Optimization of a Novel High-Performance Thin-Layer Chromatographic Method for the Concurrent Estimation of Three Proton-Pump Inhibitors and Diclofenac in Their Binary Combinations

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Key Words

Normal-phase high-performance thin-layer chromatography
Mobile-phase optimization
Omeprazole
Pantoprazole
Rabeprazole
Diclofenac

Summary

In the present study, a simple and efficient high-performance thin-layer chromatographic (HPTLC) method was developed for the separation and quantitation of three proton-pump inhibitors, omeprazole, pantoprazole, and rabeprazole, from their binary combinations with diclofenac. Using a "quality by design" approach, preliminary trials were performed on pre-coated silica gel HPTLC plates using toluene together with various alcohols (methanol, ethanol, *iso*-propanol, *n*-butanol) as the mobile phase. For better peak symmetry, ammonia was added in different volumes, and its effect on analyte retention and separation was also assessed. The mobile phase consisting of toluene–*n*-butanol–25% ammonia (3:7:0.2, *v/v*) afforded excellent separation of proton-pump inhibitors from diclofenac as well as from each other. The retardation factor (R_F) for all the separated compounds was between 0.20 and 0.80. The developed method was successfully validated as per the International Conference on Harmonization (ICH) guidelines, and the selected drugs were determined simultaneously from dosage forms without any interference from the excipients.

1 Introduction

Proton-pump inhibitors (PPIs) such as pantoprazole (PAN), rabeprazole (RAB), omeprazole (OME), and lansoprazole are extensively prescribed for the treatment of acid-related disorders such as gastric and duodenal ulcers and other hypersecretory diseases. PPIs suppress gastric acid secretion in gastric parietal cells by selective and irreversible inhibition of the gastric H^+/K^+ATPase (the proton pump) that accomplishes the final step in acid secretion. All the available PPIs differ somewhat in their pharmacokinetic and pharmacodynamic properties, which are reflected in both speed and degree of gastric acid suppression [1]. Diclofenac (DIC, [o-(2,6-dichloroanilino)phenyl]acetate) is a non-steroidal anti-inflammatory drug (NSAID) and is extensively used owing to its analgesic, antipyretic, and anti-inflammatory properties. It is considered as one of the few first choice NSAIDs employed in dealing with painful and inflammatory conditions [2]. Among other side effects, those associated with the gastrointestinal (GI) tract are uncommon, but an extensive and uncontrolled usage of NSAIDs may cause an increased risk of gastric and duodenal ulcers. DIC is a non-selective inhibitor of cyclooxygenase enzyme, which also decreases prostaglandins in the epithelium of the stomach, making it more sensitive to corrosion by gastric acid. The possible threats of peptic ulcer on long-term use of DIC requires concomitant treatment with PPIs as they have been shown to be effective in preventing the development of gastric and duodenal ulcers in high-risk patients taking NSAIDs [3].

The literature reveals that among other PPIs only three drugs, i.e., OME, PAN, and RAB, are commercially available in fixed dose combination with diclofenac. The chemical structures of these drugs are shown in **Figure 1.** Only few methods have been reported for the simultaneous quantitation of DIC in combination with either RAB [4–9] or PAN [10] based on spectrophotometry [4–6, 10] and liquid chromatography [7–9]. On the contrary, up till now few methods have been proposed based on high-performance thin-layer chromatography (HPTLC) for

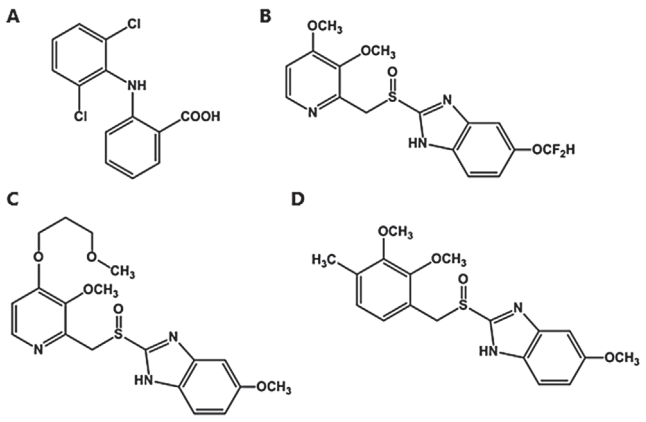

Figure 1

The chemical structures of (A) diclofenac, (B) pantoprazole, (C) rabeprazole, and (D) omeprazole.

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the analysis of PAN [11–12], RAB [13–14], and OME [15–19] either alone or in combination with other drugs; however, none of them was able to distinguish between the selected three PPIs. In addition, no efforts have been reported for the simultaneous quantitation of DIC and OME.

One of the other major advantages of thin-layer chromatography (TLC) is that it affords parallel analyses of multiple samples in a single run using small amounts of solvents as the mobile phase which reduces time and cost of analysis. The simplicity, ease of use, and flexibility in performing TLC analysis has made this technique quite attractive for pharmaceutical laboratories, especially those belonging to resource-limited countries. It also affords less consumption of organic solvents compared to high-performance liquid chromatography (HPLC) methods [14]. To the best of our knowledge, there are no published reports for the simultaneous determination of the selected drugs from their binary fixed-dose combinations using HPTLC. So, in the present study, we aimed to define optimum mobile phase and other HPTLC parameters for the separation of these drugs under normal-phase conditions, which can be further applied for their real sample analysis. The developed method was also validated according to International Conference on Harmonization (ICH) guidelines for linearity, sensitivity, selectivity, accuracy, precision, and robustness.

2 Experimental

2.1 Chemicals and Materials

Reference standard of diclofenac sodium (99.38%) was purchased from Titan Pharmaceuticals Ltd. (Mumbai, India), while omeprazole (98.71%), pantoprazole sodium sesquihydrate (99.55%), and rabeprazole sodium (98.84%) were obtained from Asutosh Pellets Ltd. (Gujarat, India). Toluene, methanol, ethanol (absolute), isopropyl alcohol, *n*-butanol, and ammonia (25%) used were of analytical grade (E. Merck, Mumbai, India). All other chemicals used were also of analytical grade (E. Merck, Mumbai, India). Aluminum-backed HPTLC plates 20 × 20 cm pre-coated with silica gel F_{254}, layer thickness 0.2 mm were procured from E. Merck KGaA (Darmstadt, Germany). The plates were washed with methanol and activated at 105°C for 20 min prior to analysis. Ten tablets of each marketed formulation for the proposed binary drug combinations viz., DIOPRA® (20 mg OME + 100 mg DIC, Cadila Pharmaceuticals Ltd., Gujarat, India), DUFEX® (20 mg PAN + 75 mg DIC, CFL Pharmaceuticals, Mumbai, India), and Dynapar PPI® (20 mg RAB + 100 mg DIC, Troikaa Pharmaceuticals Ltd., Gujarat, India) were purchased commercially from a local pharmacy store.

2.2 Instrumentation and Chromatographic Conditions

Weighing of samples was done on a Sartorius GD503 (Bradford, MA, USA) analytical balance having a readability of 0.0001 g. The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of a TLC Scanner 3, Linomat 5 auto-sprayer connected to a nitrogen cylinder, and a plate heater. Calibration standards of the proposed drug solutions and sample solutions of the marketed formulations were applied to the silica gel 60 F_{254} HPTLC plate (20 × 20 cm) by means of the Linomat 5 automatic band applicator equipped with a 100-µL Hamilton syringe and operated with settings of band length, 6 mm; distance between bands, 14 mm; distance from the plate side edge, 15 mm; and distance from the bottom of the plate, 8 mm. Once the spots were air dried, the plate was developed in a twin-trough chamber (CAMAG) previously saturated for a predetermined and optimized time with the selected mobile phase. Linear ascending method was carried out for plate development and the mobile phase was allowed to migrate a predetermined distance on the plate. The separated spots were then dried on a plate heater at 50°C. Densitometric determination of the analytes was carried out at 290 nm in the reflectance mode, using the deuterium lamp in the TLC Scanner 3; the slit dimensions were 5 mm length and 0.45 mm width, with a scanning rate of 20 mm s^{-1}. The winCATS software version 1.4.2 (CAMAG) was used to control the operating parameters through the entire analysis.

2.3 Method Development and Optimization

For planar chromatographic quantitation, two criteria must be fulfilled; adequate retention of all the analytes and good resolution ($0.2 \leq R_F \leq 0.8$) among the analyte bands. These can be very well achieved using established set of conditions, the most important ones being the type of sorbent and solvents, mobile phase composition, plate development, and mode of detection [20]. The most widely preferred and simplest form of sample application (spray-on) and plate development (ascending mode in a twin-trough chamber) were used in the present work. For sensitive recognition of the analytes, standard solutions of the analytes were prepared and scanned for their ultraviolet (UV) absorption within the range of 200–400 nm.

Mobile-phase optimization was carried out using the window diagramming approach [14]. Initially, four different aliphatic alcohols belonging to group II from Snyder's solvent classification were tried in different volume fractions in combination with toluene (group VII). TLC trials were performed and the corresponding R_F values of each compound were noted for different volume fractions of all four alcohols. Thereafter, *n*-butanol was further assessed for improved resolution upon addition of different amounts of ammonia. For each volume fraction, the effect of ammonia (within 0.0–0.5 mL) on the retention, resolution, and peak symmetry of the analytes was monitored. From the obtained results, minimum and maximum R_F value, and the resolution between the least separated pair of analytes, expressed as $(\Delta R_F)_{min}$, was evaluated against the added volume of ammonia–*n*-butanol volume fraction.

2.4 Method Validation Procedures

The working range of the developed method was evaluated by the analysis of seven standard concentrations ranging from 50 to 500 ng $spot^{-1}$ for PPIs and from 150 to 1500 ng $spot^{-1}$ for DIC. The reference standards were accurately weighed (i.e., 75.0 mg of DIC and 50.0 mg each of PAN, RAB, and OME) and transferred into separate 100-mL volumetric flasks. The powdered drugs were dissolved, mixed well, and diluted to volume with methanol. Separate series of standard solutions were prepared for PPIs and DIC within the concentration range of 10–100 µg mL^{-1} and 30–300 µg mL^{-1}, respectively. From these solutions, spots were applied on the plate representing each concentration starting with the lowest concentration to avoid carryover effect. The procedure was repeated in triplicates and the peak

area response of the separated bands was considered to establish the working range of the method [21].

During method development, solvent blank and simulated excipients were assessed for their interferences at the R_F values of the analytes. In addition, the area response of a sample representing the lowest concentration in the calibration range was obtained by three consecutive analyses, and the standard deviation (σ) of the measurement was calculated. A concentration representing 3 σ/slope was represented as limit of detection (LOD), while a concentration representing 10 σ/slope was designated as the limit of quantitation (LOQ) for each analyte.

Accuracy of the method is generally expressed as recovery (or sometimes bias) for the analysis of pre-analyzed sample solution spiked with standard solutions of the analyte. Recoveries were determined at 80%, 100%, and 120% of the expected samples concentration, in three replicates. From the area response values, the standard concentrations recovered were calculated and reported as % recovery at each concentration level for each drug. Intra-batch precision was studied by analyzing one spot three times at three afore-mentioned concentration levels. The inter-batch precision was done in a similar manner but by using two analysts on three different days. For the assessment of precision, the peak areas were obtained, and the percentage of relative standard deviation (% RSD) values were reported.

The variability in the peak area measurement and changes in the R_F values of the drugs was considered to study the robustness of the method upon deliberate changes in the mobile phase composition (±0.5%), the chamber saturation time, and the solvent migration distance. From the obtained results, % RSD values were calculated as an indication of method robustness.

2.5 Analysis of Real Samples

For the assessment of method performance, it was applied to resolve and quantitate the selected drugs from their binary fixed-dose formulations. For each binary combination, ten tablets were accurately weighed and grounded to a fine powder. A weight equivalent to 5.0 mg of OME/PAN/RAB and 25.0/18.75 mg respective amount of DIC was dissolved in about 50 mL methanol. The contents of each flask were sonicated for 2 min and were transferred to a standard 100-mL flask. The contents in the flask were diluted to the mark with the same solvent. Thereafter, 4 µL of the sample solutions were applied as a band on a pre-washed HPTLC plate, and the plate was developed under the pre-defined experimental conditions. From the obtained values of area response, the % accuracy and % RSD values were calculated as representatives of the method performance.

3 Results and Discussion

3.1 Method Development

To select the analytical wavelength for quantification of the drugs, the standard solutions were prepared representing 10 µg mL^{-1} and their UV absorption spectra were acquired. Upon assessment of their overlaid spectra, it was observed that all the selected drugs exhibited more or less traceable absorbance at 290 nm which was selected as the analytical wavelength for further analysis.

In the present study, aluminum-backed HPTLC plates 20 × 20 cm pre-coated with silica gel F_{254} were used. A brief literature survey of the reported methods suggested that the majority of the developed methods were based on non-polar solvents with low elution strengths in combination with intermediate-polar or polar solvents [22]. Usually, three solvents from different selectivity groups are tried in combination for optimization of the mobile phase; however, a more complex mobile-phase system results in problems due to solvent demixing and vapor-phase saturation. Hence, in the present work, method development was initiated with a binary solvent system consisting of toluene (group VII solvent) and four aliphatic alcohols (group II solvents) in different volume fractions based on Snyder's solvent classification system [23]. Although there are several reports which employ chloroform-based mobile phase, it was omitted due to its carcinogenic and hazardous nature [24–25]. A negligible migration of the analytes was observed when they were run on-plate using neat toluene. On the contrary, the selected alcohols gave more or less the same elution pattern for all the analytes. However, it was observed that the addition of alcohol to toluene affected the retention behavior of the analytes in a different manner. So in the subsequent phase of the study, toluene and alcohols were evaluated in different binary combinations for the effective separation of the analytes.

Mobile-phase selection was based on the same approach as proposed by *Shewiyo* et al. [26]. Instead of the tedious and laborious trial and error approach, a systematic mobile-phase optimization approach was followed for efficient resolution among the selected analytes. In doing so, the trials were carried out and the worst results were determined for each mobile phase composition, which forms the basis for selection of the optimal mobile phase. A number of experiments were carried out using different alcohol fractions ranging from 0 to 0.9, and the corresponding R_F values were noted for each fraction **(Figure 2).** Based on these findings, it was found that as the

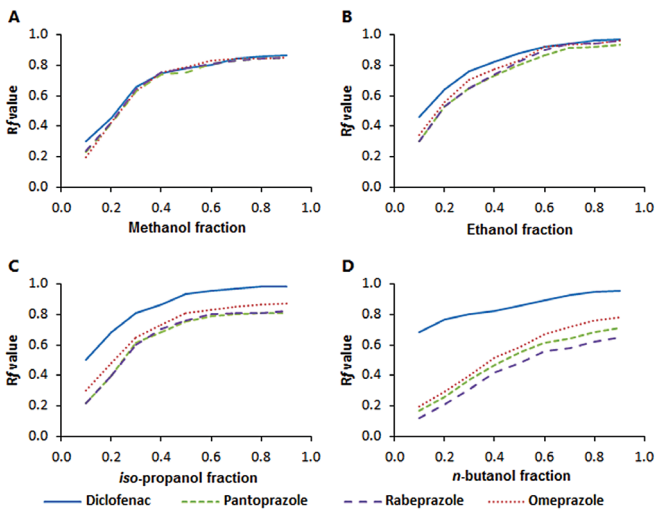

Figure 2

Variation in the retardation factors (R_F) of the selected drugs obtained using solvent mixtures of toluene with varying fractions of (A) methanol, (B) ethanol, (C) *iso*-propanol, and (D) *n*-butanol.

polarity of the alcohol increases, the resolution between the analytes becomes poor. At higher volume fractions, the R_F values were greater than 0.80 for all the alcohols. In addition, none of the solvent systems strictly fulfilled the criterion of R_F values between 0.20 and 0.80 region for the analytes, which is considered as the most reproducible region. Methanol and ethanol, in every volume fraction with toluene, showed a similar elution pattern for the analytes. Moreover, there was a gradual increase in the migration distance with increase in alcohol fractions. On the other hand, *iso*-propanol and *n*-butanol gave encouraging results with regards to differential movement of the analytes. This may be attributed to the periodic selectivity parameters of solvents belonging to group II of Snyder's classification system [23]. Although the *n*-butanol–toluene combination resulted in little diffusion of all the analyte peaks (at all volume fractions) and trivial retention of DIC (R_F ~ 0.90, at higher volume fractions), it was selected for further optimization as it provided the best resolution amongst the selected alcohols.

In the next phase of optimization, ammonia and acetic acid were added as mobile-phase additives to judge their effects on the retention of the analytes. It was found that the addition of glacial acetic acid, even in small amounts (0.1 mL), caused extensive retention/negligible elution of the PPIs with almost similar R_F values (<0.10). Probably, the acidic conditions of the mobile phase led to enhanced interaction of the ionized analytes with the unreacted silanol groups of the silica stationary phase. Conversely, ammonia resulted in better peak shape and symmetry for all the analytes at amount ≤0.5 mL, and also gave acceptable elution of the analytes within the recommended range of R_F 0.20–0.80. To define an optimum mobile-phase composition in the presence of ammonia, *n*-butanol was assessed at various volume fractions (0.50, 0.60, 0.70, 0.80, and 0.90) with varying amounts of ammonia ranging from 0.1 to 0.5 mL. The corresponding R_F values were obtained, and the absolute differences in the R_F values of closely eluting analytes (ΔR_F) were measured. From these results, the worst separation (having the least ΔR_F) was noted for all the solvent combinations [26]. For a comparative evaluation, the obtained highest and lowest R_F values and the calculated $(\Delta R_F)_{min}$ were graphically presented against the volume of ammonia/volume fraction of *n*-butanol **(Figure 3).**

The results obtained without the addition of ammonia in different toluene–*n*-butanol solvent systems (1:9, 2:8, 3:7, 4:6, and 5:5, *v/v*) gave much higher R_F values for DIC (0.78–0.90), which meant a higher risk of unreliable peak areas and erroneous quantitation due to interference by the solvent front. On the contrary, the addition of ammonia (0.1–0.5 mL) to these solvent systems led to increased retention of DIC (R_F 0.15–0.43) and PAN (R_F 0.29–0.45) compared to OME (R_F 0.65–0.69) and RAB (R_F 0.54–0.64). The separation and distribution at amounts higher than 0.5 mL ammonia led to the enhanced retention of DIC with R_F values below 0.20. This could be due to the presence of $-COO^-$ group which can be readily ionized in basic media resulting in enhanced interaction with the polar silica stationary phase.

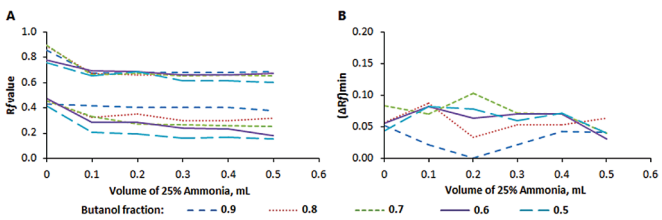

Figure 3

Chromatographic behavior of the four analytes with different mobile phases consisting of different *n*-butanol fractions with varying amounts of 25% ammonia. (A) represents minimum and maximum R_F values, while (B) shows minimum resolution between adjacent pair of analytes $(\Delta R_F)_{min}$.

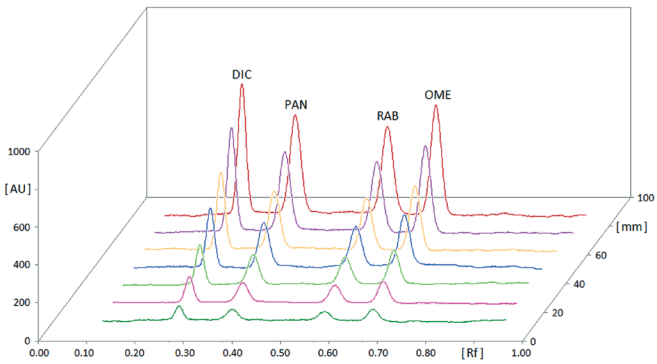

Figure 4

Overlaid HPTLC chromatograms for calibration curve concentrations of selected drugs. R_F values: diclofenac, 0.27; pantoprazole, 0.38; rabeprazole, 0.57; omeprazole, 0.67. Mobile phase: toluene–*n*-butanol–25% ammonia (3:7:0.2, *v/v*). Detection wavelength: 290 nm.

As the amount of ammonia was varied within from 0.1 to 0.5 mL, the minimum $(R_F)_{min}$ values were found to vary in a randomized manner at lower alcohol volume fractions, while the maximum R_F values were acceptable and practically unaltered for all the volume fractions of *n*-butanol (Figure 3A). From Figure 3B, it can be seen that the optimum results were obtained using the solvent systems consisting of toluene–*n*-butanol–25% ammonia in 3:7:0.2 and 2:8:0.1 (*v/v*) fractions as they both fulfill the criterion of $0.20 \leq R_F \leq 0.80$. Further, the worst resolutions between the adjacent eluting analytes (OME and RAB) were 0.10 and 0.09, respectively, being adequate for their on-plate separation. However, the mobile phase toluene–*n*-butanol–25% ammonia (3:7:0.2, *v/v*) afforded better peak symmetries for the analyte and hence a greater resolution among the analytes. As this mobile phase system provided sufficient retention and resolution between the analytes as shown in **Figure 4,** no further experiments were performed to determine a better mobile phase containing a three-solvent combination. In addition, adequate peak shapes and symmetries were found when the plates were pre-washed with methanol and activated at 105°C for 5 min by pre-saturation of the developing chambers for 30 min for better reproducibility of the R_F values.

3.2 Method Validation

Based on the peak-area results, two regression models were proposed based on linear and polynomial relationship. However, the linearity curves were found more or less pseudo-linear within the concentration range, perhaps due to the diffuse reflectance mode used for detection which never follows Beer–

Table 1

Linearity and regression parameters of the developed HPTLC method.

Parameters	Diclofenac	Pantoprazole	Rabeprazole	Omeprazole
R_F value	0.27	0.38	0.57	0.67
Working range (ng spot^{-1})	150–1500	50–500	50–500	50–500
Polynomial regression equation	$y = -0.0024x^2 + 13.0231x + 52.9179$	$y = -0.0097x^2 + 20.5386x + 13.5384$	$y = -0.0023x^2 + 15.8987x + 47.3140$	$y = -0.0089x^2 + 22.5157x - 21.8764$
Coefficient of variance (r^2)	0.9998	0.9994	0.9994	0.9991
Limit of detection (ng spot^{-1})	24.60	9.02	12.97	7.08
Limit of quantitation (ng spot^{-1})	82.00	30.07	43.24	23.59
% Recovery (mean ± SD)	100.02 ± 0.91[a]/99.60 ± ± 1.21[b]	100.10 ± 0.67[c]	100.36 ± 1.21[c]	99.64 ± 1.27[c]
Precision (% RSD)				
Intra-batch (n = 3)	0.61–1.87[a]/0.43–1.37[b]	1.16–1.43[c]	0.75–1.51[c]	0.81–1.72[c]
Inter-batch (n = 9)	0.45–1.41[a]/0.51–1.39[b]	0.60–1.68[c]	0.98–1.69[c]	0.92–1.43[c]
Robustness (% RSD)	0.76–1.29[a]/0.81–1.61[b]	1.22–1.50[c]	0.82–2.17[c]	1.63–2.18[c]

[a]Values determined at respective 80%, 100%, and 120% for 750 ng spot^{-1}
[b]Values determined at respective 80%, 100%, and 120% for 1000 ng spot^{-1}
[c]Values determined at respective 80%, 100%, and 120% for 200 ng spot^{-1}
SD, standard deviation; RSD, relative standard deviation

Lambert's law as linear functions [27]. Polynomial regression was found more superior as it offered the best fit, better correlation, and minimal residuals compared to straight-line regression; the results are shown in **Table 1.**

According to the ICH guidelines, the determination of LOD and LOQ is generally not necessary for the assay methods; however, this may provide an additional performance criterion of the developed method. In the present investigation, LOD

Table 2

Accuracy and robustness testing of the developed HPTLC method.

Analyte	Accuracy (n = 3)			Robustness testing (presented as % RSD values) (n = 5)				
	Amount of drug spotted (ng spot^{-1})	Mean amount of drug found (ng spot^{-1} ± SD)	Mean recovery (%)	Amount of drug spotted (ng spot^{-1})	Mobile phase composition (±0.5%)	Mobile phase volume (±0.5 mL)	Chamber saturation time (±2.5 min)	Migration distance (±3.0 mm)
	600	605.0 ± 6.38	100.84					
Diclofenac	750	741.6 ± 5.39	98.88	750	1.29	0.76	1.13	1.20
	900	903.0 ± 8.53	100.33					
	800	793.7 ± 7.05	9.21					
Diclofenac	1000	1006.6 ± 14.42	100.66	1000	1.25	0.81	0.89	1.61
	1200	1205.5 ± 11.69	100.46					
	160	160.4 ± 0.52	100.25					
Pantoprazole	200	201.4 ± 1.80	100.71	200	1.33	1.50	1.38	1.22
	240	238.4 ± 1.87	99.34					
	160	161.82 ± 2.36	101.14					
Rabeprazole	200	200.84 ± 2.49	100.18	200	1.75	0.82	1.22	2.17
	240	239.42 ± 2.15	99.76					
	160	160.31 ± 2.55	100.19					
Omeprazole	200	198.27 ± 2.84	99.13	200	1.65	1.63	2.18	1.70
	240	239.06 ± 1.89	99.61					

SD, standard deviation; RSD, relative standard deviation

and LOQ were determined based on the standard deviation of the area measurement, which was calculated by triplicate measurement of the lowest calibration standard. LOD/LOQ values were calculated as being 24.60/82.00, 9.02/30.07, 12.97/43.24, and 7.08/23.59 ng spot^{-1} for DIC, PAN, RAB, and OME, respectively. The calculated precision (% RSD) values were constantly less than 2%, ensuring excellent repeatability of the proposed HPTLC method (Table 1). Based on the chromatograms obtained for the blank samples, it was found that the method was selective for analysis of the selected binary drug combinations since there was no interference from the solvents and commonly used excipients.

The accuracy was found within the acceptable limits of 98.88%–101.14% for all the drugs at three concentration levels, according to ICH guidelines **(Table 2).** The results of robustness study showed % RSD values less than 3% in the measurement of peak area by inducing deliberate changes in the mobile-phase composition, the chamber saturation time, and the solvent migration distance (Table 2). Furthermore, there was a negligible change in the R_F values (±0.01) of the drugs under the studied conditions. This proved the suitability of the developed method for routine analysis of the selected drugs.

3.3 Analysis of Real Sample

The usefulness of the method was demonstrated by the analysis of real pharmaceutical samples. The chromatogram indicated that the R_F values obtained were 0.27, 0.38, 0.57, and 0.67 for DIC, PAN, RAB, and OME, respectively. The drug amounts present in the fixed dose combinations were calculated in triplicates, and these recovered amounts were compared with the claimed amounts of the individual drugs. The results of the assay **(Table 3)** indicated that the method is selective for the analysis of the chosen drugs and is free from interference from the excipients present in the formulations. In addition, the low % RSD values (less than 2%) indicated the suitability of this method for routine quality assessment of these drugs in their fixed dose combinations.

Table 3

Analysis of marketed samples of the selected binary combinations using the developed HPTLC method (*n* = 5).

Drug component	Labeled content (mg)	Mean observed content (mg ± SD)	Mean recovery (%)	RSD (%)
DUFEX®				
Diclofenac	75.0	74.61 ± 0.44	99.48	0.585
Pantoprazole	20.0	19.86 ± 0.17	99.30	0.866
Dynapar PPI®				
Diclofenac	100.0	100.29 ± 0.79	100.29	0.789
Rabeprazole	20.0	20.06 ± 0.29	100.32	1.420
DIOPRA®				
Diclofenac	100.0	99.60 ± 0.89	99.60	0.899
Omeprazole	20.0	19.79 ± 0.24	98.94	1.225

SD, standard deviation; RSD, relative standard deviation

4 Conclusion

The present study describes a systematic development of a simple and reliable HPTLC method for the separation and quantitation of DIC in binary combinations with OME, PAN, and RAB. A suitable combination of *n*-butanol and ammonia together with toluene afforded adequate retention and resolution (R_F) between the analytes, within an optimum range of 0.20–0.80. The use of ammonia facilitated the resolution of the spots as well as attaining symmetric shapes. The method was thoroughly assessed for validation parameters such as linearity, sensitivity, selectivity, accuracy, precision, and robustness. The proposed method can be readily applied for analyzing any commercially available formulation of these drugs in combination without interference from the commonly used excipients.

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