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Box-Behnken guided development of an ecofriendly RP-HPLC analytical method for simultaneous quantification of pantoprazole sodium and piperine co-loaded mucoadhesive GRDDS formulation for *H. pylori* eradication

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ABSTRACT

Helicobacter pylori (*H. pylori*) infection is the leading cause of chronic peptic ulcer disease worldwide. Many treatment options are available to treat *H. pylori* infection. However, the eradication is still a challenge due to the poor bioavailability of the currently available formulations. To improve the efficacy of therapy, novel formulations are necessary. Proton pump inhibitors (PPIs) are already a part of the treatment regimen with antibiotics. P-glycoprotein (P-gp) inhibitor was reported to have increased the efficacy of antibiotic treatment in *H. pylori* infections. To make available the P-gp inhibitor and PPI on the intestinal mucosal surface we have formulated a gastroretentive drug delivery system (GRDDS) as an adjuvant therapy with antibiotics. The objective of this study is to simultaneously estimate pantoprazole (PAN) and piperine (PIP) by reverse-phase (RP) high-performance liquid chromatography (HPLC) method from the chitosan-based sodium alginate mucoadhesive beads utilizing the design of experiments (DOEs) methodology. The HPLC settings were optimized using DOEs software. The final optimized HPLC method used a hyperclone Octadecylsilane C18 column as the stationary phase and methanol: ammonium acetate at pH 4.5 (70:30 v/v) as the mobile phase. The flow rate was 0.9 ml/minute. The validation of the developed RP-HPLC method was done as per the International Conference on Harmonization (ICH) Q2(R1) guideline. The method was linear from 0.5 to 20 µg/ml for both PAN and PIP with an R2 value of 0.999 and 0.999, respectively. The validated RP-HPLC method showed specificity for both drugs despite interference from degradation products and other GRDDS excipients. The entrapment efficiency of the final formulation was determined to be 80%–85% for PAN and 60%–67% for PIP. The novelty and merit of the DOE-based method development are that it reduces the number of trials, thereby reducing reagent wastage, and is environmentally friendly suggested by the Green Analytical Procedure Index (GAPI) tool scoring six green, six yellow, and three red.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacteria, that is classified as a type I carcinogen by the International Agency for Research on Cancer, a division of the World Health Organization [1,2]. This bacterium stays in the stomach mucosa and causes different diseases such as gastric ulcers, diarrhea, malnutrition, or other infections such as cholera or typhoid fever. For the treatment of *H. pylori* infection, a combination of antibiotics and proton pump inhibitors (PPIs) are utilized owing to the inherent anti-bacterial activity of PPIs [3].

Due to the antibiotic resistance and polypharmacy, the available treatments are not successful. The failure of

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the therapy and the antibiotic resistance have contributed to the rise in *H. pylori*-associated gastrointestinal problems [4]. The prevalence of *H. pylori* is significantly high worldwide. The resistance to antibiotics and the treatment failure can be managed by using the P-glycoprotein (P-gp) inhibitors. P-gp is a transmembrane glycoprotein with a molecular weight of 170 kDa, belonging to the extensive superfamily of ATP-binding cassette transporters. P-gp inhibitors are often used as a bioavailability enhancer because of their crucial function in modifying drug uptake and permeability. Stomach ulcers caused by *H. pylori* may be caused by an overproduction of P-gp. The role of P-gp in absorbing drugs and therapy for *H. pylori* infection was described by Damanhuri *et al*. [5] They found that P-gp-expressed rats were much more susceptible to developing *H. pylori*-induced ulcers than P-gp-inhibited rats.

The eradication of *H. pylori* involves the usage of antibiotics but the efficacy of these antibiotics can be reduced by the activity of P-gp in the stomach lining. It pumps the antibiotics out of the stomach before they are fully absorbed and can reach the site where *H. pylori* resides. By inhibiting the activity of P-gp, the antibiotics can cling in the stomach for a longer period of time and achieve higher concentrations to effectively kill *H. pylori*. Apart from bacterial eradication, P-gp inhibitors also showed a more than 90% healing rate compared to standard treatment in the case of gastric ulcers [5]. Several studies have shown that P-gp inhibitors, such as verapamil, cyclosporin A, and quinidine, can increase the effectiveness of antibiotics against *H. pylori* [6]. Piperine (PIP) is a P-gp inhibitor that stops the adhesion of *H. pylori* to the gastric mucosa and helps in the eradication of bacteria [7]. PIP has been found to possess anti-inflammatory, antioxidant, and anti-ulcer properties, which may help in the management of gastric ulcers. Some studies have shown that PIP can reduce gastric inflammation and oxidative stress, and inhibit the growth of *H. pylori*, the bacteria associated with gastric ulcers. PIP has also been shown to increase the production of gastric mucus, which could help protect the stomach lining from damage. PIP has been studied for its potential to enhance the bioavailability of other compounds, such as curcumin. The combination of pantoprazole (PAN) and PIP can increase the efficacy of the drug and cure the ulcer early. Here, in this work, the high-performance liquid chromatography (HPLC) analytical method has been developed to simultaneously quantify the PAN and PIP from the gastroretentive drug delivery system (GRDDS). Here, in Table 1, a few reported HPLC methods for PAN and PIP have been shown.

GRDDS has gained interest for localized stomach targeting and longer retention of the drug in gastric mucosa.

Table 1. The previously reported analytical HPLC method for PAN and PIP.

Drug	Column	Mobile phase	Total run time (minutes)	Flow rate (ml/minute)	Retention time (minutes)	Reference
PAN	Phenomenex ODS analytical column (150 mm 4.6 mm i.d., 5 µm particles)	Phosphate buffer pH 7.4 and ACN in a 70:30 (v/v) ratio, respectively.	20	2	PAN (6.9)	[33]
PAN and Vitamin B	Phenomenex C18 (4.6 × 150 mm, 5 µm)	Water and ACN as mobile phase in linear gradient elution mode	20	0.5	PAN (6.8), Vitamin B1 (2.7), Vitamin B6 (5.5), Vitamin B12 (3.8).	[34]
PAN	C18 column (5 µm, 4.6 × 150 mm, Zorbax Eclips Plus)	ACN and phosphate buffer (10 mm, pH 7 adjusted with 0.1 M sodium hydroxide) at 36:68 (v/v)	-	0.8	PAN (2.5)	[35]
PAN with itopride and ondansetron	C-18 column (150 × 4.6 mm × 5 µm)	Methanol: 0.01 M potassium dihydrogen phosphate buffer of pH 3.5 (80:20 v/v)	5	1	PAN (2.49), itopride (1.79) and ondansetron (2)	[36]
PAN enantiomers	Trefoil™ CEL2 column (150 mm × 3.0 mm i.d., 2.5 µm)	Methanol with 0.2 % formic acid	16	0.2	S-PAN (12.6), R-PAN (13.8)	[37]
PIP	C18 column (250 × 4.6 mm, Eurospher 100 with 5 µm)	ACN -methanol-water of 65:5:35 %	9	1	PIP(7.1)	[38]
Pregabalin and PIP	C18 Lichrospher column (250 × 4.6 mm, with 5 µm particle size)	ACN-water (pH 6.9; 70:30%, v/v) in an isocratic elution mode	10	1	Pregabalin (0.8) and PIP (2.3)	[39]
Resveratrol and PIP	Luna 5 µ 100 Å C-18(2) HPLC column	ACN: phosphate buffer (0.01% orthophosphoric acid) (55:45)	10	1	Resveratrol (3.3) and PIP (9.2)	[40]
PIP and guggulsterones	LiChroCARTWC18column (25.0 × 4.6 mm, particle size 5.0mm)	ACN and water in gradient	30	1	PIP (6.7) and guggulsterones (10.8)	[41]
Curcumin and PIP	Chromolith® SpeedROD RP-18 (50 × 4.6 mm) column	ACN –methanol–trifluoroacetic acid-water (17.6:35.3:0.1:47.0, v/v/v/v)		2.5	Curcumin (3.2) and PIP (2.1)	[42]

Chitosan and sodium alginate were utilized as the mucoadhesive polymers in this work. EUDRAGIT® L 30 D-55 is an anionic copolymer made of methacrylic acid and ethyl acrylate that is dispersed in water. This polymer was used for the enteric coating of GRDDS of PAN because PAN is not stable in the gastric environment. This allows the drug to pass through the stomach intact and be released in the more alkaline environment of the small intestine, where it can be absorbed more effectively. For the fabrication of a novel drug delivery system of PAN and PIP, simultaneous quantification of drugs is important. Therefore, it is important to find a way to estimate both PAN and PIP, simultaneously. Figure 1 and Table S1 show the structure and properties of PAN and PIP.

The objective and novelty of this study were to simultaneously estimate PAN and PIP by reverse-phase (RP)-HPLC method equipped with a photo-diode array (PDA) detector from the chitosan-based sodium alginate mucoadhesive beads utilizing design of experiments (DOEs) methodology. The DoEs is a systematic approach to determining and analyzing key aspects of a process. DoE has been employed in several steps of analytical procedures, such as preliminary screening, determining key chromatographic parameters, optimizing, and estimating robustness. This method was validated as per the International Conference on Harmonization (ICH) Q2 R1 guideline and was optimized by using of Box-Behnken design (BBD). In addition, the developed method was found to be highly stable and showed good response at low concentrations. A forced degradation study was conducted by exposing the drugs on purpose to different stress factors to evaluate their stability and the specificity of the method to estimate the pharmaceuticals among degraded products. The optimized method was applied to estimate the PAN and PIP in the mucoadhesive formulation to evaluate the entrapment efficiency.

MATERIALS AND METHODOLOGY

Gift samples of PAN (off-white powder; purity > 98%) were kindly provided by Sun Pharma of Gurgaon, Haryana, India. PIP (light yellow, purity > 97%) sourced from Sigma, Himedia Labs Pvt. Ltd. (Mumbai, India) supplied the hydrogen peroxide (30%) and the sodium hydroxide pellets (98% purity). Orthophosphoric acid (88%) was purchased from Merck Ltd. Mumbai, India. HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from Finar Ltd (Ahmedabad, India). High pure water (18.2 MΩ.cm resistivity, Milli-Q) was obtained from the Direct-Q ® 3 water purification system, Millipore Corporation, Billerica, USA. Finar Ltd. (Ahmedabad, India) supplied the 35% pure AR-hydrochloric acid. Riviera Glass Pvt. Ltd. (Mumbai, India) provided 0.22 µm membrane filters. Potassium dihydrogen phosphate (purity >98%) and sodium hydroxide were purchased from Merck Labs Pvt. Ltd. in Mumbai, India. The HyperClone Octadecylsilane (ODS) C_{18} column was purchased from Phenomenex (Hyderabad, India) and included particle sizes of 5 µm and 120 Å, and a length and width of 250 × 4.6 mm. The ingredients for the mucoadhesive beads, including sodium alginate and chitosan (purity > 98%), were obtained from Loba chemicals in Mumbai, India. Merck Labs Pvt. Ltd. supplied the calcium chloride (purity > 98%). Evonik generously provided the sample of EUDRAGIT® L 30 D-55. The HPLC-grade chemicals were employed in the method development and validation processes.

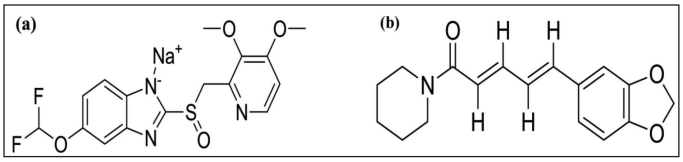

Figure 1. Structure of drugs. (a) PAN sodium. (b) PIP.

Instrumentation

To optimize and validate the chromatographic process, a Shimadzu HPLC system equipped with an LC20-AD pump, SIL20-AC HT autosampler, CTO-10 ASVP column oven, SPD-20A and SPD-M10 detectors, and LabSolutions software were used. To make the standard solution and the buffer, we used an analytical balance for the weighing purpose, that had been calibrated (a Sartorius Mechatronics CP225D, India). The mobile phase solution was filtered via a 0.22 µm membrane filter using a glass vacuum filtering assembly from Merck Millipore, Bangalore, India, and degassed in an ultrasonic bath from GT Sonic in Guangdong, China. The pH of the mobile phase was determined with the help of a calibrated digital pH meter (ELICO (Model #LI 617), Telangana, India). During the course of the sample preparation procedure, the calibrated variable micropipettes Construct (in Eppendorf, Germany) with volumes ranging from 0.2 to 10, 10 to 100, and 100 to 1,000 µl were used.

Preparation of mobile phase and standard solution

Mobile phase (MP)

The MP was composed of a 30% 10 mm ammonium acetate buffer and a 70% methanol solution. The buffer was made by dissolving ammonium acetate in Milli-Q water and then adjusting the pH with glacial acetic acid or ammonia solution to a value of 4.5. The buffer was then filtered through a 0.22 µm membrane filter before sonicating it.

Standard and sample solution

A stock solution of 1 mg/ml was prepared by dissolving correctly weighed portions of PAN (10 mg) and PIP (10 mg) into 5 ml of Milli-Q water using bath sonication. The volume was then brought up to 10 ml using more methanol. Using 1 ml of each drug's stock solution, we brought the total amount up to 10 ml to create a functional stock solution of PAN and PIP. A final concentration of 100 µg/ml was reached in the working stock solution. Different PAN and PIP concentrations were prepared from the working stock.

DoE guided HPLC method development using BBD

To find the correct wavelength for the simultaneous estimation of PAN and PIP and to achieve the desired concentration of 10 µg/ml, the working stock was further diluted. The prepared solution was analyzed using a double beam UV-Vis spectrophotomer (UV-1800, Shimadzu, Kyoto, Japan) in the range of 200–800 nm to detect absorption maxima

(λ_{max}) using water as a blank. The ammonium acetate buffer pH 4.5(10 mM) was selected on the basis of the pKa value of the PAN (3.92, 8.19) and PIP (12.22) and on the basis of preliminary trials conducted. The stationary phase of the method was selected on the basis of the literature and the properties of the drugs (hydrophobic interactions). Hyperclone ODS C_{18} column was selected as the stationary phase [8–11].

A comprehensive literature review was conducted to investigate the impact of various processing factors, including organic phase ratio, flow rate, column temperature, and injection volume, on parameters such as retention time (T_R), peak area, and resolution. Previous experiments using the one factor at a ime (OFAT) technique revealed the factors significantly influencing the separation.

The optimization process involves the precise adjustment of these key factors to establish stable testing parameters. Altering numerical values for these experimental factors requires a deep understanding of the underlying chemistry and the intricate interactions among the components. The DoE tool investigates the interaction between variables and the effect of variables on the outcomes. The DoE approach sets the range for trials and from the response of trials it selects the optimal method with robust response. The BBD and central composite design are the models used for the optimization. BBD is the response surface design that gives detailed results with the less experimental trials and hence was utilized in the current study [12,13].

This optimization procedure identified four chromatographic parameters, including buffer phase (X1), buffer pH (X2), flow rate (X3), and injection volume (X4), as independent variables, and peak area of PAN (Y1), peak intensity of PAN (Y2), peak area of PIP(Y3), peak intensity of PIP (Y4), and resolution (Y5) as the responses (Table 1).

Method validation

The ICH Q2 (R1) validation criteria were applied to the established reverse phase HPLC method for determining PAN and PIP simultaneously. The sensitivity, specificity, linearity, limits of detection (LOD), limits of quantitation (LOQ), accuracy, precision, robustness, and stability analysis were done [14,15].

Degradation studies

The objective of stress-induced degradation is to evaluate the stability of the drugs under extreme conditions. Various forms of stress, including chemical, physical, thermal, oxidative, and light stress, were applied to the drug samples. The study on stress-induced degradation performed drug solutions of 1 µg/ml for both PAN and PIP. The drug concentrations in the degradation samples were calculated per the defined protocol, and all analyses were carried out in triplicate to quantify the percentage of degradation.

Hydrochloric acid was added to samples at 0.1 and 1 M concentrations to investigate the effects on acid hydrolysis. After fully mixing 2 ml of acid in 1 ml of drug solution (1 mg/ml), it was heated to 60°C and left to react for a whole day. Using 0.1 and 1 M NaOH, the resultant solutions were neutralized. The sample was further diluted before the HPLC analysis [16,17].

PAN and PIP were tested in an alkaline medium with two different molar concentrations of NaOH (0.1 and 1 M) to evaluate their degradation behavior under pressure. After mixing 1 ml of drug solution (1 mg/ml) with 2 ml of alkali solution, the mixture was heated for 24 hours at 60°C. The solution was then neutralized with 0.1 and 1 M HCl. The sample was further diluted before the HPLC analysis [18].

1 ml of a PAN and PIP solution (1 mg/ml) was combined with 2 ml of a 3% w/v H_2O_2 solution to prepare a combination that would cause oxidative degradation using hydrogen peroxide. This combination was kept at room temperature in the dark for a whole day. The samples were then suitably diluted, and an HPLC analysis was carried out [19,20].

A drug solution (1 mg/ml) was exposed to the sun for 24 hours to investigate photolytic deterioration. The samples were then diluted, and an HPLC analysis was carried out [21].

To check the thermal degradation, 1 mg/ml of each drug's solution was mixed with 2 ml of water, and the vials were kept in an oven set to 60°C for 24 hours. After diluting the samples, HPLC was used to assess the degradation [22].

Evaluation of drug entrapment efficiency (DEE) of mucoadhesive formulation

The DEE of the new GRDDS formulation including PAN and PIP, for the eradication of *H. pylori* infection, were evaluated using the validated reverse phase HPLC technique. To develop drug-loaded mucoadhesive beads, an ionic gelation method was employed. First, sodium alginate was dissolved in distilled water at a concentration of 7% (w/v). Concurrently, a 1% acetic acid solution was employed to dissolve calcium chloride and chitosan, with continuous agitation until achieving uniformity. The sodium alginate solution was then blended with PAN and PIP until a uniform mixture was obtained. Subsequently, the drug-loaded sodium alginate solution was added drop by drop, utilizing a 26G needle, to the homogeneous mixture of calcium chloride and chitosan which contain 5% (w/v) EUDRAGIT® L 30 D-55 for the enteric coating of the beads. The resulting beads were gathered through filtration and air-dried for a duration of 8–10 hours. These prepared spherical beads were stored in an airtight container for subsequent studies [23,24].

Determination of DEE%

The PAN and PIP-loaded mucoadhesive beads underwent a process of crushing followed by immersion in 100 ml of phosphate buffer at pH 7.4. Subsequently, the suspension was sonicated at 37°C for 1 hours and allowed to stand overnight before HPLC analysis. Following centrifugation of the resultant solution, the supernatant was further diluted 30-fold using the mobile phase (diluent) before undergoing HPLC testing to determine drug content. The calculation of the percent DEE% was carried out using Equation 1.

$$DEE\% = \frac{\text{Actual drug content}}{\text{[Total drug]}} \times 100 \quad (1)$$

RESULT AND DISCUSSION

The risk of a failure of method transfer may be mitigated by first identifying and optimizing the key

chromatographic parameters, and then validating the method. Because of their significant absorption at 288 and 341 nm, respectively, PAN and PIP were analyzed using HPLC coupled to a PDA detector. The samples may be analyzed by PDA detectors at many wavelengths without having to re-analyze them. ODS C_{18} column was used to separate PAN and PIP since their log *p* values of the drugs are 2.05 and 0.28, respectively. The hydrophobic benzimidazole ring of PAN and the alkyl chain of PIP interact with the hydrophobic ODS stationary phase, despite the fact that PAN and PIP themselves are non-polar. Since PAN and PIP are retained less strongly in the stationary phase due to their hydrophobic interaction, they may be readily eluted. As a result, we employed a Phenomenex C_{18} 250 mm 4.5 mm, 5 µm column for our pilot tests. Different MPs were tested throughout method development to get the optimum separation between PAN and PIP. Trials for the method development were started with Milli-Q water (type 1 water) and ACN as a mobile phase where drug peaks were not eluted in the C_{18} 150 × 4.5 mm column. With phosphate buffer and MeOH, drugs were not eluting. After changing of column size to C_{18} 250 × 4.5 mm, both the drugs were eluting early as well as peak shape and plate count were observed to be less than the acceptance criteria. Phosphate buffer pH 6.2 and ACN were tried for the method development, where PAN showed retention with T_R 10.9 minutes and PIP showed 12.6 minutes. A combination of organic phase (ACN and MeOH) was also used but drug peaks were not eluted.

According to their respective pKa values, PAN and PIP should be in their ionized forms at a pH of 4.5. Therefore, ammonium acetate was chosen since it is a preferred buffer for keeping the pH steady between 4.5 and 5.5 [25–30]. DoE was then used to further optimize the chromatographic conditions.

DoE-guided optimization of the method using BBD

The optimization of independent variables was conducted utilizing a three-level BBD, encompassing buffer phase (X1), buffer pH (X2), flow rate (X3), and injection volume (X4) as factors. The upper and lower limits of these

Table 2. The DoE suggested independent variables and their corresponding responses.

Run	X1	X2	X3	X4	Y1	Y2	Y3	Y4	Y5
1	35	4.5	0.8	15	4.912	10.8937	4,256	27,698	17.9473
2	32.5	5	0.9	20	4.707	11.5433	4,901.67	32,798.7	20.4403
3	35	4	0.9	15	5.166	14.5097	3,937.67	22,563	22.9187
4	30	4.5	0.9	10	4.373	9.69233	2,583	16,360	17.1233
5	32.5	4	0.9	20	4.723	11.7043	5,056	31,768.7	18.492
6	35	5	0.9	15	5.127	14.5483	3,807.33	24,091.3	22.9133
7	32.5	5	1	15	4.243	10.577	3,318.33	21,811.3	19.6197
8	32.5	4.5	0.9	15	4.718	11.7985	3,971	22,599	19.6875
9	32.5	4.5	0.9	15	4.716	11.7885	4,110	23,256.5	19.9175
10	30	4.5	0.9	20	4.384	9.71	5,321.67	32,612	17.3677
11	32.5	4.5	1	20	4.264	10.642	4,635	29,908	19.8477
12	35	4.5	1	15	4.655	13.2027	3,426	21,475.3	22.8143
13	32.5	4.5	0.8	20	4.917	10.9103	5,748	37,605	18.1837
14	32.5	5	0.9	10	4.695	11.7283	2,805	15,696.7	20.6123
15	35	4.5	0.9	20	5.17333	14.6497	5,169.67	33,030.7	22.6317
16	30	5	0.9	15	4.369	9.66833	3,619	24,149	17.5403
17	32.5	4.5	0.9	15	4.717	11.786	3,991.5	27,193.5	20.1165
18	32.5	4.5	0.9	15	4.7175	11.8	3,321.5	22,346	19.9925
19	32.5	5	0.8	15	5.26233	13.1587	4,019.33	26,630.3	21.2353
20	32.5	4.5	0.8	10	5.031	11.184	2,856.33	18,059.7	18.4107
21	32.5	4	0.8	15	5.29433	13.1097	4,268	26,712	20.1967
22	30	4	0.9	15	4.376	9.61033	3,607.67	24,889.3	17.154
23	32.5	4	0.9	10	4.714	11.6817	2,472.67	16,281	20.432
24	30	4.5	0.8	15	4.912	10.8973	4,322.67	18,605.7	18.02
25	32.5	4	1	15	4.256	10.5343	3,264.33	21,674	19.0693
26	32.5	4.5	1	10	4.25167	10.626	2,275	14,784.3	19.9697
27	30	4.5	1	15	3.974	8.817	3,441	22,125	16.9773
28	32.5	4.5	0.9	15	4.717	11.803	4,063.5	15,558.5	20.016
29	35	4.5	0.9	10	5.15833	14.637	2,869	15,880.7	23.2443

critical values were determined through the OFAT method. The buffer phase was constrained to a minimum concentration of 30% and a maximum of 35%. The pH range for the buffer was specified with a minimum of 4.0 and a maximum of 5.0. Flow rates varied between 0.8 and 1.0 ml/minute, while injection volumes spanned from 10 to 20 µl. The DoEs encompassed a total of 29 distinct runs involving these independent variables, including five center points for further investigation.

These 29 different combinations of the independent variables were run through the HPLC, and the results were analyzed to determine the optimal setting. Retention time (T_R) of PAN (Y1), T_R of PIP (Y2), peak area (Y3), peak area (Y4), and resolution (Y5) were chosen as responses to optimize the chromatographic conditions. The results for each variable are listed in Table 2. The results of an analysis of variance (ANOVA) show that the model for the chosen independent variable is statistically significant at the 0.001 level shown in Table 3. BBD's response surface analysis shed light on how various independent factors affected the various chromatographic outputs.

Effect of independent variables on retention time of PAN sodium (Y_1)

The ANOVA results indicated that the Y1 is significantly affected by the independent parameters of X1, X2, and X3, but not by the X4. The ANOVA analysis yielded a quadratic equation (Eq. 2), which demonstrated a positive relationship between the X1 and Y1. If the buffer ratio is increased, PAN's T_R will also rise. Both the X2 and the X3 were shown to have a detrimental influence on Y1. The Y1 is not noticeably affected by the X4. The interaction between the X1 parameters was shown to affect the Y1 in the effect analysis. Interactions between independent factors that affected the response Y1 were also shown by the analysis.

$$Y1 = +6.15004 + 0.693972 \times A - 0.118972 \times B - 0.407722 \times C - 0.0128889 \times D - 2.12 \times AB - 0.375 \times AC + 0.00116667 \times AD - 0.0134167 \times BC - 0.00241667 \times BD + 0.0624167 \times CD \quad (2)$$

Both the 3D plot in Figure 2a and the perturbation plot in Figure 3a demonstrated how changing the buffer ratio (A) and flow rate (C) greatly affected the PAN retention time. There was a positive correlation between Y1 and X1, but a negative correlation with X2 and X3.

Effect of independent variables on retention time of PIP (Y_2)

ANOVA analysis yielded a quadratic equation (Eq. 3), which showed that X1 and X3 substantially influenced the response Y2 with $p < 0.05$. With a rise in X1, the Y2 also increased. As the factor X3 increased, the Y2 was raised. There was no discernible change in Y2 as a function of increasing X2 or X4. This analysis also found that the interplay between X1 and X3 had a significant effect on Y2 ($p < 0.05$).

$$Y2 = +11.628 + 2.00381 \times A + 0.00616667 \times B - 0.479556 \times C - 0.0324722 \times D - 0.00483333 \times AB + 1.09733 \times AC - 0.00125 \times AD - 0.00158333 \times BC - 0.0519167 \times BD + 0.0724167 \times CD \quad (3)$$

The 3D plot Figure 2b and the perturbation plot Figure 2b indicated that Y2 was considerably altered by adjusting the buffer ratio (A) and flow rate (C). Increasing the buffer ratio was shown to enhance Y2, whereas increasing the flow rate decreased Y2.

Effect of independent variables on peak area of PAN sodium (Y_3)

There is a statistically significant relationship between the independent variables X3 and X4, but only a marginal one between X1 and X2. Equation (4) derived using ANOVA revealed that an increase in X4 may increase the Y3 and a reduction in X3 can increase the Y3. In principle, increasing the injection volume increases the number of moles of analyte accessible to emit signal. A higher Y3 concentration may explain this. Similarly, as the flow rate increases, the contact duration for the analyte with the stationary phase diminishes, resulting in a small decline in response Y3. The findings demonstrated that the reaction Y3 is affected by the interaction between X3 and X4.

$$Y3 = +3583.08 - 49.9167 \times A + 387.611 \times B - 399.694 \times C + 1266.78 \times D - 201.333 \times AB - 64.25 \times AC + 55.5 \times AD - 13.0833 \times BC + 157.25 \times BD - 180.917 \times CD \quad (4)$$

Both the 3D plot in Figure 2c and the perturbation plot in Figure 3c demonstrated how changing the injection volume (D) can affect Y3. The Y3 was solely affected by the injection volume.

Effect of independent variables on peak area of PIP (Y_4)

There was a statistically significant relationship between the independent variables X3, X4, and Y4 ($p < 0.05$). An increase in X4 and a decrease in X3 both increase the Y4, as shown by the interaction equation generated from ANOVA (Eq. 5). The interaction between components X3 and X4 was also proven to be significant. There is no statistically significant relationship between X1, X2, and Y4.

$$Y4 = +23742.6 - 530.806 \times A + 76.4722 \times B - 1961.06 \times C + 8388.39 \times D + 474.25 \times AB - 2435.5 \times AC + 224.5 \times AD - 54.75 \times BC + 403.583 \times BD - 1105.42 \times CD \quad (5)$$

Figures 2d and 3d indicated that adjusting the injection volume (D) greatly affected the Y4. It was discovered that increasing the injection volume led to a larger Y4.

Effect of independent variables on resolution (Y_5)

Response Y5 was the most sensitive to changes in the independent variables among all the others considered in this research work. Resolution is defined as the temporal gap between two adjacent peaks during elution. Responses Y1 and Y2 would show the effect on the response Y5. The impact of the independent variables on the dependent variable Y5 was shown by the quadratic equation (Eq. 6) produced by the ANOVA analysis. The independent variables X1 and X2 exhibited significant reactions in the region of Y5 ($p < 0.05$). Increases in both X2 and X1 result in sharper separation of peaks.

$$Y5 = +14.1589 - 1.35028 \times A + 1.05058 \times B + 0.0322778 \times C - 0.193028 \times D + 0.28225 \times AB - 0.931833 \times AC - 0.07725 \times AD - 0.169083 \times BC - 0.03225 \times BD - 0.0445833 \times CD \quad (6)$$

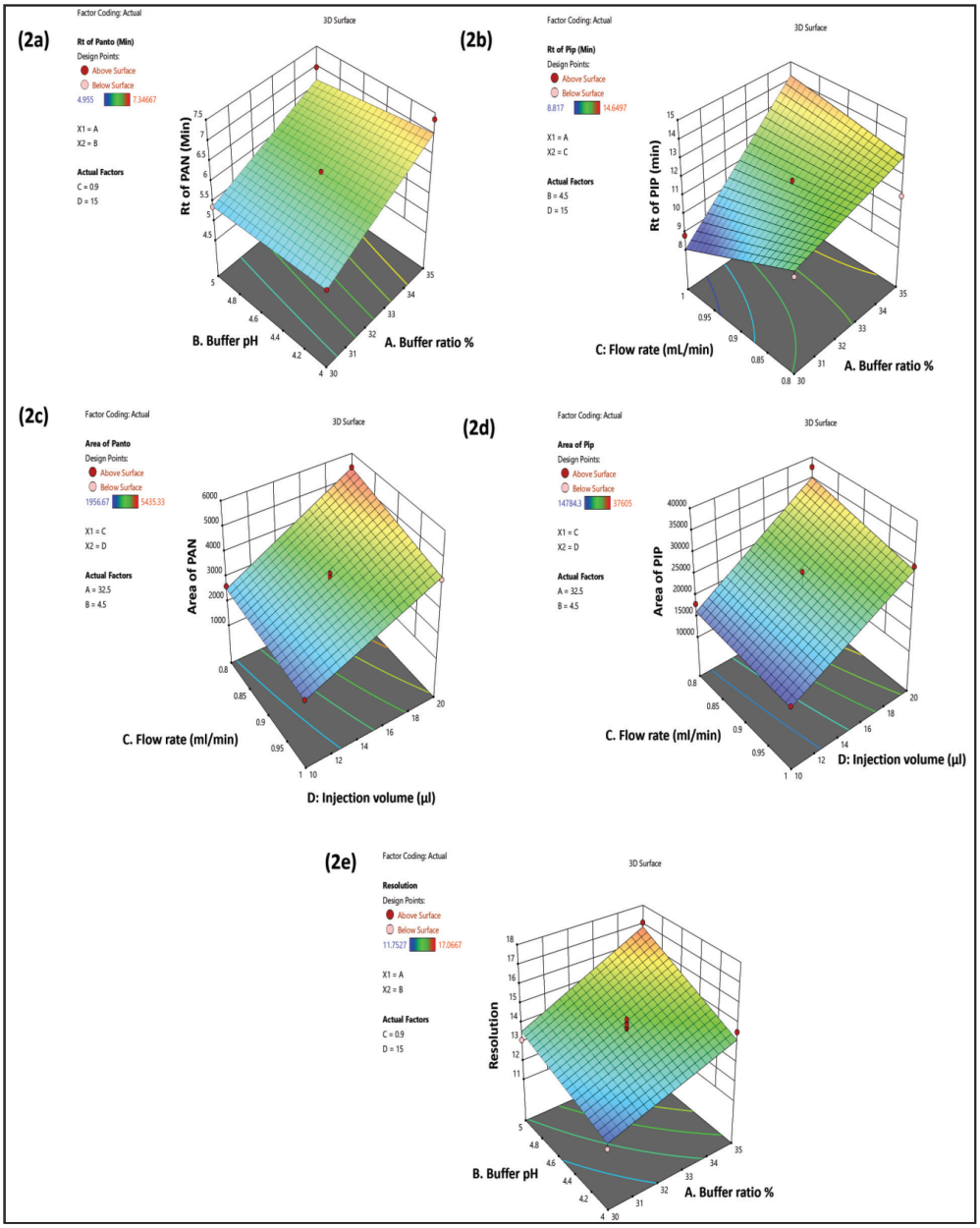

Figure 2. The 3D surface response plot showing the effect of independent variables: (a) on the retention.

Changing the X1 and X2 considerably affected the resolution, as seen in the 3D (Fig. 2e) and the perturbation plot (Fig. 3e). Higher X1 and X2 were shown to improve Y1 (Fig. 2a); Y2 (Fig. 2b); Y3 (Fig. 2c); Y4 (Fig. 2d) and Y5 (Fig. 2e).

Desirability

A desirability plot (Fig. 4) was created using the results of the ANOVA, and the conditions of the independent variables with a desirability of 0.991 were selected as the best-suggested method by the software. As a result, the following parameters have been agreed upon. Phenomenex HyperClone C_{18} column (120 Å × 250 × 4.6 mm, 5 m particle size), Isocratic elution using a mobile phase consisting of ammonium acetate and methanol (30:70) at a pH of 4.5, the column oven temperature is set at 25°C, the injection volume is 15 µl, and flow rate of 0.9 ml/minute. Under these circumstances, chromatographic runs (n = 6) were performed using a Shimadzu quaternary HPLC system equipped with an autosampler and a PDA detector at 288 nm (PAN) and 341 nm (PIP) wavelengths.

After comparing observed values for all the responses with their anticipated values from the regression model, it was determined that the relative error for all replies was less than 10%. Figure 4 shows the chromatogram produced under the ideal experimental conditions.

Method validation

Parameters including retention time, tailing factor, resolution, and theoretical plates were calculated to evaluate the

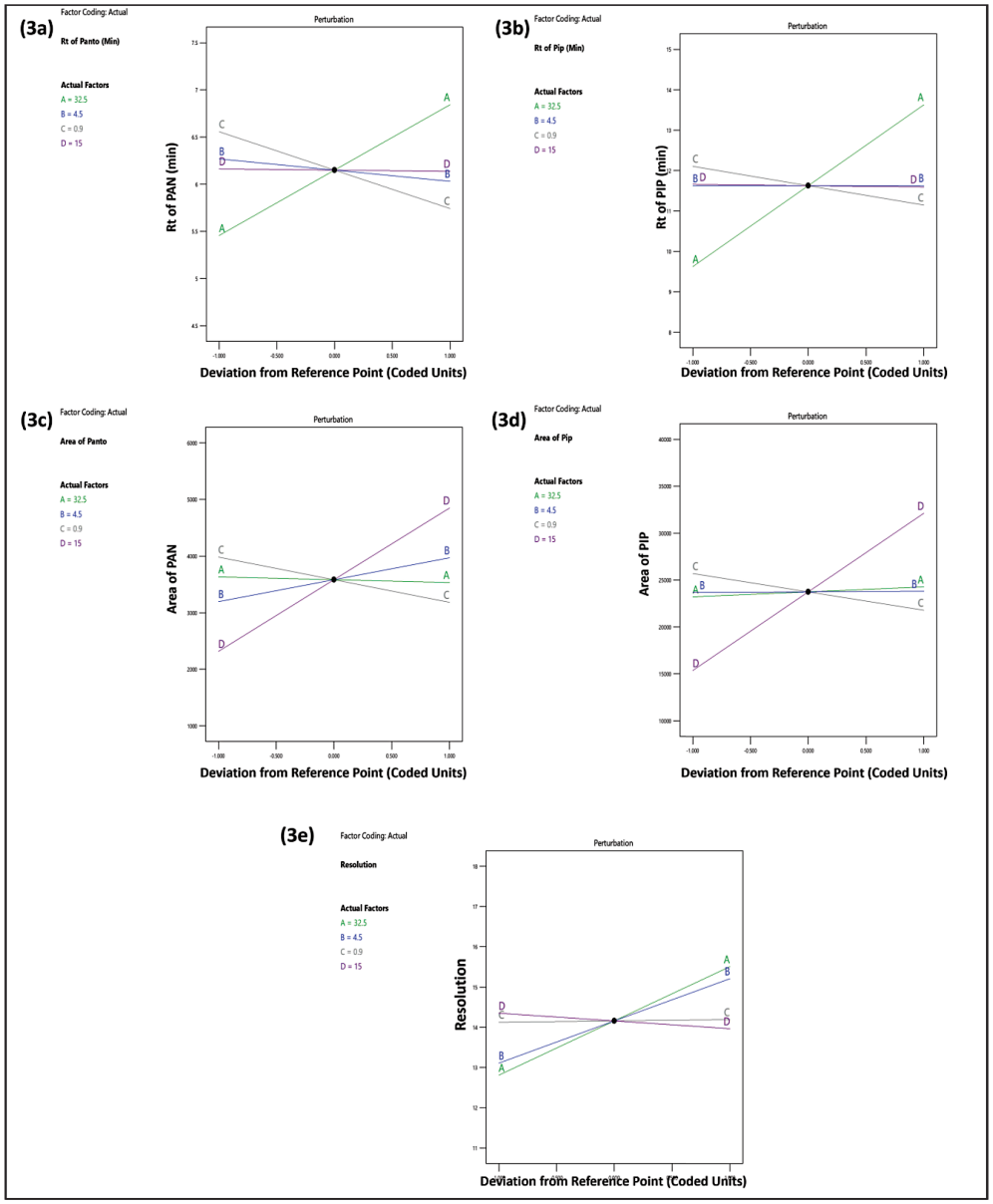

Figure 3. Perturbation plot depicting the interactions of independent variables. (a) Retention time of PAN; Y1; (b) retention time of PIP; Y2; (c) peak area of PAN (Y3); (d) peak area of PIP (Y4), and (e) resolution (Y5).

suitability of the system (refer to Table 4). The peaks of both drugs were clearly defined, as evidenced by their resolution of 14.058 ± 0.076. Table 4 presents the satisfactory results obtained from the assessed system suitability parameters.

The drug-loaded formulation (Fig. 5C) and placebo formulation (Fig. 5B) chromatograms revealed that excipients including sodium alginate, chitosan, and EUDRAGIT® L 30 D-55 did not affect the retention time of the drugs. This demonstrated the specificity of the method utilized to simultaneously quantify PAN and PIP from the prepared mucoadhesive formulations.

PAN and PIP were found to be linear in concentration from 0.5 to 20 µg/ml, with R^2 values of 0.999 and 0.999, respectively. The developed method was well-suited for the assessment of mucoadhesive formulations. For PAN and PIP, the linear equations corresponding to the calibration curve were $Y = 32{,}744x - 683.21$ and $Y = 130{,}540x - 957.44$, respectively.

The accuracy of both drugs was determined simultaneously through the measurement of percentage recoveries at three distinct concentrations (80%, 100%, and 120%). Both PAN and PIP exhibited recovery rates within the acceptable range of 90%–110%. This demonstrates that the concurrently analyzed method is suitable for the precise estimation of both substances. The accuracy of the method was assessed for both intra-day and inter-day fluctuations in PAN and PIP. The relative standard deviation (%RSD) for both intra-day and inter-day method precision was estimated to be below 2.0%. It LOD and LOQ for PAN were found to be 65 and 90 ng/ml, whereas for PIP they were 25 and 41 ng/ml, respectively (Table 4). The LOD (Eq. 7) and LOQ (Eq. 8) were calculated using the following

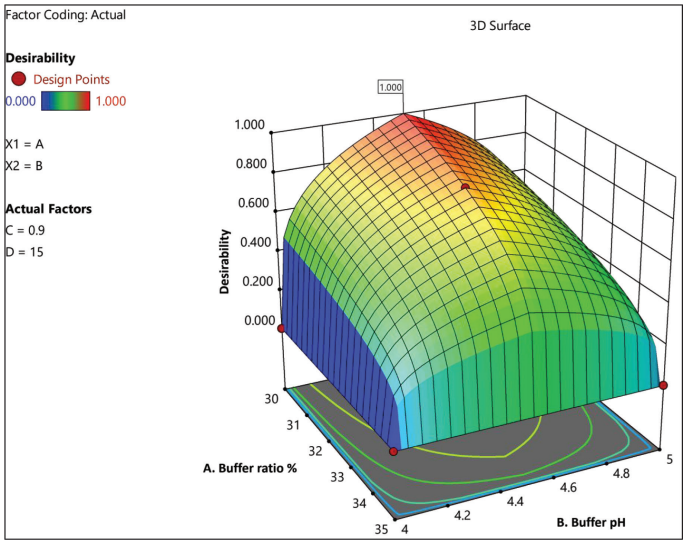

Figure 4. The 3D surface response desirability plot for the optimized analytical method.

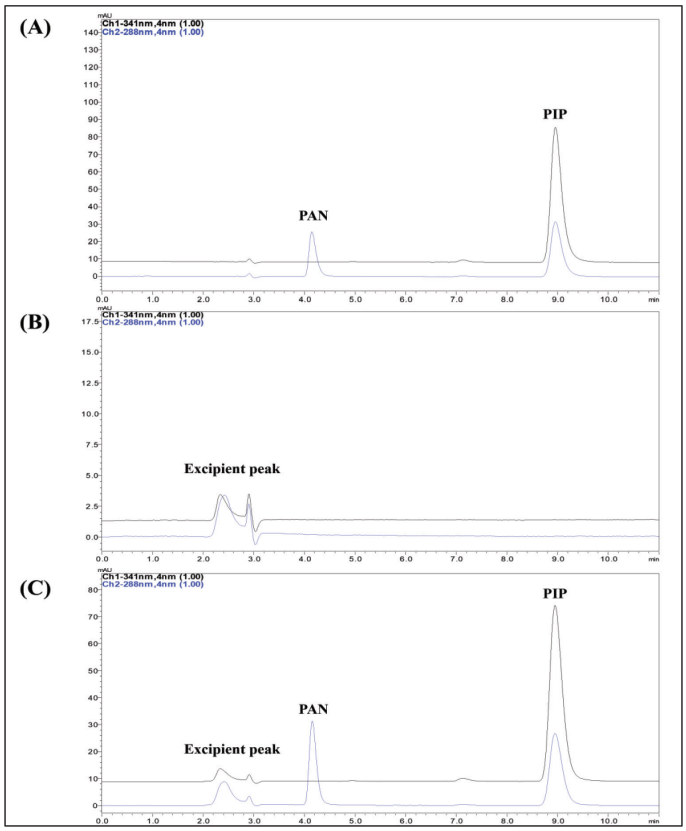

Figure 5. Chromatogram obtained at optimized conditions. (A) Chromatogram of PAN sodium and PIP. (B) Chromatogram of blank formulation. (C) Chromatogram of formulation containing PAN and PIP.

formulas, where σ refers to the SD of the response, and s refers to the slope, which is obtained from the calibration plot.

$$LOD = \frac{3.3\ \sigma}{s} \quad (7)$$

$$LOQ = \frac{10\ \sigma}{s} \quad (8)$$

The impact of modifying the buffer pH, column temperature, flow rate, injection volume, and maximum absorbance on the experiment was thoroughly examined. Notably, the retention time (T_R), peak area, tailing factor, and theoretical plates exhibited no significant variations in response to these minor adjustments. This underscores the robustness of the current analytical approach, ensuring its suitability for simultaneous testing of both medications. (Robustness data are provided as a supplementary Table S2).

The stability of 1 µg/ml stock solutions of PAN and PIP in triplicate at bench top (room temperature) was evaluated for up to 24 hours [31]. We also evaluated the stability of the autosampler at 15°C for a period of 24 hours. The percentage shift from the baseline concentration was computed. Notably, the drug recovery from the stability solutions remained within the acceptable range of 100%–105% even after 24 hours, with a RSD of under 2% (Table 4).

Forced degradation study results

A forced degradation study was conducted to assess the resistance of PAN and PIP to the formulation development environment. As a result, a comprehensive analysis of degradation was conducted. The degradation corresponding to different stress conditions is illustrated in Figure 6. The degradation process was carried out on over 87% of the PAN and 25% of the PIP under severe acidic conditions such as 1 M HCl. In contrast, degradation of PAN exceeded 80% and that of PIP was 27% under less acidic conditions such as 0.1 M HCl. PAN exhibited signs of degradation when exposed to alkaline conditions. However, the degradation of PAN and PIP at alkaline conditions was 75.71% and 16.15% at 0.1 M NaOH. The degradation of PAN and PIP on 1 M NaOH was 84.13% and 16.97%, respectively. Degradation was more than 97% for PAN and more than 30% for PIP the oxidation condition with H_2O_2. Degradation was less than 10% for both drugs at forced temperature and sunlight (UV) conditions.

Results of DEE of mucoadhesive formulation using the validated method

Formulation parameters, such as DEE, play a crucial role in various drug delivery systems, including liposomes, nanoparticles, microspheres, and other carriers like mucoadhesive beads. DEE gauges the effectiveness of a formulation in safeguarding the medication within its carrier. Entrapment efficiency, expressed as a percentage, measures the portion of the active ingredient in the formulation successfully incorporated into the carrier. DEE holds importance due to its direct impact on the effectiveness and productivity of the distribution network. Entrapment efficiency is influenced by various crucial formulation factors, including the polymer concentration, drug-to-polymer ratio, polymer solubility in the organic phase, dispersion-to-continuous phase ratio, drug-polymer interactions, and the drug's solubility in the continuous phase. The PAN and PIP concentrations within the mucoadhesive formulation were determined through the established HPLC

Table 3. ANOVA results of Box-Benhken design.

Response	T_R of PAN (Y1)		T_R of PIP (Y2)		Peak area of PAN (Y3)		Peak area of PIP (Y4)		Resolution (Y5)	
F-value	16.65		6.40		51.76		13.13		7.69	
p-value	Model	<0.0001	Model	0.0004	Model	<0.0001	Model	<0.0001	Model	<0.0001
	A	<0.0001	A	<0.0001	A	0.3301	A	0.4960	A	<0.0001
	B	0.2069	B	0.9820	B	<0.0001	B	0.9214	B	<0.0001
	C	0.0003	C	0.0921	C	<0.0001	C	0.0194	C	0.8655
	D	0.8888	D	0.9055	D	<0.0001	D	<0.0001	D	0.3177
	AB	0.7180	AB	0.9919	AB	0.0316	AB	0.7242	AB	0.3970
	AC	0.0284	AC	0.0304	AC	0.4666	AC	0.0822	AC	0.0103
	AD	0.9942	AD	0.9979	AD	0.5286	AD	0.8672	AD	0.8150
	BC	0.9330	BC	0.9973	BC	0.8813	BC	0.9674	BC	0.6096
	BD	0.9879	BD	0.9127	BD	0.0853	BD	0.7638	BD	0.9221
	CD	0.6963	CD	0.8785	CD	0.0506	CD	0.4144	CD	0.8925
R^2	0.8272		0.7805		0.9776		0.8800		0.8384	
Adjusted R^2	0.7312		0.6585		0.9651		0.8133		0.7486	

A represents the buffer ratio, B represents the buffer pH, C represents the injection volume, and D represents the flow rate. AB denotes a combination of buffer concentration and injection volume, AC denotes a combination of buffer concentration and flow rate, AD denotes a combination of buffer concentration and injection volume, BC denotes a combination of buffer pH and flow rate, BD denotes a combination of buffer pH and injection volume, and CD denotes a combination of flow.

Table 4. Validation data of the optimized analytical method.

Parameters	PAN	PIP
Retention time (min)	4.143 ± 0.010	8.969 ± 0.0.055
Tailing factor	1.601 ± 0.007	1.465 ± 0.011
Theoretical plate	3,658.593 ± 12.633	7,565.774 ± 26.039
Specificity	No interfering peaks at the T_R of PAN and PIP	
Resolution	14.058 ± 0.076	
Linearity range (µg/ml)	0.5–20	0.5–20
Regression equation	$Y = 32{,}744x - 683.21$	$Y = 130{,}540x - 957.44$
Correlation coefficient (r^2)	0.999	0.999
LOD (ng/ml)	65	25
LOQ (ng/ml)	90	41
	Accuracy (% Recovery)	
80%	97.315	99.739
100%	100.937	100.154
120%	101.404	102.696
	Precision (% CV)	
Repeatability	1.396	0.301
Inter-day	1.228	1.395
	Stability (% change)	
Bench-top	−1.34%	−1.65%
Autosampler	−0.65%	−1.39%

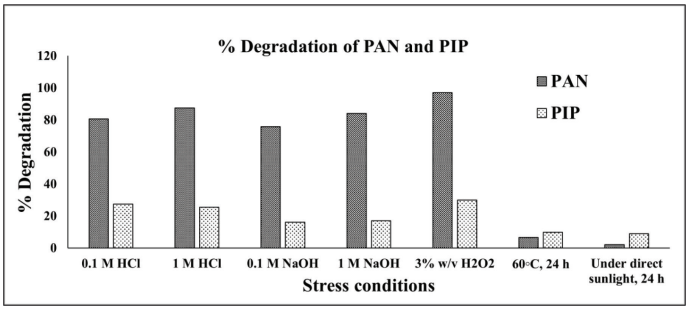

Figure 6. Column chart representation of forced degradation study results of PAN sodium and PIP showing the % degradation under each condition.

method and the % DEE was calculated using Equation (1). In preliminary experiments, lower drug entrapment levels were observed. This issue was resolved by optimizing the polymer-to-drug ratio to maximize entrapment. Ultimately, the final formulation exhibited an entrapment efficiency of 80%–85% for PAN and 60%–67% for PIP.

Greenness of analytical procedure

The environmental friendliness of the suggested analytical technique was assessed by analyzing 12 Green Analytical Procedure Index (GAPI) factors related to the sample, the reagents and compounds, and the equipment. Collection, storage, transportation, processing, extraction volume, solvent or reagent used, and supplementary reagents are all examples of independent variables. Chemicals and reagents came with their own set of risks, including the quantity of solvent/reagent needed and potential dangers to human health and safety. The energy, occupational risk, garbage, and garbage treatment

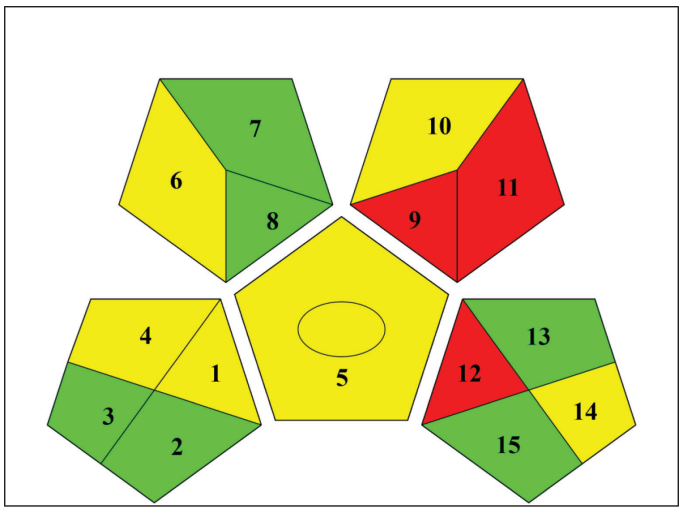

Figure 7. GAPI pictogram of proposed HPLC method for the estimation of PANand PIP from the GRDDS mucoadhesive formulation. The color red represents a high danger to the environment, while yellow and green symbolize lower danger and better greenness. Eight parameters relating to the sample (collection, preservation, transport, storage, types of processing method, scale of extraction, solvent/reagent used and additional reagent, and so on), three parameters relating to reagents and compounds (amount to solvent/reagent, health hazard, and safety hazard) and four parameters relating to instrumentation (energy, occupational hazard, waste, and waste treatment).

were considered [32]. Using the GAPI software, we created an illustration (Fig. 7) that conveys the method's friendliness to the environment. The ability to be used for both quantitative and qualitative objectives is represented by a circle in the center of a pictogram. The color red indicates a critical threat to the environment, whereas yellow and green indicate less of a threat and more greenness, respectively. As a whole, the approach was environmentally friendly, scoring six green, six yellow, and three red.

CONCLUSION

An effective RP-HPLC method was developed to simultaneously estimate PAN and PIP. A three-level Box-Behnken response surface configuration was implemented to optimize the analytical procedure. The linearity, sensitivity, accuracy, precision, and robustness were all verified in compliance with the ICH standards of the optimized analytical method. In addition, investigations into stress-induced degradation confirmed the sensitivity of the validated method to degradation products such as PAN and PIP. The method that had been validated was employed to quantify PAN and PIP using chitosan-based sodium alginate mucoadhesive particles. The components of the formulation exhibited no interaction with the retention periods of PAN and PIP. This method could be employed for the successful formulation development and analysis of these drugs.

LIST OF ABBREVIATIONS

PAN: Pantoprazole sodium; PIP: Piperine; *H. pylori: Helicobacter pylori;* GRDDS: Gastroretentive drug delivery system; ANOVA: Analysis of variance; BBD: Box-Behnken design; DoE: Design of experiments; OFAT: One factor at a time; HPLC: High performance liquid chromatography; ICH: International Conference on Harmonization; GAPI: Green Analytical Procedure Index.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

PUBLISHER'S NOTE

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