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Journal of Chromatography B, 822 (2005) 326–329
JOURNAL OF CHROMATOGRAPHY B
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Short communication

High-performance liquid chromatography method for the quantification of pantoprazole in human plasma

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Received 26 January 2005; accepted 9 June 2005
Available online 7 July 2005

Abstract

A sensitive and selective HPLC method with UV detection (290 nm) was developed and validated for quantitation of pantoprazole, proton-pump inhibitor, in human plasma. Following a single-step liquid–liquid extraction with methyl *tert*-butyl ether/diethyl ether (70/30, v/v), the analyte and internal standard (zonisamide) were separated using an isocratic mobile phase of 10 mM phosphate buffer (pH 6.0)/acetonitrile (61/39, v/v) on reverse phase Waters symmetry® C_{18} column. The lower limit of quantitation was 20 ng/mL, with a relative standard deviation of less than 4%. A linear range of 20–5000 ng/mL was established. This HPLC method was validated with between-batch and within-batch precision of 1.3–3.2% and 0.7–3.3%, respectively. The between-batch and within-batch bias was −0.5 to 8.2 % and −2.5 to 12.1%, respectively. This validated method is sensitive and repeatable enough to be used in pharmacokinetic studies.

Keywords: Pantoprazole; HPLC; Quantification; Human plasma

1. Introduction

Pantoprazole (Fig. 1), 5-(difluoromethoxy)-2-[(3,4-dimethoxy-2-pyridyl)methylsulfinyl]1H-benzimidazole, is a selective and irreversible proton pump inhibitor used in medicine as an antiulcerative agent [1]. Pantoprazole is characterized by its favourable pharmacokinetic properties and its low potential to interact with other drugs in man [2,3]. The latter is probably due to its unique metabolism as compared with other proton pump inhibitors (e.g., omeprazole, lansoprazole, rabeprazole and esomeprazole) [3–5]. Pantoprazole is metabolized by a combination of phase I and phase II metabolism [6]. Pantoprazole accumulates in the acidic compartment of the parietal cell, where it is protonated and chemically rearranged to the active inhibitor, which then covalently binds to the H^+/K^+-ATPase. This results in a long duration of action.

Several methods have been employed for the quantification of pantoprazole in human plasma and serum samples. Cass et al., [7] quantified pantoprazole enantiomers in human plasma using multidimensional high-performance liquid chromatography (HPLC) with ultraviolet detection. Limits of quantification was 200 ng/mL and a total run time higher than 20 min. Tanaka and Yamazaki [8] reported a reversed-phase HPLC method to quantify pantoprazole enantiomers in human serum using a cellulose-based chiral stationary phase and column-switching system as a sample cleanup procedure. The calibration curve for each enantiomer was linear from 100 to 5000 ng/mL and the total run time was also higher than 20 min (25 min). Huber et al., [9] reported a fully automated HPLC method for the determination of pantoprazole and its sulphone metabolite in plasma by direct injection and pre-column sample clean-up technique. Recently Peres et al., [10] reported a method based on liquid chromatography with positive ion electrospray ionization coupled to a tandem mass spectrometry (LC–MS/MS) detection for the quantification of pantoprazole in human plasma. The method has a chromatographic total run time of 4.5 min and was linear

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doi:10.1016/j.jchromb.2005.06.011

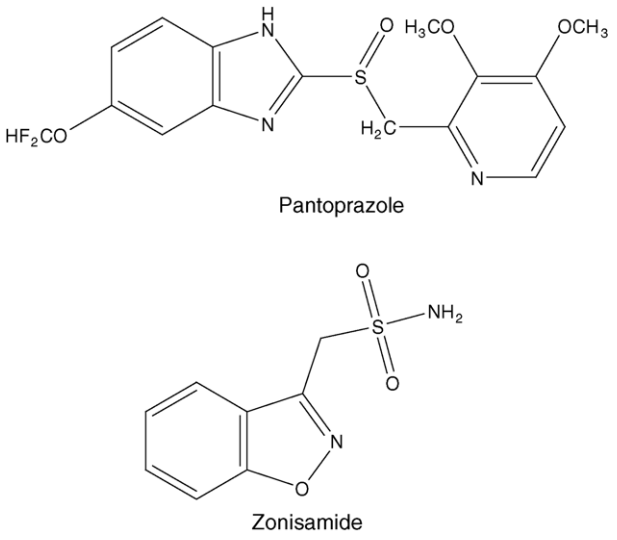

Fig. 1. Chemical structures of pantoprazole and zonisamide (I.S.).

within the range 5–5000 ng/mL. When applied in pantoprazole human plasma quantification, the limits of quantification are usually high and have a very long sample preparation and/or chromatographic time or involve expensive equipment (LC–MS/MS), which most labs may not have.

The objective of the present investigation is to establish a fully validated HPLC method with a quantification limit sufficiently low to support pharmacokinetic and bioequivalence studies of pantoprazole and also would allow quantification of a large number of plasma samples. The method reported in this paper is an accurate HPLC method to quantify the plasma concentration of pantoprazole with ultraviolet detection using liquid–liquid extraction. This method is fully validated as per FDA guidelines [11] and the LLOQ is 20 ng/mL.

2. Experimental

2.1. Chemicals

Pantoprazole drug substance was obtained from Vimta Labs (Hyderabad, India). Zonisamide (internal standard, I.S.) was from our R&D department. Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, methyl tert butyl ether, diethyl ether and potassium hydroxide pellets were purchased from Merck (Worli, Mumbai, India). HPLC Type I water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. Chromatography

The integrated high performance liquid chromatography system (LC 2010C, Shimadzu Corporation, Kyoto, Japan) was equipped with a quaternary pump, a degasser, an autosampler, an injector with a 100-μL loop, a column oven, a UV detector and a data system (Class VP version 6.12). The separation of compounds was made on a Waters symmetry® C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) at 30 °C temperature. The mobile phase was a mixture of 10 mM phosphate buffer (pH 6.0)/acetonitrile (61/39, v/v) pumped at a flow-rate of 1.0 mL/min. Detection was set at a wavelength of 290 nm.

2.3. Sample processing

A 1-mL volume of plasma was transferred to a 15-mL glass test tube, and then 50 μL of I.S. working solution (100 μg/mL) was spiked. Next a 4-mL aliquot of extraction solvent, methyl *tert*-butyl ether/diethyl ether (7/3), was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortex-mixed for 3 min using a Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The sample was then centrifuged using Multifuge 3S-R (Kendro Laboratory Products, Sorvall-Heraeus, Germany) for 3 min at 800 × *g*. The organic layer (3-mL) was quantitatively transferred to a 6-mL glass tube and evaporated to dryness using a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. Then, the dried extract was reconstituted in 250 μL of water/methanol (50/50, v/v) and a 100-μL aliquot was injected into chromatographic system.

2.4. Bioanalytical method validation

Standard stock solution of pantoprazole (2.5 mg/mL) was prepared in water/methanol (50/50, v/v). Standard stock solution of I.S. (1 mg/mL) was prepared in methanol. The I.S. working solution (100 μg/mL) was prepared by diluting stock solution with water/methanol (50/50, v/v). 50 μL of working solutions were added to 950 μL of drug-free plasma to obtain pantoprazole concentrations of 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL. The quality control samples were prepared in pool, at concentrations of 20 (LLOQ), 60 (low), 2500 (medium) and 4000 ng/mL (high), as a single batch at each concentration, and then divided in aliquots that were stored in the freezer at below −50 °C until analysis.

Recovery of pantoprazole was evaluated by comparing the mean peak areas of five extracted low, medium and high quality control samples to mean peak areas of five neat reference solutions (unprocessed). Recovery of zonisamide (I.S.) was evaluated by comparing the mean peak areas of ten extracted quality control samples to mean peak areas of ten neat reference solutions (unprocessed) of the same concentration.

3. Results and discussion

3.1. Separation

Fig. 2 shows the representative chromatograms of blank plasma, plasma samples spiked with pantoprazole at

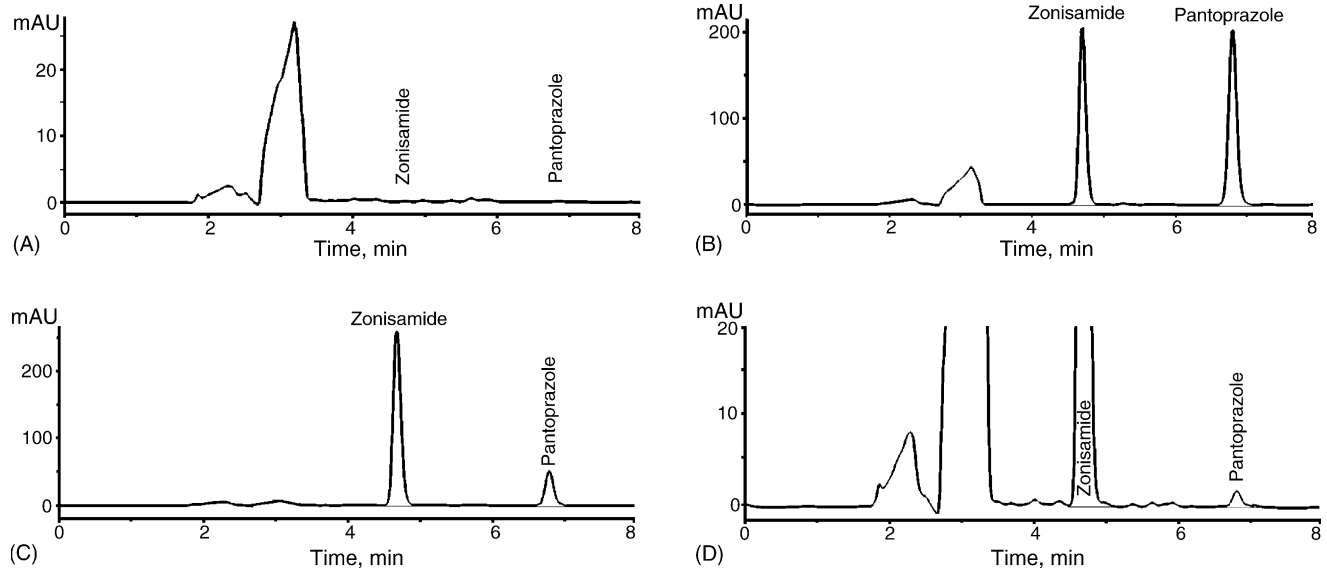

Fig. 2. Chromatograms of: (A) blank human plasma; (B) human plasma sample spiked with 2500 ng/mL of pantoprazole and I.S.; (C) plasma sample from a healthy subject following a 40 mg oral dose of pantoprazole, the plasma concentration was determined to be 498 ng/mL for pantoprazole; (D) spiked human plasma sample at LLOQ (20 ng/mL). Approximate retention times: pantoprazole = 6.8 min; I.S. = 4.6 min.

2500 ng/ml and at LLOQ (20 ng/ml), and plasma sample obtained from a healthy subject after 5 h following an oral 40 mg dose of pantoprazole. The analytes were well separated from co-extracted material under the described chromatographic conditions at retention times of 6.8 and 4.6 min, respectively. The peaks were of good shape, completely resolved one from another at therapeutic concentrations of pantoprazole. No interference with constituents from the plasma matrix was observed.

3.2. Linearity and sensitivity of the assay

The peak area ratio of pantoprazole to I.S. in human plasma was linear with respect to the analyte concentration over the range 20–5000 ng/mL. The mean linear regression equation of calibration curve for the analyte was $y = 0.00051x - 0.0042$, where y was the peak area ratio of the analyte to the I.S. and x was the concentration of the analyte. The correlation coefficient (r) for pantoprazole was above 0.999 over the concentration range used. These calibration curves were suitable for generation of acceptable data for the concentrations of the analyte in the samples during between-batch and within-batch validations.

3.3. Extraction

The extraction recovery of pantoprazole was 48.7 ± 1.9% on average, and the dependence on concentration is negligible. The recovery of internal standard, zonisamide was 54.8% at the concentration used in the assay (100 μg/mL). Recovery of the analyte and the I.S. were low, but it was consistent, precise and reproducible.

3.4. Accuracy and precision of the assay

Within-batch accuracy and precision evaluations were performed by repeated analysis of pantoprazole in human plasma. The run consisted of a calibration curve plus five replicates of each LLOQ, low, medium and high quality control samples. The accuracy values for between- and within-batch studies were within acceptable limits ($n = 3$) (Table 1). The results shown in Table 1 indicate that the assay method is reproducible for replicate analysis of pantoprazole in human plasma within the same day and reproducible on different days also ($n = 3$).

3.5. Stability

The stock solutions were stable for at least 6 months when stored at 4 °C. All the stability results were within the acceptable limits. The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. Three freeze-thaw cycles

Table 1
Accuracy and precision of the HPLC method for determining pantoprazole concentrations in plasma samples

Concentration found (ng/mL)		
	Within-batch precision ($n = 5$), (mean ± S.D.)	Between-batch precision ($n = 3$), (mean ± S.D.)
20	22.4 ± 0.7	21.6 ± 0.7
60	60.4 ± 1.4	61.2 ± 0.8
2500	2452.5 ± 18.0	2524.1 ± 62.8
4000	3951.5 ± 38.8	4005.0 ± 64.6

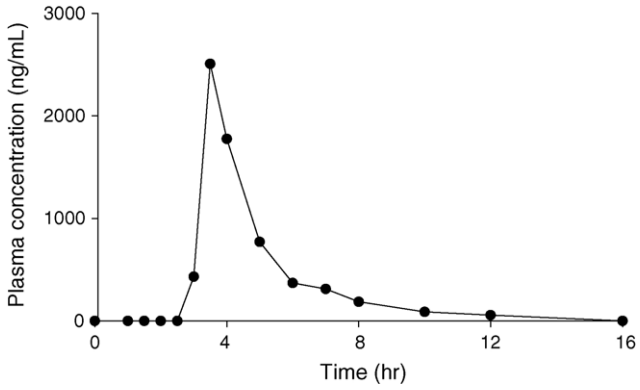

Fig. 3. Concentration vs. time profiles over 16 h of pantoprazole in human plasma from a subject receiving a single 40 mg dose of pantoprazole.

and 24 h room temperature storage for low and high quality controls samples indicated that pantoprazole was stable in human plasma under these conditions. QC samples were stable for at least 30 days if stored frozen at approximately −50 °C. Testing of autosampler stability of quality control samples indicated that pantoprazole is stable when kept in the autosampler for up to 24 h.

3.6. Application to clinical study

The present HPLC method was for the first time employed to determine the pharmacokinetic parameters of pantoprazole in subjects plasma samples of clinical studies. After a single oral dose of 40 mg pantoprazole to 18 healthy subjects, concentration versus time profiles were constructed for up to 30 h for pantoprazole quantitation. Fig. 3 shows the representative concentration–time profiles of pantoprazole in one subject following a 40 mg oral dose of pantoprazole under fasting conditions.

Acknowledgement

The authors wish to acknowledge the support received from Mr. Venkateswarlu Jasti, Managing Director, Suven Life Sciences Ltd., Hyderabad.

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