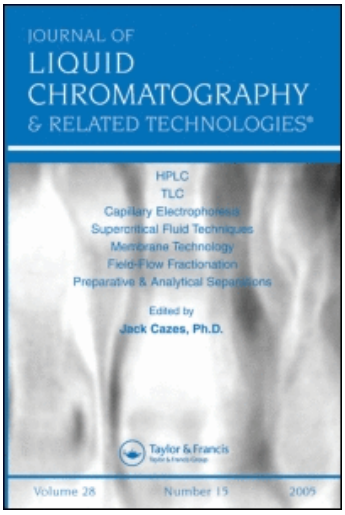

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DEVELOPMENT OF A REVERSED-PHASE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF AMOXICILLIN, METRONIDAZOLE, AND PANTOPRAZOLE IN HUMAN PLASMA USING SOLID-PHASE EXTRACTION

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ABSTRACT

A high-performance liquid chromatography method has been developed and validated for the simultaneous determination of amoxicillin, metronidazole, and pantoprazole in human plasma. Solid-phase extraction (SPE) was utilized to extract the analytes along with the internal standard, tinidazole. Baseline resolution was achieved using a 30 : 70 v/v acetonitrile–25 mM potassium phosphate buffer containing 0.25% triethylamine (pH 6.5) mobile phase, at a flow rate of 0.5 mL/min. A 5 µm phenyl column, equipped with a guard column, with detection at 230 nm constituted the HPLC system. The method yields retention times of 3.7, 4.8, 6.9, and 16.2 min for amoxicillin,

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metronidazole, tinidazole (internal standard), and pantoprazole, respectively. Limits of detection were 200 ng/mL for amoxicillin and metronidazole and 100 ng/mL for pantoprazole. Recoveries from human plasma ranged from 83–92% for amoxicillin, 81–89% for metronidazole, 85–94% for tinidazole, and 93–101% for pantoprazole. Intra-day ($n = 5$) and inter-day ($n = 15$) precision (% RSD) and accuracy (% error) for all analytes ranged from 1.11–5.97% and 0.72–13.5%, respectively.

INTRODUCTION

Helicobacter pylori (Hp) is a common bacterial infection of the gastric mucosa and is widely accepted as the most common cause of peptic ulcer disease and gastritis.[1] Current treatment for the eradication of Hp most often involves multi-drug therapy, consisting of two antibiotics and a histamine-2 antagonist or proton pump inhibitor (PPI).[2] More specifically, PPI triple therapies, which use the twice-a-day combination of a PPI plus metronidazole, 500 mg twice a day, and amoxicillin, 1 g twice a day, yield cure rates of 95–99% when patients with duodenal ulcer are prescribed triple therapy for 10 to 14 days.[1]

A review of the literature revealed that several high performance liquid chromatographic (HPLC) methods have been reported for the individual determination of amoxicillin[3–5] and metronidazole[6–9] in plasma or serum. Although, an HPLC method has been reported for the simultaneous determination of amoxicillin and metronidazole in human plasma using liquid–liquid extraction, the method requires the use of two different detector wavelengths and gradient elution analysis.[10] HPLC methods have been reported for the determination of pantoprazole in plasma or serum, including enantiomeric separations with direct injection[11–12] and column-switching sample preparation procedures.[13] However, no HPLC method has been developed for the simultaneous determination of amoxicillin, metronidazole, and pantoprazole in human plasma or gastric fluid using solid-phase extraction (SPE).

In this paper, we report an isocratic reversed-phase HPLC method to assay amoxicillin, metronidazole, and pantoprazole in human plasma using a C_{18} SPE extraction cartridge and UV detection at 230 nm. This combination of SPE and UV detection results in a method with high recoveries and good linearity, accuracy, and precision.

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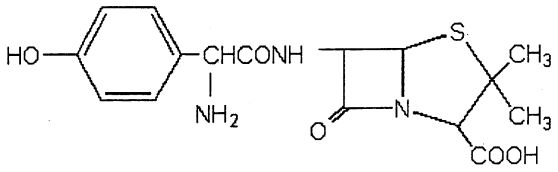
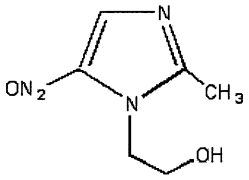
EXPERIMENTAL

Reagents and Chemicals

The structures and formulae of the compounds studied are shown in Fig. 1. Amoxicillin, metronidazole, and triethylamine were purchased from Sigma Chemical Co. (St. Louis, MO). Pantoprazole was kindly provided by Wyeth



Amoxicillin



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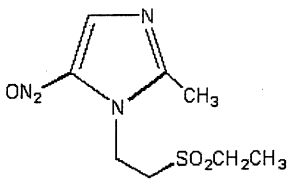
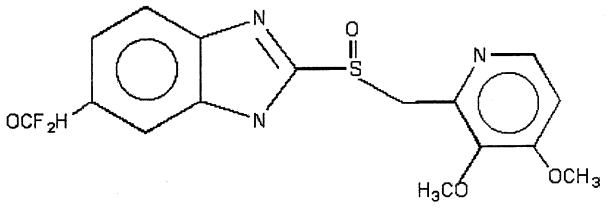


Tinidazole



Pantoprazole

Figure 1. Chemical structures of compounds studied.

Laboratories (Pearl River, NY). Acetonitrile (J.T. Baker, Phillipsburg, NJ) was HPLC grade. Monobasic potassium dihydrogen phosphate (KH_2PO_4) and concentrated phosphoric acid were Baker analyzed reagents. Varian bond-elut™ C_{18} SPE cartridges and the VAC-ELUT™ vacuum manifold were obtained from Varian Sample Preparation Products (Harbor City, CA). Oasis HLB cartridges were purchased from Waters Corp (Milford, MA). Strata™ C-18E SPE cartridges were purchased from Phenomenex (Torrance, CA). Drug-free human plasma used in this study, was purchased from Bioreclamation Inc. (Hicksville, NY) and stored at $-20^{\circ}C$ until the assay.

Chromatographic System

The HPLC chromatographic system consisted of a pump (Model 760, Micromeritics, Norcross, GA), autosampler (Model 728, Micromeritics, Norcross, GA) equipped with a 50 μL loop, and an ultraviolet variable wavelength detector (Model 481, Waters Corp., Milford, MA). Chromatographic separations were achieved on an XTerra™ phenyl column (5 μm, 15 cm × 4.6 mm i.d., Waters, Milford, MA) equipped with an XTerra™ Sentry™ guard column.

Chromatographic Conditions

Chromatographic analysis was carried out at ambient temperature ($24 \pm 1^{\circ}C$). The isocratic mobile phase was composed of a buffer solution [acetonitrile–25 mM potassium phosphate monobasic in water containing 0.25% triethylamine (pH 6.5) (30 : 70% v/v)]. The pH was adjusted with phosphoric acid. The mobile phase was filtered through a 0.45 μm nylon-66 filter (Alltech, Deerfield, IL), and degassed by sonication prior to use. The mobile phase flow rate was 0.5 mL/min and the detection wavelength was set at 230 nm. Under the chromatographic conditions described, amoxicillin eluted at 3.7 min, metronidazole eluted at 4.8 min, tinidazole (internal standard) eluted at 6.9 min, and pantoprazole eluted at 16.2 min.

Preparation of Standard Solutions

Stock solutions of amoxicillin, metronidazole, tinidazole (internal standard), and pantoprazole were prepared by dissolving appropriate amounts of each drug in water, to obtain final drug concentrations of 100 μg/mL. Working solutions were prepared by further diluting these stock solutions in 25 mM potassium phosphate buffer (pH 6.5). Calibration standards for all samples were

prepared by spiking 100 μL of drug-free human plasma with appropriate volumes of each standard solution and 20 μL of tinidazole internal standard solution, to obtain amoxicillin, metronidazole, and pantoprazole concentrations of 0.5–50 μg/mL (0.5, 1, 5, 10, 25, and 50 μg/mL) and a 2.0 μg/mL tinidazole concentration. The spiked plasma standards were then extracted from plasma.

Extraction Procedure

Extraction cartridges (Phenomenex Strata™ C18-E, 3 mL, 500 mg) were placed on a vacuum elution manifold (VAC-ELUT™, Varian Sample Preparation Products, Harbor City, CA). Each 500 mg C_{18} SPE cartridge was pre-conditioned with methanol (2 × 1 mL) and distilled water (2 × 1 mL). Care was taken to ensure that the cartridges did not run dry. One milliliter of the spiked plasma samples was loaded onto the cartridges and allowed to pass through the cartridge under mild vacuum. The cartridge was washed with water (2 × 1 mL) and dried under vacuum for 5 min. After discarding the eluent, the analytes were eluted with 1 mL methanol (4 × 250 μL) into clean 1.5 mL microcentrifuge tubes. Eluents from the cartridges were then dried at 40°C using the vacuum centrifuge (Model SC110A, Savant Instruments, Holbrook, NY, USA), and reconstituted in 1 mL mobile phase. The samples were then transferred to 1 mL injection vials, where 50 μL of sample was injected onto the HPLC column.

Assay Validation

Samples were quantified using peak area ratios of analyte to internal standard (tinidazole). The calibration curves showed good linearity in the range of 0.5–50 μg/mL for amoxicillin, metronidazole, and pantoprazole in human plasma. The regression coefficients (r^2) of calibration curves of each drug were higher than 0.99.

The limits of detection (LODs), for each analyte in plasma, were demonstrated by analysis of standard-spiked samples gradually decreasing in concentration. The LODs were determined as the concentration at which the signal/noise ratio was ~3.

The method accuracy (% error) was obtained by comparing the concentrations calculated from the calibration curves vs. concentrations added. Precision was calculated as percent relative standard deviation (% RSD). The intra-day accuracy and precision of the assay were determined by assaying three quality control samples at low (2 μg/mL, $n = 5$), medium (10 μg/mL, $n = 5$), and high (40 μg/mL, $n = 5$) concentrations for amoxicillin, metronidazole, and

pantoprazole, in three analytical runs within the same day. The inter-day accuracy and precision samples were analyzed on three different days.

To investigate the extraction efficiency of amoxicillin, metronidazole, tinidazole, and pantoprazole from human plasma, standard-spiked plasma samples were subjected to extraction and then analyzed. The resulting peak areas were compared to peak areas of samples containing equal amounts of each analyte in mobile phase.

Both processed sample and freeze–thaw stability were evaluated for amoxicillin, metronidazole, tinidazole, and pantoprazole. The stability of all four analytes stored on the autosampler tray was assessed, by injecting replicate standard-spiked plasma samples at concentrations of 1 and 10 μg/mL, at evenly spaced intervals, over a 24 hour period. Freeze–thaw stability studies were also conducted to investigate the influence of freezing and thawing on the four analytes, at concentrations of 1 and 10 μg/mL in spiked drug-free plasma. The spiked drug-free plasma samples were assayed in duplicate, and the remainder placed into a −20°C freezer for 24 hours. After 24 hours, samples were thawed and a further aliquot was assayed in duplicate. This was repeated until three freeze-thaw cycles were completed.

RESULTS AND DISCUSSION

The goal of this study, was to develop an isocratic HPLC assay for the analysis of amoxicillin, metronidazole, and pantoprazole in human plasma and gastric fluid. Since pantoprazole is more lipophilic than either amoxicillin or metronidazole, it was more challenging to obtain a short run time under isocratic conditions. Initial studies to develop an isocratic HPLC method for this mixture, involved the use of C_{18} and C_8 columns with various mobile phases containing acetonitrile– or methanol–aqueous phosphate buffers. In almost every system studied, amoxicillin and metronidazole eluted at the solvent front with poor peak symmetry, while pantoprazole eluted after 30 min.

Our attention turned to the use of a Waters XTerra™ phenyl column (Milford, MA), since it takes advantage of both silica and polymer packing materials; thereby, separating compounds with widely different physical and chemical properties. Also, XTerra™ particles replace one third of the surface silanol groups with methyl groups, resulting in the most homogenous coverage of any reversed-phase material, yielding sharp, symmetrical peaks for basic compounds. It was found, that the phenyl column reduced the retention of pantoprazole to about 16 min, while resolving amoxicillin and metronidazole away from the solvent front, under the described conditions. Although, the phenyl column did improve peak shape for the basic analytes, it was still necessary to use an amine modifier (0.25% triethylamine) in the mobile phase to obtain sharp,

symmetrical peaks. Repeated injections could be made in less than 20 min, allowing a reasonably high sample throughput. The described HPLC conditions also separated amoxicillin, metronidazole, and pantoprazole from the endogenous materials in human plasma (Fig. 2). Several different classes of drugs and the sulfone metabolite of pantoprazole were tested by the described HPLC conditions, to see if they interfered with the analysis (Tables 1 & 2).

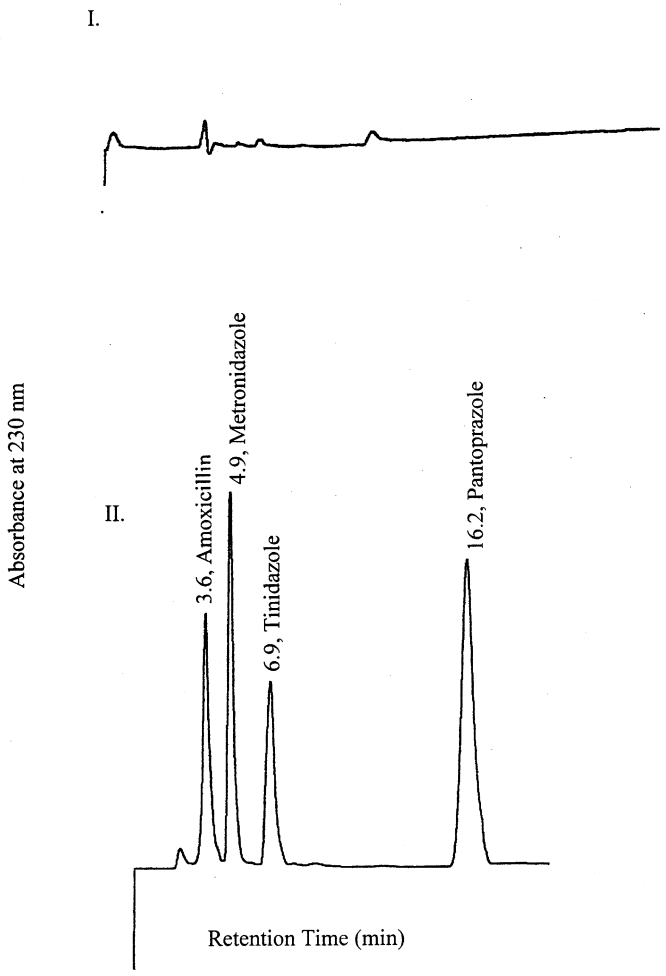


Figure 2. Representative chromatograms of blank plasma (I) and human plasma spiked with 5 μg/mL of amoxicillin, metronidazole, and pantoprazole (II). Tinidazole was used as the internal standard.

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Table 1. The Intra- and Inter-Day Precision (% RSD) and Accuracy (% Error) of Amoxicillin, Metronidazole, and Pantoprazole in Human Plasma

		Intra-Day ($n = 5$)			Inter-Day ($n = 15$)		
Analyte	Conc. Added (μg/mL)	Conc. Found (μg/mL)	% RSD	% Error	Conc. Found (μg/mL)	% RSD	% Error
Amoxicillin	2	1.90 ± 0.11	5.97	5.05	2.16 ± 0.04	1.93	6.39
	10	11.35 ± 0.28	2.48	13.5	9.96 ± 0.53	5.34	4.12
	20	19.86 ± 0.37	1.85	0.72	19.64 ± 0.84	4.26	4.76
Metronidazole	2	2.03 ± 0.06	2.81	1.32	2.03 ± 0.04	1.82	3.05
	10	9.11 ± 0.24	2.64	8.90	9.54 ± 0.19	1.95	3.29
	20	21.26 ± 0.40	1.86	6.32	21.73 ± 0.24	1.11	7.77
Pantoprazole	2	2.10 ± 0.03	1.65	4.94	2.17 ± 0.03	1.18	9.54
	10	9.51 ± 0.17	1.77	4.85	10.29 ± 0.26	2.54	4.77
	20	18.66 ± 0.22	1.16	6.70	19.27 ± 0.39	2.01	5.01

Table 2. Retention Times of Amoxicillin, Metronidazole, Tinidazole, Pantoprazole, and Other Drugs

Drug	Retention Time (min)	Drug	Retention Time (min)
Amoxicillin	3.7	Ibuprofen	>20
Metronidazole	4.8	Acetaminophen	5.2
Tinidazole	6.9	Naproxen	12.6
Pantoprazole	16.2	Nifedipine	>20
P-sulphone metabolite	8.9	Ranitidine	4.1
Guaifenesin	7.8	Cimetidine	5.9
Theophylline	4.4	Famotidine	5.4
Codeine	4.9		

Solid-phase extraction, using a series of different extraction cartridges, such as Varian Bon-Elut™, C18, Oasis™ HLB, and Phenomenex Strata™ C18-E were evaluated for the plasma and gastric fluid sample clean-up procedure. It was determined that under the conditions described in the experimental section, Strata™ cartridges produced the highest recoveries of the drugs (>80%), as well as cleaner assay samples. An attempt was made to employ the extraction procedure for gastric fluid samples; however, the recovery of amoxicillin and metronidazole in gastric fluid was lower than in plasma (30% vs. 80%). Recovery of pantoprazole in gastric fluid could not be determined, due to its instability at pH 2.

Using the described solid-phase extraction procedure and HPLC method, calibration curves were linear over the 0.5–50 μg/mL concentration range with r^2 values greater than 0.99 for amoxicillin, metronidazole, and pantoprazole in human plasma. The LODs were found to be 200 ng/mL for amoxicillin and metronidazole, and 100 ng/mL for pantoprazole. The intra-day ($n = 5$) precision and accuracy for amoxicillin (spiked concentrations of 2, 10, and 20 μg/mL) were in the range of 1.80–5.97% (RSD) and 0.73–13.5% (error), respectively, and for metronidazole (spiked concentrations of 2, 10, and 20 μg/mL) 1.86–2.81% (RSD) and 1.32–8.90% (error), respectively. At the same spiked concentrations, intra-day ($n = 5$) precision and accuracy for pantoprazole were 1.16–1.77% (RSD) and 4.85–6.70% (error), respectively. The plasma recoveries were high and reproducible, ranging from 83–92% for amoxicillin, 81–89% for metronidazole, 85–94% for tinidazole, and 93–101% for pantoprazole. At 2, 10, and 20 μg/mL, inter-day ($n = 15$) precision and accuracy for amoxicillin ranged from 1.93–5.34% (RSD) and 4.12–6.39% (error), data for metronidazole ranged from 1.11–1.95% (RSD) and 3.05–7.77% (error), and pantoprazole ranged from 1.18–2.54% (RSD) and 4.77–9.54% (error). The results from the validation of the method in human plasma, are shown in Fig. 2. The stability of each drug stored

on the autosampler was assessed up to 24 hr. The lack of instability for this period of time, allows a large batch of samples to be processed in one assay. Moreover, none of the drugs degraded after three freeze–thaw cycles.

CONCLUSION

A sensitive and efficient method for the extraction and simultaneous analysis of amoxicillin, metronidazole, and pantoprazole in human plasma has been developed and validated. This method yields high recoveries, good linearity, precision, and accuracy, within the range of 0.5–50 μg/mL. Although, not necessary for concentrating our samples, the solid-phase extraction procedure provided excellent sample clean up. Thus, the method is applicable for pharmacokinetic studies after single or multiple doses of each drug.

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