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Method validation strategies involved in non-targeted metabolomics

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ABSTRACT

Non-targeted metabolomics is the hypothesis generating, global unbiased analysis of all the small-molecule metabolites present within a biological system, under a given set of conditions. It includes several common steps such as selection of biological samples, sample pre-treatment, analytical conditions set-up, acquiring data, data analysis by chemometrics, database search and biological interpretation. Non-targeted metabolomics offers the potential for a holistic approach in the area of biomedical research in order to improve disease diagnosis and to understand its pathological mechanisms. Various analytical methods have been developed based on nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) coupled with different separation techniques. The key points in any analytical method development are the validation of every step to get a reliable and reproducible result and non-targeted metabolomics is not beyond this criteria, although analytical challenges are completely new and different to target methods. This review paper will describe the available validation strategies that are being used and as well will recommend some steps to consider during a non-targeted metabolomics analytical method development.

1. Introduction

Metabolomics was defined by Oliver Fiehn as the global unbiased analysis of the small-molecule metabolites present within a biological system in an identified and quantified manner [1]. In parallel, Jeremy Nicholson coined the term metabonomics, which is defined as the comprehensive and simultaneous profiling of metabolites and their effective changes resulting from different conditions such as diet, life style, genetic or environmental factors [2]. Currently both terms are used interchangeably. This methodology offers the potential for a holistic approach to clinical medicine, as well as improving disease diagnosis and understanding of pathological mechanisms. The exact definition of the metabolome is in some debate, however in general can be thought of as the complete compliment of all the low-molecular weight molecules (<1500 amu) present in the biological compartment in a particular physiological state under a given set of environmental conditions [3]. Metabolomics may also be the methodology for biomarker discovery. Biomarker was defined in 1998 by the National Institutes of Health Biomarker Definitions Working group as: "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" [4]. To make this definition more clear in the year of 2010 the World Health Organization suggested that a biomarker is "*almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The response may be functional and physiological, biochemical at the cellular level, or a molecular interaction*" [5]. A biomarker could be anything such as, physical traits (body temperature, blood pressure, etc.) or presence of biological molecules in tissues or body fluids. The fundamental goal of biomarker identification in biomedical research is the discovery of a molecular signature which can correlate with a specific disease type that can be used as early diagnostic tools in clinical practice [6]. This type of marker requires high sensitivity and specificity. The metabolomics field has a key role in screening chemical markers and this approach can mainly be divided in two categories, targeted and non-targeted. Targeted metabolomics consists of the quantification of one or a set of known metabolites, which are generally related to a specific pathway or biological activity [1]. It enables exact quantification of the metabolite by employing authentic analytical standards and only focus on the changes of the quantitated metabolites [7,8]. On the other hand, non-targeted approach is the data driven, rapid high-throughput analysis of all possible metabolites present in a given set of samples without any prior knowledge of the metabolites [9]. Compared to targeted approach, in non-targeted metabolomics, it is not

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possible to quantify due to the larger number of variables and because the identity of the metabolites is often unknown [10–12]. The key advantage of global approach over targeted approach is, that it enables novel areas of metabolism to be identified [8,13]. Numerous analytical platforms have been used in non-targeted metabolomics applications, such as nuclear magnetic resonance spectroscopy (NMR), Fourier transform-infrared spectroscopy and mass spectrometry (MS) coupled to separation techniques, or using direct flow injection [2,14,15]. The potentiality of NMR in high throughput metabolomics, is the minimal sample pre-treatment requirement, obtaining highly quantitative and reproducible data, however with this technique only medium to high abundance metabolites can be detected and challenges still remain for complex mixtures. MS-based metabolomics offers analyses with high selectivity and sensitivity, moreover positive and negative ionization will increase the variety of metabolites, either using direct infusion or in combination with separation techniques choosing from liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE). Use of separation techniques also reduces the complexity of the mass spectra and delivers additional information on the physico-chemical properties of the metabolites [16]. However, MS-based techniques usually require sample preparation steps and analytical method development, which should be valid enough to get effective results. LC–MS based analysis is the most widely used analytical platform in non-targeted metabolomics due to its high sensitivity and selectivity. Separation with LC can reduce ion suppression caused by co-eluting compounds, isobaric interferences and often can separate isomers. In addition, analytical separation with LC could be benefited from lower detection limits and improved MS data quality due to reduced background noise. The combination of GC with MS provides high-resolution, analyte-specific detection, and quantification of metabolites and as well it has the capability to identify unknowns. However, a major prerequisite for GC–MS analysis is a sufficient vapor pressure and the analytes should be thermally stable. The sample pre-treatment steps for GC–MS analysis are quite long and only volatile metabolites can be analyzed, which are the main limiting factors for this technique. CE is a powerful technique for the separation of charged metabolites, offering high-analyte resolution. The combination of CE with MS makes CE–MS an ideal tool for the analysis of polar compounds present in the metabolome. However, only a few applications have been published as it is not a generally available technique in the laboratory.

Accordingly, a single analytical approach is not enough to cover the entire metabolome. Thus the integration of multiplatform approaches are necessary to circumvent this issue and MS detection coupled to separation techniques along with NMR are becoming the most relevant tools in this aspect. Multiplatform metabolomics has been applied on different biological specimens either for targeted or non-targeted analysis. The multiplatform approach was well demonstrated in a study by Psychogios et al., while establishing human serum metabolome [17]. In order to achieve coverage of 4229 metabolites, six distinct analytical platforms were used, including high-resolution NMR, GC–MS, LC–MS (positive and negative ionization) and direct flow injection in MS.

After instrumental considerations another critical step in metabolomics approach is the biostatistics (univariate and multivariate data analysis) which reflects the differences between metabolomes and characterize specific phenotypic characteristics. However the correct biological interpretation of a specific metabolite difference in non-targeted approach depends on the reliability and suitability of the entire approach (from sample treatment to biomarker identification). Hence, the term validation comes in concern. The objective of any analytical measurement is to obtain consistent and reliable data avoiding false positive and negative discoveries. Validated analytical methods play a major role in achieving this goal. Results from method validation provide the picture of the quality, reliability and consistency of analytical results, which are fundamental for any good analytical practice. Method validation has received considerable attention since many years in the literature. For targeted metabolomics, there are several validation guidelines available, especially focused on instrumental aspects, while for non-targeted approach there are no guidelines available and they will only be promoted by the research community, because regulatory agencies are mainly focused on manufactured products. However, researchers are using several alternative ways to validate non-targeted approaches. The aim of this review paper is to discuss about the different validation criteria that are being used in analytical methods for non-targeted metabolomics and as well proposing validation steps to carry out this type of analysis.

2. Concept of validation

In the mid of 1970s in order to improve the quality of pharmaceuticals, the concept of validation was first proposed by the Food and Drug Administration (FDA) [18]. According to FDA validation is "*Establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes*" [19]. A properly designed system will provide a high degree of assurance that every step, process and change has been properly evaluated before its implementation. Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. It is the process of defining an analytical requirement and confirms that the method under consideration has performance capabilities consistent with what the application requires. Results from method validation can give an overview about the method quality. Quite often method validation evolves from method development and so the two activities are closely tied.

Method validation has received considerable attention in the literature and there are several guidelines available for analytical and bio-analytical aspect and they are as follows:

a. The United States FDA established two industrial guidelines. First one for the validation of analytical methods (this guidance provides recommendations to applicants on submitting analytical procedures, validation, data and samples to support the documentation of the identity, strength, quality, purity and potency of drug substances and drug products) and second one for the validation of bioanalytical methods (this guidance applies to bioanalytical methods used for human or non-human clinical, pharmacological, toxicological studies and preclinical studies-based on bioanalytical procedures such as chromatography, immunology and microbiology) [20,21].
b. ICH developed two guidelines for method validation that were later merged in one: Q2-R1. It discusses the considered characteristics (terminology and definitions) and methodology to be used during the validation of the analytical procedures [22].
c. International Union of Pure and Applied Chemistry published "Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis". This guideline provides minimum recommendations on procedures that should be employed to ensure adequate validation of analytical methods [23].

The above-described guidelines are mainly focused on seven common parameters that should be considered during bioanalytical method validation in order to establish the method "fit-for-purpose". The definition for these seven parameters has been included in Table 1.