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ORIGINAL ARTICLE

# Determination of metronidazole in a rat stomach by HPLC for obtaining basic data of the eradication therapy of *Helicobacter pylori*

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**Abstract** In the eradication therapy of *Helicobacter pylori* changes of antibiotics as these concentrations or amount in the stomach after oral administration were not clear. A simple and accurate method for determining the concentration of metronidazole (MTZ) in homogenate of rat stomach was developed in order to obtain basic data to design a pharmaceutical preparation having targeting ability to the surface of gastric-mucosa. This method included a deproteinization process by methanol, separation with reversed-phase high-performance liquid chromatography, and detection with an ultraviolet wavelength of 370 nm. Regression analysis showed that the method was linear over a standard curve range from 5 μg/mL to 2000 μg/mL. The inter-day precision and accuracy values between the ranges were 5.0% or better and −7.5 to 5.2%, respectively. The newly developed method was applied to an analysis of gastric samples after oral administration of MTZ at a dose of 5 mg/kg. It was found that the residual MTZ in the stomach was determined within 5 h after dosing. This method is useful for monitoring MTZ in stomach after its oral administration to rats.



## 1. Introduction

Gastric *Helicobacter pylori* (*H. pylori*) infection is associated with chronic (type B) gastritis and peptic ulcer [1–3]. *H. pylori* is often observed to adhere to the antral epithelium of the human stomach and gastric metaplasia in the duodenum. Gastric and duodenal ulcers are believed to develop as the result of damage to the gastric mucosa by cytotoxic substances (ammonia, cytotoxin, etc.) produced by *H. pylori* [4]. Recent studies have provided

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evidence that cytotoxin-associated gene A product (cagA)-positive *H. pylori* plays a causal role for the development of gastric carcinoma [5–7]. Therefore, the eradication of *H. pylori* is considered to be very important. The primary eradication therapy for *H. pylori* in Japan which is a triple therapy using amoxicillin, clarithromycin and proton pump inhibitor was started in 2000 with the therapy approved for health insurance coverage [8]. However, because of the increase of incomplete eradication, the second-line eradication therapy was approved in 2007 [8]. In the eradication therapy, metronidazole (MTZ) is used in the place of clarithromycin. The *H. pylori* strains with clarithromycin resistance significantly increase more and more, so the importance of the second-line eradication therapy also increases [8]. The pharmaceutical preparations used in the eradication therapy were not developed for the eradication therapy. For example in Japan, the pharmaceutical preparations, Pasetocin Tablets 250 for amoxicillin and Flagyl for MTZ, were developed for various bacterial infections in 1981 and 1963, respectively. These preparations were designed to have the effect through the blood circulation after GI absorption. For *H. pylori* eradication therapy, an intragastric local attack is expected [9]. From the point of view of pharmaceutical preparations design, the preparations used are not optimal preparations [10].

In order to design the pharmaceutical preparations, the information of drug amount and/or concentrations in stomach was very important in this case. However, the information for the amount and/or concentration of drugs in stomach after oral administration of antibiotics used for the eradication therapy was not enough for both of human and experimental animals. Therefore, we tried to develop the determination method of MTZ in rat stomach to study its intragastric behavior of rats. Many kinds of determination methods of MTZ using HPLC have been reported [11–13]. However, these methods could not apply to its determination from rat stomach, because the samples have many peaks from feed and its digests which were not constant. In order to determine MTZ in stomach further investigations were required.

This paper describes a simple high-performance liquid chromatographic (HPLC) procedure for determining the intragastric amount of MTZ in rats, and the result of the preliminary study on its intragastric behavior in rats.

## 2. Materials and methods

### 2.1. Chemicals and solvents

Metronidazole (MTZ, Fig. 1) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of special reagent grade or HPLC grade.

### 2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Model LC-10AS pump, equipped with a Model SCL-10A system controller, a Model SPD-10A UV spectrophotometric detector, a Model CTO-10A column oven, a Model C-R7A Chromatopac, and a Model SIL-10A autoinjector, all from Shimadzu corpolation (Kyoto, Japan). The mobile phase was methanol–water–perchloric acid (60%)—sodium perchlorate monohydrate (50:950:1:5, V/V/V/W). The chromatographic column was a YMC Pack AM12S05 ODS (150 mm × 6 mm I.D., particle diameter 5 μm) obtained

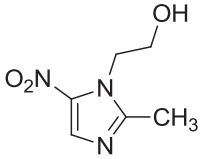


**Fig. 1** Chemical structure of metronidazole.

from YMC Co. Ltd. (Kyoto, Japan). The flow rate, wavelength for determination, and temperature of the column were 1 mL/min, 370 nm, and 40 °C, respectively. The wavelength was chosen because MTZ had absorbance at around 370 nm and peaks monitored around 370 nm from endogenous and feed were smaller.

### 2.3. Animal study and preparation of stomach homogenate

Male Sprague-Dawley rats were used. All rats (270–330 g body weight) were allowed free access to water and food. MTZ was dissolved in a 0.5% methylcellulose solution to make the solution with a concentration of 25 mg/mL of MTZ. A 0.2 mL/kg of the solution was administered orally. The stomachs were withdrawn from the abdomen after the rats were anesthetized with ether. When the stomachs were withdrawn, the esophagus of the upper side of the stomach and the duodenum of the lower side of the stomach were ligated. The stomachs withdrawn were washed out with saline and weighed. A five times saline of each stomach as weight was added to the stomach and that was homogenized. All animal experiments were carried out according to 'Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University'.

### 2.4. Preparation of standards

Rat stomachs not administered the drug were withdrawn, washed out with saline, and weighed. The drug-free homogenates were prepared according to the method described above. MTZ (100 mg) was dissolved in 50 mL of the drug-free homogenate. This homogenate was used as the standard homogenate.

### 2.5. Calibration curve samples

The standard homogenate of MTZ was diluted with the drug-free homogenate to obtain homogenates containing MTZ at the concentrations of 5, 10, 25, 50, 100, 500, 1000, and 2000 μg/mL. For the daily standard curves, a 5 mL of the homogenate at each concentration was prepared, and the curves were prepared by analyzing duplicate at each concentration.

### 2.6. Assay procedures

Stomach homogenates which were 1.0 mL or 2.5 mL were added to glass tubes, and the weight of the homogenates was measured. Exactly twice volume of methanol, 2.0 or 5.0 mL, as much as the volume of stomach homogenates was added to the tube cooled in an ice-bath. The mixture was stirred on a vortex mixer for 10 s and centrifuged at 3000 rpm for 10 min; 10 μL of the supernatant was injected into the chromatograph. The animal study and assay were performed on the same day.

### 2.7. Calculations

The peak area of MTZ in the HPLC profiles was measured. A calibration curve was established by comparing the peak-area

against the concentration of MTZ in the standards. The slope and intercept of the curve were calculated using weighted ($1/Y^2$) linear regression. The concentration of MTZ in the experimental samples was calculated using the equation $X$ (μg/mL)$=(Y-b)/a$, where $Y$ is the peak area of MTZ in an experimental sample and $b$ (intercept) and $a$ (slope) are constants generated by linear regression analysis of the calibration curve data.

### 2.8. Recovery

The relative recovery of MTZ was determined by comparing the peak-area ratio obtained from the treatment of the stomach homogenates standards with that of the standard solutions prepared with physiological saline solution at the equivalent concentrations, 10, 50, and 100 μg/mL.

### 2.9. Calculations of MTZ in rat stomach

Concentrations of MTZ in the homogenates were calculated from the calibration curve. After the concentration was calculated, the amount of MTZ in stomach was calculated from the weight of homogenates used for assay, and the weight of the homogenate prepared. The amount of MTZ in the homogenate prepared was equal to MTZ in the rat stomach. The remaining percent (%) was calculated from MTZ in the rat stomach and the oral dose of MTZ (mg) for each rat.

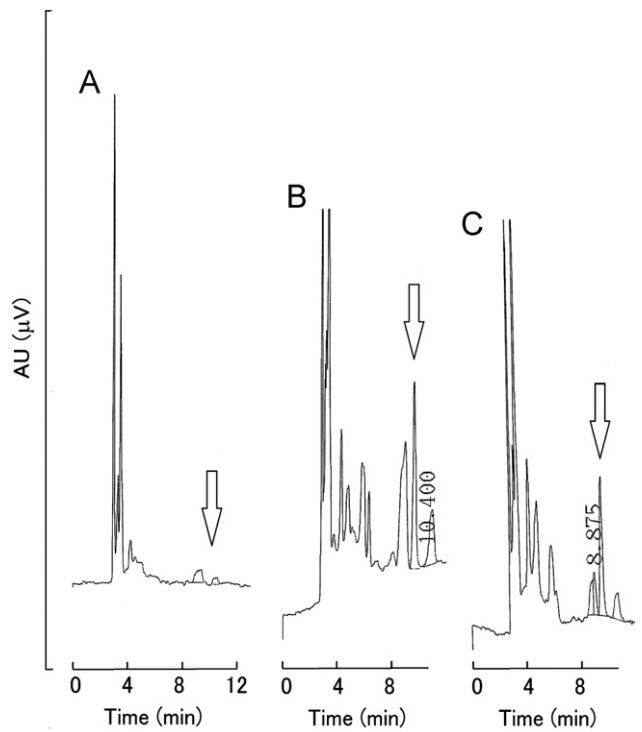


**Fig. 2** HPLC chromatograms of stomach homogenates from rats containing MTZ (A) drug-free stomach; (B) gastric sample spiked with MTZ at 25 μg/mL; (C) gastric sample from a rat 1 h after oral administration of MTZ (concentration of MTZ is 14.9 μg/mL). The arrow shows the peak of MTZ.

## 3. Results and discussion

### 3.1. Measurement of MTZ by HPLC

Typical chromatograms of MTZ obtained under the conditions described above are shown in Fig. 2. The retention time of MTZ was approximately 9.7 min. The chromatogram of drug-free stomach homogenate showed no detectable interference by endogenous substances in the stomach.

Linear regression analysis gave a slope, intercept and correlation coefficient of $Y=347.616X-49.117$, $r=0.999$, and weight$=1/Y^2$, as the calibration curve. The intra-day precision and accuracy were determined by analyzing five replicates at each drug concentration. The precision and accuracy of this method are shown in Table 1. The precision was found to range from 0.7% to 3.6%. The accuracy value ranged from −6.8% to 4.8%.

The inter-day precision and accuracy were determined by analyzing duplicates at each standard concentration over six

**Table 1** Intra-day precision and accuracy of the measurement of metronidazole in rat stomach.

| Actual concentration (μg/mL) | Concentration found (μg/mL) (mean±SD, $n=5$) | Precision (%) | Accuracy (%) |
|---|---|---|---|
| 5 | 4.9±0.1 | 1.3 | −1.5 |
| 10 | 10.1±0.3 | 2.7 | 0.6 |
| 25 | 26.0±0.4 | 1.5 | 4.1 |
| 50 | 52.4±0.9 | 1.7 | 4.8 |
| 100 | 104.6±3.8 | 3.6 | 4.6 |
| 500 | 497.4±3.5 | 0.7 | −0.5 |
| 1000 | 975.4±13.3 | 1.4 | −2.5 |
| 2000 | 1863.3±45.7 | 2.5 | −6.8 |

Precision and accuracy values were calculated by the following equations.
Precision (%)=(SD/mean) × 100.
Accuracy (%)=((concentration found—actual concentration)/actual concentration) × 100.

**Table 2** Inter-day precision and accuracy of the measurement of metronidazole in rat stomach.

| Actual concentration (μg/mL) | Concentration found (μg/mL) (mean±SD, $n=12$) | Precision (%) | Accuracy (%) |
|---|---|---|---|
| 5 | 4.9±0.2 | 3.4 | −1.1 |
| 10 | 10.1±0.5 | 5.0 | 0.7 |
| 25 | 25.5±0.4 | 1.7 | 2.1 |
| 50 | 51.4±0.8 | 1.6 | 2.8 |
| 100 | 105.2±1.8 | 1.7 | 5.2 |
| 500 | 509.2±5.4 | 1.1 | 1.8 |
| 1000 | 986.6±13.3 | 1.3 | −1.3 |
| 2000 | 1850.7±33.1 | 1.8 | −7.5 |

Precision and accuracy values were calculated by the following equations.
Precision (%)=(SD/mean) × 100.
Accuracy (%)=((concentration found—actual concentration)/actual concentration) × 100.

**Table 3** Intragastric remaining MTZ after its oral administration to rats at the dose of 5 mg/kg.

| Time after oral dosing (h) | Concentration of MTZ in homogenates (μg/mL) | MTZ in the stomach (mg)[a] | Remaining percent in the stomach[b](%) |
|---|---|---|---|
| 0.0833 | 21.5 | 1.26 | 88.3 |
| 1 | 14.9 | 0.83 | 56.2 |
| 3 | 13.4 | 0.42 | 29.9 |
| 5 | 7.2 | 0.17 | 11.6 |

[a]The amount of MTZ in the stomach was calculated from the weight of homogenates used for assay, and the weight of the homogenate prepared.
[b]The remaining percent (%) was calculated from MTZ in the rat stomach and the oral dose of MTZ (mg) for each rat.

different days. The result for the calibration curve is shown in Table 2. The precision from 5 to 2000 μg/mL was 5.0% or better. The accuracy ranged from −7.5% to 5.2%. The values of the intra-day and inter-day precision and accuracy were acceptable. The lower limit of quantification was established from the validation data as shown in Tables 1 and 2, because the precision was lower than 10% and the accuracy was existent from −10% to 10% at 5 μg/mL.

The recovery rates of each concentration (100, 50, and 10 μg/mL) were 91.9±1.8% (mean±SD, $n=3$), 93.7±5.0%, and 90.5±4.0%, respectively. The minimum inhibitory concentration (MIC) of MTZ for *H. pylori* was reported as 8 μg/mL [14]. Therefore, this method is useful to quantify gastric concentration of MTZ.

### 3.2. *Remaining MTZ in the stomach of rats*

Table 3 shows the remaining MTZ after oral administration to rats at the dose of 5.0 mg/kg. The MTZ apparently decreased with time. This method was found to be useful for determining MTZ in the stomach.

## 4. Conclusion

We developed a fast, simple and accurate method for measuring MTZ in the stomach of rats. This method involves deproteinization using methanol and reversed-phase high-performance liquid chromatography with ultraviolet detection. It has been applied to an analysis of MTZ in the stomach after the oral administration of MTZ to rats at a dose of 5 mg/kg.

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