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Metabolomic application in toxicity evaluation and toxicological biomarker identification of natural product

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ABSTRACT

Natural product plays a vital role in disease prevention and treatment since the appearance of civilization, but the toxicity severely hinders its wide use. In order to avoid toxic effect as far as possible and use natural product safely, more comprehensive understandings of toxicity are urgently required. Since the metabolome represents the physiological or pathological status of organisms, metabolomics-based toxicology is of significance to observe potential injury before toxins have caused physiological or pathological damages. Metabolomics-based toxicology can evaluate toxicity and identify toxicological biomarker of natural product, which is helpful to guide clinical medication and reduce adverse drug reactions. In the past decades, dozens of metabolomic researches have been implemented on toxicity evaluation, toxicological biomarker identification and potential mechanism exploration of nephrotoxicity, hepatotoxicity, cardiotoxicity and central nervous system toxicity induced by pure compounds, extracts and compound prescriptions. In this paper, metabolomic technology, sample preparation, data process and analysis, and metabolomics-based toxicological research of natural product are reviewed, and finally, the potential problems and further perspectives in toxicological metabolomic investigations of natural product are discussed.

1. Introduction

Natural product has a long history of use to prevent and treat diseases since the appearance of civilization, and the popularity is increasing for health care and chronic disease treatment. In ancient times, natural product was extremely significant to resist the attack of diseases. Even in this seemingly advanced era, natural product is an essential part as medications and prodrugs, directly or indirectly treating diseases. The resources of natural product are plants, animals and minerals. However, safety issues of natural product has been raised, including the identification of plant materials and active principles, the method of preparation, dosing regimens, the potential to interact with other herbal remedies and conventional drugs, and assurances that herbal products are genuine and do not contain toxins or contaminants [1]. Without knowing toxic compositions, concentrations and mechanisms, the use of natural product may be unhealthy, harmful, and even lethal. Feasible and efficient steps need to be carried out to solve this problem before toxins cause physiological or pathological damages. Metabolomics appears ideal for this purpose. Metabolomics has minimal damage to the body due to little sample needed, which make it convenient compared to proteomics and genomics.

Metabolomics, first put forward by professor Nicholson in 1999 [2], is defined as systematically qualitative and quantitative analysis of metabolites in a given organism or biological sample and

Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; AA, aristolochic acid; AAN, aristolochic acid nephropathy; AC, *Aconitum carmichaelii*; AF, *Aristolochia fangchi*; AM, *Aristolochia manshuriensis*; AR, *Alismatis rhizoma*; ATO, Arsenic trioxide; BFAJT, Bu-Fei-A-Jiao-Tang; EK, *Euphorbia kansui*; FA, *Fructus Aristolochia-contorta*; GC-MS, gas chromatography-mass spectrometry; GR, *Glycyrrhizae Radix et Rhizoma*; HPLC, high performance liquid chromatography; HYZ, Huang-Yao-Zi; JDJ, JingDaJi; LysoPC, lysophosphatidylcholines; MGS, Morning Glory Seed; MSOP, MS-based orthogonal projection; NJT, Niuhuang Jiedu Tablet; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA, principal component analysis; PGs, prostaglandins; PLA2, Phospholipase A2; PLS-DA, partial least squares-discriminant analysis; PR, *Platycodonis Radix*; RR, *Rhei Radix et Rhizoma*; SPE, solid-phase extraction; SR, *Scutellariae Radix*; ST, *Stephania tetrandra*; TMAO, trimethylamine-*N*-oxide; UPLC, ultra performance liquid chromatography; VB, Venenum Bufonis; XF, *Xanthii Fructus*; ZSASW, Zhusha Anshen Wan.

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then quantificationally describes changes of endogenous metabolites before and after stimulations or disturbances [3]. Metabolomics is suitable for observing abnormal changes of endogenous metabolites before the appearance of physiological or pathological damages. Since the metabolome represents the physiological or pathological status of organisms [4], metabolomics can be used in toxicity evaluation [5] and toxicological biomarker identification.

2. Metabolomic technology

Frequently-used technologies in metabolomics are proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-MS, high performance liquid chromatography (HPLC)-MS, ultra performance liquid chromatography (UPLC)-MS and LC-solid-phase extraction (SPE)-NMR. The different metabolomic techniques have been applied to different herbal medicine research [6]. ^{1}H NMR allows the rapid, high-throughput and automated analysis of crude extracts, and then quantitatively detects metabolites in many different groups [7,8], as well as provides structural information including stereochemical details [9]. However, ^{1}H NMR fails to obtain valid data when the concentrations of metabolites in complex sample are lower [10]. LC-SPE-NMR can solve such problems. Due to the enrichment effect of SPE, the sensitivity can be improved. Compared to MS-based approaches, NMR is less sensitive and its data is limited that was metaphorically described as 'the tip of the iceberg' [7,8,11]. GC-MS is particularly suitable for the detection of thermally stable volatile metabolites (or metabolites with volatile derivatives). GC-MS, with high resolution and sensitivity, is usually used for quality control and qualitative and quantitative determination of active components in natural product [12]. However, the application range of GC-MS is limited. Only volatile metabolites in sample can be directly analyzed, but most metabolites are non-volatile that we might lose the information.

With continuous technical updates of LC-MS, the utilization is more frequent. LC has the ability to isolate different kinds of metabolites in a complex system. MS can provide structural information to help to identify metabolites. LC-MS costs less and provides more details of large and submerged portions than NMR [11]. LC-MS allows the analysis of thermally labile non-volatile metabolites [13]. The molecules that can be detected by LC-MS range from polar sugars and non-aromatic organic acids [14] through to various lipids [15]. The ability of LC-MS to analyze various kinds of metabolites depends strongly on the ionization source and the chromatographic method that is used to separate a complex mixture of analytes [1]. UPLC from Waters Corporation, with sub-2 μm chromatographic particles and a fluid system capable of operating at pressures up to 15,000 psi, elevates better chromatographic resolution than traditional HPLC operated with larger particle [16]. MS^E can obtain highly accurate parent ion and fragment ion information in one analytical run. MS^E provides parallel alternating scans for acquisition at either low collision energy to obtain precursor ion information, or ramping of high collision energy to obtain full-scan accurate mass fragment, precursor ion and neutral loss information [17,18]. UPLC-MS^E is considered to be appropriate for metabolomic study, especially for large-scale untargeted metabolic profiling in complex biological sample.

3. Sample preparation

Urine, serum, plasma and tissue are common analytical sample. Minimal sample preparation steps are supposed to perform on urinary samples to decrease the loss of potential biomarkers, as urine is a complex sample including various endogenous and exogenous acidic, basic, and neutral compounds with high polarity. Urine sample needs to be centrifuged at 13,000 rpm to remove solid and diluted with deionized water before metabolomic analysis. SPE can extract substance with special properties.

Plasma and serum samples usually can be restored in −80 °C for 24 months or in −20 °C for 1 month. Prior to analysis, plasma and serum samples need be thawed at room temperature. Acetonitrile or methanol is added to serum and vortex-mixed vigorously for several minutes. The mixture is then centrifuged at 13,000 rpm for minutes at 4 °C. The supernatant were pipetted out, then analyzed directly or lyophilized.

Tissue is harvested after in situ cardio perfusion. Then, the tissue is immediately washed with physiological saline and stored at −80 °C for the following metabolomic study. The sample is homogenized in acetonitrile in an ice bath. Samples were then vortex-mixed for minutes, and put on ice in between. Following centrifugation (13,000 rpm, 4 °C), the supernatant is removed and then lyophilized. The extract was resuspended before analysis.

4. Data process and analysis

A flow chart of a typical targeted and untargeted metabolomics experiment is shown in Fig. 1. As analytical instruments progress, data acquisition is not the challenge of metabolomic research. NMR and MS-based technology can output data automatically and directly, and the corresponding software can help to process and analyze data. Before analysis, the evaluations of the method including stability, accuracy, precision and reproducibility need to be done. As described in Fig. 1, metabolomics can be divided into targeted metabolomics and untargeted metabolomics. Targeted metabolomics usually quantify the most abundant metabolites and use simpler statistical approach than untargeted metabolomics, while untargeted metabolomics usually use multivariate statistical analysis, such as principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

The basic principles of data analysis are comparing different metabolic expression between control group and model group, and then assessing the significance of difference by statistic methods. PCA is an unsupervised method that can summarize the information in an experimental dataset using a small number of orthogonal latent variables obtained by searching the direction of maximum variance in data set. PCA does not always extract hidden information which explains system behavior, as this may not correspond to the information summarized in the latent variables. Supervised techniques, like PLS-DA and OPLS-DA, may be preferred for these cases. PLS analysis maximizes the product of variance matrix of measured variables and correlation of measured data with properties of interest [19]. OPLS is an extension of PLS and has similar objectives to Orthogonal Signal Correction but is integrated directly in the modeling, which allows an easier validation of orthogonal components. The common software for metabolomic analysis includes the Shimadzu Class-VP software (allowing PCA analysis), CIMCA-P software (allowing PCA and PLS-DA analysis), and Micromass MarkertLynx Application software (allowing peak detection and peak alignment).

The identification of metabolites is considered as a big challenge in untargeted metabolomics. NMR and MS are the main technology platforms in metabolomic research, and increase the possibility to analyze metabolic pathway of micromolecules. The updating corresponding commercial software is crucial for identifying