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Using precursor ion scan of 184 with liquid chromatography-electrospray ionization-tandem mass spectrometry for concentration normalization in cellular lipidomic studies

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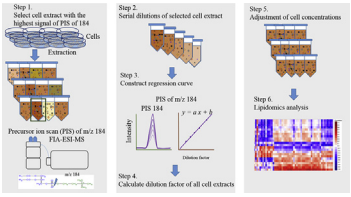
HIGHLIGHTS

- This study developed a PIS of 184 method to normalize cell concentration for lipidomic studies.
- We validated the PIS of 184 method.
- The PIS of 184 method showed good accuracy with better convenience in comparison with conventional normalization methods.
- We applied the PIS of 184 method to a cellular lipidomic study on OGD-treated neuron cells to study ischemia-induced neuron injury.

GRAPHICAL ABSTRACT



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ABSTRACT

Cellular lipidomic studies have been favored approaches in many biomedical research areas. To provide fair comparisons of the studied cells, it is essential to perform normalization of the determined concentration before lipidomic analysis. This study proposed a cellular lipidomic normalization method by measuring the phosphatidylcholine (PC) and sphingomyelin (SM) contents in cell extracts. To provide efficient analysis of PC and SM in cell extracts, flow injection analysis-electrospray ionization-tandem mass spectrometry (FIA-ESI-MS/MS) with a precursor ion scan (PIS) of *m/z* 184 was used, and the parameters affecting the performance of the method were optimized. Good linearity could be observed between the cell extract dilution factor and the reciprocal of the total ion chromatogram (TIC) area in the PIS of *m/z* 184 within the dilution range of 1- to 16-fold ($R^2 = 0.998$). The calibration curve could be used for concentration adjustment of the unknown concentration of a cell extract. The intraday and intermediate precisions were below 10%. The accuracy ranged from 93.0% to 105.6%. The performance of the new normalization method was evaluated using different numbers of HCT-116 cells. Sphingosine, ceramide (d18:1/18:0), SM (d18:1/18:0) and PC (16:1/18:0) were selected as the representative test lipid species, and the results showed that the peak areas of each lipid species obtained from different cell numbers were within a 20% variation after normalization. Finally, the PIS of 184 normalization method

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was applied to study ischemia-induced neuron injury using oxygen and glucose deprivation (OGD) on primary neuronal cultured cells. Our results showed that the PIS of 184 normalization method is an efficient and effective approach for concentration normalization in cellular lipidomic studies.

1. Introduction

Lipids play many important roles in a living body, such as the maintenance of electrochemical gradients, subcellular partitioning, signal transduction, energy storage, protein trafficking and membrane anchoring [1]. Lipidomics focuses on studying the metabolisms and functions of lipids, and it is a rapidly growing field. Cellular lipidomic studies have been favored approaches for various biomedical studies and the investigation of disease mechanisms [2–6]. To provide fair comparisons of studied cells, performing concentration normalization before statistical analysis is essential.

Lipidomics arose after metabolomics, and current concentration normalization strategies for cellular lipidomic studies mainly use strategies that are similar to those used in cellular metabolomics. These methods can be simply divided into statistical normalization or experimental normalization. Statistical normalization uses instrumental signals for data normalization, and the total ion count [7], median fold change (MFC) [8] and sum of peak areas [9] methods are frequently used approaches. Sysi-Aho *et al.* developed a normalization method using optimal selection of multiple internal standards (NOMIS) for signal calibration, which considered the effect of the chemical diversity of molecular species [10]. Although these methods provide the advantage of normalization without requiring additional experiments, the differences in the ionization efficiencies of each metabolite and the signal saturation problem may have imperfect calibration performance when electrospray ionization methods are used for the analysis [11–13].

Experimental normalization, which is also called pre-acquisition normalization, aims to ensure that similar overall sample amounts or concentrations are being analyzed. Normalization methods that are used in cellular metabolomic studies, including controlling the number of cells [9], the total protein concentration [14], or the DNA concentration [15], have also been widely used in cellular lipidomic studies. Although these methods are widely used, some limitations still exist. Because protein extraction procedures introduce interferences, such as salts and surfactants, additional samples are required for normalization purposes in protein concentration normalization methods [11,16]. Although the DNA normalization method could share the same sample used for metabolomics studies, it requires additional time for the extraction of DNA [15]. The cell number normalization method does not require any extraction procedures [9], but this method is not commonly used for primary cultured cells. All of the currently used normalization methods show some limitations, and there is no method specifically designed for cellular lipidomic studies. With the growing importance of the lipidomics field, it is important to develop a more effective and efficient concentration normalization method for cellular lipidomic studies.

The mammalian cell membrane is mainly constructed of phospholipid bilayers, and phosphatidylcholine (PC) and sphingomyelin (SM) are two of the most abundant phospholipids in the membrane, which comprise to about 50% of the membrane lipids [17–20]. Considering similar membrane lipid composition is needed to maintain cell structure, and the homeostasis of PC has also been proven to play an important role in the maintenance of membrane function [21]. The present study develop a flow injection analysis-electrospray ionization–triple quadrupole mass spectrometry (FIA-ESI-MS/MS) method for concentration normalization in cellular lipidomic studies by monitoring phosphatidylcholine (PC) and sphingomyelin (SM) content. Both PC and SM have a phosphocholine head group and would be fragmented to generate a specific ion of m/z 184 in positive polarity monitoring MS/MS analysis when collision energy is applied [22,23]. By measuring the total precursor ion signals, which generate product ions with m/z 184, the total PC and SM in the sample could be obtained, and the value can be used to correlate to the cell number for concentration normalization in lipidomic studies. The performance of the precursor ion scan of m/z 184 (PIS of 184) normalization method was evaluated using the HCT-116 cell line, and the results were also compared with other currently used normalization strategies.

The FIA-ESI-MS/MS normalization method was also applied to a primary neuronal cell culture to demonstrate its applicability to various cell types. Oxygen and glucose deprivation (OGD) of primary neuronal cultured cells was selected as our test model, and it is a frequently used *in vitro* model to study ischemia-induced neuron injury [24]. Because OGD treatment will cause cell death, optimal concentration normalization before lipidomic analysis would be crucial to provide a fair comparison between pre- and post-OGD treatment. Successful application showed that the proposed method represents a simple and effective approach for concentration normalization in cellular lipidomic studies.

2. Material and methods

2.1. Chemicals

MS-grade methanol (MeOH) and water were purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain), and LC-MS-grade isopropanol (IPA) was purchased from J.T. Baker (Phillipsburg, NJ, US). MS-grade formic acid (99%) was obtained from Sigma (St. Louis, MO, US), and MS-grade ammonium acetate was purchased from Merck (Darmstadt, Germany). The DNA concentration assay reagents, including ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and potassium acetate, were obtained from Sigma, ethanol (EtOH) was obtained from J.T Baker, Tris was obtained from Omics Bio (Taipei, Taiwan), and sucrose was obtained from Bio Basic Canada Inc. (Amherst, NY, US).

2.2. Cell culture and OGD treatment

The colon cancer HCT-116 cells were cultured in RPMI-1640 medium (Gibco, Gaithersburg, MD, US) with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, US) at 37 °C in 5% CO_2, and they were sub-cultured when 80–90% confluence was reached. The medium was renewed every two days. The non-small cell lung cancer (NSCLC) PC9, PC9 gefitinib-resistant (PC9-GR), Madin-Darby canine kidney (MDCK), MDCK with multiple drug resistance subtype 1 (MDR1-MDCK), lung adenocarcinoma HCC827, HCC827 gefitinib-resistant (HCC827-GR), ovarian cancer OVCAR-3, and SKOV-3 cell lines were also used to verify this model. The HCT-116 cells were kindly