Journal of the Korean Magnetic Resonance Society **2013**, *17,* 1-10
DOI 10.6564/JKMRS.2013.17.1.001

Applications of NMR spectroscopy based metabolomics: a review

Dahye Yoon, Minji Lee, Siwon Kim and Suhkmann Kim*

Department of Chemistry, Pusan National University, Busan, 609-735, Korea

Received May 31, 2013; Revised June 06, 2013; Accepted June 10, 2013

Abstract Metabolomics is the study which detects the changes of metabolites level. Metabolomics is a terminal view of the biological system. The end products of the metabolism, metabolites, reflect the responses to external environment. Therefore metabolomics gives the additional information about understanding the metabolic pathways. These metabolites can be used as biomarkers that indicate the disease or external stresses such as exposure to toxicant. Many kinds of biological samples are used in metabolomics, for example, cell, tissue, and bio fluids. NMR spectroscopy is one of the tools of metabolomics. NMR data are analyzed by multivariate statistical analysis and target profiling technique. Recently, NMR-based metabolomics is a growing field in various studies such as disease diagnosis, forensic science, and toxicity assessment.

Keywords NMR, metabolomics, multivariate statistical analysis, target profiling

Introduction

Metabolomics, the systematic study of the full complement of metabolites in a biological sample, has become increasingly popular and significant in the life sciences.[1-3] Metabolomics allows for a comprehensive assessment of organism responses in the situation of the immediate environment changes, taking into account genetic regulation, altered kinetic activity of enzymes, and changes in metabolic reactions.[4-7] Compared with genomics or proteomics, metabolomics reflects changes in phenotype reactions.[8] The omic sciences are complementary as ‘‘upstream’’ changes in genes and proteins, however, metabolomics are measured ‘‘downstream’’ as changes in cellular metabolism.[9-10] Metabolomics is also a terminal view of the biological system, in the central dogma, not allowing for representation of the genes and proteins that are up or down regulated.[11]

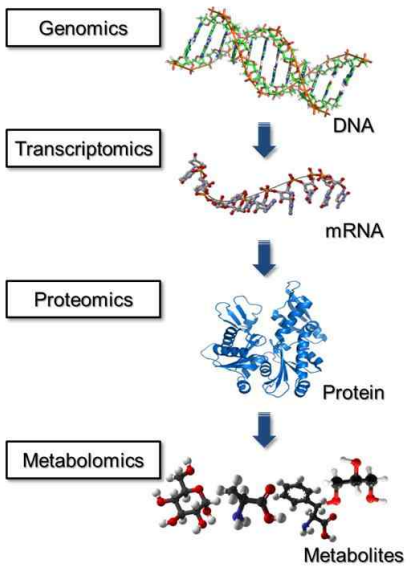

Figure 1. Schematic diagram of central dogma

Together with other omics, metabolomics provides additional information on specific reactions occurring in organism, enabling us to understand some of the metabolic pathways in pathological processes.[12-16]

* Address correspondence to: **Suhkmann Kim,** Department of Chemistry, Pusan National University, Busan, 609-735, Korea; E-mail: suhkmann@pusan.ac.kr

Metabolomics is the study of low molecular weight organic molecules within a cell, tissue, and bio fluids and how their composition varies with an external stressor. These molecules include endogenous metabolites, an important role in biological systems, which consist of amino acids, fatty acids, carbohydrates, vitamins, peptides, nucleic acids, organic acids, and lipids.[1,2,8] The metabolome reflects past events that include whole metabolism and the interaction with the environment, whereas the genome reflects the real and potential functional information of organism. Interactions with the environment (exposure to drugs or chemicals) or the onset of a disease disrupts a homeostasis at different levels of the biological organization, including the metabolome.[11] These metabolites, biological markers or biomarkers, are measurable internal indicators of molecular and/or cellular alterations that may appear in an organism after or during exposure to a toxicant and possible disease. Despite the significant advances in analytical technologies, the discovery of biomarkers in biological fluids remains a significant challenge.[8] Currently, metabolomics is an emerging field in a wide variety of studies such as toxicology, drug discovery, nutrition, cancer, diabetes, natural product discovery and environmental stress.[3,17,18] NMR and MS are the most commonly used techniques for measuring the metabolome.[11] Each technique has distinct advantages and disadvantages. MS determines the composition of molecules based on the mass-to-charge ratio in charged particles. For example, LC/MS is highly sensitive, typically at the picogram level, and permits highly specific multiple metabolite identification at low concentrations. However, MS sensitivity is dependent on metabolite pK and hydrophobicity. Similarly, the methods of extraction, quenching, and sample storage conditions can affect and potentially modify metabolite structure, thereby confounding already complex data sets and introducing greater sample-to-sample variability.[22-26] NMR exploits the behavior of molecules when placed in a magnetic field, allowing the identification of different nuclei based on their resonant frequency.[20] Although MS is more sensitive than NMR, NMR spectroscopy has the advantages of being relatively robust across many samples, relatively fast in addition to being a non-destructive process.[22,27] Compared with other analytical

Table 1. Comparison of NMR and Mass for analytical tool in metabolomics

	NMR	Mass spectroscopy
Sensitivity	Less sensitive, but will be enhanced with higher field strength, high performance probe	High sensitivity and specificity
Sample measurement	The entire metabolites in one sample analyzed in one measurement	Need different chromatography techniques according to metabolites in one sample
Sample destructive	Non-destructive; sample can be recovered	Destructive to sample
reproducibility	Very high reproducibility	Moderate
Sample preparation	Minimal sample preparation required	Need derivatization/ chemical modification
Experimental time	Average analytical time of 5~10 minutes	more than 10minutes for simplest analysis
Tissue samples	HR-MAS NMR tissue samples analyzed directly	Requires tissue extraction
Experiment cost	Low per experiment cost	Requires high per experiment
Number of detectable metabolites in urine sample	40-100 depending on spectral resolution	Could be more 500
Molecular dynamic, molecular diffusion	NMR can be used to probe the molecular diffusion and dynamics	No
Quantitative analysis	Quantitative	Not fully quantitative without appropriate standards

approaches, NMR spectroscopy can be considered as a fast and reproducible method in metabolomics research. In general NMR-based metabolomics utilizes a combination of spectroscopic data collection and multivariate data analysis to look for patterns within groups of spectra and to monitor metabolic behaviors.

By applying NMR metabolomics techniques to biological samples, it is possible to detect and quantify NMR resonances arising from hundreds of small metabolites, providing a comprehensive picture of an organism's metabolic status at a given period of time. Low sensitivity is an inherent disadvantage of NMR spectroscopy. Metabolites in the mM and mid to high uM range can be detected, meaning that about 100 metabolites can be detected in urine samples and even fewer in serum and intact tissue samples.[27-29]

Magic angle spinning (MAS) is an NMR technique in which a small quantity of intact tissue can be placed in the spectrometer and analyzed. Extracts of tissues can and have been routinely studied using biofluid NMR techniques.[19] However, with any extraction procedure, questions remain about how representative the extract is the same with the original sample and the potential for secondary (nonbiological) reactions induced by the extraction procedure.[27-29] High-resolution magic-angle spinning (HRMAS) NMR spectroscopy of intact samples provides an efficient way to monitor the metabolites present in a tissue without preparation steps such as extraction.[30] The technique has been particularly popular to aid in the correlation between metabolic profiling of biofluids and the histology of specific tissues, with ^{1}H-HRMAS NMR spectroscopy being routinely used to provide metabolic information on small intact tissue samples, including kidney, liver, brain and cultured cells. By spinning samples at high speed (typically 2,000–6,000 Hz) at the magic angle to the magnetic field, spectra can be obtained that approach the resolution of solution-state NMR.[22]

The technique is carried out by placement of a few milligrams of intact tissue into a specially designed rotor, which is spun at high speed within the bore of the magnet. NMR spectra are composed of hundreds of NMR signals related to major metabolites.

To extract useful information from complicated NMR data, pattern recognition methods such as multivariate statistical analysis and target profiling

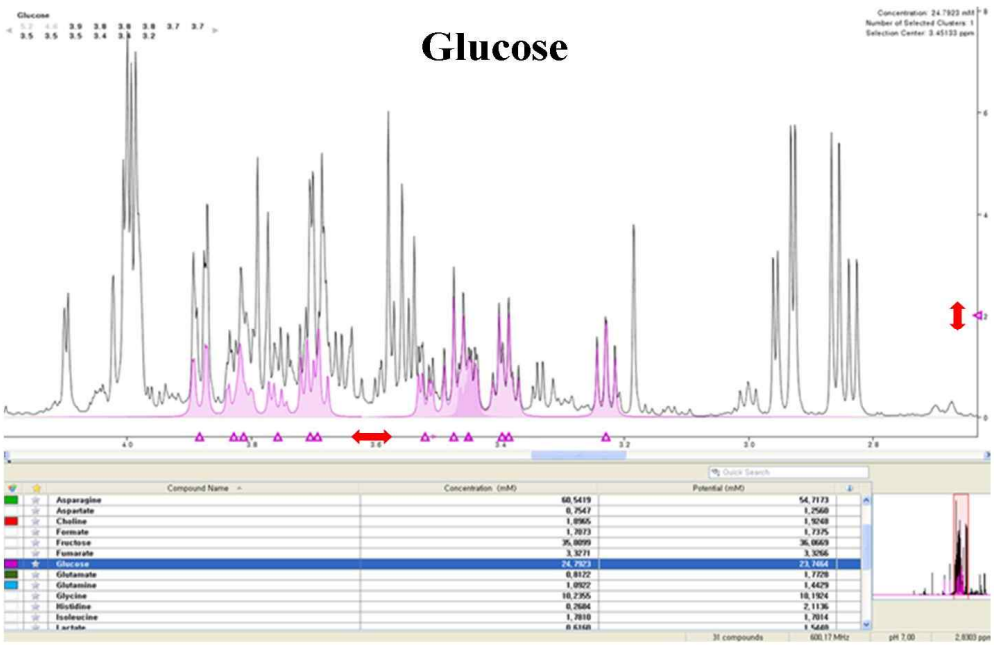

Figure 2. An example of the compound identification using Chenomx

techniques are usually utilized. Multivariate methods are important to metabolomics studies since one biomarker often will not be sufficiently specific for a given condition by itself.[11] The integrated area under the curve of each of small regions (referred to as ‘‘bins’’ or ‘‘buckets’’) is calculated, and these values serve as variables. A 0.04 ppm-wide region is a typical bin width which will produce 200 to 250 “buckets’’ of data from the typical 10 ppm NMR spectrum. Certain regions of the spectrum, such as those containing water and urea resonances (for urine), are typically excluded from the binning process. A preliminary step often relies on the use of unsupervised analyses such as principal component analysis PCA. This descriptive method does not require any information about the nature of samples.1 PCA plots are extremely powerful for rapid identification of inherent clusters in the data (which may be suggestive of a common effect or mechanism), assessment of dose-related and time-related changes, and the identification of individual outliers. Explicative analyses or classifications are performed by using supervised tools such as PLS (projection to latent structures or partial least squares) regression, PLS-discriminant analysis (PLS-DA), or OPLS (Orthogonal Projection on Latent Structure). These descriptive methods show the spectra were more separated according to information about the nature of samples.1 Multivariate methods have the advantage that biomarkers are readily identified from the model using the loading values.[11] In the other approach (targeted profiling), compounds are identified and quantified by comparing the NMR spectrum of the sample of interest to a spectral reference library obtained from pure compounds. By matching and fitting the reference peaks (which have been calibrated to an internal concentration standard; TSP(3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid), DSS(4,4-dimethyl-4-silapentane-1-sulfonic acid))to the sample peaks, it is possible simultaneously to identify and to quantify compounds. Once identification and quantification are complete, then statistical or pattern- recognition techniques can be applied to interpret the data.[33] Because quantitative metabolomics yields both compound identities and concentrations, this method provides explicit biomarker identification and immediate interpretation of metabolic pathways or processes. While small-molecule compound

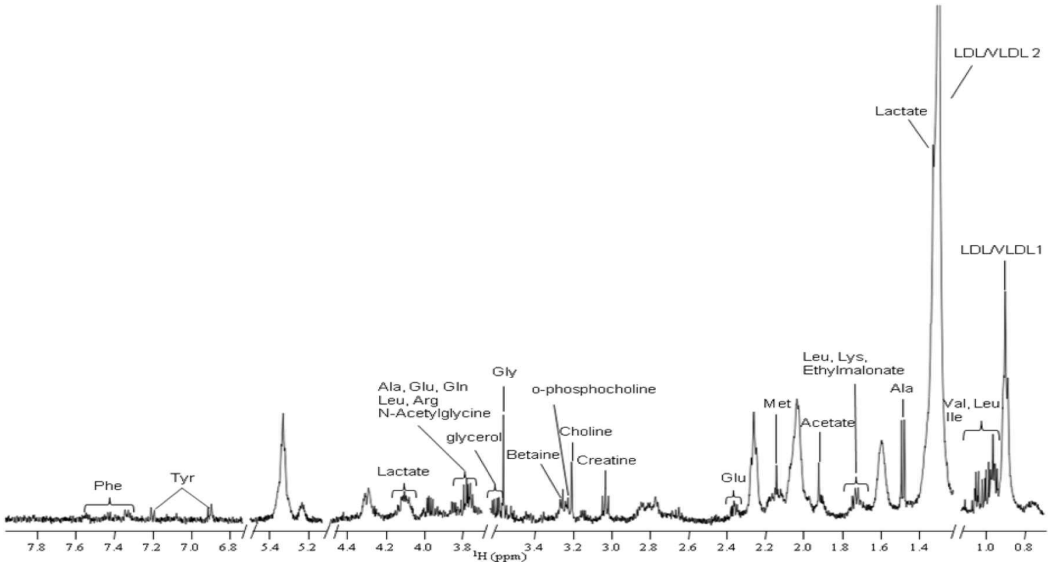

Figure 3. Normal tissue sample was selected as an example for detailed signal assignment with TSP-d_4(δ 0.00 ppm). The following metabolites were identified using the Chenomx 500 MHz library. [43]

identification has been routinely performed for many years in NMR-based metabolomics, it was largely done on compound-selective basis. Additionally, several companies (Agilent Chenomx and Bruker Biospin) have produced commercial products that implement these concepts in convenient, easy-to-use software programs.[30-33]

APPLICATIONS OF METABOLOMICS

Disease diagnosis

The metabolomics is used in disease diagnosis. Recently NMR-based metabolomics approach has advanced as a diagnostic tool for a wide variety of diseases.[22] This approach can be used in cancer diagnosis. ^{1}H NMR-based metabolomics has the capacity to detect colorectal cancer.[43] Fig.3 is HR-MAS ^{1}H NMR spectrum of colorectal normal tissue which was extracted from the patient by biopsy. The spectrum shows the metabolites which were identified by the comparison with a library. Cancer tissue had shown higher levels of arginine, betaine, glutamate, lysine, taurine and lower levels of glutamine, hypoxanthine, isoleucine, lactate, methionine, pyruvate, tyrosine relative to normal tissue. Nine cancer and normal samples from the same colorectal cancer patients were analyzed by multivariate analysis. The OPLS-DA analysis was carried out using the centering scale (Ctr scale) with SIMCA-P+ 12.0. Fig.4 shows the OPLS-DA score plot for the first two principal components from the present spectra of two groups, namely, normal and cancer. The result shows a clear classification of cancer samples and normal samples.

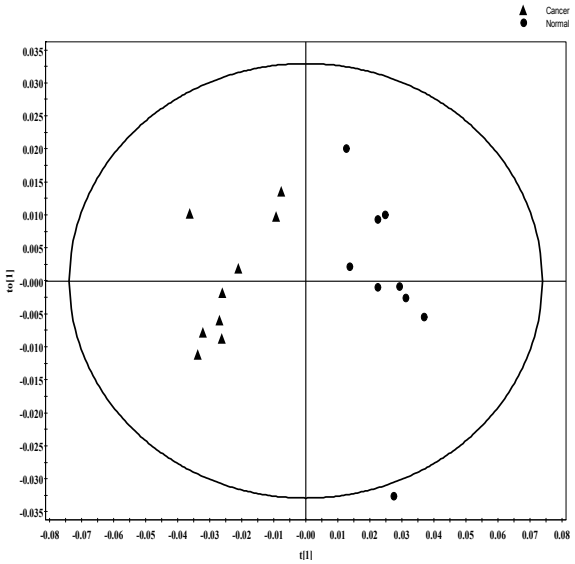

Figure 4. OPLS-DA score plot (comp.1 vs comp.2) obtained from the NMR spectra of 18 tissue samples using SIMCA-P+ 12.0. 18 samples were classified clearly into two groups. ▲: cancer ●: normal[43]

Forensic

NMR-based metabolomics also can be used in forensic science. For example, organic solvent abuse is a serious social problem, especially among solvent abuse.[44] However, there are no simple screening methods to detect glue sniffers. ^{1}H NMR measurement of their urine can be used to detect them. Toluene is the main solvent ingredient of glue but it is not soluble to water and has difficulty to detect in biofluids. Inhaled toluene is metabolized to hippuric acid in the liver and excreted in the urine. Hippuric acid is known as a good biomarker for biological monitoring of toluene exposure. Fig.5 shows the assignment of control, glue sniffer and the 2 weeks glue sniffer's urine ^{1}H-NMR spectra. Hippuric acid peaks were considerably different in glue sniffers. In glue sniffers group, the hippuric acid concentration was extremely high and the citrate concentration was decreased after glue sniffing. The amount of creatinine tends to reduce in glue sniffers' urine.

Toxicity test

NMR-based metabolomics is being applied in toxicology research. There are many applications in this field and several examples are explained as follows.

- *Silver nanoparticles(AgNPs) exposure on liver cell*

AgNps has been widely used in the commercial

products for the anti-bacterial material. However, AgNPs have been reported as toxic to the mammalian cell, lung, liver, brain and other organs.[45] Chang liver cell after the exposure of AgNPs was measured and analyzed by HR-MAS NMR spectroscopy combined with multivariate analysis.

Table 2 shows the major metabolites in the cultured Chang liver cell. The concentrations of glutathione(GSH), lactate, taurine, and glycine were decreased and most of amino acids, choline, analogues, and pyruvate were increased by the AgNPs. And the levels of the metabolites were recovered upto similar level of metabolites in the normal cell by the pre-treatment of NAC, external antioxidant. The results imply that the silver nano particles could affect the level of reactive oxygen species (ROS).

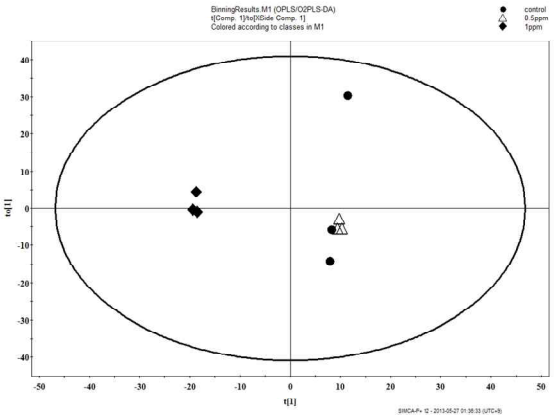

Figure 6. OPLS-DA score plot obtained from the NMR spectra of nine cultured liver cell samples using SIMCA-P+ 12.0.

-Bisphenol A exposure on liver Cell

Bisphenol A (BPA) is a important chemical in industry. BPA is used as a raw material for making plastics and resins. We are frequently exposed to BPA because there are many products made of plastics include BPA and it can dissolve to water or alcohol. BPA is widespread in our life and environment, and BPA causes some effects on health of human and wildlife. BPA is endocrine disruptor

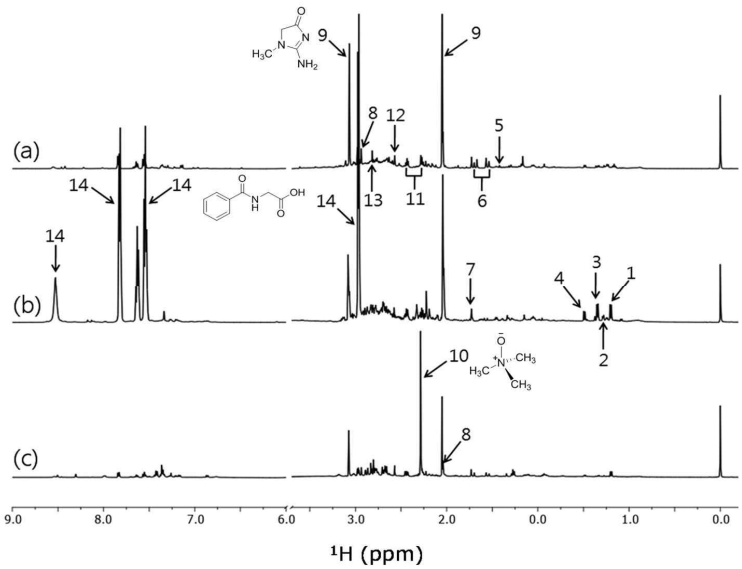

Figure 5. Representative ^{1}H NMR assigned spectra of controls, glue sniffers and the after 2 weeks glue sniffer with TSP (δ 0.00 ppm) and water resonances removed. All spectra were normalized to the TSP peak (from δ -0.026 ppm to δ 0.023 ppm). The following metabolites were identified with Chenomx. (a) control, (b) glue sniffer, (c) after 2 weeks glue sniffer. Assigned list: 1.3-hydroxybutyrate, 2.3-hydroxyisovalerate, 3.Lactate and threonine, 4. alanine, 5.succinate, 6.citrate, 7.dimethylamine, 8.creatine, 9.creatinine, 10.TMAO (Trimethylamine N-oxide), 11.taurine, 12.glycine, 13.guanidoacetate, 14.hippuric acid[44]

that modifies hormone functions by binding to the estrogen receptor also.[46] However, there are not enough studies about evident risk of BPA. BPA exposed to Chang liver cell in this experiment. The metabolites of cell were measured by HR-MAS NMR spectroscopy and analyzed by multivariate analysis.

Fig. 6 shows the difference of control group, 0.5ppm and 1ppm BPA exposed groups. The result shows the metabolites were similar in control and 0.5ppm treated group but 1ppm exposed group is distinguished in its content.

Table 2. Relative concentration of major metabolites represented in table. The concentrations of metabolites were calculated with integration of peak areas using Chenomx. The values are expressed as mean ± standard error.[45]

Metabolite	Control	AgNP	AgNP with NAC
Acetate	3.618 ± 0.314	4.479 ± 0.978	6.142 ± 0.549
Acetone	0.410 ± 0.055	1.482 ± 0.415	0.801 ± 0.176
Adipate	1.059 ± 0.034	1.395 ± 0.214	1.253 ± 0.189
Alanine	3.952 ± 0.218	4.849 ± 0.177	3.692 ± 0.240
Choline	1.409 ± 0.093	3.065 ± 0.212	1.759 ± 0.109
Creatine	3.090 ± 0.175	3.988 ± 0.247	3.675 ± 0.268
Glutamate	6.983 ± 0.623	7.806 ± 0.540	7.352 ± 0.424
Glutamine	1.752 ± 0.081	1.840 ± 0.136	2.069 ± 0.198
Glutathione	1.435 ± 0.148	1.248 ± 0.087	1.824 ± 0.163
Glycerol	1.371 ± 0.071	1.783 ± 0.190	1.882 ± 0.214
Glycine	1.934 ± 0.144	1.572 ± 0.079	2.032 ± 0.388
Hypoxanthine	1.759 ± 0.155	2.916 ± 0.154	1.655 ± 0.426
Inosine	0.602 ± 0.128	0.332 ± 0.072	0.263 ± 0.075
Isoleucine	1.473 ± 0.054	2.359 ± 0.100	1.795 ± 0.205
Lactate	51.218 ± 1.552	31.917 ± 2.435	41.727 ± 4.473
Leucine	3.315 ± 0.078	5.005 ± 0.273	4.246 ± 0.512
Methionine	0.762 ± 0.027	0.865 ± 0.086	0.821 ± 0.092
O-Phosphocholine	1.045 ± 0.236	2.275 ± 0.200	1.804 ± 0.373
Phenylalanine	0.451 ± 0.020	0.464 ± 0.105	0.484 ± 0.041
Pyruvate	0.646 ± 0.105	3.446 ± 0.639	0.785 ± 0.120
Serine	3.021 ± 0.188	3.488 ± 0.460	4.081 ± 0.830
Succinate	0.252 ± 0.026	0.244 ± 0.007	0.281 ± 0.012
Taurine	1.421 ± 0.237	0.793 ± 0.037	1.342 ± 0.239
Trimethylamine N-oxide	1.119 ± 0.058	0.965 ± 0.096	1.026 ± 0.081
Tyrosine	0.599 ± 0.048	0.634 ± 0.075	0.666 ± 0.052
Uracil	1.486 ± 0.149	4.211 ± 0.443	1.989 ± 0.089
Valine	1.514 ± 0.040	2.284 ± 0.125	1.786 ± 0.173
myo-Inositol	1.260 ± 0.220	1.675 ± 0.105	1.366 ± 0.184
sn-Glycero-3-phosphocholine	1.043 ± 0.086	2.620 ± 0.280	1.402 ± 0.276

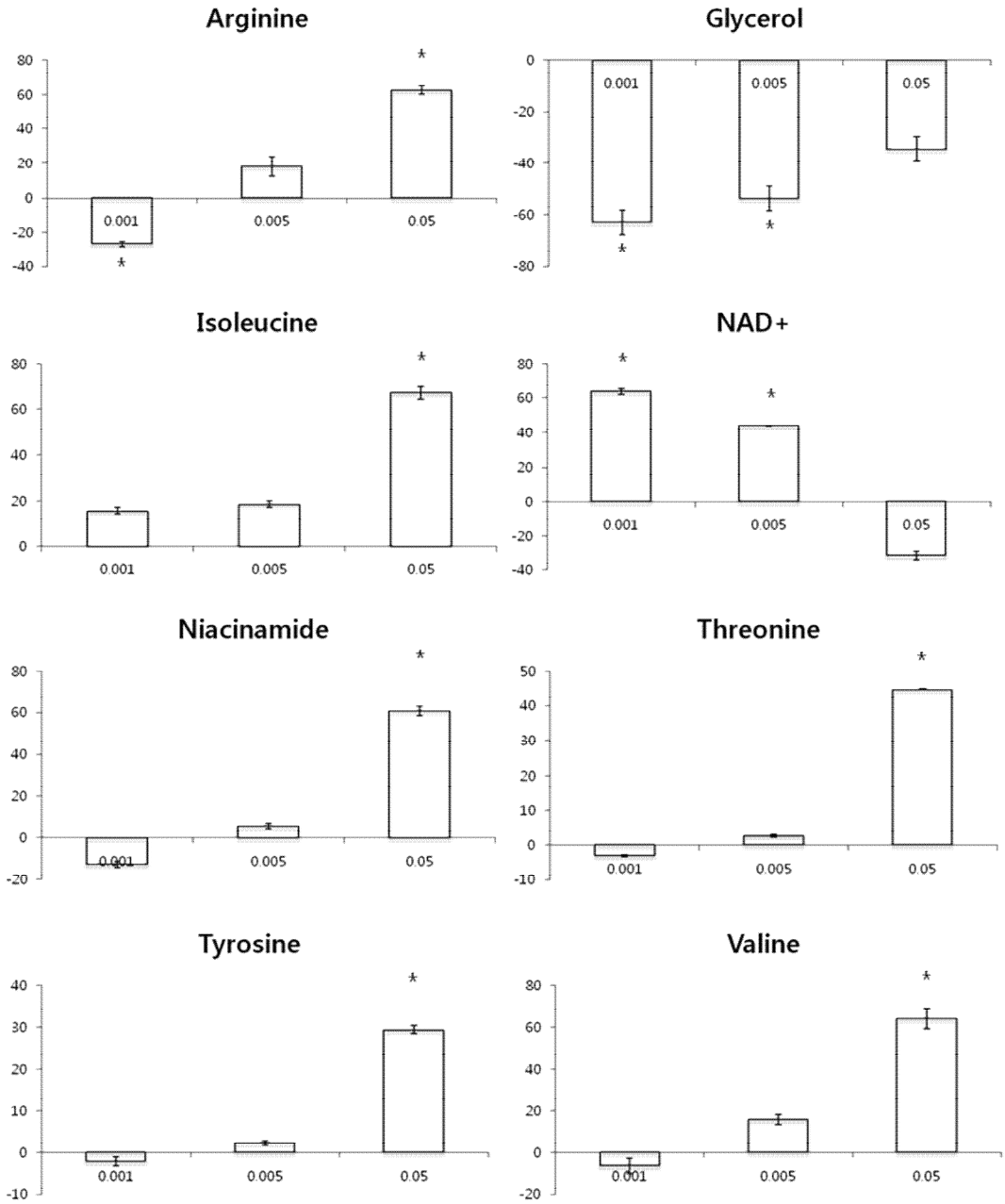

Figure 7. Percent change (%) of all identified metabolites from the t-test filtered ^{1}H NMR difference spectra of $HgCl_2$-exposed zebrafish extraction. Each percent change is shown with their standard error ('*' labeled with $p<0.05$).

-Mercury exposure to zebrafish

Heavy metals, such as mercury and lead are important environmental contaminants. These substances present severe risk to the aquatic biota and humans, even at sub-lethal concentrations.[47] Mercury is one of the most toxic heavy metals. Toxic effects include damage to the brain, kidney and lungs. Zebrafish were exposed to $HgCl_2$ to monitor the change of metabolites by the toxicity and whole body extraction was achieved and NMR-based metabolomics were utilized as an analysis tool. Fig.7 shows the changes of major metabolites in the various concentrations of mercury treatment. Some amino acids and nucleic acid are dramatically charged in the high concentration of $HgCl_2$. The result suggests that zebrafish is proper species to monitor the toxicity of heavy metal coupled with NMR-based metabolomics.

Conclusion

Metabolomics is a promising study area that changes and provides complete information on organism's metabolism. Metabolomics enable to predict interruption of metabolites changes due to disease or toxic chemicals. It is useful to develop predictive biomarkers of previous interventions as well as provide metabolic pathway disruption. The aim of this review is to introduce NMR-based metabolomics and applications of toxicology, forensic and disease diagnosis. NMR-based metabolomics with multivariate statistical analysis is a powerful technology in classifying the metabolic differences of experimental groups compared with control groups. Despite various studies in metabolomics, biomarker discovery for a number of major diseases or environmental contaminants is still in unknown. Also, NMR-base metabolomics approach has limitation in finding unique biomarkers for numerous different metabolic reactions. Thus, to extend the sensitivity of NMR techniques, it may be necessary to combine NMR-based metabolomics with more sensitive techniques such as LC-MS and/or GC-MS.

Acknowledgment

This work was supported for two years by Pusan National University Research Grant.

References

1. A. Roux, D. Lison, C. Junot, J. Heilier, *Clinical Biochemistry*. **44**, 119. (2011).
2. R.D. Beger, J. Sun, L.K. Schnackenberg, *Toxicol Appl Pharmacol*. **243**,154. (2010).
3. J.K. Nicholson, J.C. Lindon, E. Holmes, *Xenobiotica*. **29**, 1189.(1999).
4. D.A. Bennett, M.D. Waters, *Environ Health Perspect*. **108**, 907. (2000).
5. Biomarkers Definitions Working Group, *Clin Pharmacol Ther*. **69**, 89. (2001).
6. J.A. Timbrell, *Toxicology*. **129**, 1. (1998).
7. J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, *Nat. Rev. Drug Discov*. **1**, 123. (2002).
8. A. Zhang, H. Sun, P. Wang, Y. Han, X. Wang, *JOURNAL OF PROTEOMICS*. **75**, 1079. (2012).
9. J.K. Nicholson, J.C. Lindon, *Nature*. **455**, 1054. (2008).
10. H. Wang, V.K. Tso, C.M. Slupsky, R.N. Fedorak, *Future Oncol*. **6**, 1395. (2010).
11. R. Madsena, T. Lundstedtc, J. Trygga, *Analytica Chimica Acta*. **659**, 23. (2010).
12. O.A.H. Jones, D.J. Spurgeon, C. Svendsen, J.L. Griffin, *Chemosphere*. **71**, 601. (2008).
13. G.Sudama, J. Zhang, J. Isbister, J.D. Willett, *Metabolomics*. **9**, 189. (2013).

14. R.Tyagi, P. Rana, M. Gupta, A. R. Khan, M. M. Devi, D. Bhatnagar, R. Roy, R.P. Tripathi, S. Khushu, *Metabolomics*. **8**, 940. (2012).
15. R.D. Beger, T. Colatsky, *Metabolomics*. **8**, 2. (2012).
16. A. Zhang, H. Sun, X. Wang, *Anal Bioanal Chem*. **404**, 1239. (2012).
17. J.G. Bundy, M.P. Davey, M.R. Viant, *Metabolomics*. **5**, 3.(2009).
18. S.C. Gates, C.C. Sweeley, *Clin. Chem*. **24**, 1663. (1978).
19. S. Kim, *Journal of the Korean Magnetic Resonance Society*. **13**, 1. (2009).
20. J.L. Spratlin, N. J. Serkova, S. G. Eckhardt, *Clin Cancer Res*. **15**, 431. (2009).
21. J.L. Griffin, J.P. Shockcor, *Nat Rev Cancer*. **4**, 551. (2004).
22. A.M. Emwas, R.M. Salek, J.L. Griffin, J. Merzaban, *Metabolomics*. DOI 10.1007/s11306-013-0524-y
23. S. Rehman, *Molecular Biosystems*. **8**, 2274. (2012).
24. A.E.S.I. Ahmed, *Journal of Applied Polymer Science*. **119**, 709. (2011).
25. L. Corte, P. Rellini, L. Roscini, F. Fatichenti, G. Cardinali, *Analytica Chimica Acta*. **659**, 258. (2010).
26. J.K. Nicholson, I.D. Wilson, *Progress in Nuclear Magnetic Resonance Spectroscopy*. **21**, 449. (1989).
27. D. G. Robertson, *TOXICOLOGICAL SCIENCES*. **85**, 809, (2005).
28. R.S. Plumb, C.L. Stumpf, M.V. Gorenstein, J.M. Castro-Perez, G.J. Dear, M. Anthony, B.C. Sweatman, S.C. Connor, J.N. Haselden, *Mass Spectrom*. **16**, 1991. (2002).
29. N.V. Reo, Drug Chem. *Toxicol*. **25**, 375. (2002).
30. D. S. Wishart, *Trends in Analytical Chemistry*. **27**, 228. (2008).
31. D.M. Wilson, A.L. Burlingame, *Biochem. Biophys. Res. Commun*. **56**, 828. (1974).
32. R.G. Shulman, D.L. Rothman, *Annu. Rev. Physiol*. **65**, 401. (2003).
33. M.W. Weiner, H. Hetherington, B. Hubesch, G. Karczmar, B. Massie, A. Maudsley, D.J. Meyerhoff, D. Sappey-Marinier, S. Schaefer, D.B. Twieg, *NMR Biomed*. **2**, 290. (1989).
34. J.K. Nicholson, I.D. Wilson, *Nat. Rev. Drug Discov*. **2**, 668. (2003).
35. J. Trygg, E. Holmes, T. Lundstedt, *J. Proteome Res*. **6**, 469. (2007).
36. H. Serrai, L. Nadal, G. Leray, B. Leroy, B. Delplanque, J.D. de Certaines, NMR Biomed. 11, 273. (1998).
37. M. Ala-Korpela, N. Lankinen, A. Salminen, T. Suna, P. Soininen, R. Laatikainen, P. Ingman, M. Jauhiainen, M.R. Taskinen, K. Heberger, K. Kaski, *Atherosclerosis*. **190**, 352. (2007).
38. J.R. McNamara, G.R. Warnick, G.R. Cooper, *Clin. Chim. Acta*. **369**, 158. (2006).
39. S.W. Provencher, *Magn. Reson. Med*. **30**, 672. (1993).
40. B.C. Sweatman, R.D. Farrant, E. Holmes, F.Y. Ghauri, J.K. Nicholson, J.C. Lindon, *J. Pharm. Biomed. Anal*. **11**, 651. (1993).
41. H.E. Kim, Y.H. Choi, J.S. Park, H.S. Kim, J.H. Jeon, M.S. Heu, D.S. Shin, J.H. Lee, *Journal of the Korean Magnetic Resonance Society*. **16**, 91. (2012).
42. Y.K. Chae1, W. Y. Kang, S. H. Kim, J. E. Joo, J. K. Han, B. W. Hong , *Journal of the Korean Magnetic Resonance Society*. **14**, 28. (2010).
43. S. Kim, S. Lee, Y. H. Maeng, W. Y. Chang, J. W. Hyun, S. Kim, *Bull. Korean Chem. Soc*. **34**, 1467 (2013).