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The influence of culture media upon observed cell secretome metabolite profiles: The balance between cell viability and data interpretability

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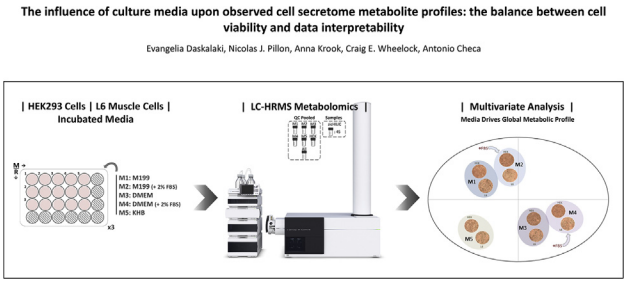
HIGHLIGHTS

- The effects of five different media combinations were tested on the metabolic profiles of HEK293 and L6 cell lines.
- The media composition drives the cluster of the observed metabolic profiles on a media-specific basis.
- The addition of fetal bovine serum affects the observed metabolic profiles.
- Incubation affects the observed metabolic profile of cell-free media.
- Experimental design guidelines are proposed for cell culture secretome metabolomics studies.

GRAPHICAL ABSTRACT



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ABSTRACT

The application of metabolomics to investigating the cell secretome has garnered popularity owing to the method's large-scale data output, biochemical insight, and prospects for novel target compound discovery. However, there are no standardized protocols for the use of cell growth media, a factor that can exert profound effects upon the detected metabolites, and thus in the interpretability of the resulting data. Herein, we applied a liquid chromatography-high resolution mass spectrometry-based metabolomics approach to examine the influence of 5 different media combinations upon the obtained secretome of two phenotypically different cell lines: human embryonic kidney cells (HEK293) and L6 rat muscle cells. These media combinations were, M1: Medium 199, M2: Medium 199 + 2% fetal bovine serum (FBS), M3: Dulbecco's Modified Eagle's Medium (DMEM), M4: DMEM + 2% FBS and M5: Krebs-Henseleit Modified Buffer (KHB). The effect of incubation (37 °C) *vs.* refrigeration (4 °C) on DMEM medium over a 24 h period was also investigated. Results were validated for a selected panel of 5 metabolites measured from an additional cell culture experiment. Metabolomics identified a total of 53 polar metabolites that exhibited differential patterns on a cell type- and medium-specific basis. We observed that choice of media was the primary contributor to the secreted metabolite profile detected. The

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addition of FBS resulted in unique detected metabolites, compared to media-only controls (M199 and DMEM alone). Glutamine and pyroglutamate were more abundant in incubated relative to refrigerated DMEM medium. The overall metabolic pattern of the metabolites from the targeted approach matched with that exhibited across M1-M5 of the metabolomics experiment, and aided in further identifying the presence of compounds that were below the limit of detection in metabolomics. Based upon these findings, we highlight the following considerations in designing a cell secretome-based metabolite profiling experiment: (1) multiple media combinations (with and without FBS) should be tested for each cell line to be investigated; (2) cell-free media combinations should be plated separately, and incubated/treated in the same experimental conditions as the cells; and (3) a compromise between cell death and metabolite detection should be identified in order to avoid batch-specific contributions from FBS supplementation.

1. Introduction

The cell is in many ways an intricate biochemical machine, with organizational structures that influence its collective driving force as a unit of life [1,2]. Ascertaining a blueprint of its phenotype begins with investigating the relatively small (<1200 Da) [3] labile metabolites, which act as fuel in this complex machine. The number of metabolites within a given system are dependent upon the number of unique cell-specific metabolomes interacting together [3–5]. Metabolomics provides a platform to analyze large-scale phenotypic changes in the cell, with liquid chromatography coupled to mass spectrometry (LC-MS) being the commonly employed technique. LC-MS enables metabolite detection from both intra- (*i.e.,* cell lysates), and extra-cellular (*i.e.,* cell secretome) matrices. From a practical stand-point, the use of cell cultures in both health and disease models provides a relatively cost-efficient approach, benefiting from controlled experimental environments, as well as less arduous ethical procedures in comparison to animal/human experiments. However, 2-D cell models can result in bioactivities that deviate from the *in vivo* response, which has resulted in the development of 3-D or spheroid cell models [6]. From a scientific perspective, the creation of novel network models (*i.e.*, from the metabolomics data), may also help to elucidate a drug's mode of action, cell to cell signaling, as well as provide new markers associated with cell toxicity [7,8].

In spite of the unique prospects of combing cell culture analysis with metabolomics, the physicochemical properties of the intra and extracellular matrices (the latter includes the media) provide formidable analytical challenges [9,10]. Detailed sample preparation methods available within industry in relation to cell culture biotechnology have already been described in detail by Causon and Hann [9]. Beyond the quenching and harvesting of cells, one major component remains when investigating the cell secretome - this being the growth media. To maintain cell growth and viability, cells are propagated in specific growth media. Culture media provides cells with sources of energy and specific compounds that regulate the cell cycle, often including amino acids, vitamins, inorganic salts, and glucose. Media content also serves to maintain pH and osmolality. In addition to growth media, most mammalian cells also require serum supplementation - with fetal bovine serum (FBS) being the most common [11]. Serum provides cells with growth factors, hormones, and attachment factors, but has the disadvantage of having an undetermined composition, which varies from batch to batch. Compounds present in either serum or culture media may potentially mask the secreted metabolites released from the cells, ultimately obscuring the profile of the secretome. An example of this analytical challenge has been noted by Creek et al. [10], whereby common supplemented media (HM11) utilized to investigate drug-induced changes in the parasite metabolome of *T. brucei* are often too enriched and therefore do not adequately reflect the *in vivo* environment of the bloodstream form of *T. brucei*. The authors proposed an adapted growth medium to ensure viability of the cells grown *in vitro*, without obscuring the desired metabolite pattern, which was guided by a non-targeted metabolomics approach (LC-MS) of the cell extracts. This could potentially be translated to the cell secretome; however, this has yet to be investigated. Another question remains regarding the use of serum. Nonnis et al. [11] utilized a LC-MS/MS approach to observe the variations in growth media combined with different concentrations of FBS (0%, 5%, and 10%) on the number of proteins in the cellular secretome of mesenchymal stromal cells (human bone marrow). The authors suggest supplementing with FBS until desired confluence, and then transferring to a serum-free medium-only environment for cell secretome collection in order to reduce analytical interferences.

Another example of an analytical challenge is in the variability exhibited across conditioned media, which is a medium containing all molecules released by cells. MacIntyre et al. [12] utilized a ^{1}H Nuclear Magnetic Resonance (^{1}H NMR)-based metabolomics approach to profile conditioned media from human foreskin fibroblasts that are used as a supplement in *in vitro* cell culture studies of human embryonic stem cells. The authors showed the effect of conditioned media on the secretory *vs.* utilization of metabolites at differing cell passaging stages [12]. Interestingly, conditioned media also evidenced metabolic changes after 2 weeks of storage at −20 °C [12].

In an attempt to apply metabolomics to the cell secretome, it becomes evident from the literature that there is no standardized method when it comes to the use of media, which is also highly dependent on which cell lines are investigated [13,14]. In the present work, we aimed to determine this variability, as well as the complex experimental outcomes induced by growth media in the cell secretome. We used LC-high resolution mass spectrometry (HRMS) based metabolomics to inspect the influence of 5 different media combinations in the obtained secretome of two phenotypically different cell lines (human embryonic kidney cells and L6 rat muscle cells). We also investigated the effect of incubating (37 °C) *vs.* refrigerating (4 °C) the same medium over a 24-h period. Finally, results were validated using a targeted panel of metabolites. We observed a strong influence from the different media combinations on the resulting cell metabolic profile and based upon these findings we propose considerations for designing cell culture media metabolomics studies.

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