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Monitoring utilizations of amino acids and vitamins in culture media and Chinese hamster ovary cells by liquid chromatography tandem mass spectrometry

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ABSTRACT

Monitoring amino acids and vitamins is important for understanding human health, food nutrition and the culture of mammalian cells used to produce therapeutic proteins in biotechnology. A method including ion pairing reversed-phase liquid chromatography with tandem mass spectrometry was developed and optimized to quantify 21 amino acids and 9 water-soluble vitamins in Chinese hamster ovary (CHO) cells and culture media. By optimizing the chromatographic separation, scan time, monitoring time window, and sample preparation procedure, and using isotopically labeled ^{13}C, ^{15}N and ^{2}H internal standards, low limits of quantitation ($\leq$0.054 mg/L), good precision (<10%) and good accuracy (100 ± 10%) were achieved for nearly all the 30 compounds. Applying this method to CHO cell extracts, statistically significant differences in the metabolite levels were measured between two cell lines originated from the same host, indicating differences in genetic makeup or metabolic activities and nutrient supply levels in the culture media. In a fed-batch process of manufacturing scale bioreactors, two distinguished trends for changes in amino acid concentrations were identified in response to feeding. Ten essential amino acids showed a zigzag pattern with maxima at the feeding days, and 9 non-essential amino acids displayed a smoothly changing profile as they were mainly products of cellular metabolism. Five of 9 vitamins accumulated continuously during the culture period, suggesting that they were fed in access. The method serves as an effective tool for the development and optimization of mammalian cell cultures.

1. Introduction

Amino acids and vitamins play important roles in human health, nutrition and production of therapeutic proteins in biotech industry [1–3]. Cultured Chinese hamster ovary (CHO) cells have become the dominant system for producing therapeutic proteins including monoclonal antibodies. Because of significant improvements in media composition and process control, the volumetric productivity has increased more than 100-fold in the past two decades [4]. In order to further enhance productivity of recombinant cell lines and control quality of the produced therapeutic proteins, precise control of the manufacturing processes through accurate measurements of process parameters, including composition of nutrients in both culture media and mammalian cells, is required.

Base media (used at time zero) and feed media (added during the cell culture) used in CHO cell cultures are nutrient mixtures containing a large number of components such as amino acids, vitamins, salts, sugars, and trace levels of minerals [5,6]. Some nutrients are susceptible to degradations under various conditions, such as light, antiviral treatments and long-term storage. For a good control of processes to produce good product quality and high yield, it is important to monitor concentrations of intracellular metabolites and media components before and during feeding. A typical mammalian cell culture process consists of the cell growth phase and protein production phase [7,8]. Both phases are a dynamic process, and nutrient requirements vary at different times of the phases. As cells grow and then produce, some components can be consumed more than the others [5]. Consequently, limitation of certain critical nutrients can lead to cell starvations or excess of others can result in accumulations of wasteful and toxic chemicals. Supply of optimal nutrients at all the stages is critical for cell growth and product yield and quality. Therefore, it is essential to quantitatively mea-

Abbreviations: CE, collision energy; ESI, electrospray ionization; HFBA, heptaflurobutyric acid; IS, internal standard; LC, liquid chromatography; LC–MS/MS, liquid chromatography tandem mass spectrometry; LOQ, limit of quantitation; MS, mass spectrometry; QQQ, triple-quadrupole mass spectrometer; RT, retention time; RSD, relative standard deviation; SRM, selected reaction monitoring.

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sure nutrient consumption and intracellular metabolite generation during cell culture processes and to provide accurate results for optimizing nutrient levels for better cell growth and higher product yield [9].

It has been a challenging analytical problem to directly measure amino acids and water soluble vitamins simultaneously because of their diverse chemical structures and properties, sample matrix interference, and trace levels especially vitamins [10–12]. Various techniques, such as thin-layer chromatography [13], liquid chromatography (LC) with detection based on absorption or emission of light [14], nuclear magnetic resonance spectroscopy [15], gas chromatography coupled to mass spectrometry (MS) [16] and LC coupled to MS [17,18], have been employed to measure metabolites. For rapid, selective and sensitive analysis of the metabolites in a complex matrix of cell culture media and cell extracts, we developed a method utilizing ion pairing reversed-phase liquid chromatography connected on-line through electrospray ionization (ESI) interface to a triple quadrupole (QQQ) mass spectrometer using selected reaction monitoring (SRM). The SRM mode of a QQQ instrument offers a molecular weight and structure specific measurement for a given analyte, thus providing additional sensitivity and specificity [19].

Many metabolomics studies using a targeted approach with the LC–MS/MS technique have been reported [20–22]. However, these methods were used for the purpose of profiling, not absolute quantitation. In this paper, we report a high-throughput, sensitive, and reproducible targeted method for quantifying amino acids and vitamins in cell culture media and CHO cells. Since the number of analytes was relatively small (30 compounds) in this study, a low-end QQQ instrument was employed, which allowed us to achieve the analytical objectives at a low cost. The techniques to maximize analytical capability of the low-end QQQ instrument are described in this paper. The developed method was expandable for monitoring a larger number of metabolites during the same 32-min run (not shown here). It is potentially further expandable to monitor hundreds of metabolites using a higher-end QQQ mass spectrometer.

2. Experimental

2.1. Materials

Amino acid standard H and heptafluorobutyric acid were purchased from Thermo Scientific (Rockford, IL, USA), and 9 vitamin standards (niacinamide, calcium D-pantothenate, pyridoxal HCl, pyridoxine HCl, biotin, thiamine HCl, folic acid, riboflavin, and cyanocobalamin) and three other amino acids (glutamine, asparagine and tryptophan) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Deuterated niacinamide and pyridoxine HCl were purchased from CDN Isotope (Pointe-Claire, Quebec, Canada), ^{2}H labeled pyridoxal HCl and biotin, ^{13}C labeled Ca D-pantothenate, thiamine HCl, folic acid, and riboflavin from Sigma Chemical Co., and uniformly ^{13}C and ^{15}N labeled amino acid mix from Cambridge Isotope, Inc. (Woburn, MA, USA). LC/MS grade methanol and water used for LC mobile phases were purchased from Sigma Chemical Co. Water used for sample preparation was purified with a Milli-Q Filtration System (Millipore, Billerica, MA, USA) and had a minimum 18 MΩ-cm resistivity.

2.2. Standard preparation

Vitamin stock solutions were prepared in water except riboflavin prepared in 1N HCl and folic acid prepared in 0.1N $NaHCO_3$. The stock solution of asparagine, glutamine, and tryptophan were prepared in 0.1N HCl. The highest concentration standard mixture (High Standard) consisted of 21 amino acids (250 μM for 20 main amino acids and 125 μM for cystine) and 9 vitamins (5 mg/L niacinamide, 5 mg/L Ca D-pantothenate, 2 mg/L pyridoxal HCl, 2 mg/L pyridoxine HCl, 1 mg/L biotin, 5 mg/L thiamine HCl, 5 mg/L folic acid, 1 mg/L riboflavin, and 5 mg/L cyanocobalamin), and the other 6 standard mixtures were prepared by diluting the High Standard by 1/2, 1/5, 1/12.5, 1/50, 1/100, and 1/500 with 20 mM HCl, respectively. The internal standard (IS) stock solutions containing ^{2}H, ^{13}C and ^{15}N labeled isotopes were prepared in 20 mM HCl as described above. An IS mixture prepared from the stocks was added to each of the seven standard mixture solutions at a ratio of 1:4 (IS:standard, v/v), resulting in approximately 20% of the High Standard.

2.3. Sample preparation

The cell culture medium samples were taken from bioreactors and centrifuged at 3000 × *g* for 5 mins to remove the cells. Immediately after the centrifugation, supernatants were collected and diluted two times in water to prevent component precipitation and stored at −80 °C until analysis. Before the analysis, frozen medium samples were thawed, diluted properly with 20 mM HCl and mixed with the IS mixture. During sample analysis, all of the standards and samples were stored in the auto-sampler tray at 4 °C.

Sample preparation procedure for the determination of intracellular amino acids and vitamins was adapted from Sellick et al. [23]. Briefly, cells at day 14 from the large scale bioreactors were harvested by centrifugation at 3000 × *g*. The cell pellets were re-suspended in 0.5 mL of ice cold methanol, flash frozen with dry ice and ethanol bath for 2 min, thawed for 2 min, and vortexed for 2 min. After centrifuged at 800 × *g* for 1 min, the supernatants were collected. The methanol extraction was repeated once and the supernatants were combined. The pellets were then extracted once with 0.5 mL of ice-cold Milli-Q water, frozen, and thawed to obtain additional hydrophilic metabolites. After centrifugation at 800 × *g* for 1 min, the supernatant from the water extraction was combined with the methanol supernatants. The pooled extracts were centrifuged at 15,000 × *g* for 1 min to remove any insoluble precipitate before drying with a speed-vac. Before analysis, the dried extracts were re-suspended in 0.5 mL of 20 mM HCl with the IS mixture. A 10 μL of aliquot was injected for the analyses. During the sample analysis, samples were stored in the auto-sampler tray at 4 °C.

2.4. Instrumentation

An LC system (1100 series, Agilent Technologies, Santa Clara, CA, USA) composed of pumps, degassers and autosampler was coupled to the mass spectrometer. A reversed phase C18 column (Atlantis T3, 2.1 × 150 mm, 3 μm particle size, or Acquity BEH, 2.1 × 100 mm, 1.7 μm particle size with a flow rate of 0.18 mL/min, Waters Corporation, Milford, MA, USA) was used for the component separation. The mobile phases used were water (A) and methanol (B) in 0.2% heptafluorobutyric acid (HFBA) [27]. Mobile Phase B increased from 0 to 30% in 19 min, to 95% in 2 min and was hold for 4 min and reduced to 0% for 7 min with total running time of 32 min. LC conditions included autosampler tray temperature at 4 °C, column temperature at 50 °C, injection volume of 10 μL, and mobile phase flow rate of 0.32 mL/min. On line with the LC, mass spectrometric analyses were performed on a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an ESI source operated in a positive-ion mode. The ESI spray voltage was set at 3500 V and the capillary temperature set at 350 °C. Nitrogen was used as the sheath gas at 45 arbitrary units and as the auxiliary gas at 20 arbitrary units, and argon as the collision gas at 1.2 mTorr. Scan time for each SRM transition was 0.05 s. To maximize the number of scans for individual