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Review

Metabolic Fingerprinting in Toxicological Assessment Using FT-ICR MS

Mina Hasegawa[1], Mika Ide[2], Mitsuru Kuwamura[1], Jyoji Yamate[1], and Shigeo Takenaka[1]

[1]Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–58 Rinkuouraikita, Izumisano, Osaka 598-8531, Japan
[2]Safety Research Laboratory, Mitsubishi Tanabe Pharma Corporation, 1–1–1, Kazusa-Kamatari, Kisarazu-shi, Chiba 292-0818, Japan

Abstract: Detection of the toxicity of a candidate compound at an early stage of drug development is an emerging area of interest. It is difficult to determine all of the effects of metabolism of a compound using traditional approaches such as histopathology and serum biochemistry. The goal of a metabolomics approach is to determine all metabolites in a living system, with the potential to detect and identify biomarkers involved in toxicity onset. Here, we summarize the metabolic fingerprints for detection and identification of metabolic changes and biomarkers related to drug-induced toxicity using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). (J Toxicol Pathol 2010; **23**: 67–74)

Key words: metabolomics, metabolic fingerprinting, toxicological assessment, FT-ICR MS, NMR, MS

Introduction

Analysis using the "omics" (transcriptomics, proteomics and metabolomics) has become widespread in the post-genomics era, with one objective being the large-scale quantitative determination of mRNAs, proteins and metabolites to establish gene function and ascertain the connectivity within the "omics" hierarchy[1]. Metabolomics can be defined as an attempt to measure all the metabolites within a cell, tissue or organism with a genetic modification, in response to a physiological stimulus, or under specific conditions at a specified time[2]. Metabolites are the end-products of a biological system and changes in metabolites can therefore be regarded as the final response of an organism to changes in gene expression. Genome-wide expression of genes is amplified gradually through the hierarchy of the transcriptome, proteome and metabolome. Changes in the concentrations of enzymes (and the transcripts that encode them) may have only small effects via metabolic pathways, but can have substantial effects on the concentration of metabolic intermediates. Consequently, the metabolome may be more sensitive to perturbations compared with either the transcriptome or proteome[3].

The technologies that enable performance of metabolomics have recently increased in power[4]. Transcriptomics and proteomics are based on target chemical analysis of biopolymers composed of 4 different nucleotides (transcriptome) or 20 amino acids (proteome). The chemical similarity of these compounds facilitates high-throughput analysis. In contrast, the metabolome has a large variety of chemical structures and properties, from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, organic amino and non-amino acids, hydrophobic lipids, and complex natural products[5]. This complexity makes it difficult to determine the complete metabolome simultaneously[6]. Therefore, it is common to perform quantitative determination of one or a few metabolites related to a specific metabolic pathway after extensive sample preparation and separation from the sample matrix using chromatographic separation and sensitive detection, which is refered to as "target analysis". The following two metabolomic approaches are common: i) metabolic profiling to identify and quantify metabolites related through similar chemistries or metabolic pathways using chromatographic separation before detection with minimal metabolite isolation after sampling and ii) metabolic fingerprinting for rapid global analysis of crude samples or extracts for classification or screening of samples without identification and quantification.

The technologies for metabolomics can also be classified into categories based on detection using mass

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Mailing address: Shigeo Takenaka, Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–58 Rinkuouraikita, Izumisano, Osaka 598-8531, Japan
E-mail: takenaka@vet.osakafu-u.ac.jp

spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared (FT-IR) spectroscopy. The high sensitivity of MS makes it an important method for measuring metabolites in complex biosamples. Gas chromatography (GC) and GC-MS are used for quantitative metabolic profiling, with GC first used over 20 years ago for disease diagnosis[7]. There has been rapid growth in metabolomic applications based on GC-MS for analysis of volatile and thermally stable polar and nonpolar metabolites, while developments in liquid chromatography (LC)-MS and capillary electrophoresis (CE)-MS have broadened the applicability of MS-based metabolomics. Tandem MS and accurate mass (time of flight) methods are normally used to validate the identities of unknown metabolites. NMR and FT-IR spectroscopy are used for structural analysis of compounds, but have relatively low sensitivity compared with MS. Nevertheless, NMR- and FT-IR-based metabolic profiling have been used successfully in many fields because both methods are highly quantitative and reproducible.

The utility of metabolomics for toxic evaluation of compounds with NMR was comprehensively assessed by the Consortium for Metabonomic Toxicology (COMET), which was founded by five major pharmaceutical companies and Imperial College London, U.K.[8]. The main objectives of COMET were to assess and develop methodologies to generate a metabolomics database using NMR analysis of rodent urine and blood serum dosed with hepato- and nephrotoxins for preclinical toxicological screening of candidate drugs and to build an expert system for prediction of the site and mechanism of toxicity. This approach was used successfully to determine metabolic interspecies variation between rats and mice dosed with a toxin[9]. The goal of the second COMET project (COMET 2) is to improve on the knowledge obtained in COMET by detailed testing of hypotheses in metabolomics using NMR and LC-MS[10].

The discovery of biomarkers is a major objective in determining the toxicity of drug and identification of toxic biomarkers through a metabolomics approach is an emerging area. There are many reports of potential biomarkers in administration of a variety of drugs, and identification of toxicological biomarkers may contribute to assessment of the mechanisms of toxicity in drug development.

Metabolic Fingerprinting with FT-ICR MS for Biomarker Identification and Toxicity Evaluation

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) can be used to obtain an ultra-high resolution (>100,000) mass spectrum in about one second. Separation of the metabolites is possible with this method alone, eliminating the need for time-consuming chromatography and derivatization. Identification of putative metabolites, or the class to which they belong, can be achieved by determining the elemental composition of the metabolite based upon the ultra-high mass accuracy[11,12]. The utility of this approach has been demonstrated in phenotyping of plants, in which the ripening process has been investigated in strawberries and transgenic mutant tobacco plants[13]. In this review, we summarize our recent findings for metabolic fingerprints of rat urine using FT-ICR MS.

Drug-induced phospholipidosis

We used phospholipidosis (PLD), which is a well-examined drug-induced symptom, to evaluate the utility of FT-ICR MS for toxicological metabolomics[14]. PLD can be induced by cationic amphiphilic drugs (CADs)[15] and is characterized by an abnormal intracellular accumulation of phospholipids and multilamellar bodies, as observed by electron microscopy[16]. Amiodarone (AMD), perhexiline, fluoxetine and gentamicin induce PLD in humans[15], and PLD in animal models has been observed with CADs at doses far in excess of those used clinically. Histopathological examinations using light and electron microscopy have traditionally been used to detect PLD in drug-treated animals, but these methods are time-consuming and cannot be performed in humans.

Urine samples from rats treated orally with AMD at 300 mg/kg/day for 3 days were analyzed using an FT-ICR MS instrument equipped with a 7.0-T actively shielded superconducting magnet (IonSpec, Lake Forest, CA, U.S.A.). Higher intensity signals at *m/z* 192.06676 and 212.00239 and a lower intensity signal at *m/z* 178.05101 were observed in the AMD-treated urine samples compared to the controls (Fig. 1). A loading plot revealed that these metabolites contributed to discrimination among the samples. The ions at *m/z* 178.05101, 192.06676 and 212.00239 were identified as hippurate (HA), phenylacetylglycine (PAG) and indican (IDN), respectively. PAG is the end product of phenylalanine metabolism in rodents[17] and has been reported as a biomarker using NMR[18] and LC-MS[19]. Elevation of urinary PAG after treatment with a CAD such as AMD that causes phospholipid accumulation may be related to changes in gut flora or the integrity of the intestine[20]. IDN is derived from the action of intestinal bacteria containing tryptophanase, which converts tryptophan to indole. Indole is then absorbed and conjugated in the liver to form IDN, which was also shown to be a urinary PLD biomarker on the loading plot. AMD treatment has been linked to changes in the gut or the integrity of the intestine that result in increased absorption of the PAG precursor, phenylacetate (PA), leading to an elevated level of urinary PAG[20]. A decrease in urinary HA levels has been detected in urinary metabolic fingerprinting for AMD-induced PLD using NMR[21], consistent with the findings from FT-ICR MS. The decrease in urinary HA has been proposed as a nonspecific marker of toxicity that reflects a complex combination of factors such as dietary intake and gut microbial metabolism[22,23]. These results show that urinary metabolic fingerprinting with FT-ICR MS can be

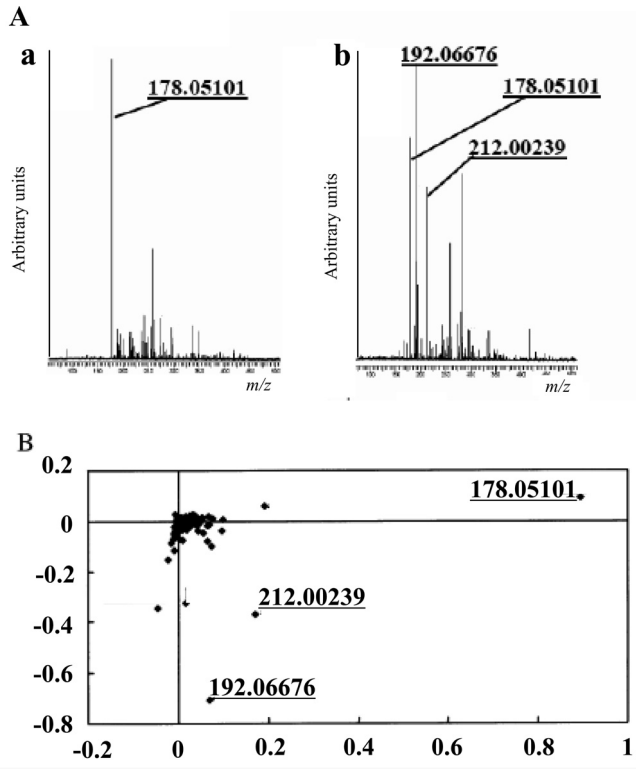

Fig. 1. (A) FT-ICR MS spectrum and (B) loading plot for amiodarone-treated rats urine. (A) Pre-dose (a) and 24 hours after final dosing (b). The intensity of the ions at *m/z* 192.06676 and 212.00239 increased and that for the ion at *m/z* 178.05101 decreased at 24 hours after final dosing compared wtih the pre-dose levels. (B) The loading plot revealed that the metabolites causing PCA exhibited *m/z* 178.05101, 192.06676 and 212.00239.

used to detect potent biomarkers for AMD-induced PLD and suggest that this method is applicable to toxicological assessment of compounds.

Drug-induced hepatotoxicity

The liver is the organ that is most exposed to drugs, with the concentration in the liver often being higher than the peak plasma concentration. The liver is also the major site for metabolism of xenobiotics that can lead to formation of active metabolites. Thus, evaluation of liver toxicity is particularly important in development of new therapeutic compounds. Here, we describe the urinary metabolic fingerprint of rat urine following treatment with two hepatotoxins, thioacetamide (TAA) and α-naphthylisothiocyanate (ANIT), to investigate the relationship between histopathology and serum biochemistry and to search for possible biomarkers involved in hepatic toxicity[24,25].

TAA-induced acute hepatic injury in rats: Rats were administered a single intraperitoneal injection of TAA (Day 0) at a dose of 300 mg/kg body weight. ALT and AST in serum both increased on Day 1, indicating that hepatocyte injury had occurred. These values decreased on Day 3 and were almost at pre-dose levels on Day 5. Histopathologically, TAA-induced centrilobular necrosis of hepatocytes accompanied by a small amount of mononuclear cell infiltration was observed on Day 1 (Fig. 2b). Hepatocyte injury and infiltration of mononuclear cells became more prominent in the centrilobular area on Day 3 (Fig. 2c), and the majority of infiltrating cells were reactive with anti-ED1 (Fig. 2d). Hepatocyte injury and cell infiltration had almost completely disappeared on Days 5 (Fig. 2e) and 7 (Fig. 2f).

In negative ion spectra, the intensities of the ions at *m/z* 178.05038, 191.01967, 212.00226, 242.01320 and 258.99494 decreased on Days 1 and 3 compared to the pre-dose intensities (Fig. 3A). In positive ion spectra, the intensity of the ion at *m/z* 401.20737 increased on Day 1, that at *m/z* 266.05390 increased on Day 3, and that at *m/z* 429.23882 decreased on Day 1 compared to the pre-dose intensities (Fig. 4A). The PCA scores plot showed that the negative ion compositions of TAA-treated rat urine samples on Days 1 and 3 differed from the pre-dose compositions, with the largest change seen on Day 1 (Fig. 3B). By Day 5, the composition was close to that in pre-dose samples (Fig. 3B). ALT and AST are well-known serum biochemical markers for hepatocyte injury and increased levels of these markers were seen on Day 1, with subsequent decreases on Day 3. The shift on the PCA scores plot for the negative ions showed a similar pattern for changes of ALT and AST. The positive ion compositions of the TAA-treated rat urine samples on Days 1 and 3 also differed from those in the pre-dose samples, but were similar to the pre-dose compositions on Days 5 and 7 (Fig. 4B). Histopathologically, hepatocyte injury accompanied by cell infiltration was seen on Day 1 and these lesions were more prominent on Day 3. These results indicate that the changes in positive ion compositions might reflect the histopathological changes.

The loading plot suggested that the negative ions at *m/z* 178.05038, 191.01967, 192.06628, 212.00226, 220.14673, 242.01320, 258.99494 and 303.06000 and the positive ions at *m/z* 266.05390, 401.20737 and 429.23882 were potential biomarkers for acute hepatic injury. The ions at *m/z* 178.05038, 192.06628, 212.00226 and 242.01320 were identified as HA, PAG, IDN and 3-methyldioxyindole sulfate, respectively. The alterations of HA and IDN depended on administration of TAA, which caused changes in the distribution of the bacterial flora. Decrease of an ion at *m/z* 242 has been reported in urinary metabolic fingerprinting in ANIT-induced intrahepatic cholestasis using LC-MS[26], and this ion may be due to 3-methyldioxyindole sulfate. Therefore, these findings suggest that alteration of urinary 3-methyldioxyindole sulfate may correlate with hepatotoxicity.

The positive ion at *m/z* 266.05390 has the empirical formula $[C_9H_{13}N_3O_4K]^+$ (calculated $[M+K]^+$ =266.05377, Δ*m/z* 0.00013). The best candidate for this ion in the compound library was the potassium ion (K^+) adduct of deoxycytidine (dCyt). MS/MS analysis of *m/z* 266.05390 in TAA-treated rat urine gave rise to an ion at *m/z* 150.0 that

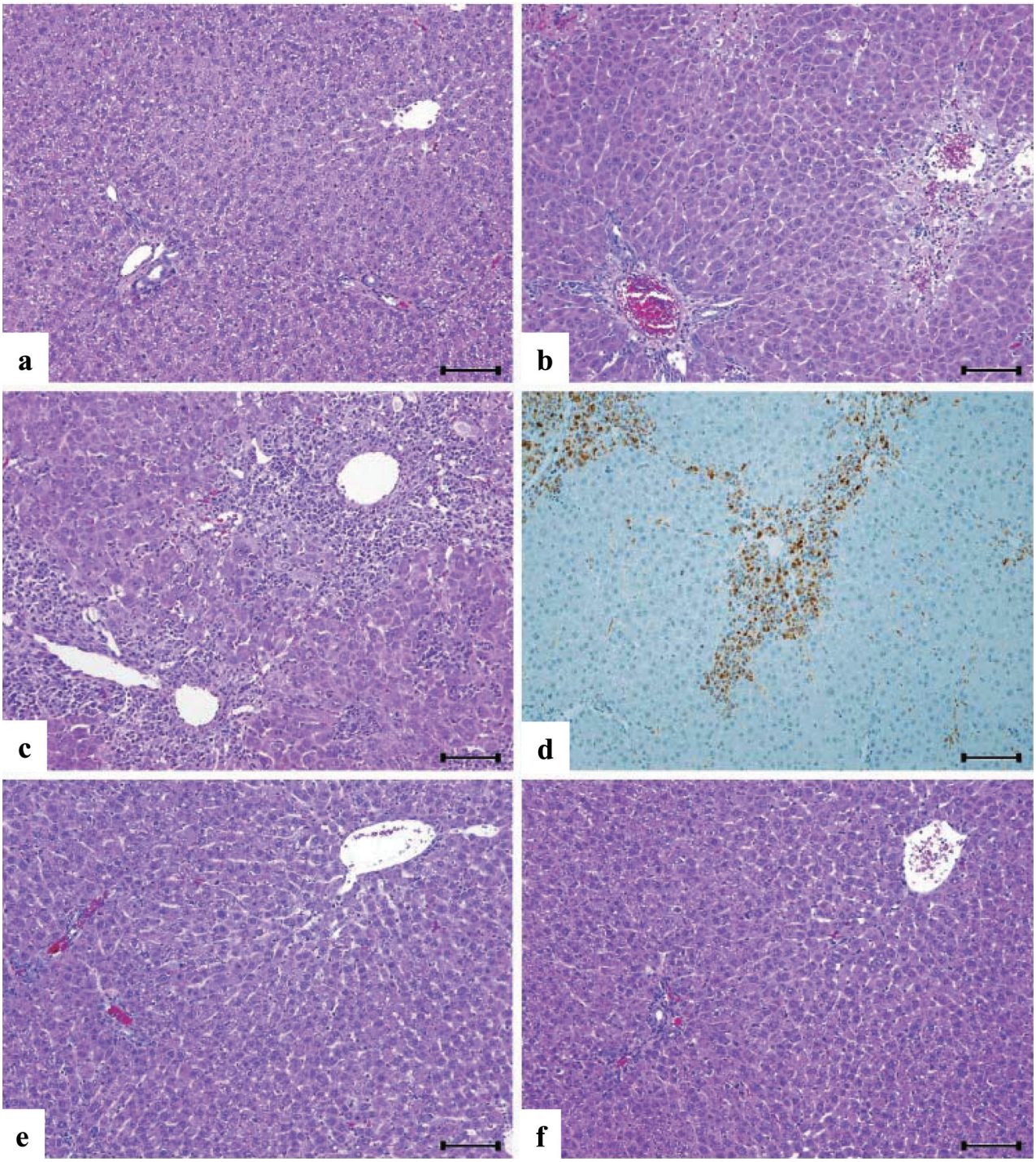

Fig. 2. Histopathological examination of the liver in thioacetamide-treated rats. (a) Normal histology of the control rat liver; HE stain, bar=60 μm. (b) Hepatocyte injury and infiltration of a few mononuclear cells in the centrilobular area of a treated rat on Day 1; HE stain, bar=60 μm. (c) More prominent hepatocyte injury and cell infiltration in the centrilobular area on Day 3; HE stain, bar=60 μm. (d) The majority of infiltrated cells showed a positive reaction with ED1 (a rat macrophage specific antibody) on Day 3; immunohistochemistry counterstained with hematoxylin, bar=60 μm. (e) Injured hepatocytes and cell reactions disappeared in the affected centrilubular area on Day 5; HE stain, bar=60 μm. (f) The affected areas disappeared and recovered the features of the control liver on Day 7; HE stain, bar=60 μm.

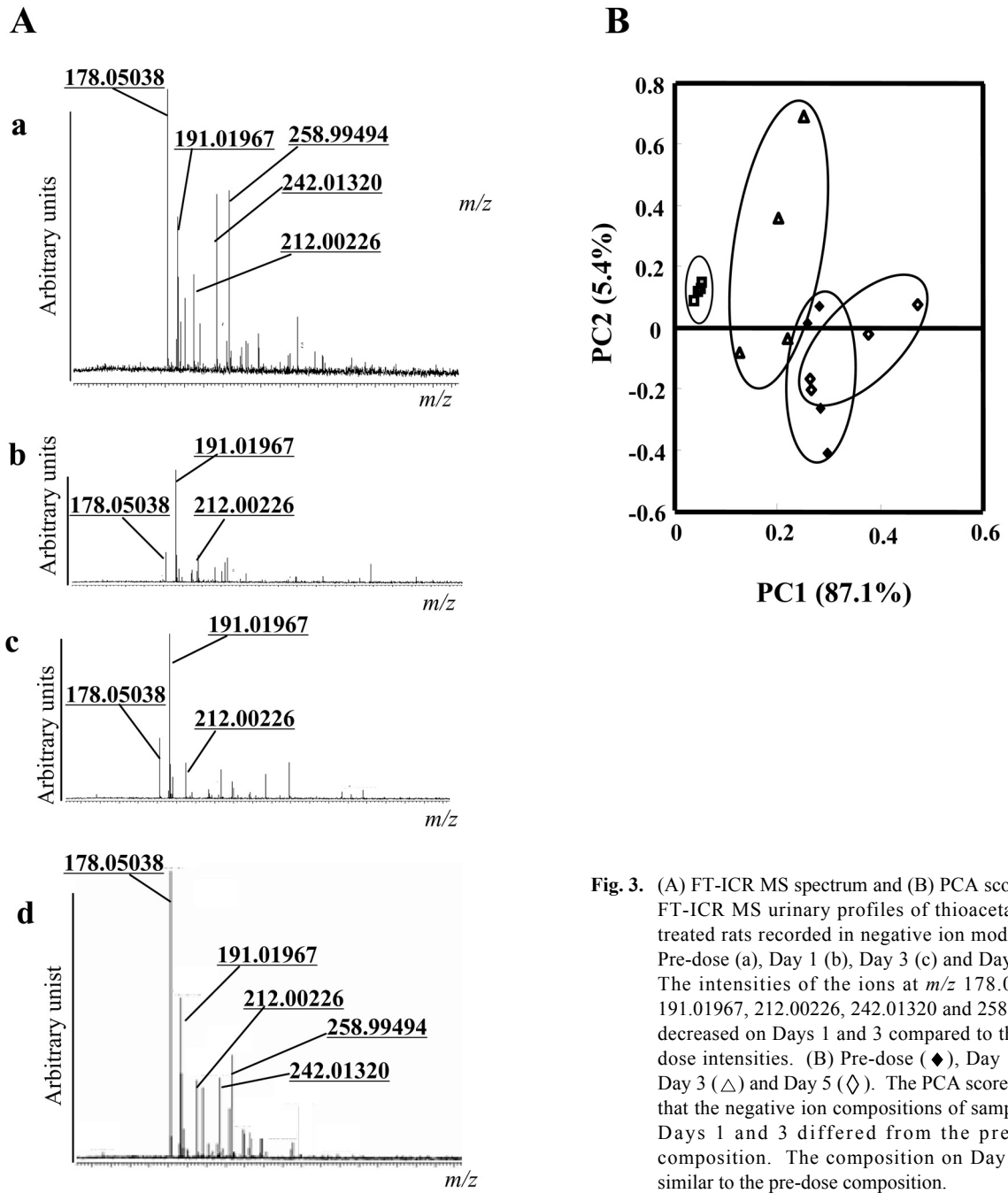

Fig. 3. (A) FT-ICR MS spectrum and (B) PCA scores for FT-ICR MS urinary profiles of thioacetamide-treated rats recorded in negative ion mode. (A) Pre-dose (a), Day 1 (b), Day 3 (c) and Day 5 (d). The intensities of the ions at *m/z* 178.05038, 191.01967, 212.00226, 242.01320 and 258.99494 decreased on Days 1 and 3 compared to the pre-dose intensities. (B) Pre-dose (♦), Day 1 (□), Day 3 (△) and Day 5 (◊). The PCA scores show that the negative ion compositions of samples on Days 1 and 3 differed from the pre-dose composition. The composition on Day 5 was similar to the pre-dose composition.

resulted from loss of deoxyribose (–116), and the MS/MS spectrum of *m/z* 266.05390 in dCyt-spiked urine was identical to that for the TAA-treated sample. These data are consistent with the hypothesis that *m/z* 266.05390 in the TAA-treated urine was the K^+ adduct of dCyt. Phagocytosis of cell debris by macrophages leads to release of dCyt from these cells *in vitro*, and normal rat tissues including the liver and kidney have no dCyt deaminase activity for conversion of dCyt to deoxyuridine[27]. An increased number of macrophages were seen on Days 1 and 3 in TAA-injected rats, in parallel with development of hepatocyte injury, and therefore macrophage infiltration may be responsible for the occurrence of dCyt in the urine on Days 1 and 3. The majority of macrophages in the injured liver were reactive with ED1. Antigens recognized by ED1 occur on the membranes of cytoplasmic granules, and especially on phagolysosomes of macrophages, and the degree of ED1 expression depends on the phagocytic activity[27]. The increased number of ED1-reactive macrophages on Days 1 and 3 reflects increased debris from injured hepatocytes. A significant amount of dCyt released from infiltrated macrophages was excreted in urine on Day 3, indicating a close relationship between hepatic macrophage infiltration and the appearance of dCyt in urine after TAA

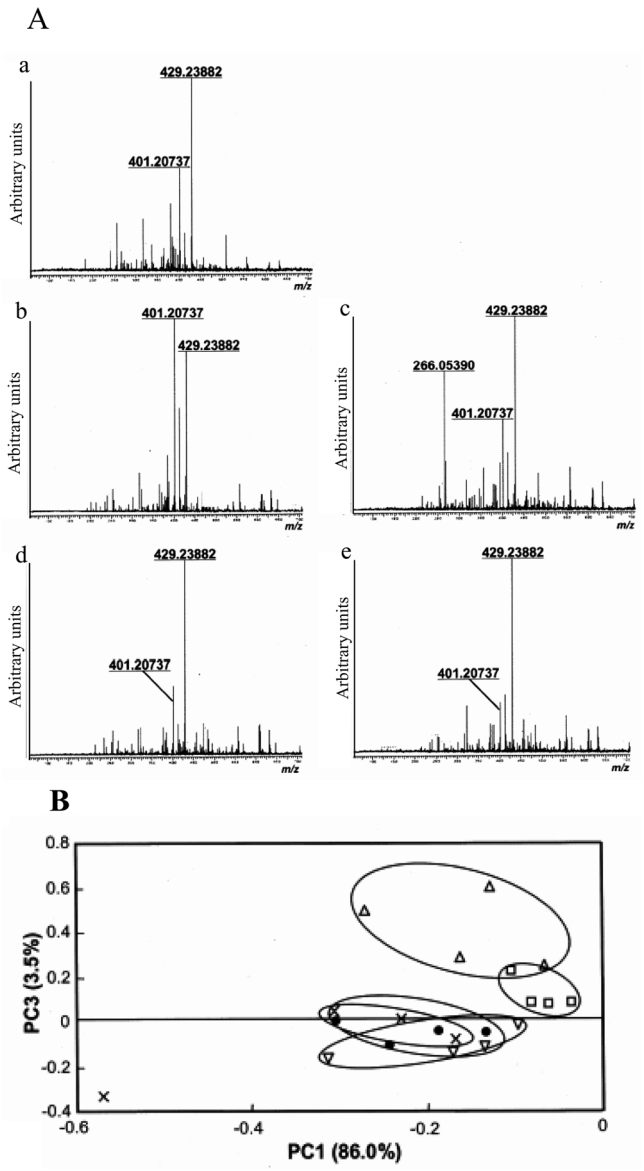

Fig. 4. (A) FT-ICR MS spectrum and (B) PCA scores for FT-ICR MS urinary profiles of thioacetamide-treated rats recorded in positive ion mode. (A) Pre-dose (a), Day 1 (b), Day 3 (c), Day 5 (d) and Day 7 (e). The intensity of the ions at *m/z* 266.05390 on Day 3 increased compared to the pre-dose levels. (B) Pre-dose (♦), Day 1 (□), Day 3 (△), Day 5 (×), Day 7 (◊). The PCA scores show that the positive ion compositions of the samples on Days 1 and 3 differed from the pre-dose composition. The composition on Day 5 was similar to the pre-dose composition.

administration.

ANIT-induced intrahepatic cholestasis in rats: Rats were administered a single oral injection of ANIT (Day 0) at a dose of 100 mg/kg body weight. ALT and AST in serum increased on Day 1, and these increases were more pronounced on Day 2. Total bilirubin (TBIL) was markedly increased on Day 2, and a lesser increase in alkaline phosphatase (ALP) was also observed. These levels then decreased on Day 4. In the ANIT-treated rats, bile duct degeneration and necrosis accompanied by peribiliary infiltration and edema of neutrophils, and bile duct obstruction by degenerative epithelium were seen on Day 1. On Day 2, regenerative bile ducts with edema and inflammation around portal tracts, and bile duct proliferation were observed. Degenerative vacuolation and single cell necrosis of hepatocytes were evident on Days 1 and 2. Bile duct proliferation and increased mitosis of hepatocytes were observed on Day 4.

In negative ion spectra, the intensities of the ions at *m/z* 178.05168 and *m/z* 192.06730 decreased at 0–48 h and 7–31 h, respectively, and those of the ions at *m/z* 512.26845 and 514.28485 increased at 7–55 h compared to the pre-dose intensities. The PCA scores plot showed that the biochemical compositions of the ANIT-treated rat urine samples at 7–24 h, 24–31 h, 31–48 h and 48–55 h differed from the pre-dose composition, with the maximum difference at 24–31 h. The compositions of the urine samples at 55–72 h and 72–96 h were almost the same as the pre-dose composition. Increased levels of AST and ALT (serum markers for hepatocyte injury) and ALP and TBIL (markers for cholestasis) were seen on Day 2. However, there were no serum biochemical findings at 24–31 h, whereas the shift on the PCA scores plot indicated differences in the urine composition at this time. Therefore, these results suggest that urinary metabolic fingerprinting with FT-ICR MS is more sensitive than traditional serum biochemistry methods.

The loading plot indicated that the ions at *m/z* 178.05168, 192.06730, 195.05139, 242.01323, 512.26845 and 514.28485 were potential urinary biomarkers for intrahepatic cholestasis. The ions at *m/z* 178.05168, 192.06730, 242.01323, 512.26845 and 514.28485 were identified as HA, PAG, 3-methyldioxyindole sulfate, taurocholic acid (TA) with one double bond, and TA, respectively. TA and TA with one double bond are both bile acids, and an increase in the levels of urinary bile acids is a well-known feature of intrahepatic cholestasis[25]. Histopathologically, cholestasis-induced bile ductar cell necrosis and subsequent membrane breakdown caused by ANIT results in elevation of urinary bile acids after 7–55 h. In previous urinary metabolic fingerprinting in ANIT-induced intrahepatic cholestasis, changes in PAG have been found by LC-MS[26] and a decrease in urinary HA has been shown using NMR[29, 30].

The levels of 3-methyldioxyindole sulfate, a tryptophan metabolite, were lower at 0–24 h and higher at 31–55 h, but the relationship of this metabolite with hepatic toxicity is unclear. A decrease of the ion at *m/z* 242 has been found in urinary metabolic fingerprinting in ANIT-induced intrahepatic cholestasis using LC-MS[25], and a similar decrease was observed in rat urine following TAA-induced hepatic injury. These results indicate that decrease in urinary 3-methyldioxyindole sulfate may be correlated with hepatotoxicity. Further study is required to examine the relationship between the toxicity of ANIT with potential

biomarkers including PAG, HA and 3-methyldioxyindole sulfate.

Conclusions

A number of metabolomics studies using NMR and/or MS have been performed to detect metabolic alterations induced by administration of compounds to assess their toxicity. The results shown above indicate that urinary metabolic fingerprinting with FT-ICR MS can be used to detect metabolic changes and to identify potential biomarkers induced by compounds with different mechanisms of toxicity. Therefore, metabolomics approaches including metabolic fingerprinting have the potential to reveal onset of toxicity at an early stage of drug development. To reach this goal, improvements in the technology for metabolomics are required to allow detection of the whole metabolome with a high degree of accuracy. Improved informatics approaches, including database construction, are also needed for identification of biomarkers, with follow-up studies to validate biomarker concentrations in biofluid and metabolic pathways *in vivo*. These advances will allow future application of toxicological assessment in personalized medicine.

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