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Short communication

Simultaneous determination of 19 intracellular nucleotides and nucleotide sugars in Chinese Hamster ovary cells by capillary electrophoresis

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ABSTRACT

Twelve nucleotides and seven nucleotide sugars in Chinese Hamster ovary (CHO) cells were determined by capillary electrophoresis (CE). The CE operating conditions of buffer pH value, ion strength, capillary temperature, polymer additive and cell extraction method were investigated. Optimum separation was achieved with 40 mM sodium tetraborate buffer (pH 9.5) containing 1% (w/v) polyethylene glycol (PEG) at a capillary temperature of 22 °C. Acetonitrile and chloroform were used for intracellular extraction. This method can be used to monitor intracellular carbohydrate metabolism.

1. Introduction

Chinese Hamster ovary (CHO) cells are one of the most common cell lines used for the production of complex recombinant biotherapeutics [1]. CHO cells are capable of post-translational modifications like glycosylation which influences critical properties such as stability and bio-activity [2]. One of the factors influencing protein glycosylation in cells is the intracellular levels of nucleotides and nucleotide sugars, as they are essential components and precursor molecules in the biosyntheses of glycoproteins [3].

Different approaches to determine nucleotides include ion-exchange, ion-pairing chromatography [4–6], high-pH anion-exchange chromatography [7], and capillary electrophoresis (CE) [8–12]. In recent years, attempts have been made with hyphenated techniques like liquid chromatography/mass spectrometry [13–15] and CE/mass spectrometry [16–19] for nucleotides as well. Ion-exchange chromatography and reversed phase liquid chromatography has been used for the determination of nucleotide sugars [20–24]. Ion-pairing chromatography [25,26] coupled with mass spectrometry and nuclear magnetic resonance [27] has likewise been applied to the determination of nucleotide sugars. CE has also been used for the separation and determination of nucleotide sugars in two reports [28,29] with promising results.

As nucleotides and nucleotide sugars coexist in biological samples, the development of a method to simultaneously determine both groups of compounds is logical. This would reduce time and streamline analytical procedures in the laboratory. There have been a few reports of the simultaneous determination of intracellular nucleotides and nucleotide sugars in a single run using liquid chromatography [30–32]. However, the general separation performance of liquid chromatography methods was insufficient. Although the potential of highly efficient electrokinetic separation is promising, the application of this technique to simultaneous determination of intracellular nucleotides and sugar–nucleotides has not been reported.

The sample preparation, including cell lysis and following extraction of target components, is crucial to this assay. Unfortunately, no efficient method is available for CE so far. Ethanol could be used to lyse and extract nucleotides and nucleotide sugars from CHO cells [3]. However, the recovery for nucleotides was poor. Perchloric acid extraction [3,30] resulted in the presence of impurities and the high ionic strength, which caused broad peaks and long migration times. The use of Triton X-100 and acetonitrile was reported as well [29] but longer migration times were observed after cell extraction. In this paper, a method based on CE separation was developed and successfully applied to the simultaneous determination of intracellular nucleotides and sugar–nucleotides.

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2. Materials and methods

2.1. Materials

Target components included 12 nucleotides [adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP), cytidine 5′-monophosphate (CMP), cytidine 5′-diphosphate (CDP), cytidine 5′-triphosphate (CTP), guanosine 5′-monophosphate (GMP), guanosine 5′-diphosphate (GDP), guanosine 5′-triphosphate (GTP), uridine 5′-monophosphate (UMP), uridine 5′-diphosphate (UDP), uridine 5′-triphosphate (UTP)] and 7 nucleotide sugars [UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal), UDP-*N*-acetyl-glucosamine (UDP-GlcNAc), UDP-*N*-acetyl-galactosamine (UDP-GalNAc), GDP-fucose (GDP-Fuc), GDP-mannose(GDP-Man), CMP-*N*-acetyl-neuraminic acid (CMP-NeuAc)]. All of above chemicals and GDP-glucose (GDP-Glc), sodium tetraborate, ammonium tetraborate, boric acid, Triton X-100 were from Sigma–Aldrich (St Louis, MO, USA). Polyethylene glycol (PEG, MW = 20,000) was from Fluka (Buchs, Switzerland). Perchloric acid, acetonitrile, chloroform and ethanol were from Merck (Darmstadt, Germany). Other reagents were of analytical-reagent grade and water (≥18.2 MΩ) used throughout the experiments was generated from a Sartorius system (Goettingen, Germany).

2.2. Intracellular extraction of nucleotides and nucleotide sugars

CHO cells were grown in-house. Cells (3×10^7 cells) were pelleted for 5 min using a centrifuge at 1000 rpm at 4 °C. The supernatant was removed and 10 mL of ice-cold PBS buffer was added to the cell pellet. The sample vial was then vortexed and cells were re-suspended. Cells were centrifuged at 1000 rpm at 4 °C again. This process was repeated twice. The cell samples were kept in an ice-bath in-between washing steps and centrifugation. Subsequently, 5 μL of 5 mM GDP-Glc was added as an internal standard. To lyse and extract cells, 10 μL of 5% Triton X-100 and 300 μL acetonitrile were added. Supernatant was obtained after centrifugation at 9500 rpm for 10 min at 4 °C. Supernatant was stored at −20 °C overnight to allow for the separation of an aqueous phase from organic phase and the lower aqueous phase was collected. A double volume of chloroform was added to remove the lipid soluble compounds further [33,34]. This mixture was then centrifuged at 14,000 rpm for 10 min at 4 °C. The upper aqueous phase was collected for CE analysis.

2.3. CE separation method

All nucleotides and sugar–nucleotides standards were prepared in pure water and stored at −20 °C. Stock sodium tetraborate solution (100 mM) was dissolved in water. The running buffers for CE were diluted from stock solution to suitable concentrations. PEG was added to running buffers to give polymer solutions with the appropriate concentrations. This polymeric solution was sonicated for 10 min to facilitate dissolution and the pH values of buffer solution were adjusted using either 1 M NaOH or 600 mM boric acid. All running buffers were freshly prepared.

The separation was performed on a Beckman PA800 CE system (Fullerton, CA, USA). Total lengths of untreated fused silica capillary were 49 or 75 cm (38 or 64 cm to detection window, respectively). The inner diameter was 50.0 μm. Before daily runs, the capillary was rinsed for 10 min with 1 M NaOH, 10 min with water, and 10 min with running buffer. Running buffer was refreshed after every three runs for reproducible migration times. A wash cycle starting with 1 M NaOH for 5 min, followed by running buffer for 5 min was carried out after each CHO cell extract run. Samples were introduced from inlet side at 0.5 psi for 15 s. The separations were performed under a constant voltage of 30 kV and detection visualized at 260 nm.

3. Results and discussion

3.1. Optimization of CE separation

Borate buffer has proved to be effective for CE separation of nucleotide sugars and nucleotides [8,9,28,29]. The effects of pH value in 50 mM sodium tetraborate buffers on the mobilities of nucleotide sugars and nucleotides are shown in Fig. 1. Smaller mobility differences among UDP-GlcNAc, GDP-Man and GDP-Fuc were observed when pH went below pH 9.0 (Fig. 1(a)), which should be avoided in the subsequent method optimization. The trends of nucleotide mobilities under different pH values were more complicated, as shown in Fig. 1(b). Generally, the mobilities of nucleotides increased with buffer pH values and usually were higher than that of nucleotide sugars since sugar parts contributed quite small amounts of charges compared with their relevant sizes. The slowest nucleotide (AMP) co-migrated with the fastest sugar–nucleotide (UDP-Gal) until pH 9.56. Among nucleosides with same phosphate chain length, mobilities typically increased with the order of adenosine, cytidine, guanosine and uridine. The migration orders of nucleotides were sensitive to the change of buffer pH value. At pH 9.2, ATP co-migrated with ADP, and GDP co-migrated with UMP. The separations were achieved at pH 9.5, and the mobilities of GDP and UMP exceeded the mobilities of CDP and CTP at the same time.

The pH value of 9.5 was chosen with the separation of most target components, except CDP and CTP. A concentration of 40 mM

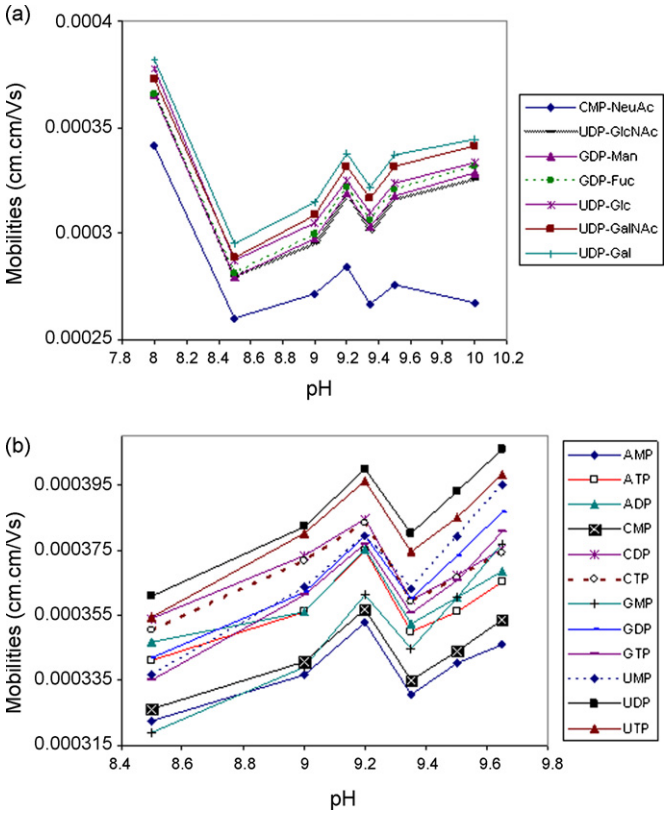

Fig. 1. Mobilities of nucleotides and nucleotide sugars measured in 50 mM sodium tetraborate buffer. Total length of capillary: 49 cm. Sample loading: 0.50 psi for 15 s. Separation voltage: 30 kV (20 kV when pH values were lower than 9.0). Capillary temperature: 25 °C. Detection: 260 nm.

Table 1
Quantification data of the analysis method

Analytes	CE separation method					Intracellular extraction method		
	R.S.D. (n = 6, %, areas)	R.S.D. (n = 6, %, times)	LOD (μM)	Linearity (R^2)	Recovery[a] (%)	R.S.D. (n = 6, %, areas)	Recovery[b] (%)	Bench-top stabilities[c], R.S.D. (n = 3, %, areas)
CMP-NeuAc	1.42	0.28	2.9	0.9996	103.3	4.33	92.2	7.25
UDP-GlcNAc	2.49	0.54	3.5	0.9994	102.1	3.98	93.5	15.9
GDP-Man	0.94	0.55	2.2	0.9991	96.7	5.54	90.1	N/A
GDP-Fuc	0.60	0.57	1.5	0.9991	94.5	5.87	90.7	N/A
UDP-Glc	2.10	0.57	7.5	0.9999	102.3	5.39	85.5	6.95
UDP-GalNAc	1.39	0.60	1.7	0.9998	99.6	5.22	82.7	7.92
UDP-Gal	2.06	0.62	2.0	0.9994	103.4	5.15	86.3	11.3
AMP	0.89	0.38	2.4	0.9999	102.0	4.73	83.5	8.05
CMP	3.46	0.38	3.9	0.9996	102.0	4.83	83.7	18.2
GMP	2.11	0.77	4.6	0.9985	106.6	6.30	81.4	13.82
ATP	0.79	0.57	1.2	0.9998	96.8	6.55	78.2	9.81
ADP	3.52	0.35	1.0	0.9999	98.6	4.46	79.1	7.54
GTP	2.80	0.70	8.5	0.9958	103.1	5.81	75.8	7.51
CTP	2.01	0.64	3.2	0.9997	95.6	4.64	72.1	10.7
CDP	1.65	0.42	3.3	0.9996	97.2	5.43	73.6	18.2
GDP	1.18	0.68	2.0	0.9996	98.3	3.84	75.9	7.13
UMP	3.18	0.89	7.5	0.9985	106.1	4.03	71.6	4.38
UTP	3.61	0.84	5.8	0.9997	98.2	3.09	70.1	12.7
UDP	2.65	1.20	2.9	0.9994	98.9	3.57	69.2	14.3

[a] Standards were spiked into sample solution before CE separation.
[b] Standards were spiked into cell pellets before cell extraction.
[c] To evaluate the analyte degradation during the cell extraction, three replicates of CHO cells stored in ice-bath were extracted successively after every 4 h. The concentrations of GDP-Man and GDP-Fuc were lower than their detection limits.

of borate buffer was found to be acceptable for both high resolution and short analysis time.

To improve the separation of CDP and CTP, a buffer additive, PEG (MW = 20,000) was therefore chosen [35,36] to reduce the analyte absorption on the capillary inner wall. Six PEG concentrations ranging from 0.1 to 2.0% (w/v) were tested. Better separations were achieved with higher PEG concentrations. However, too high a polymer concentration would introduce a strong size-exclusion effect. At a PEG concentration of 2.0% (w/v), co-migration of UDP-GlcNAc and GDP-Man was observed. Therefore, 1.0 % (w/v) PEG concentration was chosen for the separation of all target compounds. The addition of polymer into running buffer also benefited the actual sample analysis, since it reduced the absorption of proteins and peptides in the sample matrix.

The effect of capillary temperature was investigated in the range of 18–30 °C. Better separation of CDP and CTP was observed at 22 °C (Fig. 2). Therefore, the final optimized conditions were achieved at 22 °C using 40 mM sodium tetraborate buffer with 1.0% (w/v) PEG polymer (MW = 20,000), pH 9.50.

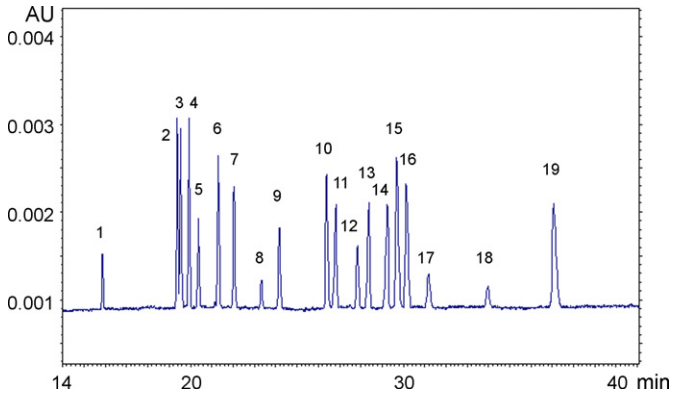

Fig. 2. Separation of 19 target compounds. Buffer: 40 mM borate buffer (pH 9.50) with 1.0% (w/v) PEG addition. Total length of capillary: 75 cm. Separation temperature: 22 °C. Peaks from left to right in eletropherogram (A): 1, CMP-NeuAc; 2, UDP-GlcNAc; 3, GDP-Man; 4, GDP-Fuc; 5, UDP-Glc; 6, UDP-GalNAc; 7, UDP-Gal; 8, AMP; 9, CMP, 10, GMP; 11, ATP; 12, ADP; 13, GTP; 14,CTP; 15, CDP; 16, GDP; 17, UMP; 18, UTP; 19, UDP. Other separation conditions as in Fig. 1.

3.2. CHO cell extraction and validation of the analytical method

A cell extraction method was developed with the simultaneous introduction of Triton X-100 and acetonitrile into the extraction buffer. Samples were kept at 4 °C or lower temperatures during the whole procedures. Cell lysis and extraction was carried out in PBS buffer to maintain moderate pH environment. Before CE analysis, chloroform was introduced to remove the lipid soluble compounds further. A mixture of standards was subjected to this extraction process; the concentrations of different compounds did not change significantly after the whole procedure. Fig. 3 shows the electropherogram of CHO cell extract. Nucleotide sugars and nucleotides were separated and identified successfully. In order to verify the feasibility of the method further, all standards (5.0×10^{-7} mol each) were added into the cell pellets before extraction. The recoveries of the extraction method were found to be 82–93% for nucleotide sugars and 69–83% for nucleotides. The variations resulting from

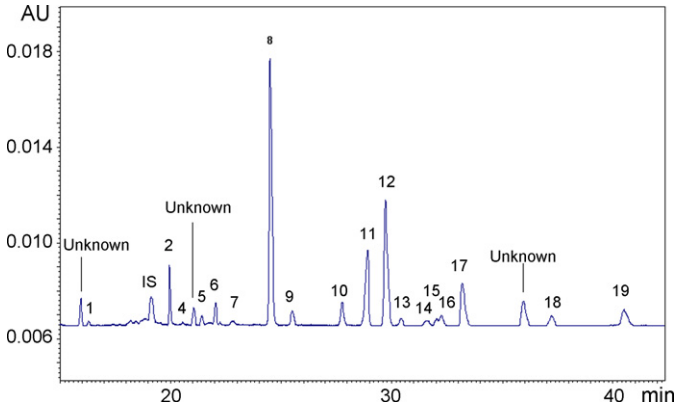

Fig. 3. Analysis of CHO cell extract. Separation conditions and peak identifications as in Fig. 2.

the fluctuations of EOF, sample loading amount and cell extraction were minimized by adding GDP-Glc as an internal standard (IS). Standard mixtures at five concentration levels, from 10 to 100 μM, exhibited a good linear relationship. Detection limits (S/N = 3) of analytes ranged from 1.0 to 8.5 μM. Relevant data about accuracy, precision and stability of CE separation and cell extraction methods were listed in Table 1.

4. Conclusions

A CE separation method for 12 common nucleotides and seven nucleotide sugars in CHO cells was established. The intracellular extraction method were developed and evaluated. The method established provides a useful platform for monitoring intracellular metabolism in CHO cells.

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