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Optimized Conditions for the Enzymatic Hydrolysis of α-Hydroxytriazolam-Glucuronide in Human Urine

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The optimum conditions for the enzymatic hydrolysis of α-hydroxytriazolam (α-OHTRZ)-glucuronide, one of the major metabolites of triazolam in human urine, were determined. β-Glucuronidases from *Escherichia coli* (*E. coli*), bovine liver, *Helix pomatia* (*H. pomatia*) and *Patella vulgata* (*P. vulgata*) were used, and the parameters studied were amounts of enzyme used, temperature and pH range. α-OHTRZ was extracted with hexane/dichloromethane (1 : 1, v/v) and quantified using a high-performance liquid chromatography with a UV detector set at 230 nm. A preliminary study showed that β-glucuronidase from *H. pomatia* gave a poor recovery compared with the other three enzymes. The optimal conditions (amounts of enzymes, temperature and pH range) for 1 ml of urine were as follows; β-glucuronidase from *E. coli* (100 U, 37°C, pH 5.5–7.8), bovine liver (100 U, 45°C, pH 5.0–5.5), and *P. vulgata* (300 U, 60°C, pH 3.8–4.5). Among these enzymes, β-glucuronidase from *E. coli* was the most effective for the hydrolysis of α-OHTRZ-glucuronide in terms of efficiencies and the wide pH range tolerated. Incubation for 90 min with β-glucuronidase from *E. coli* was sufficient for hydrolysis of α-OHTRZ-glucuronide at clinical dose. α-OHTRZ-glucuronide in human urine can be hydrolyzed rapidly and effectively using this method.

Key words —— triazolam, glucuronide, hydrolysis, β-glucuronidase

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INTRODUCTION

Triazolam is a triazolobenzodiazepine derivative, and one of the most frequently prescribed hypnotics in Japan. It is a potent hypnotic with a short duration of action in human. After a single oral dose of ^{14}C-triazolam, the major urinary metabolites were found to be α-hydroxytriazolam (α-OHTRZ) and 4-hydroxytriazolam (4-OHTRZ), accounting for 69% and 11% of the urinary ^{14}C-activity, respectively. These were mostly conjugated with glucuronic acid.[1]

Triazolam is frequently encountered in forensic toxicological analysis and its ingestion is confirmed by detection of α-OHTRZ in urine. A hydrolysis step is required for the analysis of α-OHTRZ in urine, because it is impossible to analyze α-OHTRZ using a gas chromatography-mass spectrometry without hydrolysis of its conjugated form and it is easy to obtain standard of free α-OHTRZ in comparison with conjugated one.

Hydrolysis is generally performed either with the enzyme or by acid treatment. The former is performed under more moderate conditions and generally gives cleaner extracts than the latter.[2] For α-OHTRZ-glucuronide, acid hydrolysis causes the elimination of formaldehyde from α-hydroxymethyl position.[3] Therefore, β-glucuronidase would be more appropriate for the hydrolysis of α-OHTRZ-glucuronide.

For the effective and speedy enzymatic hydrolysis, it is necessary to optimize hydrolytic conditions (enzyme origin, substrate, pH, temperature, amounts of enzyme required, and reaction time). Some of the frequently used β-glucuronidases are from *Escherichia coli* (*E. coli*), bovine liver, snail intestinal juice (*Helix pomatia*, *H. pomatia*) and *Patella vulgata* (*P. vulgata*). Borrey *et al*. reported on optimum hydrolytic conditions of lormetazepam-glucuronide with β-glucuronidase from *H. pomatia,*[4] and Meatherall optimized the hydrolytic conditions for six glucuronides of benzodiazepines or its metabolites.[5] However, the optimization of hydrolytic condition of α-OHTRZ-glucuronide has not been performed.

In this study, we report on the optimization of conditions for the hydrolysis of α-OHTRZ-glucuronide in human urine using β-glucuronidases. We also report on the optimal incubation time for hydrolysis at clinical dose.

Table 1. Buffers Used in the Enzymatic Hydrolysis

pH	buffer
3.3	0.2 M citric acid/0.1 M disodium hydrogenphosphate buffer
3.8, 4.5, 5.0, 5.5	0.5 M sodium acetate buffer
6.0, 6.8, 7.3, 7.8	0.2 M sodium phosphate buffer
8.5, 9.0	0.2 M boric acid/0.05 M borax buffer

MATERIALS AND METHODS

Chemicals and Reagents —— α-OHTRZ was obtained from Sigma (St. Louis, MO, U.S.A.). 4-OHTRZ and nitrazepam were supplied by Upjohn Co. (Kalamazoo, MI, U.S.A.). β-glucuronidase from *E. coli*, Type IX-A (No. G 7396); bovine liver, Type B-1 (No. G 0251); *P. vulgata*, Type L-II (No. G 8132) were obtained from Sigma. β-Glucuronidase/aryl sulfatase from *H. pomatia* (No. 34742) was obtained from Calbiochem (San Diego, CA, U.S.A.). The activities of β-glucuronidases used in this study were determined by the method reported by Fishman *et al.*[6] All other chemicals used in the experiments were of analytical reagent grade.

Collection of Sample —— Blank urine samples were obtained from a healthy volunteer and stored at –20°C prior to use. Triazolam (Halcion, Pharmacia K.K., Tokyo, Japan) was orally administered as a single dose (0.25 mg or 0.5 mg) to healthy volunteers. Urine samples were collected 8–9 hr after the administration and stored at –20°C prior to use. Informed consent was obtained from the volunteers.

Enzymatic Hydrolysis —— One milliliter of enzyme solution in an appropriate buffer was added to 1 ml of the urine sample, and the mixture was incubated. The enzymatic reaction was stopped by adjusting the pH to approximately 10.5 with a 3 M sodium hydroxide solution by cooling in an ice bath. The amounts of enzyme, temperature and pH varied in experiments that were performed to determine the optimized conditions. The buffers used in the enzymatic hydrolysis are shown in Table 1.

Extraction —— For the extraction of α-OHTRZ, the method described by Edeki *et al.*[7] was used with minor modifications as follows. One milliliter of 0.2 M carbonate buffer (pH 10.5) was added to the hydrolyzed urine samples. α-OHTRZ was extracted twice with 3 ml portions of hexane and dichloromethane (1 : 1, v/v) for 5 min. After centrifugation of the solution at $1000 \times g$ for 5 min, the organic layer was transferred to a tapered glass tube and evaporated to dryness under nitrogen gas. The residues were reconstituted with 100 μl of mobile phase containing 5 μg/ml nitrazepam as an internal standard (IS) and 20 μl aliquot was injected into the HPLC.

Apparatus and Chromatographic Conditions —— The HPLC system consisted of a Gulliver series liquid chromatograph (JASCO, Tokyo, Japan) equipped with a UV detector set at 230 nm for quantitative analysis and with a time-of-flight mass spectrometer (LCT, Micromass, Manchester, U.K.) for qualitative analysis, respectively. Chromatographic separation was performed with a Mightysil RP-18 column (2.0×150 mm, 5 μm, Kanto Chemical, Tokyo, Japan). HPLC separations were carried at 40°C. The mobile phase was 50 mM ammonium acetate (pH 4.0)-methanol (57 : 43, v/v) and pumped at a flow-rate of 0.2 ml/min.

RESULTS AND DISCUSSION

For the examination of optimal hydrolysis conditions, the urine sample obtained from a volunteer administered 0.25 mg of triazolam orally was used. Figure 1 illustrates typical chromatograms obtained from the extract of the urine sample with and without hydrolysis. α-OHTRZ, 4-OHTRZ and nitrazepam (IS) peaks were well separated from each other without any interfering peaks. 4-OHTRZ could be detected only by mass spectrometry. α-OHTRZ and 4-OHTRZ were not detected without hydrolysis. The calibration curve for α-OHTRZ was linear in the range 25–1000 ng/ml in urine with a correlation coefficient of 0.9999.

Figure 2 shows the β-glucuronidases dosage-dependent increase in total α-OHTRZ (originally existed and freed by hydrolysis). Total α-OHTRZ reached a plateau at 300 U/ml urine for *E. coli* and bovine liver, and at 1000 U/ml urine for *P. vulgata*. In the case of *H. pomatia*, a plateau was not attained even at 2500 U/ml urine. In subsequent examinations, smaller amounts of β-glucuronidases than the amounts required for reaching a maximum (100 U/

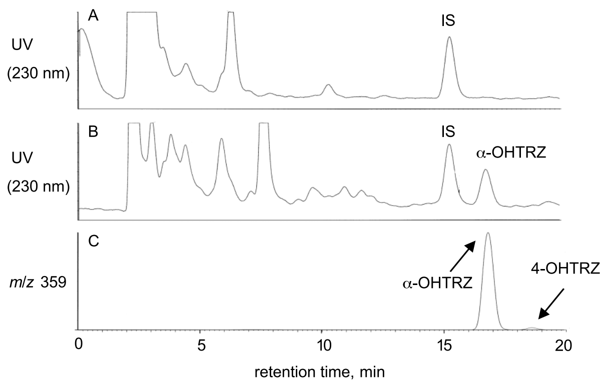

Fig. 1. HPLC Chromatograms of the Urine Extract with and without Hydrolysis

Hydrolysis was performed at pH 6.8 for 2 hr with β-glucuronidase from *E. coli* (100 U/ml urine). A: chromatogram at 230 nm without hydrolysis, B: chromatogram at 230 nm with hydrolysis, C: mass chromatogram (*m/z* 359, $[M+H]^+$ for α-OHTRZ and 4-OHTRZ) with hydrolysis. IS: nitrazepam.

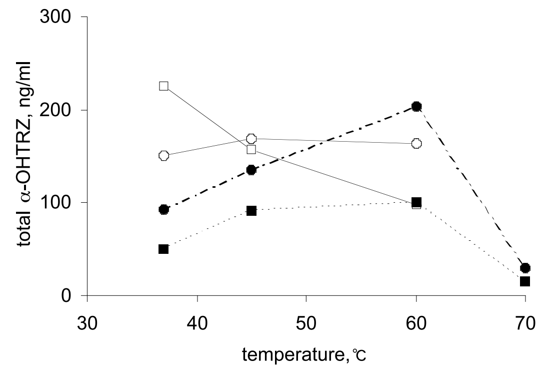

Fig. 3. Effect of Incubation Temperature of the Hydrolysis of α-OHTRZ-Glucuronide with β-Glucuronidases from *E. coli* (□), Bovine Liver (○), *P. vulgata* (●) and *H. pomatia* (■)

Each sample was incubated for 2 hr at the foresaid "Sigma-recommended pH." The amount of enzymes added was 100 U/ml urine for *E. coli* and bovine liver, 300 U/ml urine for *P. vulgata* and *H. pomatia*. Each data point represents the mean of duplicate determinations.

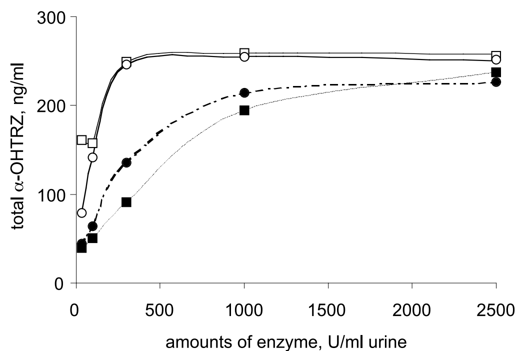

Fig. 2. Dose Dependent Increases in Total α-OHTRZ with β-Glucuronidases from *E. coli* (□), Bovine Liver (○), *P. vulgata* (●) and *H. pomatia* (■)

Urine samples were incubated at 45°C for 2 hr at "Sigma-recommended pH" for each enzyme (pH 6.8 for *E. coli*, pH 5.0 for bovine liver and *H. pomatia*, pH 3.8 for *P. vulgata*). Each data point represents the mean of duplicate determination.

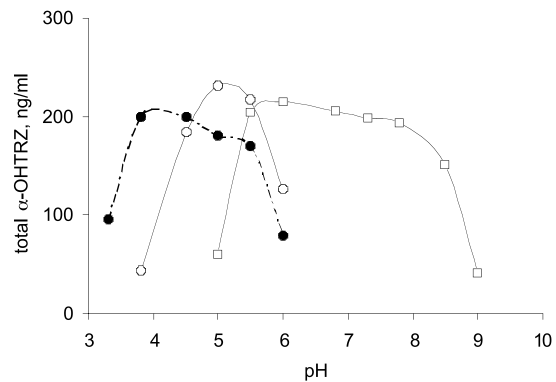

Fig. 4. Effect of pH on the Hydrolysis of α-OHTRZ-Glucuronide with β-Glucuronidases from *E. coli* (□), Bovine Liver (○), *P. vulgata* (●)

Each sample was incubated for 2 hr under the optimized conditions (*E. coli*: 100 U/ml urine and 37°C, bovine liver: 100 U/ml urine and 45°C, and *P. vulgata*: 300 U/ml urine and 60°C). Each data point represents the mean of duplicate determinations.

ml urine for *E. coli* and bovine liver, and 300 U/ml urine for *P. vulgata* and *H. pomatia*) were used, because the possibility that total α-OHTRZ may increase according to optimization of hydrolysis conditions was considered.

Figure 3 shows the effect of incubation temperature on the activity of β-glucuronidase from four different sources. Optimal recovery occurred at 37°C for *E. coli* and at 60°C for *P. vulgata*. No significant difference in hydrolytic efficiency was found among the temperatures tested for β-glucuronidase from bovine liver. The use of β-glucuronidase from *H. pomatia* resulted in a poor recovery of α-OHTRZ, therefore, it was omitted from any further study.

Figure 4 shows the effect of pH on the hydrolysis of α-OHTRZ-glucuronide at the optimized temperature. Optimal recovery occurred at the following pH values; 5.5–7.8 for *E. coli*, 5.0–5.5 for bovine liver and 3.8–4.5 for *P. vulgata*. Our findings were in agreement with "Sigma-recommended pH"[8] (pH 6.8 for *E. coli*, pH 5.0 for bovine liver, pH 3.8 for *P. vulgata*).

To determine the optimum incubation time, urine specimens obtained from four volunteers (triazolam dosage: 0.25 mg for two and 0.5 mg for the other two) were used. We selected the β-glucuronidase from *E. coli* in the three types of enzymes, because

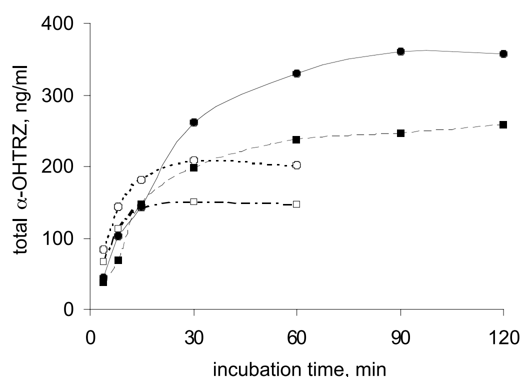

Fig. 5. Total α-OHTRZ Versus Incubation Time with β-Glucuronidase from *E. coli* under the Optimized Conditions (100 U/ml urine, 37°C, pH 6.8)

Urine specimens were obtained from four healthy volunteers orally administrated triazolam (0.25 mg for two volunteers and 0.5 mg for the other two volunteers). Triazolam dosage, □ and ○: 0.25 mg, ● and ■: 0.5 mg.

it showed a relatively wide optimal pH range and a high activity. Figure 5 shows the effect of incubation time when 100 U/ml urine of β-glucuronidase from *E. coli* was used under the optimized conditions. Total α-OHTRZ reached a plateau at 30 min for a 0.25 mg dose of triazolam and 90 min for a 0.5 mg dose.

Optimum conditions for the hydrolysis of 4-OHTRZ-glucuronide, another major metabolite of triazolam, were not examined in this study, because the concentration was too low to quantify in the urine sample. However, where 5 ml of urine sample (0.5 mg dosage) was examined as a preliminary study, we confirmed that total 4-OHTRZ reached a plateau under the optimized hydrolytic conditions (β-glucuronidase from *E. coli*, 100 U/ml urine, 37°C, pH 6.8) for 90 min (data not shown). Thus, it is suggested that 4-OHTRZ-glucuronide is substantially hydrolyzed under the conditions optimized for α-OHTRZ-glucuronide.

In conclusion, α-OHTRZ-glucuronide can be practically hydrolyzed using β-glucuronidases from *E. coli*, bovine liver and *P. vulgata*. β-Glucuronidase from *E. coli* may be the most appropriate for α-OHTRZ-glucuronide in these enzymes. Under the conditions optimized for β-glucuronidase from *E. coli*, α-OHTRZ-glucuronide can be quantitatively hydrolyzed within 90 min at least at clinical dosage.

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