Stereoselective Acyl Glucuronidation and Glucosidation of Pranoprofen, a 2-Arylpropionic Acid Derivative, in Mice *in Vivo*

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Following the oral administration of $RS(\pm)$ -pranoprofen to mice at a dose of 25 mg/kg, 10.7% of the acyl glucuronide and 46.4% of the acyl glucoside of pranoprofen were excreted in the urine within 24 h. The recovery of acyl glucoside in the urine decreased relative to that of acyl glucuronide at increasing doses (100, 200 mg/kg). Following the oral administration of $S(+)$ -pranoprofen to mice at a dose of 25 mg/kg, 5.0% of the acyl glucuronide and 56.5% of the acyl glucoside were excreted in the urine within 24 h, while 10.8% of the acyl glucuronide and 13.9% of the acyl glucoside were excreted after the oral administration of $R(-)$ -pranoprofen, respectively. The absolute configuration of the aglycone of acyl glucuronide was almost $R(-)$ -enantiomer (92.5—96.1%) in the 0—24 h urine, whereas that of acyl glucoside contained 15.3—24.7% of $S(+)$ -enantiomer after the oral administration of $R(-)$ -pranoprofen. On the other hand, only the $S(+)$ -isomer was found as the aglycone of both acyl glucuronide and glucoside after the oral administration of $S(+)$ -pranoprofen.

The present results showed that stereoselective conjugation was observed in glucosidation in mice. Nevertheless, a dose-dependent shift in glucuronidation and glucosidation was found for both the administrations of $S(+)$ - and $R(-)$ -enantiomers as well as $RS(\pm)$ -pranoprofen. Also a chiral inversion of $R(-)$ -enantiomer to $S(+)$ -antipode may occur slightly but significantly in mice.

Keywords — drug metabolism; glucuronidation; glucosidation; stereoselectivity; dose dependent shift; pranoprofen; mouse; *in vivo*

Introduction

An orally active non-steroidal anti-inflammatory drug, 2-(5*H*-[1]benzopyrano[2,3-*b*]pyridin-7-yl)propionic acid (pranoprofen), belonging to the class of 2-arylpropionic acid derivatives, has been widely used in diseases such as rheumatoid arthritis.

Previous studies have demonstrated that pranoprofen was metabolically conjugated with sugars at a carboxylic moiety in mammals *in vivo*.¹⁻³⁾ Nevertheless, conjugations with different sugars were observed among species, *i.e.*, pranoprofen was mainly glucuronidated in rats, guinea pigs, rabbits³⁾ and human subjects,⁴⁾ whereas it was predominantly glucosidated in mice.³⁾

The conjugation of xenobiotics with glucuronic acid is well known as phase II metabolism, but glucosidation has been considered to be a novel metabolic pathway in mammals. As instances for glucosidation of xenobiotics in mammals, the recent investigations described (a) *N*-glucosidation of 3-(4-pyrimidinyl)-5-(4-pyridyl)-1,2,4-triazole⁵⁾

and phenobarbital,⁶⁾ (b) phenolic *O*-glucosidation of *p*-nitrophenol⁷⁾ and (c) alcoholic *O*-glucosidation of pantothenic acid⁸⁻¹⁰⁾ and metabolically hydroxylated indeloxazine.^{11,12)} Also we first demonstrated acyl glucosidation of pranoprofen as a major urinary metabolite in mice.^{1,2)} Furthermore, we demonstrated that glucosidation was a predominant pathway at low doses, whereas glucuronidation was preferential at high doses.¹³⁾

It is well known that 2-arylpropionic acid derivatives having a chiral center at the α -position of propionic acid moiety possess the stereoisomer of $S(+)$ - and $R(-)$ -enantiomers, and that the pharmacological effects and pharmacokinetics are markedly different between enantiomers.¹⁴⁻¹⁶⁾

The present report describes the urinary excretion of acyl glucuronide and glucoside following the oral and intravenous administration of $S(+)$ -, $R(-)$ - and $RS(\pm)$ -pranoprofen at various doses, and the enantiomeric composition of aglycones of their conjugates.

Materials and Methods

Materials — Racemic pranoprofen, *S*(+)-pranoprofen ($[\alpha]_D^{20} = +45.9^\circ$) and *R*(-)-pranoprofen ($[\alpha]_D^{20} = -44.6^\circ$) were synthesized and supplied by the Research Laboratories, Yoshitomi Pharmaceutical Industries, Ltd. The optical purities of *S*(+)-enantiomer and *R*(-)-antipode are 98.4% and 97.9%, respectively, measured using high performance liquid chromatography (HPLC).

β -Glucuronidase (from *Helix Pomatia*, Type H-1, 38000 units/g) and β -glucosidase (Emulsin, from Almond, 707000 units/g) were purchased from Sigma Chemical Co. (St. Louis, MO). D-Saccharic acid-1,4-lactone and glucono- δ -lactone were purchased from Sigma Chemical Co. and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. All other chemicals were of the highest purity available from standard commercial sources.

Amberlite XAD-2 resin (Organo, Ltd., Tokyo, Japan) and Seppack C₁₈ column (Waters Associates, Milford, Massachusetts, U.S.A.) were washed with methanol and distilled water prior to the experiments. For the silicagel plates, a commercial product (precoated Kiesel-gel 60 F₂₅₄, Merck, 0.25 mm thickness) was used.

Animals — Male ddY mice weighing 20–30 g supplied by the Kyushu Laboratory Animal Center (Fukuoka, Japan) were used. The animals had free access to food and water throughout the experiments. Pranoprofen was suspended in 0.5% methylcellulose and orally administered directly by intubation at a single dose of 25, 100 or 200 mg/kg for the quantitative determination of urinary excretion and 300 mg/kg for the isolation of metabolites, respectively. In the case of intravenous administration, pranoprofen dissolved in dilute sodium hydroxide (pH9–10) was injected into the tail vein at a dose of 25 mg/kg.

Thin-Layer Chromatography (TLC)

Samples were spotted on the silicagel plates and chloroform–methanol–85% formic acid (30:5:1, v/v/v) was used as a solvent system. The substances on the plates were detected by ultraviolet (UV) lamp (Manasuru Kagaku Kogyo, Ltd., Tokyo, Japan, short wave length 253.6 nm) and

the Dragendorff reagent.

Isolation of Pranoprofen Acyl Glucuronide and Glucoside — Following the oral administration of *RS*($\pm$)-pranoprofen to mice at a dose of 300 mg/kg, a 0–24 h urine sample was collected into a receiver containing 100 mM ammonium acetate buffer pH 4.0 to prevent urine from being converted into alkalinity. The urinary pH obtained was 4–5.

0–24 h urine was passed through a glass column containing 100 ml of Amberlite XAD-2 resin, and the unadsorbed materials were removed by a 500 ml water-wash. 200 ml of methanol was then passed through the column and collected. Acyl glucuronide and glucoside were isolated from the methanol eluates by preparative TLC (Fig. 1). Furthermore, each conjugate was purified by preparative HPLC, and the identification of these conjugates was carried out by the enzymatic hydrolysis. These isolated conjugates were used for the quantitative determination of urinary conjugates by HPLC.

Enzymatic Hydrolysis of Acyl Glucuronide and Glucoside — Each conjugate was dissolved in 0.07 M acetate buffer pH 5.0 and then β -glucuronidase and β -glucosidase were added to the medium, after which the mixtures were incubated at 37 °C for 4 h. The effects of D-saccharic acid-1,4-lactone and glucono- δ -lactone on the hydrolysis of each conjugate were also de-

Fig. 1. A Thin-Layer Chromatogram of Mouse Urinary Metabolites after Oral Administration of *RS*($\pm$)-Pranoprofen (300 mg/kg)

TLC was carried out for methanol eluates obtained from Amberlite XAD-2 resin. Solvent system: chloroform–methanol–85% formic acid (30:5:1, v/v/v), detection: UV lamp (253.6 nm), A, pranoprofen; B, C, unknown metabolites; D, pranoprofen acyl glucoside; E, pranoprofen acyl glucuronide.

terminated.

Urinary Excretion of Acyl Glucuronide and Glucoside — Following the oral and intravenous administration of *S*(+)- and *R*(-)- and *RS*($\pm$)-pranoprofen at a dose of 25, 100 or 200 mg/kg, 0–24 h urine samples were collected into the receiver containing 1 ml of 100 mM ammonium acetate buffer pH 4.0, respectively.

After each urine sample was diluted with distilled water to 8–10 ml, 1–2 μ l of a supernatant (with the insolubles removed by centrifugation) was directly injected into HPLC, and the acyl glucuronide and glucoside were quantitatively determined from the calibration curves. The calibration curves were prepared by the use of acyl glucuronide and glucoside isolated from mouse urine. At the time of the determination of the conjugates, it was confirmed that there were no interfering substances in the urine from the additions of the control and the alkali hydrolyzed urine into HPLC.

Enantiomeric Composition of Aglycones of Urinary Conjugates — Following the administration of *S*(+)-, *R*(-)- and *RS*($\pm$)-pranoprofen orally at 25, 100 or 200 mg/kg, and intravenously at 25 mg/kg, the 0–24 h urine samples were collected and 0.8 ml from each urine sample was pooled ($n = 3$).

Each pooled sample was passed through a Sep-pack C₁₈ column and after washing the column with 6 ml of distilled water the adsorbed materials were eluted with 2 ml of methanol. Acyl glucuronide and glucoside in the methanol eluates were isolated by the preparative TLC mentioned above. Then, each conjugate was dissolved in 0.5 ml of 1 N sodium hydroxide and the solution was heated at 70 °C for 30 min. 0.5 ml of 1 N hydrochloric acid and 2 ml of 100 mM ammonium acetate buffer pH 4.0 were added to the reaction mixture, and the hydrolyzed aglycone was extracted with 3 ml of chloroform by shaking. 1.5 ml of each chloroform extract was evaporated to dryness and the residue was dissolved in a small amount of ethanol. The enantiomeric composition of the aglycones was calculated from the determination of *S*(+)- and *R*(-)-enantiomers by the injection of a part of the ethanol solution into HPLC equipped with a Chiralcell OD column (Daicel Chemical Indus-

tries, Ltd., Tokyo, Japan) according to the following method. It was confirmed that chiral inversions of *S*(+)- and *R*(-)-pranoprofen did not occur by treatment with 1N sodium hydroxide.

HPLC Analysis — A Shimazu LC-3A HPLC was used. Chromatography was performed on a TSK-GEL ODS 80TM column (Tosoh, Tokyo, Japan, 4.6 $\times$ 150 mm) connected to a TSK-GEL ODS-120T column (Tosoh, Tokyo, Japan 4.6 $\times$ 150 mm) for the quantitative determination and on a YMC-Pack SH-343 S-15 ODS column (Yamamura Chemistry, Tokyo, Japan, 20 $\times$ 250 mm) for the purification of acyl glucuronide and glucoside. The mobile phases were 100 mM ammonium acetate buffer pH 4.0–acetonitrile (4:1,v/v) and (3:1,v/v) with flow-rates of 1 and 6 ml/min, respectively.

For the separate determination of *S*(+)- and *R*(-)-enantiomers, chromatography was carried out on a Chiralcell OD column (Daicel Chemical Industries, Ltd., Tokyo, Japan, 4.6 $\times$ 250 mm) and the mobile phase was 0.5 mM perchloric acid buffer pH 5.0–acetonitrile–water (60:44:50,v/v/v) with a flow-rate of 1 ml/min.

The detection of samples was carried out using a fluorescence detector (Shimazu RF-530, excitation wave length; 300 nm, emission wave length; 370 nm).

Results

Confirmation of Acyl Glucuronide and Glucoside

Both isolated glucuronide and glucoside were easily and quantitatively hydrolyzed to produce pranoprofen in 1 N sodium hydroxide solution at 70 °C for 30 min. Acyl glucuronide and glucoside were hydrolyzed to pranoprofen with both β -glucuronidase and β -glucosidase. For both enzymes, hydrolysis of acyl glucuronide exhibited the inhibition by D-saccharic acid-1,4-lactone and that of acyl glucoside by glucono- δ -lactone. The hydrolysis did not show the inhibition for acyl glucuronide by glucono- δ -lactone and for acyl glucoside by D-saccharic acid-1,4-lactone as described previously.¹⁾ These results show that the isolated conjugates were acyl glucuronide and

glucoside, respectively.

Urinary Excretion of Acyl Glucuronide and Glucoside

Following the oral administration of $RS(\pm)$ -pranoprofen to mice at 25 mg/kg, 10.7% of acyl glucuronide and 46.4% of acyl glucoside were excreted in urine within 24 h after dosing. When $S(+)$ - and $R(-)$ -pranoprofen were administered, 5.0% and 10.8% of acyl glucuronide and 56.5% and 13.9% of acyl glucoside were excreted in urine during the same period, respectively. No marked difference in urinary excretion of acyl glucuronide was observed between the two enantiomers, but the excretion of acyl glucoside in the urine was remarkable for the administration of $S(+)$ -enantiomer. This result demonstrates that a large difference was shown in the excretion of acyl glucoside between the administration of $S(+)$ - and $R(-)$ -isomers (Fig. 2). Excretion of total conjugates in the urine after the administration of $R(-)$ -pranoprofen was much

Fig. 2. Dose-Dependent Shift of Acyl Glucuronidation and Glucosidation after Oral Administration of $S(+)$ -, $R(-)$ - and $RS(\pm)$ -Pranoprofen

○, acyl glucuronide; ●, acyl glucoside.

less than that after the administration of $S(+)$ -pranoprofen. Furthermore, urinary excretion of acyl glucuronide and glucoside after the intravenous administration of $S(+)$ - and $R(-)$ - and $RS(\pm)$ -pranoprofen was similar to that after the oral administration, respectively (Fig. 3).

In regard to urinary excretion of acyl glucuronide and glucoside after the administration of $RS(\pm)$ -pranoprofen, a close agreement between observed values and calculated values which were estimated by the urinary excretion after the administration of $S(+)$ - and $R(-)$ -enantiomers, was obtained.

Dose-Dependent Shift in Acyl Glucuronidation and Glucosidation

In all cases of the administration of $S(+)$ -, $R(-)$ -isomer and $RS(\pm)$ -pranoprofen, urinary excretion of acyl glucoside decreased relative to

Fig. 3. Enantiomeric Composition of Aglycones of Pranoprofen Acyl Glucuronide and Glucoside in Mouse Urine after Administration of $S(+)$ -, $R(-)$ - and $RS(\pm)$ -Pranoprofen

GN, acyl glucuronide; GS, acyl glucoside. □, $S(+)$ -enantiomer; [*], $R(-)$ -enantiomer.

acyl glucuronide at increasing doses, showing no stereoselective difference of a dose-dependent shift in acyl glucuronidation and glucosidation between the two enantiomers (Fig. 2).

Enantiomeric Composition of Aglycones of Urinary Conjugates

Although aglycones of urinary acyl glucuronide contained an excess of *R*(-)-enantiomer (92.5–96.1%), 15.3–24.7% of *S*(+)-antipode resided in aglycones of acyl glucoside after the administration of *R*(-)-enantiomer. On the other hand, aglycones of urinary acyl glucuronide and glucoside mostly originated from the *S*(+)-form after the administration of *S*(+)-enantiomer.

In the case of the administration of *RS*(±)-pranoprofen, *S*(+)-enantiomer was far more marked in aglycones of acyl glucoside; in contrast, *R*(-)-enantiomer was also largely found in that of acyl glucuronide (Fig. 3).

Discussion

The profens have a chiral center within their structures that exhibit optical activity and contain a pair of stereoisomers. It is well known that their stereoisomers might show different behaviors in terms of chiral inversion and glucuronidation among enantiomers.^{14–20} This time, stereoselective disposition of pranoprofen in mice was investigated after the administration of *S*(+)- and *R*(-)- or *RS*(±)-pranoprofen.

Although no remarkable difference in the urinary excretion of acyl glucuronide was observed between the administration of *S*(+)- and *R*(-)-enantiomers, acyl glucoside was excreted more extensively for *S*(+)-enantiomer than for *R*(-)-antipode (Fig. 2). This finding strongly indicates that the stereoselective glucosidation might predominantly occur toward *S*(+)-pranoprofen.

A small amount of both conjugates in the urine after the administration of *R*(-)-pranoprofen was not caused by the repression of absorption since the urinary recovery of acyl glucuronide and glucoside after the oral administration was similar to that after the intravenous administration (Fig. 3). A smaller amount of urinary recovery of total conjugates for *R*(-)-isomer indicates the possibility that biliary ex-

cretion shows a stereoselectivity as well as other 2-arylpropionic acids.¹⁸⁾

In the previous study,¹³⁾ we demonstrated a dose-dependent shift in acyl glucuronidation and glucosidation of *RS*(±)-pranoprofen. In all cases of the administration of *S*(+)-, *R*(-)- or *RS*(±)-pranoprofen, glucosidation decreased relative to glucuronidation at increasing doses, and there was no stereoselectivity on a dose-dependent shift in acyl glucuronidation and glucosidation. But this dose-dependent shift might be owing mainly to *S*(+)-pranoprofen because the urinary recovery was less for *R*(-)-isomer than for *S*(+)-antipode (Fig. 2).

On the other hand, Fournel *et al.*¹⁶⁾ reported that metabolic chiral inversion of 2-phenylpropionic acid from *R*(-)-isomer to *S*(+)-antipode did not occur in mice. But we found that a small but significant metabolic chiral inversion from *R*(-)-isomer to *S*(+)-antipode might occur in mice because 15.3–24.7% of *S*(+)-enantiomer was detected in the aglycone of acyl glucoside after the administration of *R*(-)-pranoprofen. As well as other 2-arylpropionic acids,^{14–18)} it seemed that a chiral inversion of pranoprofen occurred only from *R*(-)-isomer to *S*(+)-antipode, since *R*(-)-aglycone was not detected in both acyl glucuronide and glucoside after the administration of *S*(+)-pranoprofen (Fig. 3).

Furthermore, no interaction of glucuronidation or glucosidation might be observed between the two enantiomers since the observed value of recovery of acyl glucuronide and glucoside after the administration of *RS*(±)-pranoprofen was correspondent to the estimated value which was calculated from the recovery of each conjugate after the administration of *S*(+)- and *R*(-)-pranoprofen, respectively.

From the present findings we concluded that no stereoselectivity was observed in a dose dependent shift in acyl glucuronidation and glucosidation of pranoprofen, but stereoselective conjugation did occur in acyl glucosidation in mice. This is the first instance for stereoselective acyl glucosidation in mammals.

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