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# A simple high performance liquid chromatography assay for simultaneous determination of omeprazole and metronidazole in human plasma and gastric fluid[1]

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**Abstract**

Antibiotics which are actively secreted into gastric fluid may be more efficacious in the eradication of *Helicobacter pylori* in peptic ulcer disease. Other agents used in the treatment of this disease such as omeprazole or other anti-secretory agents may alter the secretion and/or distribution characteristics of antibiotics. In order to test the applicability of these concepts to metronidazole, a sensitive and specific high performance liquid chromatography (HPLC) assay was developed to quantitate omeprazole in plasma, and metronidazole in plasma and gastric fluid. The HPLC system consisted of a multi-phase column combining anion exchange and reversed phase separation (OmniPac Pax-500, Dionex), and a variable wavelength UV detector set at 254 nm. The mobile phase was a mixture of 0.1 M sodium phosphate buffer:methanol:acetonitrile (60:20:20) with final pH adjusted to approximately 7.0. Metronidazole and omeprazole were extracted by adsorption onto a $C_2$-bonded silica gel solid phase extraction column, and eluted with methanol. The extract was dried, reconstituted in a solution of acetyl salicylic acid (ASA), and then injected into the HPLC system. Under these conditions, metronidazole, omeprazole and ASA were well separated and recoveries in plasma were greater than 80%. Omeprazole could not be measured in gastric fluid because of rapid decomposition. Using 0.3 ml of sample, the assay sensitivity was less than 0.1 μg $ml^{-1}$ and linear up to 10 μg $ml^{-1}$. Both intra- and inter-assay CV were greater than 15%. It was applied successfully in determining metronidazole concentrations in clinical samples of plasma and gastric fluid. 

*Keywords:* Metronidazole; Omeprazole; HPLC; Plasma; Gastric fluid; Pharmacokinetics; *Helicobacter pylori*

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## 1. Introduction

*Helicobacter pylori* (Hp) is accepted as the most important cause of gastritis and peptic ulcer disease [1]. Treatment of duodenal ulcers is now most frequently aimed at eradication of Hp with the use of antibiotics [2]. Because of the resistance of this organism, multidrug therapy is required [3]. To date, the most effective treatment appears to be a combination of two antibiotics and a histamine-2 antagonist ($H_2$-antagonists) or proton pump inhibitor [4,5].

It has been hypothesized that efficacy of antibiotics may be related to their ability to achieve high concentrations in the gastric mucosa [6]. It is possible that antibiotics which are absorbed in the small intestine and subsequently actively secreted into the gastric lumen might be more effective agents [2]. Cimetidine has been shown to increase concentrations of weakly basic antibiotics in the gastric lumen and mucosa [7], suggesting that antibiotic concentrations in the gastric mucosa may be modulated by $H_2$-antagonists. The effect of other anti-secretory agents has not been investigated.

In order to determine the effect of omeprazole on the gastric secretion of metronidazole in humans, sensitive and specific assays are needed to measure these drugs in plasma and gastric fluid. Measurement of metronidazole or omeprazole separately has been described most frequently by high performance liquid chromatography (HPLC) coupled with ultraviolet detection [8–12]. Simultaneous determination of these two drugs has not been reported. This paper describes the development and validation of a simple and specific HPLC assay coupled with a solid phase extraction which allows simultaneous measurement of omeprazole and metronidazole in plasma, and of metronidazole in gastric fluid.

## 2. Experimental

### 2.1. Chemicals and solvents

Omeprazole and metronidazole were received as gifts from Astra Hässle AB, Sweden (via Astra Pharma, Ont., Canada) and Rhône-Poulenc Pharma, Que., Canada, respectively. The external standard acetyl salicylic acid (ASA) was generously supplied by Merck Frosst Canada, Que., Canada. Their chemical structures are shown in Fig. 1. All other drugs were obtained from their respective manufacturers. Solid phase extraction (SPE) cartridges (Chromosep® 500 mg $C_2$) were purchased from Chromatographic Specialties, Ont., Canada. All solvents were HPLC grade (BDH, N.S., Canada) and other chemicals reagent grade (VWR Scientific, Canada).

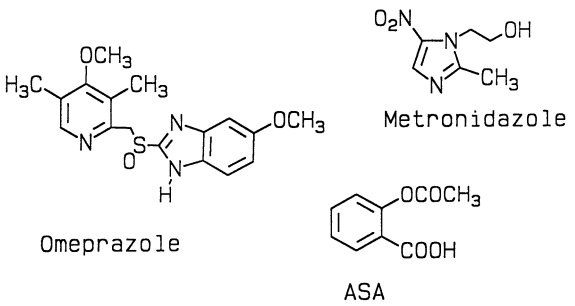


Fig. 1. Chemical structures of omeprazole, metronidazole and internal standard acetyl salicylic acid (ASA).

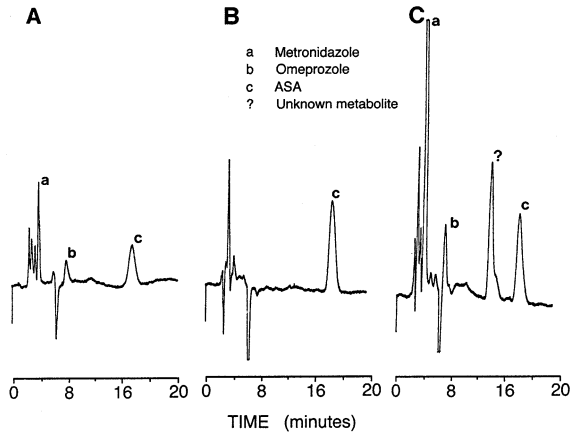


Fig. 2. HPLC-UV chromatograms of omeprazole and metronidazole in plasma of a healthy volunteer after 20 mg omeprazole b.i.d. for 7 days, and a single i.v. dose of 500 mg of metronidazole. (A, standard solution containing 10 ng each of metronidazole and omeprazole + 50 ng of ASA; B, volunteer plasma before receiving omeprazole and metronidazole; C, the same volunteer plasma 1 h after the single i.v. dose of metronidazole).

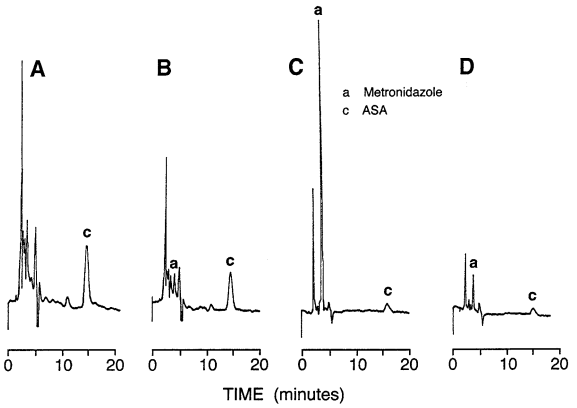


Fig. 3. HPLC-UV chromatograms of metronidazole and ASA in gastric juice samples of a healthy volunteer before and after 20 mg omeprazole b.i.d. for 7 days, and a single i.v. dose of 500 mg of metronidazole. (A, before metronidazole administration; B, standard sample containing 1 μg $ml^{-1}$ of metronidazole; C, sample collected 2-h after metronidazole from a volunteer before omeprazole; and D, the same 2-h sample from the volunteer after omeprazole).

### 2.2. Instrumentation

The HPLC system consisted of a Beckman model 114M solvent delivery module, a 163 variable wavelength UV detector set at 254 nm, and a model 427 integrator with chart speed set at 0.25 cm $min^{-1}$ (Palo Alto, CA). The injector was Rheodyne model 9125 equipped with a 100-μl peek injector loop (SPE, Ont., Canada). The analytical column was a multi-phase stationary phase column combining anion exchange and reversed phase separation mechanisms (Omnipac Pax-500, Dionex, Sunnyvale, CA) with a $C_{18}$ reversed phase cartridge guard column (LicroCart®, E.M. Merck, Darmstadt, Germany). The mobile phase was a mixture of methanol:acetonitrile:0.1 M sodium phosphate buffer (20:20:60), with final pH = 2.3 (adjusted by adding 85% phosphoric acid). The system was operated at room temperature isocratically at a flow rate of 0.7 ml $min^{-1}$ and pressure at 1.70 kpsi. The sensitivity of the detector-integrator was set at 0.04 AUFS.

### 2.3. Extraction of metronidazole and omeprazole in plasma and gastric fluid

Each 500 mg $C_2$ SPE cartridge was pre-conditioned with methanol (2 × 1 ml), distilled water (3 × 1 ml), and then 1.0 M pH 7 potassium phosphate buffer (2 × 1 ml). To each pre-conditioned cartridge was added 0.3 ml of sample or standard. For plasma samples, 0.3 ml of 0.1 M sodium phosphate buffer (pH 7.0) was added, while 0.3 ml of 1.0 M potassium phosphate buffer (pH 7.0) was added to each gastric fluid sample. The mixture was allowed to pass through the cartridge under mild vacuum (10–15“ Hg). The cartridge was washed with water (3 × 1 ml), and dried under vacuum for 20 min. Metronidazole and omeprazole were desorpted with 1 ml of methanol into a cone shape borosilicate glass culture tube (Kimax®, VWR Scientific Canada), and the extract was then dried under a stream of purified nitrogen at 55°C (Thermolyne Dri-Bath, Fisher Scientific, Canada). The residue was stored at − 20°C until analysis. Each sample was reconstituted in 0.1 ml of a mixture of methanol:acetonitrile:distilled water (20:20:60) containing 5 μg $ml^{-1}$ of ASA as external standard. Aliquot (1–50 μl) was injected into the HPLC system. Each SPE cartridge was cleaned by acetonitrile (3 × 1 ml) and then water (3 × 1 ml), and could be reused up to three times.

### 2.4. Standard solutions

Plasma and gastric fluid samples were obtained from four healthy volunteers and pooled together for the validation study. Stock solutions of metronidazole and omeprazole of 1 mg $ml^{-1}$ were prepared in methanol. These solutions were diluted with glass distilled water to 0.1, 0.05, 0.025, 0.01, 0.005 and 0.001 mg $ml^{-1}$ by serial dilution. An aliquot of these (0.2 ml) was added to 1.8 ml of plasma or gastric fluid to produce standard samples of 10, 5, 2.5, 1, 0.5 and 0.1 μg $ml^{-1}$.

### 2.5. Data analysis

Standard curves were constructed for metronidazole and omeprazole in plasma and gastric

Table 1
Recovery of metronidazole and omeprazole from plasma and gastric fluid

| Biologic fluid/drug | Metronidazole (%) | | Omeprazole (%) | |
|---|---|---|---|---|
| | 1 μg ml$^{-1}$ | 5 μg ml$^{-1}$ | 1 μg ml$^{-1}$ | 5 μg ml$^{-1}$ |
| Plasma | 82.5 ± 2.0[a] | 90.3 ± 7.4 | 84.1 ± 7.2 | 87.7 ± 8.9 |
| Gastric fluid | 48.0 ± 1.7 | 59.2 ± 7.2 | Not detected | |

[a] Each value represents mean ± S.D. of four replicates.

Table 2
Standard curve data for metronidazole and omeprazole in plasma and gastric fluid

| Concentration (μg ml$^{-1}$) | Metronidazole (plasma)[a] | Metronidazole (gastric fluid)[a] | Omeprazole (plasma) |
|---|---|---|---|
| Blank | nd | nd | nd |
| 0.1 | 0.14 ± 0.015[b] | 0.11 ± 0.018 | 0.07 ± 0.0073 |
| 0.5 | 0.69 ± 0.10 | 0.57 ± 0.030 | 0.47 ± 0.054 |
| 1 | 1.47 ± 0.11 | 0.98 ± 0.068 | 1.09 ± 0.079 |
| 2.5 | 3.29 ± 0.10 | 2.61 ± 0.054 | 2.60 ± 0.13 |
| 5 | 5.96 ± 0.78 | 4.70 ± 0.44 | 4.90 ± 0.24 |
| 10 | 12.82 ± 0.39 | 10.12 ± 0.94 | 10.02 ± 1.14 |
| Mean CV | 8.7% | 8.8% | 8.4% |
| $r^2$ | 0.9983 | 0.9985 | 0.9994 |
| Slope | 0.0013 | 0.0005 | 0.0007 |
| Intercept | 0.0355 | −0.0071 | 0.0309 |

[a] Data are after background subtraction due to the presence of a small amount of endogenous materials.
[b] Each value represents mean ± S.D. of four replicates.

Table 3
Accuracy and precision of assay

| Biologic fluid/drug | Concentration spiked (μg ml$^{-1}$) | Concentration found (μg ml$^{-1}$) | % Difference from theoretical value |
|---|---|---|---|
| Plasma metronidazole | 10 | 10.12 ± 0.31[a] | 1.20 ± 0.036% |
| | 5 | 4.70 ± 0.61 | 6.04 ± 0.79% |
| | 1 | 1.05 ± 0.079 | 5.15 ± 0.39% |
| Gastric fluid metronidazole | 10 | 10.12 ± 0.94 | 1.20 ± 0.11% |
| | 5 | 4.70 ± 0.44 | 5.93 ± 0.55% |
| | 1 | 0.98 ± 0.068 | 1.77 ± 0.12% |
| Plasma omeprazole | 10 | 10.02 ± 1.14 | 0.2 ± 0.023% |
| | 5 | 4.90 ± 0.24 | 1.94 ± 0.095% |
| | 1 | 1.09 ± 0.079 | 8.73 ± 0.63% |

[a] Each value represents mean ± S.D. of four replicates.

fluid from 0.1 to 10 μg ml$^{-1}$ using standard linear regression analysis. Peak height ratios of metronidazole or omeprazole to the external standard ASA were plotted against standard concentration of the analytes by the equation $y = mx + b$, where $y$ was the peak height ratios, $x$ was the concentration of the analytes, and $m$ and $b$ were the slope and $y$-intercept of the standard curve, respectively. Each concentration was determined in quadruplicate.

### 2.6. Clinical samples

Plasma and gastric fluid samples were collected from two healthy volunteers after a single 500-mg metronidazole (Flagyl®, Baxter, Ont., Canada) given intravenously over a 30-min period before and after 20 mg b.i.d. p.o. for 7 days of omeprazole (Losec®, Astra Pharma, Ont., Canada). Plasma samples were immediately separated from red blood cells after collection via an intravenous teflon catheter, and gastric samples by a nasogastric tube placed in stomach. The samples were frozen at $-20$°C until analysis.

## 3. Results

### 3.1. Specificity, recovery, sensitivity and linearity

Metronidazole, omeprazole and the external standard ASA were well separated from each other with retention times of 4.1, 7.6 and 16.2 min, respectively (Fig. 2A). There was a small amount of endogenous materials in plasma and gastric juice which could interfere with the measurement of metronidazole especially at low concentrations ($<0.1$ μg ml$^{-1}$; Fig. 2B and Fig. 3A). Recovery of metronidazole and omeprazole from plasma by the described solid phase extraction procedure was above 80%. Recovery of metronidazole from gastric fluid was about 50% (Table 1). Concentrations of omeprazole could not be determined in gastric fluid because of its instability in acid. Using 0.3 ml of plasma or gastric fluid sample, limit of quantitation of the assay was less than 0.1 μg ml$^{-1}$ for metronidazole and omeprazole. The standard curves were linear from 0.1 to 10 μg ml$^{-1}$ ($r^2 = 0.9983 - 0.9994$; Table 2).

Table 4
Retention times of metronidazole, omeprazole, ASA and other drugs

| Drug | Retention time | Drugs | Retention time |
|---|---|---|---|
| Metronidazole | 4.1 | Nifedipine | >30 |
| Omeprazole | 7.6 | Amiodarone | >30 |
| ASA | 16.2 | Captopril | 25.2 |
| Doxycycline | 13.3 | Cimetidine | >30 |
| Tetracycline | 5.2 | Ranitidine | <3 |
| Indomethacin | >30 | Minocycline | <3 |
| Diltiazem | >30 | | |

### 3.2. Precision and accuracy

Intra- and inter-assay variations determined over a 1-month period were less than 15%. Accuracy of the assay was greater than 90% between 1 and 10 μg ml$^{-1}$ (Table 3).

### 3.3. Clinical samples

Plasma concentrations of omeprazole in the two healthy volunteers after 20 mg b.i.d. for 7 days as determined 2 h after the last dose were 0.80 and 1.16 μg ml$^{-1}$. Before receiving omeprazole, concentrations of metronidazole in plasma and gastric fluid were 3.28 and 7.18 μg ml$^{-1}$; and 74.8 and 38.7 μg ml$^{-1}$, respectively. After omeprazole, plasma and gastric fluid concentrations of metronidazole became 5.62 and 12.3 μg ml$^{-1}$; and 13.9 and 20.1 μg ml$^{-1}$, respectively.

## 4. Discussion

Most previous HPLC assays for metronidazole or omeprazole described their separation using reversed phase columns [8–10,12,13]. However, when a $C_8$ or $C_{18}$ column was investigated during the initial method development, selectivity factor ($\alpha$) of omeprazole to metronidazole was greater than 20 in various conditions tested [12]. This is attributed to the profound difference of the physico-chemical properties between the two drugs such that omeprazole is considerably more lipophilic than metronidazole. Thus simple reversed phase chromatography is impractical for simultaneous determination of these two drugs in pharmacokinetic studies. It was found that using the Omnipac Pax-500 column which is based on a multi-phase separation combining anion exchange and reversed phase chromatography, the selectivity factor ($\alpha$) was decreased to below 2 under the described conditions. Repeated injections could be made in less than 20 min allowing a reasonably

high sample throughput. The described HPLC conditions also separated metronidazole and omeprazole from most of the endogenous materials in plasma and gastric fluid (Figs. 2 and 3). Several different classes of drugs tested by the described HPLC conditions did not interfere with the analysis (Table 4). The Omnipac Pax-500 column utilizes a multi-phase stationery phase which combines ion-exchange and reversed-phase mechanisms in a single packing. The Pax-500 substrate consists of highly cross-linked (55%) and solvent compatible beads which have a macroporous core (60 Å pore size) with high surface area for reversed-phase separation. It is also coated with polymeric latex consisting of 4% cross-linked beads serving as ion-exchange sites [14]. The advantages of using the multi-phase column are versatility, wide range of solvent and pH (pH 0–14) compatibility. These factors are useful for separation and simultaneous measurement of compounds with widely different physical and chemical properties.

Extraction of omeprazole could be accomplished by liquid–liquid extraction using methylene dichloride under mildly alkaline conditions [10,13]. On the other hand, metronidazole in plasma could be isolated by solid-phase extraction using silica gel or deproteinization with trichloroacetic acid [8,9] or methanol containing zinc sulfate ($ZnSO_4$) [12]. In our laboratory, solid phase extraction using different sorbent materials was tested. It was found that under the conditions as described in the experimental section, silica gel bonded with a two-carbon chain ($C_2$) consistently produced good recovery for metronidazole and omeprazole ($>80\%$), whereas silica gel bonded with an 18 carbon ($C_{18}$) or cyano propyl function (CN) were not satisfactory ($<10\%$ recovery). ASA was not extracted by any of these three sorbents, and is therefore used as an external standard to compensate for system variability. Recovery of metronidazole in gastric fluid was lower than in plasma (50% vs. $>80\%$). The reason may be related to an inherent difference in the extraction procedure, its stability in acid as well as the matrix effect. Omeprazole could not be recovered in gastric fluid because of its instability in acid [11].

## 5. Conclusion

In summary, the assay achieved sufficient sensitivity, reproducibility and specificity to measure simultaneously omeprazole and metronidazole in plasma, and metronidazole in gastric fluid for pharmacokinetic studies after single or multiple doses. The assay is simple, cost saving and provide high throughput which would facilitate pharmacokinetic studies involving large number of samples.

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