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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

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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 study, we 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 as rat plasma in previous report. This is the first report to quantify HA and PAG easily and accurately as potential biomarkers to monitor PLD status. This assay would be useful tool for monitoring PLD in toxicological studies by non-invasive sampling.

1. Introduction

Phospholipidosis (PLD) is a lysosomal storage disorder to accumulate excessive amounts of phospholipids within diverse cell types [1–4] 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 the 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 [5–7]. 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 [8], 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 biomark-

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ers for PLD [9,10]. The specificity and mechanistic relevance of these biomarkers with DIPL have been explored [11,12], but there still remain some limitation in applying them as authentic DIPL markers. Recently, we 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 [13]. 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 DIPL 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 [14]. 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 [15–17]. On the other hand, only a few publications are reported for quantification of PAG in biofluids [18] and there are no reliable simultaneous quantification procedure for HA and PAG. In this study we have developed 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.

2. Materials and methods

2.1. 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.

2.2. 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/h and a 12-h 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.

2.3. Drug administration and urine sample collections

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 h during the daytime and stored frozen at −80 °C until analysis.

2.4. 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).

2.5. 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 600 μL 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