




Development and validation an HPLC - UV method for determination of esomeprazole and pirfenidone simultaneously in rat plasma: application to a drug monitoring study

Emrah Dural¹ , Sema Tülay Köz^{2,3} , Süleyman Köz^{4,5} 

¹Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Sivas, Turkey

²Sivas Cumhuriyet University, Faculty of Medicine, Department of Physiology, Sivas, Turkey

³Bahçeşehir University, Faculty of Medicine, Department of Physiology, İstanbul, Turkey

⁴Sivas Cumhuriyet University, Faculty of Medicine, Department of Nefrology, Sivas, Turkey

⁵Medipol Health Group, Department of Nefrology, İstanbul, Turkey

ORCID IDs of the authors: E.D. 0000-0002-9320-8008; S.T.K. 0000-0002-3809-1070; S.K. 0000-0001-5036-0475

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ABSTRACT

Background and Aims: It has been observed that the combined treatment of esomeprazole and pirfenidone provides increased efficacy in the treatment of pulmonary fibrosis disease, recently. The aim of this study is to develop a simple, sensitive, and reliable high-performance liquid chromatography method to be used in drug monitoring to increase the effectiveness of esomeprazole and pirfenidone in treatment and to reduce their adverse effects.

Methods: Separation was conducted with a C18 reverse-phase column (4.6 mm x 250 mm, 5 µm) used as a mobile phase prepared with the phosphate buffer (10 mM KH₂PO₄ and 10 mM K₂HPO₄) and acetonitrile (60:40, v/v) by an isocratic flow (1 mL/min). Mobile phase pH was adjusted to 3.0. Ultraviolet detection was accomplished at 305 nm. The column oven was held at 35°C to ensure an efficient analytical separation.

Results: Analytical recovery of esomeprazole was between 92.43 and 105.36% and for pirfenidone it was found between 89.56 and 104.32%. Accuracy values of esomeprazole and pirfenidone were determined between (-2.90) - 4.22 and (-4.45) - 5.78, respectively. Precision (RSD%) was ≤7.89. The quantification limit was determined as 0.58 and 0.36 ng/mL. Plasma esomeprazole and pirfenidone levels were found as 0.87-8296.87 ng/mL (612.99±2212.20, mean ± standard deviation) and 0.45-238.60 ng/mL (61.44±76.35, mean ± standard deviation), respectively.

Conclusion: Unexpectedly high RSD values were observed in both plasma (360.88%) and dose-rated results (89.61%) of esomeprazole, and pirfenidone were thought to be related to individual metabolism differences.

Keywords: Esomeprazole, pirfenidone, rat plasma, method validation, HPLC-UV

Address for Correspondence:

Emrah DURAL, e-mail: emrahdural@cumhuriyet.edu.tr

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INTRODUCTION

Esomeprazole, 6-methoxy-2-[(S)-(4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfanyl]-1H-benzimidazole (Figure 1a), is a part of the novel gastric proton pump inhibitors (Liu et al., 2017; Sebaiy, Hassan, & Elhennawy, 2019). It provides decreased stomach acid secretion through inhibition of the H⁺/K⁺-ATPase in the parietal cells of the stomach. It has better oral bioavailability than S-enantiomer. Esomeprazole is widely used in the treatment of many acid-related disorders such as peptic disease, gastroesophageal reflux, and in the prevention of the adverse reactions of non-steroidal anti-inflammatory drugs (Çelebi et al., 2016; Franke, Hepp, Harder, Beglinger, & Singer, 2008; Johnson, 2003; Liu et al., 2017). In addition, remarkable results have been reported in various studies showing that its anti-fibrotic activities for both liver and lung recently (Eltahir & Nazmy, 2018; Ghebre & Raghu, 2016; Ghebremariam et al., 2015). Pulmonary fibrosis disease has a prevalence of 494.5 cases per 100,000 and an incidence of 93.7 cases per 100,000 (Raghu et al., 2014). Although this disease often occurs over the age of 60, it may occur at earlier ages in familial idiopathic pulmonary fibrosis patients (Hodgson, Laitinen, & Tukiainen, 2002; Marshall, Puddicombe, Cookson, & Laurent, 2000; Nadrous, Myers, Decker, & Ryu, 2005). The median survival rate is only 3-4 years from the time of diagnosis (Raghu et al., 2014).

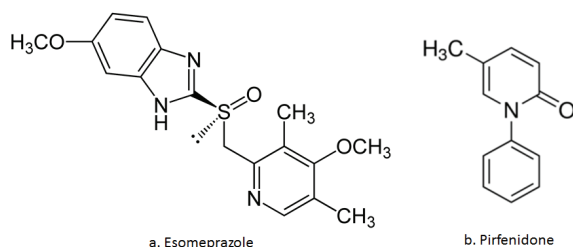


Figure 1. Chemical structures of esomeprazole (a) and pirfenidone (b).

Pirfenidone is a novel agent, 5-methyl-1-phenylpyridin-2-(1H)-one, approved for mild to moderate idiopathic pulmonary fibrosis by FDA in 2014. It is a non-peptide, orally active small molecule (185.22 g/mol) that is in use also as an antioxidant and anti-inflammatory agent. It is an orphan drug in Europe and Japan (Parmar, Desai, & Vaja, 2014). A study which conducted a phase III multi-national clinical trial has shown that it has beneficial effects on patients with various stages of idiopathic pulmonary fibrosis disease. It has been reported that this agent could reduce lung fibrosis in drug-fibrotic *in vivo* studies, including the pirfenidone-hamster and the cyclophosphamide-mouse models (Iyer et al., 1995; Kehrer & Margolin, 1997). Although it is a commonly well-tolerated agent and has a favourable benefit-risk profile, gastrointestinal problems, photosensitivity reactions and rashes are its important adverse reactions that have been seen commonly (Khan, Shirkhedkar, Chaudhari, & Pawara, 2019).

In this study, we aimed to develop a high performance liquid chromatography (HPLC) method for the monitoring of these agents from rat plasma based on solid phase extraction pretreatment due to the anti-fibrotic effects of esomeprazole and

pirfenidone detected in both combined and individual treatments (Ghebremariam et al., 2015). There are some analytical methods developed for the determination of esomeprazole from different matrices in the literature. These are based on spectrophotometric (Prabu et al., 2008), capillary electrophoresis (Estevez, Flor, Boscolo, Tripodi, & Lucangioli, 2014), gas chromatography (Raman, Reddy, Prasad, & Ramakrishna, 2008), and liquid chromatography that coupled with an ultraviolet detector (Jain, Jain, Charde, & Jain, 2011; Kayesh & Sultan, 2015; Talaat, 2017), photodiode array detector (Sebaiy et al., 2019), and tandem mass spectrometry (Gopinath, Kumar, Shankar, & Danabal, 2013). Also, some analytical methods were reported for the determination of pirfenidone from the different matrix which includes pharmaceutical dosage forms and plasma. These are spectrophotometric (Thorat, Padmane, Tajne, & Ittadwar, 2016), spectrofluorometric (Sambhani & Biju, 2018), capillary electrophoresis (Sotgia et al., 2020), high-performance thin layer chromatography (Thorat et al., 2016), gas chromatography (Ma et al., 2017), and HPLC with an ultraviolet detector (Bodempudi, Babur, & Reddy, 2015; Parmar et al., 2014; Ravisankar, Anusha Rani, Devadasu, & Devala Rao, 2014; Thorat et al., 2016), a photodiode array detector (Bodempudi et al., 2015) and a mass spectrometry detector (Tong et al., 2010; Wen et al., 2014) methods.

However, long analysis times, complex sample preparation protocols, and high sample quantities needed for analysis limit their use. At the same time low sensitivity, precision and accuracy of these methods may cause restriction of their use in the analyses. Although several liquid chromatographic methods were established for the determination of esomeprazole and pirfenidone, according to our investigation, there is no study that includes the simultaneous analysis of esomeprazole and pirfenidone by HPLC in rat plasma in the literature.

The aim of this study is to develop a simple, rapid and reliable HPLC analysis method for determination of esomeprazole and pirfenidone and to validate it in terms of linearity, repeatability, sensitivity, recovery, and robustness according to ICH Q2(R1) guidelines (ICH, 2005). This simple reproducible, efficient extraction method provided the determination of esomeprazole and pirfenidone levels from rat plasma without any process of the deproteinization and derivatization. It was used in the study of simultaneous monitoring of esomeprazole and pirfenidone levels in plasma samples from 14 rats treated by oral gavage. In addition, our study aimed to determine the plasma esomeprazole and pirfenidone concentrations of rats simultaneously by the chromatographic method to be developed. Also, it was aimed to analyze the relationship between drug doses and blood results statistically.

MATERIAL AND METHODS

Chemicals and reagents

Esomeprazole (Figure 1a) and pirfenidone (Figure 1b) analytical standards were purchased from Shandong Zhi Shang Chemical Co. Ltd. (Jinan, China) and Wuhan Benjamin Pharmaceutical Chemical Co. Ltd. (Wuhan, China), respectively. The Sep-Pac® Vac 1 cc (100 mg) solid-phase C18 cartridge was obtained from Waters (Dublin, Ireland). Ultragradient grade acetonitrile,

ethanol and methanol were bought from Carlo-Ebra (Val-de-Reuil, France). Triethylamine ($\geq 99\%$) was purchased from Sigma-Aldrich (Brussels, Belgium). Orthophosphoric acid (H_3PO_4), sodium hydroxide (NaOH), sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) which are analytical grade were bought from Merck (Darmstadt, Germany). Carboxymethyl cellulose was purchased from Biokim & Wenda Chemicals (İzmir, Turkey). Bovine serum albumin was purchased from Solarbio Life Science (Beijing, China). Polytetrafluoroethylene (PTFE) membrane filter (47 mm diameter, 0.45 μm pore size) was obtained from Millipore (Massachusetts, USA). An MRC ultrasonic bath (Harlow, UK), ACP-250H model, was used for the preparation of the mobile phase. Elga Purelab Water Purification System (Lane End, UK) was employed to supply ultra-pure water.

Instrumentation and chromatographic parameters

Agilent (Hewlett-Packard) 1100 series (California, USA) HPLC system equipped with a degasser (G1322A), a gradient-quad pump (G1311A), a manual injector (Rheodyne, 7725i) with a 20 μL loop volume, a column thermostat (G1316A, Colcom), and an UV detector (G1314A, VWD) was used for separation and quantification of esomeprazole and pirfenidone in rat plasma. The system control and integration of the produced chromatographic data was achieved by a Chemstation 08.03 software (Palo Alto, USA). A stainless steel end-cap C18 reverse-phase (RP) analytical column (4.6 mm x 250 mm i.d., 5 μm p.s.) (USA) was successfully employed for the separation and quantitation of esomeprazole and pirfenidone.

Chromatographic conditions were determined after optimization studies on the analytical column, column thermostat temperature, mobile phase content and detector wavelength selection. The best analytical separation result was achieved from the C18 RP ACE-3 column (4.6 mm x 250 mm i.d., 5 μm p.s.) out of 3 tested columns which were the RP-C18 Waters column (4.6 mm x 250 mm i.d., 5 μm p.s.) and the RP-C18 Zorbax column (4.6 mm x 150 mm i.d., 3 μm p.s.).

The minimum column back pressure (≤ 95 bar) and enough peak resolution were gained after the oven temperature was set at 35°C. Although different wavelengths (220, 246, 254, 270 and 294 nm) were tested for the determination of esomeprazole and pirfenidone in plasma, the highest peak sharpness and lowest matrix interference were obtained from 305 nm. The UV spectrums of esomeprazole and pirfenidone were given in Figure 2.

The mobile phase buffer was prepared with KH_2PO_4 (10 mM) and K_2HPO_4 (10 mM) containing 0.1% triethylamine, then the pH was set at 3.0 by orthophosphoric acid (0.1 M) and then filtered by a PTFE membrane. Then, this solution was mixed with acetonitrile (60:40, v/v) and then it was degassed with an ultrasonic bath, for 30 mins. The mobile phase was applied isocratically to the column with 1.0 mL/min constant flow. Determination of analytes concentration in the quality control and rat blood samples were carried out using linear regression of response esomeprazole and pirfenidone peak area versus to their concentrations with the ultraviolet detector set at 305 nm.

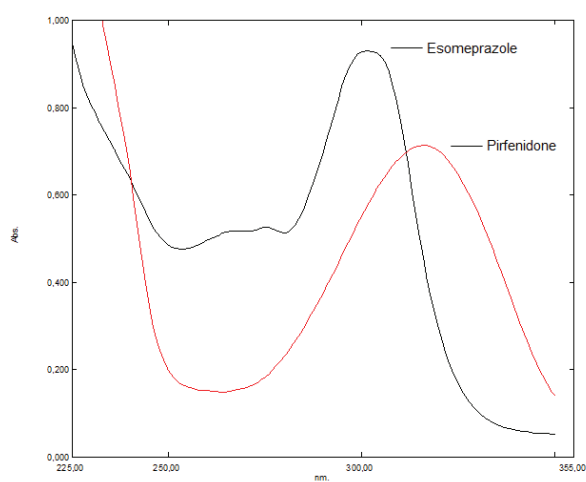


Figure 2. Overlapping UV spectra of esomeprazole and pirfenidone (200 $\mu\text{g}/\text{mL}$) that are between 255 and 355 nm.

Preparation of stock standard solutions and working standards

Simulated plasma was used in all stages during the development of the solid-phase extraction (SPE) procedure and optimization and validation of this analytical method. It was prepared as follows: 20 mg of KCl , 0.8 g of NaCl , 20 mg of KH_2PO_4 , 135 mg of Na_2HPO_4 and 4 g of bovine serum albumin were weighed and dissolved in 100 mL of ultrapure water. The final pH was adjusted to 7.4 with 1 M NaOH or 1 M orthophosphoric acid solutions (Mercolini, Mandrioli, Amore, & Raggi, 2008). It was stored as 500 μL in a 1.5 mL micro tube at -18 °C until use. The stock solution of esomeprazole and pirfenidone was prepared in methanol as 1 mg/mL and stored at -18°C until use. It has been observed to be chemically stable for at least 1 month. Working solutions of esomeprazole and pirfenidone were prepared weekly from the main stock solution in methanol as 0.25, 0.50, 1.00, 2.00 and 5.00 $\mu\text{g}/\text{mL}$ concentrations. Working standards were prepared daily, and they were used to add to simulated plasma samples prior to analysis. Quality control samples of esomeprazole and pirfenidone were freshly prepared in simulated plasma samples to provide concentrations of 25, 50, 100, 200 and 500 ng/mL. Likewise, plasma quality control standards spiked with 25, 100 and 500 ng/mL of esomeprazole and pirfenidone were prepared to measure the repeatability values of the method. Also, the same protocol was used in the preparation of limit of detection (LOD), limit of detection (LOQ), recovery and robustness test samples.

Preparation of quality control samples and real plasma samples

Simulated plasma samples have been used in the forming of the quality control samples used during the development and validation process of this method. The collected real rat blood centrifuged at 4000 rpm for 5 mins to separate the plasma. Quality control plasma and real patient plasma samples were stored at -20°C until the analyses were carried out. Working solutions were checked chromatographically for purity before experiments, were utilized as quality control specimens and were checked for the stability before and after the injections of every sample set.

The extraction procedure was carried out with a glass solid-phase apparatus (12 wells) coupled with the vacuum pump according to the following steps:

- i. In the initial step, the cartridge was conditioned with 1 mL acetonitrile;
- ii. Equilibrium was achieved with 1 mL water and 1 mL methanol;
- iii. The sample constituted 0.5 μ L plasma with 10 μ L ISTD (1 mg/mL) and 10 μ L esomeprazole and pirfenidone STDs (for quality control samples) was applied to the cartridge;
- iv. The cartridge adsorbent was washed with 1 mL (two times) water;
- v. The eluting was carried out with 1 mL acetonitrile (two times) for 5 mins at 75 kPa. All liquid in the cartridge was completely collected with a constant flow;

Finally, the collected extraction liquid (approximately 2 mL) was evaporated under nitrogen. The remains, after being reconstituted in 200 μ L of the mobile phase, were injected into the analysis system as a volume of 20 μ L.

Validation of the analysis method

The developed analytical method was validated in relation to its specificity and selectivity, linearity, accuracy and precision, sensitivity (LOD and LOQ), recovery and robustness. Intraday and inter-day validation protocol were applied considering reproducibility of the method to obtain accurate and precise measurements in accordance with ICH Q2R1 guidelines (ICH, 2005),

Specificity and selectivity

The method showed excellent chromatographic specificity without any endogenous interference in the retention times of esomeprazole and pirfenidone (4.2 and 6.1 mins, respectively) in simulated plasma. Blank (Figure 3a), spiked (Figure 3b) and real sample (Figure 3c), chromatograms were exhibited a high chromatographic resolution that conducted in 7.0 mins.

Linearity

After chromatographic conditions were established, matrix-based calibration curves of esomeprazole and pirfenidone were plotted concentrations over the concentration range 25-500 ng/mL versus peak-area of them. The calibration points (n=5), which were 25, 50, 100, 200 and 500 ng/mL composed of 3 individual replicates and were prepared by a standard addition method in simulated plasma and injected to HPLC.

Accuracy and precision

The accuracy defined as the relative error (RE%) was calculated as the percentage difference between the added and found esomeprazole and pirfenidone quantity by 5 individual replicates both intraday and inter-day. The precision, which is defined as relative standard deviation (RSD%), was calculated by five separate replicates of esomeprazole and pirfenidone both intraday and inter-day. Five replicated spiked samples were assayed intraday and inter-day at the three different concentrations (25, 100 and 500 ng/mL).

Robustness

The robustness test was performed with 200 ng/mL of esomeprazole and pirfenidone, which is the approximate medium concentration of the calibration interval. The response of the method over the changes in UV wavelength (± 3 nm) value, mobile phase flow rate (± 0.1 mL/min), mobile phase solvent content ($\pm 5\%$) and, column temperature ($\pm 5^\circ\text{C}$) was evaluated.

Sensitivity

LOD and LOQ were calculated according to the ICH recommendations based on the standard deviation of the response, and the slope of the calibration graph. 25 ng/mL was used as the lowest calibration point in a sensitivity test of esomeprazole and pirfenidone.

$\text{LOD} = 3.3 \frac{\sigma}{S}$; $\text{LOQ} = 10 \frac{\sigma}{S}$ (σ : The standard deviation of the response; S: The slope of the calibration curve).

Recovery

The recovery of the extraction procedure was calculated by comparing the results obtained from the extracted samples with the results of the unextracted samples which were directly prepared. This test was performed by adding 5 individual replicates of spiked samples at low, middle and high concentrations (25, 100 and 500 ng/mL, respectively) of esomeprazole and pirfenidone. The extraction procedure was carried out as described before in the sample preparation step.

Collection of plasma samples

Approximately 1 mL of femoral vein blood sample was taken from Wistar Albino rat 2 hrs before the Wistar Albino rat whose plasma had a steady state concentration was put down. The rats included in this study were treated with esomeprazole (50 mg/kg/day) and pirfenidone (100 mg/kg/day) by oral gavage prepared in 1 mL solution which has agents (esomeprazole and pirfenidone) dissolved in 1% carboxymethyl cellulose and 10% ethanol. In this study, 14 blood samples were obtained from 14 individual rats involved in this research.

The rats in this study were treated by oral gavage with 1 mL of solution containing agents (esomeprazole and pirfenidone) dissolved in 1% carboxymethyl and 10% ethanol. One mL rats whole blood sample was put in a vacuum tube (BD Vacutainer®) which contains K_2EDTA (5.4 mg) and was centrifuged at 3000 rpm for 10 mins on the same day. Then, the obtained plasma that had at least has 0.5 mL volume was transferred in a micro experiment tube and it was stored in a freezer at -86°C until the analysis. Plasma samples were analysed in less than 1 month.

Ethics committee approval: The ethical decision of this research was approved by the Animal Experiments Local Ethics Committee of Sivas Cumhuriyet University, with the 2016-03 decision number, dated on 14 January 2016. The research was conducted in accordance with the Declaration of Helsinki and its subsequent revisions.

Statistical analysis: All statistical analyses performed using the IBM Statistical Package for the Social Sciences (SPSS) 23.0. The Spearman non-parametric test was used to determine correlation.

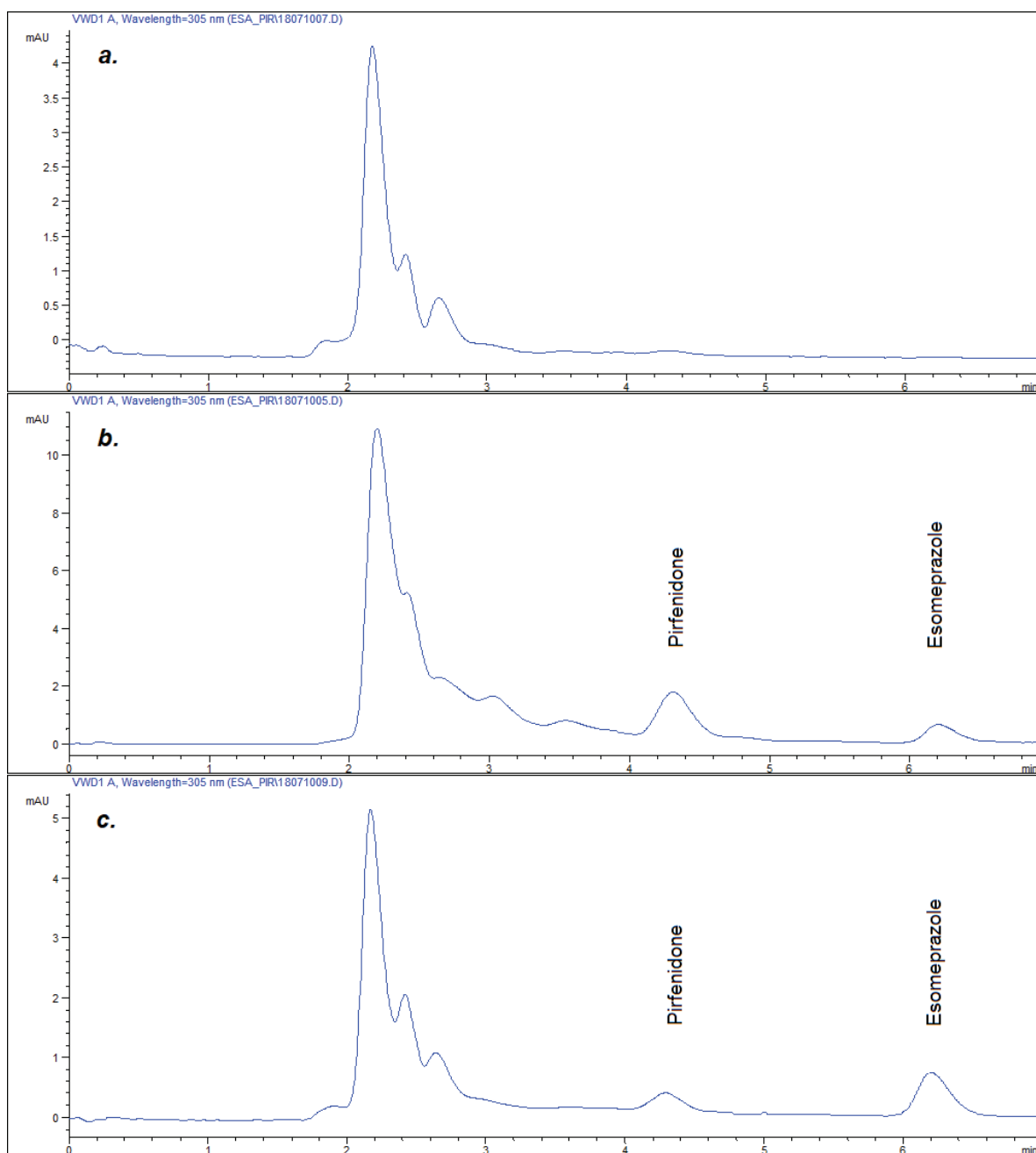


Figure 3. a. A chromatogram which belongs to blank-simulated plasma employed to prepare the quality control standards in all validation tests. b. A chromatogram sample containing 200 ng/mL esomeprazole and pirfenidone standards prepared with the standard addition method used as a quality control sample. c. A sample chromatogram belongs to a real plasma obtained from a rat treated with esomeprazole and pirfenidone.

RESULTS AND DISCUSSION

Method validation

Validation procedures were conducted considering ICH-Q2(R1) guidelines during the all of the test steps (ICH, 2005)

Linearity

The calibration curves of the esomeprazole and pirfenidone were constructed with excellent determination coefficient values which are $r^2 \geq 0.9986$ at 5 points ($n=3$) separately between 25 and 500 ng/mL concentrations by the standard addition method.

The correlation values of the method observed at the individual different 5 calibration points was quite good. The wide linear range has also had a positive effect on the use of the method. Since the obtained real blood results were shown very high standard deviation.

System suitability parameters showed that the method has a good resolution (R_s), selectivity (α), capacity factor (k') and theoretical plate number (N) for the determination of esomeprazole and pirfenidone successfully from rat plasma as it can see in Table 1. The column dead time was obtained by dividing

Table 1. Chromatographic characteristics, system suitability parameters and sensitivity values of the developed method.

Analyte	Retention time (min, t_R)	Linear range (ng/mL)	Calibration equation	Determination coefficient (r^2)	Capacity factor (k')	Theoretical plate number (N)	Specificity factor (α)	Resolution (R_s)	T_f	LOD (ng/mL)	LOQ (ng/mL)
Esomeprazole	4.2	25 - 500	$y = 226.69x + 95.92$	0.9986	1.91	7079.19	7.64	12.00	1.046	0.19	0.58
Pirfenidone	6.1	25 - 500	$y = 220.55x + 11.238$	0.9997	1.0	5831.41	4.00	7.16	1.125	0.12	0.36

Equations: Capacity factor (k') = $\frac{t_R - t_0}{t_0}$; Theoretical plate number (N) = $16 \left(\frac{t_R}{W_t} \right)^2$; Resolution (R_s) = $\frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k+1)}$; Specificity factor (α) = $\frac{k_2}{k_1}$; Peak symmetry (T_f) = $\frac{a+b}{2a}$
 Abbreviations: t_R : retention time of the analyte peak; t_0 : retention time of mobile phase peak; W_t : peak width; a : the distance from the leading edge of the peak to the peak midpoint; b : The distance from the back edge of the peak to the peak midpoint

the packed column volume by the flow rate (Column Spacing/ Dwell Time) and is given in Table 1 in minutes.

Sensitivity

The results of LOD and LOQ values, which were obtained from the measurement of individual 10 quality control (QC) samples, were demonstrated in Table 1. It was observed that the sensitivity values, especially LOQs, covered all the real plasma sample esomeprazole and pirfenidone results. Although the minimum concentrations of esomeprazole and pirfenidone determined from plasma were 0.19 and 0.12 ng/mL, it was observed that the LOQ values were determined as 0.58 and 0.36 ng/mL, respectively. Therefore, the method is suitable for reliable analyzes for low concentrations of esomeprazole and pirfenidone.

Precision and accuracy

The data obtained from the accuracy and precision tests, performed in intraday and inter-day with quality control standards established in the blank plasma samples by standard addition method, showed low RSD% values ≤ 7.59 and $\leq 7.89\%$ for interday and intraday respectively. Also low RE% average values were observed between (-4.45)–5.78% for inter-day and (-2.05)–5.69% for intraday test values (Table 2). The obtained repeatability results showed that the method has excellent precision and accuracy values not only for intraday but also for inter-day analyses.

Recovery

Recovery test results which are done at 25, 100 and 500 ng/mL were between 89.56 and 105.36% with the results given in Table 2. The method has a highly successful analytical result with the average recovery values at 97.44 and 93.83% for esomeprazole and pirfenidone, respectively. Recovery values obtained in the extraction procedure have demonstrated excellent efficiency. It was observed that the extraction procedure was not complicated and had no need for sophisticated instruments.

Robustness

No significant changes in the analytical signals were observed upon changing the UV wavelength value (± 3 nm), mobile phase flow rate (± 0.1 mL/min), mobile phase organic solvent ingredient ($\pm 5\%$), and column temperature ($\pm 5^\circ\text{C}$). All robustness results were given on Table 3. In addition to that, change of analysts, columns, sources of chemicals and/or solvents did not lead to significant changes in chromatographic signals and results, either.

Stability

The stability of quality control (QC) simulated plasma samples (25, 100 and 500 ng/mL) prepared with a standard addition method and esomeprazole and pirfenidone analytes in stock solutions under several conditions were assessed. The stability of the stock solutions at room temperature was evaluated during 1, 2, 3 and 4 week periods. The stability test of freeze-thaw was carried out by three QC samples after operating five repeated freeze-thaw periods. The stability test in the long-term was carried out for 1, 2 and 3 months using QC samples maintained at -20°C . Neither significant decrease related to analytes peak area nor degradation which could be seen in chromatograms were observed in the concentration of esomeprazole and pirfenidone in three different conditions. The relative standard deviations which were observed in all sample results were less than 6.8%.

These investigations have shown that there is not an investigation study in which esomeprazole and pirfenidone were simultaneously analyzed by a validated HPLC-UV method which has a solid phase extraction method in the literature. However, studies in the literature focusing separately on the analysis of esomeprazole and pirfenidone by HPLC are summarized below.

In Jain et al (2011) study, an HPLC method was developed for the determination of esomeprazole. Analytical separation was achieved by a C18 (4.6 mm x 150 mm i.d., 5 μm) column at

Table 2. Confidence parameters that include intraday, inter-day precision and accuracy and recovery values. These results were obtained from individual samples (n=5) formed in simulated plasma. Intraday reproducibility experiments were performed sequentially on the same day. Inter-day repeatability experiments were performed sequentially on consecutive 5 days.

Analytes	Expected concentration (ng/mL)	Intra-day repeatability			Inter-day repeatability			Recovery%
		Observed concentration \pm SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	Observed concentration \pm SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	
Esomeprazole	25	2.85 \pm 0.31	6.31	4.22	2.75 \pm 0.47	4.59	2.49	92.43
	100	4.49 \pm 0.92	4.48	- 2.05	4.37 \pm 1.53	7.43	- 2.90	94.54
	500	49.15 \pm 1.13	3.27	- 1.93	48.39 \pm 0.92	4.33	- 0.78	105.36
Pirfenidone	25	2.88 \pm 0.43	7.89	5.69	2.85 \pm 0.58	7.59	5.78	89.56
	100	5.59 \pm 1.56	5.53	3.65	4.37 \pm 1.53	6.43	- 3.82	104.32
	500	48.55 \pm 1.13	4.76	- 0.93	48.39 \pm 0.92	3.33	- 4.45	96.61

Table 3. Robustness data of the described method representing as the RSD% value. These results were obtained in the analysis of three variable points calculated by independent (n=5) analyzes.

Analytes	Mobile phases solvent content (\pm 5%)	UV wavelength (\pm 3 nm)	Flow rate (\pm 0.1 mL/min)	Column temperature (\pm 5 °C)
Esomeprazole	3.82 \pm 0.12	4.85 \pm 0.19	2.81 \pm 0.14	2.12 \pm 0.08
Pirfenidone	4.65 \pm 0.14	4.31 \pm 0.12	1.92 \pm 0.07	2.89 \pm 0.10

25°C. Acetonitrile and phosphate buffer (pH:7.0) in the ratio of 50:50 (v/v) was used as a mobile phase for HPLC. The flow rate was 0.5 mL/min. Quantitation was applied at 300 nm. Method was found to be linear ($r=0.998$) in the concentration range between 50 – 250 μ g/mL. The retention time was 5.661 min. Mean recovery was 97.75%. Accuracy was < 2.0 (RSD%) (Jain et al., 2011).

Kayesh and Sultan (2015) established an HPLC-ion pair method to determine esomeprazol in pharmaceutical formulation. As a mobile phase, tetrabutylammonium hydroxide (7.7 mM) and n-heptane sulfonic acid-Na salt (20 mM), acetonitrile and methanol (3:1:1, v/v) was isocratically applied to a C18 octadecyl-silica column (4.6 mm x 250 mm i.d., 5 μ m p.s.) (Kayesh & Sultan, 2015).

Sebaiy et al. (2019) developed a method for esomeprazole in human plasma. Protein precipitation was used for preparation of samples. Separation was carried out on a C18 column (4.6 mm x 150 mm i.d., 5 μ m p.s.). Acetonitrile and 25 mM KH_2PO_4 (25:75, v/v) was used as a mobile phase. LOD was 40 ng/mL and LOQ was found as 130 μ g/mL. A DAD detector was set at 230 nm. An aliquot of 200 μ L plasma was precipitated MeOH. Then, the mixture was centrifuged at 5500 rpm for 15 mins. The obtained supernatant liquid was fitted with a 0.45 μ m PTFE filter. Then, it was applied to the analytical system.

Method was found to be linear between 0.5 μ g/mL to 50 μ g/mL ($r=1$) concentrations. Recovery was between 98.38% and 101.14%. Accuracy was observed as -5.27 %. Precision (RSD%) was observed between 0.78 and 15.79% (Sebaiy et al., 2019).

Talaat (2017) established a micellar HPLC-UV method employing a VP-ODS column (4.6 mm x 150 mm i.d.) in separation. The mobile phase consisted of 0.1 M sodium dodecyl sulfate, 10% n-propanol, 0.3% triethylamine in 0.02 M orthophosphoric acid (pH 3.5). The flow rate was 1.0 mL/min. The method was found to be linear between 1 and 20 μ g/mL. The UV detector was set at 280 nm (Talaat, 2017).

Ravisankar et al. (2014) established a RP-HPLC method to determine pirfenidone in pharmaceutical dosage forms. Separation was carried out on a C18 column (4.6 mm x 250 mm i.d., 5 μ m p.s.). The mobile phase was composed of acetonitrile: water (50:50, v/v). The flow rate was 1.0 mL/min and UV detection was accomplished at 315 nm. The method was found linear ($r=0.999$) in the range of 2-10 μ g/mL. Recovery was found to be 99.60% to 99.80%. Precision (RSD%) was less than 2% (Ravisankar et al., 2014).

Parmar et al. (2014) developed an HPLC method for determination of pirfenidone from its pharmaceutical formulations. The separation was achieved isocratically on a reversed-phase C18

column with a mobile phase consisting of acetonitrile: water (35:65, v/v) at a flow rate of 0.7 mL/min. The UV detector was set at 317 nm. The method was found linear between 0.2 and 5.0 µg/mL. Recovery was in the range of 98 and 102% and precision was found < 2% (RSD%) (Parmar et al., 2014).

In Bodempudi et al. (2015) study, an HPLC based chromatographic method was developed for the determination of pirfenidone in the drug substance. Separation was achieved with a C18 column (4.6 mm x 250 mm i.d., 5 µm p.s.) using 0.02 M KH₂PO₄ buffer and acetonitrile as mobile phase. The flow was 1.0 mL/min and detection was achieved at 220 nm. The method was found linear (r²=0.9985) between 47 and 382 ng/mL. LOD was found 14 ng/mL and LOQ was calculated as 9.4 µg/mL (Bodempudi et al., 2015).

In the study of Thorat et al. (2016) an HPLC method for the determination of pirfenidone from tablet dosage form was established. Analytical separation was carried out using an isocratic technique on a reversed phase C18 column (4.6 mm x 150 mm i.d., 5 µm p.s.), with phosphate buffer: acetonitrile (pH 3.5) 72:28 v/v as a mobile phase at flow rate 1 mL/min. The method was linear (r=0.9964) between 5-70 µg/mL. Recovery was between 99.2 and 101.3%. Precision was found as ≤0.6751 (RSD%) (Thorat et al., 2016).

Wang et al. (2006) established an HPLC method for determination of pirfenidone as an analytical reagent in rat plasma. Rat plasma samples (150 µL) were precipitated with 10% (v/v) perchloric acid solution, then centrifuged. Obtained supernatant was applied to HPLC. This method was found to be linear between 0.15 and 76.67 µg/mL. The separation was carried out on a C18 column (4.6 mm x 250 mm i.d., 5 µm p.s.) using acetonitrile – water containing 0.2% acetic acid (23:77, v/v) as a mobile phase at 1 mL/min flow-rate. Detection was performed at 310 nm. The accuracy (RE%) was observed in ranges from (-2.6) to 7.9% and the precision (coefficient of variation) was found ≤ 4.5% (Wang et al., 2006).

In More et al. (2019) study, an HPLC analysis method for determination of pirfenidone was developed. The flow rate was 1 mL/min. The method was found linear (r=0.9989) over the range of 5-25 µg/mL. Recovery was between 98 to 100%. The separation was applied with a C18 column (4.6 mm x 250 mm i.d., 5 µm p.s.) at 30 °C and a photodiode array detector was set at 317 nm. Intraday and interday precision were found ≤ 0.65% (More, Dalwate, Chandramore, Jadhav, & Jain, 2019).

Measurement of esomeprazole and pirfenidone levels in rat plasma samples

The developed HPLC method was employed for monitorization of the esomeprazole and pirfenidone levels in plasma samples belong to 14 rats that were treated with esomeprazole (50 mg/kg/day) and pirfenidone (100 mg/kg/day). The esomeprazole and pirfenidone dose values to be applied to rats were determined according to the results of the previous studies in our laboratory.

Plasma samples were treated with the solid-phase extraction method described in Section 2.4 and made ready for HPLC

analysis. No problem was observed in the samples for the quantification of the analytes. Additionally, peak purity showed that no analytical interference was encountered in the endogenous substances. The daily used esomeprazole and pirfenidone amounts, both their plasma levels and their dose-proportional plasma levels with the descriptive statistical analysis results for the obtained data were given in Table 4.

Table 4. Sample results which involved rat plasma esomeprazole and pirfenidone concentrations that are unrevised and dose-proportional.

Sample number	Esomeprazole (50 mg/kg/day)		Pirfenidone (100 mg/kg/day)	
	Plasma concentration (ng/mL)	Plasma concentration /Dose (ng/mL/mg)	Plasma concentration (ng/mL)	Plasma concentration /Dose (ng/mL/mg)
S.01	27.89	0.56	4.08	0.04
S.02	3.49	0.07	1.36	0.01
S.03	8296.87	165.94	-	-
S.04	202.21	4.04	4.08	0.04
S.05	6.97	0.14	39.84	0.40
S.06	7.85	0.16	45.28	0.45
S.07	5.23	0.11	3.62	0.04
S.08	1.74	0.04	1.81	0.02
S.09	2.62	0.05	0.45	0.01
S.10	0.87	0.02	53.43	0.53
S.11	9.59	0.19	129.04	1.29
S.12	12.20	0.24	122.70	1.22
S.13	1.74	0.04	238.60	2.39
S.14	2.62	0.05	154.39	1.54
Mean (x̄)	612.99	12.26	3.75	0.61
SD (σ)	2212.19	44.24	11.14	0.76
RSD%	360.89	360.89	297.48	124.27

Abbreviations: SD stands for "Standard Deviation"; RSD is "Relative Standard Deviation".

In this study, it was aimed to establish a method by focusing especially on the expected esomeprazole and pirfenidone concentrations in rat plasma. Therefore, these concentrations were taken into account in establishing the method and performing validation tests. For this purpose, in our study, a narrow linear range of 5 and 50 ng/mL was preferred for esomeprazole and pirfenidone, and a strong determination coefficient (r≥0.9986) was obtained in both analytes. The LOQ obtained was found to be the lowest value (≤ 0.58 ng/mL) detected in the literature. Chromatographic analysis was completed in 7 mins total without any endogenous intervention into the plasma sample. The value obtained from the precision test as ≤7.89% during the

day and between days is compatible with the literature. Nice values between (-4.45) and 5.78 (RE%) were obtained from intraday and inter-day accuracy tests. The average recovery values from the application of the preferred solid-phase extraction in preparing the samples for HPLC analysis were 93.83 to 97.44% for esomeprazole and pirfenidone, respectively. The data obtained from the robustness test carried out according to the change in mobile phase content, mobile phase flow rate, column temperature and UV value was ≤ 4.85 (RSD%).

Obtained plasma esomeprazole and pirfenidone and their dose rated results were statistically analyzed with Spearman non-parametric test. The results showed that there was no statistically significant relationship between the analyzed results ($p>0.05$).

CONCLUSION

We strongly recommend this validated method to be used in routine therapeutic drug analysis of esomeprazole and pirfenidone. Also, it can be adapted to human plasma for monitoring overdose/poisoning caused by these drugs. Furthermore, since the method is established in the range of 25 to 500 ng/mL, it can be used in case of compliance problems with these drugs. The proposed method can be easily applied in routine therapeutic drug monitoring (TDM) studies of esomeprazole and pirfenidone. Also, it can be preferred in bioequivalence, pharmacovigilance and pharmacokinetics studies.

In this study, it was observed that both plasma-esomeprazole levels and plasma-esomeprazole concentration corrected according to daily drug doses ($\mu\text{g}/\text{mL}/\text{mg}$) has very high RSD% value which is 360.89%. In addition, both plasma-esomeprazole levels and plasma-esomeprazole corrected according to daily drug doses ($\mu\text{g}/\text{mL}/\text{mg}$) had significantly high RSD% results which were 297.48 and 124.27%, respectively. These results are both pharmacologically and toxicologically significant, and indicate that the potential to cause serious health problems for esomeprazole treatment.

Since these observed unexpected plasma esomeprazole and pirfenidone concentrations are thought to be related to the polymorphism of the CYP2C19 and CYP2A1 enzymes which are responsible for the biotransformation of esomeprazole and pirfenidone, the polymorphisms of the respective enzymes in the collected blood samples and its relationship with the plasma results obtained are planned to be investigated.

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