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RESEARCH PAPER

A preliminary study of rapid-fire high-throughput metabolite analysis using nano-flow injection/Q-TOFMS

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Abstract

In this study, we demonstrated nano-flow injection analysis (nano-FIA) with quadrupole time-of-flight mass spectrometry (Q-TOFMS) for 17 highly polar intermediates produced during glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway (PPP). We optimized the analytical conditions for nano-flow injection/Q-TOFMS, and set the flow rate and ion source temperature to 1000 nL/min and 150 °C, respectively. Under optimal conditions, a single run was finished within 3 min, and the RSD value of 50 sequential injections was 4.2%. The method also showed quantitativity of four stable-isotope-labeled compounds ($r^2 > 0.99$), demonstrating its robustness, high repeatability, and specificity. In addition, we compared three sample-preparation methods for rodent blood samples and found that protein precipitation with threefold methanol was the most effective. Finally, we applied the method to plasma samples from the serotonin syndrome (SS) model and control rats, the results of which were evaluated by principal component analysis (PCA). The two groups showed clearly separated PCA score plots, suggesting that the method could successfully catch the differences in metabolic profiles between SS and control rats. The results obtained from our new method were further validated by using the established gas chromatography/tandem mass spectrometry method, which demonstrated that there were good correlations between the two methods ($R = 0.902$ and 0.958 for lactic acid and malic acid, respectively, each at $p < 0.001$), thus proving the validity of our method. The method described here enables high-throughput analysis of metabolites and will be of use for the rapid analysis of metabolic profiles.

Keywords High-throughput metabolite analysis · Nano-flow injection analysis · Quadrupole time-of-flight mass spectrometry · Serotonin syndrome model

Introduction

Endogenous metabolites in biological fluids show a high diversity in their chemical structures and properties. It is therefore difficult to analyze the entire metabolome using a single analytical method. Mass spectrometry (MS) is a core technology in metabolomics due to its high sensitivity and selectivity. In general, to identify metabolites by MS, it is necessary to use chromatographic techniques, e.g., gas chromatography (GC) [1], liquid chromatography (LC) [2], or capillary electrophoresis (CE) [3, 4]; these techniques are powerful tools for the analysis of metabolites because retention time information can be obtained via chromatography, which is helpful in identifying target compounds. However, chromatography takes a long time and requires tedious validation methods. In addition, to detect metabolites in biological fluids, a multi-step sample preparation is mandatory prior to instrumental analysis. In particular, the extraction, concentration, and derivatization steps can potentially bias analytical results, leading to their

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misinterpretation. Simple sample preparation and rapid data acquisition methods are therefore required for metabolomics [5] with some simple methods such as ambient ionization MS having already been used for this purpose [6–13]. Ambient ionization MS techniques, such as desorption electrospray ionization (DESI) [6], direct analysis in real time (DART) [7], probe electrospray ionization (PESI) [8–11], and paper spray ionization (PSI) [12, 13], have been developed, which allow rapid analysis with minimal sample preparation. Our research group has also succeeded in developing a combination of PESI and tandem mass spectrometry (MS/MS) [9–11]. Ambient ionization MS has been successfully applied to high-throughput analysis though special ion sources are necessary for ambient ionization and there are some difficulties associated with performing ambient ionization techniques.

Flow injection analysis (FIA) is one of the simplest classical techniques and involves the injection of samples directly into a mass spectrometer. FIA has been recently applied to the analysis of metabolites [14–18], and an advantage of FIA is that it eliminates the need for chromatographic separation, allowing high-throughput data acquisition within 30 s per sample. The combined use of an autosampler with FIA enables the successful performance of high-throughput analysis of multiple samples. There is also no requirement for additional apparatus to conduct FIA/MS.

FIA/MS is conventionally operated at flow rates ranging from a few hundred μL/min to 1 mL/min. However, the sensitivity of conventional FIA/MS is insufficient for the detection of compounds at low concentrations. Although it is necessary to perform sample preparation steps such as concentration in order to detect trace-level compounds, unstable metabolites may decompose during sample preparation [19]. Thus, it is important to improve the sensitivity of FIA/MS and also to simplify the sample preparation steps. In general, the sensitivity of LC/MS can be increased by reducing the flow rate, and the nano-flow rate, which ranges from several hundred nL/min to a few μL/min, can actually enhance the efficiency of desolvation, ionization, and ion transfer, resulting in improvements in sensitivity [20]. Nano-flow LC/MS (nano-LC/MS) has been recently applied not only to proteomics [21, 22] but also to the analysis of small molecules [23, 24]. Nano-LC/MS enables the detection of endogenous molecules at low concentrations in biological fluids such as serum and plasma. Thus, it is expected that nano-flow injection analysis (nano-FIA) will enable the highly sensitive analysis of endogenous molecules. However, it is essential for FIA/MS to be combined with a highly selective mass spectrometer, because FIA/MS lacks chromatographic separation. In addition, to perform FIA/MS, it is preferable that the mass spectrometer used can execute high-speed data acquisition. Quadrupole time-of-flight mass spectrometry (Q-TOFMS) meets such demands, allowing both accurate mass measurement and high-speed data acquisition. Q-TOFMS has been widely used for the identification of drugs [25, 26] and endogenous metabolites [27–29] in biological fluids.

In this study, we developed nano-flow injection/Q-TOFMS (nano-FI/Q-TOFMS) for the analysis of intermediates produced during glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway (PPP). We also applied this method to the serotonin syndrome (SS) model and control rats to validate its practicality.

Experimental section

Reagents

Authentic standards of the intermediates produced during glycolysis, the TCA cycle, and the PPP were purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, USA). Stable isotopes of glucose 6-phosphoric acid-$^{13}C_6$, citric acid-D_4, succinic acid-D_6, and fumaric acid-D_4 were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA), while L-glutamic acid-$^{13}C_5$, $^{15}N_1$ was obtained from Taiyo Nippon Sanso Corporation (Tokyo, Japan). HPLC-grade methanol and chloroform were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Milli-Q water (Merck KGaA, Darmstadt, Germany) was used throughout the experiments.

Animal experiments and sample collection

Serum samples were obtained from ICR mice (male, 10 weeks old) under isoflurane anesthesia. Animal experiments using SS model rats were performed according to our previous report [30]. This animal study was approved by the Animal Experimental Committee of Nagoya University Graduate School of Medicine (No. 27356).

Sample preparation

Evaluation of sample preparation methods

To evaluate appropriate sample preparation methods, we compared protein precipitation (PP) or liquid-liquid extraction (LLE) procedures. Frozen mouse serum was thawed on ice, and then PP or LLE procedures were carried out. For PP, cold methanol was added to samples, where the volume ratio of methanol to serum was changed to 1:1 or 3:1 (v/v). After vortexing, followed by centrifugation, at 19,000×*g* at 4 °C for 3 min, the supernatant was collected. For LLE, cold chloroform-methanol solvent mix (2:1, v/v) was added to the samples. After vortexing, ultrapure water was added to each sample and they were further vortexed. After centrifugation, the upper layer was collected in a fresh tube and evaporated using a centrifugal concentrator (CVE-3100, EYELA,

Tokyo Rikakiki Co., Tokyo, Japan). The dried residue was reconstituted with a 75% aqueous solution of methanol. All samples were then filtered through 0.22-μm polyethersulfone (PES) membrane filter capsules (Merck KGaA) prior to analysis.

Evaluation of the method's quantitativity

Five microliters mixed internal standard (IS) solution (glucose 6-phosphoric acid-$^{13}C_6$, citric acid-D_4, succinic acid-D_6, and fumaric acid-D_4) was added to 45 μL of mouse serum. The final concentrations of spiked ISs were as follows: 1, 5, 10, and 25 μg/mL for glucose 6-phosphoric acid-$^{13}C_6$ and fumaric acid-D_4; 5, 10, and 25 μg/mL for citric acid-D_4; and 0.1, 0.2, 0.5, 1, 5, 10, and 25 μg/mL for succinic acid-D_6, respectively. The spiked serum and blank mouse serum were subjected to the extraction procedure described above.

Preparation of plasma samples from the SS model and control rats

Frozen plasma was thawed on ice, then 5 μL L-glutamic acid-$^{13}C_5$, $^{15}N_1$ (100 μg/mL) aqueous solution was added to 45 μL of plasma. The samples were treated with threefold volume methanol, as described above.

Analytical conditions and data analysis

Nano-FIA was performed using a ParadigmMS4 pump system (Michrom BioResources Inc., Auburn, CA, USA) and a PAL autosampler system (CTC Analytics AG, Zwingen, Switzerland). The flow rate was set to 1000 nL/min and a 50% methanol solution was used as the mobile phase.

Mass spectrometry was performed using a TripleTOF 5600 quadrupole time-of-flight mass spectrometer (Sciex, Framingham, MA, USA) equipped with a CaptiveSpray Ionization (CSI) source (Bruker, Bremen, Germany). The ionization voltage was set to 4 kV, and the heated interface temperature was set to 150 °C. The exact masses of the product ions and other MS parameters for each compound are shown in Table 1. Calculation of peak areas of the analytes was carried out using MultiQuant ver. 3.0.2 software (Sciex, Framingham, MA, USA). Multivariate analysis was performed using SIMCA-P+13 software (Umetrics, Umeå, Sweden), and box-and-whisker plots were made using Excel (Microsoft, Redmond, WA, USA). Statistical differences among the intermediates were evaluated using Welch's *t* test. The *p* values were adjusted to control multiple comparisons using the false discovery rate (FDR) procedure proposed by Benjamini and Hochberg [31], and the adjusted values were expressed as *q* values (with a significance level at 0.05). A GCMS-TQ8040 gas chromatograph-triple quadrupole tandem mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used to validate the results obtained from the newly developed method. Details of the analytical conditions used for GC/MS/MS were given in our previous reports [32, 33]. The Pearson correlation coefficient was calculated using the R software package (version 3.6.1) [34].

Results and discussion

Development of nano-FI/Q-TOFMS

We first analyzed authentic standard solutions of various metabolites using nano-FI/Q-TOFMS to optimize the analytical conditions (Table 1). Following optimization of the flow rate, total ion current (TIC) peak areas of three representative metabolites were inversely proportional to the flow rate used for the method, although the reproducibility was extremely low at a flow rate of 500 nL/min (see Electronic Supplementary Material (ESM) Fig. S1). As already mentioned, nano-flow improves the efficiency of desolvation, ionization, and ion transfer, resulting in higher sensitivity [20]. However, it is difficult to regulate the actual flow rate of nano-FIA if the flow rate is extremely low, which makes ESI become unstable. Also, the peak area values at each flow rate did not change based on any changes in ion source temperature (ESM Fig. S1). Consequently, we determined that the optimal flow rate and ion source temperature were 1000 nL/min and 150 °C, respectively, the latter being the default temperature of CSI. Next, we compared three different injection volumes (1, 2, and 5 μL). As shown in ESM Fig. S2, the largest peak area was obtained with an injection of 5 μL. However, the reproducibility was low with a 5-μL injection, and we set the injection volume to 2 μL.

In this study, we used a CSI source which can perform ESI in an enclosed space, which makes it possible to introduce all ions into the mass spectrometer [35]. To optimize declustering potential (DP), which is a drift voltage at the ion entrance, we continuously ramped up the DP from − 150 to 0 V and monitored the intensity of product ions derived from the three metabolites (ESM Fig. S3). Their intensities increased slightly depending on the voltage, with the highest intensities observed at 0 V, demonstrating that the drift voltage did not contribute to ion transfer in the CSI source. Thus, we selected 0 V for the DP in our method; this can also avoid in-source CID, which is advantageous for generating precursor ions.

To compare the sensitivity of nano- and conventional FIA, we analyzed standard solutions using both methods. As shown in Fig. 1, a higher sensitivity for fructose 6-phosphoric acid was obtained using nano-FIA compared with that obtained by conventional FIA. Moreover, wider peak widths were obtained with nano-FIA, allowing the simultaneous detection of a large number of analytes because many SRM transitions can be set to a method. As shown in ESM

Table 1 A list of target compounds in rodent blood

	Compound name	Polarity	Exact mass (*m/z*)			Collision energy (V)	Mass accuracy of the product ions (mDa)
			Precursor ion	>	Product ion		
Glycolysis							
1	Glucose 1-phosphoric acid/glucose 6-phosphoric acid/fructose 6-phosphoric acid	Negative	259	>	96.969	−22	0.9
2	Fructose 1,6-bisphosphoric acid	Negative	339	>	96.969	−30	0.8
3	Glyceraldehyde 3-phosphoric acid	Negative	169	>	96.969	−14	0.3
4	3-Phosphoglyceric acid/2-phosphoglyceric acid	Negative	185	>	96.969	−19	0.8
5	Pyruvic acid	Negative	87	>	43.018	−9	6.1
6	Lactic acid	Negative	89	>	43.018	−14	3.6
TCA cycle							
7	Citric acid/isocitric acid	Negative	191	>	111.008	−15	1.0
8	α-Ketoglutaric acid	Negative	145	>	101.024	−12	0.8
9	Succinyl-CoA	Negative	866	>	408.011	−51	1.3
10	Succinic acid	Negative	117	>	73.029	−14	1.2
11	Fumaric acid	Negative	115	>	71.013	−9	1.5
12	Malic acid	Negative	133	>	71.013	−22	1.5
13	Oxaloacetic acid	Negative	131	>	87.008	−12	0.4
Pentose phosphate pathway							
14	6-Phosphogluconic acid	Negative	275	>	96.969	−24	1.0
15	Ribose 5-phosphoric acid/ribulose 5-phosphoric acid	Negative	229	>	96.969	−19	0.2
16	Sedoheptulose 7-phosphoric acid	Negative	289	>	96.969	−29	0.6
17	Erythrose 4-phosphoric acid	Negative	199	>	96.969	−16	0.2

Table S1, the peak areas of 22 target metabolites showed values that were 7.6 to 66 times higher following nano-FIA compared with conventional FIA.

Optimization of sample preparation

We investigated whether the sample preparation method could affect the results of nano-FIA, by comparing three different procedures. As shown in ESM Fig. S4, it was apparent that the PP with threefold volume methanol provided the best peak shape, while the other procedures resulted in poor peak shapes. In FIA, the sample is diluted by diffusion in the mobile phase, and this leads to matrix effects of diverse magnitudes across the peak, and a split peak shape can be observed at high matrix concentrations, especially at the peak center [37]. This is likely what is causing the peak distortions in PP with equal volume methanol and LLE results, where these samples were not sufficiently diluted in sample preparation (ESM Fig. S4). It was therefore determined that PP using threefold volume methanol was the most straightforward and suitable sample preparation method for nano-FIA.

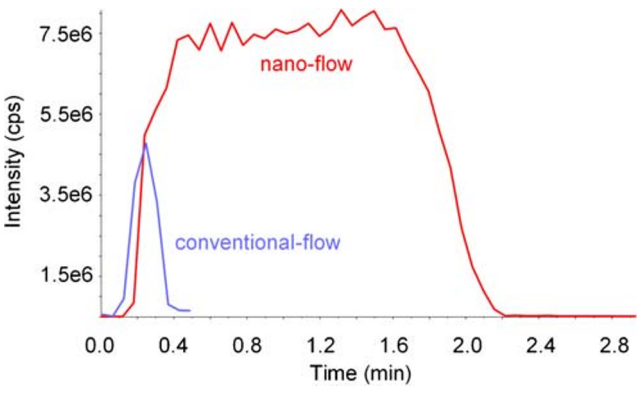

Fig. 1 Total ion current (TIC) chronograms [36] of fructose 6-phosphoric acid using conventional- and nano-flow analyses. Injection volume, 2 μL; flow rate, 0.1 mL/min for conventional flow and 1000 nL/min for nano-flow

Detection and identification of metabolites in biological fluids

Next, we investigated whether our new nano-FI/Q-TOFMS method could identify the metabolites in mouse serum. We analyzed mouse serum in accordance with the optimized method. All compounds could be detected using the negative ionization mode, and the mass accuracies of their product ions were lower than ca. 6 mDa (Table 1). Generally, intermediates of the PPP are not retained in a reversed-phase column because they are highly hydrophilic. They can also be easily

adsorbed onto the surface of the stainless-steel parts of a column because they have a phosphoric acid moiety within their molecules [38]. Previously, CE/MS [39, 40] or hydrophilic interaction chromatography (HILIC) [41, 42] column- and metal-free [43] column-based LC/MS have been used for the analysis of phosphorylated organic compounds. In contrast, our system was able to detect the intermediates of the PPP much more simply, as described earlier.

Evaluation of the analytical method

To evaluate the linearity of the regression line ranging from 0.1 to 25 μg/mL, stable-isotope-labeled glucose 6-phosphoric acid-$^{13}C_6$, citric acid-D_4, succinic acid-D_6, and fumaric acid-D_4 were added to mouse serum and pretreated with methanol for protein precipitation, where triplicate analyses were performed for each point though one outlier, which was determined by the Smirnov-Grubbs test, was found at 25 μg/mL for glucose 6-phosphoric acid-$^{13}C_6$, and thus, the outlier was omitted from construction of the regression line. The regression coefficient (r^2) of each line was greater than 0.99 (Fig. 2), which indicates that nano-FI/Q-TOFMS shows an acceptable quantitativity.

In addition, we calculated lower limit of detection (LLOD) values using the regression lines by the following equation: LLOD = 3.3σ/S, where σ is the standard deviation of blank serum and S is a slope of the regression line. The LLOD values of glucose 6-phosphoric acid-$^{13}C_6$, citric acid-D_4, succinic acid-D_6, and fumaric acid-D_4 were 2.2, 2.0, 0.11, and 0.78 μg/mL, respectively, demonstrating satisfactory sensitivity.

As mentioned earlier, glucose 6-phosphoric acid-$^{13}C_6$ is generally adsorbed onto the surface of stainless-steel columns, which may result in low repeatability, although our system showed good linearity (r^2 = 0.999) and repeatability (RSD = 1.5–19%) for glucose 6-phosphoric acid-$^{13}C_6$.

We further evaluated the robustness and stability of the system. We performed 50 continuous analyses, where we monitored L-glutamic acid-$^{13}C_5$, $^{15}N_1$ in supernatant obtained from mouse serum following protein precipitation. As shown in Fig. 3, the peak areas of L-glutamic acid-$^{13}C_5$, $^{15}N_1$ did not fluctuate during the analyses (RSD = 4.2%) and there was no decrease in sensitivity. These results demonstrated that nano-FI/Q-TOFMS is a highly robust system with high repeatability.

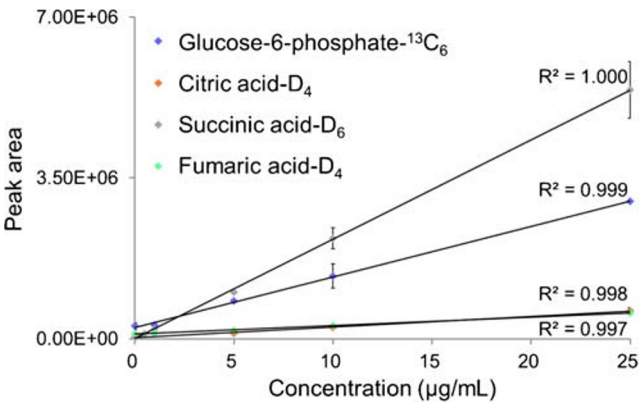

Fig. 2 Regression lines for glucose 6-phosphoric acid-$^{13}C_6$, citric acid-D_4, succinic acid-D_6, and fumaric acid-D_4. Concentration ranges of the stable-isotope-labeled compounds were as follows: glucose 6-phosphoric acid-$^{13}C_6$ and fumaric acid-D_4, 1–25 μg/mL; citric acid-D_4, 5–25 μg/mL; succinic acid-D_6, 0.1–25 μg/mL. These were spiked to mouse serum (n = 3 of each)

Application to plasma samples of the SS model and control rats

To evaluate the practical applicability of our system, plasma samples from the SS model and control rats (n = 6 of each) were analyzed by nano-FI/Q-TOFMS. The raw data are shown in ESM Table S2, and multivariate analysis was applied to the normalized data with IS. The model and control groups were clearly separated in their principal component analysis (PCA) score plots, demonstrating that there is a difference in the metabolic profiles of the SS model and control groups (Fig. 4a). The SS model group was widely plotted PCA score plots, which may be due to individual differences resulting from the syndrome; this was supported by the results of their rectal temperature measurements [30]. Additionally, the loading plots showed that the levels of all metabolites were higher in the model group (Fig. 4b). Most of the metabolites were significantly elevated in the model rats (ESM Fig. S5), suggesting SS-induced upregulation of catabolism, which may be due to abnormal muscle contractions such as myoclonus [44]. A significant test also demonstrates that fumaric acid and sedoheptulose 7-phosphoric acid were not significantly different between two groups (ESM Table S3).

To validate the present results, the established GC/MS/MS-based metabolome analysis [32, 33] was applied to plasma samples taken from the SS model rats [30], and the results

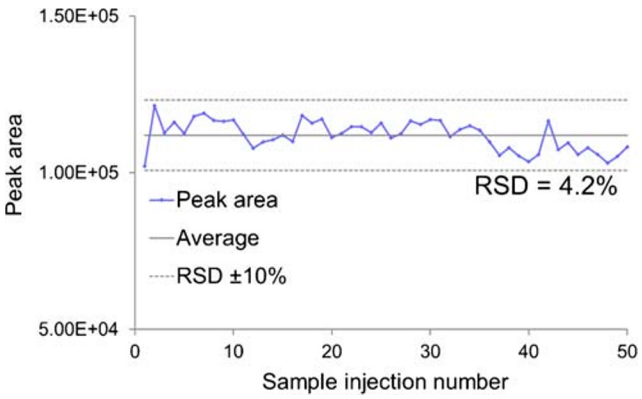

Fig. 3 Fluctuations of the peak areas of glutamic acid-$^{13}C_5$, $^{15}N_1$ spiked in mouse serum

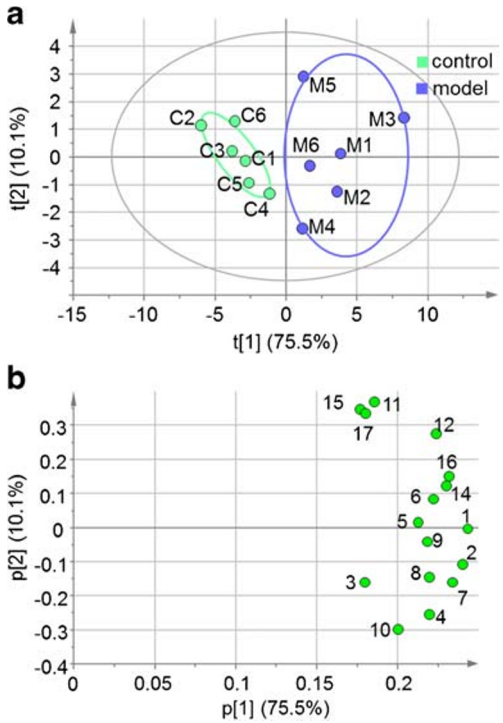

Fig. 4 Principal component analysis (PCA) score plots (**a**) and loading plots (**b**). The numbers in the loading plots correspond to the compounds in Table 1. Unit variance scaling was used and the ellipse in the score plots shows Hotteling's T^2 (< 95%)

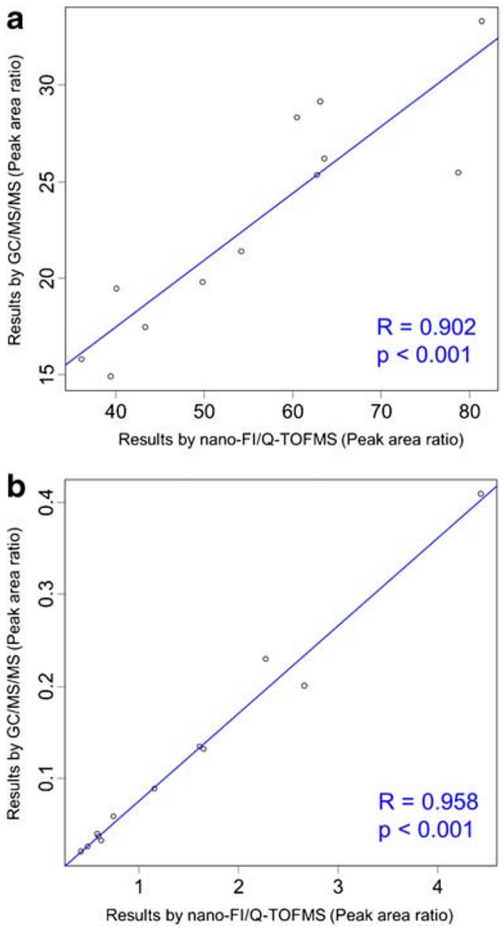

Fig. 5 Correlations between the results by GC/MS/MS and nano-FI/Q-TOFMS. **a** Lactic acid and **b** malic acid. The units of *X*-axis and *Y*-axis are peak are ratio (target compound/ISTD)

of representative metabolites such as lactic and malic acids were compared. As shown in Fig. 5, the results between nano-FI/Q-TOFMS and GC/MS/MS were closely correlated for both lactic and malic acids, proving that nano-FI/Q-TOFMS can obtain similar results to GC/MS/MS.

Limitations of the study and future perspectives

Although the nano-FI/Q-TOFMS method described here enabled us to perform high-throughput analysis of endogenous metabolites related to central energy metabolism in blood samples, it is essential to increase the number of target metabolites if we are to expand the present method to metabolome analysis.

It is impossible to differentiate between isomers by the present method when they produce the same product ion spectra. However, the combined use of ion mobility spectrometry (IMS) with MS has been recently applied to the analysis of small molecules [45, 46]. Thus, the use of IMS could compensate for the lack of chromatographic separation in FIA/MS. Furthermore, non-targeted metabolome analysis could be performed using a data-independent acquisition (DIA) method based on sequential window acquisition of all theoretical mass spectra (SWATH) analysis.

Conclusion

We successfully achieved high-throughput metabolite analysis using a nano-FI/Q-TOFMS approach. We proved that this method is robust and has high repeatability, with a single run being completed within 3 min. We applied the method to samples from the SS model and control rats, and demonstrated that it can easily discriminate between their metabolic profiles. In particular, it was possible to accurately determine highly polar intermediates of the PPP in spite of their tendency to adsorb onto the surface of stainless steel. In addition, sample preparation for this method was finished with minimum steps in comparison with the number required for LC/MS and GC/MS. The present method allows high-throughput analysis, which can prevent bias arising from the degradation of target compounds. Thus, it is expected that this method is able to be a compensatory method for the chromatographic analysis of metabolites. Although the number of target compounds in this preliminary study was limited, we plan to increase this number and this work is currently underway.

Author contributions K. Zaitsu conceived the idea and supervised the experiments. K. Taki and K. Zaitsu designed the experiment and wrote the manuscript. K. Taki performed all the experiments, and K. Taki and K. Zaitsu executed the bioinformatics analysis. K. Zaitsu, Y. Hayash, and S. Noda performed the animal experiments. K. Zaitsu and T. Taki discussed the results. A. Ishii and H. Tsuchihashi commented on the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict interest.

Animal experiments The animal study was approved by the Animal Experimental Committee of Nagoya University Graduate School of Medicine (No. 27356). We executed animal experiments in accordance with the Regulations on Animal Experiments in Nagoya University and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No. 71 of the Ministry of Education, Culture, Sports, Science and Technology in Japan, 2006).

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