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Metabolic profiling study of ketoprofen-induced toxicity using ^{1}H NMR spectroscopy coupled with multivariate analysis

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Abstract **:** ^{1}H nuclear magnetic resonance (NMR) spectroscopy of biological samples has been proven to be an effective and nondestructive approach to probe drug toxicity within an organism. In this study, ketoprofen toxicity was investigated using ^{1}H-NMR spectroscopy coupled with multivariate statistical analysis. Histopathologic test of ketoprofen-induced acute gastrointestinal damage in rats demonstrated a significant dose-dependent effect. Furthermore, principal component analysis (PCA) derived from ^{1}H-NMR spectra of urinary samples showed clear separation between the vehicle-treated control and ketoprofen-treated groups. Moreover, PCA derived from endogenous metabolite concentrations through targeted profiling revealed a dose-dependent metabolic shift between the vehicle-treated control, low-dose ketoprofen-treated (10 mg/kg body weight), and high-dose ketoprofen-treated (50 mg/kg) groups coinciding with their gastric damage scores after ketoprofen administration. The resultant metabolic profiles demonstrated that the ketoprofen-induced gastric damage exhibited energy metabolism perturbations that increased urinary levels of citrate, *cis*-aconitate, succinate, and phosphocreatine. In addition, ketoprofen administration induced an enhancement of xenobiotic activity in fatty oxidation, which caused increase levels of *N*-isovalerylglycine, adipate, phenylacetylglycine, dimethylamine, betaine, hippurate, 3-indoxylsulfate, *N*,*N*-dimethylglycine, trimethyl-*N*-oxide, and glycine. These findings demonstrate that ^{1}H-NMR–based urinary metabolic profiling can be used for noninvasive and rapid way to diagnose adverse drug effects and is suitable for explaining the possible biological pathways perturbed by nonsteroidal anti-inflammatory drug toxicity.

Keywords **:** Metabolomics; ^{1}H NMR; Multivariate analysis; Ketoprofen toxicity; Gastrointestinal damage

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INTRODUCTION

Metabolomics, the multitargeted analysis of endogenous metabolites from biological fluids, is increasingly being used as a noninvasive method for supervising pathogenesis and toxicity tests[1, 2]. In most cases, metabolomic investigations are conducted using ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy and liquid chromatography or gas chromatography/mass spectrometry. ^{1}H-NMR–based metabolomics in particular have several strengths for metabolic profiling, including observation of all high-abundance metabolites in a single "all-in-one" analysis, quantitative analysis with a high degree of reproducibility, relatively high-throughput and automated analysis, robust and established NMR technology with minimal instrument down time, and low cost on a per-sample basis[3].

Recently, we reported that ^{1}H-NMR–based metabolomics approach could be used with surrogate biomarkers to monitor and explain the adverse effects of naproxen, a nonselective nonsteroidal anti-inflammatory drug (NSAID)[2]. These medications frequently induce gastrointestinal (GI) tract damage despite being medically prescribed in the general population to reduce fever and inflammation[4-6]. However, the pathogenesis of NSAID-induced GI tract damage is incompletely understood. Moreover, the pathogenesis of GI damage is followed by a multistage pathogenic event in which intestinal permeability, reactive oxygen species, gastric motility, luminal contents, neutrophils, and the microcirculation each play a role in the inflammation and ulcer development[7-9]. Therefore, multitargeted analysis is needed to further understand the mechanism of NSAID toxicity.

In this study, we used ^{1}H-NMR spectroscopy-based profiling of urinary metabolites to investigate the dose-dependent effects of ketoprofen, another nonselective NSAID, on metabolism and GI damage in rats. We also examined the mechanism underlying ketoprofen toxicity and compared the pathways between naproxen- and ketoprofen-induced gastric damage.

EXPERIMENTAL

Animals and treatment

Male Sprague–Dawley (SD) rats (50–300 g) were kept in an animal facility accredited by the Korea Food and Drug Administration (KFDA, Unit No. 000996; Seoul, Korea) in accordance with the International Animal Care Policies of the Association for Assessment and Accreditation of Laboratory Animal Care. All animals were provided with a standard irradiated chow diet (Purina Mills Inc., Seoul, Korea) and water *ad libitum*. Upon study commencement, the rats were housed in a specified pathogen-free state at 23 ± 1°C at relative humidity of 50 ± 10% under a 12-h light/dark cycle. Before dosing, the rats were fasted overnight and transferred to metabolic cages designed specifically for separate collection of urine and feces. Each rat then received an oral dose of vehicle (0.5% Tween-80 in autoclaved tap water) or ketoprofen (10 and 50 mg/kg body weight; Sigma, USA). There were 10 rats in the control group and in each dosage group. The experimental protocol was approved by the Institutional Animal Care and Use Committeeof the KFDA.

Sample collection

After drug administration, urine samples were collected from the rat cages over a 7-h period and were then immediately separated into aliquots and stored at –74 °C until analysis. The animals were euthanized with CO_2 and their stomachs were removed and scored for hemorrhagic damage. Scoring was performed by considering the size and depth of each lesion, and the value of which was added to give an overall gastric damage score for each rat[10]. All animal-related processes were performed as outlined in *A Good Practice Guide to the Administration of Substances and Removal of Blood Including Routes and Volumes*[11].

^{1}H-NMR experiments

Urine samples were centrifuged (20 min at 15,000 × *g* at 4 °C) to remove any solid debris. Aliquots (200 μL) of the supernatant fractions were added to microcentrifuge tubes containing 60 μL of 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) in D_2O and 340 μL of 0.2 M sodium phosphate buffer (pH 7.0) containing 0.018% NaN_3. After mixing, samples were adjusted to pH 7.0 and analyzed by ^{1}H-NMR spectroscopy on a 600-MHz Varian NMR System (Varian Inc., Palo Alto, CA, USA). Solvent suppression of residual water signals was achieved using the NOESYPRESAT pulse sequence in which the residual water peak is irradiated during the relaxation delay (1.5 s) and the mixing time (0.1 s). For each sample, ^{1}H-NMR spectra were collected using 64

scans containing 67,568 data points at a spectral width of 8445.9 Hz with an acquisition time of 4.0 s and a relaxation delay of 2.0 s.

Data analysis

The ^{1}H-NMR spectra were phased and baseline-corrected using Chenomx NMR Suite 6.1 (Chenomx Inc., Edmonton, Canada). The spectrum was referenced to DSS. Signal intensities in each spectrum were calculated by integrating 0.005 ppm sections. After the spectral regions containing the water and urea peaks (4.66–5.04 and 5.45–6.30 ppm, respectively) were removed, the spectra from 0.58 to 10.45 ppm were converted into ~1728 points using MATLAB (R2008a; The Mathworks Inc., Matick, MA, USA). The resultant data were imported into SIMCA (SIMCA-P+ 12; Umetrics AB, Umeå, Sweden), where principal component analysis (PCA) was performed using Pareto-scaled data. The Chenomx NMR Suite 6.1 (Chenomx Inc.) was used to identify metabolites and obtain concentration data. One-way ANOVA with Bonferroni's multiple comparison test for concentration data was carried out using GraphPad Prism (version 5 for Windows; GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Gastric damage

The gastric damage score from each rat after ketoprofen administration showed that the extent of gastric damage was dose-dependent over the entire ketoprofen dose range used in this study (0–50 mg/kg body weight) (Figure. 1). At 0, 10, and 50 mg/kg ketoprofen, the damage scores were 0.001 ± 0.003, 0.032 ± 0.0072, and 0.107 ± 0.022, respectively, indicating that the extent of damage in the groups treated with high (50 mg/kg) doses of ketoprofen was at least 100 times that of the vehicle-treated group.

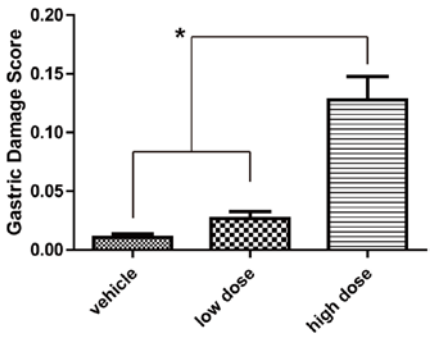

Figure. 1. Gastric damage scores for Vehicle-treated rats, low-dose (10 mg/kg body weight) ketoprofen-treated rats, and high-dose (50 mg/kg body weight) ketoprofen-treated rats. *P < 0.05, tested by one-way ANOVA test and Bonferroni's multiple comparison test. Columns represent means ± S.E.M.

^{1}H-NMR spectroscopy and pattern recognition analysis

Representative 600-MHz ^{1}H-NMR spectra of the urine samples obtained from vehicle- and ketoprofen-treated rats are shown in Figure 2. These spectra were used to identify numerous endogenous metabolites—including 3-hydroxybutyrate,1-methylnicotinamide,3-indoxylsulfate, adipate, allantoin, betaine, choline, citrate, phosphocreatine, creatinine, dimethylamine, glycine, hippurate, *N*,*N*-dimethylglycine, *N*-isovalerylglycine, pantothenate, phenylacetylglycine, putrescine, succinate, taurine, trigonelline, trimethylamine N-oxide, *cis*-aconitate—in the samples. The spectral data from the urine samples of the vehicle- and ketoprofen-treated rats indicate that the ketoprofen toxicity induced metabolic alterations in the rats.

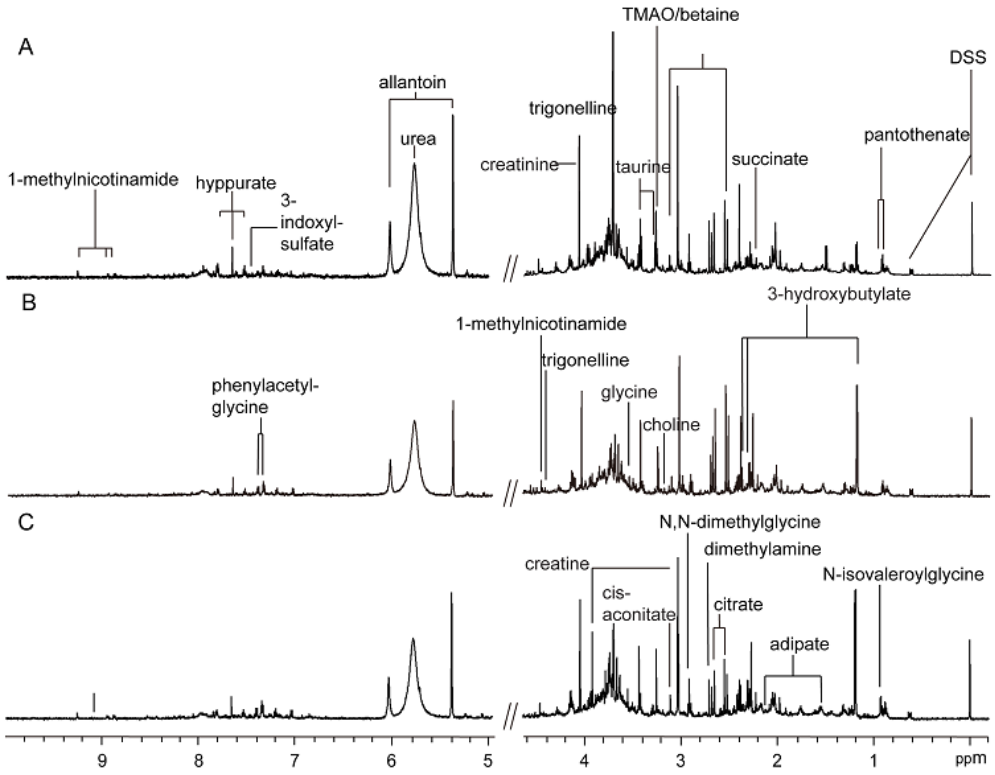

Figure. 2. Representative 600-MHz ^{1}H-NMR spectra of urine from rats treated with vehicle (A) or low- (B) or high-dose (C) ketoprofen.

To investigate the differences in the metabolite levels of the vehicle- and ketoprofen-treated groups, PCA analysis was applied to all of the groups (Figure 3). The PCA score plot derived from the NMR spectra showed clear separation between the vehicle- and ketoprofen-treated groups along the principal component 1 (PC1).

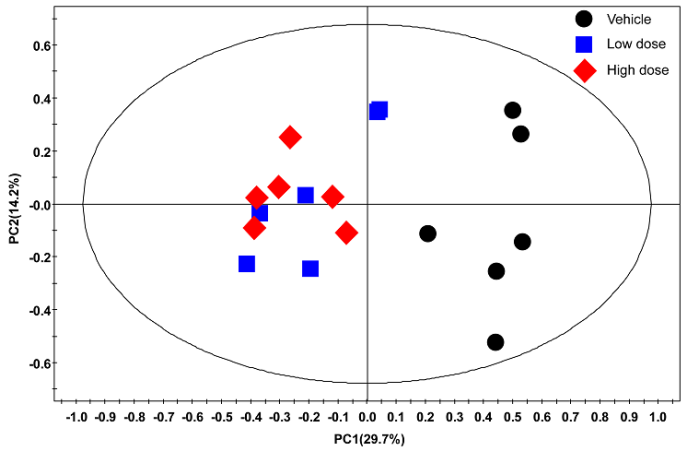

Figure. 3. PCA score plot (A) of urinary ^{1}H-NMR spectra obtained from rats treated with vehicle (black circle), low-dose (10 mg/kg) ketoprofen (blue box), or high-dose (50 mg/kg) ketoprofen (red diamond). Metabolic differences between the vehicle- and ketoprofen-treated groups are clearly evident. $R^2X = 0.44$; $Q^2 = 0.268$.

Targeted metabolic profiling for identifying novel markers

Endogenous metabolites were first identified from the ^{1}H-NMR spectra using the Chenomx library and then quantified. PCA score plots of the urinary metabolite concentrations obtained from the control and ketoprofen-treated rats (Figure 4A) revealed a dose-dependent shift from the vehicle-treated group (left) to the high-dose ketoprofen-treated group (right). This dose-dependent movement in targeted profile showed clearer pattern that seen in global profile.

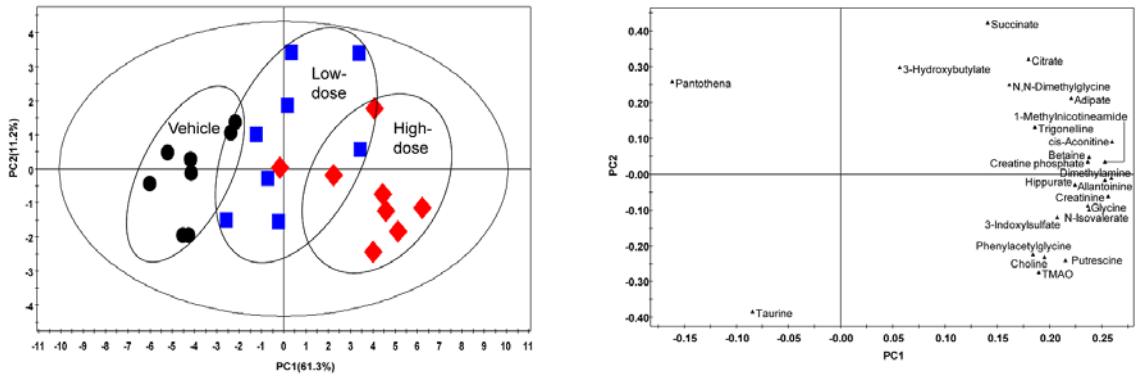

Figure. 4. PCA score (A) and loading plots (B) of urinary metabolite concentrations obtained by targeted profiling of urine from vehicle- and ketoprofen-treated rats. Metabolic patterns of the treated groups are grouped by colored circles (black circle, vehicle group; blue box, low-dose group; red diamond, high-dose group). The left and right sides of the loading plot represent the higher metabolite levels in the vehicle-treated and high-dose ketoprofen-treated groups. The loading plots were produced from the score plot, which shows the differentiation between the groups. $R^2X = 0.733$; $Q^2 = 0.547$. TMAO, trimethylamine N-oxide.

A loading plot was generated to identify the metabolites responsible for the differentiation in the score plots (Figure 4B). This plot shows increased levels of pantothenate and taurine in the vehicle-treated rats and increased levels of 3-hydroxybutyrate, 1-methylnicotinamide, 3-indoxylsulfate, adipate, allantoin, betaine, choline, citrate, phosphocreatine, creatinine, dimethylamine, glycine,

hippurate, *N*,*N*-dimethylglycine, *N*-isovalerylglycine, phenylacetylglycine, putrescine, succinate, trigonelline, trimethylamine N-oxide, and *cis*-aconitate in the ketoprofen-treated rats.

To further investigate the significance of these metabolites, we investigated the statistical significance of metabolite level variations. Variations in the concentrations of endogenous urinary metabolites that were perturbed in the ketoprofen-treated groups are shown in Figure 5. Among these metabolites, we found that levels of citrate, *cis*-aconitate, and succinate were significantly higher in the ketoprofen-treated groups than in the vehicle-treated group (Figure 5A). Because these 3 compounds are intermediates in the citric acid cycle, these changes indicate changes in energy metabolism. In addition, the level of phosphocreatine, an emergency energy regulator[12], was significantly elevated in dose-dependent manner (Figure 5A). These increased energy metabolites indicate that the body consumes large amounts of energy while mounting its cytoprotective response[13] in an acute inflammatory state. This result was consistent with that of naproxen-induced toxicity, which also showed increase levels of citrate, *cis*-aconitate, and phosphocreatine. In addition, 1-methylnicotinamide is significantly increased in a dose-dependent manner to protect against GI tract inflammation[14] (Figure 5A).

We observed that levels of metabolites produced from fatty acid oxidation, such as *N*-isovalerylglycine, adipate, and phenylacetylglycine, were significantly higher in the ketoprofen-treated groups than in the vehicle-treated group (Figure 5B). Additionally, metabolites affected by xenobiotics—such as dimethylamine, betaine, hippurate, 3-indoxylsulfate, *N*,*N*-dimethylglycine,

trimethylamine N-oxide, and glycine—were also increased in the ketoprofen-treated groups (Figure 5C). With regard to fatty acid metabolism, hepatic microsomal and peroxisomal long-chain CoA ligases have been implicated in the formation of acyl-CoA thioesters, which cause a toxic effect of carboxylic acid xenobiotics of 2-acrylpropinate NSAIDs, which need enantiospecific metabolic inversion from the inactive *R* (-) to the pharmacologically active *S* (+) enantiomer[15]. Therefore, an enhancement of xenobiotic activity in fatty acid oxidation for the bioactivity of ketoprofen may have a toxic effect on gastric damage. Moreover, levels of pantothenate, which is critical in the metabolism and synthesis of carbohydrate, protein, and fats, were significantly lower in the ketoprofen-treated groups than in the vehicle-treated group[16] (Figure 5D), which is also consistent with the results seen in the naproxen-induced pathway. However, tryptophan catabolism, a novel pathogenesis of gastric damage induced by naproxen, could not be observed in the ketoprofen-induced results.

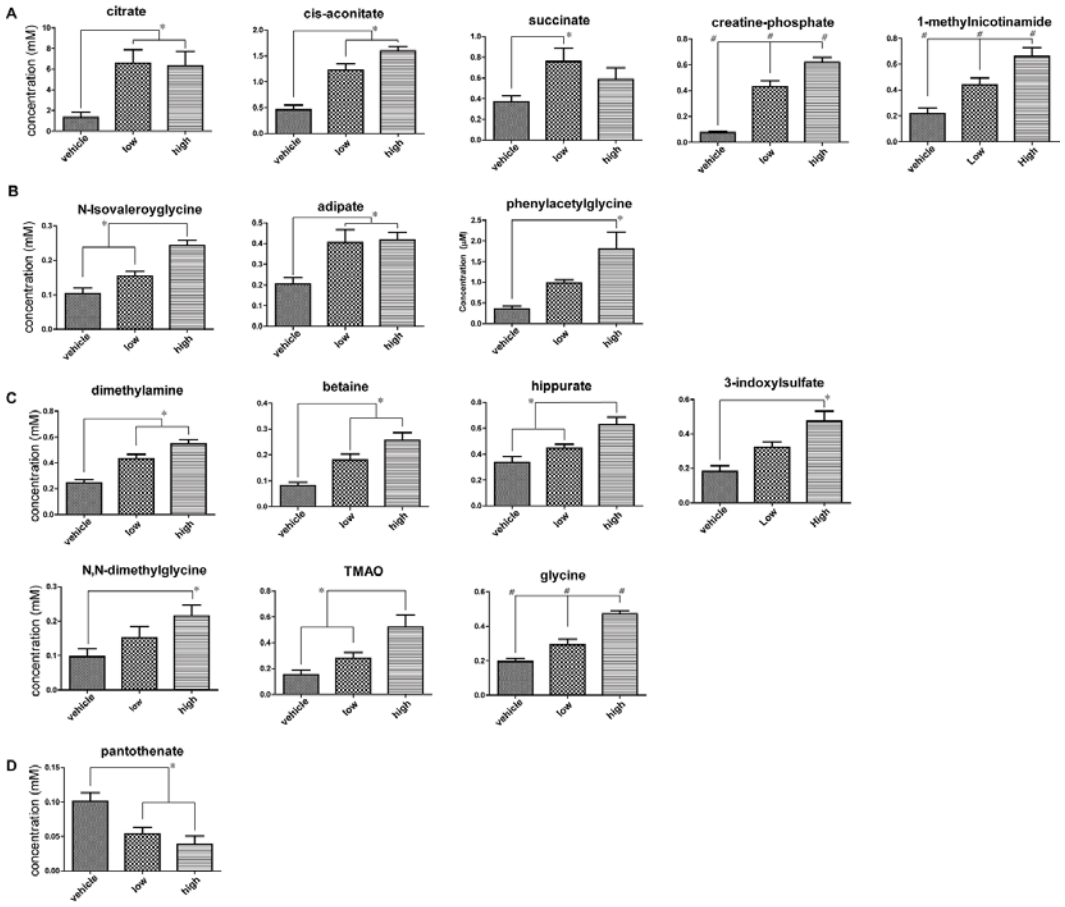

Figure. 5. Urinary metabolites exhibiting statistically significant changes in urinary concentration after naproxen administration in rats. $^{*}P < 0.05$ for each pair; $^{\#}P < 0.05$ for all pairs.

In conclusion, the results of this study demonstrate that ^{1}H-NMR–based metabolomics can be used to effectively distinguish metabolic differences according to the extent of gastric damage. Furthermore, global and targeted metabolic profiling via ^{1}H-NMR–based metabolomics is a

noninvasive and novel approach for monitoring drug toxicity and understanding the typical biological pathways involved in a variety of NSAIDs.

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