doi:10.2533/chimia.2016.732 Chimia 70 (2016) 732–735 © Swiss Chemical Society

FH HES

Universities of Applied Sciences

Fachhochschulen – Hautes Ecoles Spécialisées

Nucleotide and Nucleotide Sugar Analysis in Cell Extracts by Capillary Electrophoresis

Blanka Bucsella, Antoine Fornage, Catherine Le Denmat, and Franka Kálmán*

*Correspondence: Prof. Dr. F. Kálmán, E-mail: franka.kalman@hevs.ch, HES-SO Valais, University of Applied Sciences, Sion, Wallis, Institute of Life Technologies, Route du Rawyl 47, CH-1950 Sion 2

Abstract: In biotechnological processes the intracellular level of nucleotides and nucleotide sugars have a direct impact on the post-translational modification (glycosylation) of the therapeutic protein products and on the exopolysaccharide pattern of the cells. Thus, they are precursors and also key components in the production of glycoproteins and glycolipids. All four nucleotides (at different phosphorylation stages) and their natural sugar derivatives coexist in biological samples. Their relative ratios depend on the actual conditions under which the cells are grown. Therefore, their simultaneous determination at different time points and different cell culture conditions in biotechnological samples is of interest in order to develop the optimal cell culture process. In our study capillary electrophoresis (CE) combined with UV detection @ 260 nm was selected for the separation and quantification of the complex nucleotide mixture of the structurally very similar nucleotides and nucleotide sugars in cell extracts. The high separation efficiency of CE as well as its insensitivity to the complex cell matrix makes this method superior to commonly used HPLC methods. In our study eleven nucleotides and six nucleotide sugars were analyzed. A robust and reproducible analysis system was developed. As background electrolyte borate (40 mM, pH 9.5) was used containing 1% PEG (MW 35'000 Da) which enhanced resolution. In order to obtain high reproducibility in terms of migration time, mandatory for the unambiguous identification of the single compounds in the complex cell extract mixtures, dynamic coating was also employed. The method was tested for CHO cell extracts where three sugar nucleotides and seven nucleotides were identified and quantified using GDP-Glc as internal standard.

Keywords: Capillary electrophoresis · Cell extracts · Direct detection · Nucleotide · Nucleotide sugar

1. Introduction

Efficacy of therapeutic proteins may depend on their post-translational modifications, especially their glycosylation.[1] It was reported that the nucleotide and sugar nucleotide pool in mammalian cells has a direct impact on the glycan pattern of the glycoproteins.[2–6] Two different types of glycosylation, the O- and N-types, can be distinguished. The O-linked glycans are attached to the hydroxyl group of serine or threonine *via* N-acetylgalactosamine (GalNac). In all N-linked oligosaccharides, N-acetylglucosamine (GlcNAc) is linked to the amide nitrogen of asparagine. Due to the fact that 50% of the therapeutic proteins are manufactured by mammalian cell lines and the biological activity of them is glycan pattern dependent, in the biopharmaceutical industry interest has arisen to investigate and understand the regulation and biosynthesis of the glycosylation process in order to obtain optimal cultivation conditions. The mammalian glycosylation process (the glycan synthesis and the glycan attachment to the protein backbone) takes place in the endoplasmic reticulum (ER) and the Golgi apparatus with sugar nucleotides (activated monosaccharaides) as precursors.[5] These sugar nucleotides are uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), uridine diphosphate glucose (UDP-Glc), uridine diphosphate N-acetylgalactosamine (UDP-GalNAc), uridine diphosphate galactose (UDP-Gal), guanosine diphosphate mannose (GDP-Man), guanosine diphosphate fucose (GDP-Fuc), cytidine monophosphate N-acetylneuraminic acid (CMP-Neu5Ac), uridine diphosphate glucoronic acid (UDP-GlcA) and cytidine monophosphate N-glycolylneuraminic acid (CMPNeu5Gc). In the biosynthesis of the sugar nucleotides the ribonucleotide triphosphates (adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphosphate (UTP)) also play a key role *via* nucleic acid synthesis, cellular growth, and energy metabolism.[6] Further mono- and diphosphate nucleotides also participate in the nucleotide sugar biosynthesis and energy metabolism such as adenosine diphosphate (ADP), adenosine monophosphate (AMP), cytidine diphosphate, cytidine monophosphate (CMP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), uridine monophosphate (UMP), and uridine diphosphate (UDP).

Several extraction procedures exist to extract nucleotides/nucleotide sugars from mammalian cells based on acetonitrile (ACN), methanol and perchloric acid (PCA) solvent systems.[7,8] A systematic investigation was carried out by Dietmair and others who concluded that the use of 50% ACN was sufficient to extract all nucleotide/nucleotide sugars with a high recovery from CHO cells.[7] For their qualitative and quantitative analysis of the cell extracts reliable and high-resolution analytical methods are necessary. Recently, high-performance anion-exchange chromatography (HPAEC), reverse phase ion pairing high-performance liquid chromatography and capillary electrophoresis methods have been used.[9–14] These methods have some drawbacks such as the long analysis time, poor, not sufficient separation efficiency and non-reproducible separation times.[9,11,13] The aim of this work was to set up a robust separation and quantification method that can be applied for the analysis of CHO cell extracts in order to optimize the cell culturing conditions for a defined industrial therapeutic glycoprotein. For this reason a suitable capillary electrophoretic method was selected and further optimized.[10] The optimized separation system was tested for the quantification of the nucleotides and nucleotide sugars in CHO cell extracts.

2. Experimental

2.1 *Materials*

All standard nucleotides (AMP, ADP, ATP, CMP, CTP, GMP, GTP, UMP, UDP, UTP) and nucleotide sugars (GDP-Glc, UDP-GlcNAc, UDP-Glc, UDP-GalNAc, UDP-Gal, UDP-Gal) were supplied by Sigma-Aldrich (Buchs, Switzerland) except

GDP, which was obtained from Carbosynth (Berkshire, UK). Boric acid was purchased from Fluka (Buchs, Switzerland) and poly(ethylene glycol) MW 35'000 Da from Sigma-Aldrich (Buchs, Switzerland). Ready-to-use solutions for dynamic coating of the capillary were taken from the CEofix Carbo Kit, Analis SA (Namur, Belgium).

2.2 *Instrumentation*

All electrophoretic separations were performed on an Agilent 7100 3D CE instrument equipped with a DAD detector and with external gas pressure (air 5.5 bars). The detection wavelength was 260 nm. The length of the fused bare silica capillary was 67.5 cm/76 cm, its internal diameter 50 µm, (BGB, Boeckten, CH). The capillary was thermostated at 18 °C. Each new capillary was conditioned with 1M NaOH, distilled water and the background electrolyte for 20 min each. Before daily use the capillary was flushed with dynamic coating (solutions: initiator, conditioner, accelerator) from CEofix Carbo Kit (Analis SA, Namur, Belgium) following the procedure of the manual. Before each injection the capillary was conditioned with initiator for 90 s at 3.4 bars and for 180 s at 3.4 bars with accelerator. The inlet electrode was washed in run buffer and the capillary was rinsed for 180 s at 3.4 bars with background electrolyte. Samples were injected 15 s at 0.3 bars. Separation was performed at 30 kV (normal polarity) for 60 min with a ramp of 1 min from 0 to 30 kV. At the end 10 kV was applied with both capillary ends in conditioner for 120 s followed by a wait time of 120 s.

2.3 *Preparation of the Electrolytes and Standard Solutions*

The background electrolyte (BGE) borate stock solution (200 mM) was prepared by weighing 12.36 g boric acid in a 250 mL beaker and adding 150 mL water. Boric acid was dissolved under agitation. 1M NaOH was added to obtain a pH between 6 and 8. The solution was filled up to 250 mL in a volumetric flask and filtered through a 0.45 µm membrane. The final BGE was prepared in a 200 mL beaker weighing 2.00 g polyethylene glycol and adding 40 mL 200 mM borate stock solution and about 150 mL water. The pH was adjusted to 9.5 with 1M NaOH. The solution was filled up to 200 mL in a volumetric flask with distilled water and filtered through a 0.45 µm membrane filter.

To prepare the standards, an approximately 1 mM stock solution was prepared for each standard, separately. 5 mg nucleotide/nucleotide sugar was weighed into a 10 mL volumetric flask and filled up to the mark with distilled water. Until further usage it was stored at –18 °C.

To test linearity 5–50 µM standard mix solutions containing all standards were prepared; *i.e.* a 50 µM standard mix solution contained 50 µL from each of the 16 1 mM standards and 25 µM from GDP-Glc I mM (applied as internal standard) filled up with 175 µL distilled water. The standard mix solutions were stored at –18 °C.

2.4 *Samples*

The samples were provided by the Biotechnology Group of the Institute of Life Technologies (University of Applied Sciences, Sion, Wallis) from CHO cell cultures to perform preliminary tests. The extraction method was carried out on the freshly harvested CHO cells (contained 3×10^7 cells) according to the protocol of Dietmair *et al*.[7] The extracts were stored at –18 °C until further analysis.

2.5 *Data Analysis*

For quantification the response factor of each nucleotide/nucleotide sugar was calculated as the ratio between the peak area and concentration. This ratio is a constant for each nucleotide and nucleotide sugar. The relative response factor was applied for concentration determination of each compound in cell extracts, 25 µM GDP-Glc was used as internal standard.

3. Results and Discussion

3.1 *Effect of Dynamic Coating on the Reproducibility of the Migration Time*

An initial CE separation system was set up based on the method of Fen *et al*.[10] Separation efficiency was acceptable, but migration time reproducibility after 5 successive injections of a standard mix was not sufficient, *e.g.* a 6% RSD (n = 5) was measured for the migration time of UDP-GalNac (for mobility 1%), which is not sufficient for unambiguous identification and quantification of the different nucleotides and nucleotide sugars in cell extracts without spiking each standard (which would result in a very high work load) (Fig. 1). Therefore, dynamic coating was tested to control the electroosmotic flow (EOF) and to gain a robust separation system with smaller shifts in migration. The principle behind the dynamic coating strategy is that the capillary is rinsed with a polycation buffer (depicted as 'initiator') that is able to cover the silanol groups of the capillary wall. This is followed by a rinsing step with a polyanion buffer (depicted as 'accelerator') results in a double layer on the capillary wall which provides a constant charge density on the capillary wall and with that a constant EOF among different analysis.[15] After applying the coating procedure the RSD (n = 5) in migration time for UDP-GalNa was 0.3%, for mobility 0.2%, sufficient for unambiguous identification of the compounds.

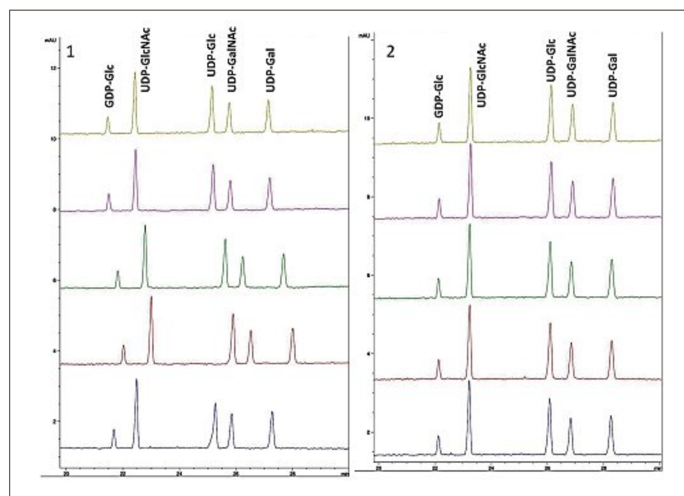

Fig. 1. Electropherograms of five successive injections of a mixture of standards (15 µM GDP-Glc (IS), 50 µM each as UDP-GlcNAc, UDP-Glc, UDP-GalNAc, UDP-Gal); conditions see experimental part, temperature here: 15 °C; 1: without dynamic coating 2: employing the dynamic coating CEofix procedure.

3.2 *Optimization of the Separation System*

In order to obtain the ideal separation parameters for the 16 nucleotides/nucleotide sugars with the application of the dynamic coating, the pH and the concentration of the background electrolyte, the capillary temperature, and the applied voltage were optimized. As an example the optimization of the electrolyte pH is depicted on Fig. 2. With 40 mM pH 9.5 borate buffer full baseline separation of all standards investigated was achieved.

The optimal conditions for the separation are 18 °C capillary temperature, 30kV, BGE: 40 mM borate pH 9.5, detection at 260 nm, injection: 15 s, 0.3 bar. For the calibration an internal standard was applied (25 µM GDP-Glc) and the relevant response factors

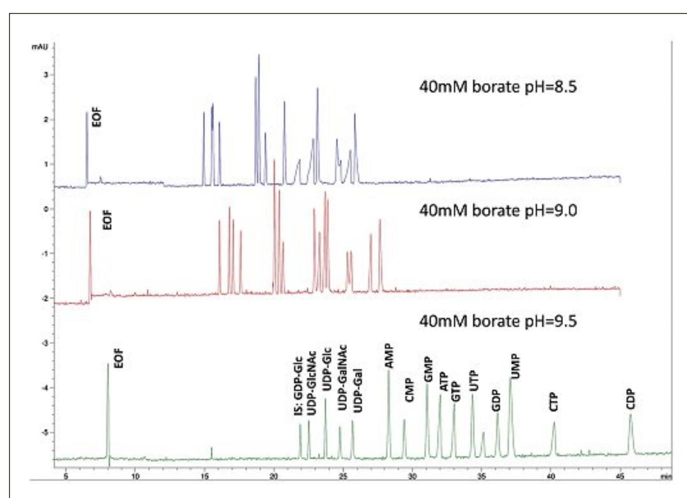

Fig. 2. Electropherograms of a mixture of all 16 standards at different pHs of the background electrolyte (40 µM each all standards, 25 µM GDP-Glc (IS); conditions see experimental part; separations are employing the dynamic coating CEofix procedure.

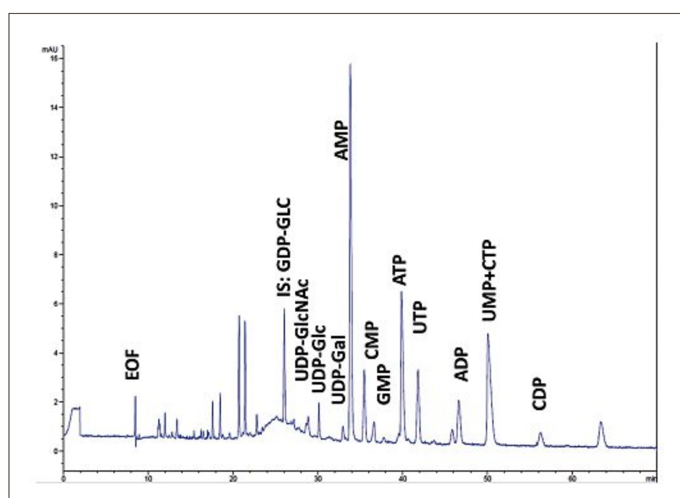

Fig. 3. Electropherogram of the acetonitrile extracted nucleotide and nucleotide sugars from CHO cells. Conditions see experimental part; separations are employing the dynamic coating CEofix procedure.

were used for quantification of each nucleotide or nucleotide sugar in the cell extracts (Table 1). The calibration was set up in the range from 5 to 50 µM.

3.3 *Testing the Optimized System with Cell Extracts*

In order to test the optimized method extracts were obtained from CHO cells and analyzed without derivatization or concentration step. The electropherogram is depicted on Fig. 3.

Under the conditions employed eleven nucleotide/nucleotide sugars were detected and quantified in the CHO extracts. Since standards and extracts are dissolved in different solvents, a shift in EOF and migration time is observed between extracts and standard solutions, but based on electrophoretic mobilities all compounds can be unambiguously identified without extra spiking experiments. It is seen that more peaks than in the standard mix are obtained, which correspond probably to nucleotides/nucleotide sugars for which no standards were available. In the cell extracts ATP and AMP presented in the highest concentration. Separation of UMP and CTP in the cell extracts was not possible due to some matrix effects on the separation system.

Table 1. Migration time values, electrophoretic mobilities and response factors (calculated from the areas applying GDP-Glc as internal standard) for the standard nucleotides and nucleotide sugars (n = 3)

Standards	Migration time [min]		μ_{EP} [cm^2/Vs]		Response Factor [GDP-Glc]	
	Mean	RSD%	Mean	RSD%	Mean	RSD%
IS: GDP-Glc	22.40	0.3	-2.22	0.2	1.00	0.0
UDP-GlcNAc	23.24	0.3	-2.26	0.2	0.76	3.7
UDP-Glc	24.45	0.3	-2.32	0.2	1.24	3.0
UDP-GalNAc	25.50	0.3	-2.38	0.2	0.74	2.9
UDP-Gal	26.92	0.3	-2.42	0.2	0.89	4.3
AMP	28.94	1.1	-2.51	0.4	2.26	4.0
CMP	30.44	1.1	-2.55	0.4	1.10	4.5
GMP	32.24	0.8	-2.61	0.4	2.08	5.1
ATP	33.29	1.9	-2.64	0.7	2.09	8.9
GTP	34.11	2.3	-2.67	0.8	2.61	44.2
UTP	35.48	1.2	-2.69	0.4	2.89	32.9
ADP	36.29	2.3	-2.72	0.7	0.95	4.7
GDP	37.20	1.4	-2.74	0.5	2.07	22.7
UMP	37.76	1.5	-2.75	0.5	2.20	32.0
CTP	39.93	1.4	-2.76	0.5	2.20	32.9
CDP	43.88	3.3	-2.83	0.9	1.51	5.6
UDP	46.60	3.0	-2.91	0.7	2.29	8.9

4. Conclusion

A capillary electrophoresis method was optimized for the separation and quantification of 16 nucleotides and nucleotide sugars with the application of dynamic coating. The assay is straightforward and simple, it does not need derivatization and direct detection is possible at 260 nm with a LOQ of about 3 μM under the conditions employed. The analysis system was tested on CHO cell extracts and appeared to be high resolution and robust for quantification of nucleotides and nucleotide sugars in biotechnological samples. The nucleotides and nucleotide sugars are stable at room temperature at least 48 h in the acetonitrile extracts. As next steps the optimization of the sample preparation/extraction method is planned to achieve higher sensitivity.

Acknowledgements

This work was financially supported by HES-SO RESEARCH Funds for Interdisciplinary Projects (ITV16socle_Bio-En-Bac project No. 59170). CEofix™ Carbo kits were kindly provided by Analis SA (Namur, Belgium).

Received: September 15, 2016

[1] N. Jenkins, L. Murphy, R. Tyther, *Mol. Biotechnol.* **2008**, *39*, 113.
[2] N. S. C. Wong, L. Wati, P. M. Nissom, H. T. Feng, M. M. Lee, M. G. S. Yap, *Biotechnol. Bioeng.* **2010**, *107*, 321.
[3] M. Gawlitzek, H. S. Conradt, R. Wagner, *Biotechnol. Bioeng.* **1995**, *46*, 536.
[4] G. B. Nyberg, R. R. Balcarcel, B. D. Follstad, G. Stephanopoulos, D. I. C. Wang, *Biotechnol. Bioeng.* **1999**, *62*, 336.
[5] H. Lodish, A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, J. Darnell, 'Protein Glycosylation in the ER and Golgi Complex', in 'Molecular Cell Biology', 4th Ed., W. H. Freemann, New York, **2000**.
[6] D. M. Karl, D. B. Craven, *Appl. Environ. Microbiol.* **1980**, *40*, 549.
[7] S. Dietmair, N. E. Timmins, P. P. Gray, L. K. Nielsen, J. O. Krämer, *Anal. Biochem.* **2010**, *404*, 155.
[8] L. D. Fairbanks, G. Jacomelli, V. Micheli, T. Slade, H. A. Simmonds, *Biochem. J.* **2002**, *366*, 1, 265.
[9] N. Tomiya, E. Ailor, S. M. Lawrence, M. J. Betenbaugh, Y. C. Lee, *Anal. Biochem.* **2001**, *293*, 129.
[10] H. T. Feng, N. Wong, S. Wee, M. M. Lee, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2008**, *870*, 131.
[11] R. Lehmann, M. Huber, A. Beck, T. Schindera, T. Rinkler, B. Houdali, C. Weigert, H. Häring, W. Voelter, E. D. Schleicher, *Electrophoresis* **2000**, *21*, 3010.
[12] D. Friedecký, J. Tomková, V. Maier, A. Janošt'ková, M. Procházka, T. Adam, *Electrophoresis* **2007**, *28*, 373.
[13] I. J. Del Val, S. Kyriakopoulos, K. M. Polizzi, C. Kontoravdi, *Anal. Biochem.* **2013**, *443*, 172.
[14] T. Ryll, R. Wagner, *J. Chromatogr. B Biomed. Sci. Appl.* **1991**, *570*, 77.
[15] G. Vanhoenacker, F. De l'Escaille, D. De Keukeleire, P. Sandra, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2004**, *799*, 323.