Intestinal Absorption of Azetirelin, a New Thyrotropin-Releasing Hormone (TRH) Analogue. I. Possible Factors for the Low Oral Bioavailability in Rats

Isao Sasaki,*a,b Takuya Fujita,a Masahiro Murakami,a Akira Yamamoto,a Eiji Nakamura,c Hajime Imasaki,d and Shozo Muranishi*a

Department of Biopharmaceutics, Kyoto Pharmaceutical University,a Misasagi, Yamashina-ku, Kyoto 607, Japan, Pharmaceutical Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd.,a 180 Osumi, Yazi-cho, Shizuoka 425, Japan and Applied Pharmacology and Development Laboratories, Yamanouchi Pharmaceutical Co., Ltd.,a 1–1–8 Azasawa, Itabasi-ku, Tokyo 174, Japan. Received January 13, 1994; accepted May 17, 1994

Absorption of azetirelin, a new thyrotropin-releasing hormone (TRH) analogue, from the gastrointestinal (GI) tract was evaluated. The bioavailability of this compound after oral administration was considerably poor in rats. Studies were undertaken to elucidate the mechanisms for this low oral bioavailability of azetirelin. The plasma azetirelin levels following intravenous and hepatoporal vein injection were virtually identical over the dose range of 0.02–0.1 mg/kg, indicating a minor contribution of the hepatic first-pass metabolism of this drug. Azetirelin was stable against peptide hydrolyses both in luminal fluid and intestinal mucosal homogenates, whereas its degradation occurred when incubated with cecal contents under an anaerobic condition. In addition, complete degradation of azetirelin during the GI transit was disclosed by analyzing the fecal sample collected after oral administration of [14C]azetirelin. These results suggested that gut bacteria may be responsible for the hydrolysis of azetirelin in the GI tract. The low intestinal permeability of azetirelin was revealed by a modified everted gut experiment in various segments of the rat intestine. The poor membrane transport characteristics of azetirelin may be due to its high hydrophilicity. From these results, it was suggested that the insufficient oral bioavailability of azetirelin may be mainly attributed to its low intestinal permeability due to a lack of lipophilicity, and also to the degradation of the peptide by intestinal microflora.

Keywords azetirelin; bioavailability; intestinal absorption; intestinal microflora; metabolism; everted gut experiment

Azetirelin, (N-hydroxy-4-oxo-2-azadiynylcarbonyl)-L-histidyl-L-proline amide, Fig. 1), is a newly synthesized thyrotropin-releasing hormone (TRH) analogue which shows selective action on the central nervous system (CNS). It was reported that the thyrotropin (TSH)-releasing activity of azetirelin is less than that of TRH.1) However, the activating effects of azetirelin on the CNS, such as anti-pentobarbital sleep and anti-reserpine hyperthermia, are about 10–100 times more potent and 8–36 times longer-lasting than those of TRH in mice. 2) Unlike the rapid enzymatic inactivation of TRH in the body, azetirelin is extremely stable in plasma and is degraded much more slowly than TRH in brain homogenate.23) This increased metabolic stability is considered a primary reason for the potent and longer-lasting pharmacological properties of azetirelin.

On the other hand, the low oral bioavailability of TRH and some of its analogues have already been demonstrated by other authors.3) Yokohama et al. reported that the oral bioavailability of TRH was less than 2% in rats, less than 13% in dogs, and was about 2% in humans.30) They also demonstrated that TRH was stable against enzymatic degradation in the gastrointestinal (GI) tract, while it was absorbed only from the upper region of the small intestine by a carrier-mediated transport mechanism.4) Vickers et al. reported that the bioavailability of L-pyro-2-amino-adipyl-L-histidyl-L-thiazolidine-4-carboxamide (MK-771), a TRH analogue, was similarly low.3) They have concluded that this is due to poor intestinal permeability rather than to a metabolic problem.

In the present study, we have evaluated the bioavailability of azetirelin following oral administration to rats. In addition, the hepatic first-pass metabolism and the stability of azetirelin in the GI tract were examined to explain its insufficient absorption characteristics. We also investigated the intestinal transport of azetirelin by using modified everted gut experiments.

MATERIALS AND METHODS

Chemicals Azetirelin and [14C]azetirelin were synthesized in the Central Research Laboratories of Yamanouchi Pharmaceutical Co., Ltd. The specific activity of the labeled compound was 2.73 MBq/mg. TRH was purchased from Peptide Institute, Inc. (Osaka, Japan). 5(6)-Carboxyfluorescein (CF) was obtained from Eastman Kodak Co., Ltd. (Rochester, NY, U.S.A.). Acetaminophen was obtained from Yoshitomi Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals and solvents were

Fig. 1. Chemical Structure of Azetirelin

(*) indicates the location of the radiocarbon of the labeled compound.

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of reagent grade and were used without further purification.

**Animals** Male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan), weighing 250—330 g, were used in the study. Unless otherwise stated, rats were fasted overnight with free access to water before use.

**Oral Administration to Rats** Azetrelin was dissolved in saline and administered orally to rats at doses of 10 and 25 mg/kg. Dosing volume was 4 ml/kg. At each designated time interval after administration, four rats were lightly anesthetized with ether and blood was collected from the jugular vein. The blood samples were centrifuged for 20 min at 3000 rpm to obtain plasma (0.5 ml). After extraction with methanol, the plasma concentration of azetrelin was determined by radioimmunoassay according to the method of Nakamura *et al.* The peak plasma concentration ($C_{\text{max}}$) and the peak plasma concentration time ($T_{\text{max}}$) were obtained from the mean plasma concentration—time curves. The area under the plasma concentration—time curve ($AUC$) was calculated by the trapezoidal rule and then added to the value of plasma concentration at the time last detected, divided by the terminal elimination rate constant, which was calculated by the least squares method with a semi-logarithmic scale. Mean residence time (MRT) was calculated by means of moment analysis.

**Estimation of the Hepatic First-Pass Effect** Rats were anesthetized intraperitoneally with sodium pentobarbital (32 mg/kg). After a midline incision, azetrelin dissolved in saline solution was administered by injection into the femoral vein or the hepatoportal vein at doses of 0.02, 0.05, and 0.1 mg/kg ($n = 4$). The dosing volume was 1 ml/kg. Blood samples were collected periodically from the jugular vein up to 90 min. The plasma concentration of azetrelin was determined as described above. The $AUC$ value was calculated by the linear trapezoidal method up to the last sampling time.

**Stability of Azetrelin in Luminal Fluid and Intestinal Mucosal Homogenates** Luminal fluid was collected from the whole small intestine of rats according to the method of Takada *et al.* Intestinal mucosal homogenates were prepared according to the method of Hashizume *et al.* with some modifications. The mucosal surface of the small and the large intestine of rats were scraped off and collected, respectively. Homogenates were prepared by homogenizing the mucosa in ice-cold phosphate buffered saline (PBS) using a Polytron homogenizer (Kinematica GmbH, Switzerland). After centrifuging the homogenates at 5000 rpm for 10 min, the resulting supernatants were diluted with PBS to adjust the protein concentration to 10 mg/ml and stored at −80 °C until use. The protein concentration was determined by the method of Lowry *et al.*, using bovine serum albumin as a standard. For stability studies, 100 µl of the luminal fluid or the intestinal mucosal homogenates were incubated with 400 µl of 0.1 mM azetrelin PBS solution at 37 °C. At predetermined times up to 180 min, 50 µl of samples were withdrawn from the incubation mixture, to which was added 150 µl of acetonitrile to precipitate the tissue proteins, thereby terminating the reaction. Fifty-microliters of 30 µg/ml l-phenylephrine hydrochloride solution, the internal standard, was then added. After centrifugation, 10 µl of supernatant was injected into the HPLC.

**Stability of Azetrelin in Cecal Contents** Fresh cecal content from non-fasted rats was suspended in two-fold its volume of bicarbonate buffer (NaHCO₃, 9.240 g; Na₂HPO₄·12H₂O, 7.125 g; NaCl, 0.470 g; KCl, 0.450 g; CaCl₂·2H₂O, 0.073 g; MgCl₂·6H₂O, 0.087 g/l). The pH of the buffer was adjusted to 7.0 by bubbling with CO₂ gas prior to use. The suspension was filtered through four layers of gauze. Stability studies were conducted by incubating 0.5 ml of the suspension with the same volume of azetrelin or TRH buffer solution in a sealed test tube under a CO₂ atmosphere at 37 °C. The final concentration of the drugs in the incubation mixture was 0.1 mM. At predetermined times, 50 µl samples were taken and analyzed by HPLC as described above. In a control experiment, the suspension was boiled and sterilized by direct heating before use.

**Excretion of Radioactivity after Oral Administration of [¹⁴C]Azetrelin** Rats were dosed orally with 1 mg/kg of [¹⁴C]azetrelin. Urine, feces, and exhaled air were collected up to 72 hr from rats housed individually in glass metabolism cages (Natsume Seisakusyo Co., Japan). For the biliary excretion studies, a polyethylene cannula (i.d. 0.58 mm, o.d. 0.96 mm, Natsume) was inserted into the bile duct under ether anesthesia. Bile samples were collected from the rats placed in Bollman's cages for 24 hr postdosing. Aliquots of urine and bile samples were added to Aquasol-2 scintillant (New England Nuclear, NEN, Boston, MA, U.S.A.). Aliquots of fecal samples were air-dried, ground, and oxidized to CO₂ in a sample oxidizer (Packard Instrument Co., model 360B). The exhaled air was trapped by Oxisorb-CO₂ absorber (NEN) and aliquots were dissolved in Oxiprep-2 scintillant (NEN). The radioactivity of these samples was determined using a liquid scintillation counter (Packard, model 4640). The fecal samples collected over 24 hr postdosing were homogenized with 4-fold their volume of distilled water. After centrifugation, the supernatant was examined by TLC-radiochromatography. A silica gel plate (Kieselgel 60 F254, df = 0.25 mm, E. Merck) was used. The solvent system employed was chloroform, methanol, and ammonia water (6:4:1, v/v).

**Intestinal Transport of Azetrelin** Transport of azetrelin, CF, and acetaminophen from the intestine was studied by the modified method of Barr and Riegelman, as reported previously. Rats were anesthetized with pentobarbital (32 mg/kg). The whole intestine was washed with saline and quickly removed. The intestine was divided into the duodenum (below the pylorus), jejunum (5 cm away from the ligament of Treitz), ileum (above the cecum), and colon (below the cecum). Each segment was everted and both ends of the everted intestine were cannulated with silicone tubes (i.d. 3 mm, o.d. 5 mm, Natsume) to make a 5 cm loop. As serosal fluid, 0.5 ml of pH 6.5 isotonic phosphate buffer solution containing 10 mM glucose was introduced. The everted loop was placed in the mucosal fluid (5 ml of pH 6.5 buffer solution containing 0.1 mM drug), which was bubbled with 5% CO₂-95% O₂ at 37 °C. Periodically, the whole serosal solution was collected and immediately replaced by the buffer solution.
containing no drug.

For the determination of azetirelin, 200 \( \mu l \) of the sample solutions were mixed with 200 \( \mu l \) of acetonitrile and 50 \( \mu l \) of 30 \( \mu g/ml \) 1-phenylephrine hydrochloride solution, the internal standard. After centrifugation, 10 \( \mu l \) of the supernatant was injected into the HPLC. For the determination of acetaminophen, the procedures were almost the same except for the use of 60 \( \mu g/ml \) of 2-acetaminophen as an internal standard solution. The concentration of CF was determined on a spectrofluorometer (Hitachi, 650-10S) after dilution of the aliquots with 0.2 mM sodium bicarbonate buffer (pH 10). The excitation and emission wavelengths were 520 and 490 nm, respectively.

The apparent permeability coefficient \( (P_{app}) \) of each compound was calculated according to the following equation:

\[
P_{app} = \frac{dC}{dt} \times \frac{1}{V(C_0 \times A)}
\]

where \( \frac{dC}{dt} \) is the change in concentration per unit time (\( \mu mol/ml \cdot s \)), \( V \) is the volume of the serosal fluid (ml), \( C_0 \) is the initial concentration of the mucosal drug solution (\( \mu mol/ml \)), and \( A \) is the surface area of the intestinal membrane (cm\(^2\)).

HPLC Conditions Azetirelin, TRH and acetaminophen were assayed by a reverse-phase HPLC procedure on a Nucleosil ODS column (150 \( \times \) 4.6 mm, 5 \( \mu m \)). The HPLC system used consisted of a liquid chromatograph pump (Hitachi, 635), a variable wavelength absorption monitor (Shimadzu, SPD-6A), a sample injector (Kyowa-seimitsu Co., Ltd., KSP-600), and a data processor (Shimadzu, C-R2AX). The mobile phase for azetirelin and TRH was a mixture of 0.01 M sodium 1-heptanesulfonate, acetonitrile, and methanol (87:8:5, v/v) adjusted to pH 4 with phosphoric acid. The mobile phase for acetaminophen consisted of water, acetonitrile, and methanol (88:6:6, v/v). The flow rate was 1.0 ml/min, and the column effluent was monitored at 220 nm (azetirelin and TRH) or 254 nm (acetaminophen).

Statistical Significance Results were expressed as the mean ± S.E. of at least 3 experiments. Statistical analyses were performed using the Student’s t-test.

RESULTS

Oral Bioavailability of Azetirelin in Rats Figure 2 shows the mean plasma concentration-time curves following the oral administration of azetirelin at doses of 10 and 25 mg/kg. The pharmacokinetic parameters were calculated and are summarized in Table I. The absorption lag time was not apparent after dosing, and \( T_{max} \) was within 0.5 h at 10 and 25 mg/kg. The \( C_{max} \) and \( AUC \) values increased almost in proportion to the dose. The systemic availability of azetirelin following oral administration was calculated by comparing the \( AUC \) with that after intravenous administration,\(^{10}\) and was estimated to be less than 2% at both 10 and 25 mg/kg. These results indicate that the oral bioavailability of azetirelin is extremely poor.

Contribution of the Hepatic First-Pass Metabolism to the Intestinal Absorption of Azetirelin Figure 3 shows the ratio of the average \( AUC \) values plotted against dose following either hepatoportal vein or intravenous injection of azetirelin to rats. The plasma azetirelin levels following hepatoportal vein injection increased in proportion to the dose (data not shown) and were virtually identical to those after intravenous administration. Consequently, the ratio of the average \( AUC \) values, \( AUC_{portal}/AUC_{i.v.} \), was close to 1 over the dose range studied (0.02—0.1 mg/kg).

Stability of Azetirelin in Luminal Fluid and Intestinal Mucosal Homogenates The stability of azetirelin against
luminal digestive enzymes was determined by incubating azetirelin with luminal fluid collected from the rat small intestine. The results are shown in Fig. 4A. Azetirelin was found to be stable in the luminal fluid, and more than 90% of the intact drug still remained at 180 min. Similar results were also obtained in the stability studies with mucosal homogenates of the small and large intestine, as shown in Fig. 4B. These results indicate that azetirelin is extremely stable against luminal digestive enzymes and mucosal peptide hydrolases in the rat intestine.

**Stability of Azetirelin in Cecal Contents** Degradation of azetirelin by bacterial metabolism was examined by incubating cecal contents of non-fasted rats with an azetirelin solution under an anaerobic condition. Figure 5 shows the time course of degradation of azetirelin and TRH in the suspension of cecal contents. Azetirelin was gradually degraded, and only about 30% of the intact drug remained after 180 min. The degradation rate of TRH was much faster than that of azetirelin, and no intact drug was found within 30 min. The susceptibility of these drugs to proteolysis in cecal contents was evaluated by their half-life, which was calculated from the first order rate constant obtained from the semilogarithmic plots shown in Fig. 5. The half-lives of azetirelin and TRH hydrolysis were 95 and 4 min, respectively. On the other hand, no degradation of azetirelin was observed when the cecal contents were sterilized by boiling prior to the experiments. This result suggests that intestinal microflora are responsible for the degradation of azetirelin.

**Excretion of Radioactivity after Oral Administration of [14C]Azetirelin to Rats** The cumulative percentages of radioactivity of orally administered [14C]azetirelin recovered in the urine, feces, exhaled air, and bile are presented in Fig. 6. By 72 h after dosing, 67.4% of the total radioactivity was exhaled as 14CO2, whereas the recovery of radioactivity in the urine and feces were 5.6% and 21.9%, respectively. The biliary excretion within 24 h postdosing was almost negligible (0.54%), indicating that...
most of the radioactivity in the feces is originated from unabsorbed azetirelin. Figure 7 shows the thin-layer autoradiogram of $[^{14}C]$azetirelin in fecal homogenates collected over 24 h after dosing. It was demonstrated that no intact drug remained in the feces. This indicates that unabsorbed azetirelin was completely degraded during the GI transit.

**Intestinal Permeability of Azetirelin by in Vitro Everted Gut Experiments** Figure 8 shows the time course of azetirelin transport across the various segments of rat intestine. The transport of azetirelin in the ileal segment seems to be slightly greater than that in the other segments. The $P_{app}$ of azetirelin in each segment was calculated and summarized in Table II. There was no significant difference between these segments regarding the intestinal transport of azetirelin. The $P_{app}$ value of azetirelin in the jejunal segment was compared with those of CF, a poorly absorbable model compound,$^{11}$ and acetaminophen, which is known to be an easily absorbable drug after oral administration. The results are summarized in Table III. As is evident from the table, the permeability of azetirelin was comparable to that of CF, and was much lower than that of acetaminophen. Table III also shows the logarithms of the distribution coefficient ($\log D$) of these compounds between octanol and a pH 6.5 phosphate buffer solution used in the transport studies. Again, the $\log D$ value of azetirelin ($-2.1$) was similar to that of CF ($-2.8$), indicating that azetirelin is a highly hydrophilic compound.

**DISCUSSION**

The present study has demonstrated that the oral bioavailability of azetirelin, a TRH-like tripeptide, was poor in rats and almost equal to that reported for TRH.$^{30}$ In our pilot studies, similar results were also obtained in other species such as dogs and humans.

There are several possible reasons for the low oral bioavailability of peptide and protein drugs, such as extensive hepatic first-pass metabolism, instability due to the presence of luminal and membrane-related enzymes, and impermeability across the intestinal mucosa.$^{1,2}$ In the present study, we examined each of these factors to elucidate the mechanisms involved in the low oral bioavailability of azetirelin.

The plasma azetirelin levels following intravenous and hepatoportal vein injection were compared in order to estimate the extent of first-pass metabolism in the liver. The results obtained here showed that the average $AUC$ values following administration by either routes were virtually identical regardless of the dose (Fig. 3). It has become apparent, therefore, that the hepatic first-pass effect is small and is not responsible for the low oral bioavailability of azetirelin.

From the results of our pilot study, azetirelin was found to be stable in the gastric environment. The present study showed that azetirelin was also stable against peptide hydrolyses both in luminal fluid and intestinal mucosal homogenates (Fig. 4). Similar findings were also noted for TRH by Yokohama et al.$^{41}$ Safran et al.$^{13}$ and Moss et al.$^{14}$ On the other hand, the bacterial metabolism of azetirelin and TRH in the GI tract was suggested, since the degradation of these drugs was observed in the cecal contents of non-fasted rats (Fig. 5). Vickers et al.$^{10}$ reported that MK-771 was also metabolized by gut microflora.$^{34}$ Thus, bacterial metabolism in the GI tract may be a common fate of orally administered TRH and its analogues. It is worth noting that the degradation rate of TRH was much faster than that of azetirelin. A possible explanation is the difference in their susceptibility to bacterial enzymes, since azetirelin is known to be more stable than TRH against enzymatic degradation in the body.$^{21}$

Further evidence of the bacterial metabolism of azetirelin in the GI tract was obtained by an in vivo excretion study. Extensive degradation of azetirelin during GI transit was directly demonstrated by TLC analysis of the fecal sample (Fig. 7).

The major route of excretion after the oral administration of $[^{14}C]$azetirelin was via expired air, where 67.4%
of the total radioactivity was excreted in 72 h. The urinary excretion was only 5.6% (Fig. 6). On the other hand, Nakamura et al. reported that 66.1% of the total dose was excreted as an intact drug from the urine after intravenous administration of unlabeled azetrelin.\(^\text{10}\) Thus, the results obtained here suggest that the metabolic fate of azetrelin after oral administration is quite different from that after intravenous administration. This may also be explained by the presystemic metabolism of azetrelin, probably by gut microflora, in the GI tract.

In recent years, several examples of drug metabolism by intestinal microflora have been discussed in relation to their low bioavailability after oral administration.\(^\text{13}\) Our study also implies the contribution of bacterial metabolism to the low oral bioavailability of azetrelin, since a large portion of orally administered azetrelin may reach the lower part of the intestine, because of its high stability against digestive enzymes and its poor intestinal permeability; thus, it is subjected to bacterial metabolism. Further insights about the mode of metabolism of azetrelin by intestinal microflora remain to be investigated.

The high hydrophilicity of azetrelin may be one of the main reasons for its poor intestinal permeability, which is essentially similar to the case of TRH. The log \(D\) value of azetrelin between octanol and pH 6.5 buffer solution is only \(-2.1\) (Table III), and this is almost identical to that of TRH (\(-2.46\) in octanol/pH 7.4 buffer solution).\(^\text{16}\) These values are far from being favorable for intestinal absorption.\(^\text{17}\)

The involvement of a carrier-mediated mechanism in the intestinal absorption of TRH was discussed by Yokohama et al.\(^\text{45}\) They demonstrated that the absorption of TRH occurs only in upper region of the small intestine. We found, however, no such regional specificity in the intestinal transport of azetrelin (Table II). Therefore, it seems likely that the absorption mechanism of azetrelin is different from that of TRH. The details of the intestinal transport mechanism of azetrelin may be discussed in a subsequent publication.

In conclusion, the data presented here indicate that the low oral bioavailability of azetrelin is attributed to its poor membrane permeability due to its lack of lipophilicity, as well as the degradation of the peptide by gut microflora, which seems to inhibit its absorption in the lower part of the intestine.

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