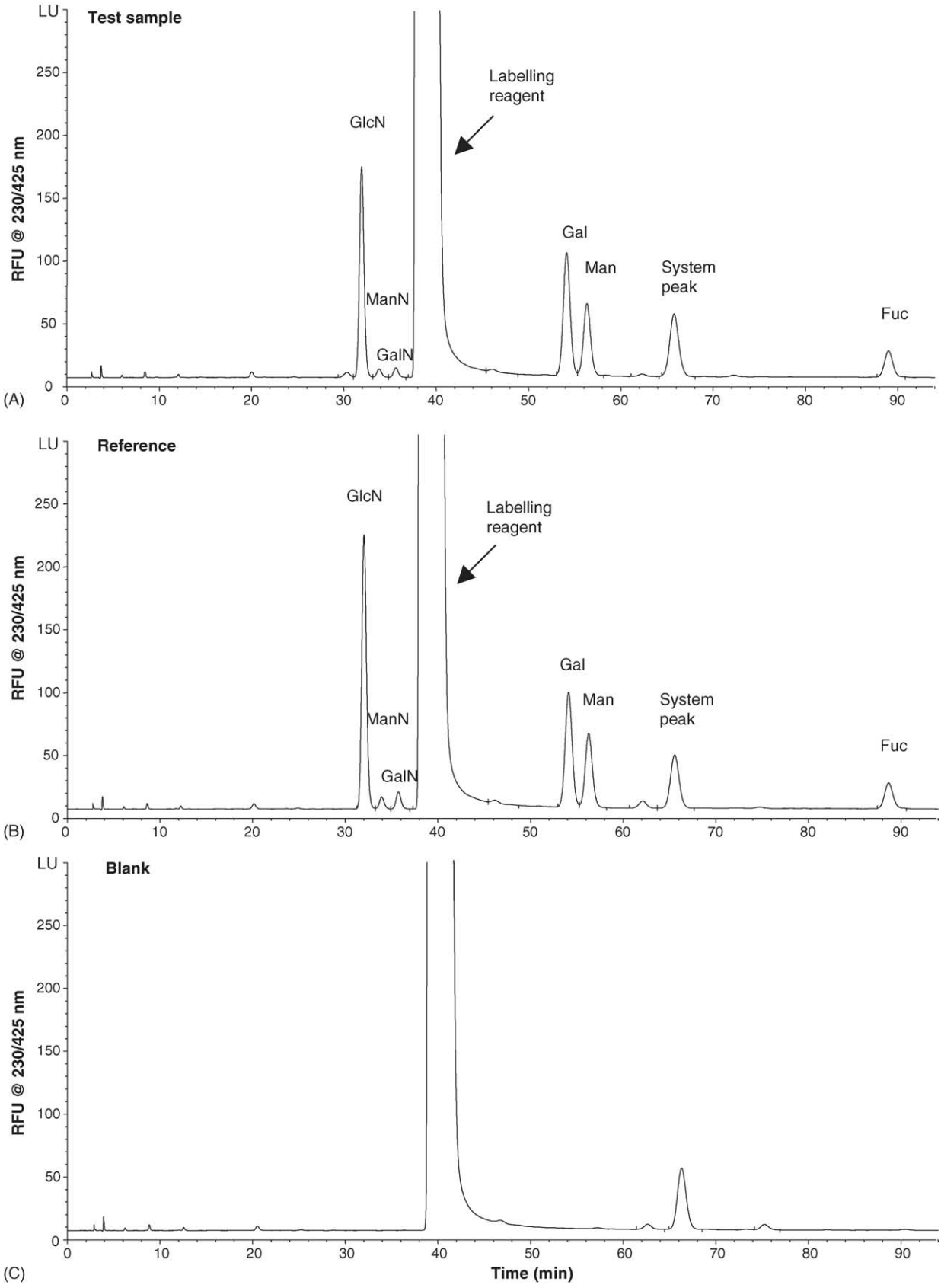

Fig. 5. RP-HPLC separation of AA-labelled monosaccharides on a Hypersil® BDS C_{18} column using 50 mM sodium acetate pH 4.1/methanol mobile phase, for other parameters, see Section 2, separation system II. (A) glycoprotein test sample; (B) HPLC-standard solution 2 (GlcN, GalN, Gal, Man, Fuc); (C) PBS-buffer.

Table 4
Summary of RP-HPLC method validation

Test	Acceptance criteria	GlcN	GalN	Gal	Man	Fuc
Accuracy						
Recovery (%)	80–120	96.1	99.3	96.9	98.9	100.2
S_{rel} (%, $n = 6$)	≤15	12.3	8.2	5.5	2.6	3.5
Linearity						
y-intercept	≤25%	3.2	0.8	1.4	0.4	0.3
Slope	–	330.7	415.9	318.1	317.9	360.1
Correlation coefficient, *r*	≥0.990	0.997	0.999	0.999	0.998	0.997
Precision of whole process						
S_{rel} (%, $n = 6$) for test sample	≤10	4.2	2.0	3.2	1.8	2.5
S_{rel} (%, $n = 6$) for reference solution	≤10	3.8	6.1	4.9	2.3	2.1
Precision of injection						
S_{rel} (%, $n = 6$)	≤5	3.2	4.3	3.0	2.0	4.5
Intermediate precision						
S_{rel} (%, $n = 4$)	≤10	6.6	4.4	2.3	2.7	4.3
Specificity	Chromatographic peaks separated, no indication of interferences	√	√	√	√	√
Limit of quantitation (LOQ)[a] (pmol)		5	5	12.6	12.6	15
Peak/noise ratio	≥10	14	17	26	26	32
S_{rel} (%, $n = 6$)	≤20	6.9	2.6	1.5	3.8	2.2

For conditions, see Section 2, separation system II.
[a] Per injection.

The specificity of the assay was established through method development and reconfirmed during validation. All reaction and hydrolysis by-products, particularly the derivatization reagent, are well separated from the target analytes. Epimers of the amino-monosaccharides do not disturb the quantitation, placebo does not exhibit any interference (Fig. 5). Robustness testing in terms of slightly changed derivatization/chromatographic conditions was not tested and is subject to future investigations.

In conclusion, the assay described here is suitable for the quantitative monosaccharide analysis of a given glycoprotein drug substance in a routine lot release environment based on its excellent performance results obtained for linearity, accuracy, repeatability/precision, stability of solution, limit of quantitation and specificity as demonstrated above.

4. Conclusions

A CZE-LIF and a RP-HPLC-fluorescence assay were developed for the sensitive, quantitative determination of the monosaccharide content of a given glycoprotein drug substance in PBS buffer, providing the molar ratio of individual monosaccharides to protein, to be used as a measure for the consistency/stability of glycoprotein drug substance preparations. In both assays the monosaccharides are released from the glycoprotein by acidic hydrolysis followed by their labelling with a fluorophore for sensitive detection: for CZE with APTS, for RP-HPLC with AA. Both methods are reproducible, use state of the art, common instrumentation and chemicals. Sample clean up is not required prior to analysis. Both methods show similar sensitivity and both methods proved to be reproducible and robust. Although the assays were developed and the RP-HPLC assay was validated for a particular drug substance glycoprotein matrix, they can be applied in monosaccharide studies in general, including other glycoproteins and antibodies.

The advantage of the CZE assay is the fast analysis time and the use of inexpensive and common silica capillaries. Further, advantageous is the fact that in the reaction medium used for labelling with APTS no side reactions occur. The drawback of the CZE assay is that for efficient, sensitive APTS-labelling reacetylation of the amino-monosaccharides has to be performed, which is an additional sample preparation step. For the glycoprotein under investigation the reacetylation of the amino-monosaccharides was not satisfactory, resulting in too low recoveries of the amino-monosaccharides. For glycoproteins containing only neutral monosaccharides the assay should be applicable and can be validated without tedious optimization.

The main advantage of the RP-HPLC method using AA-labelling is that derivatization of hexosamines does not require re-*N*-acetylation of amino-monosaccharides. The drawback of the AA-labelling is, that in methanol–acetate–borate reaction medium epimerization of the amino-monosaccharides occurs, resulting in two peaks for every amino-monosaccharide. The ratios of XN?/GalN; ManN/GlcN and GlcN/ManN proved to be constant and independent on

the hexosamine concentration. In the developed chromatographic system the epimers of the amino-monosaccharides are well separated from the target analytes and do not disturb the quantitation of the amino-monosaccharides.

References

[1] International Conference on Harmonization, Guideline on the validation of analytical procedures: methodology, Fed. Reg. 62 (1997) 27464.
[2] J.R. Ramussen, M. Bergh, S. Hirani, Patent WO8907641 (1989).
[3] G. Hunt, W. Nashabeh, Anal. Chem. 71 (1999) 2390.
[4] S. Ma, W. Nashabeh, Anal. Chem. 71 (1999) 5185.
[5] K. Kanazawa, K. Ashida, M. Itoh, H. Nagai, H. Sasaki, M. Fukuda, Biol. Pharm. Bull. 22 (4) (1999) 339.
[6] T. Toyoda, T. Itai, T. Arakawa, K.H. Aoki, H. Yamaguchi, J. Biochem. 128 (2000) 731.
[7] N.E. Robinson, A.B. Robinson, PNAS 98 (22) (2001) 12409.
[8] N. Viseux, X. Hronowski, J. Delaney, B. Domon, Anal. Chem. 73 (2001) 4755.
[9] S. Elliott, T. Lorenzini, S. Asher, K. Aoki, J. Egrie, Nature Biotechnol. 21 (2003) 414.
[10] A. Braun, R. Gassman, K. Kraus, G. Lorenzi, u. Weigel, Pharm. Acta Helv. 71 (1996) 447.
[11] V.E. Bichsel, V. Curcio, R. Gassman, H. Otto, Pharm. Acta Helv. 71 (1996) 439.
[12] G.N. Saddic, M.B. Ebert, S.T. Dhume, K.R. Anumula, Methods Mol. Biol. 194 (2002) 23.
[13] T. Patel, R. Parekh, B. Moellering, C. Prior, Biochem. J. 285 (1992) 839.
[14] P. Hermentin, in: F. Lottspeich, H. Zorbas (Eds.), Bioanalytik, Spektrum Verlag, Heidelberg, Berlin, 1998, pp. 485–536.
[15] K.R. Anumula, Anal. Biochem. 220 (1994) 275.
[16] K.R. Anumula, Anal. Biochem. 283 (2000) 17.
[17] A. Guttman, J. Chromatogr. A 763 (1997) 271.
[18] F.A. Chen, T.S. Dobashi, R.A. Evangelista, Glycobiology 8 (1998) 1045.
[19] S. Kim, S. Kim II, K.-S. Ha, S.-H. Leem, Exp. Molec. Med. 32 (2000) 141.
[20] S.C. Churms, J. Chromatogr. 500 (1990) 555.
[21] M.F. Chaplin, J.F. Kennedy (Eds.), Carbohydrate Analysis: A Practical Approach, IRL Press, Oxford, 1986, pp. 43–51.
[22] C. Chiesa, R.A. O'Neil, C.G. Horváth, P.J. Oefner, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996.
[23] S. Hase, J. Chromatogr. Lib. 55 (1995) 555.
[24] A. Fox, S.L. Morgan, J. Gilbert, in: C.J. Biermann, G.D. McGinnis (Eds.), Analysis of Carbohydrates by GLC and MS, CRC Press, Boca Raton, FL, 1989.
[25] M.R. Hardy, R.R. Townsend, Y.C. Lee, Anal. Biochem. 170 (1988) 54.
[26] R.R. Townsend, A. Manzi, R.K. Merkle, M.F. Rhode, M. Spellman, A. Smith, S.A. Carr, ABRF News 8 (1997) 14.
[27] T. Soga, D. Heiger, Anal. Biochem. 261 (1998) 73.
[28] F.N. Lamari, R. Kuhn, N.K. Karamanos, J. Chromatogr. B 793 (2003) 15.
[29] S. Honda, T. Ueno, K. Kakehi, J. Chromatogr. 608 (1992) 289.
[30] S. Honda, S. Suzuki, A. Nitta, S. Iwase, K. Kakehi, Methods, Compan. Methods Enzymol. 4 (1992) 233.
[31] S. Suzuki, R. Tanaka, K. Takada, N. Inoue, Y. Yashima, A. Honda, S. Honda, J. Chromatogr. A 910 (2001) 319.
[32] P. Jackson, Biochem. J. 270 (1990) 705.
[33] C. Chiesa, C.G. Horváth, J. Chromatogr. 645 (1993) 337.
[34] C. Chiesa, R.A. O'Neil, Electrophoresis 15 (1994) 1132.
[35] M. Larsson, R. Sundberg, S. Folestad, J. Chromatogr. A 934 (2001) 75.
[36] F.A. Chen, R.A. Evangelista, Anal. Biochem. 230 (1995) 273.
[37] S. Hase, S. Hara, Y. Matsushima, J. Biochem. 85 (1979) 217.
[38] D.K. Toomre, A. Varki, Glycobiology 4 (1994) 653.
[39] H. Schweiger, P. Oefner, C. Huber, E. Gill, G. Bonn, Electrophoresis 15 (1994) 941.
[40] R.A. Evangelista, M.-S. Liu, F.A. Chen, Anal. Chem. 67 (1995) 2239.
[41] C. Prakash, I.K. Vijah, Anal. Biochem. 128 (1983) 41.
[42] P. Jackson, Anal. Biochem. 216 (1994) 243.
[43] G.B. Harland, G. Okafo, P. Matejtschuk, I.C. Sellick, G.E. Chapman, P. Camilleri, Electrophoresis 17 (1996) 406.
[44] Cs. Horváth, W. Melander, I. Molnár, J. Chromatogr. 125 (1) (1976) 129.
[45] A. Guttman, F.A. Chen, R.A. Evangelista, N. Cooke, Anal. Biochem. 233 (1996) 234.
[46] M. Fountoulakis, H.-W. Lahm, J. Chromatogr. A 826 (1998) 109.
[47] J. Samuel, M.E. Tanner, Nat. Prod. Rep. 19 (2002) 261.
[48] T.J. Donohue, K. Blades, M. Helliwell, Chem. Abstr. 131 (1999) 299623a.