Nasal Absorption Kinetic Behavior of Azetirelin and Its Enhancement by Acylcarnitines in Rats

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Received June 3, 1997; accepted October 16, 1997

Purpose. The long-term stability and nasal absorption characteristics of a basic nasal formulation of azetirelin, a thyrotropin-releasing hormone analog and its absorption enhancement by incorporation of acylcarnitines in the formulation were investigated.

Methods. The long-term stability of basic nasal azetirelin formulations at 25 °C was predicted by calculation from the Arrhenius plot of the data on 6 months' storage at 40, 50 and 60 °C. Nasal azetirelin absorption characteristics were kinetically examined by intranasal administration to rats, determination of plasma azetirelin level by radioimmunoassay, and fitting the data to a two-compartment model including absorption rate.

Results. Basic nasal azetirelin formulations of pH 4.0 and pH 5.1 were predicted to be highly stable. Residual azetirelin after 2 years storage at 25 °C was greater than 95%. Nasal absorption characteristics of this formulation in the pH 4.0–6.3 range showed pH-dependency, with pH 4.0 showing the highest absolute bioavailability (Bioav) of 17.1%. This nasal Bioav was 21 times greater than that of oral administration (0.8%). Acylcarnitines with 12 or more carbon atoms in the acyl chain greatly enhanced nasal absorption of azetirelin: Bioavs with lauroylcarnitine chloride (LCC) and palmitoylcarnitine chloride were 96.9% and 72.9%, respectively. This enhancement by LCC plateaued at the low concentration of 0.1%.

Conclusions. The basic nasal azetirelin formulation at pH 4.0 is stable and shows adequate absorption, with nasal absorption having greater Bioav than oral absorption. The 12-carbon acylate LCC was the strongest enhancer among acylcarnitines and provided near-total delivery of the administered dose to the blood.

KEY WORDS: azetirelin; thyrotropin-releasing hormone analog; stability; nasal absorption; acylcarnitines.

INTRODUCTION

Azetirelin is a novel analog of the tripeptide thyrotropin-releasing hormone (TRH) in which the pyroglutamyl moiety of TRH is substituted by an (oxo-azetidinyl) carbonyl moiety. The activating effects of azetirelin on the CNS, such as its inhibition of pentobarbital-induced sleep and reserpine-induced hypothermia, are about 10–100 times more potent and 8–36 times longer-acting than those of TRH in mice (1). Unlike TRH, which undergoes rapid enzymatic inactivation in the body, azetirelin is extremely stable in plasma and is degraded much more slowly than TRH in brain homogenates (2). This increased metabolic stability of azetirelin may confer greater pharmacological potency and efficacy over TRH. Although azetirelin has strong potential pharmacological properties when administered by intravenous injection, its estimated oral bioavailability is reported to be very low, around only 2% (3).

In recent years, the systemic delivery of peptides and proteins by nasal administration as a non-parenteral route has received growing attention. However, development of clinically useful peptides and proteins has met with limited success. Larger molecules in particular show little or no systemic absorption upon nasal administration and require the use of effective and safe enhancers. Among enhancers studied to date, however, polyoxyethylene 9-lauryl ether, deoxycholate, sodium tauro-24, 25-dihydrofusidate, and lysophosphatidylcholine have been recently shown to produce irreversible or severe side effects on mucociliary transport rate, nasal morphology and ciliary beat frequency (4). In contrast, acylcarnitines are endogenous amino acid-like compounds which play a role in the cellular mitochondrial transport system by carrying fatty acids across the mitochondrial membrane (5). The local mucosal safety of palmitoylcarnitine has been recently demonstrated. This compound's enhancement of drug absorption (6) and mucosal transepithelial electrical resistance (7) were reversible, and no mucosal morphological change was reported (6,7).

The aim of the present study was to investigate the nasal absorption profile of azetirelin using a rat model. First, the long-term stability of basic nasal azetirelin formulations was tested at various pHs and storage temperatures. Second, the potential nasal azetirelin absorption in these solutions was assessed. Finally, the enhancing ability of acylcarnitines on nasal absorption of azetirelin was investigated using various acylcarnitines.

MATERIALS AND METHODS

Chemicals and Drug Solutions

Azetirelin (N^{α}-[((S)-4-oxo-2-azetidinyl) carbonyl]-L-histidyl-L-prolineamide dihydrate, MW=384.39, pKa=6.2) was synthesized in the Central Research Laboratories of Yamanouchi Pharmaceutical Co., Ltd. DL-Carnitine hydrochloride (CHC), DL-octanoylcarnitine chloride (OCC), DL-lauroylcarnitine chloride (LCC), palmitoyl-DL-carnitine chloride (PCC), sodium taurocholate (TRC), phenylephrine hydrochloride, and sodium 1-heptansulfonate were purchased from Sigma Chemical Co., Ltd. (USA). All other reagents used were of reagent grade.

The basic nasal formulation contained azetirelin (50 mg/mL) in 0.01 M isotonic citrate buffer, with benzalkonium chloride (0.01%) as a preservative. After incorporation of azetirelin and acylcarnitines, all solutions were adjusted for pH with NaOH or HCl. PCC was incorporated in the basic formulation before use.

Stability Studies

Three basic nasal solutions at pH 4.0, 5.1 and 6.3 were prepared. All sample solutions were sealed in 5-mL glass ampoules and stored in an oven at 40, 50 or 60 °C for 6 months. At specified storage times, samples were collected and stored in a freezer at −20 °C until HPLC analysis. pH values were also measured in each sample to confirm pH stability.

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0724-8741/98/0100-0077$15.00/0

HPLC Analysis

HPLC analysis was carried out by the internal standard method. Samples were diluted 50 times and mixed with an equivalent volume of 0.1% phenylephrine hydrochloride (internal standard). Ten milliliters of this mixture was injected into a Nucleosil $_5C_{18}$ HPLC column (5 μm, 4 mm×150 mm, Chemco Science Co., Ltd., Japan) for separation of analytes and detection at 220 nm. The mobile phase was a mixture of 2% sodium 1-heptansulfonate : acetonitrile : methanol (50:3:3), pumped at 1.5 mL/min at less than 40 °C. The concentration of azetirelin was determined by comparing the peak area ratio (drug/internal standard) of sample with that of standard solutions from the calibration curve.

Animal Experiments

All experiments were conducted in adherence to the "Principles of the Animal Ethical Committee of Yamanouchi Pharmaceutical Co., Ltd.". Male Fischer rats (135–182 g, 8 weeks) were fasted for 20 h before administration. Anesthesia was induced by intraperitoneal injection of sodium pentobarbital (Nembutal®; Abbott Laboratories, USA) at 50 mg/kg 10 min before administration and maintained with additional injections at 40 mg/kg. No surgical operation was conducted.

For intranasal administration, the nasopalatine was closed with a cyanoacrylate adhesive agent (Aron Alpha A®; Sankyo Co., Japan). Polyethylene tubing (PE10) connected to a 5-μL micro-syringe was then inserted about 4 mm into the right nasal cavity. A buffered azetirelin solution sample (1 mg/20 μL/kg) was administered within 1 min. For oral administration, a buffered azetirelin solution sample (10 mg/4 mL/kg) was delivered to the stomach through a sound within 1 min. For intravenous administration, azetirelin solution (1 mg/mL/kg) in saline (0.9% NaCl) was injected into the jugular vein. At each time point, a rat was sacrificed and blood was collected from the inferior vena cava with a heparinized syringe. Plasma was separated by centrifugation at 3000 rpm for 10 min at 4 °C and stored at −20 °C until assay. After extraction of a 0.5 mL plasma sample with methanol, the plasma concentrations of azetirelin were determined by radioimmunoassay (8).

Pharmacokinetic Analysis

For pharmacokinetic analysis of the overall absorption behavior of azetirelin in rats, a two compartment analysis model including a nasal (or oral) compartment was used. Equations 1 and 2 were derived by integration:

$$C_1 = Ae^{-\alpha \times t} + Be^{-\beta \times t} = \frac{D_1}{V_c}\left[\frac{(K_{21}-\alpha)}{(\beta-\alpha)}e^{-\alpha \times t} + \frac{(K_{21}-\beta)}{(\alpha-\beta)}e^{-\beta \times t}\right] \quad (1)$$

$$C_2 = \frac{F_a \times D_2 \times K_a}{V_c}\left[\frac{(K_{21}-K_a)}{(\alpha-K_a)\times(\beta-K_a)}e^{-K_a \times (t-\mathrm{Lag}T)} + \frac{(K_{21}-\alpha)}{(K_a-\alpha)\times(\beta-\alpha)}e^{-\alpha \times (t-\mathrm{Lag}T)} + \frac{(K_{21}-\beta)}{(K_a-\beta)\times(\alpha-\beta)}e^{-\beta \times (t-\mathrm{Lag}T)}\right] \quad (2)$$

where V_c is the volume of the central compartment; α and β are the first-order macro-rate constants describing the disposition of the drug; K_{12} and K_{21} are the first-order rate constants for the transfer of drug between central and peripheral compartments; C_1 and C_2 are the concentrations of drug administered intravenously and intranasally (or orally), respectively; D_1 and D_2 are the amounts of drug administered; and LagT, F_a, and K_a are the lag time, fraction absorbed, and first-order absorption rate constant of nasal (or oral) absorption, respectively. Absolute bioavailability (Bioav) was calculated as $F_a \times 100$. Absorption-duration time, the time to absorption of 95% of total possible azetirelin absorption ($T_{95\%}$), was calculated as LagT − (ln 0.05/K_a).

Intravenous pharmacokinetic parameters were estimated by fitting Eq. 1 to the average data of rats. Using intravenous parameters of V_c, K_{12}, K_{21}, α and β, intranasal (or oral) absorption pharmacokinetic parameters (LagT, Fa, and Ka) were estimated by fitting Eq. 2 to the average data sets. Computation was carried out using the nonlinear least-squares regression analysis program NONLIN84® (Statistical Consultants, USA) on a VAX6210 digital computer (Digital Equipment Corp., USA). Hartley and Levenberg's modification of the Gauss-Newton method was used.

RESULTS AND DISCUSSION

Degradation Kinetics of Basic Nasal Azetirelin Formulation

In order to optimize a basic nasal formulation, the stability of buffered azetirelin solutions was tested. Preliminary studies showed that azetirelin was not sufficiently stable at pHs greater than 7.0. Therefore, the pH range of the buffer was set between 4 and 6. Figure 1 (inset) shows representative stability profile sets for azetirelin in pH 4.0 citrate buffer solution containing a preservative at different temperatures (40, 50 and 60 °C) for 6 months. The linear relationship between logarithmic percentage remaining and storage time indicates pseudo-first-order degradation kinetics. The degradation rate constant was calculated from the slope of the graph by linear regression analysis. Regression lines were linear for all pH values studied, with a correlation coefficient (r) > 0.957.

The Arrhenius plot was constructed from these observed first-order degradation rate constants at three temperatures (Fig. 1). This plot shows a good linear relationship between logarithmic degradation rate (K_{deg}) and the reciprocal of absolute temperature (T): r = 1.000 for pH 4.0, 0.999 for pH 5.1, and 0.997 for pH 6.3 (Table 1). Activation energy (E_a) was evaluated according to Arrhenius equation: ln K_{deg} = ln A − (E_a/RT), where A is the frequency factor and R is the gas constant. From these Arrhenius parameters, the percent remaining ($R_{\%}$) of azetirelin at specified temperature and storage time (t) conditions can be predicted by the equation $R_{\%} = 100 \times \exp^{-\{A \times \exp(-E_a/RT)\} \times t}$. Long-term stability of basic nasal azetirelin formulations was predicted at 25 °C which is average room temperature. Therefore it is of greatest importance to assure product stability at this temperature. It was found that $R_{\%}$ was pH-dependent, greater than 95% residual azetirelin was predicted after 2 years' storage for the pH 4.0 (95.1%) and 5.1 (97.6%) formulations, but less than 90% for the pH 6.3 (89.1%).

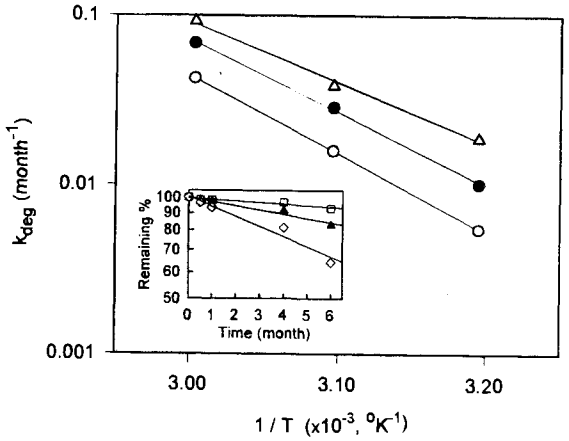

Fig. 1. Arrhenius plot for the basic nasal formulation at three different pHs: (●) pH 4.0, (○) pH 5.1, and (△) pH 6.3. (Inset) Representative sets of stability profiles for azetirelin in pH 4.0 buffer solution at different temperatures: (□) 40°C, (▲) 50°C and (◇) 60°C.

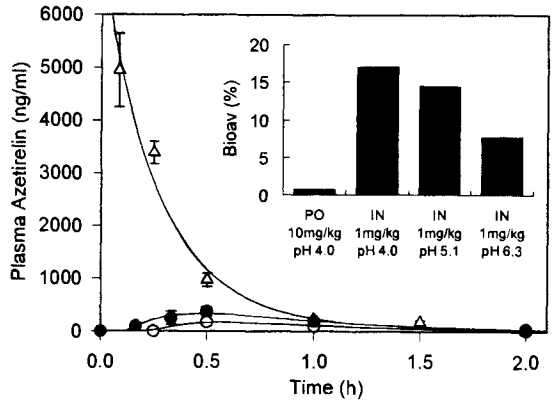

Fig. 2. Time course of blood azetirelin concentrations after intravenous (1 mg/kg), intranasal (1 mg/kg), and oral (10 mg/kg) administration of azetirelin in rats. (△) intravenous (r = 0.970), (●) intranasal (r = 0.983), (○) oral (r = 0.970). Solid curves were calculated by computer-fitting. Each point represents the mean ± SE of 3 to 5 rats. (Inset) pH dependency on intranasal NT 36 absorption of basic nasal formulation.

Nasal absorption of azetirelin in rats was subsequently investigated using these stable pH formulations.

Comparison of Nasal and Oral Kinetic Absorption of Azetirelin

Nasal absorption of azetirelin was compared with oral absorption in rats. Azetirelin was administered intravenously (1 mg/kg), nasally (1 mg/kg, pH 4.0), or orally (10 mg/kg, pH 4.0), and plasma azetirelin levels were determined by radioimmunoassay. The plasma azetirelin profiles (Fig. 2) were well-fitted to the two compartment analysis including nasal (or oral) absorption pharmacokinetic parameters (LagT, Fa, and Ka). Even though time points were obtained from only single rats, reasonably good fits between the predicted and observed points were obtained, with r = 0.991 for intravenous, between 0.939 and 0.983 for nasal, and 0.970 for oral data (Table 2).

At the same buffered drug solution of pH 4.0, absolute bioavailability (Bioav) after nasal administration (17.1%) was 21 times higher than that after oral administration (0.8%). This low oral bioavailability of azetirelin was thought to be due mainly to a lack of lipophilicity Also degradation of the peptide by intestinal microflora but not peptide hydrolases both in luminal fluid and intestinal mucosal homogenates was another possibility (3). In pilot studies in humans, the oral bioavailability of azetirelin was about 2% as low as seen in rats. Similar results have been demonstrated for TRH (9). Oral bioavailability of TRH in humans and rats was as low as that of azetirelin (2% or less). Further, nasal bioavailability of TRH both in humans (10) and rats (11) was similarly high, at about 20%. Since azetirelin is an analog of TRH, its pharmacokinetic properties can be assumed to be very close to those of TRH. Therefore, nasal delivery of azetirelin in humans using nasal basic azetirelin formulation at pH 4.0 is predicted to have a bioavailability of about 20%.

Table 1. Arrhenius Parameters and Prediction of Long-Term Stability of Azetirelin[a]

	Arrhenius Parameters			Calculated Values at 25 °C		
	Ea	A		Residual %		
	kcal/mol	1/month	r	0.5 years	1 year	2 years
pH 4.0	19.8	6.54E + 11	1.000	98.7	97.5	95.1
pH 5.1	21.2	3.77E + 12	0.999	99.4	98.8	97.6
pH 6.3	16.4	5.49E + 09	0.997	97.2	94.4	89.1

[a] Each parameter was calculated by computer-fitting.

Effect of pH on Nasal Absorption of Basic Nasal Azetirelin Formulation

The nasal absorption of azetirelin shows pH dependency. Bioav decreased from 17.1% to 7.8% as pH increased from

Table 2. Pharmacokinetic Parameters after Intravenous (1 mg/kg), Oral (10 mg/kg), and Intranasal (1 mg/kg) Administration of Azetirelin in Rats[a]

	A ng/mL		B ng/mL	α 1/h	β 1/h		r
Intravenous	6840		229	3.81	0.950		0.991
	Promoter %	pH	Bioav %	Ka 1/h	LagT min	$T_{95\%}$ h	r
Oral	—	4.0	0.8	2.45	14.9	1.47	0.970
Nasal							
BAS1	—	4.0	17.1	2.2	7.8	1.51	0.983
BAS2	—	5.1	14.5	1.4	5.0	2.18	0.989
BAS3	—	6.3	7.8	2.9	7.2	1.15	0.939
Nasal							
CHC	1	4.0	10.3	22.7	9.7	0.29	0.988
OCC	1	4.0	10.7	13.6	5.1	0.31	0.999
LCC	0.03	4.0	12.6	11.0	3.5	0.33	0.999
LCC	0.1	4.0	82.9	43.5	7.9	0.18	0.996
LCC	1	4.0	96.9	11.5	0.0	0.26	0.996
LCC	3	4.0	80.5	44.2	8.1	0.20	0.995
PCC	1	4.0	72.9	15.8	0.0	0.19	0.996
TRC	1	4.0	36.6	10.2	7.8	0.42	0.976

[a] Each parameter was calculated by computer-fitting to the mean of 3 to 5 rats.

4.0 to 6.3 (Fig. 2 (inset)). This greater Bioav of azetirelin at low pH coincides with those of other peptides after intranasal administration, for example insulin in dogs (12) and secretin in rats (13). The dissociation of the peptide hexamer to the monomer at pH 2–3 in the case of insulin, and damage to nasal mucosa at pH 2.9 in the case of secretin respectively were thought to be the primary causes of the greater Bioav of these peptides at low pH. Azetirelin is as small a peptide as the TRH tripeptide, and the self-association of TRH is not reported to date. On our histological examination, no morphological change in rat or rabbit nasal mucosa were observed after intranasal administration of the buffered azetirelin formulations at pH 4.0, 5.1 and 6.3. In the nasal mucosa, the same types of aminopeptidases exist as in the intestinal mucosa (14). The activity of these aminopeptidases was reported to be pH dependent, with maximum enzyme activity at neutral pH and reduced activity towards a low pH of 3.5 (15). Enzymatic hydrolysis of azetirelin in the nasal mucosa may be affected by pH, and thereby may affect its bioavailability. On the other hand, cationic species contributing to the nasal absorption of a drug at low pH has also been proposed for salmon calcitonin (SCT) (16). Considering that the pK_a value of azetirelin is 6.2, cationic species of this peptide may be better able to interact with the negative charge of the nasal epithelium at a pH less than neutral.

Enhancing Ability of Acylcarnitines on Nasal Absorption of Azetirelin

The stability and nasal absorption data for the basic nasal azetirelin formulation indicated that its optimal pH was pH 4.0. The basic formulation of pH 4.0 was used to investigate the ability of acylcarnitines to enhance nasal absorption of azetirelin in rats.

Specific plasma azetirelin profiles (Fig. 3 (inset)) were obtained after intranasal administration of formulations containing one from a family of acylcarnitines (1%) with different numbers of carbon atoms in the acyl chain (n) of n = 0 (CHC), n = 8 (OCC), n = 12 (LCC), and n = 16 (PCC). These profiles

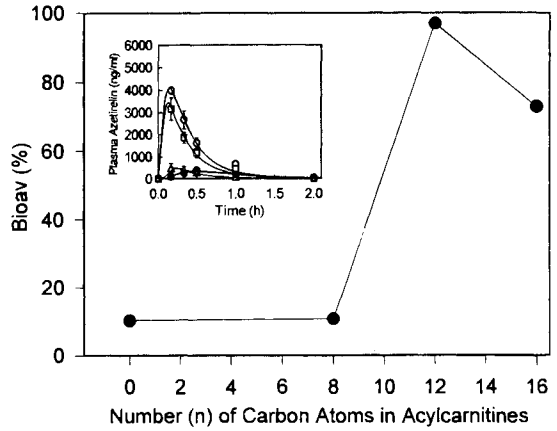

Fig. 3. Effect of the number (n) of carbon atoms in the acyl chain of acylcarnitines (1%) on nasal absorption of azetirelin (1 mg/kg) in rats. (Inset) Time course of blood azetirelin concentration after intranasal (1 mg/kg) administration of azetirelin in rats. (●) CHC (r = 0.988), (△) OCC (r = 0.999), (○) LCC (r = 0.996), (□) PCC (r = 0.996). Solid curves were calculated from computer-fitting. Each point represents the mean ± SE of 3 or 4 rats.

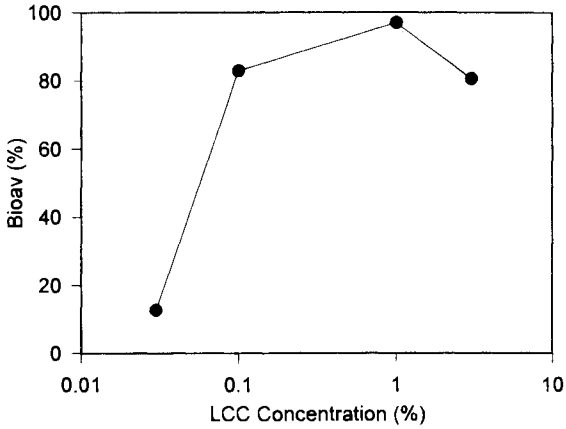

Fig. 4. Dependency of nasal azetirelin (1 mg/kg) absorption in rats on the concentration of LCC. Each point represents the mean of 3 or 4 rats.

fit well into the two compartment model, including nasal absorption pharmacokinetic parameters, in the r range of 0.988 − 0.999. Results showed a clear structure-dependency of acylcarnitines on Bioav (Fig. 3). LCC and PCC strongly enhanced nasal azetirelin absorption (Bioavs of 96.9% and 72.9%, respectively), whereas OCC and CHC did not (10.7% and 10.3%, respectively). This enhancement by LCC and PCC was much greater than that seen with 1% TRC (36.6%, Table 2). TRC, a bile salt, is well-known to enhance the nasal absorption of drugs, but is also toxic to nasal mucosa (4). Importantly, long-chain acylcarnitines produced near-total intranasal delivery of a small peptide (azetirelin) to the blood. This observed bell-shaped enhancement of azetirelin absorption as a function of acyl chain length of acylcarnitines was also observed in the membrane perturbing capacity of acylcarnitines as previously reported (17). There appeared to be a critical chain length (n = 12) for acylcarnitine activity in the perturbation. In order to partition into the nasal mucosa and perturb the lipid order, acylcarnitines must possess a critical chain length. If the length becomes longer, then incorporation into the lipid bilayer of the membrane may become unfavorable.

The dose-dependency of acylcarnitines was also investigated using LCC (Fig. 4). Bioavs with LCC at a concentration of 0.1–3% were markedly greater (80.5–96.5%) than that at 0.03% (12.6%). Enhancement plateaued at 0.1%. Interestingly, this maximum and saturated enhancement with LCC coincides with our previous results for SCT, a 32-amino acid: LCC was the strongest acylcarnitine enhancer of nasal SCT absorption, and enhancement was saturated at the low concentration of 0.1% (15). Taking into account the fact that the critical micelle concentration of LCC is 0.03%, it has been suggested that acylcarnitine micelle formation plays a key role in their enhancement of nasal azetirelin absorption, as was seen with SCT (15). The reason for the decrease in Bioav at 3% of LCC may relate to the increase in the number and size of the cationic micelles of LCC (pKa = 4.85). These micelles may block water channels in the tight junction of nasal mucosa and inhibit the paracellular transport of azetirelin. Acylcarnitine reportedly loosens these tight junctions, enhancing drug absorption (7).

Absorption-duration times ($T_{95\%}$s) for acylcarnitines were in the range of 0.19–0.33 h, whereas those for oral and intranasal

administration without acylcarnitines were 1.47 h and 1.51 h, respectively. The important characteristics of nasal azetirelin formulated with acylcarnitines are their short absorption-duration and very high efficiency in delivery. These findings suggest that the greatly enhanced intranasal administration of azetirelin using acylcarnitines will be an alternative non-parenteral delivery method.

In conclusion, the following results were found concerning the nasal absorption of azetirelin: 1. Basic nasal azetirelin formulations at pH 4.0 and pH 5.1 are predicted to be stable for at least 2 years at 25 °C, using an Arrhenius plot. 2. Then nasal absorption of azetirelin in rats is pH-dependent. Among pH 4.0–6.3 formulations, intranasal administration at pH 4.0 had the highest absolute bioavailability of 17.1%, which was 21 times greater than that of oral administration (0.8%). 3. Acylcarnitines with 12 or more carbon atoms in the acyl chain enhanced the nasal absorption of azetirelin. In particular, the absolute bioavailability was maximally enhanced by LCC, at 96.9%.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Professor A. Tsuji, Kanazawa University, for his stimulating and helpful suggestions, and Mr. E. Nakamura and Mr. I. Sasaki for their technical radioimmunoassay assistance.

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