Journal of Pharmaceutical and Biomedical Analysis 113 (2015) 137–150

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Review

An overview of fecal sample preparation for global metabolic profiling

Olga Deda[a], Helen G. Gika[b,*], Ian D. Wilson[c], Georgios A. Theodoridis[a]

[a] Department of Chemistry, Aristotle University of Thessaloniki, 54124, Greece
[b] Department of Chemical Engineering, Aristotle University, Thessaloniki 54124, Greece
[c] Department of Surgery and Cancer, Imperial College, Exhibition Road, South Kensington, London SW7 2AZ, UK

ARTICLE INFO

Article history:
Received 3 February 2015
Accepted 5 February 2015
Available online 14 February 2015

Keywords:
Feces
Metabolomics
Metabonomics
Disease biomarker discovery
Gut microflora

ABSTRACT

The global metabolic profiling of feces represents a challenge for both analytical chemistry and biochemistry standpoints. As a specimen, feces is complex, not homogenous and rich in macromolecules and particulate, non-digested, matter that can present problems for analytical systems. Further to this, the composition of feces is highly dependent on short-term dietary factors whilst also representing the primary specimen where co-metabolism of the host organism and the gut-microbiota is expressed. Thus the presence and the content of metabolites can be a result of host metabolism, gut microbiota metabolism or co-metabolism. Successful sample preparation and metabolite analysis require that the methodology applied for sample preparation is adequate to compensate for the highly variable nature of the sample in order to generate useful data and provide insight to ongoing biochemical processes, thereby generating hypotheses. The current practices for processing fecal samples for global metabolic profiling are described with emphasis on critical aspects in sample preparation: e.g., homogenization, filtration, centrifugation, solvent extraction and so forth and also conditions/parameter selection are discussed. The different methods applied for feces processing prior to metabolite analysis are summarized and illustrated using selected examples to highlight the effect of sample preparation on the metabolic profile obtained.

Contents

1. Introduction

Global metabolic profiling, known by the terms metabolomics and metabonomics, is being increasingly employed to determine metabolic phenotypes, provide novel biomarkers of various conditions and generate hypotheses. Metabolomics is defined in the Oxford English dictionary as "the scientific study of the set of metabolites present within an organism, cell, or tissue" whilst metabonomics is "the quantitative measurement of the dynamic multi parametric metabolic response of living systems to physiological stimuli or genetic modification" [1]. This simultaneous detection, identification and quantification of a large number of metabolites, often at extremely varied concentrations, is technically very demanding so each link of this chain must be studied and optimized. The most widely used analytical platforms are nuclear magnetic resonance (NMR) spectroscopy, gas chromatography mass spectrometry (GC–MS) and liquid chromatography mass spectrometry (LC–MS) [2–4].

Numerous studies reflect the need to improve the detection, the identification and particularly data processing, in order to produce

* Corresponding author. Tel.: +30 2310996224.
E-mail address: gkikae@auth.gr (H.G. Gika).

http://dx.doi.org/10.1016/j.jpba.2015.02.006
0731-7085/

reliable data from metabolic profiling studies [5–11]. However, it could be argued that the process of the sample preparation prior to metabolomics analysis has not yet received the necessary attention [12]. As a result, there is no universal approach in the preparation of samples for holistic analysis. Limiting factors are the matrix diversity and also sample physiological variation [13]. There is arguably a significant need for the development of robust, repeatable methods for sample preparation. An optimal sample preparation method should be short, reproducible and capable of extracting all metabolites while avoiding sample alteration through the process [14].

In the life sciences, the specimens of interest are biofluids such as plasma, serum, urine, bile etc., and tissue. Another interesting specimen which, to date, has not been the subject of major research in metabolomics is feces. In fact, stool material is the most accessible biospecimen for research, aimed at revealing the effect of gut microbiota on host metabolism. Trillions of microbiota coexist in the human or animal intestine, creating a human–microbe hybrid that has been termed a "super-organism" [15]. The population of many hundreds of species of gut bacteria plays a significant role in the life of host, affecting the balance between health and disease [16,17]. The gut microflora can be categorized in four major phyla: Firmicutes and Actinobacteria (Gram-positive) and Bacteroidetes and Proteobacteria (Gram-negative). Firmicutes and Bacteroidetes dominate the mammalian intestine [17] and are responsible for creating the appropriate nutrient environment [18], regulating fat storage [19] and keeping the mucosal immune system of the host healthy [20,21]. The basic functions performed by the gut microbiota include bile salt metabolism, synthesis of vitamins, digestion and fermentation of non-digestible polysaccharides and proteins, and stimulation of the immune function [22]. Recent investigations have examined the effects on the gut microbiota of antibiotic treatment [21] and probiotics [23]. There are cases where ingestion of probiotics has a negative effect, such as when these are administered to premature infants they can cause sepsis [24]. The gut microflora exhibits a pronounced effect on entero-hepatic recirculation, as observed in various studies. The role of the gut microflora in chronic gastrointestinal disease has been reviewed [25,26].

In the analysis of feces ^{1}H NMR spectroscopy has been the major analytical tool. NMR is a non-destructive technique, with less parameters to optimize, in comparison to chromatographic methods where many aspects must be taken into account, in order to have, a sensitive robust and stable chromatographic system. GC–MS, has also found use due to the high sensitivity and resolution it provides and the potential for structural identification of candidate biomarkers [27]. LC–MS although the most widely applied method in metabolomics analysis [3], has not yet been widely used in fecal analysis.

The aim of the present review is to summarize the processes applied for sample preparation in metabolomics analysis of stool. The reported methods vary from simple to rather extensive processes employing a number of steps and techniques (homogenization with different approaches, lyophilization, mixing, centrifugation, filtration, metabolite extraction, derivatization and so forth). Few studies have investigated the influence of the various sample preparation steps on the finally obtained metabolic profile. The present review also aims to highlight the key points in such multi-step processes. Metabolomics based biomarker discovery is challenging but when this is performed in feces, there is one additional source of complexity: biomarkers within such a complex specimen could be a result of metabolism from gut microflora.

2. Sampling and storage of fecal samples

Irrespective of the subsequent method of analysis, the first step in fecal metabolite profiling is the collection and storage of the samples. Feces from laboratory animals are obtained directly from their intestine after sacrifice [23], or as pellets from their cages [28]. Following collection, common practice includes storage of the samples at −80 °C, −40 °C or −20 °C, sometimes after snap freezing with liquid nitrogen [16,29]. Some studies have investigated the effect of the storage conditions [30,31] but there is no consensus whether storage may contribute as a differentiation factor or not [25]. In the case of human samples, specific instructions are typically given to patients regarding sample collection. In a study on IBS (Inflammatory Bowel Syndrome) and UC (Ulcerative Colitis) [32], patients were given a sample kit that contained sterilized plastic bags into which the samples were placed. These bags were then sealed with a clip and placed directly in vessels with ice before transportation to the laboratory. In other cases, feces were collected prior to surgery or endoscopic examination [33].

3. Fecal sample preparation for ^{1}H NMR analysis

Sample preparation can be a critical point in global metabolic profiling irrespective of the method of analysis. Because of the heterogeneous nature and complexity of feces, inefficient extraction, or poor reproducibility, will be reflected in the quality of the resulting ^{1}H NMR spectra. The conversion of raw, untreated, fecal samples to extracts suitable for analysis should be performed in such a way as to maximize metabolite extraction and avoid bias or editing of the fecal metabolome [25]. An optimized preparation method for metabolomics analysis should be non-selective and reproducible [30]. An obvious example of how fecal metabolite profiles can be affected is sample homogenization, particularly in the case of fecal pellets from rodents, as this greatly affects extraction efficiency. Disaggregating the sample enables better penetration of the extracting buffer/solvent throughout the sample, resulting in more efficient extraction (Deda et al., in preparation). Sonication and mechanical smashing, by using a mechanical crusher, are steps that may also affect the metabolic profile obtained from feces whilst the use of a "tissuelyser" results in more homogeneous extracts. Such factors have been examined by Saric et al. [31] and Wu et al. [16], as discussed below in detail.

The most commonly used extracting solvent [21,31,32,34] is phosphate buffered saline (PBS; 1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). PBS is prepared directly in D_2O [28] or distilled water [21,34]. The use of deuterated water for the preparation of buffer improves spectral quality (Deda et al., in preparation), but is also associated with an increased cost per sample. The pH value of the extraction solvent should also be considered carefully as pH alteration may lead to the degradation of some metabolites (e.g. through hydrolysis) [16] and at the same time may affect extraction efficiency of ionized or ionizable metabolites. In the literature, the ratio of feces weight to PBS volume (mg μL^{-1}) varies from 1:2 [21,34] to 1:50 [32], without explanation of the specific ratio selected.

Following extraction, another important factor is the removal of particulates by centrifugation and in most cases two centrifugation cycles are used. The first centrifugation is performed after extraction with PBS and the second immediately before pipetting the extracts into the NMR tubes for spectroscopic analysis. The centrifugation duration varies, spanning between 1 min [32], 10 min [30], 15 min [28,33,34], 30 min [23], 1 h [21], up to 2 h [35].

Another common practice to remove particulate is filtration. Usually, a cell strainer filter is used first, followed by centrifugation through a syringe filter. Filtration was not found to greatly affect the metabolic profile, but leads to cleaner, particulate free extracts, thereby helping to obtain improved shimming of the NMR spectrometer and ensuring a better quality of the NMR spectrum. For techniques such as LC–MS, filtration, is essential (as