Investigation of Phenylacetylglycine and Hippuric Acid in Plasma as Potential Biomarkers for Drug-induced Phospholipidosis.

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Abstract

The research on potential biomarker discovery for drug-induced phospholipidosis revealed novel plasma and urine biomarkers to monitor phospholipidosis status in noninvasive way. Utilization of these biomarkers can avoid drug-induced phospholipidosis and it is critically beneficial to improve quality of life for patients who suffer from drug-induced toxicity. In this thesis, I investigated metabolomics research to identify phenylacetylglycine (PAG) to hippuric acid (HA) ratio in plasma as potential indicator to monitor phospholipidosis in rats and established a highly sensitive and reliable assay method.

In chapter 1, I investigated biomarker discovery in rat urine after phospholipidosis inducing drugs administration. Metabolomics study revealed PAG to HA ratio in urine was increased in time and dose dependent manners and it was well correlated with histopathological observation. These urine biomarkers were applied to plasma since the dynamics of these metabolites in urine were expected to linked with their plasma concentrations. The HA and PAG concentrations and PAG to HA ratios were monitored before and after treatment with amiodarone, a well-known phospholipidosis inducing drug. The PAG to HA ratio showed clear dose and time dependent increases after amiodarone administration. And the increment of the PAG/HA ratio decreased in a time dependent manner after the dosing period and this was consistent with the results from the histopathological evaluation.

To confirm the utility of their potential biomarkers, a reliable and robust analytical method development is important. In chapter 2, I developed and validated a quantification method by using liquid chromatography-tandem mass spectrometry

(LC/MS/MS) for simultaneous quantification of HA and PAG in rat urine. The established analytical method showed good precisions and accuracies confirmed by the assessments for intra- and inter-day assay validation procedures.

In summary, these novel, non-invasive and highly quantitative biomarkers to monitor drug-induced phospholipidosis status is critically beneficial to avoid drug derived serious toxicity to improve the quality of life for patients.

Abbreviations

CAD	Cationic amphiphilic drug
DIPL	Drug induced phospholipidosis
HA	Hippuric acid
LC/MS/MS	liquid chromatography-tandem mass spectrometry
NMR	Nuclear magnetic resonance
PAG	Phenylacetylglycine
PLD	Phospholipidosis
SRM	Selected reaction monitoring

General Introduction

Drug induced phospholipidosis (DIPL) is a lysosomal storage disorder characterized by an abnormal accumulation of phospholipids in cells such as hepatocytes, lymphocytes and macrophages and tissues (Anderson and Borlak, 2006; Reasor and Kacew, 2001; Tengstrand et al., 2010). In these cells, myeloid bodies can be observed using transmission electron microscopy (TEM) (Josepovitz et al., 1985; Mortuza et al., 2003). Myeloid bodies occur naturally in the late endosomes/lysosomes of tissues where they act as storage vesicles for secreted and undigested lipids and proteins (Schmitz and Müller, 1991). Excess undigested components in lysosome leads the accumulation of myeloid bodies and other inclusions in the cells in DIPL (Mohammad and Haoxing, 2014). Currently more than 50 drug candidates and marketed drugs including anti-depressants, antianginal, antimalarial, and cholesterol-lowering agents have been reported to cause DIPL and most of them that cause DIPL are cationic amphiphilic drugs (CADs) (Lüllmann et al., 1978, Halliwell, 1997, Reasor, 1989). Amiodarone, an antiarrhythmic drug with CAD structure used to treat and prevent irregular heartbeats, is well-known to induce phospholipidosis. Amiodarone has numerous side effects to various tissues including lung, thyroid, eye, liver and skin, especially it causes fatal severe pulmonary fibrosis toxicities (Baumann et al., 2017). While it's quite important to monitor the state of phospholipidosis, TEM method has limited utility to monitor in humans because of the invasive nature of acquiring patient tissue biopsy samples. A qualified biomarker of DIPL in the blood, plasma or urine is needed to provide a more routine, non-invasive, and cost effective means to monitor DIPL in the clinic.

There are approximately 50 congenital lysosomal metabolic disorders like Gaucher

disease, Fabry disease and Niemann-Pick disease (Grabowski, 2012, Kaminsky and Lidove, 2014). These diseases are caused by lysosomal dysfunction as a consequence of deficiency of lysosomal enzymes required for the sphingolipids metabolism to accumulate glycolipids or phospholipids like glucosylceramide, galactocerebroside and sphongomyeline (Segatori, 2014). And the histopathological findings also observe myeloid bodies in the cells of these diseases (Mahmud, 2014, Liu et al., 2014). In that sense there are several similarity points between DIPL and lysosomal disorders. On the other hand, the cause of a majority of lysosomal disorders are clearly identified as single genetic mutation of specific lipid metabolism, whereas the mechanism of DIPL has not been extensively studied and is not well understood yet (Hostetler and Matsuzawa, 1981; Joshi et al., 1988; Reasor and Kacew, 2001; Xia et al., 2000). Additionally, unlike lysosomal disorders, there are various species of lipids accumulated in DIPL. From these factors, the possible mechanism of DIPL should be participated not only phospholipids metabolism but also biosynthesis of the phospholipids and other homeostasis of the lipid components.

Metabolomics is the comprehensive analytical research for small molecules, such as sugar, amino acid and lipid components to explore the biological signatures of living systems to pathophysiological stimuli or genetic modification (Wei, 2011). Among so-called "omics" approach including genomics, transcriptomics and proteomics, metabolomics is to examine the final downstream product of the central dogma and is closest to the functional phenotype of the cell or organism. The metabolome is thus also closer and more susceptible to external perturbations such as drug treatment.

Furthermore, well established metabolic pathway map with around 4000 estimated small molecules can lead the mechanism hypothesis easily compared with that by the millions or tens of thousands of proteins, transcripts and genes (Leader et al., 2011). The other advantage of metabolomics approach is their concentrations, unlike other "omics" measures, directly reflect the underlying biochemical activity and state of cells and/or tissues. However, in terms of constructing an absolute quantification of endogenous metabolite remains as technical issues since coexisting substances interfere with metabolite in biological samples to hamper sensitivity and selectivity (Annesley 2003, Mallet et al., 2004). Thus, highly accurate and robust metabolite assay method development and validation is quite important for testing the hypothesis of biological alteration by external stimulations.

In this thesis, I investigated biomarker discovery by metabolomics approach using nuclear magnetic resonance (NMR) in rat urine after administration of phospholipidosis inducing drugs of amiodarone, chloroquine, quinacrine, tamoxifen and fluoxetine. The metabolomics analysis revealed that hippuric acid (HA) and phenylacetylglycine (PAG) levels were well correlated with histopathologic changes in DIPL in rats, such as foamy macrophage accumulation and vacuolated lymphocyte numbers. Simultaneous quantification methods for HA and PAG in rat urine was successfully developed and validated using liquid chromatography-tandem mass spectrometry (LC/MS/MS). By using the established analytical method I confirmed the PAG/HA ratio showed clear dose and time dependent increases after amiorarone administration and it decreased after dosing period that also reflect the histopathologic findings. Since phenylalanine,

an essential amino acid for animals including human, is known to be a precursor for both HA and PAG, its two major metabolic alterations, such as inhibition of beta-oxidation at phenylalanine to HA pathway by PLD-inducing drugs and concomitant acceleration of a compensation pathway to PAG, may be considered to be underlying mechanism for the change in PAG to HA ratio.

Chapter 1

Biomarker discovery for drug-induced phospholipidosis:

phenylacetylglycine to hippuric acid ratio in urine and plasma as potential

markers

Abstract

Drug-induced phospholipidosis (DIPL) is one of significant concerns in drug safety assessment; however, its mechanism and predictive biomarkers are still not well elucidated. In this chapter, I have applied metabolomics approach, based on nuclear magnetic resonance (NMR), to exploration for novel index that reflects a DIPL status using rat urine after administrations of well-known phospholipidosis inducing drugs of amiodarone, chloroquine, quinacrine, tamoxifen and fluoxetine, and both hippuric acid (HA) and phenylacetylglycine (PAG) levels were well correlated with histopathologic changes in DIPL in rats, such as foamy macrophage accumulation and vacuolated lymphocyte numbers, and the ratio in plasma was increased in time and dose dependent manners. Taking reproducibility of data and convenience for sampling into consideration, the ratio of PAG to HA in plasma is expected to be practical marker in monitoring DIPL in rats.

Introduction

Phospholipidosis (PLD) is a lipid storage disorder in which excess phospholipids accumulates within many cell types such as hepatocytes, lymphocytes and macrophages (Drenckhahn et al., 1983, Farrell, 2002, Ploemen et al., 2004, Rudmann et al., 2004). The risk for drug-induced PLD is one of the significant concerns in drug development, especially in safety assessment, because more than 50 cationic amphiphilic drugs (CADs), including antidepressants, antianginal, antimalarial, and cholesterol-lowering agents, have already been reported to induce PLD so far (Lüllmann et al., 1978, Halliwell, 1997, Reasor, 1989). CADs are thought to induce PLD by inhibiting lysosomal phospholipase activity, but its mechanism has not been extensively studied and is not well understood yet (Hostetler and Matsuzawa, 1981; Joshi et al., 1988; Reasor and Kacew, 2001; Xia et al., 2000). Moreover, it is still ambiguous whether drug-induced PLD represents benign adaptive responses or toxicity-related events. The absence of a non-invasive biomarker has made it difficult to study PLD *in vivo*. Electron microscopic observation has long been employed as the most reliable method for identifying phospholipidotic cell damage (Drenckhahn et al., 1976). Since histopathological evaluation is relatively non-quantitative, time consuming and an expensive procedure, it is considered to be an impractical screening tool for rapid toxicity assessment. Furthermore, it is also difficult to monitor PLD in clinical studies without use of an invasive methodology such as tissue biopsy. Therefore, development of a non-invasive diagnostic tool for PLD is highly desirable in pre-clinical and clinical studies for the

development of new drugs.

Currently there are several candidates for a non-invasive biochemical marker for PLD. Since CADs are known to induce PLD in the lymphocytes of animals and humans (Lullmann-Rauch, 1979; Dake et al., 1985), vacuolated lymphocytes in peripheral blood have been considered as a potential diagnostic biomarker for PLD (Rudmann et al., 2004). Using a nuclear magnetic resonance (NMR) based metabolomics approach, urinary and plasma phenylacetylglycine (PAG) has been proposed as a potential biomarker for PLD (Nicholls et al., 2000); however, the mechanism behind it has not been fully elucidated (Delaney et al., 2004). On the other hand, a liquid chromatography-mass spectrometry (LC/MS)-based approach identified the elevation of serum bis(monoglycero)phosphate (BMP) in PLD induced by drug administration (Mortuza et al., 2003); and di-docosahexanoyl (C22:6)-BMP was proposed to be a potential marker of drug-induced PLD in rats (Liu et al., 2014).

So as to be practical biomarker for safety assessment, it is essential to elucidate its link to drug-induced toxicity and assess its predictability at least in pre-clinical studies. In this report, I have found new biomarker candidates of drug-induced PLD, hippuric acid (HA) and phenylacetylglycine (PAG), in rat urine by using NMR spectrometry. Then the ratio of urinary PAG to HA was confirmed to reflect the disease state in rats with the administration of PLD-inducing drugs. Furthermore, an alternate analytical method to determine urinary and plasma concentrations of HA and PAG was established with liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) technology. Using the LC/MS/MS-based protocol thus established for

robust but convenient quantification, I evaluated the drug-induced alterations in HA and PAG levels not only in urine but also in plasma, which can be easily collected in monitoring biomarkers for PLD.

Materials and Methods

Regents

Amiodarone, chloroquine, quinacrine and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluoxetine and two reference standards, hippuric acid (HA) and phenylacetylglycine (PAG) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). As the internal standard (IS) in the LC/MS/MS analyses, hippuric acid-d5 (HA-d5) was purchased from C/D/N ISOTOPES (Quebec, Canada) and phenylacetylglycine-d4 (PAG-d4) was prepared in house. Deuterium oxide (D2O) and sodium 3-(trimethylsilyl)-propionate-2,2,3,3,-d4 (TSP-d4) were purchased from ISOTEC.INC (Miamisburg, OH, USA). Acetonitrile, methanol (HPLC grade) and formic acid, ammonium formate and ammonium acetate (regent grade) were also obtained from Wako. All other solvents with the highest purity grades were obtained from commercial sources and used without further processing.

Animals

Five or six weeks old Crl: CD (SD) rats were purchased from Charles River Japan, Inc. (Tokyo, Japan). The animals were individually housed in metal cages in a clean booth and were allowed free access to tap water and a powdered laboratory diet (CE-2, CLEA Japan, Inc., Tokyo, Japan). The racks were placed in an animal room under the following conditions: temperature of 20-26 °C, a relative humidity of 40-70%, air exchange at 8-25 times/hour and a 12-hour light/dark cycle (lights on from 7:00 a.m. to

7:00 p.m.). After s 7-day acclimation period, the animals were randomly assigned into control and treatment groups based on their body weight. All the procedures in animal handling and bleeding are assessed and approved by Animal Care and Use Committee in Takeda Pharmaceutical Company Limited.

Drug administration and sample collection

Three to five male rats (6-7 weeks old) were used for each dose group. All test compounds were suspended in 0.5 w/v % methylcellulose solutions, and the dosing suspension was administered in the morning into the stomach of rats via a catheter. The vehicle was also administered to control rats in the same manner. The volume administered to each animal, 10 mL/kg for each dosage level, was adjusted based on the body weight on the first day before dosing.

For the studies for blood smears, histopathological observation and ^{1}H-NMR analysis, amiodarone (100, 300 and 1000 mg/kg/day), chloroquine (25, 75 and 250 mg/kg/day), tamoxifen (100, 300 and 1000 mg/kg/day), quinacrine (60, 200 and 600 mg/kg/day) or fluoxetine (30, 100 and 300 mg/kg/day) was administered once daily for 3 consecutive days. The highest dosing corresponds to approximate 1/2-1/3 of LD_{50} of each compound. After the final dose, urine samples were collected in cooled plastic bottles for 6 hours. Drinking water and laboratory diet was removed during urine sampling. The urines were centrifuged and resultant supernatants were stored at -70 °C until ^{1}H-NMR analysis. Twenty-four hours after the final administration, blood was collected for smear preparation and all the animals underwent euthanasia for necropsy examination.

For the purpose of acquisition of the toxicokinetic parameters , the respective doses for these drugs were administered singly to the other three rats and blood samples were collected at 1, 3, 6 and 24 hours after dosing.

For the LC/MS/MS analysis of urine, rats were dosed once daily for 7 consecutive days with amiodarone, chloroquine, tamoxifen or quinacrine (300, 75, 100 and 60 mg/kg/day, respectively). After the final administration, the urine samples were collected for 4 hours during the daytime and stored frozen at -80 ˚C until analysis. For the analysis of the plasma, fluoxetine or amiodarone was dosed once daily for 3 consecutive days. Fluoxetine was administered at 10, 30 and 100 mg/kg/day and the blood samples were collected at 24 hours after the final dosing. On the other hand, blood samples were taken serially in the morning (9 am), afternoon (1 pm) and evening (5 pm) on 5 and 3 days before dosing and then amiodarone was administered at 100 and 300 mg/kg/day at 9 am. Blood samples were collected consecutively at pre-dosing (9 am), 4 hours (1 pm), 8 hours (5 pm) and 24 hours after the first and third dosing days. The blood at 48 and 168 hours after the final dosing was also collected. All the blood samples were collected from the tail vein under ether anesthesia and centrifuged to obtain plasma. The resultant plasma samples were stored frozen at -80 ˚C until analysis.

Blood smears, histopathology and toxicokinetics

Blood smears stained with May-Giemsa were examined microscopically to count the number of vacuolated lymphocytes out of 300 cells in the microscopic analysis. The organs and tissues (lung, lymph node, liver and spleen) from all animals were sampled,

examined and fixed in 10 vol % neutral buffered formalin. Brain was also evaluated in the chloroquine, fluoxetine or quinacrine-treated groups. The histopathologic examination was conducted in a blinded fashion based on the following criteria: foam cell infiltration was seen in the lung and the mesenteric lymph nodes, increased tingible body macrophages was seen in the spleen and vacuolization was seen in the hepatocytes, Kupffer cells and bile duct epithelial cells in the liver, the tubular cells of the kidney and cerebellar Purkinje cells. Drug concentrations in plasma were evaluated by high-performance liquid chromatography (HPLC) system to calculate the toxicokinetic parameters.

^{1}H-NMR spectroscopy and data analysis

To neutralize the urinary samples, 250 µL of a phosphate buffer solution (0.2 M Na_2HPO_4/0.2 M NaH_2PO_4, pH 7.4) were added to 500 µL of urine and left to stand for 10 min. Precipitates was removed by centrifugation at 13,000 rpm for 10 min at 10 °C. Aliquots of the clear supernatants (600 µL) were mixed with 60 µL of 11mM TSP-d4/D_2O solution as an internal standard, then 1H NMR spectra were measured at 599.59 MHz on a Unity INOVA 600 spectrometer (Varian, Inc. CA, USA) at 298 K. The water resonance was suppressed by using 1-D Nuclear Overhauser Enhancement Spectroscopy (NOESY) pulse sequence with irradiation during a 1s relaxation delay and a 100 ms mixing time. Spectra were acquired using 64 free-induction decays (FIDs) into 64 K data points, and a spectral width of 8,000 Hz. Exponential line broadening of 0.2 Hz was applied prior to Fourier transformation. Spectra were phased manually,

corrected for baseline distortion and referenced to TSP automatically using an ACD/Spec Manager (ACD Labs, Canada). Subsequently all NMR spectra were data reduced to 246 integrated regions corresponding to the chemical shift range of δ10-0.16 with region width of 0.04 ppm. The spectral region 4.60-6.12 ppm was excluded to remove variability due to suppression of water resonances and cross-saturation effects. The intensities of the NMR signals were estimated manually, and normalized using the creatinine signal (δ 4.06).

LC/MS/MS and data analysis

For the preprocessing of the urinary samples for HA and PAG quantification with LC/MS/MS, 20 µL aliquots of rat urine were mixed with 10 µL of IS solution and 1 mL of water/acetonitrile (1:1, v/v). After removal of precipitants by centrifugation, 20 µL of the supernatant were further diluted with 1 mL of solvent (10 mmol/L ammonium formate/acetonitrile/formic acid, 475:25:1, v/v/v). In case of the plasma samples, 50 µL of aliquots were mixed with 10 µL of IS solution and 450 µL of acetonitrile, centrifuged, and then 50 µL of the supernatants were diluted with 150 µL of solvent. The samples thus prepared were injected into a LC/MS/MS system, equipped with an SIL-HT*c* autosampler and LC-10AD*vp* pump system (Shimadzu, Kyoto, Japan). The analytical column used was an L-Column ODS (2.1 × 50 mm, 5 µm, Chemicals Evaluation and Research Institute, Tokyo, Japan) and the flow rate was 0.2 mL/min at 40°C. Mobile phase A (MP-A) consisted of 10 mmol/L ammonium formate/formic acid (500:1, v/v) and mobile phase B (MP-B) consisted of acetonitrile/formic acid (500:1, v/v). The gradient

started with 5% MP-B and was linearly increased to 60% within 3 minutes, and then increased to 80% for following 0.2 minutes. This condition was kept from 3.2 to 5 minutes and then it was cycled back to the initial conditions over 0.1 minutes. The total analysis time was 10 minutes. Final chromatographic retention times for HA, PAG, HA-$d5$ and PAG-d_4 were between 3.5 and 4 minutes.

The quantification of analytes was performed by electrospray LC/MS/MS in the selected reaction monitoring (SRM) mode on a API3000 or API4000 tandem quadrupole mass spectrometer with a turbo ion spray configuration, operated in the positive ionization mode, with Analyst controlling software (AB Sciex, ON, Canada). Source conditions were typically as follows (API3000/API4000): ion spray voltage 4200 V/5200V, turbo probe temperature 450 ˚C/550 ˚C, unit resolution on Q1 and Q3. Heated gas (air), nebulizer gas (air) and curtain gas (N_2) flows were set to 7 or 5, 1.04 L/min/60 unit and 0.95 L/min/70 unit, respectively. Multipliers were set to 2000 V, and the dwell times for HA, PAG and their corresponding ISs HA-d_5 and PAG-d_4 were 150 ms. For the SRM analysis, the following ion transitions were obtained: HA mass-to-charge ratio value (*m/z*) 180 → 105, PAG *m/z* 194 → 91, HA-d_5 *m/z* 185 → 110 and PAG-d_4 *m/z* 198 → 93. Sensitivity was optimized for each compound by varying collision cell pressure, declustering potential, focusing potential (for API3000 use only) and collision energy in the SRM mode and maximizing ion intensity. The standard curves (50 - 5000 µg/mL for HA, 5-1000 µg/mL for PAG in urine, 0.02 - 10 µg/mL for HA and PAG in plasma) gave correlation coefficients >0.99 and coefficient of variations ranging within 15%.

Statistical analysis

The mean values of the percentage of vacuolated lymphocytes out of 300 cells for each sample in the blood smear, HA and PAG concentrations in the urine and plasma of CAD treated animals versus those of control group were compared by Williams test or Dunnett multiple comparison test and were considered significant at $p < 0.025$ and 0.05, respectively.

Results

Histopathology and toxicokinetics evaluation of PLD-related changes in CAD-treated rats

In one or more organs in the CAD-treated rats, PLD-related histopathological changes were observed as follows: accumulation of foamy macrophages in the lungs; and medullary sinus of the mesenteric lymph nodes, vacuolization of the hepatocytes, Kupffer cell and biliary ducts in the liver, white pulp of the spleen, neurocytes and Purkinje cells in the brain (Table1). These histopathological changes were characterized by the accumulation of multilamellar bodies and/or accumulation of electron thick dense bodies in the cytoplasm of the various cell types as shown in Figure 1. In all the groups except for the amiodarone low dose (100 mg/kg), the percentages of vacuolated lymphocytes were significantly higher than that in control group and the increase was dose-dependent. Therefore, the minimum toxic doses in histopathological evaluation were determined to be 300 mg/kg for amiodarone, 25 mg/kg for chloroquine, 100 mg/kg for tamoxifen, 60 mg/kg for quinacrine and 30 mg/kg for fluoxetine in a 3-day short term exposure. Results of the toxicokinetic parameters in plasma for each CAD compound are summarized in Table 3. While the high dose range was selected for the purpose of this study, the maximum concentration (Cmax) and the area under the curve (AUC) were mostly correlated in a dose dependent manner.

Determination of urinary PAG and HA by ^{1}H-NMR analysis

^{1}H-NMR spectra analysis was carried out for all spectra through normalization of the signal intensity by the creatinine signal in each spectrum at δ4.06. Figure 2 shows typical NMR charts indicating the changes in the resonances in rat urine after dosing amiodarone (once daily for 3 days). As shown in Figure 2, the results indicated that intensities of the resonance at δ3.98(d), δ7.56(t), δ7.64(t), δ7.84(d), which were assigned as hippuric acid (HA), in inverse relation to the dose.   The same tendency was observed in all CADs-treated animals. On the other hand, the intensities of the resonances at δ3.68(s), δ3.76(d), δ7.36(m), δ7.43(m), δ7.48(m), which were assigned as phenylacetylglycine (PAG), increased dose-dependently, whose tendency was also observed in all CADs-treated animals. The ratios of PAG to HA (PAG/HA ratio) calculated from the NMR signal intensity are summarized in Table 2, indicating that the values of PAG/HA ratio were significantly higher than that of the control group except for the lowest dose groups of amiodarone and chloroquine. The values increased in a dose dependent manner in all the CADs-treated animals, and it coincided well with the increment in the percentages of vacuolated lymphocytes shown in Table 1.

Quantitative analysis of HA and PAG in urine and plasma by LC/MS/MS

Since the results obtained through histopathological evaluation and NMR analysis of the urine samples suggested to us that the PAG/HA ratio in urine could be a surrogate for the PLD-related histopathological changes in CAD-treated rats, I then tried quantification of the urinary PAG and HA by LC/MS/MS. After 7 days of multiple oral

administration of amiodarone, chloroquine, tamoxifen, or quinacrine (300, 75, 100, 60

mg/kg/day, respectively) to rats, the concentrations of HA and PAG in urine were

determined and the PAG/HA ratios were calculated (Figure 3). PAG concentration was

significantly increased in the amiodarone-treated rats in compared with the controls

and slightly increased in the rats treated with quinacrine or tamoxifen, but did not

change in chloroquine treated group (Figure 3B). The same tendency was also observed

in the PAG/HA ratio (Figure 3C). Although these results supported the idea that the

PAG/HA ratio indicates the state of PLD, its reliability might be hampered by very large

inter-individual variability in both urinary PAG and HA concentrations. As is the case

in other urinary biomarkers, PAG and HA concentrations might require normalization

with creatinine.

Since the dynamics of HA and PAG in urine were expected to linked with their plasma

concentrations, which do not require any normalization, I next performed the

quantitative determination of HA and PAG in plasma. While the concentrations of HA

and PAG in plasma were much lower than those in urine, their plasma levels were

successfully determined by LC/MS/MS. In the amiodarone-treated rats, plasma

concentrations of HA tended to decrease, those of PAG significantly increased, and the

ratio of HA to PAG significantly increased in a dose-dependent manner (Figure 4).

Multiple dosing of fluoxetine also provided the same tendency, PAG/HA ratio in plasma

was significantly high in 100 mg/kg/day group (Figure 5). Additionally, in our

preliminary experiments, a toxic dose of phenobarbital as the negative control did not

change the PAG/HA ratio while imipramine as another PLD inducing drug showed a

significant increase of the PAG/HA ratio in plasma. These results of the PAG/HA ratio in plasma correlated with the results of the histopathological studies shown in Table 1 and those of the PAG/HA ratio in urine determined by ^{1}H-NMR analysis as shown in Figure 2 and Table 2, indicating the potency of the PAG/HA ratio in plasma as a marker for PLD induced by CAD administration.

Intra- and inter-day variation of HA and PAG and time course study after amiodarone treatment

To evaluate time-course changes in the PAG/HA ratio in plasma, amiodarone was administrated once daily for 3 consecutive days and blood samples were collected serially after the first and third dosing. Concurrently, blood samples were also collected 5 and 3 days prior to administration to determine the variability of the HA and PAG concentrations in the timing of sampling. As shown in Figure 6, the PAG/HA ratio in the evening was higher than that of morning due to the decrease in HA but there was no increase in the PAG levels. This result strongly suggested to us that it is important to match the sampling time point to avoid the influence by daily fluctuations in the HA concentrations. The variability of the HA and PAG concentrations and PAG/HA ratio at different sampling times and days was checked further with no intervention control group (Figure 7), and this result suggests to us that the baseline of PAG/HA ratio is stable in the morning.

The HA and PAG concentrations and PAG/HA ratios in the morning were monitored before and after treatment with amiodarone (Figure 8). The PAG/HA ratio showed clear

dose and time dependent increases after amiorarone administration. The increment of the PAG/HA ratio in the higher dose group (300 mg/kg/day) was sustained after the dosing period ended. On the other hand, the ratio in the lower dose group (100 mg/kg/day) rapidly decreased in a time dependent manner after the dosing period and this was consistant with the results from the histopathological evaluation. Trends to decrease in HA and increase in PAG levels were observed but were not clear enough to show dose-dependency. It was noteworthy that PAG kept increasing in the higher dosing group after the dosing period (Figure 8B), and this is considered to contribute to the sustained PAG/HA ratio.

Discussion

The evidence for the presence of PLD is obtained through histopathological examination of animal tissues at pre-clinical stage; however, each CAD tends to induced different distribution of PLD as shown in Table 1. Therefore, the mechanism and process underlying PLD development are considered to be complex and might differ from one drug to another. There are no predictive biomarkers for drug-induced tissue PLD other than lymphocyte vacuolation, but morphological observation would not be suitable for screening purposes. Therefore, biochemical biomarkers are still being explored for drug safety assessment.

Metabolomics is one of popular technologies in the latest toxicology testing (Robertson et al., 2011). To identify biomarker candidates for CAD-induced PLD, I initially conducted statistical analysis of the NMR spectra of urinary samples and have successfully separated the CAD-dosed groups from the control group by principle component analysis. The dominant factors were citrate and α-ketoglutarate, two components of Krebs cycle, but it seemed to be difficult to generate a hypothesis for PLD mechanism with only these major energy metabolites. Therefore, I pursued manual checking of the NMR spectra of urinary samples and found increases in PAG-related and decreases in HA-related signals (Figure 2). The increase of PAG in urine matched well with the previous report on PAG as biomarker candidate for PLD in rats (Delaney et al., 2004, Hasegawa et al., 2007, Doessegger et al., 2013). On the other hand, the relationship between PLD and HA had not been elucidated. The key finding of this

study was that the ratio of PAG to HA in urine correlated well with CAD-induced PLD. LC/MS/MS analysis focused on PAG and HA was applied to urine and plasma, and the link between the PAG/HA ratio and PLD was validated. A similar global metabolomic approach with LC/MS was applied to the analysis for aristolochic acid-induced nephrotoxicity in rats and both PAG and HA were also reported to change concomitant with many other metabolites; however, they did not focus on the PAG/HA ratio in their analysis (Zhao et al., 2015).

HA and PAG are known to be metabolites derived from phenylalanine, but the metabolic pathway seems to be complicated because of the contribution of microbiota in the gut to the process. For example, it is reported that antibiotic-induced bacterial suppression reduced the excretion of mammalian-microbial urinary cometabolites including HA and PAG (Swann et al., 2011). On the other hand, phenylalanine is well-known to be essential amino acid that cannot be synthesized *de novo* in animals; therefore, the amounts of phenylalanine and its metabolites, HA and PAG, in the body could also be affected by food intake. The evaluation of the circadian variation in the plasma HA and PAG concentrations (Figure 6 and 7) revealed that HA tends to be much higher in the morning which corresponded well with the feeding pattern of rats as a nocturnal animal. I also have found that plasma concentrations of HA in rats were significantly decreased in fasting rats compared with fed rats in a preliminary experiment.

Based on the results in the present study and literature, I propose the hypothesis that the catabolism of phenylalanine, as simplistically illustrated in Figure 9, might be

perturbed in PLD-induced rats as the mechanism behind the changes in HA and PAG. While tyrosine synthesis solely depends on phenylalanine hydroxylase (PAH) at the first step, a CAD inducer like amiodarone does not affect the PAH activity (Delaney et al., 2004). On the other hand, it is known that phenylalanine is deaminated by phenylalanine dehydrogenase to form phenylpyruvate. Phenylpyruvate is further metabolized by phenylpyruvate decarboxylase to form phenylacetaldehyde and oxidized by aldehyde dehydrogenase to form phenylacetate. Eventually, phenylacetate is conjugated with glycine to form PAG to be excreted in rats or conjugated with glutamine to form phenylacetylglutamine in human and primates (Doessegger et al., 2013). Alternatively, phenylpyruvate is also metabolized to phenyllactate, which is converted eventually to form benzoic acid. In this pathway, the cinnamic acid that is formed by dehydration of phenyllactate is metabolized to benzoic acid via β-oxidation. It is known that amiodarone inhibits the mitochondrial β-oxidation of fatty acids (Fromenty et al., 1990; Fromenty and Pessayre, 1995; Kaufmann et al., 2005; Spaniol et al., 2001; Waldhauser et al., 2006). Hence, the inhibition of β-oxidation in this pathway would cause a decrease in HA level. Furthermore, the inhibition of the pathway leading to HA might cause a compensatory increase in the alternate metabolic pathway of phenylpyruvate, resulting in the increase in PAG. It has been suggested that the inhibition of β-oxidation is related to a dysfunction in lipid metabolism, which is the cause of PLD (Fromenty and Pessayre, 1995). HA decrease and PAG increase in the plasma could be an index for inhibition of β-oxidation by drugs; therefore, they could be a surrogate marker for PLD. Although levels of phenylalanine and its metabolism could

be affected by food intake or gut flora (Delaney et al., 2004), the PAG/HA ratio is a simple index for the effects on metabolic balance in drug-induced PLD.

Tables and Figures

Table 1. Summary of histopathological changes and vacuolated lymphocyte ratio in drug-induced phospholipidosis.

Compound	Dose	Histopathology (Plsis/steatosis)					Vacuolated lymphocyte (%)	
		Lung	Lymph node	Liver	Spleen	Brain	mean	SD
Control	0 mg/kg	-	-	-	-	ND	1.55	1.32
Amiodarone	100 mg/kg	-	-	-	-	ND	1.25	1.89
	300 mg/kg	+	+	-	-	ND	32.50	5.74 *
	1000 mg/kg	+	+	-	+	ND	39.75	10.24 *
Chloroquine	25 mg/kg	-	+	-	-	-	4.50	1.91 *
	75 mg/kg	-	+	-	+	-	24.25	10.56 *
	250 mg/kg	-	+	-	+	-	66.25	3.94 *
Tamoxifen	100 mg/kg	+	+	-	-	ND	20.25	5.85 *
	300 mg/kg	+	+	+	-	ND	38.75	12.20 *
	1000 mg/kg	+	+	+	-	ND	39.50	17.62 *
Quinacrine	60 mg/kg	+	-	+	-	-	14.00	7.75 *
	200 mg/kg	+	+	+	-	-	31.75	9.60 *
	600 mg/kg	+	+	+	-	-	52.33	5.86 *
Fluoxetine	30 mg/kg	+	-	-	-	-	7.25	2.87 *
	100 mg/kg	+	+	+	-	+	28.00	10.42 *
	300 mg/kg	+	+	+	-	+	30.75	9.84 *

+: Phospholipidosis/steatosis,   -: no abnormality,   ND: not determined

*: Significantly different from the control group; $p < 0.025$ (Williams)

Table 2. ^{1}H-NMR signal ratio of PAG to HA (PAG/HA ratio) in urine of CAD-treated rats

Compounds	Dose mg/kg	PAG/HA ratio
Control	-	0.12 ± 0.04
Amiodarone	100	0.16 ± 0.06
	300	0.52 ± 0.15*
	1000	1.27 ± 0.56*
Chloroquine	25	0.15 ± 0.05
	75	0.24 ± 0.07*
	250	0.69 ± 0.25*
Tamoxifen	100	0.31 ± 0.07*
	300	1.2 ± 0.28*
	1000	1.43 ± 0.87*
Quinacrine	60	0.28 ± 0.09*
	200	0.92 ± 0.38*
	600	4.42 ± 2.51*
Fluoxetine	30	0.19 ± 0.04*
	100	0.37 ± 0.19*
	300	0.56 ± 0.51*

Results are expressed as the mean ± SD.

* Significantly different from the control group $p < 0.025$ (Williams test).

Table 3. Pharmacokinetic parameters in plasma after a single administration of CAD compounds.

Compounds	Dose mg/kg	Tmax (h)			Cmax (µg/mL)			AUC$_{0-24h}$ (µg·h/mL)		
Amiodarone	100	4.0	±	1.7	1.21	±	0.70	17.0	±	8.3
	300	2.7	±	0.0	2.46	±	0.58	34.3	±	7.9
	1000	2.3	±	1.2	1.84	±	0.29	30.3	±	2.2
Chloroquine	25	6.0	±	0.0	0.05	±	0.01	0.5	±	0.1
	75	5.0	±	1.7	0.12	±	0.03	2.2	±	0.5
	250	5.0	±	1.7	0.22	±	0.03	4.4	±	0.6
Tamoxifen	100	5.0	±	1.7	0.85	±	0.11	13.5	±	2
	300	5.0	±	1.7	1.37	±	0.26	25.5	±	3.8
	1000	3.0	±	0.0	1.85	±	0.73	33.5	±	18.9
Quinacrine*	60	3.0			0.01	±	0.02	0.0	±	0.1
	200	13.5			0.04	±	0.03	0.5	±	0.5
	600	8.7	±	13.3	0.21	±	0.11	1.9	±	0.2
Fluoxetine	30	2.3	±	1.2	0.44	±	0.02	5.3	±	0.7
	100	4.0	±	1.7	0.76	±	0.25	12.7	±	6.7
	300	5.0	±	1.7	1.37	±	0.20	24.2	±	3.3

Results are expressed as the mean ± SD.

* Plasma concentrations were less than quantification limit through all the time-points at 60 mg/kg (n=2) and 200 mg/kg (n=1) in quinacrine dosing groups.

Figure 1. Vacuolated lymphocyte and foamy cells accumulation in the lung in

CAD-treated rats. Blood smear (a)CAD-treated rat (b) normal rat: Histopathology of (c)

CAD-treated rat (d) normal rat.
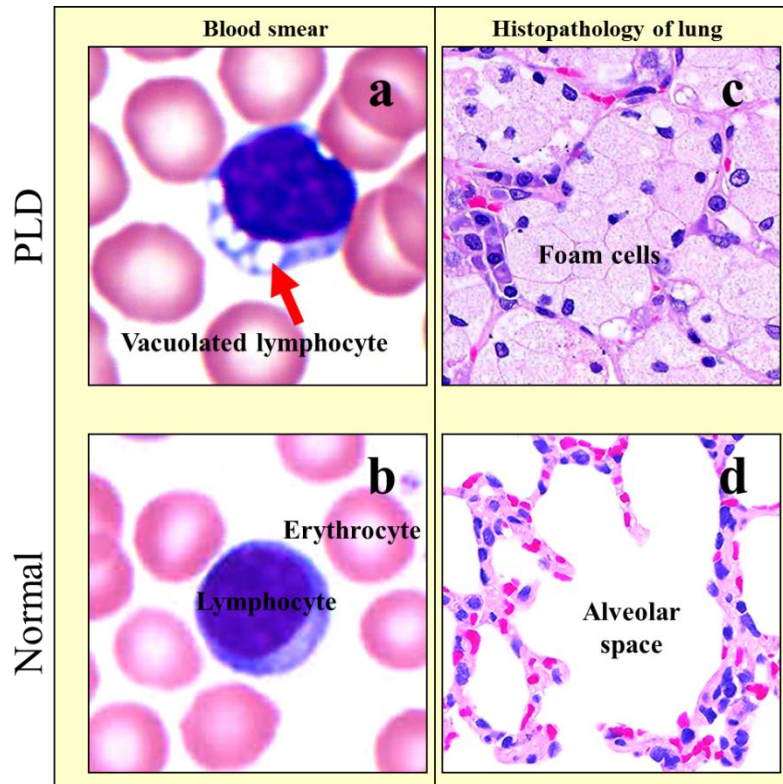
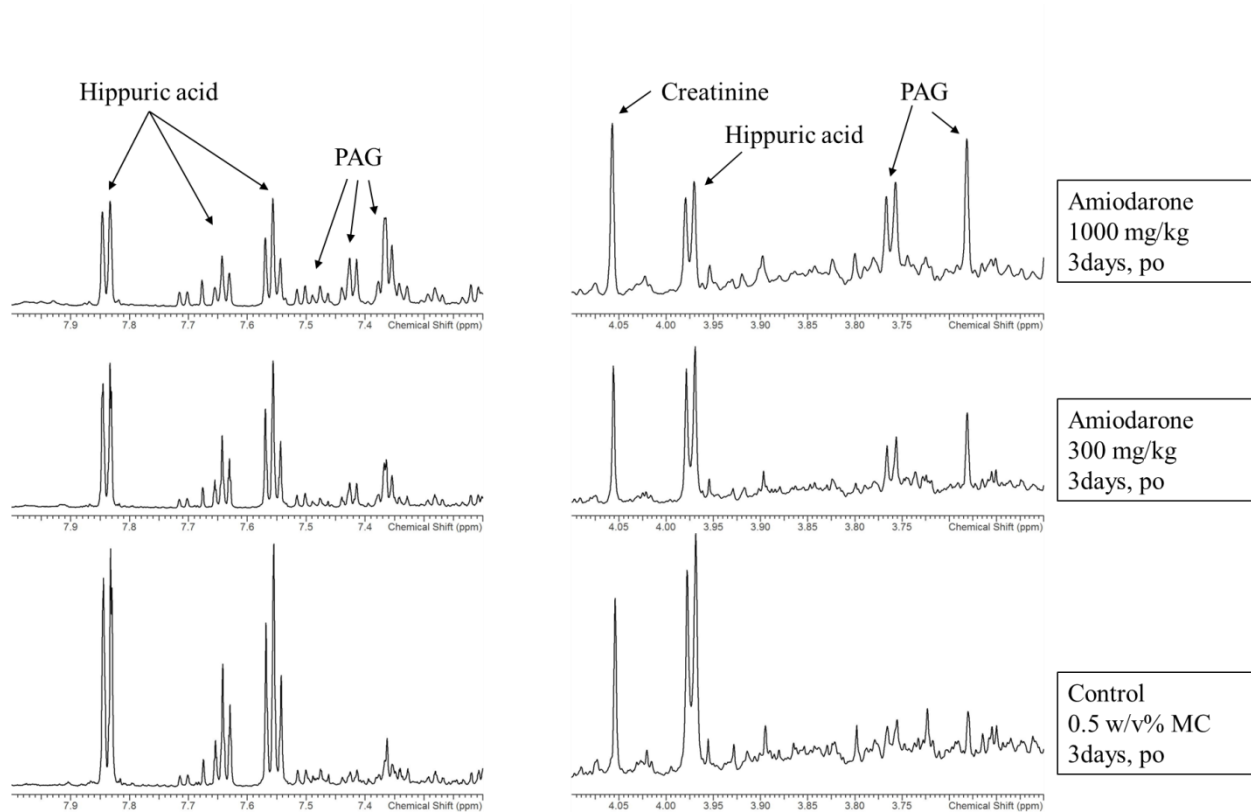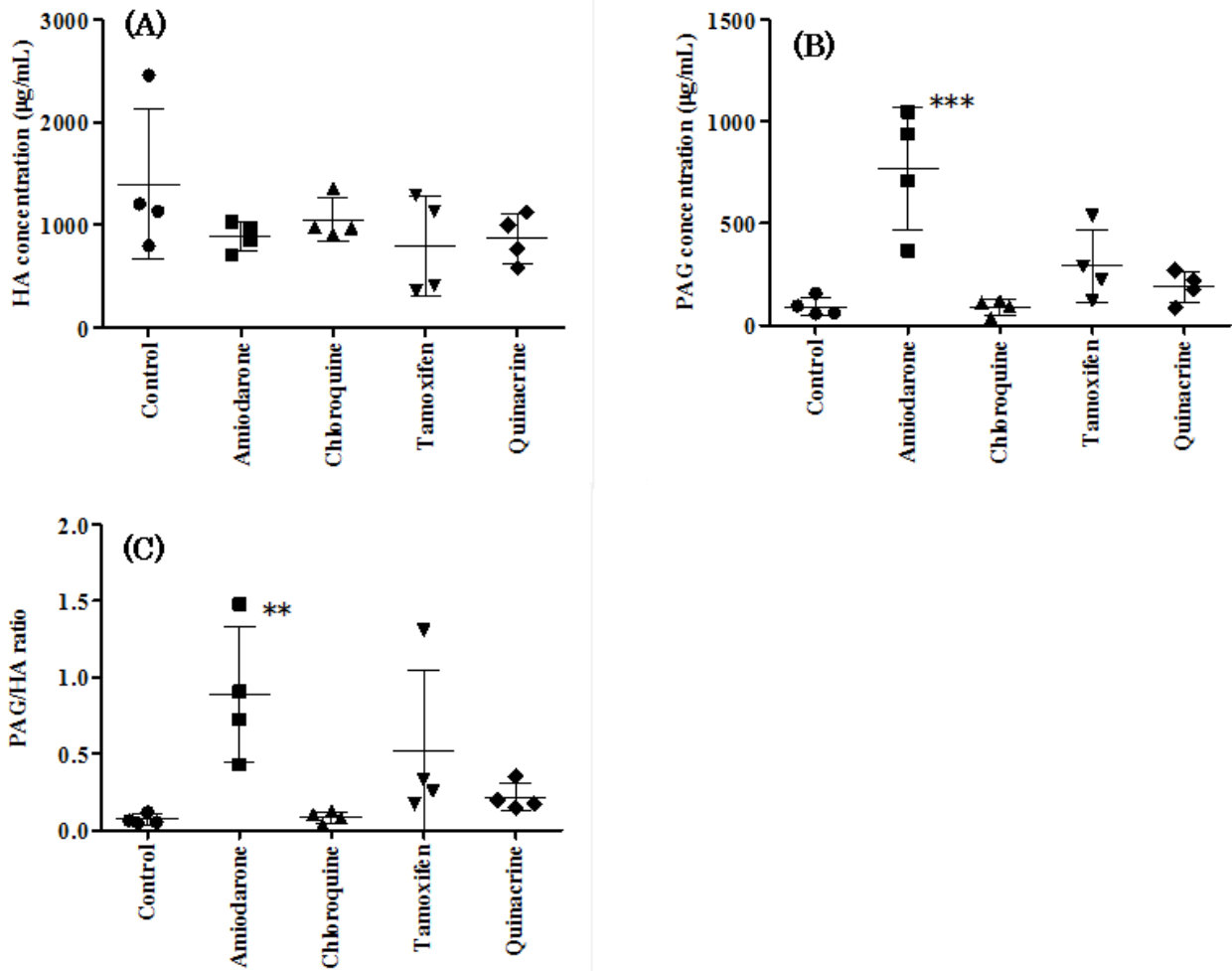**Figure 2.** Changes of ^{1}H-NMR signal intensity of rat urine after the last dose of amiodarone.

Figure 3. Urinary concentrations of HA (A) and PAG (B) and concentration ratio of PAG to HA (C) after 7-day administration of CADs.

Statistical analysis was performed using Dunnett multiple comparison test; significance denoted as *p<0.05, **p<0.01, ***p<0.001 from control.

Figure 4. Plasma concentrations of HA (A) and PAG (B) and concentration ratio of
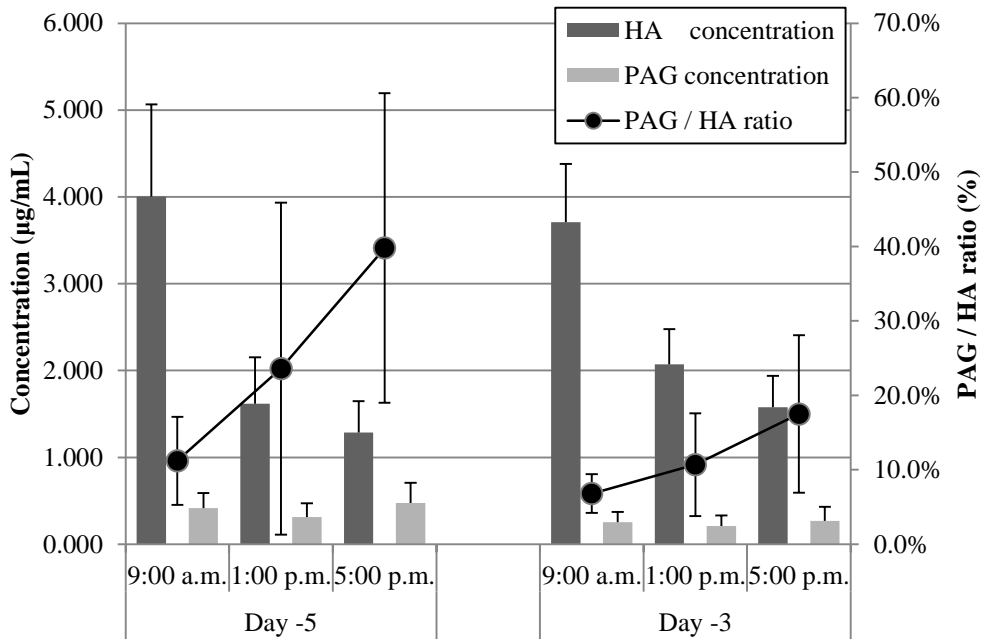
PAG to HA after 3-day administration of amiodarone.
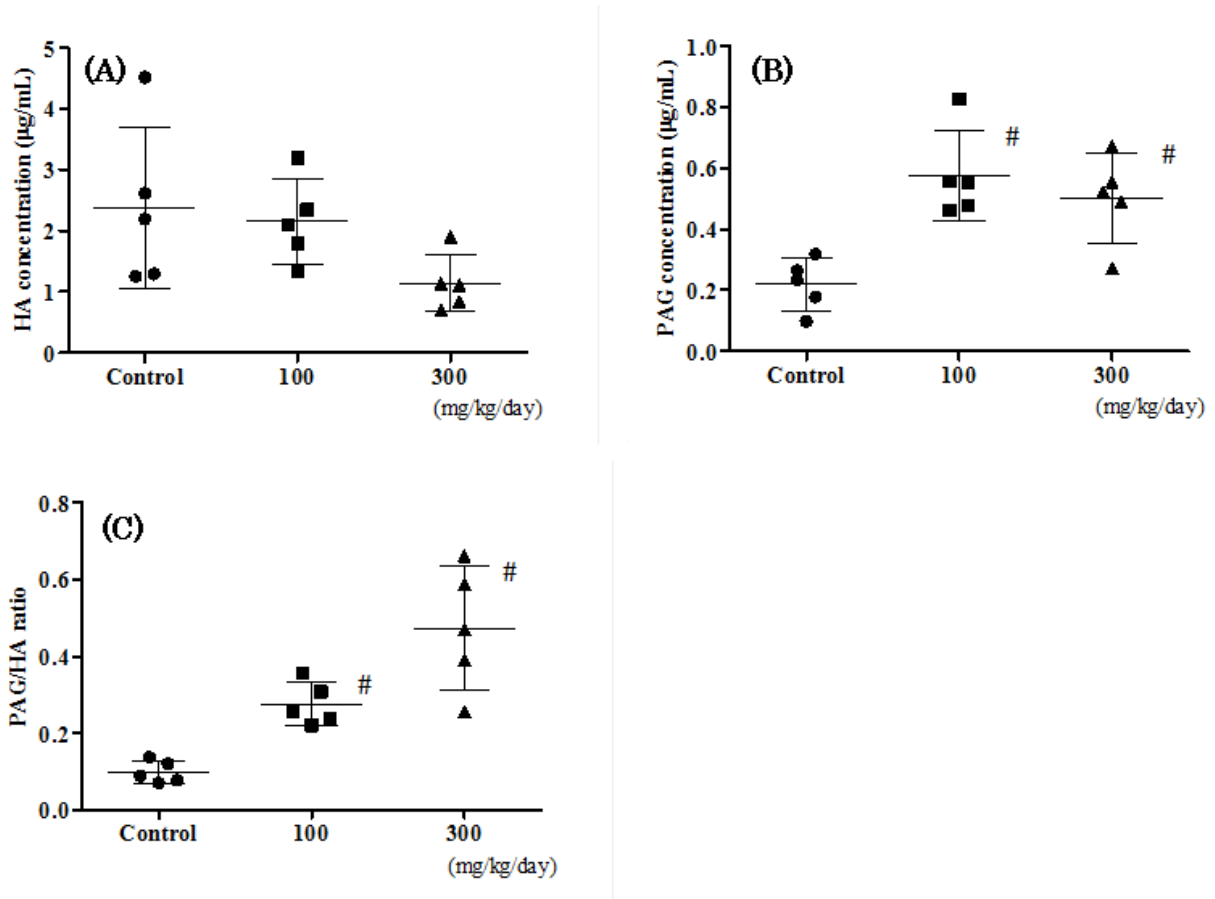
Statistical analysis was performed using Williams test; significance denoted as #

p<0.025 from control.

Figure 5. Plasma concentrations of HA (A) and PAG (B) and concentration ratio of

PAG to HA after 3-day administration of fluoxetine.
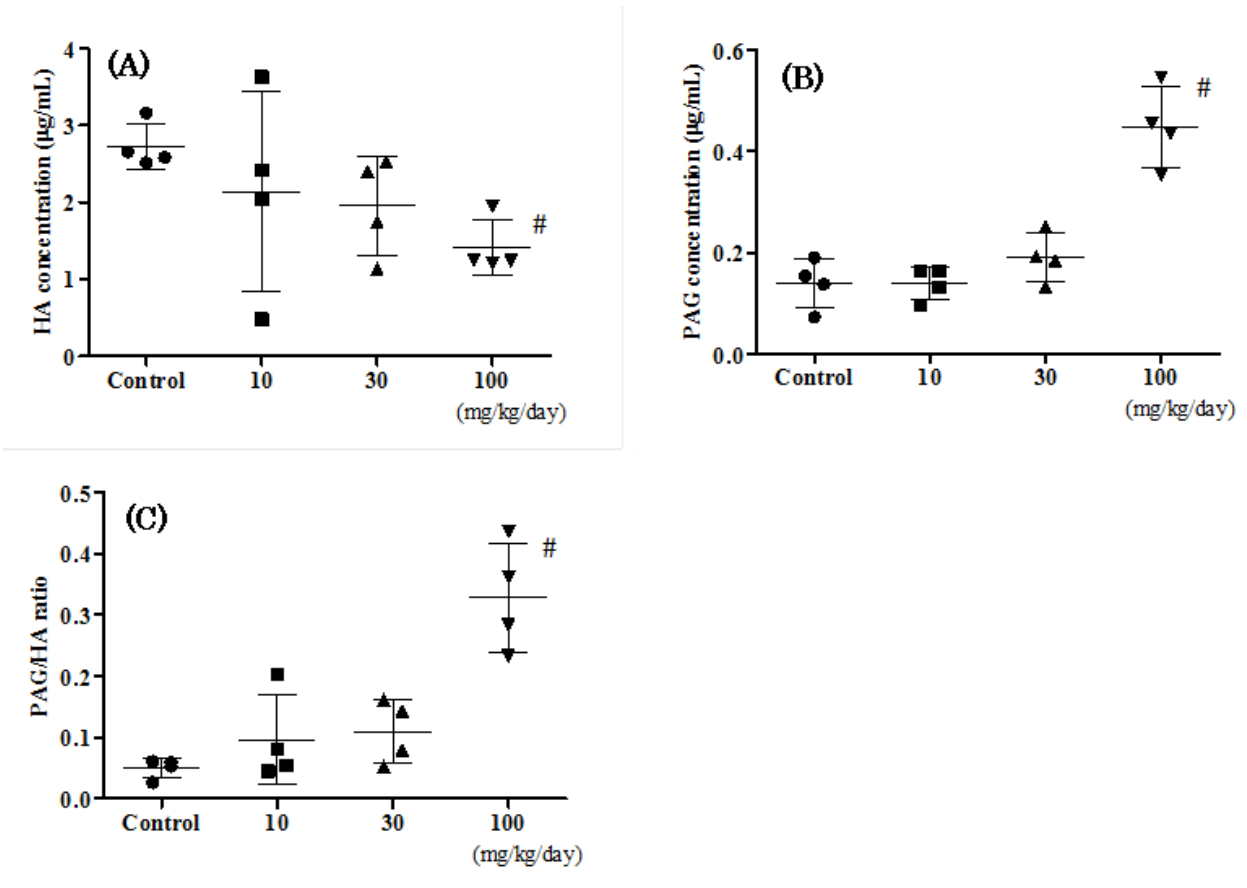
Statistical analysis was performed using Williams test; significance denoted as #

p<0.025 from control.

Figure 6. Plasma concentrations of HA and PAG and concentration ratio of PAG to HA at different time points (9:00 am, 1:00 pm and 5:00pm) 5 and 3 days prior to administration.

Figure 7. Transition of the plasma concentrations of HA and PAG and concentration

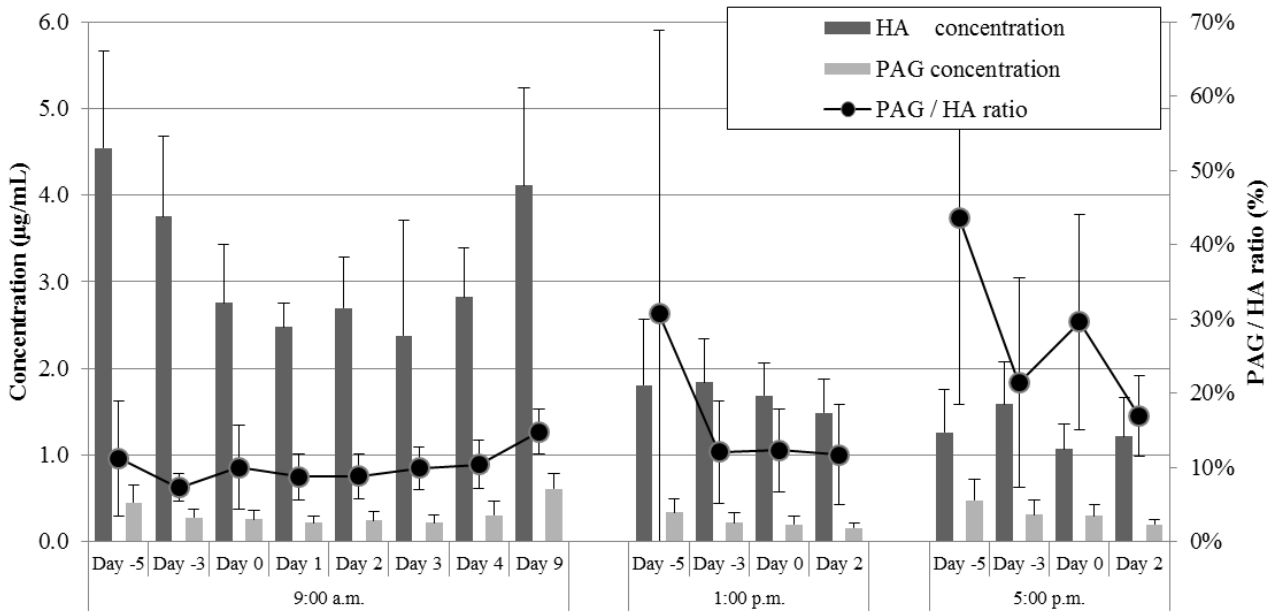ratio of PAG to HA in no intervention control group.
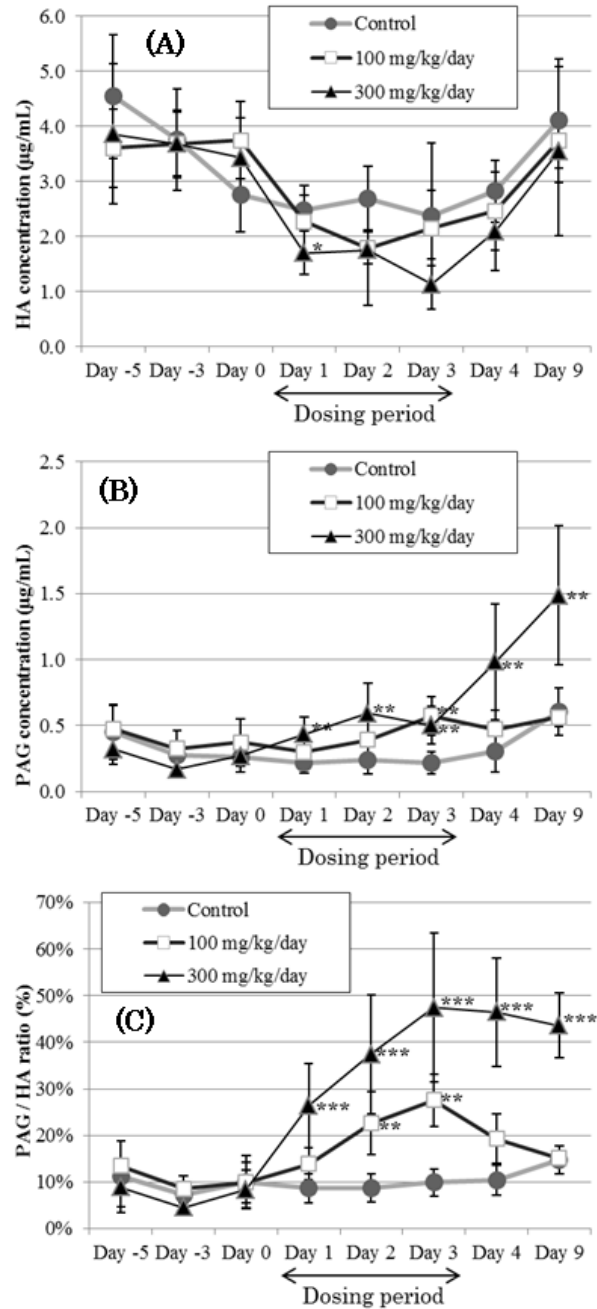
Figure 8. Plasma concentrations of HA (A) and PAG (B) and concentration ratio of

PAG to HA (C) before, during and after amiodarone administration.

Statistical analysis was performed using Dunnett multiple comparison test;

significance denoted as *p<0.05, **p<0.01, ***p<0.001 from control.

Figure 9. Metabolic pathway of L-phenylalanine

Figure 9. Metabolic pathway of L-phenylalanine
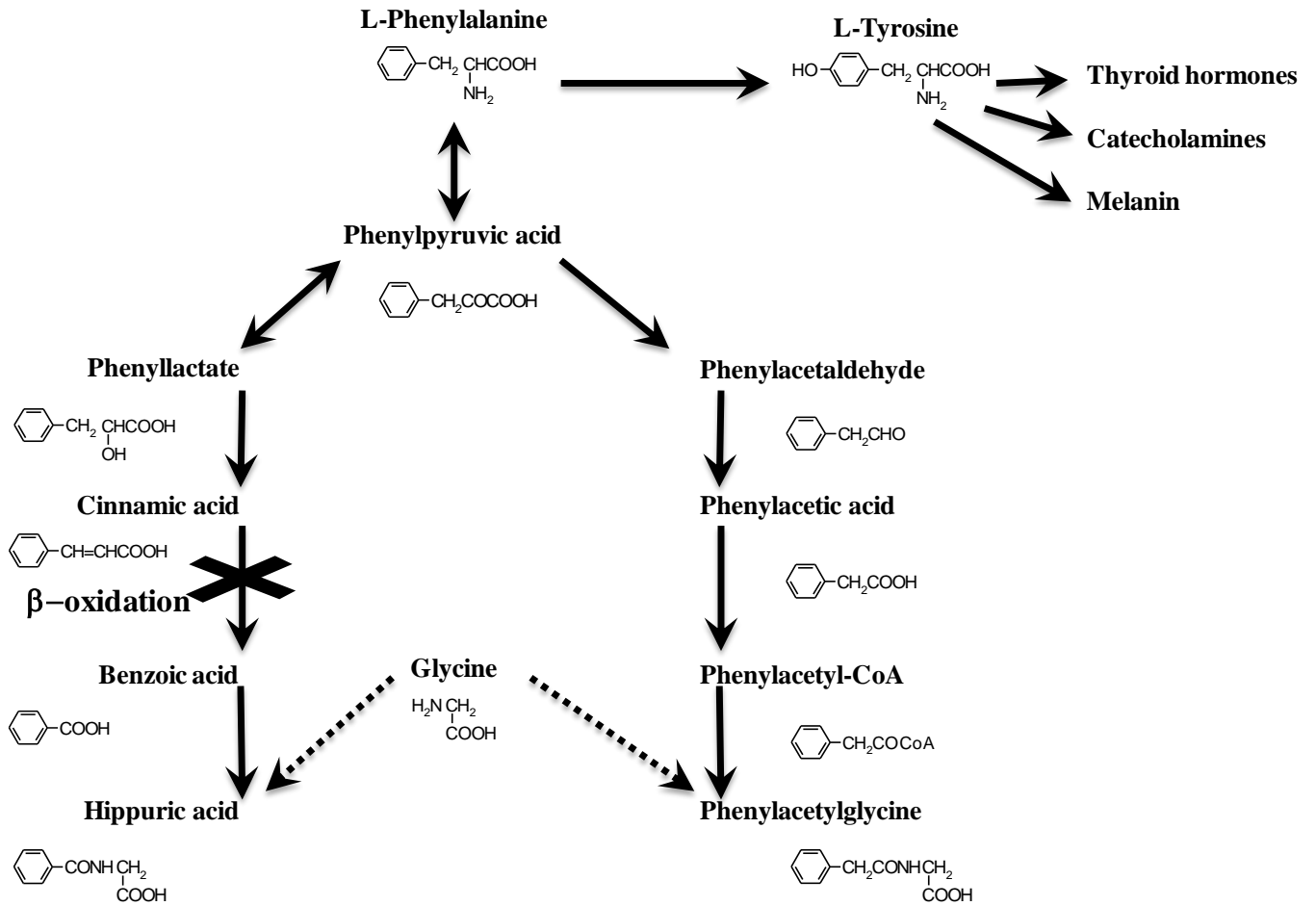
Chapter 2

Method development and validation for simultaneous quantitation of

endogenous hippuric acid and phenylacetylglycine in rat urine using liquid

chromatography coupled with electrospray ionization tandem mass

spectrometry

Abstract

Urinary hippuric acid (HA) and phenylacetylglycine (PAG) are biomarker candidates for drug-induced phospholipidosis (PLD). To confirm their utility in preclinical and clinical settings, it is essential to develop and validate their quantification method in advance. In this chapter, I have applied liquid chromatography-tandem mass spectrometry (LC/MS/MS) for simultaneous quantification of HA and PAG in rat urine, and matrix based ion suppression was assessed by post-column infusion assay. Effective sample dilution reduced matrix effect of urine to be negligible level and calibration curves showed good correlation between those in urine diluent and buffer alone. Reliability of this assay was confirmed by the assessments for intra- and inter-day precisions and accuracies of quality control samples. The method was applied to rat urine after multiple oral administrations of PLD-inducing drugs, and the changes in HA and PAG concentrations and their ratio were successfully detected. This assay would be useful tool for monitoring PLD in toxicological studies by non-invasive sampling.

Introduction

Phospholipidosis (PLD) is a lysosomal storage disorder to accumulate excessive amounts of phospholipids within diverse cell types (Drenckhahn et al., 1983, Farrell, 2002, Ploemen et al., 2004, Rudmann et al., 2004) and then organs/tissues affected by PLD exhibit histopathological changes and inflammatory reactions. The primary characteristic of PLD is cytoplasmic vacuoles observed by standard histopathological examination, but the authentic morphological hallmark of PLD is the appearance of multilamellar bodies under electron microscope finding. Since lysosomes are organelle responsible for metabolizing waste materials to be excreted, the substances which are normally broken down and excreted would be trapped inside the cells under PLD.

Risk for PLD induction is one of significant concerns in drug development, as it is called drug-induced PLD (DIPL); because more than 50 cationic amphiphilc drugs (CADs), including antidepressants, antianginal, antimalarial, and cholesterol-lowering agents, have been reported to induce PLD not only in animals but also in humans (Lüllmann et al., 1978, Halliwell, 1997, Reasor, 1989). DIPL and its progress are difficult to monitor due to invasive nature of tissue samples acquisition and it is not possible to predict which tissues will be affected. In most cases, risk of DIPL has been first identified in histopathological examination, as a part of general toxicity studies at late discovery stage. To select lead and develop candidate compounds without PLD concern at earlier stages, readily accessible biomarker is preferred for routine assessment. Vacuolated lymphocyte in the peripheral blood is useful screen for the

detection of PLD (Drenckhahn et al., 1976), but it requires histopathological skills for quantification. Biochemical index has long been explored and bis(monoglycero)phosphate (BMP) and phenylacetylglycine (PAG) were proposed as potential biomarkers for PLD (Mortuza et al., 2003, Nicholls et al., 2000). The specificity and mechanistic relevance of these biomarkers with DIPL have been explored (Delaney et al., 2004, Mesens et al., 2012), but there still remain some limitation in applying them as authentic DIPL markers. I have identified dose-dependent increase of PAG and concomitant decrease of hippuric acid (HA) in urine and plasma of CADs-treated rats by ^{1}H-NMR analysis. PAG, HA and PAG/HA ratio was well correlated with histopathological changes in PLD in rats. Phenylalanine is known to be a precursor for both HA and PAG, its two major metabolic alterations, such as inhibition of beta-oxidation at phenylalanine to HA pathway by PLD-inducing drugs and concomitant acceleration of a compensation pathway to PAG, are considered to be underlying mechanism for the change in PAG to HA ratio. Taking reproducibility of data and convenience for sampling into consideration, the ratio of PAG to HA in plasma was validated further to be practical marker in monitoring drug-induced PLD in rats. On the other hand, their application to routine measurement of urinary sample is still needs some optimization.

In general, single urinary biomarker measurements require to be presented as ratio to urinary creatinine to control for variations in urine volume excreted (Wasung et al., 2015). The simultaneous measurement for PAG to HA ratio might enable us to skip normalization process, because ratio to urinary creatinine for each metabolite can be

compensated in the calculation. On the other hand, the degree of accuracy in absolute quantification of each metabolite is still remaining as technical issue even in PAG to HA ratio measurement. Since coexisting substances interfere with urinary metabolite to hamper sensitivity and selectivity, pre-analytical sample processing needs to be incorporated into analytical procedure. Dilution would be preferred rather than extraction, because recovery rate needs to be argued in any of extraction procedures. Therefore, selection of appropriate matrix for sample and standard dilution would also be important in establishing reliable method.

There are several publications to quantify HA and other metabolites with various separation and detection procedures (Laryea et al., 2010, Moein et al., 2014, Remane et al., 2015) . On the other hand, only a few publications are reported for quantification of PAG in biofluids (Stanislaus et al., 2012) and there are no reliable simultaneous quantification procedure for HA and PAG. In this chapter, I describe development of a method for simultaneous quantification of rat urinary HA and PAG as potential biomarkers for DIPL using high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS). Degree of matrix based ion suppression and linearity of calibration curve were assessed in addition to robustness and reproducibility. The method was also validated with representative CADs known to induce PLD.

Materials and Methods

Regents

Hippuric acid (HA) and phenylacetylglycine (PAG) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) as reference standards. Hippuric acid-*d5* (HA-*d5*) was purchased from C/D/N ISOTOPES (Quebec, Canada) and phenylacetylglycine-*d4* (PAG-*d4*) was prepared in house as internal standards (ISs). Amiodarone, imipramine and tamoxifen were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, and regent grade formic acid, ammonium formate and ammonium acetate were obtained from Wako. All other solvents with the highest purity grades were purchased from commercial suppliers and used without further processing.

Animals

Five weeks old Crl: CD (SD) rats were purchased from Charles River Japan, Inc. (Tokyo, Japan). The animals were individually housed in metal cages in a clean booth and were allowed free access to tap water and a powdered laboratory diet (CE-2, CLEA Japan, Inc., Tokyo, Japan). The racks were placed in an animal room under the following conditions: temperature of 20-26 °C, a relative humidity of 40-70%, air exchange at 8-25 times/hour and a 12-hour light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.). After 7 days acclimation period, animals were randomly assigned into control and treatment groups based on body weight. All the procedures in animal

handling are assessed and approved by Animal Care and Use Committee in Takeda Pharmaceutical Company Limited.

Drug administration and urine sample collection

Four male rats (6 weeks old) were used for each dosing group. All test compounds were suspended in 0.5 w/v % methylcellulose solutions, and the dosing suspension was administered in the morning into the stomach of rats via catheter. The vehicle was also administered to control rats in the same manner. The volume administered to each animal, 10 mL/kg for each dosage level, was adjusted based on the body weight on the first day before dosing.

The test compounds were administered once daily for 7 consecutive days with amiodorone (300 mg/kg/day), chloroquine (75 mg/kg/day), tamoxifen (100 mg/kg/day), quinacrine (60 mg/kg/day), perhexiline (200 mg/kg/day) or imipramine (100 mg/kg/day). After the final administration, the urine samples were collected for 4 hours during the daytime and stored frozen at -80 °C until analysis.

Preparation of standard solutions

HA and PAG stock solutions, containing 10 mg/mL HA or 1 mg/mL PAG in acetonitrile/water (1:1, v/v), were mixed and serially diluted in acetonitrile/water (1:1, v/v) to prepare standard solution ranging from 50 to 5000 µg/mL for HA and 5 to 500 µg/mL for PAG. PAG stock solution was also used as 1000 µg/mL standard solution. A mixture of 1000 µg/mL HA-*d5* and PAG-*d4* working solution for IS was prepared in

acetonitrile/water (1:1, v/v).

Sample preparation

Twenty micro liters of 6 individual rat urine for matrix-based calibration standard or 10 mmol/L ammonium acetate buffer for buffer-based calibration standard were mixed with 10 µL of the IS solution, 20 µL of each standard solution (in the case of calibrators), or water/acetonitrile (1:1, v/v, unspiked urine samples), and diluted with 1 mL of water/acetonitrile (1:1, v/v). After mixing and centrifugation, 20 µL of the supernatant was further diluted with a 1 mL of a mixture of mobile phases (MP-A/MP-B, 95:5, v/v). The diluted solution was injected into a LC/MS/MS system.

For preparing quality control samples (QCs), the initial control rat urine sample determined the concentration from the buffer-based calibration curve was qualified as QC-I. Two hundred forty micro liter and 60 µL of a mixture of 5000 µg/mL of HA and 500 µg/mL of PAG solution was evaporated under a stream of nitrogen gas and the residue was dissolved in 600µL of QC-I to provide the QC-H (QC-I + 2000 µg/mL for HA, QC-I + 200 µg/mL for PAG) and QC-M (QC-I + 500 µg/mL for HA, QC-I + 50 µg/mL for PAG), respectively. The QC-H sample was diluted 20-fold in 10 mmol/L ammonium acetate solution to prepare QC-L ((QC-I + 2000)/20 µg/mL for HA, (QC-I + 200)/20 µg/mL for PAG). To investigate matrix effect using a post column infusion system, three different dilution rate urine samples and no matrix sample were prepared. Twenty micro liters of 6 individual rat urine or 10 mmol/L ammonium acetate solution were diluted according to the sample preparation method described above except for addition

of standard solution and IS solution to obtain c.a. 2700-fold diluted urine and its matrix-free sample. Other individual aliquots of 20 µL of the rat urine were diluted with 80 or 980 µL of mixture of mobile phases (MP-A/MP-B, 95:5, v/v) to prepare 5 and 50-fold diluted urine samples, respectively.

Mass spectral instrumentation

The quantification of the analytes was performed by electrospray LC/MS/MS in the selected reaction monitoring (SRM) mode on a API3000 tandem quadrupole mass spectrometer (MDS SCIEX, ON, Canada) with a turbo ion spray configuration, operated in the positive ionization mode, with Analyst controlling software. Source conditions were typically as follows: ion spray voltage 4200 V, turbo probe temperature 450 ˚C, unit resolution on Q1 and Q3. Heated gas (air), nebulizer gas (air) and curtain gas (N2) flows were set to 7, 1.04 and 0.95 L/minutes, respectively. Multipliers were set to 2000 V, and the dwell times for HA, PAG and their corresponding ISs HA-*d5* and PAG-*d4* were 150 ms. For the SRM analysis, the following ion transitions were obtained: HA mass-to-charge ratio value (m/z) 180 → 105, PAG m/z 194 → 167, HA-*d5* m/z 185 → 110 and PAG-*d4* m/z 198 → 93. Sensitivity was optimized for each compound by varying declustering potential, focusing potential and collision energy in the SRM mode and maximizing ion intensity. For our instrument, at a collision cell pressure of 12 bit (N2), declustering potential, focusing potential and collision energy were typically as follows: HA 21 V, 150 V, 17 V, PAG 26 V, 120 V, 29 V, HA-*d5* 21 V, 100 V, 19 V, and PAG-*d4* 26 V, 150 V, 25 V.

HPLC separation method

For the quantitative method, a liquid chromatography system was composed of a Shimadzu (Kyoto, Japan) SIL-HTc autosampler and LC-10ADvp pump system. The analytical column was a Chemicals Evaluation and Research Institute (Tokyo, Japan) L-Column ODS (2.1 × 50 mm, 5 μm) at a flow rate of 0.2 mL/minuets at 40°C. Mobile phase A (MP-A) consisted of 10 mmol/L ammonium formate/formic acid (500:1, v/v) and mobile phase B (MP-B) consisted of acetonitrile/formic acid (500:1, v/v). The gradient started with 5% MP-B and linearly increased to 60% within 3 minutes, then increased to 80% for following 0.2 minutes. This condition was kept from 3.2 to 5 minutes and then it was cycled back to the initial conditions over 0.1 minutes. The total analysis time was 10 minutes. Final chromatographic retention times for HA, PAG, HA-*d5* and PAG-*d4* were between 3.5 and 4 minutes. To confirm the specificity of the assay, additional two columns of different separation mode were also used. A CAPCELL PAK UG120 Ph column (2.0 × 50 mm, 5μm, Shiseido, Co. Ltd., Tokyo, Japan) and CAPCELL PAK UG80 NH2 column (2.0 × 50 mm 5μm, Shiseido) were used under the same LC gradient condition described above.

Post-column infusion method

The purpose of the post-column infusion examination is to verify the validity of the quantification of endogenous components in urine by using buffer-based caliburation curves. A post column infusion system was used on the quantitative analysis method.

Ten micro liters of different dilution rate urine samples and matrix-free solvent were injected into the LC/MS/MS system. A mixture of 1 μg/mL HA-d5 and PAG-d4 in acetonitrile/water (1:1, v/v) solution were continuous infused with a Harvard Pump 11 syringe pump (South Natic, MA, USA) at a flow rate of 10 μL/minutes between the analytical column and the MS source.

Quantification

Peaks on the chromatograms, detected using the SRM mode, were identified based on the retention time and the mass number of the monitoring ions. The concentrations of HA and PAG were determined from the peak area ratios of the analytes to each IS using the internal standard method. The calibration curve was obtained by a 1/C weighted least-squares linear regression on the ratios of the peak areas of the analytes to those of the IS versus the theoretical concentrations of the analytes in the buffer based calibration standards:

$$Y = a \times Ctheor + b,$$

where Y, Ctheor, a, and b are the peak area ratio, the spiked concentration of the analyte, the slope, and the Y-intercept, respectively. The concentrations of HA and PAG in rat urine (Cobs) were calculated from the equation for the calibration curve of each analyte:

$$Cobs = (Y - b) / a$$

The linearity of the method was investigated using the sample preparation procedure described above for HA and PAG. Buffer-based calibration curves (eight points) were

prepared in concentration ranges of 50-5000 µg/mL for HA and 5-1000 µg/mL for PAG. Precision, accuracy and stability were determined by running standard QCs at four different concentrations covering the calibration range, on the same (intra-day) and on different days (inter-day variability).

Results

Specificity of SRM chromatogram targeting HA and PAG in urine

At first, I have checked whether there were any other peaks overlapping with HA and PAG in LC/MS/MS chromatogram of rat urine, which makes quantitative analysis difficult. Five individual rat urine samples were analyzed by LC/MS/MS system equipping three columns of different functional groups (octadecylsilyl, phenyl and amino). In each SRM chromatogram targeting HA and PAG, there was no notable peak except for the peaks corresponding to HA and PAG. These findings strongly indicated that the peaks detected at SRM channel selected for HA and PAG were free of interfering components.

Assessments of matrix effect

Matrix-dependent signal suppression or enhancement (matrix effect) is a major drawback in quantitative analysis by LC/MS/MS. In this study, the extent of matrix effect and assay reliability were assessed by following two experiments: (1) monitoring post column infused HA and PAG at corresponding SRM channel with the subsequent injection of serially diluted urine or buffer, (2) comparison of the slopes of calibration curves in the presence or absence of urine.

In the first experiment, deuterium-labeled HA and PAG (HA-*d5* and PAG-*d4*, Figure 10) were used as tracers for SRM to differentiate signals from those of endogenous HA and PAG in urinary sample. HA-*d5* and PAG-*d4* solution were continuously infused, and

then diluents of rat urine (5-, 50-, 2700-fold) or buffer alone were injected into the LC/MS/MS system. SRM channels selected were m/z 185 → 110 for HA-*d5* and m/z 198 → 93 for PAG-*d4*. Figure 11 shows schematic of the post column infusion experimental set up. Ion suppression which was attributed to urinary matrix was estimated by the comparison of SRM chromatograms; and typical post column infusion chromatograms for each analyte were shown in Figure 12. At the retention time of approximately 3.5-4.0 minutes, corresponding to those of HA and PAG, significant signal suppression was observed by the injections of 5- and 50-fold urinary diluents. On the other hand, the chromatogram of 2700-fold diluted urine was almost identical to that of buffer alone except for earlier (approximately 1 min) timing. The integrated ion intensity ratios for HA-*d5* and PAG-*d4* within 3.5-4.0 minutes window were 99.7% and 101.7% (against buffer alone) for the 2700-fold diluted urine samples, whereas the 50-fold and 5-fold diluted samples showed 58.3% and 32.5% for HA-*d5* and 68.0% and 30.2% for PAG-*d4*, respectively. Urines from six individual rats showed same trend; therefore, ion suppression by matrix effect of urine would be able to be excluded in this sample preparation procedure with an appropriate dilution.

Next, I have assessed the interference with slopes of calibration curves by urinary matrix. Calibration curves were obtained with matrix (2700-fold diluted urine)-based and buffer-based standards as a pair for six individual rats and serially diluted standards (50-5000 µg/mL for HA and 5-1000 µg/mL for PAG). As shown in Table 4, the slopes for HA and PAG thus obtained were almost identical regardless of the presence of matrix. Y-intercepts of matrix-based calibration curves for HA and PAG were higher

than those of buffer-based calibration curves due to the presence of the endogenous HA and PAG. These endogenous HA or PAG were calculated by dividing Y-intercept by slope, and compared with the quantified value calculated from the buffer-based calibration curve (Table 5). The concentrations thus calculated matched well in each pairs; therefore, buffer-based calibration standards can be used to determine the quantity of HA and PAG in rat urine instead of matrix-based calibrations.

Linear range and accuracy

The linearity of buffer-based calibration curves were also assessed within the ranges 50-5000 µg/mL for HA and 5-1000 µg/mL for PAG. The precision and accuracy of the data for intra- and inter-day variability were evaluated using quality control samples (QCs) prepared with n=5 at four different concentration levels, covering the calibration ranges used for HA and PAG. Back-calculated HA and PAG concentrations of the quality control samples assayed in three separate runs are shown in Table 6. The intra-day precision (coefficient of variation, C.V.) and accuracy (relative error, R.E.) were between 0.8 to 1.9% and -5.3 to -0.5% for HA and between 1.3 to 2.7% and -6.1 to -3.7% for PAG, respectively. Inter-day precision and accuracy ranged between 0.8 to 2.2% (C.V.) and -4.8 to -1.9% (R.E.) for HA and between 0.6 to 3.0% (C.V.) and -4.2 to -3.1% (R.E.) for PAG, respectively. Therefore, the assay was confirmed to be very robust and reproducible.

Stability assessments

The stability of HA and PAG in stock solution, biological matrix, and analytes for LC/MS/MS analysis were evaluated. Stock solutions and working solutions of HA, PAG, HA-*d5* and PAG-*d4* in water/acetonitrile (1:1, v/v) were confirmed to be stable for 24 h at room temperature and for 45 days at 5 ˚C. The analytes were stable for 49 h in the glass assay vial set in the autosampler at 10 ˚C. In rat urine, HA and PAG were stable for 24 h in an ice-water bath and for 60 days at -80 ˚C. At least three freeze and thaw cycles did not show any interference with stability of HA and PAG.

Method comparison

Since HA is one of the major endogenous components of urine, there are several quantitative analytical methods for HA with various separation and detection procedures of HPLC-DAD, GC/MS and LC/MSMS (Laryea et al., 2010, Moein et al., 2014, Remane et al., 2015). GC/MS analysis needs time consuming sample derivertization process and HPLC-DAD method often has a problem of lack of specificity compared with MS detection method. Regarding LC/MS/MS analysis, the critical issue for this method development is to avoid the matrix based ion surpression and to meet the situation authors adopted unique sample extraction/dilution procedures. However there still have problems concerning analytical robustness or validity of using alternative blank matrix (Laryea et al., 2010, Moein et al., 2014). My analytical procedure in this report precisely demonstrated the assay validity by specificity confirmation, matrix effect evaluation and intra- and inter-day assay validation for HA

and PAG simultaneously.

Application to toxicology study in rats

The method was applied to diagnose PLD state in the toxicity study. After multiple oral administration of PLD inducing drugs to rats, the concentrations of HA and PAG in urine were determined by the described method (Figure 13). The mean urinary concentrations of HA treated with amiodarone, chloroquine, tamoxifen, quinacrine, perhexiline and imipramine were 894.9, 1054.7, 800.3, 869.3, 1207.2 and 888.9 µg/mL, respectively, which were slightly lower than that of control samples (1401.3 µg/mL). The concentrations of PAG treated with amiodarone (765.9 µg/mL) was significantly higher than that of control samples (92.5 µg/mL), and PAG level treated with chloroquine, tamoxifen, quinacrine, perhexiline and imipramine were 89.2, 292.6, 188.0, 132.0 and 291.9 µg/mL. Although the concentrations of HA and PAG after drug treatment except for PAG in amiodarone group were not significant compared with control group, The increase trend for HA and decrease trend for PAG were observed. The proportions of the PAG to HA in amiodarone group were also significantly higher than that of control. We assumed its reliability might be hampered by very large inter-individual variability in both urinary PAG and HA concentrations. As is the case in other urinary biomarkers, normalization with creatinine should be required.

Discussion

There have been two hypotheses to explain the mechanisms underlying DIPL; the first one is direct binding of CADs to phospholipids to form indigestible complex by lysosome (Halliwell, 1997) and the other one is inhibition of phospholipase activity by the formation of lamellar body in lysosome (Reasor and Kacew, 2001). They were based on possible interaction of CADs to phospholipid layer of the lysosome, but both of them are not sufficient to predict which metabolic pathway and tissues will be affected by DIPL. Under such circumstances, the biomarker for DIPL is still limited to the consequence of histopathological change even though it is identified to be biochemical metrics. For example, di-docosahexaenoyl (22:6)- Bis(monoacylglycerol)phosphate (di-22:6-BMP) was reported to be a reliable biomarker of DIPL that can be monitored in the plasma and urine (Baronas et al., 2007, Mesens et al., 2012, Tengstrand et al., 2010); however, BMP is a lysosomal phospholipid which is practically identified to increase in the damaged tissues of animals and humans with DIPL and Niemann–Pick type C (NPC) disease (Besley and Elleder, 1986, Harder et al., 1984, Rouser et al., 1968, Tengstrand et al., 2010). Recently, I have identified that PAG, HA and their ratio in plasma and urine can be biomarker for DIPL and implicated their possible link to the inhibition of β-oxidization by metabolomic approach (Kamiguchi et al., 2016). Since phenylalanine is the precursor for PAG and HA, catabolism of phenylalanine might be perturbed by CADs as discussed previously (Kamiguchi et al., 2016).

The plasma PAG/HA showed good correlation with CAD-induced PLD, however, urinary sample showed large inter-individual variability in the previous study

(Kamiguchi et al., 2016). Normalization with creatinine might be one possible solution, but the degree of accuracy in absolute quantification of each metabolite is another technical issue. In this study, we have identified that coexisting substances in urine interfere with PAG and HA to hamper their sensitivity and selectivity by post-column infusion SRM chromatograms (Figure 12). Pre-analytical sample processing might need to be incorporated into analytical procedure but dilution with the buffer successfully reduced sample ion suppression to negligible level. The calibration curve generated with buffer-based dilution series showed good linearity with those from matrix (urine)-based dilution. The robustness and reproducibility were also confirmed by intra- and inter-day precision and accuracy tests. Finally, the method was successfully applied to rat urine after multiple oral administrations of drugs which induce PLD and the PAG to HA ratio were clearly higher than that of control (Figure 13). From the viewpoint of animal welfare, screening for DIPL risk with spot urine is preferred because it can be set as a part of routine pharmacology study but not independent toxicology test.

In silico analyses and in vitro assays were also proposed to detect or screen potential phospholipogenic compounds (Chatman et al., 2009). As a whole, standardized strategy for risk management of DIPL has long been highly desired but uncertainness of the pathological significance of DIPL hampers its establishment (Chatman et al., 2009). In addition to it, only a few compounds such as amiodarone, gentamicin, chloroquine, 4,4-diethylaminoethoxyhexestrol and telithromycin has been reported to cause concurrent toxicity with PLD in humans (Chatman et al., 2009); and, this makes the situation to be highly complicated. Therefore, disease pathway analyses of DIPL with

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these toxic compounds on humans would still be indispensable; and biomarkers selected would be keys for them. It might not be absolutely consistent in experimental condition and sample matrix but plasma PAG to HA ratio increased prior to di-22:6-BMP increment in urine of amiodarone treated rat (Liu et al., 2014). Since the focus of this study is to establish quantitation method and its validation, time course study with urinary sample has not been conducted. Further studies with combinatory use of urinary PAG to HA ratio and di-22:6-BMP as biomarkers would enables us to understand DIPL process further; and, would be base for understanding concurrent toxicity with PLD in humans.

Tables and Figures

Table 4. Comparison of slopes for HA (A) and PAG (B) between the buffer-based standard curves and matrix-based standard curves obtained from six individual rat plasma.

(A)

| No. | Slope | | b/a |
	Buffer-based standard (a)	Matrix- based standard (b)	(%)
1	0.0021062	0.0021322	101.2
2	0.0021062	0.0020863	99.1
3	0.0021062	0.0022252	105.6
4	0.0021326	0.0021068	98.8
5	0.0021326	0.0020765	97.4
6	0.0021326	0.0021011	98.5
Mean		0.0021214	
S.D.		0.0000543	
C.V. (%)		2.6	

(B)

| No. | Slope | | b/a |
	Buffer-based standard (a)	Matrix- based standard (b)	(%)
1	0.0020456	0.0020753	101.5
2	0.0020456	0.0020839	101.9
3	0.0020456	0.0020796	101.7
4	0.0020331	0.0020599	101.3
5	0.0020331	0.0020785	102.2
6	0.0020331	0.0021024	103.4
Mean		0.0020799	
S.D.		0.0000138	
C.V. (%)		0.7	

Table 5. Comparison of the concentrations of endogenous HA (A) and PAG (B),

calculated from buffer-based standard curves and matrix-based standard curves.

(A)

| No. | Concentration (μg/mL) | | b/a |
	Buffer-based standards (a)	Matrix-based standards (b)	(%)
1	2310	2240	97.0
2	1990	2040	102.5
3	2210	2060	93.2
4	2000	2040	102.0
5	1970	2050	104.1
6	2020	2030	100.5

(B)

| No. | Concentration (μg/mL) | | b/a |
	Buffer-based standards (a)	Matrix-based standards (b)	(%)
1	90.9	90.3	99.3
2	117	116	99.1
3	76.0	78.1	102.8
4	111	110	99.1
5	108	106	98.1
6	109	105	96.3

Table 6. Back-calculated HA and PAG concentrations of quality control samples assayed in three separate batch runs.

		Concentration (μg/mL)											
		Day 1 (Intra-day)				Day 2				Day 3			
No.	Nominal	C_{HA} (QC-I)	4140 (QC-L)	2640 (QC-M)	4140 (QC-H)	C_{HA} (QC-I)	4160 (QC-L)	2660 (QC-M)	4160 (QC-H)	C_{HA} (QC-I)	4080 (QC-L)	2580 (QC-M)	4080 (QC-H)
1		2180	4040	2550	3810	2160	4080	2500	3960	2100	4140	2620	4040
2		2130	4110	2590	3960	2200	3920	2540	3910	2010	4130	2590	3950
3	Observed	2170	4120	2590	3900	2110	4090	2460	3890	2090	4200	2470	3950
4		2070	4150	2570	3940	2180	3700	2620	3820	2160	4130	2540	3970
5		2140	4190	2570	3970	2170	3950	2490	3940	2060	3810	2510	3890
Mean (n=5)		2140	4120	2570	3920	2160	3950	2520	3900	2080	4080	2550	3960
S.D.		40	60	20	70	30	160	60	50	60	150	60	50
C.V.(%)		1.9	1.5	0.8	1.8	1.4	4.1	2.4	1.3	2.9	3.7	2.4	1.3
R.E.(%)		-	-0.5	-2.7	-5.3	-	-5.0	-5.3	-6.3	-	0.0	-1.2	-2.9

Inter-day		Concentration (μg/mL)			
(3 days)	Nominal	C_{HA} (QC-I)	4130 (QC-L)	2630 (QC-M)	4130 (QC-H)
Mean (n=3)		2130	4050	2550	3930
S.D.		40	90	30	30
C.V. (%)		1.9	2.2	1.2	0.8
R.E. (%)		-	-1.9	-3.0	-4.8

QC-L values were corrected with dilution factor, 20.

		Concentration (μg/mL)											
		Day 1 (Intra-day)				Day 2				Day 3			
No.	Nominal	C_{PAG} (QC-I)	310 (QC-L)	161 (QC-M)	310 (QC-H)	C_{PAG} (QC-I)	308 (QC-L)	159 (QC-M)	308 (QC-H)	C_{PAG} (QC-I)	308 (QC-L)	159 (QC-M)	308 (QC-H)
1		112	285	153	301	106	306	154	294	110	269	148	292
2		115	300	154	300	107	305	156	290	110	287	158	311
3	Observed	107	285	158	296	116	307	155	291	107	291	149	298
4		110	287	151	301	105	300	154	297	109	316	155	298
5		109	297	157	291	112	312	159	297	107	289	158	316
Mean (n=5)		111	291	155	298	109	306	156	294	109	290	154	303
S.D.		3	7	3	4	5	4	2	3	2	17	5	10
C.V.(%)		2.7	2.4	1.9	1.3	4.6	1.3	1.3	1.0	1.8	5.9	3.2	3.3
R.E.(%)		-	-6.1	-3.7	-3.9	-	-0.6	-1.9	-4.5	-	-5.8	-3.1	-1.6

Inter-day		Concentration (μg/mL)			
(3 days)	Nominal	C_{PAG} (QC-I)	309 (QC-L)	160 (QC-M)	309 (QC-H)
Mean (n=3)		110	296	155	298
S.D.		1	9	1	5
C.V. (%)		0.9	3.0	0.6	1.7
R.E. (%)		-	-4.2	-3.1	-3.6

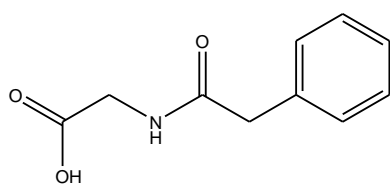QC-L values were corrected with dilution factor, 20.

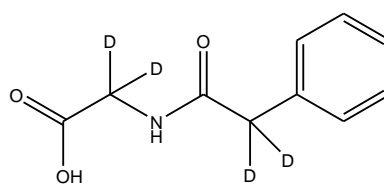Figure 10. Chemical structures of HA, PAG, HA-*d*5 and PAG-*d*4
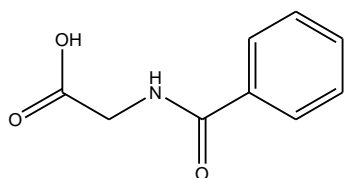
Hippuric acid
(HA)
MW 179
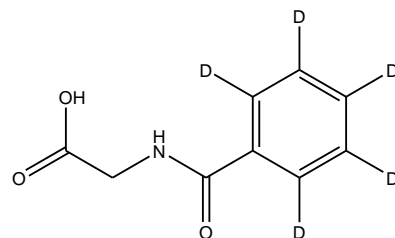
Hippuric acid-d5
(HA-*d5*)
MW 184

Phenylacetylglycine
(PAG)
MW 193

Phenylacetylglycine-d4
(PAG-*d4*)
MW 197

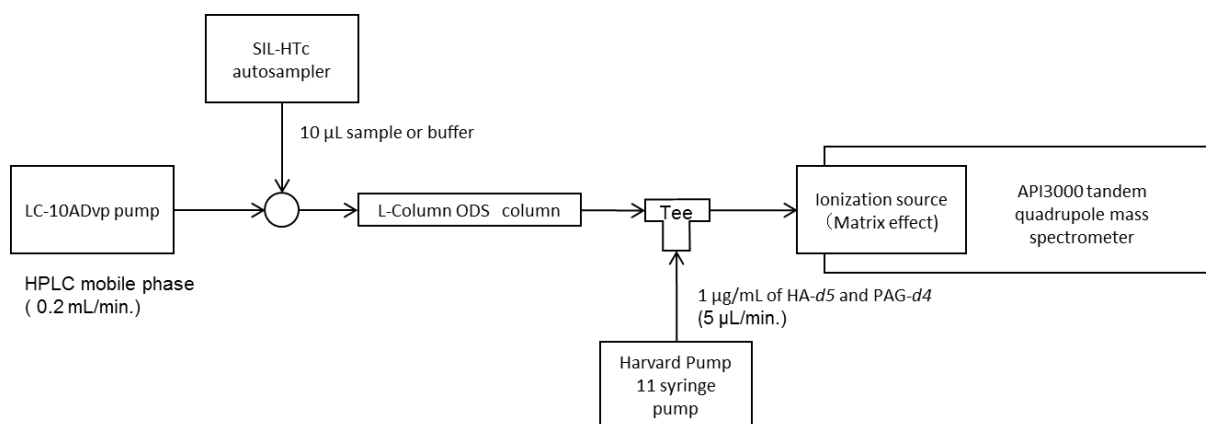**Figure 11. Scheme of the post-column infusion instrumentation**

Figure 12. Post-column infusion SRM chromatograms for HA-d5 (A), and PAG-d4 (B) obtained after injecting diluted rat urine and buffer samples. These chromatograms are plotted the mean intensity of six individual rat urine samples.
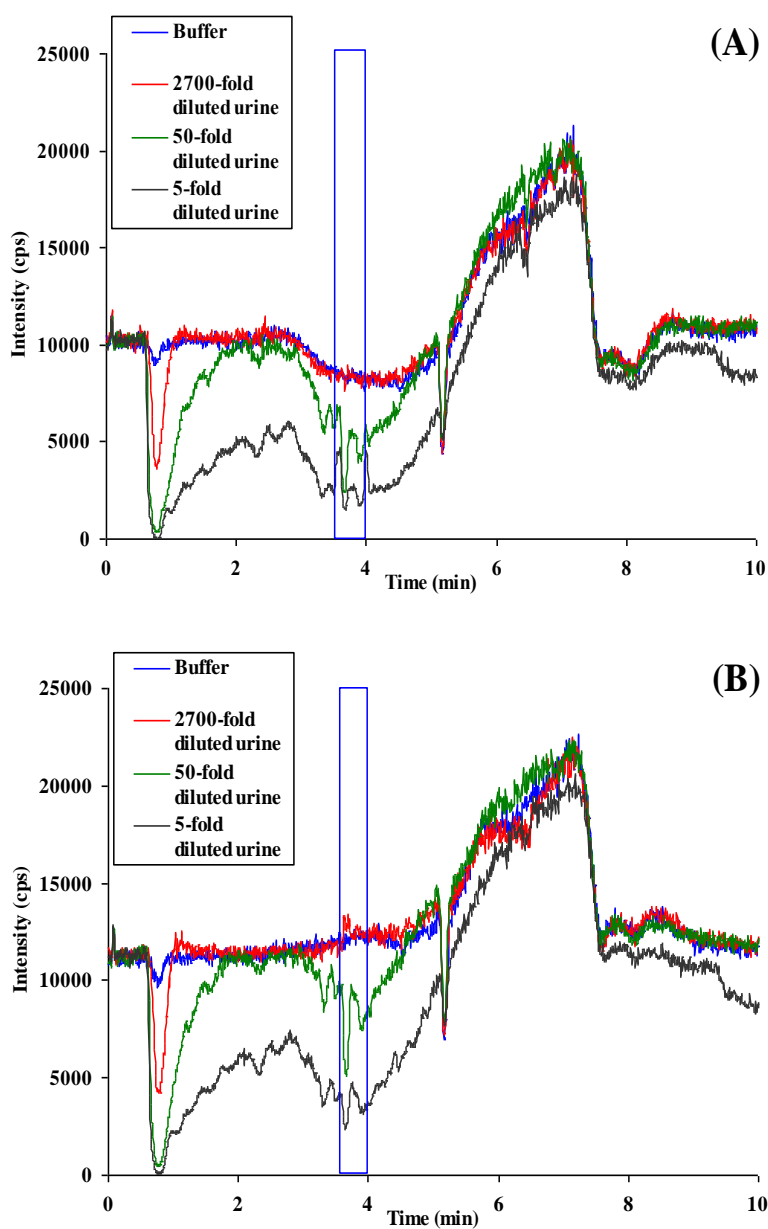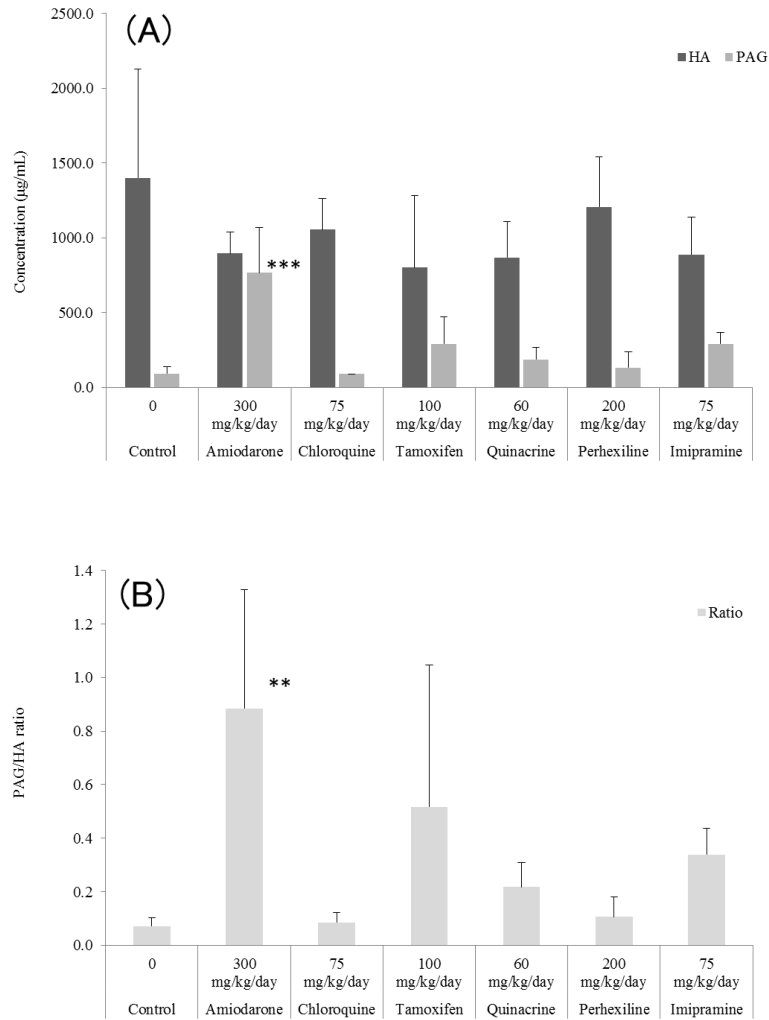
Figure 13. Urinary concentrations of HA and PAG (A) and proportions of the PAG to HA (B) after multiple administrations of PLD inducing drugs.

Statistical analysis was performed using Dunnett's multiple comparison test; significance denoted as **p<0.01, ***p<0.001 from control.

General Discussion

In this thesis, I investigated the potential biomarkers in non-invasive way to monitor the status of drug-induced phopholipidosis with a metabolomics approach. In Chapter 1, the biomarker exploration study in urine and plasma of rats after administrations of various PLD inducing drugs was investigated to discover the changes in PAG to HA ratio were well correlated with histopathologic changes in DIPL. I discussed the hypothesis of the metabolic perturbation of phenylalanine as precursor component of both HA and PAG. In Chapter 2, a simultaneous quantification methods for HA and PAG in rat urine was developed and validated using LC/MS/MS and discussed the advantage of combination of the two metabolic components to use as DIPL biomarker in a biological aspect.

DIPL is one of significant concerns for medication not only in drug research and development process but also in medical front. Especially, diagnosis and prognosis of DIPL at earlier stage by non-invasive method are still challenging because its mechanism and predictive biomarkers are not well elucidated. In Chapter 1, I firstly investigated the histopathological examination with well-known PLD inducing drugs of amiodarone, chloroquine, tamoxifen, quinacrine and fluoxetine. The result showed that each drug tends to induce toxicity in different tissues that is assumed the different drug distribution and molecular mechanism. One of the concepts of biomarkers from biological fluids like urine, blood and plasma is some biological signatures of pharmacological changes in a body should be reflected in systemic circulation or excretion. Metabolomics approach in this study successfully discovered HA and PAG as potential biomarkers of DIPL from rat urine and plasma. Since these endogenous

components are natively regulated for maintaining the fundamental vital processes, these levels are affected by not only the drug effect but also complex biological mechanism. I revealed the HA concentration in plasma in control rats was much higher in the morning than that in the evening that corresponds well with the feeding pattern of rats as a nocturnal animal. On the other hand, PAG level was not fluctuated compared with HA that suggested the PAG homeostasis is insusceptible to external stimulations in normal condition. It is known that both HA and PAG are catabolites of essential amino acid phenylalanine. The circadian rhythm of HA is dominantly affected by ingestion of phenylalanine as diet whereas PAG is considered to be a metabolite of minor metabolic pathway in the body. The phenylalanine catabolism is started from deamination by phenylalanine dehydrogenase to form phenylpyruvate. Phenylpyruvate metabolism is branched to eventually form phenylacetate and benzoic acid then conjugated by glycine to form PAG and HA, respectively. Between these two pathways, the conversion to phenylacetate should be the rate limiting step thus the plasma PAG level is not affected food consumption. In the other pathway, cinnamic acid that is formed by dehydration of phenyllactate is metabolized to benzoic acid via β-oxidation. The evidence that amiodarone inhibits the mitochondrial β-oxidation of fatty acids (Fromenty et al., 1990; Fromenty and Pessayre, 1995; Kaufmann et al., 2005; Spaniol et al., 2001; Waldhauser et al., 2006) indicates the decrease in HA level in plasma might be substituted the molecular mechanism of PLD formation. Further estimation may lead that the inhibition of the pathway leading to HA might cause a compensatory increase in the alternate metabolic pathway of phenylpyruvate, resulting in the increase in PAG.

HA decrease and PAG increase in the urine and plasma could be an index for inhibition of β-oxidation and they could be a potential surrogate marker for PLD.

To confirm and verify the utility of the HA and PAG as potential biomarkers, it is quite important to setup a quantitative and robust analytical procedure. However, there remains several technical issues to investigate absolute concentration of endogenous metabolites in biological fluids because there are always existed 'unknown' concentration of endogenous components themselves and other analytical coexisting substances interfere with metabolite in biological samples to hamper sensitivity and selectivity. In Chapter 2, I developed and validated a quantification method by using LC/MS/MS procedure for simultaneous quantification of HA and PAG in rat urine. The originally constructed post-column infusion procedure successfully evaluated the sample-derived analytical interference that the coexisting substances in undiluted urine drastically interfered with PAG and HA to hamper their sensitivity and selectivity. The calibration curve generated with buffer-based dilution series showed good linearity with those from matrix (urine)-based dilution. The robustness and reproducibility were also confirmed by intra- and inter-day precision and accuracy tests.

Currently there has been reported several potential candidates of non-invasive biomarkers to monitor DIPL, for example di-22:6-BMP. However, BMP is a lysosomal phospholipid which is practically identified to increase in the damaged tissues of animals and humans with DIPL and Niemann–Pick type C (NPC) disease. One of the advantages for DIPL biomarker to monitor PAG to HA ratio is that both HA and PAG are generated from the same precursor phenylalanine so that this indicator is less

affected by other external factors. The mechanism and process of drug-induced PLD are considered to be complex and might differ from one drug to another; therefore, additional studies would be required to confirm the hypothesis with metabolic flux analysis using stable isotopes and/or key enzyme inhibitors. Detailed validation, such as time-course studies with CADs other than amiodarone and concomitant monitoring of histopathological status, would also be required before practical use of the PAG/HA ratio as a toxicological index. To apply this hypothesis in the clinic, it should be confirmed whether phenylacetylglutamine, an alternate of PAG in humans and primates as described above, could be used as an index. Since the PAG/HA ratio in plasma showed good correlation with CAD-induced PLD in this study, subsequent studies on this biomarker are expected to contribute largely in predicting and understanding drug-induced PLD.

In summary, these novel, non-invasive and highly quantitative biomarkers to monitor DIPL status is critically beneficial to avoid drug derived serious toxicity to improve the quality of life for patients.

Acknowledgements

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