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Analytical method development and validation of simultaneous estimation of rabeprazole, pantoprazole, and itopride by reverse-phase high-performance liquid chromatography

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ABSTRACT

A simple, selective, rapid, and precise reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of rabeprazole (RP), pantoprazole (PP), and itopride (IP) has been developed. The compounds were well separated on a Phenomenex C_{18} (Luna) column (250 mm × 4.6 mm, dp = 5 μm) with C_{18} guard column (4 mm × 3 mm × 5 μm) with a mobile phase consisting of buffer containing 10 mM potassium dihydrogen orthophosphate (adjusted to pH 6.8): acetonitrile (70:30 v/v) at a flow rate of 1.0 mL/min and ultraviolet detection at 288 nm. The retention time of RP, PP, and IP were 5.35, 7.92, and 11.16 minutes, respectively. Validation of the proposed method was carried out according to International Conference on Harmonisation (ICH) guidelines. Linearity range was obtained for RP, PP, and IP over the concentration range of 2.5–25, 1–30, and 3–35 μg/mL and the r^2 values were 0.994, 0.978, and 0.991, respectively. The calculated limit of detection (LOD) values were 1, 0.3, and 1 μg/mL and limit of quantitation (LOQ) values were 2.5, 1, and 3 μg/mL for RP, PP, and IP correspondingly. Thus, the current study showed that the developed reverse-phase liquid chromatography method is sensitive and selective for the estimation of RP, PP, and IP in combined dosage form.

1. Introduction

Rabeprazole (RP), [2-[[[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl]-methyl] sulfinyl]-1*H*-benzoimidazole] (Fig. 1), is used for the treatment of severe gastroesophageal reflux disease (GERD) and peptic ulcer. Pantoprazole (PP), [6-(difluoromethoxy)-2-[[(3, 4-dimethoxy-2-pyri-dinyl)-methyl] sulfinyl] 1-benzoimidazole] (Fig. 1), is used for the treatment of erosive esophagitis associated with GERD. Both RP and PP are proton pump inhibitors and they inhibit gastric acid secretion by targeting the gastric acid pump H^+ K^+ adenosine

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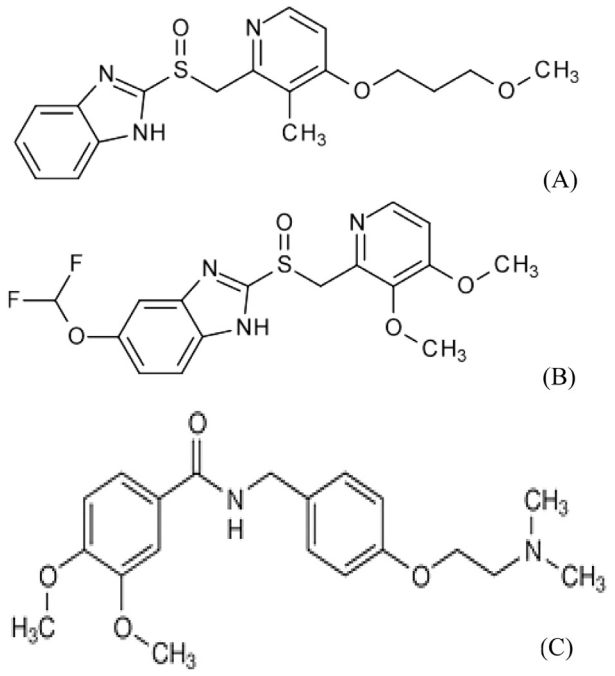

Fig. 1 – Chemical structures of (A) rabeprazole, (B) pantoprazole, and (C) itopride.

triphosphatase of the parietal cell. Proton pump inhibitors also are effective in treating patients with Zollinger-Ellison syndrome [1]. Itopride (IP), [N-[4-[2-[dimethyl amino ethoxy] phenyl] methyl]-3,4-dimethoxybenzamide (Figure 1), inhibits the dopamine D_2 receptor at the parasympathetic nerve ends and increases the release of acetylcholine, thereby increasing the esophageal and gastrointestinal motility. It also exerts an antiemetic action [2].

The combination of PP inhibitor and IP is widely available in the market for the treatment of gastrointestinal disorders. In general, these kinds of multicomponent dosage forms are useful for effective therapy and augment patient compliance. A range of analytical techniques, such as ultraviolet (UV)-visible spectrophotometry, high-performance liquid chromatography (HPLC), high-performance thin layer chromatography (HPTLC), and liquid chromatography-mass spectrometry (LC-MS) were reported in the literature for the determination of RP [3–10], PP [11–16], and IP [17–20] in dosage forms and biological samples in separate as well as in combination. Nevertheless, there is no information on the HPLC method for the concurrent determination of these drugs in combined dosage forms. The current study describes a simple, precise, and accurate reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of RP, PP, and IP in combined dosage forms.

2. Materials and methods

2.1. Chemicals

Acetonitrile (HPLC grade), orthophosphoric acid [analytical reagent (AR) grade], sodium hydroxide (AR grade), and ammonium acetate (AR grade) were purchased from E. Merck (India) Ltd. Worli, Mumbai, India. All active pharmaceutical ingredients (APIs) RP, PP, and IP as reference standards were obtained from Medley Pharmaceuticals Limited, Mumbai, India (99.7–99.9% purity).

2.2. Equipment

Shimadzu's HPLC quaternary system with UV-visible detector (LC-10AT VP) and 7725i injector was used for method development and validation.

2.3. Method development and optimization of chromatographic conditions for separation

The chromatographic condition was optimized by using different columns, mobile phase composition, pH (6.6, 6.8, and 7.0), wavelength (285, 288, and 290), flow rate (0.9, 1.0, and 1.1), column temperature (ambient to 45°C), and injection volume (10, 20, 30, and 50 μL).

2.4. Sample preparation

2.4.1. Chemical form of the APIs

RP sodium is a white to slightly yellowish-white solid. PP sodium is a white to off-white crystalline powder. IP hydrochloride is a white crystalline solid.

2.4.2. Preparation of RP, PP, and IP stock solutions

Stock solution was prepared by weighing 10 mg each of RP, PP, and IP standards in a 10-mL volumetric flask, dissolving in mobile phase, and diluting to volume with the same mobile phase up to 10 mL and retained as stock solution. Further dilutions were made with mobile phase.

2.4.3. Preparation of RP, PP, and IP standard dilutions

One milliliter from the stock solutions of RP, PP, and IP were transferred into a 10-mL volumetric flask separately and further diluted up to 10 mL with the solvent. Then 1 mL of RP, 1.5 mL of PP, and 2 mL of IP solutions were transferred into a 10-mL volumetric flask and diluted up to 10 mL separately to attain the final concentrations of 10 μg/mL, 15 μg/mL, and 20 μg/mL of RP, PP, and IP, respectively.

2.4.4. Preparation of mixed standard solutions

From the aforementioned standard stock solution, mixed standard solution was prepared by dissolving appropriate concentration of the stocks in the mobile phase and used for the estimation of individual drugs from the combination.

2.4.5. Preparation of the sample solution

The label claim of the dosage form includes 10 mg of RP sodium, 40 mg of PP sodium, and 50 mg of IP hydrochloride.

Twenty tablets of RP, PP, and IP available as combination dosage forms were weighed and powdered. An amount of the powder equivalent to one tablet was weighed accurately and mixed with the mobile phase in a 100-mL volumetric flask, sonicated for 5 minutes and filtered through 0.2 μ membrane filter to remove insoluble matter. One milliliter of the filtrate

was then diluted to 100 mL with mobile phase in a volumetric flask.

2.4.6. *Method for the estimation*

With the optimized chromatographic conditions, a steady baseline was recorded. After stabilization of the baseline for about 30 minutes, successive aliquots of the standard solution of the same concentration were injected and chromatogram was recorded until the reproducibility of the peak areas was satisfactory. This procedure was repeated using the sample solution so that duplicate injection of the sample solution was bracketed by injection of the standard solution.

The response factor of the standard peak and sample peak was obtained and the amount of each drug in the sample was determined. This procedure was repeated six times.

The concentration of each drug in the multicomponent dosage form was calculated using the formula (1):

$$\text{Concentration of drug} = \frac{\text{Response factor of the sample}}{\text{Response factor of the standard}} \times \text{Concentration of standard} \quad (1)$$

2.5. *Validation of the method*

The developed method was validated for as per ICH Q2 (R1) guidelines [21] for various parameters such as accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantitation (LOQ), and stability.

2.5.1. *Accuracy*

The accuracy of the RP-HPLC method was evaluated by selecting three different concentrations—lower quantitation limit (LQC), medium quantitation limit (MQC), and higher quantitation limit (HQC). In each concentration, a minimum of six injections were given and the amount of the drugs present, percentage recovery, and related standard deviation were calculated. The percentage recovery was calculated using the formula (2):

$$\text{Percentage recovery} = \frac{[b-a]}{c} \times 100 \quad (2)$$

Where a is the amount of the sample drug, b is the amount of sample drug and the standard drug and c is the amount of standard drug added.

2.5.2. *Precision*

The precision of the developed method was studied by performing interday and intraday variations. Intraday variations were studied by consecutively injecting the standard and sample solutions for six times on the same day. Interday variations were studied by estimating the drugs present in the multicomponent dosage forms on three different days. Six injections of standard and sample solutions were made every day. The amount of each drug, percentage content, standard deviation, and percentage coefficient of variation were calculated.

2.5.3. *Linearity and range*

The six series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient.

2.5.4. *LOD and LOQ*

The LOD and LOQ of RP, PP, and IP were determined by injecting progressively lower concentrations of the standard solutions into the HPLC column using the optimized chromatographic conditions in accordance with 3.3 s/n and 10 s/n criteria, respectively, where s/n indicates signal-to-noise ratio.

2.5.5. *Robustness*

For demonstrating the robustness of the method, slight variations in the optimized conditions were done and the standard solution was injected. The variations made were ±2% in the ratio of acetonitrile in the mobile phase, ±0.2 unit in the pH of the buffer, ±0.1 mL/min in the flow rate, ±5°C in the column temperature, and ±1 nm in the wavelength. The separation factor, retention time and peak asymmetry were calculated.

2.5.6. *Stability*

The mobile phase, standard solution, and the sample solution were subjected to long-term (3 days) stability studies. The stability of these solutions was studied by storing the standard solution for 3 days and observing for changes in the separation, retention, and asymmetry of the peaks, which were then compared with the pattern of the chromatogram of freshly prepared solution.

3. Results and discussion

Proper selection of the HPLC method depends on the nature of the sample (ionic or ionizable or neutral molecule), its molecular weight, and solubility. The drugs selected for the current study are polar in nature; hence, RP-HPLC was selected for its separation because of its simplicity and suitability.

3.1. *Optimization of the chromatographic condition*

Method optimization for the simultaneous estimation of the combination of RP, PP, and IP in multicomponent dosage forms was carried out.

3.1.1. *Column selection*

Experiments with different columns were conducted to achieve best separation of analyte peak with other blank and placebo peaks. It was found that the peak shape, retention time, tailing factor, and column efficiency were good with Phenomenex C_{18} column (250 × 4.6 mm, dp = 5 μm) with C_{18} guard column (4 mm × 3 mm × 5 μm).

3.1.2. *Mobile phase composition*

On the basis of the solubility study, 10 mM potassium dihydrogen orthophosphate was decided as the buffer preparation to be used. A mixture of 10 mM potassium dihydrogen orthophosphate and the organic solvents in different proportions were tested, as variation in the mobile phase

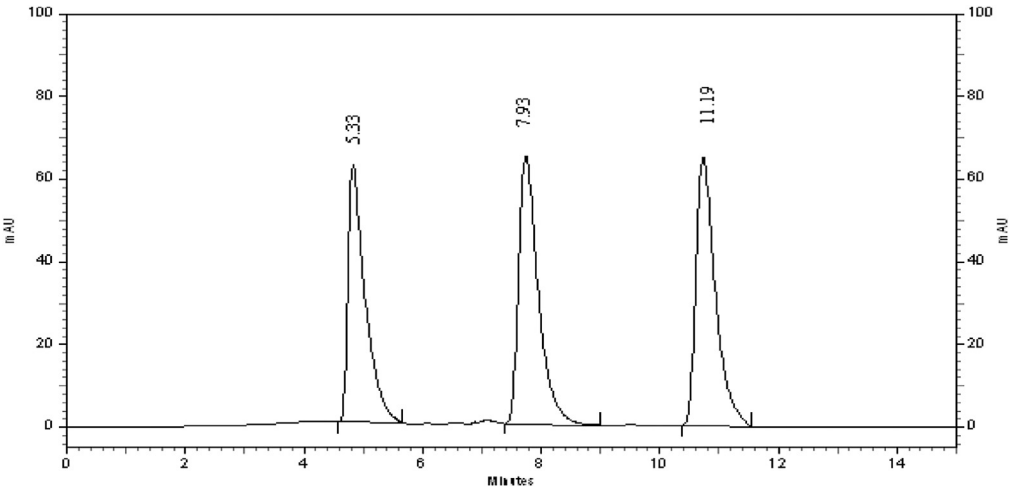

Fig. 2 – The representative chromatogram. The simultaneous estimation of rabeprazole, pantoprazole, and itopride, the peaks at the retention time of 5.33, 7.93, and 11.19 minutes, respectively.

composition led to substantial changes in the chromatographic performance. Decreasing the organic modifier content resulted in decrease in the retention time of the analyte but had no effect on analyte response. When experiments were performed with methanol instead of acetonitrile as organic modifier in the mobile phase, late elution of analyte with peak tailing and increased column pressure were observed. Hence, acetonitrile was selected as an organic modifier. Many trials on the composition of buffer and organic solvents were made to decide the ultimate composition of the mobile phase as buffer:acetonitrile (70:30).

Based on the peak shape, peak symmetry, and retention time, the flow rate of 1 mL/min, and ambient column temperature were also optimized.

3.1.3. *Detection wavelength*

The sensitivity of a HPLC method with UV detection depends on the proper selection of detection wavelength, which can be determined by recording overlaid UV spectra. In the current study, solutions containing 10 μg/mL of RP, 15 μg/mL of PP, and 20 μg/mL of IP were prepared in mobile phase and scanned under 200–400 nm of UV region to record the overlaid UV spectra.

3.1.4. *pH of the buffer*

pH plays an important role in achieving the chromatographic separation as it controls the elution properties by controlling ionization characteristics. The pKa values for RP and PP were 5 and 3.8, respectively. Potassium dihydrogen orthophosphate buffer (10 mM) was selected based on the solubility studies. Various trials on pH were made to determine the optimized pH at which the APIs are separated well. At pH 6.8, peak shape, peak tailing and theoretical plate count were found to be satisfactory; hence, 6.8 was decided as the pH of the buffer. A tolerable limit of pH 6.8 ± 0.1 was optimized using a pH meter.

In order to determine the adequate resolution and reproducibility of the proposed method, suitability parameters including retention time, plate number, and tailing factor were investigated and were found to be 5.33 min, 8670, 0.9 for RP; 7.93, 8550, 1.05 for PP; and 11.19, 7980, 1.09 for IP, respectively, which indicates the method suitability.

The optimized chromatographic conditions are mobile phase concentration—buffer (10 mM KH_2PO_4): Acetonitrile (ACN) 70: 30% v/v, pH 6.8, 288 nm as detection wavelength, 1.0 mL/min flow rate, ambient column temperature, 50 μL injection volume.

Table 1 – Accuracy (recovery, %) studies expressed in concentration (μg/mL) of rabeprazole, pantoprazole, and itopride at three different concentrations.

Study no.	Rabeprazole			Pantoprazole			Itopride		
	2.5	10	25	1	15	30	3	20	35
1.	97.9	102.2	104.67	98.76	100.78	102.01	99.97	99.92	99.93
2.	96.55	98.12	99.43	99.15	101.53	101.98	100.02	100.07	101.98
3.	96.24	103.16	102.55	99.67	99.18	99.59	100.05	100.12	102.01
4.	98.65	106.08	101.68	101.25	98.76	99.43	99.92	99.98	101.78
5.	95.07	101.07	103.09	101.87	102.08	98.68	99.98	99.89	101.56
6.	101.1	100.33	105.05	100.45	101.86	98.75	99.95	99.91	100.65
Avg	97.58	101.82	102.74	100.19	100.69	100.07	99.98	99.98	101.31
%RSD	2.13	2.70	2.06	1.21	1.40	1.53	0.04	0.09	0.83

Avg = average; RSD = relative standard deviation.

Table 2 – Intraday and interday assay precision analysis data of the proposed method.

Actual concentration	Measured concentration (μg/mL), RSD (%) ($n = 6$)	
	intraday	interday
Rabeprazole		
2.5	2.56, 0.82	2.49, 0.66
25	24.65, 0.89	24.86, 0.88
45	45.09, 0.92	44.89, 0.99
Pantoprazole		
1	0.99, 0.42	0.95, 0.99
15	14.95, 0.22	14.92, 0.76
30	30.02, 0.88	29.89, 0.86
Itopride		
3	2.99, 0.84	3.09, 0.65
20	19.86, 0.95	20.09, 1.00
35	34.86, 0.62	35.00, 0.92

RSD = relative standard deviation.

3.2. *Validation of method*

3.2.1. *Specificity*

The specificity of the existing method of analysis by HPLC is shown in Figure 2; the complete and clear separation of RP, PP, and IP was observed without any interference in retention time.

3.2.2. *Accuracy*

The accuracy of the method was determined by recovery experiments. Recovery studies were carried out with six injections and three different concentrations. The percent recovery, mean, and relative standard deviation (% RSD) were calculated and presented in Table 1. APIs with concentration 2.5, 10, and 25 μg/mL of RP; 1, 15, and 30 μg/mL of PP; and 3, 20, and 35 μg/mL of IP were prepared. The test solution was injected six times for each spike level and the assay was performed as per the test method. Analysis of the results has shown that the percentage recovery values were close to 100 % and also the RSD values were less than ±5%. The accuracy and reliability of the developed method was established.

3.2.3. *Precision*

The precision of the method was demonstrated by interday and intraday variation studies at various concentrations: 2–25 μg/mL for RP, 1–30 μg/mL for PP, and 3–35 μg/mL for IP, and their data are summarized in Table 2. The lower RSD% values (<1.00) indicate good precision of the developed method.

3.2.4. *Linearity and range*

Six series of standard solutions were selected for assessing linearity range (2.5–25 μg/mL for RP, 1–30 μg/mL for PP, and 3–35 μg/mL for IP). The calibration curve was plotted using response factor versus concentration of the standard solution. From the calibration curve, the slope and intercept were calculated. The data obtained from the linearity range are depicted in the graph and the results show the Y intercept as $187.6x - 11.31$ for RP, $82.77x + 1216$ for PP and $75.29x + 1374$ for IP with higher correlation coefficient r value 0.994, 0.978, and 0.991 for RP, PP, and IP respectively.

3.2.5. *LOD and LOQ*

The LOD and LOQ of the compounds were determined by injecting progressively lower concentrations of the standard solutions into the HPLC column using the optimized chromatographic conditions. The LOD values were found to be 1, 0.3, and 1 μg/mL for RP, PP, and IP, respectively. The LOQ values were found to be 2.5, 1, and 3 μg/mL for RP, PP, and IP, respectively.

3.2.6. *Robustness*

The robustness was evaluated by making slight variations in the optimized conditions such as flow rate, pH of mobile phase, column temperature, wavelength, and percentage of organic solvent. The mixed standard solution was injected in five replicates and %RSD of assay was calculated for each condition. The results obtained (Table 3) as a cause of small

Table 3 – Robustness study of the proposed method.

Factor	Level	Mean (Assay, %) RSD ($n = 3$)		
		Rabeprazole	Pantoprazole	Itopride
Flow rate (mL/min)	0.9	98.5, 1.12	98.8, 0.55	97.6, 1.11
	1.0	99.0, 0.68	98.2, 0.82	96.8, 0.95
	1.1	98.7, 0.96	99.3, 0.86	99.3, 0.92
pH of mobile phase	6.6	98.7, 0.96	99.6, 0.95	99.6, 0.82
	6.8	98.4, 1.82	98.3, 0.75	98.4, 0.99
	7.0	98.8, 0.95	97.5, 0.89	99.3, 0.66
Column temperature	25	98.8, 0.96	98.9, 0.82	97.5, 0.95
	30	98.4, 1.25	98.6, 1.02	97.6, 0.94
	35	99.8, 1.28	97.3, 0.98	99.6, 0.60
Wavelength	287	100.2, 0.64	100.8, 0.87	98.4, 1.09
	288	98.8, 0.95	98.4, 0.78	99.5, 0.97
	289	97.2, 0.88	98.8, 0.95	98.9, 1.08
Percentage organic solution	28	99.2, 0.92	99.6, 0.95	99.5, 0.52
	30	97.6, 0.88	101.3, 0.55	98.7, 0.96
	32	97.6, 1.08	99.2, 0.83	98.8, 0.96

RSD = relative standard deviation.

Table 4 – Solution stability studies.

Study no.	(Percentage deviation between actual and stored recovery)		
	Rabeprazole	Pantoprazole	Itopride
1	2.58	0.25	0.46
2	2.32	0.19	0.35
3	2.28	0.17	0.62
4	2.49	1.78	0.92
5	1.05	0.36	0.55
6	1.08	0.89	0.53
Average	0.61	0.30	0.36

deliberate variations in the method parameters has proven that the analytical method is robust.

3.2.7. *Stability*

The stability of the drug solutions was assessed by maintaining the solution at room temperature for 3 days and observing for changes in the chromatographic pattern as well as the content of the solution on comparison with the freshly prepared solution. The results were expressed in terms of percent deviation between actual and stored recovery (Table 4).

4. Conclusion

A convenient and rapid simultaneous RP-HPLC method has been developed for the estimation of RP, PP, and IP. Best separation was achieved on a Phenomenex C_{18} (250 mm × 4.6 mm internal diameter (i.d.), 5 μ) with C_{18} guard column (4 mm × 3 mm × 5 μm), 10 mM potassium dihydrogen orthophosphate (adjusted to pH 6.8): acetonitrile (70:30 v/v) at a flow rate of 1.0 mL/min as mobile phase and 288 nm as detection wavelength. The method was validated in terms of accuracy, precision, specificity, linearity, robustness, and solution stability according to ICH guidelines. The proposed method is simple, fast, accurate, and precise for the simultaneous quantification of RP, PP, and IP in bulk drugs and finished products as well as for routine analysis in quality control.

Conflicts of interest

The authors declare that there is no conflict of interest.

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