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