In Vivo and in Vitro Binding of (−)-Hydroxyhexamide, a Major Metabolite of Acetohexamide, to Rabbit Serum

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The in vivo and in vitro bindings of (−)-hydroxyhexamide, a major metabolite of acetohexamide, to rabbit serum were examined by using an ultrafiltration method. The in vivo serum protein binding of (−)-hydroxyhexamide was much lower than the in vitro serum protein binding. The in vitro serum protein binding of (−)-hydroxyhexamide was strongly displaced by the addition of acetohexamide. Furthermore, the in vitro serum protein binding of (−)-hydroxyhexamide in the presence of acetohexamide and (−)-hydroxyhexamide at the same concentrations as those found 1.0 h after acetohexamide administration was approximately similar to the in vivo serum protein binding of (−)-hydroxyhexamide. These results lead us to conclude that acetohexamide, the parent drug of (−)-hydroxyhexamide, plays an important role in the in vivo serum protein binding of (−)-hydroxyhexamide.

Keywords — (−)-hydroxyhexamide; serum protein binding; rabbit serum; acetohexamide; pharmacologically active metabolite; displacement; ultrafiltration method

Introduction

Acetohexamide is used as an antidiabetic drug. This drug has been reported to be biotransformed to (−)-hydroxyhexamide in humans. The in vivo and in vitro bindings of (−)-hydroxyhexamide were examined by using an ultrafiltration method. A similar result was observed in the oxidative metabolism of acetohexamide in rabbits. Since (−)-hydroxyhexamide is known to be more pharmacologically active than the parent drug, it is important clinically to have a detailed knowledge of its serum protein binding. We have already studied the binding of (−)-hydroxyhexamide to human serum albumin. The purpose of the present study is to elucidate the mechanism of the difference between in vivo and in vitro bindings of (−)-hydroxyhexamide to rabbit serum.

Materials and Methods

Chemicals — Acetohexamide was supplied by Shionogi & Co., Ltd. (Osaka, Japan). The racemate of hydroxyhexamide, (±)-hydroxyhexamide, was synthesized from acetohexamide according to the method of Girgis-Takla and Chroneos. The melting point was 143–146 °C. The infrared (IR) spectrum (in KBr, JASCO IRA-A spectrometer) showed a hydroxy band at 3520 cm⁻¹. The proton nuclear magnetic resonance (¹H-NMR) spectrum (60 MHz, JEOL PMX-60 spectrometer) showed a broad hydroxy proton peak at 3.58 ppm. All other chemicals were of reagent grade.

Isolation of (−)-Hydroxyhexamide — (−)-Hydroxyhexamide, [α]²⁰_D = −19.9 ° (0.5%, CHCl₃), was isolated from the enzyme reaction mixture after incubation at 37 °C for 2.0 h. The cytosolic fraction of rabbit liver homogenate was used as enzyme preparation. After heat treatment, the enzyme reaction mixture was centrifuged at 3000 rpm for 10 min. The supernatant fluid was adjusted to pH 3.0 with 5N HCl, saturated with NaCl, and extracted 3 times with an equivalent volume of benzene-ethyl acetate (1:1, v/v). The residue was dissolved in a small amount of hot ethanol and H₂O was carefully added to precipitate a crude metabolite. The crude metabolite was chromatographed on a silica gel column with stepwise eluent of benzene - aceton - acetic acid (90:10:5, v/v) and benzene-acetone-acetic acid (70:30:0.5, v/v). Fractions eluted with the latter were evaporated, and the resulting metabolite, (−)-hydroxyhexamide, was recrystallized from 30% ethanol. The enantiomeric purity of (−)-hydroxyhexamide was determined by the ¹H-NMR spectrum (400 MHz, JEOL GX-400 spectrometer) of diastereomer derived by the
reaction of (−)-hydroxyhexamide with (R)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Aldrich Chem. Co., Milwaukee, U.S.A.). The (−)-hydroxyhexamide was found to be almost 100% in the enantiomeric purity. The details will be described in our next publication.

Animal Experiments — Male albino rabbits weighing 2.5–3.2 kg were used. Acetohexamide at a dose of 50 mg/kg was intravenously administered to the rabbits. The acetohexamide injection was prepared by dissolving the drug in saline solution containing the same molar amount of NaOH. Blood samples were collected periodically from the ear vein. The blood was centrifuged at 3000 rpm for 15 min to obtain the serum for analysis.

Protein Binding Experiments — In vivo and in vitro protein binding experiments were carried out by means of the ultrafiltration method described previously. The in vivo protein binding of acetohexamide or (−)-hydroxyhexamide was determined for the serum obtained 1.0 h after intravenous bolus administration of acetohexamide to rabbits. The in vitro protein binding of acetohexamide or (−)-hydroxyhexamide was determined for the serum prepared by adding the drug or metabolite.

Analytical Methods — The concentration of acetohexamide and (−)-hydroxyhexamide were measured by high-performance liquid chromatography (HPLC). After the addition of 1N HCl (0.5 ml), the serum (0.1 ml) or the ultrafiltrate (0.1 ml) was extracted with 5.0 ml of benzene-ethyl acetate (1:1, v/v) containing fenbufen (Lederle Labs., Pearl River, U.S.A.) as the internal standard. The extract (4.0 ml) was evaporated in vacuo and the residue was dissolved in acetonitrile (0.3 ml) to subject to HPLC. HPLC was carried out using a Hitachi 655A-11 HPLC apparatus equipped with a LiChrosorb RP-18 column (250 × 4 mm i.d., Cica-Merck) and a Hitachi 638-41 UV monitor. Acetonitrile-0.2% acetic acid (47:53, v/v) was employed as a mobile phase at a flow rate of 1.0 ml/min.

Statistical Analysis — Statistical significance of difference between means was determined by using the unpaired Student's t-test.

Results

Serum Concentration of Acetohexamide and (−)-Hydroxyhexamide after Acetohexamide Administration

The time course of the serum concentrations of acetohexamide and (−)-hydroxyhexamide after intravenous bolus administration of acetohexamide to rabbits are shown in Fig. 1. (−)-Hydroxyhexamide at a high serum concentration appeared rapidly after acetohexamide administration. This may be because the enzyme which catalyzes the metabolic reduction of acetohexamide is present in various rabbit tissues, as reported previously. However, the serum concentration of (−)-hydroxyhexamide was lower than that of acetohexamide in all the range of 0.2 to 3.0 h.

In Vivo and In Vitro Serum Protein Binding

Figure 2 shows the in vivo and in vitro bindings of (−)-hydroxyhexamide to rabbit serum. The in vivo binding was much lower than the in vitro binding. It is interesting to note that a

![Graph](image-url)

Fig. 1. Time Course of Serum Concentration of Acetohexamide (AH) and (−)-Hydroxyhexamide ((−)-HH) after Intravenous Bolus Administration of Acetohexamide to Rabbid

○, acetohexamide; ●, (−)-hydroxyhexamide. Each point represents the mean ± S.E. of 4 rabbits.
Protein Binding of (−)-Hydroxyhexamide

Fig. 2. *In Vivo* and *in Vitro* Binding of (−)-Hydroxyhexamide ((−)-HH) to Rabbit Serum

○, *in vitro*; ●, *in vivo*. In the case of the *in vitro* binding, each point represents the mean ± S.E. of 3—6 experiments.

Fig. 4. Effect of Acetohexamide (AH) on the *in Vitro* Binding of (−)-Hydroxyhexamide ((−)-HH, 100 μg/ml) to Rabbit Serum

Each point represents the mean ± S.E. of 3 experiments.

marked difference is observed between the *in vivo* and *in vitro* bindings of (−)-hydroxyhexamide to rabbit serum. Figure 3 shows the *in vivo* and *in vitro* bindings of acetohexamide to rabbit serum. The *in vivo* binding was slightly low as compared with the *in vitro* binding. In addition, since no significant difference was found between the *in vitro* bindings of (−)-hydroxyhexamide (91.5 ± 0.7%, 100 μg/ml, mean ± S.E., n = 4) and (±)-hydroxyhexamide (90.8 ± 0.5%, 100 μg/ml, mean ± S.E., n = 4) to rabbit serum, it is possible to assume that (−)-hydroxyhexamide has the same binding capacity as the (±)-isomer to rabbit serum.

Fig. 3. *In Vivo* and *in Vitro* Binding of Acetohexamide (AH) to Rabbit Serum

○, *in vitro*; ●, *in vivo*. In the case of the *in vitro* binding, each point represents the mean ± S.E. of 3 experiments.

Fig. 5. Effect of (−)-Hydroxyhexamide ((−)-HH) on the *in Vitro* Binding of Acetohexamide (AH, 100 μg/ml) to Rabbit Serum

Each point represents the mean ± S.E. of 4—6 experiments.
The displacing effect of acetohexamide on the in vitro serum protein binding of (−)-hydroxyhexamide, and of (−)-hydroxyhexamide on the in vitro serum protein binding of acetohexamide are shown in Figs. 4 and 5, respectively. Acetohexamide caused a marked decrease in the in vitro binding of (−)-hydroxyhexamide to rabbit serum (Fig. 4). On the other hand, (−)-hydroxyhexamide slightly decreased the in vitro binding of acetohexamide to rabbit serum (Fig. 5).

In order to elucidate the mechanism of the marked difference between in vivo and in vitro bindings of (−)-hydroxyhexamide to rabbit serum, the in vitro serum protein binding of (−)-hydroxyhexamide was examined in the presence of acetohexamide and (−)-hydroxyhexamide at the same concentrations as those found 1.0 h after acetohexamide administration. As shown in Fig. 6, the obtained result was approximately similar to the in vivo result illustrated in Fig. 2.

Discussion

The (−)-hydroxyhexamide concentration in serum after acetohexamide administration to rabbits was determined by HPLC of Takagishi et al.6 The HPLC does not separate (−)-hydroxyhexamide and the (+)-isomer. However, we have demonstrated that (−)-hydroxyhexamide is isolated from the urine after acetohexamide administration to rabbits2 and (−)-hydroxyhexamide with almost 100% in enantiomeric purity is isolated from the enzyme reaction mixture of rabbit liver. Thus, the metabolite in the rabbit serum determined by the HPLC was regarded as (−)-hydroxyhexamide.

It has been reported that metabolites can displace the parent drugs from their binding sites on serum proteins.5,9,10 On the other hand, information concerning the displacement of metabolites from their binding sites by the parent drugs has been very limited. This is because most metabolites, except metabolites of drugs like procainamide, propranolol and imipramine,11 are pharmacologically inactive. If metabolites are pharmacologically active and have a relatively high binding capacity, the displacement of metabolites by the parent drugs will be significant clinically.

In the present study, we have found that (−)-hydroxyhexamide, a pharmacologically active metabolite, highly binds to rabbit serum and is strongly displaced from its binding sites by the parent drugs. Moreover, when acetohexamide was administered intravenously to rabbits, the serum concentration of acetohexamide was high as compared with that of (−)-hydroxyhexamide. On the basis of these findings, the displacement of (−)-hydroxyhexamide from its binding sites by acetohexamide seems to be the reason that the in vivo binding of (−)-hydroxyhexamide to rabbit serum is much lower than the in vitro binding. This is also supported from the data with respect to the in vitro binding of (−)-hydroxyhexamide to rabbit serum in the presence of both acetohexamide and (−)-hydroxyhexamide at the same concentrations as those found 1.0 h after acetohexamide administration. However, in the case of the in vivo binding of (−)-hydroxyhexamide to rabbit serum, the displacing effect of metabolites other than (−)-hydroxyhexamide or the pH change of the serum by hypoglycemia may be involved, although further investigations should be con-
ducted.

In conclusion, there is a marked difference between the \textit{in vivo} and \textit{in vitro} bindings of (−)-hydroxyhexamide to rabbit serum. Since (−)-hydroxyhexamide is a pharmacologically active metabolite and displaced strongly from its binding sites by the parent drug, it is important to determine its binding capacity for pharmacokinetic and pharmacodynamic analyses according to the \textit{in vivo} method.

References