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Research Article

Simultaneous Estimation and Validation of Levocetirizine, Pseudoephedrine and Ambroxol in Bulk and Combined Tablet Dosage Form by HPTLC.

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ABSTRACT
Objective: To develop and subsequently validate a new simple and sensitive high performance thin layer chromatographic (HPTLC) method for estimation of Levocetirizine, Pseudoephedrine and Ambroxol simultaneously, from a bulk drug and combined dosage form. Method: The separation of drugs was carried out on Merck HPTLC aluminium sheets of silica gel 60 F_{254} as stationary phase and the chromatogram was developed using Ethyl-acetate: methanol: ammonia (8: 1: 0.5 v/v/v) as the mobile phase. Result: Levocetirizine, Pseudoephedrine and Ambroxol showed R_f values 0.1 ± 0.02, 0.39 ± 0.05, and 0.73 ± 0.05 respectively, when scanned densitometrically at 212 nm using Camag TLC Scanner. The described method was linear over a concentration range of 100 ng $spot^{-1}$ to 700 ng $spot^{-1}$, 600 ng $spot^{-1}$ to 4200 ng $spot^{-1}$ and 1200 ng $spot^{-1}$ to 8400 ng $spot^{-1}$ for the Levocetirizine, Pseudoephedrine and Ambroxol respectively. Results of analysis were validated according to International Conference on Harmonization ICH Q2B guidelines statistically, and by recovery studies. The limit of detection (LOD) for Levocetirizine, Pseudoephedrine and Ambroxol were found to be 25 ng $spot^{-1}$, 40 ng $spot^{-1}$ and 35 ng $spot^{-1}$ respectively. The limit of quantification (LOQ) were found to be 60 ng $spot^{-1}$, 87 ng $spot^{-1}$ and 71 ng $spot^{-1}$ for Levocetirizine, Pseudoephedrine and Ambroxol respectively. Conclusion: The results of the study showed that the proposed HPTLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Levocetirizine, Pseudoephedrine and Ambroxol bulk drug and in its pharmaceutical dosage form.
Keywords: HPTLC, Levocetirizine, Pseudoephedrine, Ambroxol, Validation.

INTRODUCTION

Levocetirizine (LEV) is a second generation H1 antihistamines marketed for the treatment of perennial and seasonal allergic rhinitis and chronic idiopathic urticaria. It is the most active enantiomer of cetirizine and has a favorable pharmacokinetic profile. LEV is rapidly and extensively absorbed, minimally metabolized and has a volume of distribution which is lower than other compounds from the same group.[1, 2] Chemically, LEV is the active enantiomer of cetirizine. It is the L-enantiomer of the cetirizine racemate. LEV works by blocking histamine receptors. It does not prevent the actual release of histamine from mast cells, but prevents it binding to its receptors. This in turn prevents the release of other allergy chemicals and increased blood supply to the area, and provides relief from the typical symptoms of hayfever. Chemically it is 2-[2-[4-[(R)-(4-chlorophenyl)-phenyl-methyl] piperazin-1-yl] ethoxy] acetic acid, is shown in Figure 1. Pseudoephedrine (PSE) is a sympathomimetic,[3] amine commonly used as a decongestant, Chemically it is (1S,2S)-2-methylamino-1-phenylpropan-1-ol, and is official in the United States Pharmacopoeia,[4] British Pharmacopoeia,[5] and Indian Pharmacopoeia,[6] is shown in Figure 2. Ambroxol (AMB), chemically it is trans-4-(2-amino-3, 5-dibromobenzylamino) cyclohexanol, shown in Figure 3,[7] is semi-synthetic derivative of vasicine obtained from Indian shrub *Adhatoda vasica*. It is a metabolic product of bromhexine. It is used as broncho secretolytic and expectorant drug.[3] It stimulates the transportation of the viscous secretions in the respiratory organs and reduces the stand stillness of the secretions. AMB is a mucolytic agent used in the treatment of respiratory disorders associated with viscid or excessive mucus. Literature review reveals that methods have been reported for analysis of LEV in combinations,[8, 9] PSE in single drug,[10] as well as in combinations,[11, 12, 13, 14, 15] and AMB in single,[16, 17, 18] as well as in combinations.[19, 20, 21, 22, 23]

Up to now, there have been no published reports about the simultaneous quantitation of LEV, PSE and AMB by

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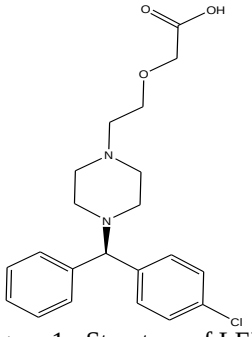

Figure 1: Structure of LEV

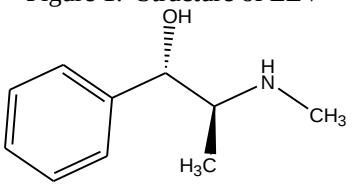

Figure 2: Structure of PSE

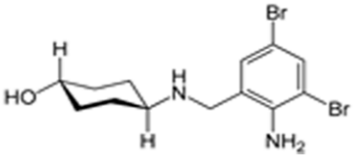

Figure 3: Structure of AMB

HPTLC in bulk drug and in tablet dosage form. This present work reports for the first time the simultaneous quantitation of LEV, PSE and AMB by HPTLC in bulk drug and in tablet dosage form. The proposed method is validated as per ICH guidelines.[24] This work gives validated HPTLC method for simultaneous estimation of LEV, PSE and AMB in combination using methanol: 0.04m potassium dihydrogen phosphate buffer of pH 3, with 0.5% Triethylamine, in the ratio of 65:35 v/v as a mobile phase. The column used was Thermo electron Co. ODS hypersil 5µ, 250 mm x 4.6 mm id with flow rate of 1 ml / min, using UV detection at 212 nm.

MATERIALS AND METHODS

Materials: Standard bulk drug samples were provided by Cipla Pvt. Ltd. Patalganga, Thane, Maharashtra, and used without further purification and certified to contain 98.55% (w/w) on dried basis. Tablets of combined dosage form were procured from the local market (Aldin D). All other reagents used were of HPLC grade, and were purchased from Merck Chemicals, India.

Instrumentation: The samples were spotted in the form of bands of 6 mm width with a Camag 100 microlitre sample (Hamilton, Bonaduz, Switzerland) syringe on silica gel precoated aluminum plate 60 F_{254}, (20 × 10 cm) with 250 mm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai using a Camag Linomat V (Switzerland). The plates were prewashed by methanol and activated at 110^0C for 5 min prior to chromatography. A constant application rate of 0.1 ml/s was employed and the space between two bands was 5 mm. The slit dimension was kept at 5 mm × 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm, each track was scanned thrice and the baseline correction was used. The mobile phase consisted of Ethyl-acetate: methanol: ammonia (8: 1: 0.5 v/v/v), and 10 ml of mobile phase was used per chromatography. Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25^0C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. Subsequent to the development, HPTLC plates were dried in current of air with the help of air dryer in a wooden chamber with adequate ventilation. The flow rate in the laboratory was maintained unidirectionally (laminar flow, towards exhaust). Densitometric scanning was performed on a Camag HPTLC scanner III in the reflectance-absorbance mode at 212 nm and operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffuse light. Evaluation was via peak areas with linear regression.

Table 1: Linearity and range of LEV, PSE and AMB by HPTLC.

Drug	Conc. (ng spot^{-1})	Average area	SD	%RSD
LEV	100	250	4	1.61
	200	480	7	1.46
	300	689	8	1.16
	400	902	11	1.22
	500	1123	17	1.51
	600	1324	19	1.43
	700	1533	22	1.43
PSE	600	1090	19	1.74
	1200	2089	31	1.48
	1800	2983	39	1.31
	2400	3888	45	1.16
	3000	4822	49	1.02
	3600	5671	63	1.11
	4200	6554	74	1.13
AMB	1200	12024	153	1.27
	2400	25284	245	0.97
	3600	37460	532	1.42
	4800	48636	674	1.38
	6000	60252	874	1.45
	7200	72588	972	1.34
	8400	83928	1103	1.31

Note: The values are mean value of 6 observations (N=6)

Selection of detection wavelength: Stock solution of the LEV, PSE, and AMB were prepared in 10 ml of methanol and UV spectra of individual drug was taken and it was found that LEV, PSE, and AMB showed maximum absorbance at 210 nm, 212 nm, and 208 nm respectively.

Table 2: Linear regression data for the calibration curve of LEV, PSE and AMB by HPTLC.

Parameters	LEV	PSE	AMB
Linearity range	100-700 ng spot^{-1}	600-4200 ng spot^{-1}	1200-8400 ng spot^{-1}
r^2	0.999	0.999	0.999
Slope	2.132	1.511	9.914
Intercept	47.14	243.1	1008

Note: The values are mean value of 6 observations (N=6)

Table 3: Intraday Precision and Inter day precision by HPTLC.

Compound	Conc. (ng spot^{-1})	Intraday Precision			Inter day precision		
		Measured Conc.	SD	% RSD	Measured Conc.	SD	% RSD
LEV	100	98.90	1.66	1.67	98.16	1.35	1.37
	400	398.05	6.58	1.65	395.24	7.28	1.84
	700	700.21	12.19	1.74	696.37	13.25	1.90
PSE	600	600.46	6.48	1.07	593.84	6.48	1.09
	2400	2375.97	33.02	1.38	2354.79	44.45	1.88
	4200	4213.96	41.72	0.98	4211.31	44.26	1.05
AMB	1200	1221.56	8.18	0.66	1216.05	11.37	0.93
	4800	4805.83	34.56	0.72	4797.76	30.24	0.63
	8400	8418.84	87.72	1.04	8414.80	81.63	0.97

Note: The values are mean value of 6 observations (N=6)

Table 4: Repeatability of sample application and Repeatability of measurement.

Drug	Conc. (ng spot^{-1})	Repeatability of sample application					Repeatability of measurement				
		Area		Avg. Area	SD	% RSD	Area		Avg. Area	SD	% RSD
		Plate 1 (n=3)	Plate 2 (n=3)				Plate 1 (n=3)	Plate 2 (n=3)			
LEV	400	902	900	901	12.76	1.42	903	888	895.5	7.22	0.80
	400	897	887	892			897	903	900		
	400	886	892	889			900	921	910.5		
	400	915	905	910			891	901	896		
	400	879	865	872			899	878	888.5		
	400	890	900	895			887	910	898.5		
PSE	2400	3858	3888	3873	52.67	1.38	3847	3849	3848	28.07	0.73
	2400	3705	3765	3735			3829	3819	3824		
	2400	3759	3799	3779			3799	3810	3805		
	2400	3827	3857	3842			3812	3898	3855		
	2400	3857	3857	3857			3819	3892	3856		
	2400	3780	3895	3838			3847	3849	3848		
AMB	4800	48936	48736	48836	284.2	0.59	48503	48299	48401	197.60	0.41
	4800	48754	48754	48754			48756	48720	48738		
	4800	48056	48056	48056			48374	48298	48336		
	4800	48765	48565	48665			48372	48928	48650		
	4800	48754	48754	48754			48948	48000	48474		
	4800	48560	48747	48654			48209	48208	48209		

Note: The values are mean value of 6 observations (N=6)

After overlain convenient point was found at 212 nm and was selected as the detection wavelength. Also In situ spectra by HPTLC were taken.

Preparation of standard stock solution: Standard stock solution of LEV (0.5 mg/ml), PSE (3 mg/ml) and AMB (6 mg/ml) was prepared by dissolving 5 mg LEV, 30 mg PSE and 60 mg AMB were weighed accurately and transferred to 10 ml volumetric flask. LEV, PSE, and AMB were dissolved in 10 ml methanol (HPLC grade). The solutions were further diluted with methanol to obtain the final concentration 100 ng/µl of LEV, 600 ng/µl PSE and 1200 ng/µl AMB respectively.

Preparation of sample stock solution: For preparing sample stock solution, twenty tablets were weighed (each containing 5 mg of LEV, 30 mg of PSE and 60 mg of AMB) and their average weight was calculated. The tablets were finely powdered and powder equivalent to 5 mg of LEV, 30 mg of PSE and 60 mg of AMB was accurately weighed in 25 ml volumetric flask and dissolved in 20 ml of methanol (HPLC grade).

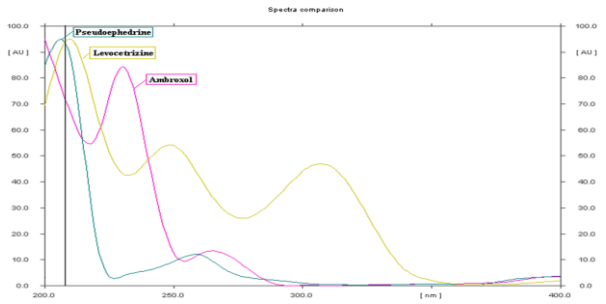

Figure 4: In situ overlay spectrum for LEV, PSE and AMB (λmax 212nm)

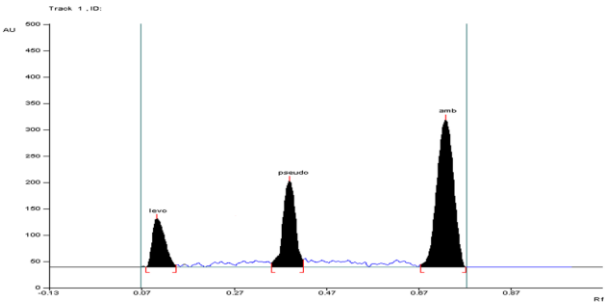

Figure 5: Densitogram of standard drugs (LEV, PSE and AMB) by HPTLC.

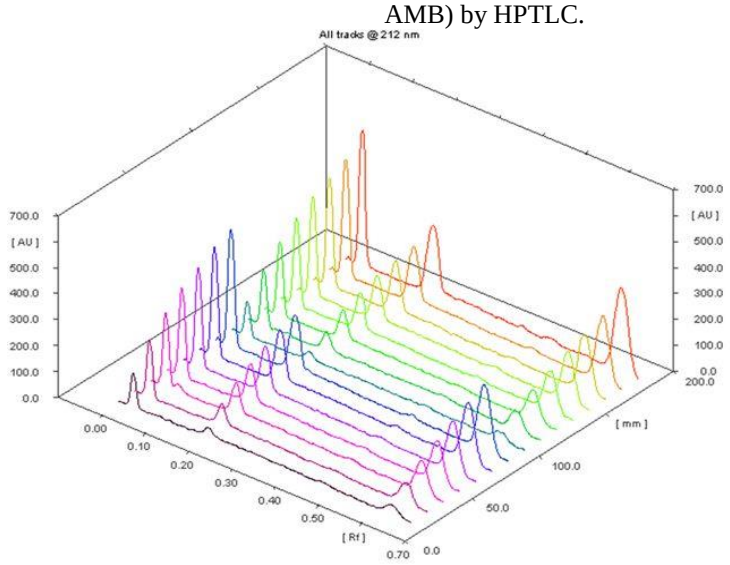

Figure 6: 3-D View of all linearity study peaks (15 peaks). [In this view Spot 1 to 7 and 8 to 14 are in ascending volume of 1µl to 7 µl respectively. Spot 15 is of 10 µl. [In 1 µl; LEV 100 ng, PSE 600 ng, and AMB 1200 ng). The plate was developed using optimized chromatographic conditions.]

The solution was sonicated for 30 min, then the solution was filtered through Whatman filter paper no. 41 and the residue was washed with methanol and volume was adjusted up to the with same solvent. The solution was further diluted with HPLC grade methanol to obtain the final concentration 100 ng/µl of LEV, 600 ng/µl PSE and 1200 ng/µl AMB respectively.

Optimization of HPTLC method: A solvent system that would give dense and compact spots with appropriate and significantly different R_f values were desired for quantification of LEV, PSE, and AMB in pharmaceutical formulations. Various solvent systems like tolune: ethyl acetate, toluene: ethyl acetate: methanol and tolune: ethyl acetate: methanol: formic acid were tried to separate and resolve spots of LEV, PSE, and AMB from their impurities and other excipients of formulations. Ethyl-acetate: methanol: ammonia (8: 1: 0.5 v/v/v) was found to result in the compact spot and best peak shape of LEV, PSE, and AMB. LEV, PSE, and AMB were satisfactorily resolved with R_F of 0.1 ± 0.02, 0.39 ± 0.05, and 0.73 ± 0.05 respectively at wavelength of 212 nm.

Validation of the method: Validation of the optimized HPTLC method as per the ICH guidelines was carried out with respect to the following parameters.

Linearity and range: Aliquots of 1, 2, 3, 4, 5, 6, 7 µl of mixed standard stock solution of 100 ng/µl LEV, 600 ng/µl PSE, and 1200 ng/µl AMB were applied on the TLC plates using semiautomatic spotter under nitrogen stream. The plates were dried, developed using the previously described mobile phase and densitometrically analyzed at 212 nm. The linear regression data for calibration curves and linear relationship over a concentration range of 100-700 ng/spot for LEV, 600-4200 ng/spot PSE, and 1200-8400 ng/spot AMB were studied. Each concentration was applied six times to the HPTLC plate.

Precision

Intra-day precision: The intra-day precision was determined by analyzing standard solution of LEV, PSE, and AMB at three different concentrations of 100, 400, 700 ng/spot of LEV, 600, 2400, 4200 ng/spot of PSE, and 1200, 4800, 8400 ng/spot of AMB, six times on the same day. Each concentration was applied and % RSD was calculated.

Inter-day precision: The inter-day precision was determined by analyzing standard solution of LEV, PSE, and AMB at three different concentrations of 100, 400, 700 ng/spot of LEV, 600, 2400, 4200 ng/spot of PSE, and 1200, 4800, 8400 ng/spot of AMB three different days over a period of one week and % RSD was calculated.

Repeatability

Repeatability of sample application: Repeatability of sample application was determined by spotting standard stock solution of LEV, PSE, and AMB in a concentration of 100, 400, 700 ng/spot of LEV, 600, 2400, 4200 ng/spot of PSE, and 1200, 4800, 8400 ng/spot of AMB, six times on TLC plate by semiautomatic spotter. After developing the plate with optimized mobile phase and scanning wavelength i.e. 212 nm, separated spots of LEV, PSE, and AMB were scanned without changing position of the plate and % RSD for sample application, peak areas were calculated.

Table 5: Robustness study of the HPTLC method.

Drug	Parameters	% RSD
LEV	Mobile phase composition (± 0.1 ml)	1.45
	Amount of mobile phase (± 0.5 %)	1.05
	Time from spotting to chromatography (± 20 min)	1.04
	Time from chromatography to scanning (± 20 min)	1.15
PSE	Mobile phase composition (± 0.1 ml)	1.06
	Amount of mobile phase (± 0.5 %)	1.02
	Time from spotting to chromatography (± 20 min)	1.25
	Time from chromatography to scanning (± 20 min)	0.95
AMB	Mobile phase composition (± 0.1 ml)	1.01
	Amount of mobile phase (± 0.5 %)	0.90
	Time from spotting to chromatography (± 20 min)	0.75
	Time from chromatography to scanning (± 20 min)	1.35

Note: The values are mean value of 6 observations (N=6)

Repeatability of measurement: Repeatability of measurement of peak area was determined by spotting standard stock solution of LEV, PSE, and AMB in a concentration of 100, 400, 700 ng/spot of LEV, 600, 2400, 4200 ng/spot of PSE, and 1200, 4800, 8400 ng/spot of AMB, six times on TLC plate by semiautomatic spotter. After developing the plate, separated spots of LEV, PSE, and AMB were scanned without changing position of the plate and % RSD for measurement of peak areas were calculated.

Limit of detection and limit of quantification: Limits of detection (LOD) and Limits of quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ. The LOD and LOQ were determined by measuring the magnitude of analytical background by injecting a blank and calculating the signal-to-noise ratio for LEV, PSE, and AMB by injecting a series of solutions until the S/N ratio 3 was obtained for the LOD and 10 for the LOQ. The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. To determine the LOD and LOQ, serial dilutions of mixed standard solution of LEV, PSE, and AMB were made from the standard stock solution in the range of 10 ng ml^{-1} – 100 ng ml^{-1} of LEV, 10 ng ml^{-1} – 100 ng ml^{-1} of PSE, and 10 ng ml^{-1} – 100 µg ml^{-1} of AMB. The samples were injected in the LC system and the chromatograms were run and a measured signal from the samples was compared with those of blank samples.

Robustness of the method: To evaluate the robustness of a HPTLC method, few parameters were deliberately varied. The robustness of the method was studied, during method development at three different concentration levels of 100 ng/spot, 400 ng/spot and 700 ng/spot for LEV, 600 ng/spot, 2400 ng/spot, 4200 ng/spot for PSE, and 1200 ng/spot, 4800 ng/spot, and 8400 ng/spot for AMB by determining the effects of small variation, mobile phase composition (±0.1 %), amount of mobile phase (±5 %), time from spotting to chromatography (±20 min) and scanning time (±20 min). Each concentration was applied in six times and % RSD was calculated.

Specificity: To confirm the specificity of the proposed method, sample stock solution of marketed formulation of LEV, PSE, and AMB at three different concentrations of 100, 400, 700 ng/spot for LEV, 600, 2400, 4200 ng/spot PSE, and 1200, 4800, 8400 ng/spot AMB were spotted on TLC plate, developed and scanned as described earlier. The peak purity of LEV, PSE, and AMB were assessed by comparing the spectra at three different levels i.e., peak start, peak apex and peak end positions of the spot.

Recovery: Recovery studies were carried out by applying the method to drug sample to which known amount of LEV, PSE, and AMB corresponding to 50, 100 and 150 % of label claim had been added (standard addition method). At each level of the amount, six determinations were performed and the results obtained were compared with expected results.

Stability in sample solution: Three different concentrations of sample solution 100 ng $µl^{-1}$, 400 ng $µl^{-1}$, 700 ng $µl^{-1}$ for LEV, 600 ng $µl^{-1}$, 2400 ng $µl^{-1}$, 4200 ng $µl^{-1}$ PSE, and 1200 ng $µl^{-1}$, 4800 ng $µl^{-1}$, 8400 ng $µl^{-1}$ AMB were stored at room temperature for 24 h. Then applied on plate, the plate was developed with optimized mobile phase and scanned at 212 nm. The standard deviation and % RSD of peak areas were calculated.

Analysis of the marketed formulation: The sample stock solution was further diluted to get sample solutions at three different concentrations i.e. 100 ng/µl, 400 ng/µl, 700 ng/µl of LEV, 600 ng/µl, 2400 ng/µl, 4200 ng/µl of PSE and 1200 ng/µl, 4800 ng/µl, 8400 ng/µl of AMB. They were spotted on the plate followed by development with optimized mobile phase and scanning at 212 nm. The analysis was repeated for six times. Each spot was resolved into three peaks in the chromatogram of drug samples, extracted from the marketed formulation. The content of drug was calculated from the peak areas recorded.

Table 6: Recovery Studies by HPTLC.

Drug	Label claim (mg/tab)	Amount Added (%)	Total amount (mg)	Amount Recovered (mg)	Recovery (%)
LEV	5	50	7.5	7.42	98.93
		100	10	10.01	100.12
		150	12.5	12.38	99.04
PSE	30	50	45	45.42	100.93
		100	60	59.23	98.71
		150	75	74.78	99.70
AMB	60	50	90	90.23	100.15
		100	120	120.31	100.25
		150	150	149.73	99.82

Note: The values are mean value of 6 observations (N=6)

RESULTS AND DISCUSSION

*Selection of analytical wavelength:*The UV spectra of LEV, PSE and AMB scanned in UV spectrophotometer shows λ-max at 210 nm, 212 nm, and 208 nm respectively. In situ UV spectra of LEV, PSE and AMB by HPTLC (Figure 4) show λ-max at 210 nm, 212 nm, and 227 nm respectively. After overlain convenient point was found at 212 nm. Therefore 212 nm was selected as scanning wavelength for HPTLC study of LEV, PSE and AMB in combination.

Table 7: Stability of LEV, PSE and AMB in sample solutions by HPTLC.

Parameter	LEV	PSE	AMB
% RSD of area	1.36	1.44	1.13

Note: The values are mean value of 6 observations (N=6)

Optimization of HPTLC method: Initially many different combinations of mobile phases were tried for the method development. tolune: ethyl acetate, Toluene: Ethyl acetate: methonal, in different proportions were tried. It was found that in the ethyl acetate: methonal the movement of the drug was good but poor peak shape was observed. In Ethyl acetate: methanol: ammonia (8: 1: 0.5 v/v/v), it shows a good resolution, sharp and symmetrical peak, of LEV, PSE, and AMB resolved with R_F 0.1 ± 0.02, 0.39 ± 0.05, and 0.73 ± 0.05 respectively, (Figure 5.) when scanned densitometrically at 212 nm. The saturation time for mobile phase was 30 min at room temperature (25°C ± 2) at relative humidity of 60 % ± 5.

*Validation of the method:*The results of validation studies on the Simultaneous estimation of LEV, PSE and AMB in the current study using optimized mobile phase and optimized chromatographic conditions for HPTLC are given below.

Linearity and range: For linearity and range of LEV, PSE and AMB by HPTLC, seven concentrations 100 ng μl^{-1}, 200 ng μl^{-1},300 ng μl^{-1}, 400 ng μl^{-1}, 500 ng μl^{-1}, 600 ng μl^{-1}, 700 ng μl^{-1} of LEV, 600 ng μl^{-1}, 1200 ng μl^{-1},1800 ng μl^{-1}, 2400 ng μl^{-1}, 3000 ng μl^{-1}, 3600 ng μl^{-1}, 4200 ng μl^{-1} of PSE and 1200 ng μl^{-1}, 2400 ng μl^{-1}, 3600 ng μl^{-1}, 4800 ng μl^{-1}, 6000 ng μl^{-1}, 7200 ng μl^{-1}, 8400 ng μl^{-1} of AMB were prepared from standard stock solution (containing 0.5 mg/ml LEV, 3 mg/ml PSE and 6 mg/ml AMB) in optimized mobile phase. Each concentration was injected in six times. The LEV, PSE, and AMB showed linear increase in area by increasing concentration in a range of 100 ng μl^{-1} to 700 ng μl^{-1} for LEV, 600 ng μl^{-1} to 4200 ng μl^{-1} for PSE and 1200 ng μl^{-1} to 8400 ng μl^{-1} for AMB (Table 1) (Figure 6)with good correlation coefficient of (r^2=0.999 for each). (Table 2) The average % RSD was in the acceptable limit.

Table 8: Applicability of the proposed method for determination in commercial tablets.

Drug	Label Claim (mg/tablet)	Amount found (mg)	Drug Content (%)	RSD (%)
LEV	5	4.81	98.02	0.88
PSE	30	29.64	98.81	1.12
AMB	60	60.01	100.01	1.52

Note: The values are mean value of 6 observations (N=6)

Precision: The Intraday precision and Inter day precision experiments are shown in Table 3, for LEV, PSE and AMB respectively. The developed method was found to be precise as the % RSD values for repeatability and intermediate precision studies were < 2%, as recommended by ICH guideline.

Repeatability: Repeatability of sample application and repeatability of measurement of peak area showed that the standard deviation and %RSD values are below the

instrumental specifications, ensuring proper functioning of HPTLC system. (Table 4)

LOD and LOQ: The signal : noise ratios 3:1 and 10:1 were considered as LOD and LOQ respectively. The limit of detection is found to be 25 ng $spot^{-1}$ 40 ng $spot^{-1}$ and 35 ng $spot^{-1}$ for LEV, PSE and AMB respectively. The limit of quantitation is found to be 60 ng $spot^{-1}$, 87 ng $spot^{-1}$ and 71 ng $spot^{-1}$ for LEV, PSE and AMB respectively.

Robustness of the method: Robustness of the method was tested by small changes in parameters and the effects on the results were examined. The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 2%. The values of %RSD are as shown in Table 5 indicates robustness of the HPTLC method.

Specificity: It was observed that excipients present in formulation did not interfere with peaks of (R_f 0.10 ± 0.02 for LEV, R_f 0.39 ± 0.05 for PSE and R_f, 0.73 ± 0.05 for AMB). The specificity of the method was also confirmed by overlaying the spectra of standard and sample of LEV, PSE and AMB recorded on TLC scanner in UV range.

Recovery studies: Recovery studies of the drugs were carried out for the accuracy parameter. These studies were carried out at three levels i.e. multiple level recovery studies. Sample stock solutions in three 10 ml volumetric flasks from tablet formulation (149.46 mg i.e. average weight of 20 tablet) was prepared. To the above prepared solutions, 50 %, 100 % and 150 % of the standard drug solutions were added. Dilutions were made and recovery studies were performed. The recovery of LEV, PSE and AMB was found that 99.36%, 99.78%, and 100.07% respectively. % Recovery was found to be within the limits as listed in Table 6.

Stability in sample solution: Three sample stock solutions containing three different concentrations 100 ng/µl, 400 ng/µl and 700 ng/µl of LEV, 600 ng/µl, 2400 ng/µl, and 4200 ng/µl of PSE and 1200 ng/µl, 4800 ng/µl, and 8400 ng/µl of AMB were prepared from sample solution and stored at room temperature for 3 days. No additional peak was found in the chromatogram that mean drug is stable in sample solutions (Table 7).

Analysis of a formulation: Three spots at R_f 0.10 ± 0.02 for LEV, R_f 0.39 ± 0.05 for PSE and R_f, 0.73 ± 0.05 for AMB were observed in the chromatogram of drug samples extracted from conventional tablets. There was no interference from the excipients present in the conventional tablets. After analysis of the formulation, the drug content found were 99.01 ± 0.45, 99.29 ± 0.74 and 100.03 ± 0.65 for LEV, PSE and AMB respectively. This indicate that there is no degradation of LEV, PSE and AMB in marketed formulation that were analyzed by this method (Table 8)

CONCLUSION

The proposed HPTLC method provide simple, accurate, and reproducible for quantitative determination of LEV, PSE, and AMB in pharmaceutical tablets, without interference from the excipients. The chromatographic method is validated according to ICH guidelines. Statistical tests indicate that the proposed method reduces the duration of analysis and appears to be equally suitable for the routine analysis in pharmaceutical formulation in quality control laboratories, where time is essential.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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