ENHANCED INTESTINAL ABSORPTION OF AMINO ACIDS AND AMINO ACID-LIKE DRUG BY POSSIBLE FORMATION OF ENAMINE IN ADMINISTERED SOLUTION BY THE PRESENCE OF ETHYLACETOACETATE

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(Received August 2, 1983)

Ethylacetoacetate promoted the colonic and the jejunal absorption of amino acids such as L-phenylalanine and D-phenylalanine when administered in aqueous solution. Enhanced absorption of these isomers of amino acid might occur *via* a formation of enamine, which was observed in aqueous solution by monitoring ultraviolet absorbance at 288 nm. Since mixture of L- or D-phenylalanine and ethylacetoacetate in aqueous solution enhanced colonic absorption of cefmetazole while the solution did not affect so much on the jejunal absorption of cefmetazole compared to the colonic absorption of it, enhancing action of enamine probably produced in aqueous solution may be more effective at the colonic compartment.

Keywords —sodium phenylalanine; sodium ampicillin; sodium cefmetazole; ethylacetoacetate; adjuvant; enamine; colon; jejunum; *in situ* loop method

It has been established that enamine formed by reacting drugs having amino moiety in the structure with β -dicarbonyl compounds are prospective for the practical purpose as prodrugs or adjuvants enhancing rectal drug absorption.¹⁻⁴⁾ Formation of enamine derivatives of amino acid-like compounds with β -dicarbonyl compounds are fairly rapid and stable in organic solvents. Reaction and behavior of enamine formation in aqueous solution are not sufficiently studied to understand the intestinal mucosal membrane permeability to enamine and to elucidate the mechanism of enhanced drug absorption in the intestinal tract. It has been reported¹⁾ that some enamines are easily hydrolyzed in aqueous solution and the reaction is accelerated in acidic solution. But, if enamines could be easily formed and fairly stable in aqueous solution, amino acid-like drugs may be transformed to enamine by incorporating β -dicarbonyl compounds in aqueous preparation before administration and can be used as aqueous microenema for rectal delivery preparation.

In the present study, enamine formation of D-, L-phenylalanine and ampicillin with ethylacetoacetate, a β -dicarbonyl compound used as flavoring agent and approved as food additive, in aqueous solution and the effect of enamine formation on the intestinal absorption of phenylalanine and ampicillin were studied. And also the effects of phenylalanine enamine of ethylacetoacetate on the rectal absorption of cefmetazole, which does not have amino moiety in the structure and does not penetrate well through intestinal mucosal membrane because of its low lipophilicity, was studied employing an *in situ* loop technique in rats.

MATERIALS AND METHODS

Materials —D-, L-Phenylalanine and ethylacetoacetate were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Sodium cefmetazole was supplied from Sankyo Co., Ltd. (Tokyo, Japan). Sodium ampicillin was supplied from Toyo-Jozo Co., Ltd. (Tokyo, Japan). Other reagents used were of analytical grade. Sodium

salts of phenylalanine was routinely prepared by neutralization method.

Preparation of Test Solution Administered — Aqueous test solution containing sodium salt of phenylalanine or ampicillin and ethylacetoacetate was incubated at 37°C for 1 h prior to administration to rats. For the preparation of drug test solution containing sodium cefmetazole, sodium cefmetazole was dissolved in the test solution immediately before experiments. Test solution was administered into each loop employing a microsyringe at a dosage volume of 200 μ l/kg.

Animals — Wistar male rats, 225 to 250 g, were fasted for 16 h prior to the experiments but water was given freely. During the experiment, rats were anesthetized with sodium pentobarbital (32 mg/kg) and placed on a hot plate kept at 38°C.

In Situ Loop Study — The jejunum and the colon were exposed by abdominal incision, and 4 cm loops of upper jejunum and colon were prepared by ligation of each end.⁵⁾ Each loop was removed at a designated time intervals after the administration. Compounds remained in loops were collected by washing with 0.001 N HCl solution and adjusted to 20 ml. The absorption of compounds was determined by the difference of remaining amounts against administered amounts in each loop. After the experiment, wet weight of each loop tissue was measured.

Analytical Method — Phenylalanine, ethylacetoacetate and cefmetazole in sample solution were measured by high performance liquid chromatography (HPLC) method according to the following condition*; a liquid chromatograph model Trirotor-II equipped with UV-detector model UVIDEC-100-III (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a column of 4.6 mm $\times$ 250 mm packed with reversed phase (silica gel C-18) were used. Methyl alcohol-0.03 M citrate buffer at pH 5.9 (20:80) was delivered as mobile phase at a 1 ml/min flow rate. Assay of sodium ampicillin was carried out by microbio-

assay described in the previous paper.¹⁾

Study of Formation of Enamine — Aqueous solution containing sodium salts of phenylalanine or ampicillin and ethylacetoacetate was incubated at 37°C under shaking. The pH of incubated solution was 9.0 to 9.5 in all experiments. Aliquots of sample solution (100 μ l) was collected at designated time intervals. The sample solution was diluted with sufficient amounts of methyl alcohol solution below 10°C and was measured at 288 nm, the maximum absorbance of the enamine synthesized from sodium phenylalanine or ampicillin and ethylacetoacetate in organic solvent,¹⁾ using a Shimadzu Spectrophotometer UV-200. Methyl alcohol did not affect the formation and hydrolysis of enamine below 10°C.

RESULTS AND DISCUSSION

Although it is well known⁶⁾ that essential amino acids are mainly absorbed by an active transport and the absorption of D-isomer is 6 times smaller than that of L-isomer from jejunum, absorption of D- and L-phenylalanine from the jejunum and the colon was compared. An unphysiological high pH should be noted since pH of administered solution was 9.0 to 9.5 due to high concentration of each sodium phenylalanine.

As shown in Fig. 1, absorption of L-phenylalanine from the jejunal loop occurred extensively and more than 95% of phenylalanine was absorbed from the loop during 30 min after administration. On the other hand, absorption of D-phenylalanine was about 25% at 30 min after the administration. And, in rat colonic loop, more than 75% of each D- and L-phenylalanine remained at the same period, as shown in Fig. 2. The absorption of sodium cefmetazole from the colonic and jejunal loop was not affected by the presence of sodium D- or L-phenylalanine in administered solution.

These results may suggest that mucosal barrier of the jejunum and the colon is not so much

* Retention time of phenylalanine, cefmetazole and ethylacetoacetate: 7.1, 10.2 and 13.5 min, respectively.

modified by the administration of alkaline test solution (pH 9.0 to 9.5) used in this study for the these compounds' absorption. Taking into account of relatively small dosage volume which is diluted with intestinal fluid, still non-physiological alkaline condition could continue for some time before physiological buffering action will operate. Since absorption of cefmetazole

used as a model of poorly absorbed drug was not so much affected even by the coadministration of 0.66 M sodium phenylalanine (about 5 to 6% absorption, see in Table II) compared to the administration of the drug alone ($5.3 \pm 2.1\%$ absorption, $n = 4$), following study was carried out without using physiological buffer.

Absorption of D-phenylalanine from the jeju-

FIG. 1. Disappearance of L-Phenylalanine ($\circ$ and $\bullet$) and D-Phenylalanine ($\triangle$ and $\blacktriangle$) in Absence ($\circ$ and $\triangle$) and Presence ($\bullet$ and $\blacktriangle$) of 1.32 M Ethylacetoacetate from Rat Jejunal Loop as a Function of Time after Administration of Aqueous Solution Containing 0.66 M Sodium L- or D-Phenylalanine at a Dosage Volume of 200 μ l/kg

Wet weight of the jejunal loops used in this study was 328 ± 32 mg. Disappearance of ethylacetoacetate was shown in symbol, $\blacksquare$. Each value represents mean $\pm$ SD ($n \geq 4$).

FIG. 2. Disappearance of L-Phenylalanine ($\circ$ and $\bullet$) and D-Phenylalanine ($\triangle$ and $\blacktriangle$) in Absence ($\circ$ and $\triangle$) and Presence ($\bullet$ and $\blacktriangle$) of 1.32 M Ethylacetoacetate from Rat the Colonic Loop as a Function of Time after Administration of Aqueous Solution Containing 0.66 M Sodium L- or D-Phenylalanine at a Dosage Volume of 200 μ l/kg

Wet weight of the colonic loops used in this study was 284 ± 21 mg. Disappearance of ethylacetoacetate was shown in symbol, $\blacksquare$. Each value represents mean $\pm$ SD ($n \geq 4$).

nal loop after administration of test solution containing 0.66 M sodium D-phenylalanine was enhanced significantly by the presence of 1.32 M ethylacetoacetate in the solution (Fig. 1), though absorption of L-phenylalanine from the jejunal loop was promoted only at first 5 min by the presence of ethylacetoacetate. This small effect against the absorption of L-phenylalanine may be due to rapid absorption of L-phenylalanine alone. On the other hand, in the colonic loop, each D- and L-phenylalanine was significantly absorbed by the presence of ethylacetoacetate in the solution and only about 30% of each of them remained in the loop at 30 min after administration (Fig. 2).

Further, enhanced absorption of D-phenylalanine examined from the loop showed dependency on the concentration of ethylaceto-

acetate in administered solution, as can be seen in Table I.

As another model drug for amino acid-like drug, absorption of sodium ampicillin from the colonic and jejunal loop was studied. As shown in Table I, absorption of ampicillin from the both loop occurred significantly by the presence of ethylacetoacetate compared to that in the absence of ethylacetoacetate in administered solution. However, ethylacetoacetate did not increase the cefmetazole absorption from the colonic loop ($5.6 \pm 1.6\%$, $n = 5$) and the jejunal loop ($6.0 \pm 2.3\%$, $n = 5$).

From above finding, a possible complex formation between phenylalanine or ampicillin and ethylacetoacetate should be considered to explain the enhanced absorption of phenylalanine and ampicillin. The fact that the ethylace-

TABLE I. *Enamine Formation Constant, K_{obs} , of Sodium Phenylalanine or Sodium Ampicillin with Ethylacetoacetate, and the Absorption of Sodium Phenylalanine and Ampicillin by the Presence of Ethylacetoacetate from Rat Jejunal and Colonic Loop with 4 cm Length at 10 min after Administration*

Composition in aqueous solution administered and K_{obs}		Absorbed percent of phenylalanine or ampicillin	
Concn. of amino acid or ampicillin (M)	Concn. of ethylacetoacetate $K_{obs}^{a)}$ (M)	At the jejunal loop	At the colonic loop
Sodium D-phenylalanine 0.66	0.00	16.8 + 4.2	7.6 + 2.6
	0.66	32.2 + 5.5 ^{b)}	37.7 + 6.1 ^{b)}
	0.99	40.6 + 6.3 ^{b)}	48.5 + 4.8 ^{b)}
	1.32	48.1 + 5.5 ^{c)}	56.1 + 4.9 ^{c)}
Sodium L-phenylalanine 0.66	0.00	—	—
	0.66	22.3	—
	0.99	25.8	—
	1.32	27.4	—
Sodium ampicillin 0.10	0.00	9.7 + 2.3	3.8 + 2.5
	0.10	16.2 + 2.7 ^{b)}	15.8 + 3.8 ^{b)}
	0.20	24.5 + 3.6 ^{c)}	22.5 + 4.2 ^{c)}

a) $K_{obs} = (\text{enamine}) / (\text{phenylalanine or ampicillin}) (\text{ethylacetoacetate})$.

b) $p < 0.01$ against without ethylacetoacetate (Student's *t*-test, $n \geq 4$).

c) $p < 0.01$ against without ethylacetoacetate and with 0.66 M ethylacetoacetate for phenylalanine or 0.10 M ethylacetoacetate for ampicillin ($n \geq 4$).

toacetate did not influence the absorption of cefmetazole may strongly support this possible complex formation described above, because cefmetazole does not have active amino group to react with β -dicarbonyl.

To study the complex formation of phenylalanine or ampicillin with ethylacetoacetate, spectrophotometric study was carried out according to the method described in the experimental section. The mixture of aqueous solution of sodium phenylalanine or sodium ampicillin and ethylacetoacetate produced the new λ max at 288 nm and the absorbance at 288 nm increased according to the incubation time at 37°C, as shown in Fig. 3 for phenylalanine. Since synthesized enamine of sodium phenylalanine or

sodium ampicillin with ethylacetoacetate showed the λ max at 288 nm, it is indicated that the above mixture should produce the enamine complex even in aqueous solution. This enamine formation reached to an equilibrium state against the starting materials at 1 h after incubation at 37°C. The calculated apparent enamine formation constants were summarized in Table I.

Since enamine was found to be in equilibrium state against the starting materials, greater absorption of phenylalanine and ampicillin observed is due to the higher fraction of enamine formed by the higher concentration of ethylacetoacetate when administered. Further, since ethylacetoacetate can be soluble in 1.32 M in the solution containing 0.66 M sodium phenylalanine in spite of the solubility of about 0.75 M in aqueous solution at 37°C, apparent increased solubility of ethylacetoacetate is due to enamine formation with phenylalanine.

However, since the colonic and the jejunal absorption of ethylacetoacetate occurred rapidly as shown in Fig. 1 and 2, gradual decrease of phenylalanine absorption rate according to the time after administration with ethylacetoacetate may be due to the decrease of enamine fraction by decrease of ethylacetoacetate concentration with rapid absorption of it alone. This result also suggest that controlling the absorption of ethylacetoacetate alone lead to a more suitable delivery of amino acid and ampicillin.

Since it has been recently reported that enamine derivatives enhance the rectal absorption of polar drugs and macromolecular drugs,^{2,3)} the effect of the mixture of sodium phenylalanine and ethylacetoacetate on the absorption of sodium cefmetazole from the rat colonic and jejunal loop was studied. As described above, the colonic and the jejunal absorption of sodium cefmetazole was not facilitated by the presence of each of ethylacetoacetate, sodium D- and L-phenylalanine. However, a remarkable absorption from the colonic loop was observed when coadministered with the mixture of ethylacetoacetate and sodium phenylalanine, as shown in Fig. 4. The absorption pattern of cefmetazole

FIG. 3. OD (Optical Density) Value Profile at 288 nm of Aqueous Solution Containing 0.66 M Sodium L- (○) or D-Phenylalanine (●) and 1.32 M Ethylacetoacetate as a Function of Time after Incubation at 37°C

OD was measured after dilution of aqueous sample with 5000 folds volume of methyl alcohol. Symbol (×) shows the OD of enamine of phenylalanine synthesized in concentration of 1.32×10^{-4} M in methyl alcohol.

was similar to that of phenylalanine. this results may indicate that the absorption of cefmetazole is dependent on the absorption of phenylalanine in the presence of ethylacetoacetate. And since absorption of phenylalanine showed dependency on the enamine formation with ethylacetoacetate, absorption of cefmetazole is related to the absorption of the enamine. That is, the higher the enamine formation in administered solution produced, the greater the enhanced cefmetazole absorption occurred, as shown in Table II.

Above results indicate that the colonic absorption of cefmetazole is controlled by the enamine fraction in administered solution.

However, the absorption of cefmetazole from the jejunal loop was not so much increased by the presence of the mixture of sodium phenylalanine and ethylacetoacetate compared to that from the colonic loop (Fig. 5). However, since absorption of phenylalanine in the presence of ethylacetoacetate from the jejunal loop occurred in the similar degree compared to that from the colonic loop, presence of enamine after administration may be not different in each loop. There-

fore, adjuvant action of enamine formed from sodium phenylalanine and ethylacetoacetate at the jejunal loop did not occur strongly compared to that in the colonic loop.

To clarify the factor(s) influencing the action of the enamine at the colonic and the jejunal loop must lead us to elucidate the possible mechanism of the enamine action. At the present time, to determine the enamine behavior in the rat body is difficult, because of its apparent rapid hydrolysis in low concentration in body compared to high concentration in administered solution. However, as we reported,⁷⁾ adjuvant action of 5-methoxysalicylate, another non-surfactant adjuvant, showed the action differences at the different position of the intestine. And the differences of adjuvant action of 5-methoxysalicylate was elucidated partly by the affinity of adjuvant to each intestinal tissue. Although the phenomena obtained in the study on adjuvant action of 5-methoxysalicylate may be considered for the adjuvant action of enamine, it must be required to conduct the physicochemical study in more detail for enamine formation

TABLE II. *Colonic Absorption of Sodium Cefmetazole by the Presence of the Mixture of Sodium Phenylalanine and Ethylacetoacetate in Administered Solution at 30 min after Administration*

Compositions in administered solution			Absorbed percent of cefmetazole
Concn. of sodium cefmetazole (M)	Concn. of sodium phenylalanine (M)	Concn. of ethylacetoacetate (M)	
0.03	D-Phenylalanine		
	0.66	0.00	5.9 ± 2.6
		0.66	34.3 ± 6.2 ^{a)}
		0.99	59.0 ± 5.9 ^{b)}
		1.32	65.2 ± 4.9 ^{b)}
	D-Phenylalanine		
	0.66	0.00	5.1 ± 2.7
		0.66	32.6 ± 4.9 ^{a)}
		0.99	52.3 ± 8.3 ^{b)}
		1.32	60.2 ± 4.2 ^{b)}

a) $p < 0.01$ against without ethylacetoacetate ($n \geq 4$).

b) $p < 0.01$ against without and with 0.66 M ethylacetoacetate ($n \geq 4$).

in aqueous solution before elucidating the mechanism of enamine's adjuvant action. And the study on the physicochemical property of enamine in aqueous solution may lead us to elucidate the behavior of enamine in the body. Therefore, we are going to report the behavior

of enamine in aqueous solution soon.

Although it is not clear as yet as to what kind of mechanism is involved in the adjuvant action of enamine enhancing the absorption of polar drugs, following conclusions were obtained in this study. 1) Possible enamine formation of amino acid-like drug with ethylacetoacetate in aqueous solution lead to new pharmaceutical ap-

FIG. 4. Disappearance of Cefmetazole from the Rat Colonic Loop after Administration of Aqueous Solution at a Dosage Volume of $200 \mu\text{l/kg}$

Administered solution contained 30 mM sodium cefmetazole and following additive; 0.66 M sodium L-phenylalanine (○), 0.66 M sodium D-phenylalanine (△), 0.66 M sodium L-phenylalanine and 1.32 M ethylacetoacetate (●) or 0.66 M sodium D-phenylalanine and 1.32 M ethylacetoacetate (▲). Each value represents mean $\pm$ SD ($n \geq 4$). Symbols, □ and ■, represent the disappearance of L- and D-phenylalanine, respectively, in the presence of ethylacetoacetate. Wet weight of the colonic loop used was 273 ± 28 mg.

FIG. 5. Disappearance of Cefmetazole from the Rat Jejunal Loop after Administration of Aqueous Solution at a Dosage Volume of $200 \mu\text{l/kg}$

Administered solution contained 30 mM sodium cefmetazole and additive symbolized contained 30 mM sodium cefmetazole and additive symbolized in Fig. 4. Each value represents mean $\pm$ SD ($n \geq 4$). Symbols, □ and ■, represent the disappearance of L-phenylalanine and D-phenylalanine, respectively, in the presence of ethylacetoacetate. Wet weight of the jejunal loop used was 347 ± 36 mg.

proach to improve the intestinal absorption of amino acid-like drugs. 2) Since adjuvant action of enamine at the colonic loop occurred strongly compared to the jejunal loop, rectal route is a suitable route for the administration of polar drugs enhanced by the enamine.

REFERENCES

- 1) T. Murakami, H. Tamauchi, M. Yamazaki, K. Kubo, A. Kamada, and N. Yata: Biopharmaceutical Study on the Oral and Rectal Administration of Amino Acid-like β -Lactum antibiotics in Rabbits, *Chem. Pharm. Bull.*, **29**, 1986–1997 (1981).
- 2) A. Kamada, T. Nishihata, S. Kim, M. Yamamoto, N. Yata: Study of Enamine Derivatives of Phenylglycine as Adjuvants for the Rectal Absorption of Insulin, *Chem. Pharm. Bull.*, **29**, 2012–2019 (1981).
- 3) T. Murakami, N. Yata, H. Tamauchi, and A. Kamada: Studies of Absorption Promoters for Rectal Delivery Preparation. II. A Possible Mechanism of Promoting Efficacy of Enamine Derivatives in Rectal Absorption, *Chem. Pharm. Bull.*, **30**, 659–665 (1982).
- 4) T. Yagi, N. Hakui, Y. Yamazaki, R. Kawamori, M. Schichiri, H. Abe, S. Kim, M. Miyake, K. Kamikawa, T. Nishihata, and A. Kamada: Insulin Suppository; Enhanced Rectal Absorption of Insulin using an Enamine Derivatives as New Promotor, *J. Pharm. Pharmacol.*, **35**, 177–178 (1983).
- 5) T. Nishihata, J. H. Rytting, A. Kamada, and T. Higuchi: Enhanced Intestinal Absorption of Insulin in Rats in the Presence of Sodium 5-Methoxysalicylate, *Diabetes*, **30**, 1065–1067 (1981).
- 6) B. G. Muck: Intestinal Absorption of Amino Acids, "Physiology of the Gastrointestinal Tract," ed. by L. R. Johnson, Raven Press, New York, 1981, pp. 1097–1122.
- 7) T. Nishihata, Y. Wada, M. Yamazaki and A. Kamada: Effect of administration sites on the enhancing action of sodium 5-methoxysalicylate against the intestinal absorption of sodium cefmetazole, *J. Pharm. Dyn.*, in preparation.