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Development and Validation of RP-HPLC Method for Simultaneous Estimation of Cyclosporine A in Combination with α-Linolenic Acid

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A suitable isocratic reverse-phase HPLC method was developed for the simultaneous estimation of cyclosporine A and α-linolenic acid (ALA) in their synthetic mixture formulated to evaluate the synergistic therapeutic effect. The reversed phase HPLC chromatographic method was developed to obtain two separate peaks within short retention time period (30 min run time), without any interference from excipients peaks. The chromatographic separation was achieved on C_{18} column (5 µm, 4.6 × 250 mm) at 50 ± 0.3 ºC. The analytes were eluted at a flow rate of 0.8 mL/min using ultraviolet detector at 210 nm with mobile phase of 1% v/v orthophosphoric acid in water: acetonitrile:2-propanol (25:60:25 v/v/v). The proposed method was validated for specificity, precision, linearity, accuracy and system suitability. All validation parameters were within the acceptable range.

Keywords: Cyclosporine A, α-Linolenic acid, Reverse-phase HPLC method.

INTRODUCTION

Cyclosporine A is used as an immunosuppressant drug in post organ transplantation to prevent rejection [1,2]. It has been used widely for treatment of certain autoimmune diseases such as severe rheumatoid, arthritis, psoriasis and inflammatory skin conditions. Formulation of cyclosporine A as micro and nanoparticulate systems have been studied in recent years to improve bioavailability by oral, ophthalmic and topical route [3-5]. In this context, a microparticulate drug delivery systems is developed comprising cyclosporine A and α-linolenic acid in proper ratio to treat disorder of eyes. Since the current research involved the development and evaluation of novel pharmaceutical dosage forms for ophthalmic administration of cyclosporine A and α-linolenic acid, the first important step was to have a suitable analytical method to quantify the drugs simultaneously in the dosage form. Hence, there was a need to carry out the method development and validation for the simultaneous estimation of cyclosporine A-autoimmune drug and α-linolenic acid-ω3 fatty acid in ophthalmic dosage form by suitable analytical method as per ICH guidelines.

Several analytical methods have been described for quantification of these drugs individually. Number of methods reported in literature for assaying cyclosporine A are based elution of cyclosporine A at high temperatures using HPLC with ultra-violet detection [6-10] and at relatively low UV absorbance wavelength [11,12].

Other methods for cyclosporine A include radioimmunoassay (RIA), which is not suitable for routine anal [13] and HPLC coupled with mass-spectrometry [14,15], which requires a very expensive equipment. The difficulty with all HPLC-UV methods is related to cyclosporine A's lack of chromophores which imply the use of short-wavelength ultraviolet light detection (*e.g.* 205, 210 nm) and many molecular species absorb energy at this wavelength [9].

USP 38 describes analysis of cyclosporine A by HPLC method [16], however no official pharmacoepoeia contains analytical method for fatty acid α-linolenic acid. The number of extraction procedures [17,18] for extraction of different fatty acids and quantification methods to determine the fatty acid composition in drying oils like linseed, walnut, borage, soybean and poppy seed are developed. The fixed oil of flax seed containing

ω-fatty acids have been extracted using ultrasound-assisted extraction, microwave assisted extraction and supercritical fluid extraction (SFE) and evaluated by GC-MS, FT-IR spectroscopy and HPTLC [18-21]. α-Linolenic acid in perilla oil was measured using the reversed-phase high performance liquid chromatography coupled with evaporative light-scattering detector (RP-HPLC-ELSD) [20]. High-performance liquid chromatography system, equipped with a photo diode array detector was established for the determination of α-linolenic acids and other unsaturated fatty acids in vegetable oils [21].

The aim of the present study was to develop a simple and reliable HPLC-UV method to quantify the cyclosporine A and α-linolenic acid in ophthalmic drops simultaneously. Moreover, the analytical method was validated for different analytical parameters.

EXPERIMENTAL

Cyclosporine A reference standard was obtained as a gift sample by Panacea Biotech, Navi Mumbai and α-linolenic acid reference standard was purchased from Clearsynth Labs Ltd. (Mumbai). Ophthalmic liquid formulation containing both drugs was prepared in-house for simultaneous estimation using RP HPLC method. HPLC grade acetonitrile, propan-2-ol and ACS grade orthophosphoric acid were purchased from Rankem. PTFE syringe filters were purchased from Millipore Corp. (USA). HPLC ready deionized water was obtained from a Mill-Q gradient A-10 water purification system, Millipore Corp. (USA).

Chromatographic conditions: The chromatographic technique was performed on LC solutions system with an auto-sampler model LC 2010 CHT (Shimadzu, Japan) 230 V equipped with a quaternary pump, column heater, PDA detector and LC solutions software. Analytical balance (AND make) and Vacuum microfiltration unit with 0.45 µ membrane filter was used in the study.

The sample separation was achieved on Unisphere C_{18} column 250 × 4.6 mm i.d. and 5 µm particle size (Unichrome, Mumbai, India). Column was maintained at 50 ± 0.3 ºC and separation was aided by mobile phase mixture of 1% v/v ortho-phosphoric acid in water:acetonitrile:2-propanol (25:60:25 v/v/v). It was filtered and degassed prior to use. The flow rate was 0.8 mL/min and the injection volume was 20 µL. PDA detector was used at wavelength 210 nm.

Preparation of mobile phase: Buffer solution 1% was prepared by mixing accurately 1 mL of concentrated ortho-phosphoric acid in 100 mL of water. Mobile phase: 60 volumes of acetonitrile and 15 volumes of propan-2-ol were mixed well with 25 volumes orthophosphoric acid (OPA) buffer and sonicated for 5 min.

Preparation of standard stock solution: Accurately weighed 10 mg of cyclosporine A and 10 mg of α-linolenic acid were transferred into a 10 mL volumetric flask separately. Diluent (5 mL) was added to both of these volumetric flasks, cyclosporine A flask was sonicated for 5 min, α-linolenic acid flask was shaken and the final volume was made up with diluent to get standard stock solution 1 and 2 respectively (1000 µg/mL of cyclosporine A and 1000 µg/mL of α-linolenic acid).

Preparation of standard working solutions: A stock solution 1 (1 mL) was diluted to 10 mL with diluent to get 100 µg/mL of cyclosporine A solution. This solution (3 mL) and the standard stock solution 2 containing α-linolenic acid (1 mL) was added to 10 mL volumetric flask and final volume was made up using diluent and labeled as standard stock solution 3 (100 µg/mL of α-linolenic acid and 30 µg/mL of cyclosporine A). Further, 1 mL of stock solution 3 was diluted to 10 mL with diluent to obtain final standard working solution (10 µg/mL of α-linolenic acid and 3 µg/mL of cyclosporine A).

Preparation of sample solution: Accurately weighed ophthalmic gel equivalent to 1 mg of α-linolenic acid and 0.3 mg of cyclosporine A was transferred to 100 mL volumetric flask and dissolved in diluent by sonicating for period of 30 min with shaking intermittently at intervals of 10 min. The flask was shaken and volume was made up to the mark with diluent to get a solution containing 10 µg/mL of α-linolenic acid and 3 µg/mL of cyclosporine A. The solution was filtered through PVDF 0.45 µ syringe filter before filling into injection HPLC vial.

RESULTS AND DISCUSSION

Method development: For determination of working wavelength, a standard stock solution containing cyclosporine A and α-linolenic acid was prepared to give concentration of 10 µg/mL of each drug. The prepared solution was scanned in UV between 200 to 400 nm using diluent as a blank. Different chromatographic conditions were tried for separation and resolution such as change in column, mobile phase ratios, flow rate, temperature, *etc.* and λ_{max} was found to be 210 nm. Unisphere C_{18} column 250 × 4.6 mm i.d. and 5 µm particle size column was found satisfactory. After several initial trials with mobile phase mixtures of methanol, acetonitrile, water, buffer and propan-2-ol in various proportions, the mobile phase mixture of 1% OPA in water:acetonitrile:propan-2-ol (25:60:25) at flow rate of 0.8 mL/min resulted in sharp and separate peaks at 210 nm. Peak purity of cyclosporine A and α-linolenic acid was checked using photodiode array detector and 210 nm was considered satisfactory for detecting both the drugs with adequate sensitivity. The chromatograms of standard and sample are shown in Figs. 1 and 2.

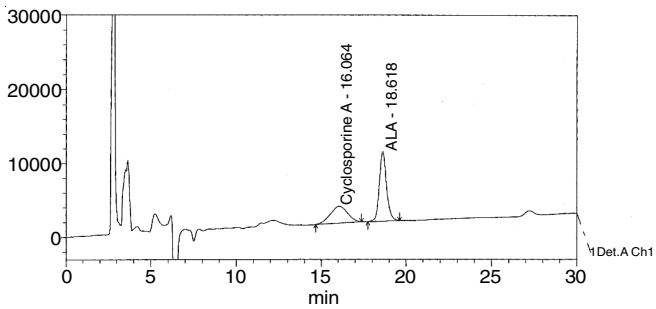

Fig. 1. Chromatogram of cyclosporine A and α-linolenic acid in standard preparation

Linearity was studied by analyzing five standard solutions, which were prepared from cyclosporine A and α-linolenic acid standard stock solutions at concentration levels from 50% to

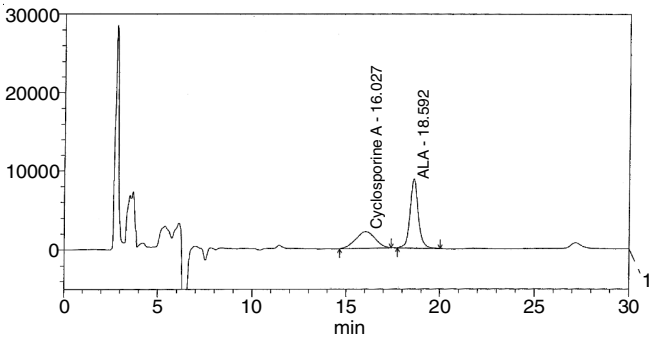

Fig. 2. Chromatogram of cyclosporine A and α-linolenic acid in sample preparation

150% of assay concentration. From standard stock solution 3, aliquots of 2.5, 4, 5, 6 and 7.5 mL were diluted to 10 mL with diluent to give concentrations of 5, 8, 10, 12 and 15 µg/mL of α-linolenic acid and 1.5, 2.4, 3.0, 3.6 and 4.5 µg/mL cyclosporine A. Calibration curve with concentrations *versus* peak area was plotted and the obtained data was treated by least square linear regression analysis. The results of linearity are tabulated as Table-1. The calibration curve of both drugs are shown in Figs. 3 and 4, which show an excellent correlation between peak area and concentration. The correlation coefficients were found to be 0.999 for α-linolenic acid and 0.998 for cyclosporine A, hence indicates linearity of developed method for both the drugs.

TABLE-1
LINEARITY DATA OF α-LINOLENIC ACID AND CYCLOSPORINE A

α-Linolenic acid		Cyclosporine A	
Conc. (µg/mL)	Peak area	Conc. (µg/mL)	Peak area
0	0	0	0
5	64533	1.5	125282
8	110377	2.4	204892
10	148587	3.0	261092
12	187081	3.6	317726
15	237666	4.5	399066

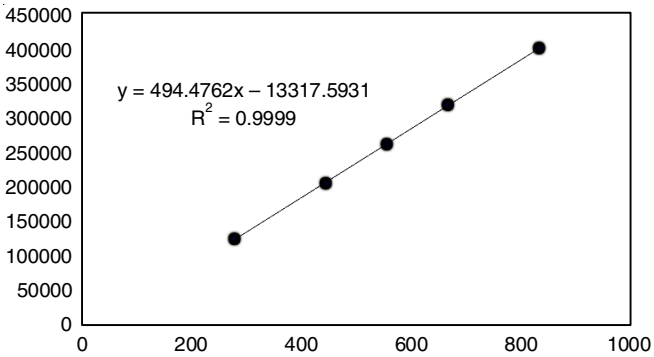

Fig. 3. Linearity chart of α-linolenic acid

Limit of detection and limit of quantification: The lower and the upper limit of detection were 1.5 and 4.5 µg/mL of cyclosporin A and 1.0 and 15.0 µg/mL of α-linolenic acid, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were established at signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ of both drugs were

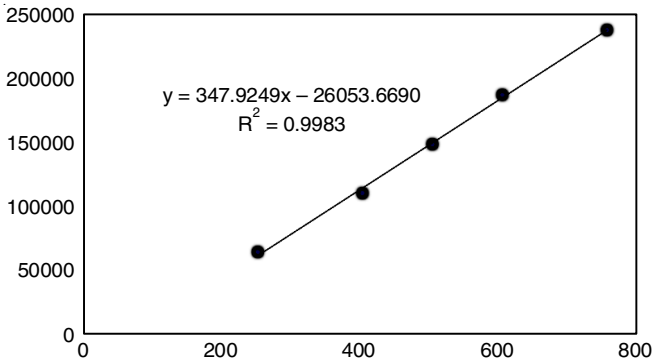

Fig. 4. Linearity chart of cyclosporine A

determined experimentally by injecting six injections of each drug. The results are shown in Table-2.

TABLE-2
LOD AND LOQ VALUES

Parameter	α-Linolenic acid (µg/mL)	Cyclosporine A (µg/mL)
LOD	1.0	1.5
LOQ	5.0	1.5

Precision (repeatability): The precision of the method was verified by repeated injection of 10 µg/mL of α-linolenic acid and 3 µg/mL of cyclosporine A (n = 6) without changing the parameters of proposed chromatographic method (Tables 3 and 4).

TABLE-3
METHOD PRECISION OF α-LINOLENIC ACID

Sample No.	Retention time (min)	Peak area	Assay (%)
1	18.6	268479	99.0
2	18.6	278596	101.2
3	18.6	269875	100.1
4	18.6	271456	100.6
5	18.6	282987	102.8
6	18.6	267482	99.5
Mean	18.6	273146	100.5
%RSD	0.0	2.28	1.35

TABLE-4
METHOD PRECISION OF CYCLOSPORINE A

Sample No.	Retention time (min)	Peak area	Assay (%)
1	16.1	159815	99.7
2	16.1	169476	98.6
3	16.1	165808	96.5
4	16.0	170691	99.6
5	16.1	164704	96.8
6	16.0	172310	100.3
Mean	16.1	168134	98.58
%RSD	0.32	2.76	1.62

Accuracy: To determine accuracy of c developed method, recovery studies were carried out by standard addition method. A known quantity of pure drugs was added to obtain concentration levels 50%, 100% and 150% of desired strengths of α-linolenic acid and cyclosporine A. The contents were then

TABLE-5
RECOVERY DATA OF α-LINOLENIC ACID AND CYCLOSPORINE A IN ACCURACY DETERMINATION

Analytes	% Level	Replicate No.	Peak area	% Recovery	% Mean recovery	% RSD
α-Linolenic acid	50	1	138597	97.2	97.2	0.52
		2	143117	97.8		
		3	136446	96.8		
	100	1	279571	99.8	99.1	0.86
		2	268812	98.2		
		3	271258	99.5		
	150	1	398287	100.2	100.5	0.36
		2	401253	100.9		
		3	399844	100.4		
Cyclosporine A	50	1	81980	101.5	100.8	0.65
		2	80967	100.2		
		3	81458	100.9		
	100	1	170467	101.0	100.4	0.47
		2	169815	100.1		
		3	170058	100.3		
	150	1	258718	101.4	100.5	0.81
		2	249547	100.3		
		3	248355	99.8		

analyzed by the proposed method. Percentage recovery results are given in Table-5.

Specificity: Specificity of proposed method was evaluated by comparing the chromatograms of mobile phase blank, Placebo solution, standard solution and sample solution containing α-linolenic acid and cyclosporine A. For this 20 µL of each of these solutions was injected into HPLC system separately and the chromatograms obtained are shown in Figs. 5-8.

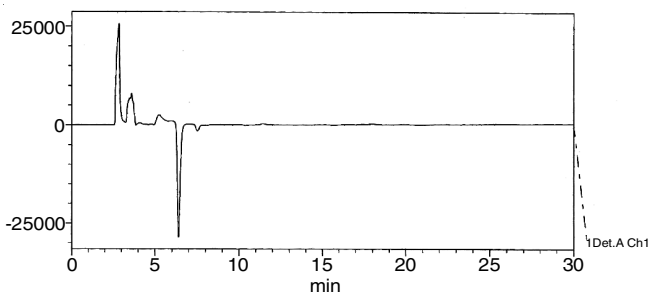

Fig. 5. Chromatogram of mobile phase blank solution

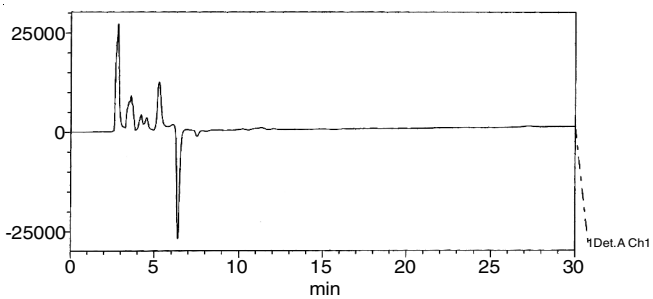

Fig. 6. Chromatogram of placebo solution

It was observed that there were no co-eluting peaks at retention time of drugs indicating no interference from excipients and other degradation products. Thus, the peaks of analytes were pure thereby confirmed the specificity of the method.

System suitability: The parameters of system suitability were evaluated by preparing the standard solution of cyclosporin A (3 µg/mL) and α-linolenic acid (10 µg/mL) as per

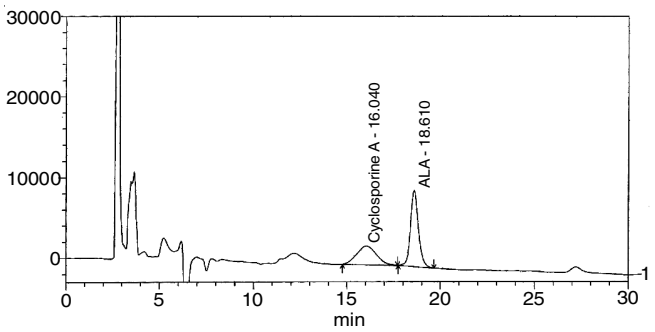

Fig. 7. Chromatogram of standard solution

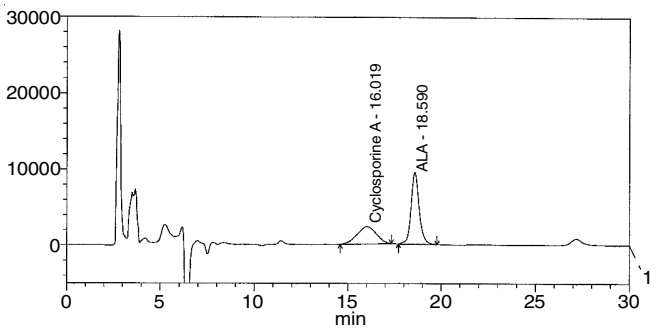

Fig. 8. Chromatogram of sample solution

test method and the solutions were injected six times into the system. The system suitability parameters like rentation time (R_t), theoretical plate count, peak tailing, theoretical plates resolution were evaluated and found within the limits (Table-6).

All the validation parameters of both drugs *viz.* α-linolenic acid and cyclosporine A are tabulated in Tables 7 and 8, respectively.

Conclusion

A new HPLC method for analysis of the drug product containing α-linolenic acid and cyclosporine A was developed and found to be applicable for simultaneous estimation of these drugs. This method was found to be accurate, precise and linear

TABLE-6
SYSTEM SUITABILITY PARAMETERS

Drug/Number of injections	Cyclosporine A			α-Linolenic acid			Resolution
	Rt (min)	Theoretical plate count	Peak tailing	Rt (min)	Theoretical plate count	Peak tailing	
1	16.1	149842	1.1	18.6	268812	1.2	1.88
2	16.1	150256	1.0	18.6	278549	1.2	1.87
3	16.1	148496	1.1	18.6	268914	1.2	1.84
4	16.1	155265	1.1	18.6	274696	1.2	1.84
5	16.1	157482	0.9	18.6	276547	1.2	1.88
6	16.1	152349	1.1	18.6	268444	1.2	1.89

TABLE-7
VALIDATION PARAMETERS OF α-LINOLENIC ACID

Parameter	Result	Acceptance criteria
System suitability		
Theoretical plates	272660	NLT 2000
Asymmetry	1.2	NMT 2.0
Retention time (min)	18.6	–
% RSD (peak area)	2.3	NMT 5.0
Specificity	Complies	No interference by excipients
Method precision		
% RSD (assay)	1.35	NMT 2.0
Linearity range (µg/mL)	5.0 To 15.0	–
Correlation coefficient (r)	0.999	NLT 0.99
Accuracy (mean % recovery)		
50%	98.2%	98-102%
100%	99.1%	
150%	100.5%	

RSD = Relative standard deviation

TABLE-8
VALIDATION PARAMETERS OF CYCLOSPORINE A

Parameter	Result	Acceptance criteria
System suitability		
Theoretical plates	152281	NLT 2000
Asymmetry	1.0	NMT 2.0
Retention time (min)	16.1	–
% RSD (peak area)	2.7	NMT 5.0
Specificity	Complies	No interference by excipients
Method precision		
% RSD (assay)	1.62	NMT 2.0
Linearity range (µg/mL)	1.5 to 4.5	–
Correlation coefficient (r)	0.998	NLT 0.99
Accuracy (mean % recovery)		
50%	100.8	98-102%
100%	100.4	
150%	100.5	

RSD = Relative standard deviation

across the analytical range. It was simple and specific with lower limits of detection and quantification for both drugs in formulation. High resolution obtained at comparatively lower temperature (50 ºC) makes this method more promising and useful.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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