Mini Review

GC/MS/MS-based targeted metabolomics for pathophysiological analysis of animal models

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Abstract Metabolomics is an essential tool for not only understanding the pathophysiology of various diseases but also for searching for initial clues to unknown toxic effects of drugs. Mass spectrometry-based metabolomics has achieved highly sensitive and selective analysis of metabolites, and gas chromatography mass spectrometry remains a gold standard because of its robustness and usability. However, it is tedious to annotate metabolites with electron ionization (EI)-based mass spectra; thus, gas chromatography tandem mass spectrometry (GC/MS/MS)-based metabolome analysis has played an important role in metabolomics. In particular, the selected reaction monitoring (SRM) mode achieves higher selectivity and an improved signal-to-noise ratio, which leads to easier metabolite identification. In this mini review, we concisely outline the pros and cons of GC/MS/MS-based metabolome analysis and provide its applications to pathophysiological analysis of disease and drug-induced toxicity in animal models based on our previous studies. Finally, future perspectives for newly developed high-throughput metabolome analysis are briefly described.

Key words: targeted metabolome analysis, gas chromatography tandem mass spectrometry

Introduction

Metabolome analysis is a powerful tool for investigating the pathophysiology of various diseases and/or toxic effects of drugs together with other omics techniques[1−4]. Currently, mass spectrometry-based platforms are widely used for metabolome analysis in various fields because of their high selectivity and sensitivity[5]. Although appropriate derivatization methods such as methoxylation and trimethylsilylation are required for gas chromatographic separation of analytes[6,7], the gas chromatography/mass spectrometry (GC/MS)-based platform has remained a gold standard for targeted metabolome analysis because GC is a robust analytical system, and a capillary column shows high separation capacity for analytes. However, metabolite annotation using electron ionization (EI)-based mass spectra can be tedious for analysts, even when automated annotation software is available for EI-based metabolome data, and there is also room for improving quantitativity, which will be mainly achieved by increasing selectivity; the selectivity of GC/MS is sometimes insufficient when targets and contaminants are co-eluted.

To obtain a higher signal-to-noise (S/N) ratio and selectivity, gas chromatography tandem mass spectrometry (GC/MS/MS)-based targeted metabolome analysis has been increasingly used in medical and toxicological fields[8−15]. Since selected reaction monitoring (SRM) mode can dra-

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matically improve selectivity and S/N ratio, GC/MS/MS, especially SRM mode analysis, provides not only high operability for metabolite annotation, which is achieved by simplifying the results, but also higher relative quantitativity. Indeed, our team successfully applied GC/MS/MS-based targeted metabolome analysis to drug-induced acute intoxication in animal models to estimate unknown toxic effects of drugs and understand their pathophysiology[4,16,17]. As described later, GC/MS/MS-based targeted metabolome analysis is extremely useful for exploratory investigation of initial clues to unknown mechanisms of various diseases and drug-induced toxicity.

Some vendors currently provide ready-to-use GC/MS/MS-based targeted metabolome platforms, and such commercially available platforms can greatly contribute to the widespread use of metabolomics in various fields. GC/MS/MS-based metabolome analysis has also become more familiar to scientists who will use metabolomics in their research. Moreover, technological advances in ionization for mass spectrometry will provide researchers with a new approach to high-throughput metabolome analysis, and ambient ionization/mass spectrometric techniques have been extended to various scientific fields including metabolomics.

In this mini review, we concisely outline the pros and cons of GC/MS/MS-based targeted metabolome analysis and introduce its application to drug-induced toxicity and animal disease models for pathophysiological analysis based on our previous studies. Additionally, we provide future perspectives using new analytical techniques, namely ambient ionization/mass spectrometry, for high-throughput metabolome analysis.

GC/MS/MS-based Targeted Metabolome Analysis

GC/MS/MS-based targeted metabolome analysis has been performed not only in chemical ionization (CI) mode but also in EI mode[8–15]. From the viewpoint of method development, the CI-based method is generally simpler than the EI-based one because CI normally generates protonated or deprotonated ions; thus, precursor ions are easily selected for each metabolite. Xu et al. used GC/positive chemical ionization (PCI)-MS/MS to analyze 37 fatty acids derived from methyl esters in the plasma of coronary artery disease patients[10]. Tsikas et al. reviewed a GC/electron capture negative ion chemical ionization (ECNICI)-MS/MS-based method for oxidized and nitrated oleic acid in biological samples[9]. Generally, negative ion chemical ionization dramatically improves the S/N ratio, which is mainly due to background noise reduction and improved ionization selectivity[18,19]. In their study, pentafluorobenzyl (PFB) ester derivatization was used to produce $[M\text{-}PFB]^-$ and $[M\text{-}H]^-$ ions, which were selected as precursor ions, improving the selectivity of the method.

Unlike CI, EI provides many fragment ions during ionization because high internal energies are supplied to the ionized molecules created by EI. Since the intensity of molecular ions ($M^{+\bullet}$) is generally weak or undetected in EI analysis, analysts sometimes need to select fragment ions as precursor ions for collision-induced dissociation. Specifically, the GC/EI-MS/MS-based method is more complex with respect to optimizing analytical conditions, such as selecting SRM transitions and setting the collision energy (CE), than that with GC/CI-MS/MS, though a large variety of SRM transitions can be selected for each metabolite, allowing the selectivity of the method to be dramatically enhanced. Tsugawa et al. reported a GC/EI-MS/MS method using SRM analysis with TMS derivatization, achieving simultaneous detection of 110 metabolites[11]. Also, Hirata et al. used a GC/EI-MS/MS-based metabolome method and determined sensitive metabolic biomarkers for pancreatic cancer in human blood[13].

To develop a GC/EI-MS/MS-based metabolome method, an enormous amount of time is required to select appropriate SRM transitions, optimize the CE for each transition, and allocate scheduled SRM. Therefore, some vendors have provided commercially available methods for GC/MS/MS-based metabolome analysis, which are optimized in advance and are thus highly practical for users who want to begin using metabolomics.

In our previous studies, we used an EI-based method for metabolome analysis[12,14,15], which is commercially available from Shimadzu Corporation (Kyoto, Japan). With this method, appropriate SRM transitions are selected in advance, and the CEs for each SRM transition and scheduled time are sufficiently optimized. Currently, ca. 500 metabolites are pre-registered for the method and can be simultaneously detected in a single run, and quantifier and qualifier ions for each metabolite are selectable, which enhances the identification accuracy for the targeted metabolites. Additionally, the retention indices of each metabolite are pre-registered in the method, and the retention times of

each metabolite can be easily adjusted by analyzing alkanes[20,21].

Based on these procedures, we can easily change the scheduled time and optimize dwell times for each transition using Excel-based software provided by the vendor. Particularly, adjusting and optimizing scheduled times for each SRM transition are time-consuming unless analysts can use such optimization software. Owing to such user-friendly tools, commercially available methods facilitate widespread use of metabolomics in various fields.

As described above, methoxylation and trimethylsilylation are generally used for metabolite derivatization in GC/MS- and GC/MS/MS-based metabolome analyses, though some researchers used other derivatization techniques to improve sensitivity or selectivity. Kvitvang et al. reported that methyl chloroformate (MCF) derivatization was applied to 67 principal metabolites including amino acids and non-amino organic acids, achieving significant sensitivity for these in urine and serum samples[8]. This suggested that there is a high potential to improve the selectivity or sensitivity by changing derivatization methods in GC/MS/MS-based targeted metabolome analysis. However, a limitation of GC/MS/MS-based metabolome analysis is that it is generally difficult to sensitively detect highly polar metabolites such as phosphate metabolites, and these cannot all be detected by GC/MS/MS. Thus, compensatory analytical techniques such as liquid chromatography tandem mass spectrometry (LC/MS/MS)-based-metabolome analysis are also necessary to expand the coverage of target metabolites.

Application of GC/MS/MS-based Targeted Metabolome Analysis to Pathophysiology of Animal Models

Pathophysiological analysis of animal disease models by GC/MS/MS-based targeted metabolome analysis

As is well known, metabolome analysis is a useful tool for investigating initial clues concerning the pathophysiology of various diseases animal models. To investigate potential biological markers, however, metabolome analyses on blood samples from animal disease models were performed in most studies, while more information is obtained by tissue metabolome analysis to achieve pathophysiological understanding of diseases models, because the blood metabolome is easily changed depending on metabolome changes in pathological and/or toxicity-affected organs. For instance, we performed metabolome analysis on serotonin syndrome (SS) rat models, where hyperthermia and abnormal muscle contraction such as myoclonus are the main symptoms of SS[15,22]. We applied GC/MS/MS-based metabolome analysis to plasma, liver and muscles of control and the SS rats and readily identified 144−195 metabolites in plasma and the tissue samples. Many plasma metabolites were significantly increased, though these have no remarkable networkability (Fig. 1a). Significantly changed metabolites in liver and muscles showed respective networkability (Fig. 1b−d), and these changes were strongly related to the SS symptoms. Consequently, these results suggest that significant changes in the plasma metabolome of the SS model rats were caused by the metabolome changes in the target tissues. We also observed site differences in metabolome changes in muscles, which were well matched to supporting results obtained from gene-expression analysis of *uncoupling protein-3* (*UCP-3*). Finally, we revealed the pathophysiology of SS based on metabolome analysis as shown in Fig. 2.

Exploratory investigation of unknown toxic effects of drugs in toxicology

In forensic toxicology, abuse of new psychoactive substances (NPSs) is a social problem worldwide[23]. To date, various NPSs have been distributed in underground markets, and cathinones and cannabinoid receptor (CBR) agonists are most frequently abused[24−26]. It is essential to evaluate the acute toxicity of NPSs in forensic toxicology, though minimal information is available on their toxicity. This is because they are generally synthesized in clandestine laboratories, and it is too difficult for forensic toxicologists to obtain information on either their chemical structures or pharmacokinetics[27]. Nevertheless, some NPSs are highly toxic and sometimes fatal to humans[28,29]. Although it is too difficult to extrapolate phenotypic changes in human from the metabolome changes in animal models, metabolomics is a useful technique for understanding NPS-induced toxicity in animal models to search important clues related to their toxic effects. In particular, the results obtained are easily understandable as phenotypic changes, which are pivotal to estimating the unknown toxic effects of NPSs on animal models[30−32]. This idea is also accepted in toxicological fields for estimating or predicting the adverse effects of drugs on animal models, and thus metabolomics has been widely applied not only to forensic toxi-

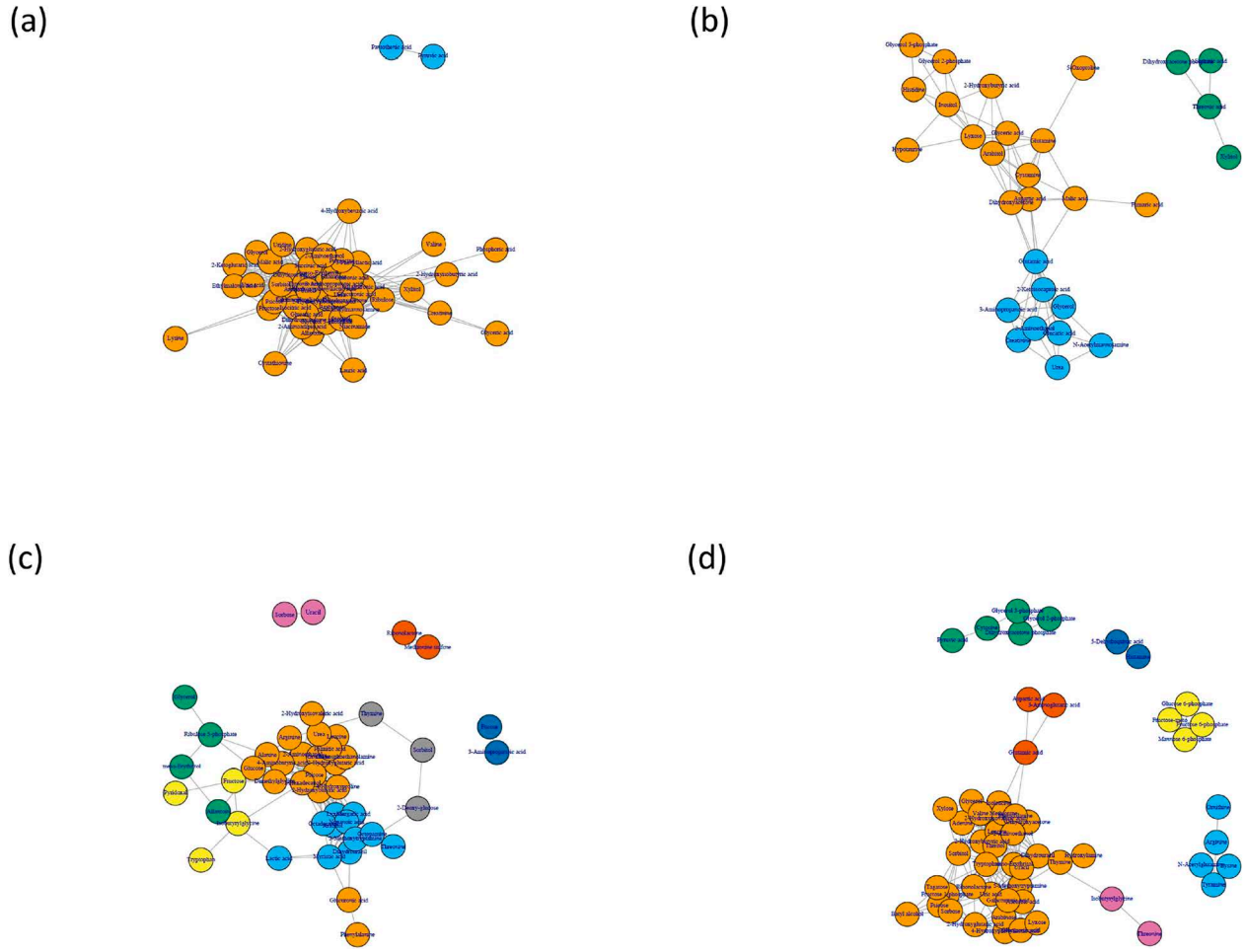

Fig. 1. Networkability of the significantly altered metabolites in each tissue.
(a) Plasma, (b) liver, (c) gastrocnemius muscle, and (d) trapezius (reprinted from ref. 15 with permission).

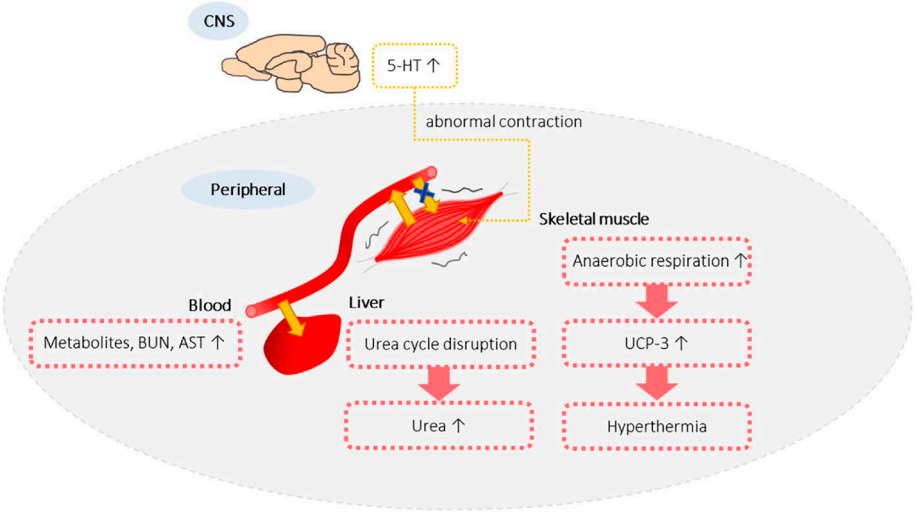

Fig. 2. Pathophysiology of the metabolome alteration observed in serotonin syndrome rat models (reprinted from ref. 15 with permission). Abnormal contraction of skeletal muscles induces up-regulation of anaerobic respiration, resulting in hyper thermogenesis via UCP-3 activation. Various metabolites were also accumulated in blood, which finally leads to urea cycle disruption in the liver and increase of BUN.

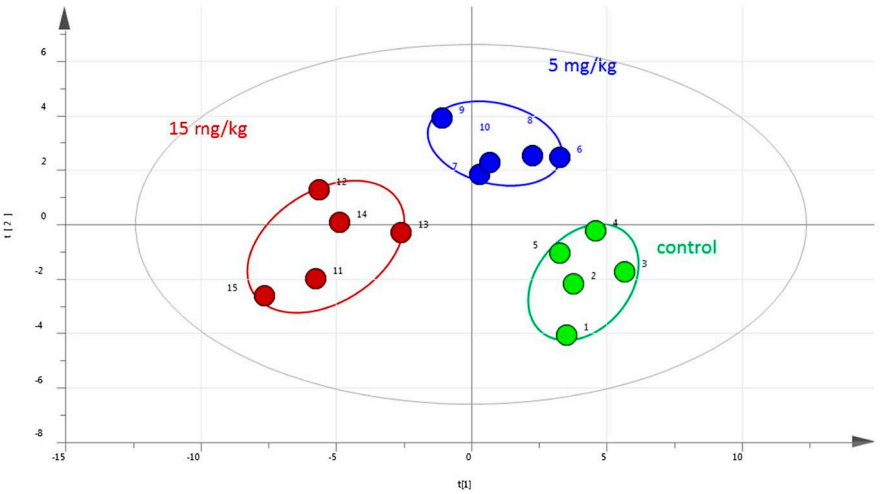

Fig. 3. Score plots of projection to latent structures discriminant analysis for control and cannabinoid receptor agonist administered mice (reprinted from ref. 14 with permission). Blue plots, 5 mg/kg administered group; red plots, 15 mg/kg administered group; green plots, control group. These groups were separated along the first principle component axis, which shows a dose-dependence effect of the agonist.

cology but also to toxicological assessment using animals. A concise review on applying metabolomics to the adverse effects of drugs has already been published[17].

In our study, a high dose of CBR agonist can induce seizure-like abnormal behavior in rodents, though the main mechanisms of this have not been elucidated; we applied GC/MS/MS-based metabolome analysis to a CBR agonist administered to rat cerebrum to investigate clues to abnormal behavior[14]. In this study, rat cerebrum was pretreated by the Bligh and Dyer extraction method, and an internal standard (IS) correction method was used for relative quantification. Various normalization methods such as IS-based and quality control-based techniques have been reported for metabolome analysis, and it is necessary to select an appropriate correction method for matching experimental conditions and/or sample features[33–35]. Based on the pre-experimental results, GC/MS/MS-based targeted metabolome analysis showed high repeatability for extracts from rat cerebrum samples. Finally, we applied projection to latent structures discriminant analysis (PLS-DA) to the obtained metabolome data, demonstrating the dose-dependent effect of the drug (Fig. 3). Based on the loading plots, the CBR agonist can induce energy metabolism and glutamatergic excitatory neurotransmission disorders in the cerebrum. Interestingly, these results are consistent with other researchers' findings that CBR agonists can induce mitochondrial dysfunction by stimulating CBRs on the outer mitochondrial membrane[36,37].

Future Perspectives for a Newly Developed Metabolome Analysis

As described above, GC/MS/MS-based targeted metabolome analysis has played a pivotal role in metabolomics because of its selectivity, operability, and robustness. Chromatographic separation is necessary for simultaneous analysis of a vast number of metabolites, though it leads to a low-throughput analytical run. Particularly, some derivatized metabolites are known to be unstable even at room temperature, and their degradation can bias the final results. Thus, there is a pressing and underlying need to develop a high-throughput analytical method for metabolomics.

Ambient ionization techniques such as direct analysis in real time (DART) and desorption electrospray ionization (DESI) are currently widely used in various fields[38–41], and many reports have been published on their applications to metabolome analysis[42–45]. Ambient ionization techniques lack chromatographic separation, resulting in lower selectivity, though their high-throughput nature is advantageous.

Our team also developed high-throughput analysis of metabolites using probe electrospray ionization tandem mass spectrometry (PESI/MS/MS) and applied this to animal model tissues[42,44]. PESI was developed in 2007, and this uses a thin probe needle for sampling and ionization units[46]. Our team combined PESI and tandem mass spectrometry, resulting in higher selectivity and S/N ratio. Particularly, our system can directly analyze tissue samples such as liver and brain without sample pretreatment,

achieving total flow rapidity for metabolite analysis. Thus, our system was introduced as the fastest method in a review article by Zampieri et al.[47]. We have extended the method to one hundred metabolites and completed direct tissue analysis within 5 min with no tedious sample pretreatment. Ambient ionization/mass spectrometry will be a new analytical tool for metabolome analysis.

Conclusion

In this mini review, we outline GC/MS/MS-based targeted metabolome analysis and its application to pathophysiological analysis of animal disease models and investigation of unknown toxic effects of drugs on animals. GC/MS/MS-based targeted metabolome analysis generally shows high selectivity and improves S/N ratio, providing user-friendly platforms for metabolome analysis. In our previous studies, GC/MS/MS-based metabolome analysis strongly helped us to interpret the pathophysiology in the animal models. For the serotonin syndrome model rats, we were able to systematically understand biological changes in the model rats. We also easily found that the abused cannabinoid receptor agonist can cause energy metabolism disruption in rat cerebrum. Although it is difficult to extrapolate biological effect on human from such animal studies, we could estimate the phenotypic changes such as toxicity by applying metabolome analysis to animal models. Additionally, we discuss future perspectives of new analytical tools, especially ambient ionization/mass spectrometry and its high-throughput nature, which will be useful for metabolome analysis as it requires no chromatographic separation. Improvement of such analytical tools will create new platforms for metabolomics, which can make metabolome analysis be easier on researchers.

Conflicts of Interest

There are no conflicts of interest to declare.

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