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A New Solid - Phase Extraction Method for Determination of Pantoprazole in Human Plasma Using High - Performance Liquid Chromatography

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Abstract

BACKGROUND: A new simple, selective and accurate high-performance liquid chromatographic (HPLC) method utilising solid-phase extraction for the determination of pantoprazole in human plasma samples has been developed.

AIM: The purpose of this paper was developing a new HPLC method suitable for the determination of pantoprazole in plasma samples, which enables simple and rapid isolation and concentration of the analysed drug.

METHODS: The chromatographic separation was accomplished on a LiChroCart LiChrospher 60 RP select B column using a mobile phase composed of 0.2 % (V/V) water solution of triethylamine (pH 7) and acetonitrile (58:42, V/V) followed by UV detection was at 280 nm. The solid-phase extraction method using LiChrolut RP-18 (200 mg, 3 ml) was applied to the obtained good separation of investigated drug from endogenous plasma components. Best results were achieved when plasma samples were buffered with 0.1 mol/L KH_2PO_4 (pH 9) before extraction, eluted and reconstituted with acetonitrile and 0.001 mol/L NaOH after extraction, respectively.

RESULTS: The standard calibration curves showed good linearity within the range of 25.0-4000.0 ng/mL with a correlation coefficient greater than 0.996. Retention times of pantoprazole and internal standard, lansoprazole was 4.1 and 6.0 min respectively. The limit of quantification was 25.0 ng/mL. For intra- and inter-day precision relative standard deviations ranged from 4.2 to 9.3%. The relative errors for stability investigations were ranged from 0.12 to -10.5%.

CONCLUSION: This method has good precision and accuracy and was successfully applied to the pharmacokinetic and bioequivalence study of 40 mg pantoprazole in healthy volunteers.

Introduction

Pantoprazole is a proton pump inhibitor used as the first-line treatment for patients with acid-peptic disorders, including erosive gastro-oesophageal reflux disease, nonerosive reflux disease and duodenal gastric ulcers. There are several investigations concerning the determination of pantoprazole in human plasma samples by high-performance liquid chromatography (HPLC). In the published methods, liquid-liquid extraction has been used for sample preparation [1], [2], [3], [4] using different solvents. The disadvantage of these methods employing liquid-liquid extraction (with grate chemicals consumption) is that they involve several steps which can yield poor separation from the endogenous plasma interferences, these methods are time-consuming (usually up to 1 h) regarding multiple steps of extraction, drying etc. Some authors proposed methods for determination of pantoprazole in human plasma using deproteinisation as sample preparation

method with acetonitrile or methanol [5], [6], [7], [8], single or automated on-line solid-phase extraction method [9], [10], [11], [12] or method by direct injection [13]. The solid-phase extraction method is less labour intensive due to the mechanism which allows extraction of the components in a single loading step. In comparison with liquid-liquid extraction, solid-phase extraction is better suited to enriching the concentration of the investigated components in plasma samples and not susceptible to problematic emulsions.

In this paper, we describe a new HPLC method suitable for the determination of pantoprazole in plasma samples employing solid phase extraction for sample preparation, which enables simple and rapid isolation and concentration of the analysed drug. This method is advantageous because of its simplicity, efficient clean-up of the complex biological matrix and shorter time of analysis, and it is suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans.

Methods

Chemicals and standards

Pantoprazole working standard and internal standard lansoprazole was supplied by Krka, d.d., Novo Mesto, Slovenia. HPLC grade methanol and acetonitrile were purchased from Across Organics, Belgium. Triethylamine, o-phosphoric acid, sodium hydroxide, potassium dihydrogen phosphate and columns for solid phase extraction (LiChrolut RP-18, 40-63 μm, 200 mg, 3mL) were obtained from Merck (Germany).

Instrumentation and chromatographic conditions

The method development was performed with a High-pressure liquid chromatographic system consisting of an autosampler Perkin Elmer LC ISS Series 200, an ultraviolet diode array detector (Perkin Elmer LC 235 C). The system was controlled, and data analysis were performed with the software package Turbochrom Version 4.1. Plus, and UV-spectrometric data were produced by TurboScan Version 2.0. The detector was set at 280 nm, and peak areas were integrated automatically by using the software. Chromatographic separation was carried out using LiChroCart LiChrospher 60 RP select B (4.0 mm x 250 mm, 5 μm).

A series of parameters, including composition and pH of mobile phase and flow rate, were tested concerning the location and shape of the peaks of pantoprazole and the internal standard in the corresponding chromatograms. The final choice of the mobile phase giving satisfying resolution and run time was 0.2 % (*V/V*) triethylamine in water with pH = 7 and acetonitrile (58:42, *V/V*). The triethylamine solution was prepared by adding 200 μL triethylamine in 100 mL water and pH of this solution [7] was adjusted by concentrated o-phosphoric acid. The mobile phase was filtered and degassed with helium and delivered at a flow rate of 1.2 mL/min. The injection volume was 50 μL.

Preparation of standards

Stock solutions of 1000 μg/mL of pantoprazole and lansoprazole were prepared in water and 0.05 mol/L NaOH respectively. These solutions were stored at +4°C, and no change in stability over 1 month was observed. The working solutions were prepared freshly every day by diluting appropriate portions of these solutions with water.

Sample preparation

Human plasma was prepared from heparinised whole blood samples. Blood samples were collected from healthy volunteers and stored at -20°C. After thawing, samples were spiked daily with stock solutions of pantoprazole and lansoprazole as an internal standard.

A 12 ports solid-phase extraction vacuum manifold (Merck) was used for sample preparation. A single extraction with LiChrolut RP-18 (40-63 μm) 200 mg, 3 mL standard PP-tubes was used to isolate the drug and internal standard from plasma samples. The cartridge was conditioned sequentially by elution with 2 mL methanol and 2 mL water. Spiked plasma sample (total volume 1.05 mL, 1 mL spiked plasma with pantoprazole and 0.05 mL internal standard) was buffered with 1 mL of 0.1 mol/L KH_2PO_4 (pH 9) and was introduced into the cartridge under vacuum at 5 psi. Water (2 mL) was used to rinse the cartridge. Elution was then performed with 0.7 mL of acetonitrile. This eluate was collected in a clean tube and was evaporated to dryness under N_2 for about 20 min at 40°C. After the reconstitution of the residue with 200 μL of 0.001 mol/L NaOH, a 50 μL volume was injected into the HPLC system.

Calibration curves

Typical calibration curves were constructed with six blank plasma samples spiked with appropriate amounts of the standard solutions. The calibration range was 25.0-4000.0 ng/mL of pantoprazole. The standard samples were prepared according to the procedure as unknown samples. Content of pantoprazole in control and unknown samples was determined by an internal standard method using weighted (1/c) calibration curves obtained by plotting

peak height ratios of pantoprazole and the internal standard against pantoprazole concentration.

Method validation

The proposed method was validated by evaluating selectivity, precision, accuracy, linearity, sensitivity, ruggedness and stability according to guidelines for Bioanalytical Method Validation, European Medicines Agency, Committee for Medicinal Products for Human Use, 2009 [14]. The selectivity of the proposed method was determined by comparing the chromatograms of diluent, standard solutions, blank plasma and spiked plasma samples. Intra-day precision values were assessed by calculating the relative standard deviation (RSD) for eight replicates at three different concentrations spiked into blank. Inter-day precision was assessed by measuring two individually prepared spiked plasma samples at three different concentration levels of pantoprazole in 6 different days. Accuracy (intro- and inter-) was determined by calculating relative error (%). Ruggedness was performed on the second HPLC column of the same type by injecting a standard solution of pantoprazole and internal standard. Relative error was calculated by comparing the mean peak height for both substances to those obtained by changing pH value of the mobile phase from 7.0 to 7.3 and from 7.0 to 6.7 or to those obtained by changing the detection wavelength from 280 nm to 275 nm and from 280 nm to 285 nm. Stability of pantoprazole in plasma was evaluated for 2, 12 and 24 hours, after one and three freeze/thaw cycles and after 1 month stored at -20°C using spiked samples at two different concentration levels prepared in duplicate.

Results

Method development

Our objectives for this work were to develop a robust, rapid and reproducible analytical assay for pantoprazole in human plasma using HPLC-UV which after validation would be appropriate for application in a clinical study evaluating the bioequivalence of various pantoprazole formulations. Therefore, a series of studies were conducted to develop a convenient and easy-to-use method for quantitative analysis of pantoprazole in plasma samples. Several HPLC method variables concerning their effect on the separation of pantoprazole and internal standard from the matrix, reducing run time and maximising resolution were investigated.

In our extensive preliminary experiments, a series of aqueous mobile phases containing buffer solutions with different pH values in combination with different modifiers including acetonitrile, 2-propanol and triethylamine with different volume fractions were tested. The presence of triethylamine in the mobile phase improved peak shape and increased the intensity of the observed pantoprazole signal. The results were most satisfactory when the mobile phase consisted of 0.2% (*V/V*) triethylamine in water with pH 7 and acetonitrile in volume fractions 58:42. A set of column packing including C8, C18 and RP select B with different lengths and particle sizes were tested and the LiChroCart LiChrospher 60 RP select B packing showed the best separation. The total chromatographic run time is 7 min, which is amenable to the high-throughput requirements of clinical study analyses. A typical chromatogram of standard solutions of pantoprazole and internal standard produced by the developed HPLC method is shown in Figure 1c. The retention time of pantoprazole and internal standard (lansoprazole) are 4.1 min and 6.0 min, respectively.

Also, to obtain satisfactory values for recovery of pantoprazole different cartridges for solid phase extraction were tested. The satisfactory values for recovery of pantoprazole and internal standard were obtained when solid phase extraction is performed on LiChrolut RP-18 tubes.

Also, to improving the extraction procedure, plasma samples were buffered with 1.0 mL of 0.1 mol/L KH_2PO_4 solution (pH 9) before introducing into the cartridges.

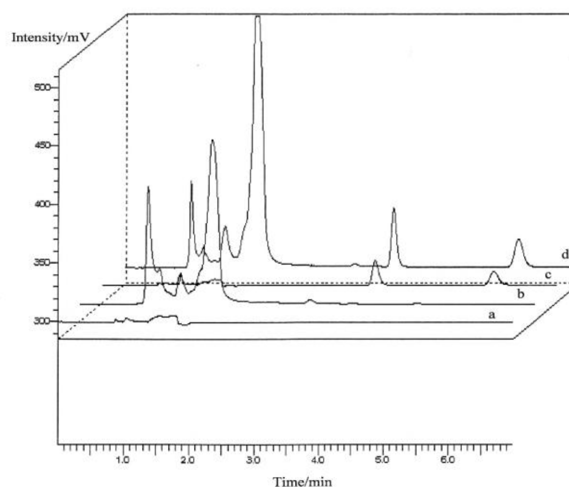

Figure 1: Chromatograms of A) diluent; B) blank plasma; C) standard solutions of pantoprazole and internal standard; D) spiked plasma sample with pantoprazole and lansoprazole

Under the chromatographic conditions described, pantoprazole and the standard internal peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Typical chromatograms of blank plasma in comparison to spiked sample are shown in Figure 1b and 1d). On the other hand, the method in this report has sufficient sensitivity and reproducibility to permit the pharmacokinetic studies. The developed HPLC method can be used for the analysis of plasma

samples from healthy volunteers after oral administration of 40 mg pantoprazole. A typical chromatogram of a plasma sample of a patient after administration of 40 mg pantoprazole is shown in Figure 2. Chromatograms showed no interfering peak at the pantoprazole and internal standard peaks position.

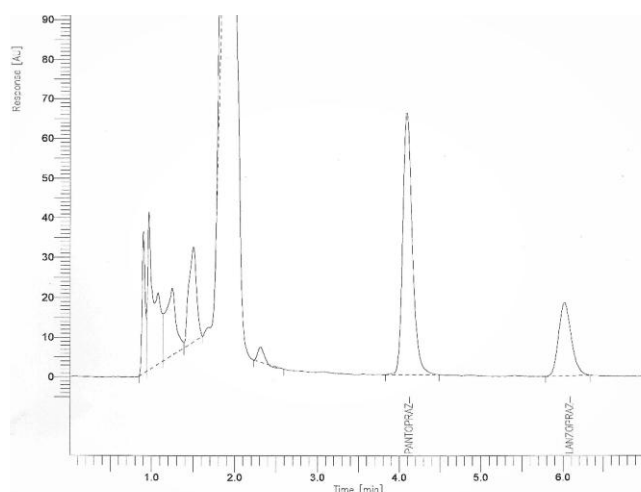

Figure 2: Chromatogram of plasma sample of a patient after administration of 40 mg pantoprazole (2h post-dose, the concentration of pantoprazole 2023.7 ng/mL)

Linearity

The method was validated using a six-point calibration curve ranging from 25.0 to 4000.0 ng/mL of pantoprazole. Respective weighted (1/c) linear regression equation was: $y = 0.001726 \cdot \gamma + 0.020017$. The correlation coefficient was routinely greater than 0.996.

Precision and accuracy

Intra-day precision and accuracy were determined by measuring individually prepared eight spiked plasma samples at three different concentration levels of pantoprazole, first near LOQ. Inter-day precision and accuracy were investigated by analysing 2 series of spiked plasma samples at low, middle and high concentration levels of pantoprazole in six different days. Then, the corresponding relative standard deviation and relative errors were calculated. The data for intro- and inter-day precision and accuracy of the proposed method are shown in Table 1. As can be seen from results presented in Table 1, for intra- and inter-day precision, RSDs ranged from 4.2 to 8.9% and from 4.9 to 9.3%, respectively. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs.

Relative errors at all three concentrations studied are less than 7.0% for intra-day accuracy and 7.3% for inter-day accuracy, and it is obvious that the method is remarkably accurate, which ensures obtaining reliable results.

The lower limit of quantification

The lower limit of quantification was defined as the lowest concentration of pantoprazole on the standard curve, which can be measured with acceptable accuracy and precision (RSD less than 20%, relative error ± 20 %, n = 6) [14]. The LLOQ was estimated using the lowest calibration standard in six different analytical days. Pantoprazole concentration of 25 ng/mL was accepted as LLOQ.

Table 1: Intra-and inter-day precision and accuracy data

Pantoprazole nominal concentration (ng/mL)	Intra-day		Inter-day	
	Mean (n = 8) observed concentration (ng/mL)	Relative standard deviation (%)	Mean (n = 12) observed concentration (ng/mL)	Relative standard deviation (%)
Precision				
50.0	47.1	8.9	46.3	9.3
750.0	697.5	7.0	743.2	5.3
2500.0	2487.7	4.2	2418.3	4.9
Accuracy		Relative error (%)		Relative error (%)
50.0	47.1	-5.9	46.3	-7.3
750.0	697.5	-7.0	743.2	-0.9
2500.0	2487.7	-0.5	2418.3	-3.3

Stability of pantoprazole in blood samples

Stability investigation of pantoprazole in plasma samples was performed by analysing two series of spiked samples at two different concentration levels (50 and 2500 ng/mL) after different storage conditions: immediately, after staying in an autosampler for 2, 12 and 24 hours, after one and three freeze/thaw cycles and after 1 month stored at -20°C. Samples were analysed against a calibration curve, obtained from freshly spiked calibration standards, and the obtained concentrations are compared to the nominal concentrations. The relative errors were calculated and for stability investigations after 2, 12 and 24 hours ranged from 0.12 to -10.5%, after one and three freeze/thaw cycles ranged from -2.76 to 7.88% and for after 1 month stored at -20°C ranged from 0.48 to 4.5%.

Discussion

The results from this investigation show that pantoprazole added to plasma samples is stable in the different storage conditions if we are taking into account that the criterion is mean concentration at each level should be within ± 15% of the nominal concentration [14].

In conclusion, the proposed HPLC method employing solid-phase extraction for sample preparation is simple and convenient for the

determination of pantoprazole in plasma samples and the total run time for the chromatographic run is less than 7 minutes. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. The quantitation of pantoprazole was not affected by any of the possible matrix interfering substances. In conclusion, this paper describes a very simple and sensitive HPLC method for the determination of pantoprazole suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans.

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