Journal of Pharmaceutical and Biomedical Analysis 148 (2018) 273–279

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

The combination of four analytical methods to explore skeletal muscle metabolomics: Better coverage of metabolic pathways or a marketing argument?

C. Bruno[a,b], F. Patin[a,b], C. Bocca[c], L. Nadal-Desbarats[b], F. Bonnier[d], P. Reynier[c], P. Emond[b], P. Vourc'h[a,b], K. Joseph-Delafont[a], P. Corcia[b,e,f], C.R. Andres[a,b], H. Blasco[a,b,*]

[a] CHRU de Tours, Laboratoire de Biochimie et Biologie Moléculaire, Tours, France
[b] UMR INSERM U930, Université François Rabelais de Tours, France
[c] Institut MITOVASC, CNRS 6015, INSERM U1083, Université d'Angers, Angers, France
[d] Université François-Rabelais de Tours, Faculté de Pharmacie, EA 6295 Nanomédicaments et Nanosondes, Tours, France
[e] Centre de Ressources et de Compétences SLA, CHU Tours, France
[f] Fédération des Centres de Ressources et de Compétences de Tours et Limoges, Litorals, France

ARTICLE INFO

Article history:
Received 1 July 2017
Received in revised form 12 October 2017
Accepted 13 October 2017
Available online 18 October 2017

Keywords:
Metabolomics
Muscle
NMR
Mass spectrometry

ABSTRACT

Objectives: Metabolomics is an emerging science based on diverse high throughput methods that are rapidly evolving to improve metabolic coverage of biological fluids and tissues. Technical progress has led researchers to combine several analytical methods without reporting the impact on metabolic coverage of such a strategy. The objective of our study was to develop and validate several analytical techniques (mass spectrometry coupled to gas or liquid chromatography and nuclear magnetic resonance) for the metabolomic analysis of small muscle samples and evaluate the impact of combining methods for more exhaustive metabolite covering.
Design and methods: We evaluated the muscle metabolome from the same pool of mouse muscle samples after 2 metabolite extraction protocols. Four analytical methods were used: targeted flow injection analysis coupled with mass spectrometry (FIA-MS/MS), gas chromatography coupled with mass spectrometry (GC–MS), liquid chromatography coupled with high-resolution mass spectrometry (LC–HRMS), and nuclear magnetic resonance (NMR) analysis. We evaluated the global variability of each compound i.e., analytical (from quality controls) and extraction variability (from muscle extracts). We determined the best extraction method and we reported the common and distinct metabolites identified based on the number and identity of the compounds detected with low analytical variability (variation coefficient < 30%) for each method. Finally, we assessed the coverage of muscle metabolic pathways obtained.
Results: Methanol/chloroform/water and water/methanol were the best extraction solvent for muscle metabolome analysis by NMR and MS, respectively. We identified 38 metabolites by nuclear magnetic resonance, 37 by FIA-MS/MS, 18 by GC–MS, and 80 by LC–HRMS. The combination led us to identify a total of 132 metabolites with low variability partitioned into 58 metabolic pathways, such as amino acid, nitrogen, purine, and pyrimidine metabolism, and the citric acid cycle. This combination also showed that the contribution of GC–MS was low when used in combination with other mass spectrometry methods and nuclear magnetic resonance to explore muscle samples.
Conclusion: This study reports the validation of several analytical methods, based on nuclear magnetic resonance and several mass spectrometry methods, to explore the muscle metabolome from a small amount of tissue, comparable to that obtained during a clinical trial. The combination of several techniques may be relevant for the exploration of muscle metabolism, with acceptable analytical variability and overlap between methods However, the difficult and time-consuming data pre-processing, processing, and statistical analysis steps do not justify systematically combining analytical methods.

1. Introduction

Metabolomics is an emerging science that has largely evolved since its development, due to impressive progress in analytical

* Corresponding author at: Laboratoire de Biochimie et Biologie Moléculaire, Hôpital Bretonneau, Bâtiment B2A, 2 Boulevard Tonnellé, 37044 Tours, France.
E-mail address: helene.blasco@univ-tours.fr (H. Blasco).

https://doi.org/10.1016/j.jpba.2017.10.013
0731-7085/

techniques and the promise of its use in the healthcare field [1–6]. This approach has been developed using a variety of analytical techniques, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. To date, 40 153 metabolites have been described, identified, and referenced in the Human Metabolome DataBase (HMDB, http://www.hmdb.ca/), based on the use of these techniques, alone, or in combination [4,5,7]. There is a large overlap in the metabolites identified by these techniques and such experiments are costly and time-consuming. It is thus not clear whether combining several analytical techniques are generally useful or more attractive for the investigation of specific tissues, characterized by several over- and under-represented biochemical pathways. Exploration of the muscle metabolome is of utmost importance for a better understanding of metabolic and/or neuromuscular diseases, but no standard method has been described [8–12]. The objectives of our study were to develop and validate several analytical techniques (GC–MS, LC–MS, and NMR) for performing metabolomic studies on small muscle samples and evaluate their combination. We also aim to purpose an "optimized" combination of methods, especially for muscular tissue in this study.

2. Materials and methods

2.1. Sample collection

Experiments were performed on medial hamstring muscle samples collected from 10 wild type mice previously used in a closed preclinical study [13]. A total of 500 mg of muscle specimens was collected from ten mice just after sacrifice. The samples were pooled and stored in 10- and 30-mg aliquots at −80 °C (Fig. 1(1)).

2.2. Sample preparation

Metabolites were extracted following two different protocols (Fig. 1), the NMR protocol and the MS protocol, for analysis by several mass spectrometry techniques, including flow injection analysis coupled with mass spectrometry (FIA-MS/MS), gas chromatography coupled with mass spectrometry (GC–MS), and ultrahigh performance liquid chromatography coupled with high-resolution mass spectrometry (LC–HRMS). Details of the extraction protocols are provided in Supplemental data. For each protocol, we systematically performed 15 different extractions protocols of muscle samples to evaluate the global variability by each technique.

2.3. NMR

A semi targeted metabolomics approach was performed by NMR. Each extract was analysed as previously described [9] on a Bruker AVANCE III 600MHZ spectrometer® (see Supplemental data for more details). We only compared the qualitative and quantitative content of muscle extracts between the two extraction protocols, as intra-assay precision has been previously evaluated [9].

2.4. FIA-MS/MS

A semi-quantitative targeted metabolic fingerprint strategy by FIA-MS/MS was performed for the determination of amino acids and acylcarnitines using the Perkin Elmer Neobase Kit (Perkin Elmer Life and Analytical Sciences, Turku, Finland). Additional data concerning sample preparation and the MS methods are detailed in Supplemental data. Analytical variability has been previously validated for routine use following the *Comité Français d'Accréditation* (COFRAC) recommendations [14] using quality controls (QC). Thus, we only evaluated the global variability of the experiment from the analysis of 15 muscle sample extracts.

2.5. GC–MS

We performed a semi-quantitative targeted approach by GC–MS (see details in Supplemental data). We only evaluated the global variability between 15 different muscle sample extracts, as this protocol has already been validated and published for cell extracts [15], and is used in routine practice for organic acid chromatography.

2.6. LC–HRMS

We used a recently published non-targeted approach [16] performed on a Dionex UltiMate 3000 UHPLC system (Dionex, Sunnyvale, USA) coupled to a Thermo Scientific Q Exactive HRMS (Thermo Fisher Scientific, Bremen, Germany). Additional data for this experiment are provided in Supplemental data. We assessed the analytical variability from 10 QC samples (obtained after pooling 10 μL of each extract) and global variability from the analysis of extracts from 15 muscle samples.

2.7. Building of the final dataset

We collected the area or concentration of all peaks detected for each experiment. We normalised metabolite levels using internal standards (IS) for each technique. In addition, we tested two additional normalisation methods, considering the dried muscle mass or the concentration of hydrophilic proteins, measured by Nanodrop™ (ThermoFisher®, Waltham, USA). Only metabolites with a coefficient of variation <30% were used to build the final dataset for further analysis [13,15,17]. We compared the methods based on the number, intensity, and variability of the area of the peaks.

2.8. Evaluation of combining analytical methods

We built a Venn diagram (Venny 2.1) to represent common and distinct metabolites identified using the various analytical techniques. The identification of corresponding metabolic pathways was performed using MetaboAnalyst (http://www.metaboanalyst.ca/).

3. Results

3.1. NMR

We identified 38 metabolites by NMR (Table A, Annexe A). Extracts from protocol NMR.b contained several contaminants, and several metabolites were extracted only using protocol NMR.a (not shown). There was no difference in peak intensity between both methods (not shown). Thus, we considered protocol NMR.a to be superior.

3.2. FIA-MS/MS

We semi-quantified 42 metabolites, regardless of the method of extraction used, but the relative abundance of several types varied between the two methods (Fig. 2). More metabolites had a CV < 30% when protocol MS.a was used than protocol MS.b (Supplemental Table A). Normalisation against the amount of water-soluble proteins enabled the analysis of a total of 37 metabolites with acceptable CV (15.5+/−6.1%) with MS.a (Table A, Supplemental table A).