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Review

Quantitative analysis of endogenous compounds

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ABSTRACT

Accurate quantitative analysis of endogenous analytes is essential for several clinical and non-clinical applications. LC–MS/MS is the technique of choice for quantitative analyses. Absolute quantification by LC/MS requires preparing standard curves in the same matrix as the study samples so that the matrix effect and the extraction efficiency for analytes are the same in both the standard and study samples. However, by definition, analyte-free biological matrices do not exist for endogenous compounds. To address the lack of blank matrices for the quantification of endogenous compounds by LC–MS/MS, four approaches are used including the standard addition, the background subtraction, the surrogate matrix, and the surrogate analyte methods. This review article presents an overview these approaches, cite and summarize their applications, and compare their advantages and disadvantages. In addition, we discuss in details, validation requirements and compatibility with FDA guidelines to ensure method reliability in quantifying endogenous compounds. The standard addition, background subtraction, and the surrogate analyte approaches allow the use of the same matrix for the calibration curve as the one to be analyzed in the test samples. However, in the surrogate matrix approach, various matrices such as artificial, stripped, and neat matrices are used as surrogate matrices for the actual matrix of study samples. For the surrogate analyte approach, it is required to demonstrate similarity in matrix effect and recovery between surrogate and authentic endogenous analytes. Similarly, for the surrogate matrix approach, it is required to demonstrate similar matrix effect and extraction recovery in both the surrogate and original matrices. All these methods represent indirect approaches to quantify endogenous compounds and regardless of what approach is followed, it has to be shown that none of the validation criteria have been compromised due to the indirect analyses.

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1. Introduction

Metabolomics is a field of science, which involves the comprehensive quantitative and qualitative profiling of multiple metabolites and their interaction with environmental variables of interest such as diet, disease, environment, or exposure to chemicals. In a simplistic view, a metabolomics experiment is a comparative analysis of samples to identify if these samples can be distinguished on the basis of quantitative and/or qualitative differences in their metabolic profiles. In general, two strategies can be followed in metabolite profiling. The first strategy targets limited number of metabolites or a subset of known metabolites that either have similar structure or involved in a specific metabolic pathway. The metabolites of interest are already selected and their absolute concentrations are measured usually to prove a hypothesis based on a priori information about the targeted metabolites. This approach is referred to as "targeted" metabolomics [1,2]. The second strategy relies on a holistic approach towards the profiling of as many metabolites as possible without a prior knowledge of the identity of these metabolites. This "non-targeted approach" produces comprehensive lists of metabolites of potential interest with relative quantification. These metabolic changes then can then be mapped to specific pathways and provide biomarkers and/or mechanistic information [3].

Eicosanoids, bile acids, and steroid hormones represent examples of classes of endogenous compounds (metabolites) that are extensively characterized via various metabolomics approaches. Comprehensive analysis of the eicosanoids metabolome helps understand their role in cell proliferation, inflammatory diseases, tissue repair, coagulation, and the immune system [4,5]. Bile acids have both pathological and physiological roles in liver diseases, regulating lipid, glucose, energy, and their own homeostasis. Due to the wide variation of the physicochemical, pathological, and physiological roles of individual BAs, quantitative metabolomics is the best approach to elucidate the mechanisms underlying the various BA functions [6–11]. Quantitative analyses of steroid hormones are used clinically for the diagnosis of cushing's syndrome, congenital steroid enzyme deficiency, and other endocrine disorders [12]. In addition, metabolomics is widely used in agricultural applications to study plant responses to environmental factors, including drought, salt, low oxygen caused by waterlogging or flooding of the soil, temperature, light, or oxidative stress [13].

The quantitative component of metabolomics analyses can be classified into relative, semi-quantitative, or absolute quantification [14,15]. In relative quantification, the effect of treatment(s) on the analytes of interest is measured by comparing the detector responses of these analytes in samples from treatment groups relative to reference or control groups. Semi-quantitative analyses are used when reference standards for the analytes of interest are not readily available; therefore, available standards of other molecules related to these analytes are used as surrogate standards to translate the detector responses into concentrations. For example, parent compounds are sometimes used as standards to quantify their metabolites [16]. The assumption is that both the analytes and the surrogate molecules have similar detector responses. In absolute quantification, however, concentrations of analytes are measured with predetermined levels of accuracy and precision using standards of the same analytes.

2. Quantitative liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis

Liquid chromatography-tandem mass spectrometry (LC–MS/MS) has become the technique of choice for quantitative analyses because of its sensitivity, selectivity, and speed [17,18]. In LC–MS, the effluent from the LC system enters the ionization source, where analytes are ionized before mass-separation by the MS analyzer. Atmospheric pressure ionization (API) sources, allow direct coupling of the LC system to the MS, where LC effluent flows directly into the ionization source. However, due to differences in ionization efficiencies in the MS source, different analytes of the same concentrations may produce different MS responses. Therefore, authentic standards are always required for the absolute quantification of analytes using LC–MS/MS.

Any components of biological matrices, primarily salts and phsphospholipids that co-elute from the chromatographic column with the analytes of interest, do compete over the same charges with the analytes in the MS source, which may suppress/enhance the signal intensity of these analytes. Therefore, the same analyte with the same concentration can produce different MS responses, when exist in different matrices. This phenomenon is known as matrix effect [19].

Typically; biological samples are prepared before analyses to extract the analytes of interest from as much as possible of the irrelevant endogenous sample components. The most popular extraction techniques used are protein precipitation (PP), liquid–liquid extraction (LLE), and solid-phase extraction (SPE). PP can be achieved by (i) decreasing the dielectric constant with the addition of an organic reagent such as methanol or acetonitrile, (ii) increasing the ionic strength, i.e. salting out effect by the addition of high concentrations of ammonium sulfate, or (iii) changing the sample pH with the addition of concentrated acids such as perchloric acid or trichloroacetic acid, or concentrated alkaline reagents like sodium or potassium hydroxide. In LLE, an organic solvent is used in which analytes have higher solubility in comparison to their solubility in the aqueous phase. The pH of the sample is adjusted so that the drug to be extracted is unionized, thus facilitating partitioning into the organic solvent. The upper organic layer is then aspirated, evaporated, and reconstituted in a solvent compatible with the LC buffer system. Trimethylbutylether, chloroform, diethylether, dichloromethane, and diethyl acetate are the commonly used organic solvents in LLE of biological samples. SPE is the most popular sample preparation method. In SPE, analytes are selectively retained to a special adsorbent via different mechanisms of interaction, such as hydrophobic, electrostatic, or size exclusion. The tremendous advances in SPE technology, which produces sorbents with a very wide diversity in selectivity, allow SPE to be suitable for the extraction of a wide spectrum of compounds with different physicochemical properties. These sorbents are commercially available in different formats such as columns, cartridges, or syringes.

Extraction recovery is then calculated to quantify the efficiency of the sample preparation method in the extraction of analytes from the matrix [20,21]. Overall absolute extraction recovery is calculated as the ratio of the analyte peak area in samples spiked before extraction compared to the corresponding peak area in untreated samples prepared in neat solution (assuming no loss of analyte due to degradation by matrix components). In LC–MS/MS, however, absolute extraction recovery does not only represent extraction efficiency of the sample preparation procedure, but also includes the matrix effect on the MS signal. Matrix effect is calculated as the ratio of analyte peak area in samples spiked after extraction compared to the corresponding peak area in untreated samples prepared in neat solution. On the other hand extraction efficiency can be quantified via the relative extraction recovery, which is the ratio of analyte peak area in samples spiked before extraction compared to the corresponding peak area in samples spiked after extraction. Therefore, Overall absolute extraction recovery = matrix effect × relative extraction recovery (extraction efficiency). For example, as shown in Fig. 1A, extraction of two different matrices spiked with one analyte at the same concentration