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HPTLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF NAPROXEN AND POMEGRANATE PEEL EXTRACT IN COMBINED DOSAGE FORM

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Running title: HPTLC method development for drugs

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ABSTRACT

Background: The cost-effective, simple and accurate method for simultaneous estimation of drugs is high-performance thin-layer chromatography (HPTLC). In this research, Naproxen and Pomegranate Peel Extract (PPE) was measured simultaneously by the HPTLC method.

Methods: The stationary phase was Precoated silica gel G60 F_{254} aluminium sheets (10 x 10 cm) while mobile phase was ethyl acetate: toluene: methanol(4:5.5:0.5 v/v/v). The chromatographic analysis was performed in reflection or absorbance mode at 240 nm. The validation of the developed method included linearity, the limit of detection, the limit of quantification, accuracy, precision, specificity and robustness as per ICH Q2 (R1) guidelines.

Results: The Rf value of Naproxen was 0.52 ± 0.01 and for Pomegranate Peel Extract was 0.83 ± 0.01. The linear regression analysis of both drugs showed a linear relationship between 200-1000 ng/band. The regression equation of Naproxen was y = 3.5847x + 253.22 with 0.9953 correlation coefficient, while regression equation of PPE was y = 3.5371x - 124.28 with 0.9928 correlation coefficient.

Conclusion: The method was accurate and precise, which can further be used to estimate the drugs simultaneously.

Keywords: Pomegranate Peel Extract, Naproxen, high-performance thin-layer chromatography, simultaneous estimation, validation.

INTRODUCTION

Osteoarthritis (O.A.) is a slow, occurring deteriorating disease. The damage to the joint becomes noticeable after many years of its occurrence(Sinusas, 2012). Osteoarthritis is a mechanical disease due to the wear and tear of cartilage upon aging. It affects more than 500 million people worldwide(Hunter et al., 2020), according to a recent survey in 2020, and the number is continuously increasing. The seven-percentage global population is suffering from O.A.

Naproxen is a nonsteroidal anti-inflammatory drug (NSAIDs) exhibiting anti-inflammatory, antipyretic and analgesic actions(Johnston, 1997). It is a propionic acid derivative, which decreases the formation of precursors of prostaglandins and thromboxane by inhibiting the enzymes cyclooxygenase I and II(Ong et al., 2007). It also inhibits platelet aggregation by reducing the formation of thromboxane A2 synthesis(Wo & Fossel, 2017).

Pomegranate is a traditional medicine for the treatment and prevention of OA(Mastrogiovanni et al., 2019). It is used for the treatment of various ailments apart from O.A. It gives an anti-inflammatory effect by protecting chondrocytes against Interleukin-1 (IL-1), which encourage cartilage damage. Pomegranate peel extract (PPE) stimulate type I procollagen synthesis and reduce the production of matrix metalloproteinase 1 (MMP-1) by dermal fibroblasts, which is responsible for damage to cartilage and the repairment cycle in OA(Ismail et al., 2012).

The combination of Naproxen and PPE was proposed for the treatment of osteoarthritis. The mixture is novel and has not been explored previously. The simultaneous estimation of a newer combination was performed by the high-performance thin-layer chromatography (HPTLC) method. HPTLC is an advanced thin layer chromatography with maximum separation efficiency of chromatographic layers(Priya et al., 2013). This method involves accurate and reproducible qualitative and quantitative evaluation of drugs(Bhusari & Dhaneshwar, 2012). The stationary phase has HPTLC plates with small particles and narrow size distribution, which provides better-quality resolution and higher detection sensitivity(Patel et al., 2011).

The simple, precise and accurate method was developed to estimate anti-arthritic drugs, Naproxen and PPE simultaneously. The chromatographic separation was performed on Precoated silica gel G60 F_{254} aluminium sheets (10 x 10 cm).

MATERIALS AND METHODS

The Pharmacopoeial grade API, excipients, and analytical grade chemicals were either procured as a gift sample or purchased from authorized vendors. In addition, distilled water was used throughout the study. Barroque Pharmaceutical Ltd. Khambhat gave Naproxen as a gift sample, and Pharmanza Herbals Ltd. Dharmaj gifted pomegranate peel extract. Ethanol, Propylene glycol, Glacial acetic acid, Methanol and Chloroform was purchased from Astron chemicals, Ahmedabad, India. Ethyl acetate, Toluene and Hexane was purchased from Chemdyes corporation, Rajkot, India

Analytical method development for estimation of drugs by HPTLC method(Alam et al., 2019; Devi et al., 2016; Guermouche et al., 2000; Jain et al., 2011; Mistry et al., 2015; Parys et al., 2020)

A. Stock solution preparation of drugs

Accurately weighed 100 mg of Pomegranate peel extract and Naproxen in 100 ml ethanol were dissolved in 100ml volumetric flasks. The obtained 1000 µg/ml stock solutions were further diluted with ethanol to get working standard solutions of drugs(Mistry et al., 2015).

B. Method of preparation of Vanillin Sulphuric Acid reagent

5 g vanillin and 5 ml concentrated Sulphuric acid were mixed, followed by dilution up to 100 ml ethanol in a 100 ml volumetric flask(Hajmalek et al., 2016).

C. Method Development(Jain et al., 2011; Mistry et al., 2015; Parys et al., 2020)

Optimization of the mobile phase was initiated by selecting various solvents like methanol, chloroform, ethyl acetate, toluene, glacial acetic acid and Hexane. Different ratios of selected solvents were placed in the development chamber before 20 minutes to each run for saturation. Prepared drug solutions (10 µl) were applied on HPTLC plates as bands of 6 mm using Linomat V. application position at 15 mm from sides and 8 mm from the bottom of the plate. The ascending technique developed plates at 8 cm migration distance and then dried them in an oven(Sethy et al., 2019). Dipping plates in vanillin sulphuric acid reagent was followed by drying in an oven at 120°C ± 2°C. Densitometric scanning in absorbance reflection mode at 240 nm using a deuterium lamp was done at slit dimensions of 6 mm × 0.30 mm, scanning speed at 20 mm/sec and data resolution at 100 µm/step. The optimum separation between spots (Resolution factor, Rs≥1.5) and migration of spots with Rf value between 0.2 to 0.8 were evaluated to achieve separation reproducibility.

Table 1 depicts chromatographic conditions used during HPTLC analysis.

Table 1 Chromatographic specifications

Sr. No	Parameters	Chromatographic specifications
1	Stationary phase	Precoated silica gel G60 F_{254} aluminium sheets (10 x 10 cm)
2	Chamber saturation time	25 minutes
3	Temperature	Room temperature
4	Migration distance	80 mm
5	Slit dimension	6.0 × 0.3 mm
6	Band Width	6 mm
7	Development Technique	Ascending
8	Plate drying	2-3 min
9	Visualizing reagent	Vanillin sulphuric acid reagent
10	Visualizing mode	Dipping method
12	Drying mode, temperature and time	120°C ± 2°C for 1-2 min

D. Selection of detection wavelength (Mistry et al., 2015)

The prepared standard drug solutions were applied on the plate in the form of bands one after another. After chromatographic development, the plate was viewed in the U.V. chamber. As the marker compounds cannot be considered at a standard wavelength, a spraying reagent was used to derivatize the plate and make it visualized(Attia et al., 2017). The plate was dipped in a solution of vanillin sulphuric acid (detecting agent) and heated at 85°C for 1-2 min. Spectra were recorded at a scan speed of 80 nm/sec over 400-800 nm. A single wavelength showing maximum absorbance was selected as a detection wavelength for the HPTLC method.

E. Method Validation

Various parameters like linearity, the limit of detection, the limit of quantification, precision, accuracy, specificity, and robustness were performed to validate the method according to ICH Q2 (R1) guidelines(Zakeri-Milani et al., 2014).

F. Linearity(Alam et al., 2019; Mistry et al., 2015; Parys et al., 2020)

In this method, linear relationships between peak area and concentration of both drugs in ng/band were measured. Total five replicate measurements in the concentration range of 200-1000 ng/band for both drugs were performed. Calibration curves in Microsoft excel were constructed by plotting the area of the band's peak versus the concentration of drugs and treated by ordinary least square regression analysis(Sivasubramanian, 2017).

G. Limit of detection (LOD) and Limit of quantitation (LOQ)(Alam et al., 2019; Mistry et al., 2015; Parys et al., 2020)

According to the ICH guideline, LOD and LOQ are calculated by the standard deviation of the response (σ) and slope of the calibration curve (S) by following formulas.

$$LOD = 3.3 \times \frac{\sigma}{S}$$

$$LOQ = 10 \times \frac{\sigma}{S}$$

H. Precision(Alam et al., 2019; Mistry et al., 2015; Parys et al., 2020)

Precision is the repeatability of results, and for the developed method, it is measured by performing three replicate measurements of the sample application and measurement of peak area. Three solutions were applied on HPTLC plates to form 200, 600 and 1000 ng/band of both drugs.

I. Accuracy(Alam et al., 2019; Mistry et al., 2015; Parys et al., 2020)

The methodological recovery study to check the recovery of the drug at a different level in the formulation was assessed by accuracy. The known standard amount to sample at 50, 100 and 150% was added and analyzed in triplicate.

J. Specificity(Alam et al., 2019; Mistry et al., 2015; Parys et al., 2020)

The specificity of the method was confirmed by analyzing peak purity and Rf value of standard drug and formulation. The spectra were noted at three different levels, i.e., peak start (S), peak apex (M) and peak-end (E) position of the spot.

K. Robustness (Alam et al., 2019; Mistry et al., 2015; Parys et al., 2020)

According to the ICH guideline, robustness means the capability of an analytical procedure to remain unaffected by minor and deliberate variations in method parameters. In this study, the following changes were considered to measure Rf and peak area:

1. Mobile phase composition (0.5 ± 0.1 ml methanol)
2. Saturation time (25 ± 5 min)
3. Solvent front (80 ± 5 mm)
4. Wavelength (240 ± 1 nm)

RESULTS AND DISCUSSION

HPTLC is a simple, rapid, sensitive, and specific method to simultaneously determine drug concentration in a sample(16). Various mobile phases trials (table 2) were taken using several solvents like chloroform: methanol, Hexane: toluene: methanol, toluene: ethyl acetate, ethyl acetate: toluene: methanol and chloroform: methanol: toluene with different ratios(Mistry et al., 2015).

Table 2 Trials of various mobile phases.

MOBILE PHASE	RATIO	DETECTION	REASON
n-Hexane: Ethyl acetate	6:4	Vanillin sulphuric acid reagent	Pomegranate Peel Extract peak not appropriate
n-Hexane: Ethyl Acetate. : Methanol	6.5:0.5:2	Vanillin sulphuric acid reagent.	Spectra of both drugs get merged
n-Hexane: Chloroform. : Methanol	7.5:0.5:1	Vanillin sulphuric acid reagent.	Pomegranate Peel Extract Rf not in range and Naproxen Rf in range Rf=0.42
n-Hexane: Chloroform : Methanol	7.5:1.5:1	Vanillin sulphuric acid reagent.	Naproxen peak observed, but Pomegranate Peel Extract peak does not appear.
n-Hexane: Ethyl.Acetate. : Toluene	7:1:1	Vanillin sulphuric acid reagent.	Pomegranate Peel Extract Rf not in range.
n-Hexane: Ethyl Acetate: Toluene	6:2:1	Vanillin sulphuric acid reagent.	The peak and Rf were appropriate, but some extra peak appears. Optimization of the mobile phase needs to be done.
n-Hexane: Ethyl.Acetate. : Toluene	6:3:1	Vanillin sulphuric acid reagent.	Both Spectra were merged.

Ethyl Acetate: Toluene: Methanol	4:5:1	Vanillin sulphuric acid reagent.	The Rf value of both drugs was in range, and the mobile phase is appropriate but needs some optimization.
Ethyl Acetate: Toluene: Methanol	**4:5.5:0.5**	**Vanillin sulphuric acid reagent.**	**Good Peak Shape (Rf Naproxen = 0.52) (Rf PPE = 0.83)**

The preliminary study was initiated with Hexane: ethyl acetate: methanol with differing ratios from the reported papers. With Hexane: ethyl acetate: methanol resulting R_f value was not in range, Hence to increase R_f non-polar solvent were tried from which suitable solvents were found to be toluene.

Good separation within the R_f value 0.2 to 0.8 was obtained using Hexane: Ethyl Acetate (6:4) ratio, but Pomegranate peel extract peak was not detected; hence, mobile phase optimization was suggested. Finally, better separation with good peak shape was observed with **Ethyl Acetate: Toluene: Methanol** in ratio **4:5.5:0.5** (figure 1), observed in the chromatogram. Hence, this mobile phase may be considered optimized.

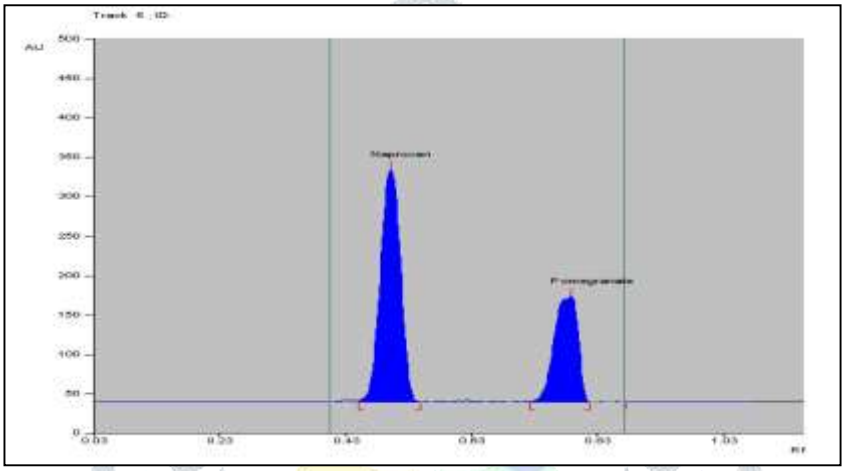

Figure 1 HPTLC chromatogram of PPE and Naproxen using optimized mobile phase Ethyl Acetate: Toluene: Methanol (4:5.5:0.5)

A. Selection of detection wavelength

As the compounds cannot be viewed at the visible wavelength (200-800 nm) in the U.V. chamber, it became necessary to use a spraying reagent to derivatize the plate and make it visualized(Guermouche et al., 2000). The U.V. spectra of both drugs in Camag TLC Scanner IV were recorded after derivatization To determine the appropriate wavelength. Both drugs showed high intensity at 240 nm (figure 2).

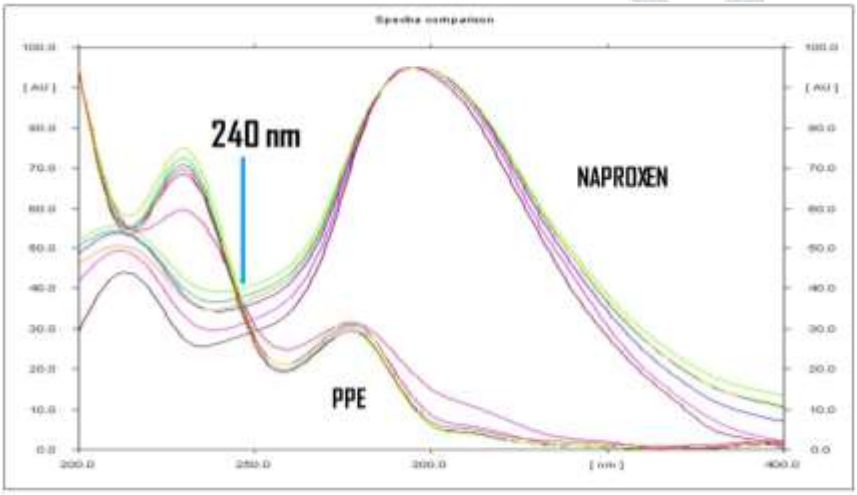

Figure 2 Detection wavelength for Naproxen and PPE

B. Method Validation

Linearity

Table 3 shows the data of concentration and peak area observed in HPTLC for both drugs.

Table 3 Calibration study of Pomegranate peel extract and Naproxen by HPTLC method.

Naproxen		Pomegranate peel extract	
Concentration (ng/band)	**Area (Mean ± S.D)**	**Concentration (ng/band)**	**Area (Mean± S.D)**
200	974.8±16.982	200	589.3±3.6400
400	1658.6±8.1161	400	1250.1±24.463
600	2372.3±75.625	600	1967.4±9.466
800	3251.4±59.085	800	2863.3±5.147
1000	3763.1±72.641	1000	3319.8±60.99

n=5, S.D = Standard deviation

The plotted calibration curve between concentration and peak area was linear over the 200-1000 ng/band range with regression coefficient (r^2) of 0.9953 and 0.9928 for Naproxen and PPE, respectively (figure 3 and 4). In addition, the 3D chromatogram of both drugs reflects linearity over the measured wavelength (figure 5).

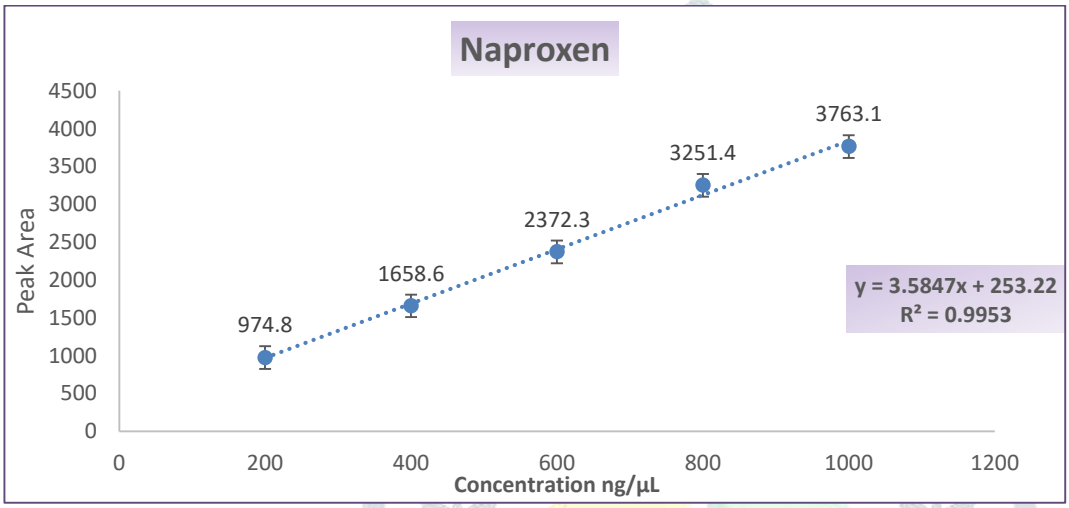

Figure 3 Calibration curve of Naproxen at 240 nm

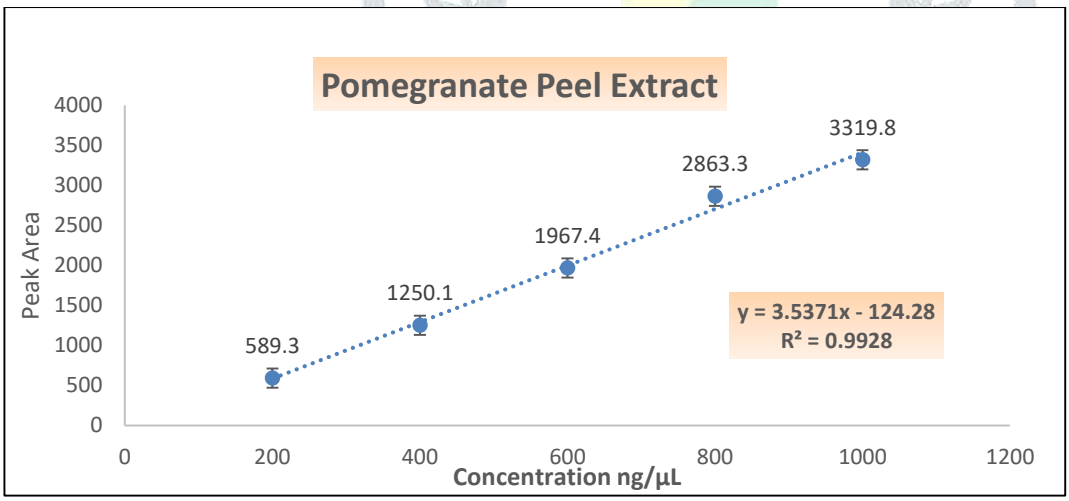

Figure 4 Calibration curve of PPE at 240 nm

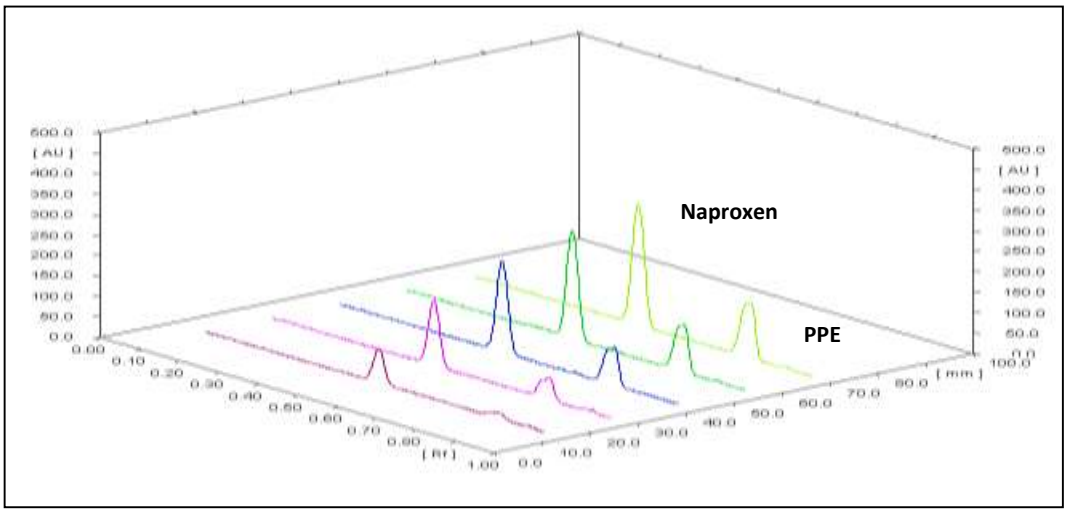

Figure 5 3D chromatogram of linearity of PPE and Naproxen

LOD and LOQ

The calibration curve data were analyzed in Microsoft excel. Linear regression parameters extracted from the analysis along with LOD and LOQ values are summarized in table 4.

LOD and LOQ of Naproxen were 0.179 ng/band and 0.526 ng/band, respectively, while Pomegranate peel extract was 0.151 ng/band and 0.472 ng/band, respectively.

Precision

The experiment was repeated a day thrice (intraday), and the amount of drug found was calculated, and % RSD is calculated similarly on another day (interday). The procedure is repeated, and the amount of drug found and % RSD is calculated. The amount of drugs found in both intraday and interday precision studies is close, revealing that the method is precise enough. (Table 5)

Accuracy

The evaluation of drug recovery at three different concentration levels, 50%, 100% and 150%, were evaluated after spiking with the standard. Table 6 showed percentage recovery between 100.8% to 102.1% for Pomegranate peel extract and 99.0% to 100.5% for Naproxen

Table 4 Linear regression parameters for Pomegranate peel extract and Naproxen by HPTLC method

Parameters	Naproxen	Pomegranate peel extract
Calibration range (ng/band)	200-1000	200-1000
Regression equation	y = 3.5847x + 253.22	y = 3.5371x - 124.28
Correlation coefficient	0.9953	0.9928
The standard deviation of slop	3.963	4.575
The standard deviation of the intercept	19.913	17.546
LOD (ng/band)	0.179	0.151
LOQ (ng/band)	0.526	0.472

Specificity

The peak purity of chosen drugs in formulations and that of the standard were evaluated by comparing spectra (figure 6 and figure 7) at peak start, peak apex and peak end, indicating specificity in the presence of other interference. Results of peak purity are depicted in Table 7.

Robustness

The considerable change in various parameters like the mobile phase composition, chamber saturation time, distance travelled, and wavelength depicted less than 2% RSD in the peak area. Rf value reflected that the method was robust(Shah et al., 2017). All results shown in table 8 reflect that selected factors remained unaffected by minor variation of studied parameters.

Table 5 Precision studies by HPTLC method.

Conc. of drug applied (ng/band)	INTRADAY		INTERDAY	
	Conc. of drug found ± SD ng/band	% RSD	Conc. of drug found ± SD ng/band	% RSD
Naproxen				
200	974.8±16.982	0.031	972.00±0.41	0.042
600	2372.3±75.625	0.020	2573.9±0.45	0.017
1000	3763.1±72.641	0.032	3764.4±0.59	0.015
Pomegranate peel extract				
200	589.3±3.6400	0.061	588.7±0.410	0.070
600	1967.4±9.466	0.052	1965.0±0.557	0.038
1000	3319.8±60.99	0.016	3320.2±0.328	0.008

Table 6 Accuracy study by spiking with the standard.

Drugs	Percentage of standard added (%)	The initial amount of drug (ng/band)	Amount Added (ng/band)	The total amount of drug taken (ng/band)	The total amount of drug found (ng/band) ± S.D.	Total percentage of the drug found (%)
Pomegranate peel extract	50	400	200	600	605.2 ±1.18	100.8%
	100	400	400	800	817.5 ±0.72	102.1%
	150	400	600	1000	1012.6 ±2.28	101.2%
Naproxen	50	200	100	300	297.8 ±0.28	99.0%
	100	200	200	400	402.8 ±0.72	100.5%
	150	200	300	500	495.7 ±2.53	99.0%

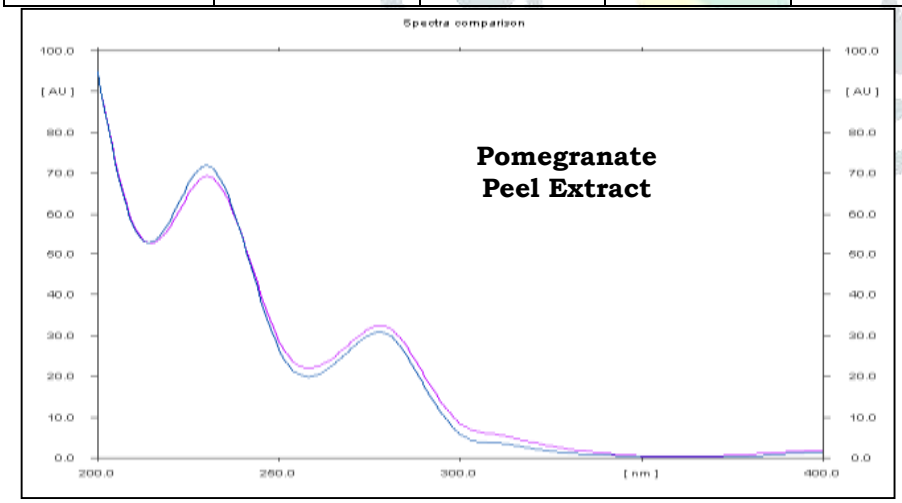

Figure 6 Overlay spectra of pure PPE with the formulation

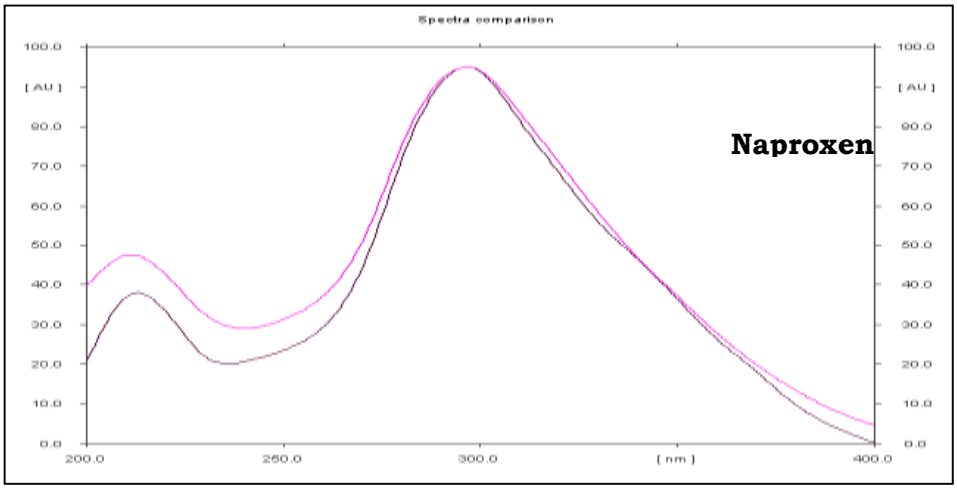

Figure 7 Overlay spectra of pure Naproxen with the formulation

Table 7 Peak Purity

	FORMULATION		STANDARD	
	Naproxen	**PPE**	**Naproxen**	**PPE**
r(S,M)	0.999894	0.999691	0.999977	0.999978
r(M,E)	0.999817	0.999129	0.999918	0.999928

Table 8 Data of Robustness study

Change in the mobile phase (0.5 ± 0.1 ml methanol)						
	Rf ± S.D.			**Peak area ± S.D.**		
	0.4 ml	**0.5 ml**	**0.6 ml**	**0.4 ml**	**0.5 ml**	**0.6 ml**
NPX	0.49 ±0.02	0.52 ±0.03	0.48 ±0.03	951.4 ±15.873	972.8 ±16.982	966.1 ±11.279
PPE	0.81 ±0.03	0.83 ±0.02	0.82 ±0.04	1419.5 ±22.346	1443.1 ±24.463	1468.5 ±27.574
Change in chamber saturation time (25 min ± 2 min)						
	23	**25**	**27**	**23**	**25**	**27**
NPX	0.51 ±0.04	0.52 ±0.03	0.48 ±0.03	944.6 ±12.283	972.8 ±16.982	962.4 ±18.148
PPE	0.79 ±0.05	0.83 ±0.02	0.81 ±0.02	1415.4 ±20.322	1443.1 ±24.463	1428.7 ±19.573
Change in solvent front (80 mm ± 5 mm)						
	75	**80**	**85**	**75**	**80**	**85**
NPX	0.54 ±0.05	0.52 ±0.03	0.46 ±0.03	929.3 ±14.572	972.8 ±16.982	962.5 ±12.679
PPE	0.78 ±0.04	0.83 ±0.02	0.74 ±0.05	1468.6 ±32.134	1443.1 ±24.463	1474.5 ±12.517
Change in wavelength (240 nm ± 1 nm)						
	239	**240**	**241**	**239**	**240**	**241**
NPX	0.46 ±0.04	0.52 ±0.03	0.46 ±0.05	994.5 ±21.634	972.8 ±16.982	951.4 ±36.531
PPE	0.74 ±0.03	0.83 ±0.02	0.80 ±0.06	1469.4 ±15.646	1443.1 ±24.463	1459.3 ±38.714

CONCLUSION

HPTLC is a new, simple, rapid and low-cost method for simultaneous drugs estimation. The technique gave reproducible results in the assessment of Naproxen and PPE in pure and combined form. ICH Q2 (R1) guidelines validated the developed method. The method was found to be sensitive, specific, reproducible, accurate and precise in validation. In addition, it is convenient to employ in routine quality control analysis of both drugs.

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