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Running Head: MS/MS measurement of albumin/AFP

Mass spectrometry measurement of albumin/alpha fetoprotein ratio as an indicator of iPSC-derived hepatocyte differentiation

Abstract

During the process of differentiation from induced pluripotent stem cells (iPSCs) to hepatocytes it is crucial to monitor the levels of cellular maturation. In the present section we present a new method to evaluate the stage of differentiation based on the monitoring of the ratio between two plasma proteins typically secreted by hepatocytes, i.e. albumin and alpha fetoprotein. This ratio shows particularly useful for the direct comparison of cells grown in different conditions, avoiding typical processes of standardisation for the cell number (i.e. variation of cell quantity due to the use of different seeding densities and different growth vessels/supports or difficulties in establishing the effective cell viability due to the use of bioreactors or other 3D devices). Our analysis is performed via liquid chromatography-tandem mass spectrometry which allows a precise, selective and reproducible quantitation also in the case of low abundant proteins.

Key words

Albumin; alpha fetoprotein; liquid chromatography; mass spectrometry; plasma proteins; bioreactor.

1 Introduction

To monitor the process of differentiation from iPSC to hepatocytes, we propose the quantitation of two plasma proteins, albumin and alpha fetoprotein (AFP), *via* liquid

chromatography coupled to tandem mass spectrometry (LC-MS/MS). Both proteins are secreted from hepatocytes. However, albumin production (negligible in foetal cells) has been shown to increase during the maturation of hepatocytes, while AFP is a marker of immature liver cells, being secreted only from hepatoblasts and foetal hepatocytes ***(1,2)***.

With the aim to perform a direct comparison between cells grown in different conditions, we demonstrate that the ratio albumin/alpha fetoprotein is a new effective indicator of the stage of hepatocyte maturation. Specifically, the use of this ratio allows to overcome typical standardisation issues, due to the presence of a different number of cells into the plate. This aspect is crucial in the case of bioreactors or other devices, where the cell count is not always possible or easily performed.

Several methods have been reported in literature for the analysis of plasma proteins from cultured cells, for instance two-dimensional electrophoresis, ELISA, RIA and mass spectrometry analysis ***(3-6)***. Among them, the separation *via* liquid chromatography followed by detection and quantitation *via* mass spectrometry presents numerous advantages. Firstly, it offers high selectivity and specificity (i.e. absence of cross interference between proteins having a similar sequence) through multiple reaction monitoring (MRM) analysis ***(7)***. Additionally, the limited presence of contaminants and matrix effects (due to the separation *via* liquid chromatography), as well as the high signal to noise ratio (which allows an accurate, precise and reproducible quantitation also of low abundant proteins), are also key advantages.

2 Materials

Tryptic peptides and their respective isotopically-labelled internal standards were purchased from GenScript (NJ, USA). Sequencing grade modified trypsin was obtained from Promega.

HPLC grade acetonitrile was purchased from Fisher Scientific, whereas water was purified using a Milli-Q system (Millipore).

The UPLC-MS/MS instrument consisted of a Waters ACQUITY UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer with an electrospray ionization source. The mass spectrometer was operated in positive ion mode and data were acquired using MassLynx V4.1 software. Chromatographic separations were conducted through a Waters ACQUITY UPLC™ BEH C18 column (1.7 μm, 2.1 x 50 mm) kept at 40 °C. The mobile phases, freshly prepared before the use, were 0.01% formic acid (A) and 0.01% formic acid in acetonitrile (B). The flow rate was 700 μL·min−1 and separations were conducted under the following chromatographic conditions: 97% solvent A for 0.2 min, decreased to 60% over 6.8 min before being further decreased to 0 in 0.01 min; solvent A was then maintained for 1.50 min at 0 before being quickly increased to 97% over 0.01 min. Column equilibration time was 1.5 min, with a total run time of 10 min. The injection volume was 20 μL. Mass spectrometric conditions were as follows: capillary voltage 2.7 kV, desolvation temperature 600 °C, cone gas flow 150 L/h, desolvation gas flow 1200 L/h, collision gas flow 0.15 mL/min and nebulizer gas flow 7 bar. Dwell time was set at 3 msec for each analyte. Stable isotope-labelled peptides were employed as internal standards. Quantitation were performed by using one unique peptide per protein and two transitions per peptide (Fig. 1). Multiple reaction monitoring (MRM) parameters are presented in Table 1.

3 Methods

3.1 Sample preparation and LC-MS/MS analysis

1. Precipitate the proteins by adding 4 volumes of methanol to 50 μL of media (15 min; -20 ºC). Negative controls: cell media; positive controls: primary human hepatocytes (see **Note 1-3**).

2. Remove the supernatant after centrifugation (5 min; 13000 rpm).

3. Dissolve the pellets at rt in 20 µL of denaturing buffer (100 mM Tris base, 6 M urea, 2 M thiourea and 2% ASB-14, adjusted to pH 7.8 with HCl) containing 1 µM of labelled internal standards (see **Note 4,5**).

4. Add 3 µL of 0.2 M 1,4-dithioerythritol freshly solubilised in the denaturing buffer (30 min; rt) (see **Note 6**).

5. Add 6 µL of 0.2 M iodoacetamide freshly solubilised in the denaturing buffer (15 min; rt) (see **Note 7,8**).

6. Add 150 µL of water to quench the reaction.

7. Add 10 µL of 0.1 mg/mL trypsin (o/n; 37 ºC) (see **Note 9**).

8. Spin (5 min; 13000 rpm) and transfer the supernatant into injection vials.

9. Inject 20 µL into the LC-MS/MS system (10).

10. Quantify the two proteins by using calibration curves prepared into the same matrix (Fig. 5).

4 Notes

1. This procedure describes the analysis of albumin and AFP from the supernatant of cells grown in culture. In the case of cells grown in a 3D system (e.g. decellularised liver scaffold), we found essential to add further purification/concentration steps, in order to remove collagen and other contaminants released from the tissues. These steps are particularly crucial in the presence of big volumes and low protein abundancy (i.e. bioreactors).

- Centrifuge 15 mL of supernatant by using 100 KDa cut-off 15 mL Amicon® Ultra centrifugal filters (25 °C; 20 min; 4000 g). Wash the concentrated solution with water (25 °C; 2 min; 4000 g). This step helps reducing the amount of collagen and other big molecular weight proteins.

- Centrifuge the filtrates by using 50 KDa cut-off 15 mL Amicon® Ultra centrifugal filters (15 °C; 20 min; 4000 g). This step helps concentrating albumin and AFP (MW between 66 and 69 KDa) removing lower MW contaminants (e.g. salts from the buffer).
- Precipitate all the remaining concentrated volume (about 200-250 µL) by adding 4 volumes of methanol, as described at point 1 of the section "Methods".

2. For the precipitation of albumin and AFP, comparable results can be obtained by using cold acetone in the place of methanol in the same conditions.

3. 4 or 9 volumes of methanol have been used at different times to improve the precipitation of albumin and AFP. Optimal results were obtained by using 4 volumes of methanol for 15 min. By increasing volumes or time, we observed a decrease in the amount of precipitated albumin and AFP, probably due to their higher solubilisation in the solvent (Fig. 2).

4. Freshly dilute the internal standards (IS) into the buffer from a 0.2 e^{-4} M stock solution in MilliQ water.

IS contain a tag that is recognised and cleaved by trypsin. This tag is useful to exclude problems with trypsin in case the unlabelled peptides is not detected.

5. Vortex and quickly spin before leaving the mixture on a shaker at rt for at least 10 min to favour the solubilisation.

6. 1,4-Dithioerythritol is essential to reduce disulphide bridges, preventing the formation of intra- and inter-molecular bonds. Reaction is performed on a shaker.

7. Iodoacetamide is used to alkylate SH residues, preventing the re-formation of SS bonds, and thus improving the digestion step with trypsin. Iodoacetamide is unstable and light-sensitive, therefore it is important to prepare a fresh solution immediately before use and perform the carbamidomethylation in the dark (e.g. using aluminium foil). Reaction is carried out on a shaker.

8. 1,4-Dithioerythritol and iodoacetamide were reacted for 45 and 90 min in total (30+15 min *vs* 60+30 min) and results were comparable (Fig. 3).

9. Tryptic digestion was performed for 30 min, 2 h, 4 h and o/n in order to choose the best condition for the digestion of albumin and AFP (Fig. 4).

10. Tryptic peptides, with sequences unique to human albumin (AVMDDFAAFVEK) and human AFP (TFQAITVTK) and their respective isotopically-labelled internal standards (EQLK.AVMDDFAAF[V(13C5,15N)]EK; NFG.TRTFQA[I(13C6,15N)]TVTK), were chosen according to the criteria reported by Liebler and Zimmerman ***(8)***. Homology with bovine (eventually present in the media) and mouse peptides (eventually from the tissue scaffold in the case of cells grown in a 3D system) were excluded by comparison of the aminoacidic sequences.

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