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Running Head: MS/MS measurement of bile acids

Measurement of bile acids as a marker of iPSC-derived hepatocytes functionality

Abstract

During the process of differentiation from induced pluripotent stem cells (iPSCs) to hepatocyte-like cells it is crucial to monitor the functionality of cells, in order to avoid an over-estimation of the level of maturation. To this end, we propose the bile acid profiling as a novel approach useful to determine the maturation of hepatocyte-like cells. The main advantages of the method are the simplicity and rapidity of test (i.e. single-step sample preparation followed by 3.5 min analysis), as well as the possibility to localise possible enzyme deficiencies by quantifying the accumulation of specific intermediates involved in the synthetic pathways.

Key words

Bile acids; liposomes; mass spectrometry; liquid chromatography; cholesterol.

1 Introduction

iPSC-derived hepatocytes are useful *in vitro* models of liver function and toxicity, as well as an alternative source of human hepatocytes ***(1,2)***. Although the production of hepatocytes from iPSCs has been extensively reported ***(3-6)*** and iPSC-derived hepatocytes seem to share many features with primary human hepatocytes, it as well known that they tend to be generally less mature ***(7)***. Foetal hepatocytes may express proteins (e.g. CYP3A4) that are

commonly used as adult markers ***(8,9)*** and therefore, it is essential to characterise cellular function in detail, in order to avoid an over-estimation of cell maturation ***(8)***.

In this context, we propose the bile acid (BA) profiling as a novel approach to monitor the stage of cellular differentiation. Indeed, BA synthesis and secretion is a specific function of hepatocytes, as 17 distinct enzymes are required to produce primary bile acids and at least 40 intermediates have been described for this biochemical pathway ***(10)***. The main advantages of our method are the rapidity and accuracy of responses which are achieved through a single-step sample preparation followed by a 3.5 min analysis with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Moreover, as the enzymes involved in BA synthesis are located in different cellular organelles, the accumulation of specific BA intermediate can potentially give useful information about any enzyme deficiency ***(10,11)*** (e.g. C27 hydroxylation occurs mainly in the mitochondria, whereas further ring structure modification is performed in the cytoplasm; side chain modification and conjugation mainly are carried out in peroxisomes ***(12)***). In some circumstances, when BAs are already present in the media, because used as nutrients for iPSC-derived hepatocytes, it is essential to incubate the cells with isotopically-labelled cholesterol prior to the measurement of the production of isotopically-labelled BAs.

2 Materials

Bile acids and their deuterium labelled internal standards were purchased from CDN Isotopes, 5-Cholesten-3β-ol-2,3,4-$^{13}C_3$ ($^{13}C_3$-cholesterol) was purchased by Sigma-Aldrich and Phospholipon® 90 (P90H) was donated by Phospholipid GmbH. HPLC grade methanol was purchased from Fisher Scientific, whereas water was purified using a Milli-Q system (Millipore).

3 Methods

3.1 Direct quantitation of bile acids from the media

1. Extract bile acids by adding 4 volumes of methanol (containing 20 nM of labelled internal standards) to 50 µL of media (15 min; -20 °C) (*see* **Note 1**). Negative controls: media; positive controls: primary human hepatocytes.

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

3. Inject 20 µL into the LC-MS/MS system and perform the quantitation by using calibration curves in methanol as previously reported ***(10)*** (Fig. 1).

3.2 Quantitation of isotopically-labelled bile acids after the administration of $^{13}C_3$ cholesterol to cells

A direct and precise quantitation of the amount of BAs secreted from the cells is not always possible as some of the media, specifically used for hepatocyte cultures, contain a variable amount of BAs. To overcome quantitation problems and perform an accurate measure of BAs effectively secreted from the cells, we have fed the cells with $^{13}C_3$-cholesterol and measured the amount of $^{13}C_3$-labelled BAs in the media. As cholesterol is not soluble in aqueous media, $^{13}C_3$-cholesterol liposomes were prepared, according to the protocol below, and administered to the cells (*see* **Note 2**).

1. Resuspend 12 mg of P90H and 2.5 mg of $^{13}C_3$ cholesterol in 2 mL of sterile physiological water in a glass bottle. Add the antimicrobial primocin.

2. Sonicate at room temperature for 10 min (power 14) (it is recommended to conduct step 1and 2 under a fumehood, in order to avoid cell contamination).

3. Let the suspension cool down at room temperature before using it to incubate the cells (*see* **Note 3**).

4. Dilute the liposomes to 2 mM in the media before filtering with a 0.4 µm filter and further dilute to 1 mM.

5. Incubate the cells for 24 hours with the liposomal suspension.

6. Harvest the media and extract the $^{13}C_3$-BAs as described at points 1 and 2 of paragraph 3.1.

7. Inject 20 µL into the LC-MS/MS system and perform the quantitation by using calibration curves prepared in methanol as previously reported ***(10)*** (*see* **Note 4,5**).

4 Notes

1. The isotopically-labelled BAs used as internal standards are: $^{2}H_4$-cholic acid, $^{2}H_4$-glycocholic acid, $^{2}H_5$-taurocholic acid, $^{2}H_4$-chenodeoxycholic acid, $^{2}H_4$-glycochenodeoxycholic acid, $^{2}H_4$-taurochenodeoxycholic acid.

2. Alternatively, isotopically labelled unconjugated BAs can be used in the place of $^{13}C_3$-cholesterol. For instance, by feeding primary human hepatocytes (positive control) with $^{2}H_6$-deoxycholic acid or $^{2}H_9$-chenodeoxycholic acid, we could measure the production of their glycine conjugates. However, these compounds resulted in high cell toxicity, by acting as powerful detergents and thus leading to cell lysis. In addition, the conjugation with glycine gives only limited information about cellular functionality.

3. Use the liposomal suspension within a few days to avoid possible deterioration. If not used within the same day, store the suspension at - 4°C in the fridge.

4. Considering $^{13}C_3$-cholesterol is used for the synthesis of BAs, it is required to add 3 mass units to all the precursor ions of the MRM panel.

5. For an accurate quantitation of $^{13}C_3$-BAs, it is important to consider the interference produced by the unlabelled BAs which are contained into the media. The calculated isotopic contribution of unlabelled BAs that can interfere with the MS signal of $^{13}C_3$-BAs is reported in table 1 (highlighted in yellow). Therefore, it results essential to firstly quantitate the

unlabelled BAs, in order to calculate their isotopic interference on $^{13}C_3$-BAs and subtract it (Fig. 2).

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