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ARTICLES

Development and Validation of RP–HPLC, HPTLC and UV-Visible Spectrophotometric Methods for Simultaneous Estimation of Alprazolam and Propranolol Hydrochloride in Their Combined Dosage Form[1]

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Abstract—Three accurate, sensitive and reproducible methods are described for the quantitative determination of alprazolam (**ALP**) and propranolol hydrochloride (**PNL**) in their combined dosage form. The first method involves an RP–HPLC separation on the C_{18} column using acetonitrile–25 mM ammonium acetate buffer and 0.2% triethylamine (pH of buffer adjusted to 4 with glacial acetic acid) in the ratio of 35 : 65 (v/v) as mobile phase. Symmetrical peaks with good separation, ALP at 9.3 min and PNL at 3.5 min, were achieved. Quantification was done with photo diode array detection at 255 nm over the concentration ranges of 0.5–50 and 10–250 μg/mL for ALP and PNL, respectively. The second method is based on the separation of drugs by HPTLC using chloroform–methanol–ammonia 7 : 0.8 : 0.1 (v/v/v) as mobile phase. Quantification was achieved using UV detection at 248 nm over the concentration range of 100–600 ng/spot and 5–30 μg/spot for ALP and PNL, respectively. The third method involves dual wavelength UV-visible spectrophotometric method. It is based on the determination of PNL at 319.4 nm using its absorptivity value and ALP at 258.2 nm after deduction of absorbance due to PNL. Quantification was achieved over the concentration range of 1–40 and 80–200 μg/mL for ALP and PNL, respectively. All methods were validated according to ICH guidelines and successively applied to marketed pharmaceutical formulation, and the results of all three methods were compared statistically as well. No interference from the tablet excipients was found.

Keywords: RP–HPLC, HPTLC, UV-visible spectrophotometry, alprazolam, propranolol hydrochloride

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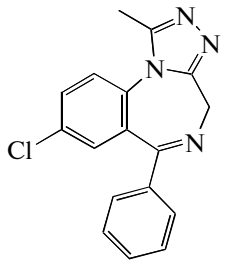
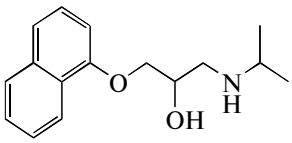
Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]-benzodiazepine) is an orally absorbed benzodiazepine. ALP is very effective in the short-term symptomatic relief of moderate to severe anxiety, essential tremor, and panic attacks [1]. Propranolol hydrochloride (-[(1-Methyl-ethyl)amino]-3-(1-naphthalenyloxy)-2-propanol) is a non selective beta blocker, completely absorbed from gastrointestinal tract.



Alprazolam



Propranolol

PNL is indicated for the management of various conditions which include hypertension, angina pectoris, tachyarrhythmia, myocardial infarction, control of tachycardia/tremor associated with anxiety. Both the drugs are official in IP, EP and USP, but PNL is also official in BP. Literature survey revealed many chromatographic and spectrophotometric methods for determination of ALP alone or in combinations with other drugs from pharmaceutical formulations and biological fluids [2–7]. Several chromatographic and spectrophotometric methods have also been reported for the determination of PNL from pharmaceutical formulations and biological fluids [8–17]. A single RP–HPLC method is reported for the determination of ALP and PNL in a combined dosage form [18]. In

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the reported RP–HPLC method, more amount of organic phase was used and the optimized mobile phase is not LC–MS compatible. Hence it cannot be extended for characterization of degradation products and impurity profiling of drugs in combination. Therefore, it was endeavored to develop an accurate, precise and sensitive alternative RP–HPLC method along with UV-visible spectrophotometric and HPTLC to estimate both the drugs simultaneously in their combined dosage form.

EXPERIMENTAL

Chemicals and reagents. A standard drug sample of ALP was provided by Astron Research Centre (Ahmedabad, India) and of PNL by Torrent Research Centre (Ahmedabad, India). The pharmaceutical dosage form used in this study was LAM–PLUS tablets labeled to contain ALP 0.25 mg and PNL 20 mg (Tes Med India Pvt Ltd). Acetonitrile (HPLC grade), chloroform (AR grade), methanol (AR grade), triethylamine (AR grade), glacial acetic acid (AR grade) and ammonium acetate (AR Grade) were purchased from S.D. Fine Chemicals (Mumbai, India). Calibrated amber colored glasswares were used throughout the work.

Instrumentation. RP–HPLC method was developed on an HPLC system consisting of a pump (JASCO PU 2080, Japan) equipped with a PDA detector and a rheodyne injector of 20 μL loop. Borwin PDA software was used for computational purpose; pH meter of model 111E/101E (Analabs Scientific Instruments Ltd, India) and range of pH 0 to 14 with resolution ±0.01 pH, accuracy ±0.01 pH was used. The HPTLC instrumentation consisted of a Linomat V sample applicator with 100 μL Hamilton syringe and TLC scanner III controlled by winCATS software (CAMAG, Muttenz, Switzerland). Merck TLC plates coated with silica gel $60F_{254}$ on aluminum sheets were used as the stationary phase. The plates were developed in a CAMAG twin trough chamber of 20 × 10 cm previously saturated for 30 min with the mobile phase. UV-visible spectrophotometric method was developed on a Shimadzu UV-visible double beam spectrophotometer, model 2400 PC series with spectral width of 1 nm, wavelength accuracy of 0.5 nm and a pair of 10 mm matched quartz cells (Shimadzu, Japan). All weighing was done on Citizen electronic balance Model CX 220 (Citizen India Ltd).

Chromatographic conditions. *RP–HPLC method.* Phenomenex C_{18} column (150 × 4.6 mm, 5.0 μm particle size) was used as stationary phase. Mobile phase composition was acetonitrile–25 mM ammonium acetate buffer and 0.2% triethylamine, pH 4 adjusted with glacial acetic acid 35 : 65 (v/v). The flow rate was 1.0 mL/min. The mobile phase was filtered through a nylon 0.45 μm, 47 mm membrane filter and degassed before use. Detection was carried out at 255 nm with PDA detector. Mixture of acetonitrile and water in ratio of 35 : 65 was used as diluent throughout the HPLC analysis.

HPTLC method. The solutions were spotted in the form of bands of 4 mm width with a Camag 100 μL sample applicator syringe on precoated silica gel aluminium Plate 60 F_{254}. The plates were activated at 110°C in oven for 20 min prior to sample application. A constant application rate of 0.1 μL/sec was employed and space between two bands was 10 mm. The spotted plate was developed in twin trough chamber previously saturated for 30 min with mobile phase consisting of chloroform–methanol–ammonia 7 : 0.8 : 0.1, (v/v/v) to a distance of 8 cm. The developed plate was dried in a current of air with the help of an air dryer. The developed spots were scanned at 248 nm with slit dimension 5 mm × 0.45 mm and scanning speed of 10 mm/sec.

Preparation of solution. *Preparation of standard stock solution*. ALP and PNL (25 mg each) were weighed accurately, transferred to individual 25 mL volumetric flasks and dissolved in methanol. The solutions were sonicated for 10 min. The flasks were shaken and volume was made up to the mark with methanol to get solutions containing 1000 μg/mL of ALP and PNL. Aliquot of 1 mL was pipetted further and diluted to 10 mL with methanol to obtain final concentration of 100 μg/mL of ALP and PNL, respectively, and labeled as standard stock solution.

Preparation of sample stock solution. A total of twenty tablets were weighed accurately and powdered. An amount of tablet powder equivalent to 100 mg of PNL (1.25 mg of ALP) was transferred to a 10 mL volumetric flask; 5 mL of methanol was added to flask and sonicated for 10 min. The solution was shaken, volume was made up to the mark with methanol and filtered through Whatmann filter paper No 41. An aliquot of 1.0 mL was pipetted out from sample stock solution and diluted to 10 mL with diluent to obtain the solution containing 1000 μg/mL of PNL (12.5 μg/mL of ALP). The above solution was further diluted to obtain the solution containing 160 μg/mL of PNL (2 μg/mL of ALP) for RP–HPLC. An aliquot of 1.6 mL and 0.1 mL of sample stock solution was accurately transferred to two individual 10 mL volumetric flasks and diluted with methanol to obtain working solutions for HPTLC and UV-visible spectrophotometry.

Method validation. *Preparation of calibration curves.* For an RP–HPLC calibration curve of ALP, solutions containing 0.5, 1, 5, 10,20 and 50 μg/mL ALP with 20 μg/mL PNL, and for calibration curve of PNL, solutions containing 10, 50, 100, 150, 200 and 250 μg/mL PNL with 20 μg/mL ALP were prepared in diluent from standard stock solution. An aliquot (20 μL) of each solution was injected under the operating chromatographic conditions.

For an HPTLC calibration curve of ALP, solutions containing 10, 20, 30, 40, 50 and 60 μg/mL ALP with 1600 μg/mL PNL and for calibration curve of PNL, solutions containing 500, 1000, 1500, 2000, 2500,

3000 μg/mL PNL with 20 μg/mL ALP were prepared in methanol. The plates were developed and scanned as described above.

Calibration curves for both methods were constructed by plotting peak areas versus concentrations of ALP and PNL, and the regression equations were calculated. Each response was the average of six determinations.

For UV-visible spectrophotometry the individual solutions containing 1, 5, 10, 20, 30 and 40 μg/mL of ALP as well as 80, 100, 120, 140, 160, 180 and 200 μg/mL of PNL were prepared in methanol and analyzed at 258.20 nm and 319.40 nm for ALP and PNL, respectively. Calibration curve was constructed by plotting absorbance versus concentration.

Accuracy (recovery). The accuracy of the method was determined by calculating recoveries of ALP and PNL by the standard addition method at three different levels (80, 100 and 120%) to preanalysed tablet sample. For RP–HPLC, known amounts of standard solutions of ALP (1.0, 1.25 and 1.5 μg/mL) and PNL (80, 100 and 120 μg/mL) were added to preanalysed sample solution containing 100 μg/mL PNL (1.25 μg/mL ALP). For HPTLC, known amounts of standard solutions of ALP (12, 15 and 18 μg/mL) and PNL (960, 1200 and 1440 μg/mL) were added to preanalysed sample solution containing 1200 μg/mL PNL (15 μg/mL ALP); 10 μL of each solution was spotted on activated silica plate. For UV-visible spectrophotometry, known amounts of standard solution of ALP (0.8, 1.0 and 1.2 μg/mL) and PNL (64, 80 and 96 μg/mL) were added to preanalysed sample solution containing 80 μg/mL PNL (1 μg/mL ALP).

Precision. The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses three times on the same day and on three different days for three different concentrations of ALP (1, 10 and 50 μg/mL) and PNL (10, 100 and 200 μg/mL) for RP–HPLC, ALP (10, 30, and 50 μg/mL) and PNL (1000, 2000, and 3000 μg/mL) for HPTLC and ALP (1, 10, and 30 μg/mL) and PNL (100, 140, and 180 μg/mL) for UV-visible spectrophotometry. Repeatability was assessed by analyzing one concentration six times. It was performed on concentration 5 μg/mL of ALP and 100 μg/mL of PNL for RP–HPLC, 30 μg/mL of ALP and 2000 μg/mL of PNL for HPTLC and 5 μg/mL of ALP and 100 μg/mL of PNL for UV-visible spectrophotometry.

LOD and LOQ were calculated using the signal to noise (S/N) ratio method. LOD was taken as the concentration of analyte at which S/N was 3. LOQ was taken as the concentration of analyte at which S/N was 10.

Robustness. The robustness of the methods was studied by analyzing the same samples of ALP and PNL with deliberate variation in the method parameters. The changes in the responses of ALP and PNL were noted. For RP–HPLC, pH of mobile phase (±0.2), flow rate (±0.2 mL), detection wavelength (±3 nm) and mobile phase composition (±5%) were deliberately changed. For HPTLC, various parameters were changed like detection wavelength (±2 nm), chamber saturation time (±3 min) and size of chamber (10 × 10 cm).

Table 1. Results (mean ± SD) of system suitability parameters for ALP and PNL by proposed RP–HPLC and HPTLC methods

Parameter	ALP	PNL
	RP–HPLC	
R_t, min	9.070 ± 0.007	3.265 ± 0.003
Asymmetry factor	1.062 ± 0.025	1.430 ± 0.031
Capacity factor	4.88 ± 0.18	1.117 ± 0.065
Theoretical plates	$(1.30 \pm 0.03) \times 10^4$	$(3.3 \pm 0.1) \times 10^3$
Resolution	–	21.27 ± 0.18
	HPTLC	
R_f	0.55 ± 0.021	0.24 ± 0.02
Peak area	$(1.086 \pm 0.009) \times 10^4$	$(4.26 \pm 0.06) \times 10^3$

RESULTS AND DISCUSSION

Determination of ALP and PNL simultaneously from combined dosage form was a challenging task as proportion of both the drugs is in the ratio of 1 : 80 for ALP and PNL in marketed dosage forms.

RP–HPLC method. To optimize various RP–HPLC parameters such as peak shape, peak symmetry, run time and resolution, several trials were taken. Symmetrical peaks with good separation (retention time, ALP = 9.3 min and PNL = 3.5 min) were obtained under conditions stated under experimental section (Fig. 1). Linear correlation was obtained between peak area and concentration for ALP and PNL in the range of 0.5–50 and 10–250 μg/mL, respectively.

HPTLC method. Experimental conditions, such as mobile phase and wavelength of detection, were optimized to provide accurate, precise, and reproducible results for simultaneous determination of ALP and PNL by HPTLC. Good separation of both the drugs (R_f values of ALP is equal 0.56 and PNL–0.24) with minimum tailing was obtained by using the conditions described under experimental part (Fig. 2). Linear correlation was obtained between peak area and concentration for ALP and PNL in the range of 100–600 ng/spot and 5–30 μg/spot, respectively.

A system suitability test for RP–HPLC and HPTLC was performed before each validation run. Five replicate injections of standard preparation were made. For RP–HPLC parameters used were asymmetry of the chromatographic peak, peak resolution, number of theoretical plates and capacity factor. For HPTLC parameters used were R_f value and peak area. Table 1 describes system suitability parameters for HPLC and HPTLC methods.

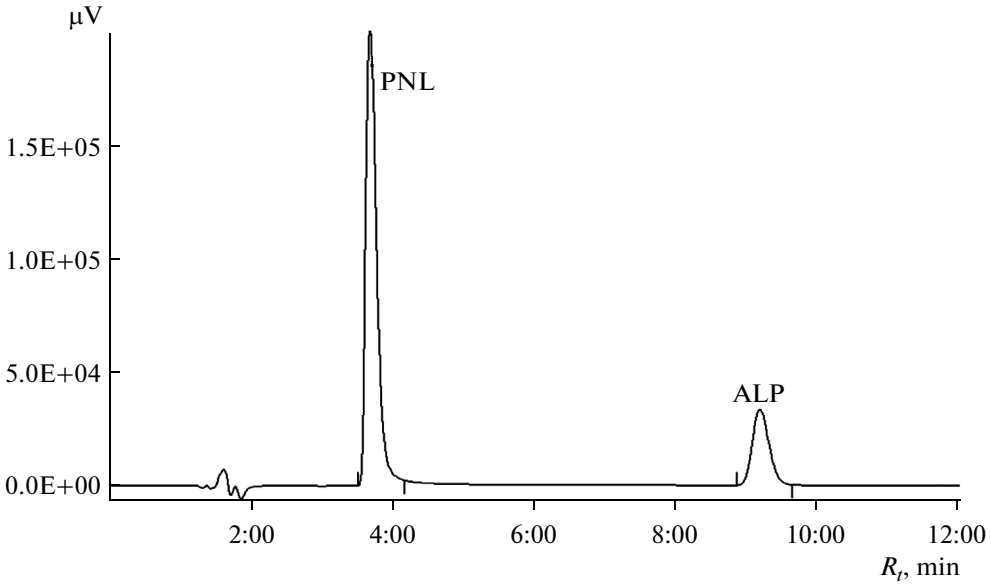

Fig. 1. HPLC chromatogram of standard mixture of ALP (20 μg/mL) and PNL (20 μg/mL) with R_t of 9.3 and 3.6 min, respectively at 255 nm.

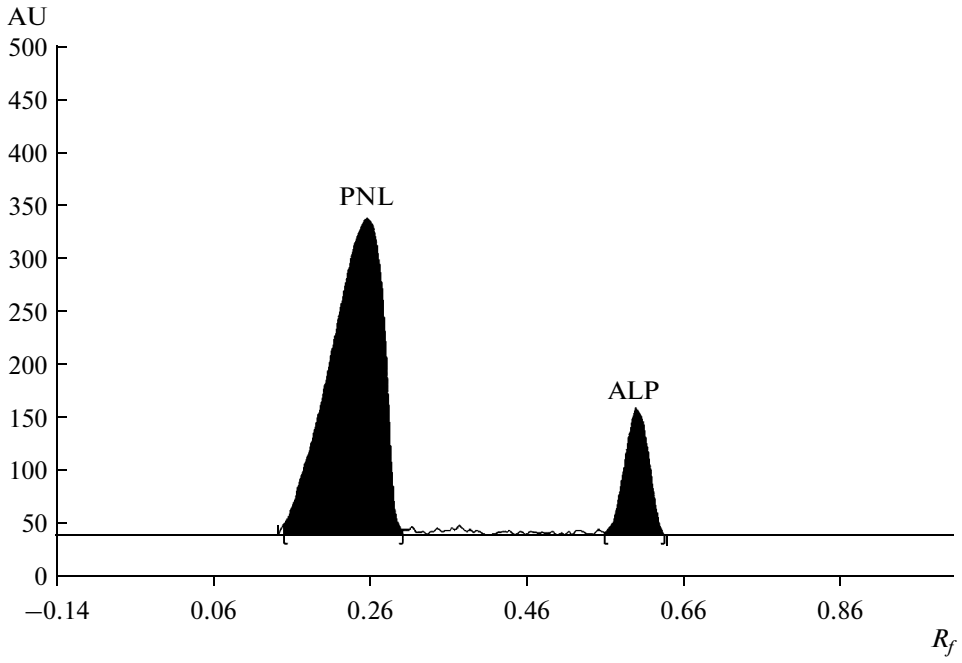

Fig. 2. HPTLC chromatogram of ALP (20 μg/mL) and PNL (1600 μg/mL) with R_f of 0.56 and 0.24, respectively from marketed tablet dosage form at 248 nm.

UV-visible spectrophotometric method. From the overlain spectra of methanolic solution of ALP and PNL, two wavelengths, 258.20 nm and 319.40 nm were selected for analysis. PNL can be estimated at 319.40 nm where ALP shows zero absorbance. ALP can be estimated by taking difference in absorbance at 319.40 nm and 258.20 nm where difference in absorbance for PNL is zero (Fig. 3). Linear correlation was obtained between absorbance and concentration for ALP and PNL in the range of 1–40 μg/mL and 80–200 μg/mL, respectively.

Method validation of proposed methods. The methods were validated in compliance with International Conference on Harmonization guidelines [19].

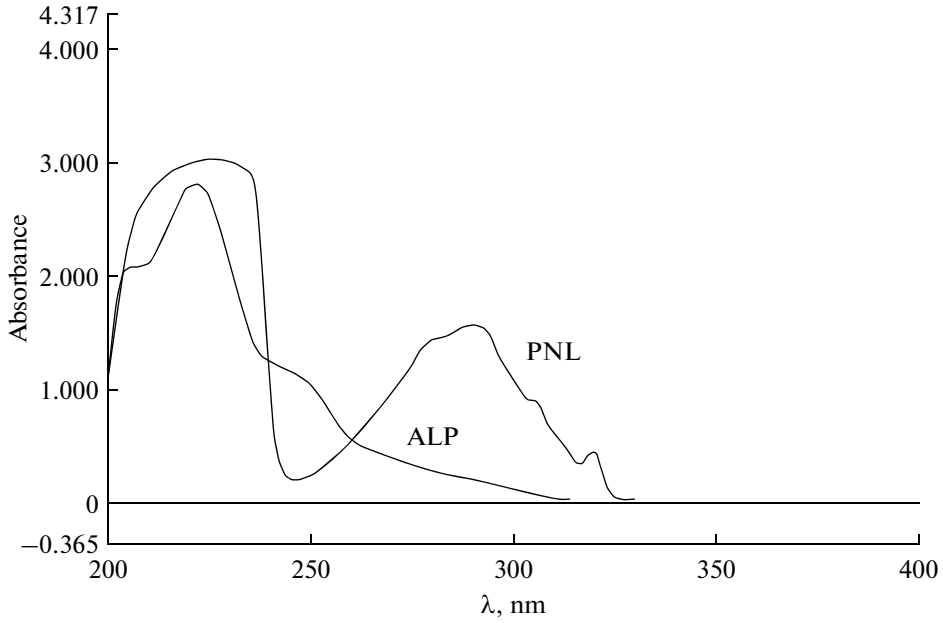

Fig. 3. Overlay spectra of ALP (1 μg/mL) and PNL (80 μg/mL).

(a) Result of *linearity* for ALP and PNL by RP–HPLC, HPTLC and UV-visible spectrophotometric methods are furnished in Table 2.

(b) *Precision*. Interday and Intraday variation in estimation of ALP and PNL showed that the RSD values was always less than 2% during analysis by all three methods. These low RSD values show good precision of the methods (Table 2).

(c) *Accuracy*. The % recovery values of ALP and PNL was obtained in the range of 98.21 ± 1.78–101.19 ± 1.98 and 98.60 ± 0.21–100.22 ± 0.42, respectively for RP–HPLC, HPTLC and UV-visible spectrophotometric methods which were satisfactory (Table 3).

(d) *Robustness*. The robustness of the methods was studied by analyzing the same samples of ALP and

Table 2. Validation parameters for proposed methods

Parameter	RPHPLC		HPTLC		UV-visible spectrophotometry	
	ALP	PNL	ALP	PNL	ALP	PNL
Linearity ($n = 6$)	0.5–50 μg/mL	10–250 μg/mL	100–600 ng/spot	5–30 μg/spot	1–40 μg/mL	80–200 μg/mL
Linear regression equation*						
intercept (c)	−8420	−19358	363.3	5795	0.009	0.015
slope (m)	67718	28934	9.063	958.7	0.028	0.005
r^2	0.999	0.998	0.997	0.996	0.999	0.999
LOD	0.12 μg/mL	0.14 μg/mL	12.0 ng/spot	0.80 μg/spot	0.12 μg/mL	1.10 μg/mL
LOQ	0.38 μg/mL	0.43 μg/mL	38 ng/spot	2.4 μg/spot	0.37 μg/mL	3.3 μg/mL
Precision (RSD, %)						
intraday ($n = 3$)	0.69–1.73	0.40–0.30	0.73–1.14	1.55–1.86	0.67–1.94	0.65–1.48
interday ($n = 3$)	1.14–1.62	1.21–1.89	0.95–1.52	1.72–1.88	0.51–1.96	0.71–1.23
Repeatability, % ($n = 6$)	1.69	1.60	1.21	1.83	1.41	0.37

* $y = mx + c$.

Table 3. Results of recovery studies of ALP and PNL by proposed methods

Method	Amount of drug taken, μg/mL		Amount of standard added		Recovery ± SD, % ($n = 3$)	
	ALP	PNL	ALP	PNL	ALP	PNL
RPHPLC	1.25	100	1	80	99.44 ± 1.09	100.22 ± 0.42
	1.25	100	1.25	100	99.27 ± 0.92	98.60 ± 0.21
	1.25	100	1.5	120	99.84 ± 1.66	99.06 ± 0.25
HPTLC	15	1200	12	960	99.17 ± 0.98	99.29 ± 0.70
	15	1200	15	1200	100.58 ± 1.32	99.50 ± 1.06
	15	1200	18	1440	99.08 ± 1.54	98.73 ± 0.50
UV-visible spectrophotometric	1	80	0.8	64	101.19 ± 1.98	98.98 ± 0.57
	1	80	1	80	98.21 ± 1.78	100.04 ± 0.31
	1	80	1.2	96	99.02 ± 1.62	99.08 ± 0.20

Table 4. Results (mg) of assay for ALP and PNL in marketed formulation by proposed methods

Method	Labeled amount		Amount found		Assay, mean ± SD, % ($n = 3$)	
	ALP	PNL	ALP	PNL	ALP	PNL
RP–HPLC	0.25	20	0.250	19.91	100.2 ± 1.2	99.5 ± 1.5
HPTLC	0.25	20	0.247	19.80	99.2 ± 1.3	99.0 ± 1.2
UV-visible	0.25	20	0.247	19.79	98.1 ± 0.02	98.95 ± 0.05

PNL with deliberate variation in the method parameters. The changes in the responses of ALP and PNL were noted.

(e) *Specificity*. For RP–HPLC method, peak purity of ALP and PNL was assessed by comparing their respective spectra at peak start, apex and end positions of the peak. The peak purity was found to be 0.9999 for both ALP and PNL. No interference was detected at the R_t of ALP and PNL in sample solution. For HPTLC method, a good correlation ($r = 0.9999$) was obtained between the standard and sample spectra of ALP and PNL respectively.

Assay of marketed formulation. The proposed methods were successfully used for the assay of commercially available combined tablet dosage form containing ALP and PNL. Typical chromatogram for marketed formulation containing ALP and PNL is depicted in Fig. 4. Six replicate determinations were performed on accurately weighed tablets. The results obtained for ALP and PNL were comparable with the corresponding labeled claim amounts. The assay values obtained by all the three methods are shown in Table 4.

Comparison of proposed methods. The assay results of ALP and PNL in their combined dosage form obtained using proposed RP–HPLC, HPTLC and UV-visible spectrophotometric methods were compared using ANOVA test. The calculated *F*-value (1.337 for ALP and 0.654 for PNL) was found to be less than the critical *F*-value (5.143) at the 0.05 significance level for both the drugs. This result shows that there is no significant difference with respect to accuracy and precision between the proposed methods.

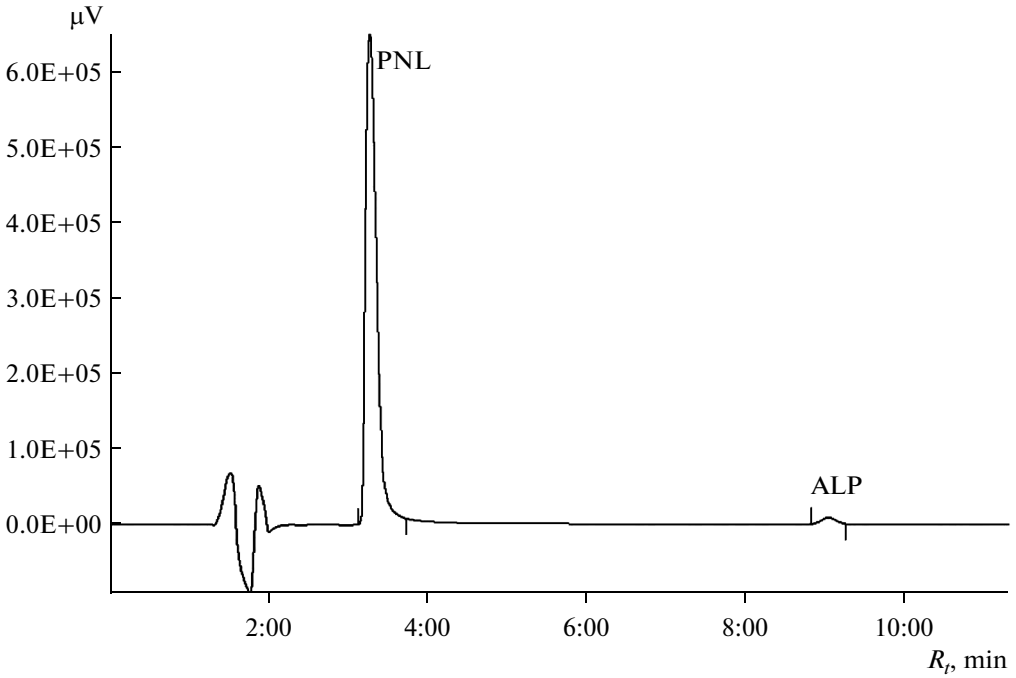

Fig. 4. RP–HPLC chromatogram of ALP (2 μg/mL) and PNL (160 μg/mL) with R_t of 9.3 and 3.5 min, respectively from marketed tablet dosage form at 255 nm.

* * *

The developed and validated RP–HPLC, HPTLC and UV-visible spectrophotometric methods provide simple, accurate and reproducible quantitative analysis for simultaneous determination of ALP and PNL in combined dosage form. The validation data and the recovery studies show that the method is free from the interference of the excipients used in the formulations. Compared to reported RP–HPLC method [18], developed method is more suitable for extensive work of drug analytes in biological fluid, characterization of degradation products and impurity profiling as it is compatible for LC–MS studies. Statistical comparison of the assay results for ALP and PNL in tablet dosage forms by the proposed methods indicate no significant difference and any of the developed method can be successfully applied for routine quality control of ALP and PNL in their combined dosage form.

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