Simultaneous Determination of Pantoprazole and Its Two Metabolites in Dog Plasma by HPLC

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Abstract

A simple and sensitive high-performance liquid chromatography (HPLC) method is developed and validated for simultaneous determination of pantoprazole and its two metabolites (pantoprazole sulfone and pantoprazole thioether) in dog plasma and applied to a pharmacokinetic study in Beagle dogs. Following a protein precipitation procedure, the samples are separated using reversed-phase HPLC (C_{18}) by a gradient of acetonitrile and ammonium acetate (pH 6.0) at a flow rate of 1.0 mL/min and quantitated using UV detection at 290 nm. Omeprazole is selected as the internal standard. The method has a lower limit of quantitation of 0.025 µg/mL for pantoprazole and its two metabolites, using 0.1-mL aliquots of plasma. The linear calibration curves are obtained in the concentration range of 0.025–10.0 µg/mL for three analytes. The intra- and interrun precision (relative standard deviation), calculated from quality control (QC) samples, is less than 13% for three analytes. The accuracy determined from QC samples is between –6.4% and 12%.

Introduction

Pantoprazole (PAN) (Figure 1), a selective and long-acting proton pump inhibitor (1), is used in the treatment of acid-related gastrointestinal disorders such as duodenal and gastric ulcers, reflux esophagitis, and Zollinger–Ellison syndrome (2). Drugs with sulfoxide moieties, such as omeprazole (3), lansoprazole (4), and PAN, can undergo oxidative metabolism by cytochrome P450 or flavin containing monooxygenase (or both) (5,6) and can undergo reductive metabolism by aldehyde oxidase or thioredoxin-linked enzymes (or both) (7). Both liver and gut flora are potential sites for formation of the sulfide metabolite (5–7). These metabolites include pantoprazole sulfone (PAN-SO_2), pantoprazole thioether (PAN-S), demethylated PAN-SO_2, and demethylated PAN-S. In order to elucidate the pharmacokinetics of PAN and its metabolites after administration of PAN to animals, it is essential to develop a method to determine the concentration of PAN and its metabolites in biological fluids. Several high-performance liquid chromatographic (HPLC) methods have been available for the determination of PAN and its metabolites in biological fluids (8–12). Although three HPLC methods (8–10) have enabled PAN and PAN-SO_2 to be determined in plasma or serum, these methods do not determine PAN-S. Furthermore, two methods among them utilized a column-switching system as a sample cleanup procedure. There are two papers that reported separation and determination of PAN enantiomers in human serum using cellulose-based chiral stationary phase (11) and tris-(3,5-dimeth-oxyphenylcarbamate) of amylose phase (12), respectively. But these methods could not be used to determine its metabolites. This paper describes a simple and sensitive gradient HPLC method for the simultaneous determination of PAN, PAN-SO_2, and PAN-S in dog plasma and applies the method to a pharmacokinetic study of PAN in Beagle dogs.

Experimental

Chemicals

PAN, PAN metabolites (PAN-SO_2 and PAN-S), and internal standard (IS) (omeprazole) were provided by Institute of Materia Medica, Shenyang Pharmaceutical University (Shenyang, China). Compound purities exceeded 99%. Methanol and acetonitrile were of HPLC grade, and other

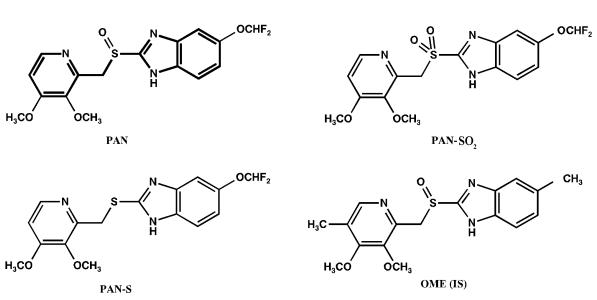

Figure 1. Structures of PAN, PAN-SO_2, PAN-S, and IS (omeprazole).

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reagents were commercially available and of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

Chromatographic system

The HPLC system (Hewlett Packard, Palo Alto, CA) consisted of a G1313A quaternary pump, a G1314A UV detector, G1313A autosampler, G1316A column oven, and vacuum degasser unit. The mobile phase consisted of a gradient mixed from acetonitrile (A) and ammonium acetate (pH 6.0, 10mM) (B) at a flow rate of 1.0 mL/min. The column was equilibrated with 35% A at time 0. After injection of the plasma sample (50 µL), the A volume ratio was linearly increased to 50% at 6 min, held constant at 50% until 10 min, then reduced to 35% at 12 min, and the column was equilibrated for 3 min before application of the next sample. The total analysis time was 15 min. Separation was accomplished at 25°C on a diamonsil C_{18} column (4.0 × 250 mm, 5 µm) (Dikma, Beijing, China). The UV detector was set at 290 nm.

Standard solutions and quality control samples

The stock standard solution of PAN, PAN-SO_2, and PAN-S was prepared by dissolving accurately weighed portions in methanol to give a final concentration of 400.0 µg/mL. The solution was then successively diluted with methanol to achieve standard working solutions at concentrations of 0.025, 0.050, 0.125, 0.250, 1.0, 2.5, 5.0, and 10.0 µg/mL for PAN, PAN-SO_2, and PAN-S, respectively. An 8.0 µg/mL IS (omeprazole) working solution was prepared by diluting the 400.0 µg/mL stock standardsolution of omeprazole with methanol.

The standard working solutions (100 µL) were used to spike blank dog plasma (0.1 mL) to give final concentrations in the range of 0.025–10.0 µg/mL. The plasma samples at concentrations of 0.050, 0.250, 2.5, and 8.0 µg/mL for PAN, PAN-SO_2, and PAN-S were used as quality control (QC) samples in developing the analytical method and during the pharmacokinetic study. All of the solutions were stored at 4°C and were brought to room temperature before use.

Sample preparation

One-hundred microliters of IS (8.0 µg/mL omeprazole prepared in methanol) was added to a 0.1-mL aliquot of plasma. The sample mixture was deproteinized with 0.2 mL acetonitrile, and the precipitate was removed by centrifugation at 3000 *g* for 10 min. Then the supernatant was transferred into a clean tube and evaporated to dryness at 40°C under a gentle stream of nitrogen, and the residue was reconstituted by 100 µL of ammonium acetate (pH 6.0, 10mM)–acetonitrile (35:65 v/v). A 50-µL aliquot of the solution was injected into the HPLC system.

Method validation

The specificity of the method was demonstrated by comparing chromatograms of six independent plasma samples from dogs, each as a blank and spiked sample. Standard curves were constructed using weighted ($w = 1/c^2$) linear least-squares regression analysis of the observed peak area ratios of PAN, PAN-SO_2, PAN-S, and IS. The unknown sample concentrations of PAN, PAN-SO_2, and PAN-S were calculated from the linear regression equation of the peak area ratio against concentrations of the calibration curves.

Accuracy and precision were assessed by determining QC samples at four concentration levels (Table I, six replicate samples of each concentration of QC samples) on three different validation runs. The accuracy was expressed as the relative error (RE) [(mean concentration found – concentration spiked)/(concentration spiked) × 100%], and the precision was evaluated by the relative standard deviation (RSD) [(standard

Table I. Summary of Precision and Accuracy from QC Samples Prepared by PPT*

Analyte	Conc. added (µg/mL)	Conc. found (µg/mL)	Intrarun RSD (%)	Interrun RSD (%)	RE (%)
PAN	0.05	0.054	11	9.8	8.0
	0.25	0.254	5.4	5.1	1.6
	2.5	2.504	3.5	4.0	0.2
	8.0	7.895	7.3	6.3	–1.3
PAN-SO_2	0.05	0.056	10	5.8	12.0
	0.25	0.264	3.6	3.9	5.6
	2.5	2.432	5.6	5.2	–2.7
	8.0	8.234	8.9	7.2	2.9
PAN-S	0.05	0.053	13	7.9	6.0
	0.25	0.234	4.9	5.4	–6.4
	2.5	2.467	6.3	3.6	–1.3
	8.0	8.325	11	5.7	4.1

* $n = 3$ run, six replicates per run.

Table II. Recovery of PAN, PAN-SO_2, PAN-S, and IS from Plasma*

Analyte		Recovery (%) 0.05 µg/mL	0.25 µg/mL	2.5 µg/mL	8.0 µg/mL
PAN	Mean	98.3	98.4	99.3	95.3
	SD†	8.4	6.9	6.7	9.2
PAN-SO_2	Mean	98.4	105.3	94.8	97.4
	SD	12.3	8.9	5.4	7.8
PAN-S	Mean	105.3	89.5	96.5	97.3
	SD	9.9	8.3	5.9	7.3
IS	Mean	–	–	–	98.6
	SD	–	–	–	4.3

* $n = 3$.
† Standard deviation.

deviation/mean concentration found) × 100%].

The absolute recovery for each analyte and IS from plasma were determined as follows: QC samples at four concentration levels of PAN, PAN-SO_2, PAN-S, and IS at one concentration level (Table II, three replicate samples of each) were treated following the protein precipitation procedure (PPT) described, and the absolute recovery was determined by comparing peak area of each drug in the treated samples with those obtained from direct injection of the untreated (stock standard solutions) drugs.

The stability of PAN, PAN-SO_2, PAN-S, and IS in the reconstituted solution obtained was assessed by placing QC samples at three concentrations and IS samples under ambient conditions for 4, 8, and 12 h.

Application of the analytical method

All animal procedures described in this report were approved by the Ethics Committee of Shenyang Pharmaceutical University and the investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). The established analytical method was used to investigate the profile of PAN, after oral administration of 6 mg/kg PAN to 8 Beagle dogs (10 kg ± 1 kg). Blood samples (0.6 mL) were collected into heparinized tubes from the jugular vein predose, and at 0, 5, 15, 30, and 45 min and 1, 1.5, 2, 3, 4, and 6 h after an oral administration. Blood samples were immediately processed for plasma by centrifuged at 3000 *g* for 10 min. The plasma concentration-time curves of PAN, PAN-SO_2, and PAN-S in plasma were plotted.

Results and Discussion

Preparation of plasma samples

Sample preparation is a critical step for the simultaneous determination of several components in biological sample by HPLC. Currently, the most widely employed biological samples preparation methodologies are liquid–liquid extraction, PPT, solid-phase extraction (SPE), and column-switching technique. The difference in polarity for PAN and its metabolites make it difficult to simultaneously extract them from plasma with organic solvents. Although the SPE and column-switching techniques (8–12) were good methods, the SPE increased the time of sample preparation and the column-switching technique required a column-switching system. As the PPT procedure has the advantages of simplicity and universality for drug molecules in plasma, thus in the paper the plasma samples containing PAN and its metabolites were prepared by the PPT procedure.

Separation and impact of pH on retention times

The retention times of PAN and IS remained largely unaffected by changes in the pH of ammonium acetate (10mM), though the retention time of PAN-SO_2 and PAN-S changed substantially with pH modulation in the range pH 4–8 (Figure 2). Under the HPLC conditions applied, PAN, PAN-SO_2, PAN-S, and IS were sufficiently separated, and the retention times were 7.3, 8.2, 8.9, and 13.2 min for IS, PAN, PAN-SO_2, and PAN-S, respectively (Figure 3).

Assay specificity

Potential interference from endogenous compounds was investigated by the analysis of six different sources of dog plasma. Figure 3 illustrates a representative chromatogram

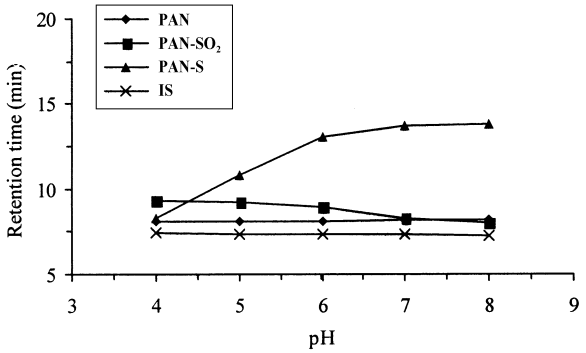

Figure 2. Effect of pH of ammonium acetate (10mM) on the retention times of PAN, PAN-SO_2, PAN-S, and IS.

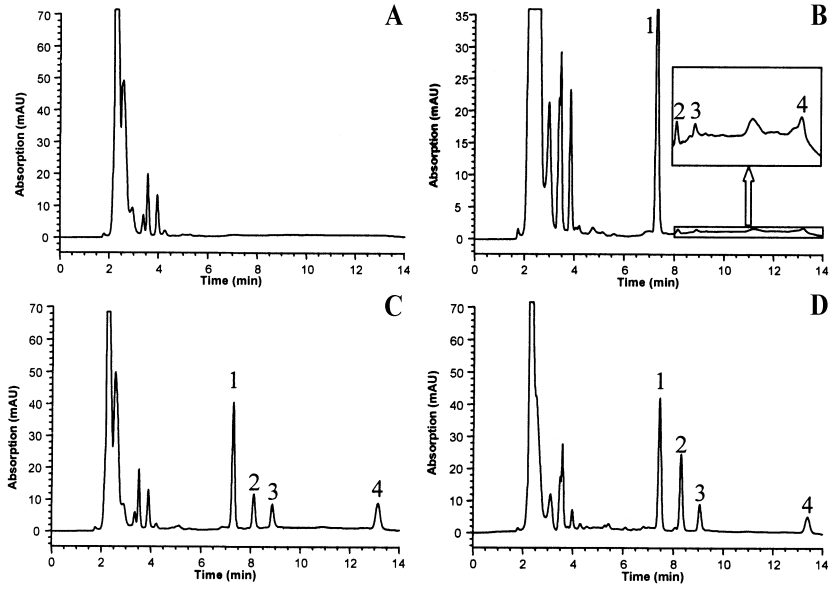

Figure 3. Representative chromatograms of PAN, PAN-SO_2, PAN-S, and IS in dog plasma obtained from (A) blank dog plasma; (B) plasma spiked with 0.025 of µg/mL PAN, PAN-SO_2, PAN-S, and 8.0 µg/mL of IS; (C) plasma spiked with 1.0 µg/mL of PAN, PAN-SO_2, PAN-S, and 8.0 µg/mL of IS; and (D) plasma sample from a dog after an oral administration of PAN (peaks: 1 = IS, 2 = PAN, 3 = PAN-SO_2, and 4 = PAN-S).

of blank plasma sample (A); two blank plasma samples, each spiked with PAN and its metabolites at 0.025 µg/mL (B), 1.0 µg/mL (C), and IS (8.0 µg/mL); and a dog plasma sample (D). A clean chromatogram shows no interference from endogenous substances with analytes or IS in plasma samples.

Calibration curve and lower limit of quantitation

The calibration curves (n = 9) for PAN and its metabolites are shown in Figure 4. The linearity of the calibration curves of the parent compound and its two metabolites was achieved at concentrations ranging from 0.025 to 10.0 µg/mL. The average correlation coefficients for PAN, PAN-SO_2, and PAN-S were 0.9981 ± 0.0006, 0.9960 ± 0.0010, and 0.9985 ± 0.0009 (n = 9), respectively.

Lower limit of quantitation (LLOQ) is defined as the lowest concentration on the calibration curve for which an acceptable accuracy within ± 15% and a suitable precision below 15% were obtained. The LLOQ from dog plasma for PAN, PAN-SO_2, and PAN-S was determined to be 0.025 µg/mL. These limits are already sufficient for pharmacokinetic studies.

Assay precision and accuracy

Table I summarizes the intra- and interrun precision and accuracy for PAN, PAN-SO_2, and PAN-S from QC samples. The results were calculated using one-way analysis of variance. The intrarun precision of PAN, PAN-SO_2, and PAN-S was measured to be below 11%, 10%, and 13%, respectively. The interrun precision of these were measured to be below 9.8%, 7.2%, and 7.9%, respectively. The interrun accuracy of PAN, PAN-SO_2, and PAN-S ranged from –1.3% to 8.0%, –2.7% to 12%, and –6.4% to 6.0%, respectively. The method was demonstrated to be accurate and reproducible for the determination of PAN, PAN-SO_2, and PAN-S in dog plasma.

Absolute recovery and storage stability

The recoveries from plasma of PAN, PAN-SO_2, and PAN-S at concentrations of 0.05, 0.25, 2.5, and 8.0 µg/mL were determined (Table II). The recoveries of PAN were 98.3 ± 8.4%, 98.4 ± 6.9%, 99.3 ± 6.7%, and 95.3 ± 9.2% at 0.05, 0.25, 2.5, and 8.0 µg/mL, respectively. Those of PAN-SO_2 were 98.4 ± 12.3%, 105.3 ± 8.9%, 94.8 ± 5.4%, and 97.4 ± 7.8%, respectively. Those of PAN-S were 105.3 ± 9.9%, 89.5 ± 8.3%, 96.5 ± 5.9%, and 97.3 ± 7.3% at four QC concentration levels, respectively. The recoveryof IS was 98.6 ± 4.3% at a concentration of 8.0 µg/mL.

According to Huber et al. (10), PAN and PAN-SO_2 in serum are stable under –15°C for 11 months. In our experiment, the stability of PAN, PAN-SO_2, PAN-S, and IS under process conditions was evaluated. It was found that PAN and IS were stable in a reconstitution solution of ammonium acetate (pH 6.0, 10mM)–acetonitrile (35:65 v/v) for approximately 4 h at room temperature, and unstable after 8 h under the same conditions, whereas PAN-SO_2 and PAN-S were stable at 12 h under the same conditions.

Application of the analytical method in pharmacokinetic studies

After an oral administration of 6 mg/kg PAN to 8 Beagle dogs, plasma concentrations of PAN, PAN-SO_2, and PAN-S were simultaneously determined by the validated HPLC method. The mean plasma concentration-time curves of PAN, PAN-SO_2, and PAN-S after administration of PAN are shown in Figure 5.

Conclusion

This paper describes a sensitive, simple, gradient HPLC method for the simultaneous determination of PAN and its two metabolites (PAN-SO_2 and PAN-S) in dog plasma. Using PPT as a sample cleanup procedure, the LLOQ of PAN and its two metabolites was 0.025 µg/mL with 0.1-mL aliquots of plasma. The method has been demonstrated to be suitable for use in pharmacokinetic studies of PAN.

Acknowledgments

This paper was supported in part by Grant 2003AA2Z347C of the 863 Program of China. The authors would like to thank Mr. Qinghe Wang, Shenyang Pharmaceutical University (Shenyang, China) for the synthesis of PAN-SO_2.

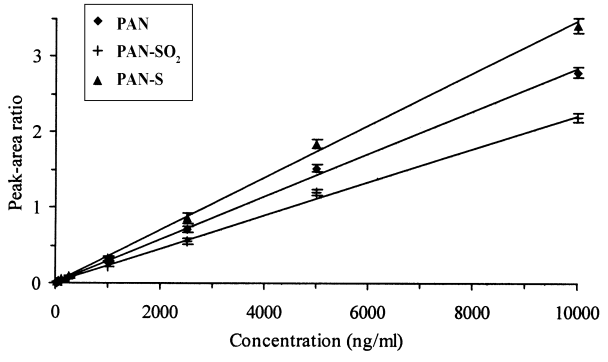

Figure 4. Standard calibration curves for PAN, PAN-SO_2, and PAN-S. Data expressed as mean ± standard deviation (n = 9).

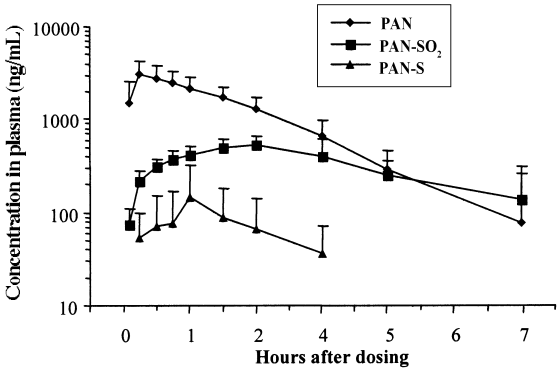

Figure 5. Mean plasma concentration-time profiles of PAN, PAN-SO_2, and PAN-S after an oral administration of 6 mg/kg PAN to 8 Beagle dogs.

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Manuscript received July 17, 2004;
revision received March 4, 2005.