Efficacy and pharmacological mechanism of pronase-enhanced low-dose antibiotics for *Helicobacter pylori* eradication

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Drs Guo and Zou made an equal contribution to this study.
This study examined the efficacy and pharmacological mechanism of pronase-assisted low-dose antibiotics to eradicate *H. pylori*. Mongolian gerbils infected with *H. pylori* received 7-day treatment (omeprazole, different concentrations of pronase, amoxicillin and clarithromycin), the efficacy was assessed using the eradication rate and the colonisation of *H. pylori*. In Mongolian gerbils that orally administered with pronase, the thickness of the gastric mucous layer (GML) was examined using immunohistochemical and Alcian Blue staining, and the concentrations of amoxicillin in gastric tissue and serum were detected using HPLC. The eradication rates were 80.0% (12/15) in the HPQG and 86.7% (13/15) in the HAG (*P* = 1.000). The antibiotic doses in the HPQG was only 1/20 of the HAG. Thirty minutes after oral treatment with pronase, the sticky protein of GML was hydrolysed, and the GML became thinner. A higher amoxicillin concentration in both the gastric tissue and serum was observed in the pronase group compared with the Am10 group. The concentration of amoxicillin in the Am10+Pr108 group in gastric tissue was 3.8 times higher compared to the Am10 group in 5 minutes. Together, these data suggest that pronase significantly reduced the dose of antibiotics used in *H. pylori* eradication. The pharmacological mechanism is likely that pronase removing the mucus layer, promoting chemical factors (i.e., gastric acid, pepsinogen) distribution and increasing the antibiotic concentrations in the deep GML, which acted on *H. pylori* collectively. Thus, pronase may enhance the level of antibiotics to eradicate *H. pylori* in the clinic.
*Helicobacter pylori* is a significant cause of chronic active gastritis, peptic ulcer disease and gastric cancer (1). Several strategies have been proposed to eradicate *H. pylori*, including the standard first-line treatment (standard triple therapy, STT), quadruple, sequential, and concomitant treatments, etc (2). However, the eradication rates for *H. pylori* infection have decreased to less than 80% in many countries (3). One of the reasons for this incomplete eradication is most likely that *H. pylori* is protected by the GML, and the effective antimicrobial concentrations cannot be achieved in the deep GML or epithelial cell surfaces where *H. pylori* is present (4-6). This is mainly caused by the barrier of the dense GML, the short residence time of the antimicrobial agents in the stomach, and the degradation of antimicrobial agents, such as amoxicillin and clarithromycin, which are degraded by gastric acid (4).

Approaches to achieve effective antimicrobial concentrations in the deep GML include increasing the administration of high doses of antimicrobial agents on a daily basis, although this is usually accompanied by adverse effects and poor patient compliance (7), and extending the residence time of antimicrobial agents in the stomach by changing the dosage form (4), however, the antibiotic dose is not reduced. A more optimal strategy is not only to reduce the antibiotic dose but also to improve the local drug concentration in the deep GML.

Pronase, a type of hydrolysable protease, is known to hydrolyse sticky proteins and to remove the surface gastric mucous layer of the stomach (8) and has also been used as a premedication for endoscopy (9). It also increased amoxicillin transfer rate from plasma to gastric aspirate by traversing the gastric mucosa (10). Here, pronase alone exhibits no inhibitory effect on *H. pylori*, although it has been reported that pronase
has an effect in promoting the eradication of *H. pylori*. However, this effect is not obvious, and the pharmacological mechanism remains unclear (11, 12). In this work, we report the effect of pronase-enhanced antibiotics on the eradication of *H. pylori* and study its pharmacological mechanism in the Mongolian gerbil model.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Pronase samples were a gift from the Beijing Tide Pharmaceutical Co., Ltd (Beijing, China). Omeprazole was purchased from Wuhan Chufengyuan Technology Co., Ltd (Wuhan, China). Amoxicillin and Clarithromycin were purchased from Southwest Pharmaceutical Co., Ltd (Chongqing, China). N-Acetyl-L-cysteine was purchased from Absin Bioscience Inc (Shanghai, China). Misoprostol was purchased from Shanghai New Hualian Co., Ltd (Shanghai, China). Primers for PCR and qRT-PCR were synthesised by BGI-Shenzhen (Shenzhen, China). The gastric mucin primary antibody was purchased from Novus Biologicals (Littleton, USA), and the secondary horseradish peroxidase antibody was purchased from SinoBio Biotech Co., Ltd (Shanghai, China). Alcian Blue stain (pH 2.5) was obtained from Zhuhai Baso Biotechnology Co., Ltd (Zhuhai, China). The urease test kit was purchased from Zhuhai Kedi Technology Co., Ltd (Zhuhai, China). HPLC-grade acetonitrile, methanol and hematoxylin were purchased from Sangon Biotech (Shanghai) Co., Ltd. All other reagents were of analytical grade.

**Animals.** Six-week-old male specific pathogen-free Mongolian gerbils (body weight 50-60 g) were purchased from the Center of Experimental Animals (Zhejiang, China) and were maintained under standard laboratory conditions (room temperature, 23 ± 2°C; relative humidity, 55 ± 5%; 12-h light: 12-h dark cycle) with free access to an autoclaved pellet diet and sterile water. After a 1-week equilibration period, these
gerbils were used in the experiments. All animal experiments were approved by the Animal Ethical and Experimental Committee of Third Military Medical University.

**Infection of Mongolian gerbils with *H. pylori***. According to our previous method (13), the *H. pylori* strain M13 that was derived from a Chinese clinical isolate and adapted to colonise the GML of Mongolian gerbils was used for inoculation in Mongolian gerbils. The bacteria were inoculated in 150 mL of brain heart infusion broth supplemented with 5% foetal bovine serum and 1% glucose with shaking at 220 rpm and 37 °C under 5% O₂ and 8% CO₂. After harvesting, the bacterial suspensions were prepared at a concentration of 2.0 × 10⁹ CFU/mL. All groups of Mongolian gerbils were inoculated with 0.4 mL of *H. pylori* suspension orally by gavage, twice a day for only one day. The gerbils were housed for 4 weeks under these described conditions. Four weeks after inoculation, ten random gerbils from all of the Mongolian gerbils were sacrificed by cervical dislocation to evaluate the colonisation rate of the *H. pylori* in the stomach, when the infection rate was 100% for the next experiment.

**Detection of *H. pylori***. Infection with *H. pylori* was assessed using bacterial culture, urease test and PCR. The stomachs were removed from the gerbils and cut into two parts. One part of the stomach was serial plated on modified Skirrow’s medium, and the agar plates were incubated for 2 days at 37 °C under 5% O₂ and 8% CO₂ in three gas incubators (Thermo Scientific). Viable colonies for each stomach were identified as *H. pylori* on the agar plates, which were positive for gerbils. Another part of the stomach was homogenised with normal saline, the mixture was identified using the urease test. In addition, the mixture were also centrifuged for 5 min at 13000 × g, and the precipitate was collected for PCR and qRT-PCR. PCR and qRT-PCR analysis of the samples was performed using a previously reported method.
The DNA was isolated from the precipitate using the Microbial Genomic DNA Extraction Kit (SunShineBio). The PCR primers used to amplify the ureC (glmM) gene of *H. pylori* were F: 5′-AAGCTTTAGGGGTGTTAGGGGTTT-3′ and R: 5′-AAGCTTACTTTCTAACACTACGC-3′. The assay was run on a S-1000 (Biorad). After a denaturation step at 94°C for 3 min; 35 cycles were performed for PCR amplification, where the cycle parameters were 94°C for 45 sec, 55°C, for 30 sec, 72°C for 30 sec; followed by an extension step at 72°C for 5 min. The PCR product was separated by electrophoresis with a 2% gel containing ethidium bromide and visualised with a UV light source. Both the urease test and PCR were positive, which were defined as positive for *H. pylori*. The colonisation of *H. pylori* was quantified by amplifying the 16S rDNA of *H. pylori*. For 16S rDNA, the primers used were F: 5′-TTTGTTAGAGAAGATAATGACGGTATCTAAC-3′ and R: 5′-CATAGGATTTC-ACACCTGACTACTAC-3′, and the TaqMan probe was P: 5′-FAM-CGTGCCAG-CAGCCGCGGT-TAMRA-3′. The assay was run on a CFX™ Real-Time System (Biorad). After a denaturation step at 95°C for 2 min, 40 cycles were performed for PCR amplification. The cycle parameters were 95°C for 5 sec and 60°C for 30 sec.

**Eradication of *H. pylori* in vivo.** Mongolian gerbils infected with *H. pylori* were assigned to 9 groups (15 animals per group). They were divided into the following groups: (i) non-treated group (NG): omeprazole 4 mg/kg; (ii) low antibiotics group (LAG): omeprazole 4 mg/kg, amoxicillin 10 mg/kg and clarithromycin 5 mg/kg; (iii) high antibiotics group (HAG): omeprazole 4 mg/kg, amoxicillin 200 mg/kg and clarithromycin 100 mg/kg; (iv) N-Acetyl-L-cysteine group (NACG): omeprazole 4 mg/kg, N-Acetyl-L-cysteine 125 mg/kg; (v) pronase and misoprostol group (PMG):
omeprazole 4 mg/kg, misoprostol 0.3 mg/kg, pronase 108 mg/kg; (vi) high pronase group (HPG): omeprazole 4 mg/kg, pronase 108 mg/kg; (vii) low pronase quadruple group (LPQG): omeprazole 4 mg/kg, pronase 27 mg/kg, amoxicillin 10 mg/kg and clarithromycin 5 mg/kg; (viii) medium pronase quadruple group (MPQG): omeprazole 4 mg/kg, pronase 54 mg/kg, amoxicillin 10 mg/kg and clarithromycin 5 mg/kg; and (ix) high pronase quadruple group (HPQG): omeprazole 4 mg/kg, pronase 108 mg/kg, amoxicillin 10 mg/kg and clarithromycin 5 mg/kg. In these groups, the gerbils were treated twice daily for 7 consecutive days. The gerbils were orally and successively administered omeprazole, pronase or N-Acetyl-L-cysteine, amoxicillin and clarithromycin mixture, with a time interval of 30 minutes, misoprostol was orally administered after taking omeprazole 15 min. Four weeks after administration, the eradication of \textit{H. pylori} was examined using bacterial culture, the urease test and PCR.

**Immunohistochemical and Alcian Blue staining of GML.** The normal gerbils were divided into three groups: the control group (n=3), which received only oral doses of omeprazole (4 mg/kg); the medium pronase group (n=3), which was successively given oral administration of omeprazole (4 mg/kg) and pronase (54 mg/kg), and the high pronase group (n=3), which was successively orally given omeprazole (4 mg/kg) and pronase (108 mg/kg), with a time interval of 30 minutes. Thirty minutes after administration, the gerbil’s stomach was removed and frozen. Frozen tissue sections were obtained, which were stained immunohistochemically with a primary antibody against gastric mucin, according to a method previously reported (16). Other frozen sections were stained using the Alcian Blue solution (17). The sections were washed with deionised water for 30 minutes, and placed in the Alcian Blue solution (pH 2.5) for 15 minutes and then rinsed briefly with water. Next,
the tissue sections were counterstained with hematoxylin staining solution for 15 seconds and rinsed briefly with water for 10 minutes. To best observe the GML, the tissue sections were immediately visualised under a microscope after staining. The thickness of the GML was defined as the distance from the outermost edge of the GML to the luminal surface of the surface mucous cells (18). The thickness of the GML was selected using a microscope for 60 randomly selected points in each of the control and treatment groups.

**Pharmacokinetics of amoxicillin.** The gerbils were divided into four groups (36 animals per group), which were treated with the following conditions: (i) the Am10 group: omeprazole 4 mg/kg; amoxicillin 10 mg/kg and clarithromycin 5 mg/kg; (ii) the Am200 group: omeprazole 4 mg/kg; amoxicillin 200 mg/kg and clarithromycin 100 mg/kg; (iii) the Am10+Pr54 group: omeprazole 4 mg/kg, pronase 54 mg/kg, amoxicillin 10 mg/kg and clarithromycin 5 mg/kg; (iv) the Am10+Pr108 group: omeprazole 4 mg/kg, pronase 108 mg/kg, amoxicillin 10 mg/kg and clarithromycin 5 mg/kg. The gerbils were orally and successively administered omeprazole, pronase, amoxicillin and clarithromycin mixture, with a time interval of 30 minutes. The amoxicillin concentration was determined in the gastric tissue and serum using HPLC (19, 20). The gastric tissue and blood samples were collected before and at 5, 10, 15, 20, 25, 30, 35, 45, 60, 75, 90 min after oral administration, with three gerbils for each sampling point. The gastric tissue was removed and washed twice in PBS. Next, it was homogenised with 0.5 mL PBS and centrifuged for 5 min at 13000 × g. After the supernatants were collected, an equal volume of methanol was added and mixed for 2 minutes. The mixture were centrifuged for 10 min at 13000 × g. The supernatant fluid was collected for HPLC analysis. Blood samples were coagulated for five hours at 4°C and centrifuged for 5 min at 5000 × g. The serum was collected, and subsequent
steps were performed according to the gastric tissue operations. The apparatus used for this study was an Agilent 1260 quaternary pump, and the HPLC method included a mobile phase consisting of a mixture of 50 mM phosphate buffer and acetonitrile (97.5 : 2.5 v/v). This solution was adjusted using concentrated phosphoric acid to pH 3.0 and pumped at a flow rate of 0.7 mL/min through a phase reverse column Agilent Zorbax Extend C18 (150 mm × 4.6 mm, 5 μm) (21, 22). The optimisation wavelength of amoxicillin detection was 210 nm.

**Statistical analysis.** The eradication rate between each group was compared using the χ² test or Fisher’s exact test. The difference in colonisation in the two groups was examined using the Independent Sample t-test. The figures represented the data from three independent experiments, and the data were expressed as the mean ± standard deviation (S.D.). Statistically significant differences between groups were defined as a P-value < 0.05. The statistical analyses were performed using SPSS 19.0 statistical software for Windows.

**RESULTS**

**The eradication of *H. pylori* in vivo.** Four weeks after administration, the effects of the eradication of *H. pylori* were examined using bacterial cultures (Table 1). The eradication rate in the LAG was 13.3% (2/15), and the HPQG achieved an 80.0% (12/15) eradication rate. There were statistically significant differences in the LAG compared with the HPQG (P < 0.05). In contrast, the HPQG showed no significant difference compared with the HAG (P > 0.05) that obtained an 86.7% (13/15) eradication rate, however, the antibiotic doses of HPQG were only 1/20 of the dose used in the HAG. In addition, The MPQG achieved a 53.3% (8/15) eradication rate, which showed no significant difference compared with the HPQG (P = 0.245). The
NACG and HPG showed a little eradication effects. The PMG and NG had no eradication effect, the eradication rate of them are 0% (0/15).

The eradication effects for the different treatments were further confirmed using the qRT-PCR method (Fig. 1). qRT-PCR can demonstrate both the effect of eradication as well the differences in the amount of colonisation. Compared with the LAG, the amount of colonisation decreased significantly in the MPQG and HPQG. There was no significant difference between the HAG and the MPQG and HPQG, where the P-value was 0.673 and 0.193, respectively. The PMG had a higher amount of colonisation than the other groups.

**Hydrolysis of pronase in the GML.** To visualise the gerbil’s GML, immunohistochemistry and Alcian Blue staining were performed and are shown in Fig. 2. The GML includes mucins and mucous polysaccharides, which are well preserved in all gerbils that were only orally treated with omeprazole. However, the mucins and mucus polysaccharides from gerbils that were treated with pronase were significantly damaged and were thinner compared to gerbils without pronase premedication. The destruction effect of the low pronase group on GML is not obvious (data not reported).

**Amoxicillin concentrations in the gastric tissue and serum.** A satisfactory separation of amoxicillin from the endogenous components in the gerbil gastric tissue and serum was obtained (Fig. 3A). In Fig. 3B, the amoxicillin concentration of the Am10+Pr108 group in the gastric tissue achieved a maximum value in 5 minutes. The value was 12.13 μg/mL. The amoxicillin concentration of the Am10 group also reached the highest in 5 minutes; however, its value was only 3.17 μg/mL. The value of the Am10+Pr108 group was 3.8 times that of the Am10 group. The amoxicillin concentration of serum in all groups became larger over time. In Fig. 3C, there was a
wave in all groups within 15 minutes, where the amoxicillin was absorbed by the
gastric tissue into the blood. The amoxicillin concentration of the gastric tissue and
serum in the Am200 group was higher compared to the other groups at the same
sampling point.

DISCUSSION

To date, there are many methods to enhance the effect of eradication for \textit{H. pylori} in
the stomach, such as increasing the dose, prolonging the duration, and developing
new drugs (23). However, the adverse effects and drug waste have become more
serious, and the development of new methods will be challenging. Thus, the treatment
of \textit{H. pylori} will be a larger issue in the future. A potentially useful method would be
to directly improve the local drug concentration in the deep GML. According to
Gotoh’s reports (11), the quadruple therapy of lansoprazole, pronase, amoxicillin and
metronidazole could improve the eradication effect of \textit{H. pylori} in human, which may
be owed to the increased delivery of antibiotics at the site of infection by virtue of its
ability to remove and disrupt the GML. However, the doses of antibiotics were 500
mg of amoxicillin and 250 mg of metronidazole, and the time of therapy, were thrice
daily for 2 weeks, which worsens the compliance due to an additional drug being
administered at the same time. On this basis, we explored the effect of pronase
enhancement of low doses of antibiotics to eradicate \textit{H. pylori} in gerbils.

To comprehensively evaluate the pronase quadruple therapy for the treatment of \textit{H.
pylori}, according to the dose of STT (omeprazole 20 mg, amoxicillin 1 g and
clarithromycin 500 mg) in humans (24), we performed a preliminary experiment to
study the effect of different doses of antibiotics to eradicate \textit{H. pylori} in the gerbil
stomach (data not reported). The therapeutic effects of MAG (a drug consisting of
Omeprazole 4 mg/kg, amoxicillin 20 mg/kg and clarithromycin 10 mg/kg were administered twice daily for 7 consecutive days and EAG (a drug consisting of omeprazole 4 mg/kg, amoxicillin 5 mg/kg and clarithromycin 2.5 mg/kg were administered twice daily for 7 consecutive days) were 40.0% (6/15) and 6.7% (1/15), respectively. The eradication of HAG and LAG are shown in Table 1. We selected an antibiotic dose of LAG with pronase was more convincing compared to other groups, because the antibiotic usage was lower and the cure rate increase greater.

In addition to the bacterial culture, urease test and PCR, we used qRT-PCR to examine the amount of colonisation of *H. pylori*. qRT-PCR is more sensitive compared to other methods. It can detect *H. pylori* even at low concentrations (25). Combined with the eradication rate and the amount of colonisation, this further demonstrates that pronase can assist antibiotics in eradicating *H. pylori*.

We studied the pharmacological mechanism underlying pronase-enhanced low-dose antibiotic treatment in *H. pylori* infection. Pronase alone does not exhibit an inhibitory effect on *H. pylori* in vitro even at concentrations of $3.3 \times 10^3$ mg/L in previous studies (data not shown), which were consistent with previous reports demonstrating that pronase did not inhibit the growth of *H. pylori* (11). Misoprostol is a mucosal protective agent(26), the eradication rate of PMG is 0% (0/15), there was significant difference between the PMG and HPG about the eradication rate ($P = 0.022$). This suggests pronase did not play a role of eradication for *H. pylori* in stomach directly. The N-Acetyl-L-cysteine that is a mucolytic agent has a similar hydrolysis mechanism of pronase, and has been used in the treatment of *H. pylori* (27, 28). In our study, the NACG obtained a 20% (3/15) eradication rate. Combination of the hydrolysis experiment of pronase and the eradication rate of HPG, it suggests that the mechanism of pronase is that removing the gastric mucus layer and likely
promoting chemical factors (i.e., gastric acid, pepsinogen) distribution and increasing
the antibiotic concentrations in the deep GML, which acted on *H. pylori* collectively.

*H. pylori* lives deep in the GML (5, 6), which is formed by two types of mucin
layers that are derived from surface mucous cells and gland mucous cells (18). There
is sufficient antibiotic concentration in the deep GML, which can effectively eradicate
the *H. pylori* (29-31). By detecting the antibiotic concentration of gastric tissue after
oral treatment with pronase, the value of amoxicillin concentration in the
Am10+Pr108 group was 3.8 times that of the Am10 group in 5 minutes. This finding
indirectly indicated that pronase promoted the diffusion and absorption of antibiotics
in gastric tissue. It showed that pronase enhanced the ability of low doses of
antibiotics to achieve an effective concentration that can eradicate *H. pylori*.

In our study, we also detected the concentrations of clarithromycin in gastric tissue
and serum according to previous studies (10, 22). However, the crest is very small,
and the detection is not sufficiently sensitive. Thus, calculation of the peak area is not
accurate. We speculate that the reason is too small oral dose. Moreover, amoxicillin
also displays the higher crest, larger peak area, and exhibits better accuracy. Thus, we
chose an amoxicillin concentration to represent the concentration-time profiles of
antibiotics in gastric tissue and serum.

In conclusion, we found that 7 days of pronase quadruple treatment with low-dose
amoxicillin and clarithromycin exhibit similar eradication rates with 20 times the dose
of antibiotics alone, most likely by the mechanism of reducing the GML thickness to
enable the distribution of chemical factors and increase the antibiotic concentration in
the deep GML, which acted on *H. pylori* collectively. Thus, this treatment can reduce
the use of antibiotics, thereby lowering costs, reducing the emergence of
drug-resistant bacteria, and improving compliance. Furthermore, using pronase
enhanced low-dose antibiotics for the eradication of *H. pylori* is recommended. If pronase is to be applied to the clinic, then additional safety evaluations and further clinical studies are required.

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**REFERENCES**


**TABLE 1.** The different therapies for *H. pylori* eradication in Mongolian gerbils

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eradication rate after 4 weeks, % (n)</th>
<th>P value</th>
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<tr>
<td>NG</td>
<td>0 (0/15)</td>
<td></td>
</tr>
<tr>
<td>LAG</td>
<td>13.3 (2/15)</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HAG</td>
<td>86.7 (13/15)</td>
<td></td>
</tr>
<tr>
<td>NACG</td>
<td>20.0 (3/15)</td>
<td></td>
</tr>
<tr>
<td>PMG</td>
<td>0 (0/15)</td>
<td></td>
</tr>
<tr>
<td>HPG</td>
<td>40 (6/15)</td>
<td>0.022&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPQG</td>
<td>33.3 (5/15)</td>
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<tr>
<td>MPQG</td>
<td>53.3 (8/15)</td>
<td>0.245&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>HPQG</td>
<td>80.0 (12/15)</td>
<td>1.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The HPQG vs. LAG.

<sup>b</sup> The HPQG vs. HAG.

<sup>c</sup> The HPQG vs. MPQG.

<sup>d</sup> The HPG vs. PMG.
FIG 1 Colonisation of *H. pylori* in the stomach using qRT-PCR analysis.
FIG 2 The GML of gerbils stained with immunohistochemistry and Alcian Blue. (1) and (4) The control group. (2) and (5) The medium pronase group. (3) and (6) The high pronase group. (A) The GML was immunohistochemically stained. The gray parts are the mucins of the GML, which were labelled by the gastric mucin antibody. (B) The GML was stained using an Alcian Blue solution. The blue part is the acid mucous polysaccharides, which were stained with Alcian Blue. The dark blue part of (A) and (B) are the nucleus of the gastric mucous cells, which were stained using the hematoxylin staining solution (original magnifications: (A) and (B) ×200; bar = 25 μm).
FIG 3 Detection of amoxicillin in the gastric tissue and serum using HPLC. (A): (1) HPLC chromatogram of the amoxicillin standard (45 µg/mL), (2) HPLC chromatogram of the drug-free gerbil gastric tissue, (3) HPLC chromatogram of the gerbil stomach after oral administration of amoxicillin (10 mg/kg), (4) HPLC chromatogram of the drug-free gerbil serum, (5) HPLC chromatogram of the gerbil serum after oral administration of amoxicillin (10 mg/kg). (B) The concentration-time profiles of amoxicillin in gastric tissue after co-administration with clarithromycin. (C) The concentration-time profiles of amoxicillin in serum after co-administration with clarithromycin.