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Method development and validation of Prevpac combination therapy drugs in spiked human plasma, Deplin and Spasmonal in pharmaceutical dosage forms by Rp-hplc methods

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Abstract
The intention of the present work is to develop simple, precise and accurate RP-HPLC methods for the estimation of Prevpac combination therapy drugs (30 mg capsules of lansoprazole; LAN, 500 mg capsules of amoxicillin; AMX and 500 mg tablets of clarithromycin; CLM) in spiked human plasma, Method A; Deplin tablets (15 mg levomefolate calcium; LMF) in pharmaceutical formulation, Method B and Spasmonal (60 mg capsules of alverine citrate; ALV) in marketed formulation, Method C. Prevpac combination therapy is employed to treat ulcer effectively, since the dosages being given separately, there is need to study the protein binding of these drugs in plasma to estimate their absorbance in plasma. The effective chromatographic separation was accomplished on phenomenex C18 (250 x 4.6 mm, 5 µm particle size) column for all the methods. The methods were optimized by isocratic elution of mobile phase constituting Acetonitrile and phosphate buffer (pH 6) in the ratio of 70:30 set at a flow rate of 0.6 mL/min monitored at 227 nm for Method A, methanol : acetate buffer (pH4.4) in a ratio of 80:20 %v/v and studied at 290 nm, Acetonitrile: Buffer pH (5) in the ratio of 80:20, %v/v for method B and Acetonitrile: Buffer pH (5) in the ratio of 80:20, %v/v and monitored at 215 nm for Method C, respectively. The liquid chromatography methods were extensively validated and all the parameters were within the acceptance criteria with correlation of 0.999, retention time less than 6 min and percentage RSD less than 2 for all the three methods. The methods were proved to be more accurate, simple, precise and rapid by statistical validation, recovery studies.

Keywords: Amoxicillin (AMX), Lansopraxole (LAN), Clarithromycin (CLM), Levomefolate calcium (LMF), Alverine citrate (ALV), Method A/B/C

Introduction
Pharmaceutical combination therapy is multiple therapies employed to treat a *single* disease achieved by prescribing/administering separate drugs, or dosage forms that contain more than one active ingredient. PREVPAC combination therapy comprise 500 mg capsules of AMX, 30 mg capsules of LAN and 500 mg tablets of CLM, employed to treat ulcer effectively. AMX is moderate-spectrum, bacteriolytic, β-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms. Lansoprazole, proton-pump inhibitor (PPI) which inhibits the stomach's production of gastric acids while Clarithromycin is used in the treatment of bacterial infections caused in middle ear. Deplin tablets constitute L-Methylfolate, Levomefolate calcium are prescribed treat low folate levels whereas Spasmonal capsules are administered as antispasmodic and an adrenergic-β3 receptor agonist.
Literature survey revealed that there were few LC methods for the estimation AMX [1-14], LAN [15-19] and CLM [20, 21] individually and in combination with other drugs but no method has been far developed for the estimation of these three drugs; no analytical methods has been developed for estimation of LMF while few LC/MS and colorimetric methods have been reported for the estimation of ALV [22, 23].
Since the Prevpac combination therapy drugs are administered separately as different dosage forms by oral route, there is necessity to study the additive effect of the drugs by studying protein binding and absorbance of these drugs in plasma. Deplin tablets and Spasmonal capsules being newer pharmaceutical agents, call for the newer method development is indeed. The resolution of the present work is to develop simple, precise, accurate and economical RP-HPLC methods for the estimation of Prevpac combination therapy drugs in spiked human plasma; Deplin and Spasmonal in pharmaceutical formulations and to validate the method according with ICH guidelines.

Materials and Methods

Equipment

SHIMADZU LC-20AD system with SPD-20A UV-Vis detector equipped with Sphinchrom software was used for method development, double-beam Perkin Elmer (LAMBDA 25) UV-Vis spectrophotometer was used for spectral measurements and ELICO pH meter was used for pH measurements.

Reagents and standards

AMX, LAN, CLM, LMF and ALV were obtained as a gift sample from Aurobindo Pharma Ltd, Hyd., Acetonitrile and Triple distilled water of HPLC grade, 0.2 M potassium dihydrogen phosphate (KH_2PO_4), 0.2 M sodium hydroxide (NaOH) and 10 M Potassium hydroxide (KOH) which are of AR grade were used for the experimental work.

Method Development

Chromatographic conditions

The chromatographic separation of all the three methods was accomplished effectively on Phenomenex C18 (250 x 4.6 mm, 5 µm particle size) column for all the methods. The optimization of the methods was achieved by isocratic elution of mobile phase constituting Acetonitrile and phosphate buffer (pH 6) in the ratio of 70:30 set at a flow rate of 0.6 mL/min monitored at 227 nm for Method A, methanol : acetate buffer (pH4.4) in a ratio of 80:20 %v/v and studied at 290 nm, Acetonitrile: buffer pH (5) in the ratio of 80:20, %v/v for method B and Acetonitrile: buffer pH (5) in the ratio of 80:20, %v/v and monitored at 215 nm for Method C, respectively. The methods were developed at ambient temperature.

Preparation of mobile phase

Method A, preparation of phosphate buffer, pH 6

125.0 ml of 0.2 M potassium dihydrogen phosphate was placed in a 250-ml volumetric flask, 14 mL volume of 0.2 M sodium hydroxide was added and then volume was made with water.

Method B, preparation of acetate buffer, pH 4.4

136 g of sodium acetate and 77 g of ammonium acetate was taken and dissolved in 1000 ml of water. 250 ml of glacial acetic acid was added to the final volume.

Method C, preparation of phosphate buffer, pH 5

6.8 g of potassium dihydrogen phosphate was diluted in 1000 ml of water and the pH was adjusted to 5.0 with 10 M potassium hydroxide.

Acetonitrile and phosphate buffers were filtered through 0.45 µm membrane filtered and sonicated before use.

Preparation of stock solutions of Method A

AMX, About 50 mg of AMX was weighed in to a 50 ml clean dry volumetric flask, dissolved and diluted to volume with diluent and sonicated for 5 min.

LAN, About 50 mg of LAN was weighed in to a 50 ml clean dry volumetric, dissolved and diluted to volume with diluent and sonicate for 5 min.

CLM, About 250 mg of clarithromycin was weighed in to a 50 ml clean dry volumetric, dissolved and diluted to volume with diluent and sonicated for 5 min.

Preparation of stock solutions of Method B (LMF)

25 mg of LMF was weighed into a 25 ml clean dry volumetric flask, 10 ml of diluents was added and sonicated for 5 min and made the final volume with diluent.

Preparation of stock solution of Method C (ALV)

50 mg of ALV into a 50 ml was weighed in a clean dry volumetric flask, 30 ml of diluent was added and made to the final volume with diluent.

Sample preparation

Commercial formulations, Method A (Prevpac: 30 mg LAN capsules, 500 mg AMX capsules and 500 mg CLM tablets), Method B (LMF tablets) and Method C (ALV capsules) were taken as twenty tablets/capsules of each and weighed, ground to powder. Average weight was calculated and transferred into 50 mL volumetric flask; dissolved in mobile phase, diluted to the mark and sonicated for 20 min, filtered through 0.45 µm Whatman filter paper, diluted to get approximate concentration each of six replicates and 20 µL was injected in to the column and chromatographed as per above mentioned conditions.

Calibration standards

Method A

Human plasma was mixed with EDTA to precipitate plasma proteins and centrifuged for 2 min. The supernatant is used further for preparing calibration standards and as blank. Calibration standards are prepared by spiking the plasma with appropriate quantities of working standard to reach 400-100 µg/mL, 100-600 µg/mL and 2000-5000 µg/mL for AMX, LAN and CLM, respectively. One mL of AMX, LAN and CLM added to 8 mL of dichloromethane mixed for 30 s and centrifuged at 3,500 rpm for 15 min. The organic layer was separated and evaporated to dryness under stream of nitrogen stream in a 50 °C water bath. The residue was dissolved in 1 mL of mobile phase and a 20 µL was injected into the HPLC system and the chromatogram was reported in fig1.

Method B/C

Different volumes of stock solutions were accurately transferred to 10 ml volumetric flasks to reach concentration range of 10-40 µg/ml for method B and 100-600 µg/ml for method C, respectively. Six replicate solutions of the above range each of 20 µL were injected into the HPLC system and the chromatogram was shown in fig 2 and 3.

Method optimization

Acetonitrile and phosphate buffer (pH 6) in the ratio of 70:30%, v/v, for method A, methanol and acetate buffer (pH 4.4) 80:20 %, v/v, for method B and acetonitrile and phosphate buffer (pH 5) 80:20%, v/v, for method C, respectively were selected as the mobile phase, as it was found ideal to resolve the peaks with retention time 3.5 ± 0.022 min 4.52±0.031 and 5.56 ± 0.021 min for AMX, CLM and LAN, respectively for method A, 4.867±0.04 min for LMF, method B and 4.18±0.03 min for methods C, respectively and shown in the fig. 4, fig. 5 and fig. 6, respectively. Detection wavelength was chosen by scanning the analytes over a range of wave length from 190-400 nm in a spectrophotometer and the suitable wavelength was found to be 227 nm for method A and 290 nm for method B and 215 nm for method C, respectively. The overlay UV spectra of both the components and their binary mixture were shown in fig. 4, fig. 5 and fig. 6 and the optimization parameters were produced in table 1.

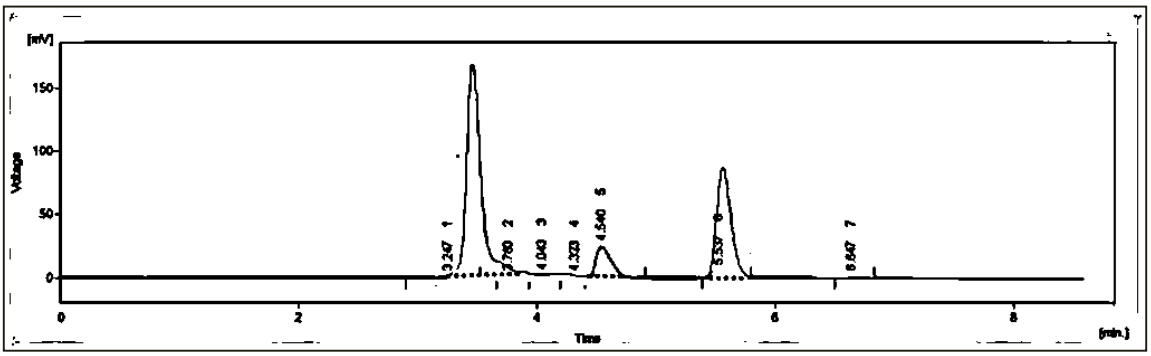

Fig 1: Chromatogram of AMX, LAN and CLM in plasma

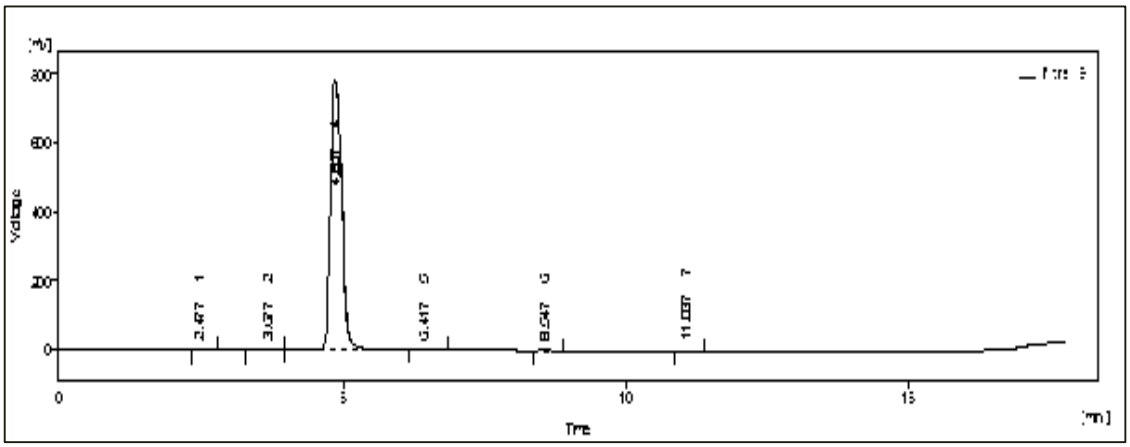

Fig 2: Chromatogram of LMF

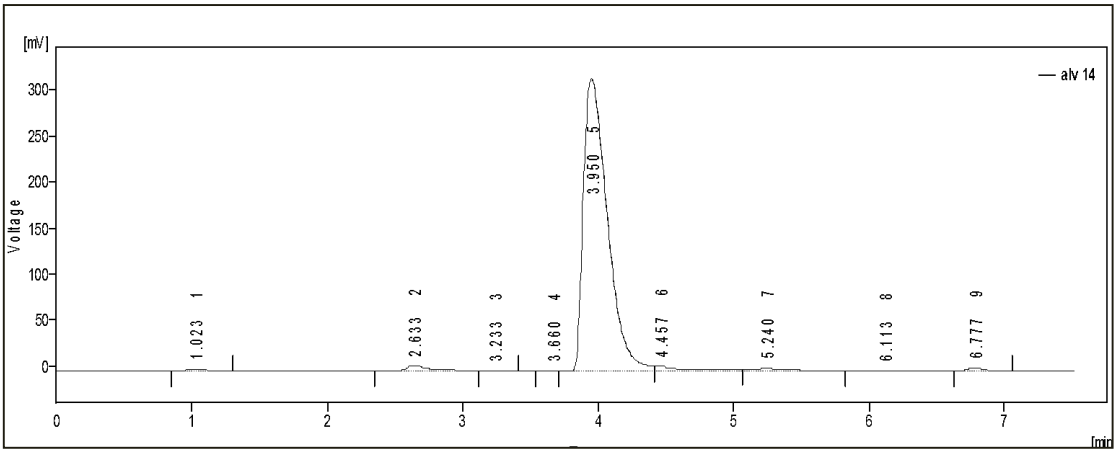

Fig 3: Chromatogram of ALV

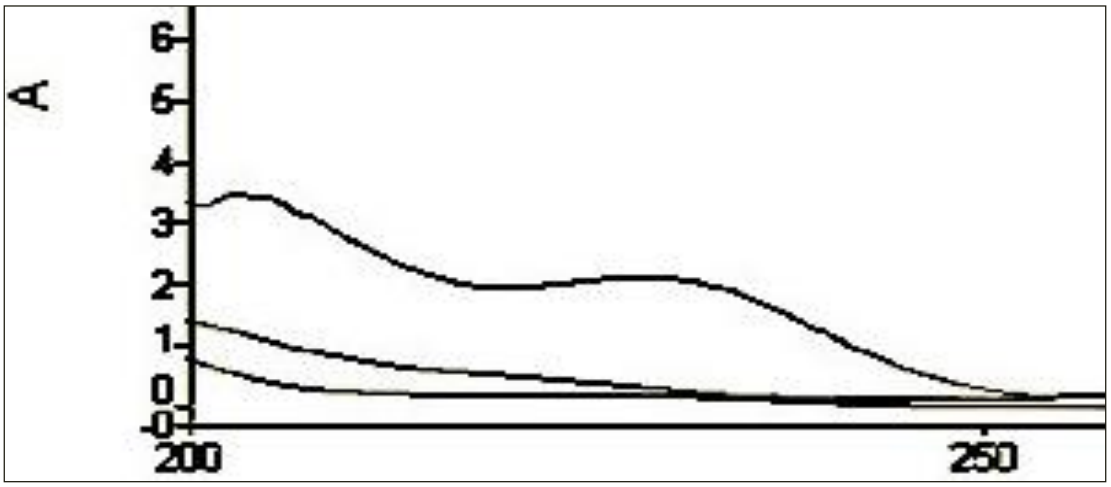

Fig 4: Overlay spectra of AMX, LAN and CLM

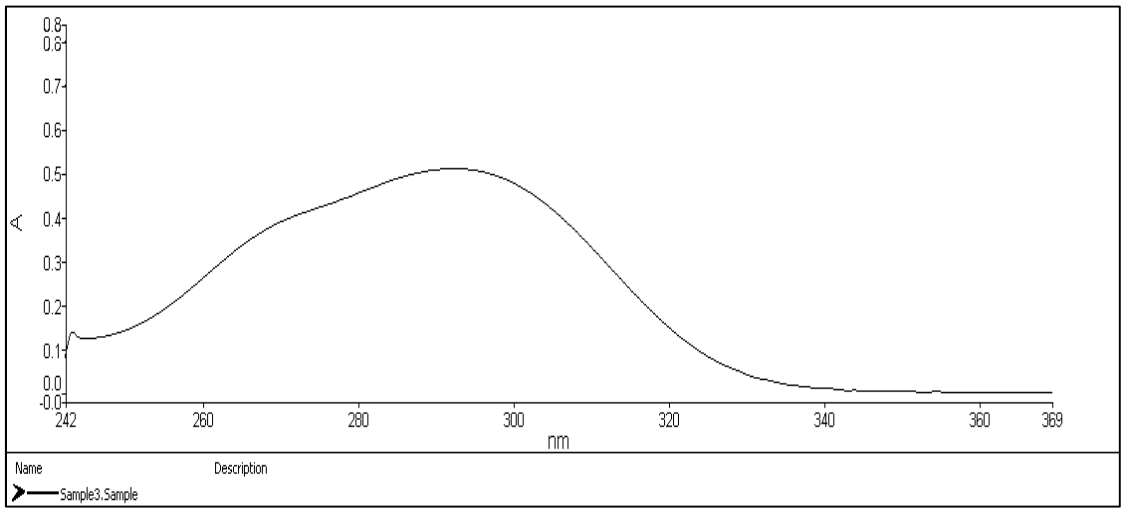

Fig 5: Absorption spectrum of LMF

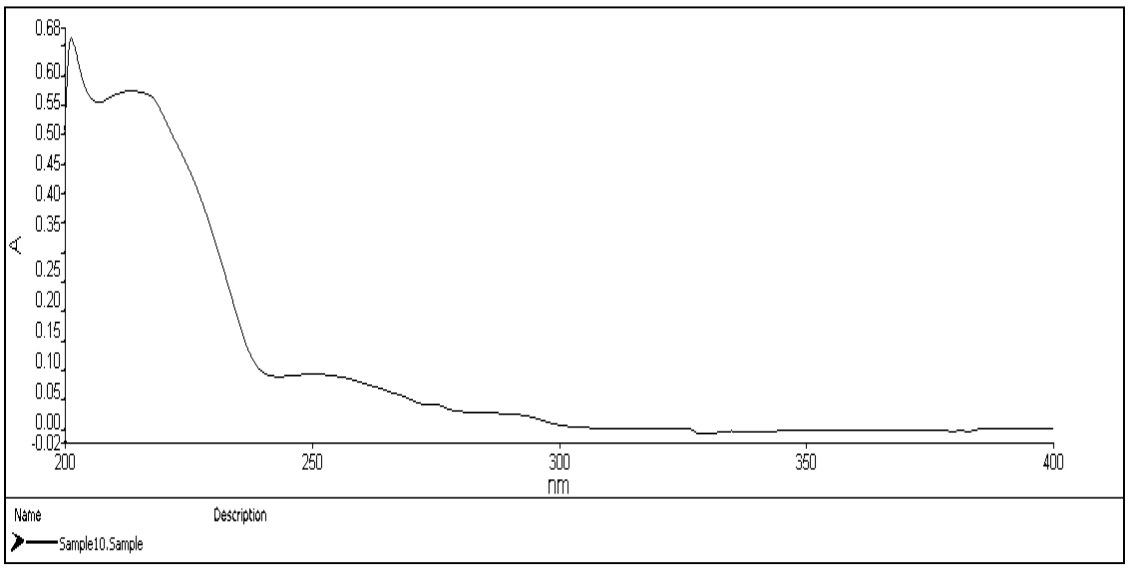

Fig 6: Overlay spectra of ALV

Table 1: Optimization parameters of Method A and Method B

Parameters	Method A			Method B	Method C
	AMX	CLM	LAN	LMF	ALV
Column	C_{18} column (250 x 4.6 mm, 5 µ)				
Run time	10 min				
Diluent	Mobile phase				Acetonitrile
Retention time	3.523	4.520	5.560	4.867	4.18
Linearity µg/mL)	400-1000	2000-5000	100-600	10-40	100-600
LOD (µg/mL)	0.41	155	1.118	1.17	11.11
LOQ (µg/mL)	23	469	3.32	3.15	33.64
Mean Area	1523	5358	334	10445	6820
SD	5.612	5.215	6.017	22.27	41.34
%RSD	0.31	1.33	0.92	0.21	0.60
Theoretical plates	8,826	19,342	25,235	7830	9274
Tailing factors	0.9	0.8	0.5	1.01	1.01
Resolution factor	_	4	3.43	-	-
Symmetry factor	1.07	1.03	0.99	0.99	1.03

Results and Discussions

Method validation

After development of HPLC methods, method A, method B and method C were carried out with respect to several parameters like precision, accuracy, linearity, robustness, ruggedness to ensure that the developed method copes with all the requirements for the intended purpose.

System suitability studies

Standard solutions of AMX, LAN and CLM (Method A); LMF (Method B) and ALV (Method C) were prepared, standards set as per test method and was injected five times in to HPLC system. The chromatograms were recorded and system suitability parameters were given in table 1.

Linearity

Chromatograms were recorded by preparing series of solutions and calibration graph was plotted with area in the Y-axis and concentration of standard solution on the X-axis as follows and results were presented in table 2 and pictured in fig. 7, fig. 8, fig. 9, fig. 10 and fig. 11.

Table 2: Results showing linearity values of method A, method B and Method C

S. No	Method A						Method B		Method C	
	AMX		LAN		CLM		LMF		ALV	
	Conc. (µg/ml)	Area, mv	Conc. (µg/ml)	Area, mv	Conc. (µg/ml)	Area, mv	Conc. (µg/ml)	Area, mv	Conc. (µg/ml)	Area, mv
1	400	997	100	1679	2000	230	10	4269	100	2138
2	500	1253	200	3528	2500	282	15	6231	200	3891
3	600	1501	300	5591	3000	340	20	8457	300	5916
4	700	1751	400	7303	3500	393	25	10648	400	8022
5	800	1998	500	8905	4000	450	30	12338	500	9859
6	900	2231	600	10503	4500	502	35	14555	600	11097
7	1000	2455	-	-	5000	555	40	16623	-	-

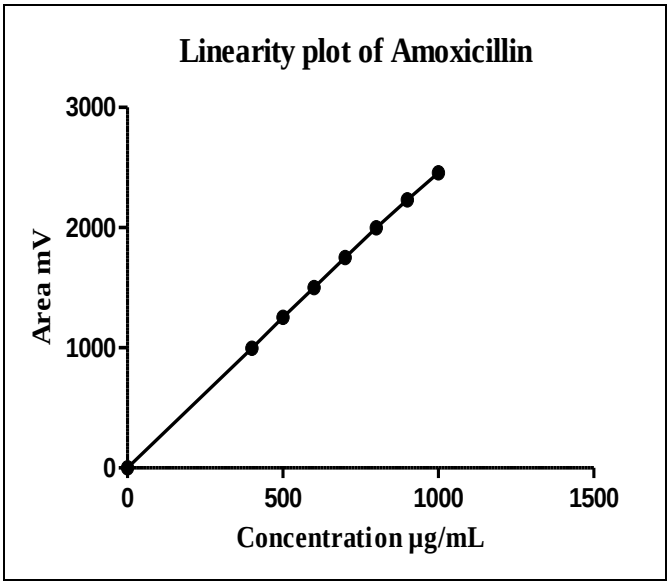

Fig 7: Linearity plot of AMX

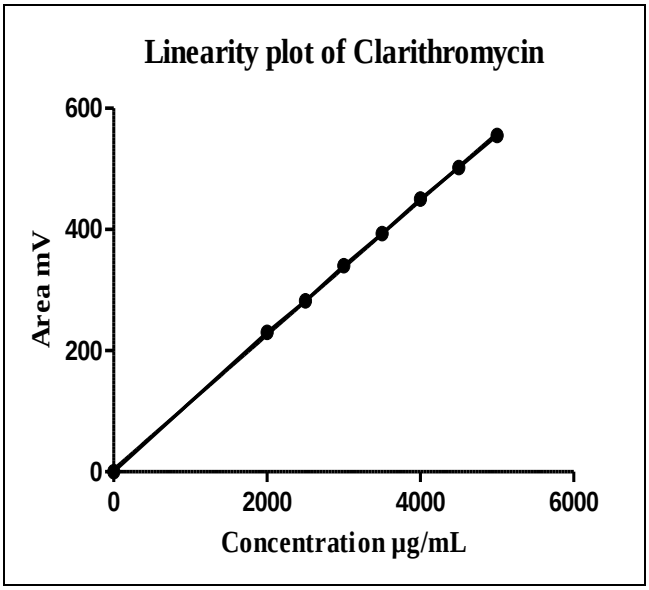

Fig 9: Linearity plot of CLM

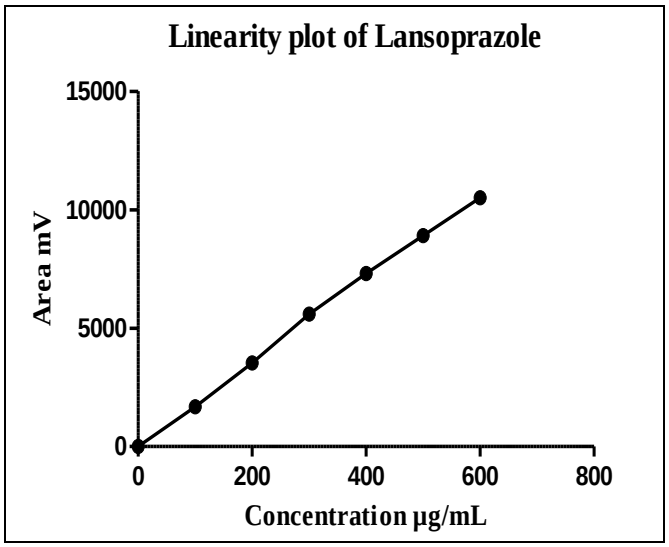

Fig 8: Linearity plot of LAN

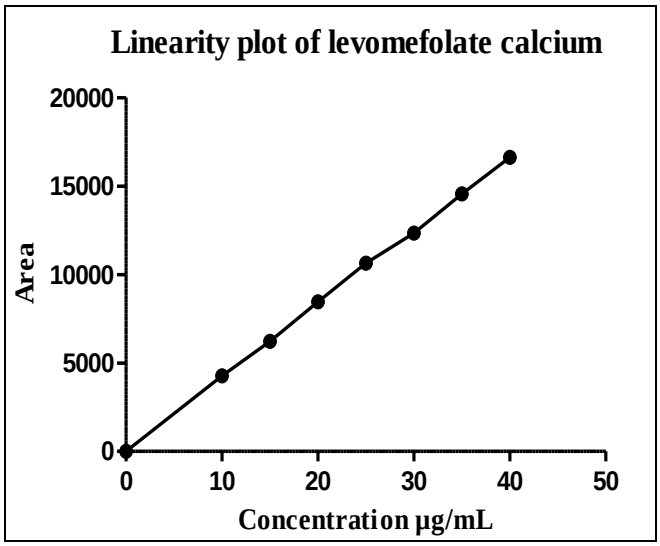

Fig 10: Linearity plot of LMF

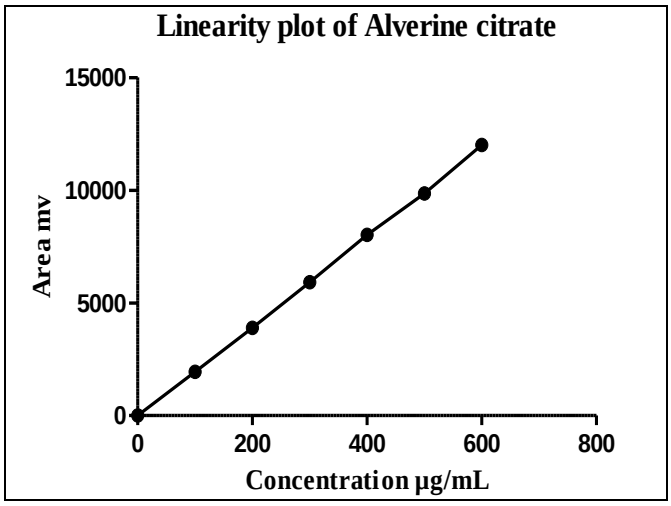

Fig 11: Linearity plot of ALV'

Precision

Method precision and system precision were preformed preparing six replicates of each standard and sample solutions, respectively and the results were presented in table 3.

Table 3: Results showing method precision and system precision

Parameter	Method Precision					System Precision				
	Method A			Method B		Method A			Method B	Method C
	AMX	LAN	CLM	LMF	ALV	AMX	LAN	CLM	LMF	ALV
Sample 1	98.5	98.9	100.1	99.97	99.98	1751	7303	388	4900	8686
Sample 2	100.2	99.6	98.6	99.68	100.6	1747	7300	394	4867	8588
Sample 3	99.3	97.8	100.2	98.99	100.03	1754	7309	386	4967	8695
Sample 4	99.4	100.1	98.7	100.03	99.19	1757	7299	391	5124	8687
Sample 5	98.6	98.8	99.4	100.1	101.06	1761	7304	398	5023	8693
Sample 6	100.3	99.5	99.3	101.02	100.4	1754	7301	390	4978	8682
Mean	99.3	99.1	99.3	99.97	100.2	1754	7303	391	8440	8671.833
SD	0.7	0.8	0.7	0.065	0.63	5.020	3.93	4.665	28.45	41.34449
% RSD	0.765	0.8	0.765	0.6	0.6	0.28	0.058	1.19	0.337	0.47

SD, Standard Deviation; RSD, Relative Standard Deviation

Accuracy

To evaluate the accuracy of the proposed methods recovery studies were carried out by standard addition method, where a known amount of each drug is added to, a pre analyzed tablet powder at 3 levels of 50%, 100%, 150% of method A, method B and method C, respectively. At each level recovery studies were carried out in triplicate and expressed as percent recoveries. The results were presented in table 4 for Method A and Method B, respectively.

Table 4: Results of Accuracy studies of Method A and Method B

S. NO.	Method A						Method B		Method C	
	AMX		LAN		CLM		LMF		ALV	
	% Recovery*	% RSD	% Recovery*	% RSD	% Recovery*	% RSD	% Recovery	% RSD	% Recovery	% RSD
50%	99.99	0.35	99.17	0.7	100.1	0.15	100.6	0.67	99.87	0.21
100%	99.5	0.36	99.85	0.7	99.93	0.51	100.7	0.49	99.97	0.15
150%	100.4	0.25	99.78	0.3	99.99	0.19	100.5	0.76	99.93	0.23

*Three determinations

Robustness

Robustness is measured by reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Results were reported in table 1

Ruggedness

Ruggedness is measured by studying system to system/Analyst to Analyst/column to Column variability and the results were shown in table 1.

Limit of detection and Limit of quantification

The LOD and LOQ of the developed methods were determined by analyzing progressively lower concentrations of the standard solutions using optimized chromatographic conditions. The results were shown in table 1

Conclusion

The proposed methods were found to be sensitive, economical, accurate and precise and suitable for quantification in marketed dosage forms without any interferences.

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