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CHEMOMETRIC ASSISTED RP-HPLC METHOD DEVELOPMENT & VALIDATION OF CYPROHEPTADINE HCL AND TRICHOLINE CITRATE

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ABSTRACT:

Special, effective high pressure liquid chromatography method has been developed for the simultaneous quantification of Cyproheptadine and Tricholine Citrate. By using Waters HPLC e-2695 quaternary pump with a PDA detector of 2998 instrument the chromatographic separation of Cyproheptadine and Tricholine Citrate was achieved on the column of Luna C_{18} (250x4.6mm, 5 µ) using an isocratic elution with a buffer containing water and acetonitrile at a rate of 90:10 as a mobile phase with a flow rate of 1 ml/min at ambient temperature. A detector wavelength of 245 nm utilizing the PDA detector was given in the instrumental settings. The linearity was studied between the concentration range of 0.5-3 µg/ml of Cyproheptadine and 68.75-412.5µg/ml of Tricholine Citrate was injected. The plotted calibration curves were linear with a regression coefficient of R^2> 0.999, indicates that the linearity was within the limit. As a part of method validation the parameters like specificity, linearity, accuracy, ruggedness, and robustness were determined and the results were found to be within the allowable limit. The method developed was found to be applicable to routine analysis and to be used for the measurement of both active pharmaceutical ingredients (i.e., Cyproheptadine and Tricholine Citrate). Validation of the proposed method was carried out according to an International Conference on Harmonization (ICH) guidelines. Since, there is no HPLC method reported in the literature for the estimation of Cyproheptadine and Tricholine Citrate, there is a need to develop quantitative methods under different conditions to achieve improvement in specificity, selectivity etc.

KEYWORDS: Cyproheptadine, Development, RP-HPLC, Tricholine Citrate, Validation

INTRODUCTION:

Cyproheptadine, sold under the brand name Periactin among others, is a first-generation antihistamine[1] (Church et al., 2011; Lee HE et al., 2014) with additional anticholinergic[2] (Nair et al., 2004; Fox et al., 2014), antiserotonergic[3] (Lindley et al., 2000), and local anesthetic[4] (Krøigaard et al., 2005; Philbin et al., 1990) properties. Cyproheptadine is a very potent antihistamine or inverse agonist[5] (Nutt et al., 2017; Berg et al., 2018) of the H_1 receptor[6] (de Graaf et al., 2011; Shimamura et al., 2011). At higher concentrations, it has anticholinergic, antiserotonergic, and antidopaminergic activities. Of the serotonin receptors[7] (Iqbal et al., 2012), it is an especially potent antagonist of the 5-HT_2 receptors[8] (Eison et al., 1996), and this underlies its effectiveness in the treatment of serotonin syndrome. Cyproheptadine is known to be an antagonist or inverse agonist of all of the receptors listed in the adjacent table. Cyproheptadine has weak antiandrogenic activity[9] (Pucci et al., 1997) Cyproheptadine is a tricyclic benzocycloheptene and is closely related to pizotifen and ketotifen as well as to tricyclic antidepressants. Cyproheptadine is used in cats as an appetite stimulant[10] (Agnew et al., 2014) and as an adjunct in the treatment of asthma. Possible adverse effects include excitement and aggressive behavior. The elimination half-life of cyproheptadine in cats is 12 hours. Cyproheptadine is a second line treatment for pituitary pars intermedia dysfunction in horses[11] (Durham, 2017).

Tricholine Citrate is a bile acid[12] (Wilcox et al., 2014) binding agent. It removes bile acids from the body. The liver then produce more bile acids using cholesterol, as a result, the levels of cholesterol in the body is lowered. Tricholine Citrate is used in the treatment of high cholesterol[13] (Al-Allaf et al., 2010) Tricholine citrate can cause acute toxicity, GI upset, nausea[14] (Kranke et al., 2002) or vomiting[15] (Koch, 2000) and mild to moderate forms of skin rashes[16] (Boyd et al., 2007).

MATERIALS AND METHOD:

Chemicals:

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Acetonitrile, HPLC-grade formic acid, water, were purchased from Merck India Ltd, Mumbai, India. APIs of Cyproheptadine, Tricholine Citrate standards were procured from Glenmark, Mumbai.

The Instrumentation:
Waters alliance liquid chromatography (model 2695) monitored with empower 2.0 data handling system and a detector of photo diode array (model 2998) was used for this study.

Method optimization:
To optimize the chromatographic conditions, different ratios of phosphate buffer and the acetonitrile in the mobile phase with isocratic mode was tested. However the mobile phase composition was modified at each trial to enhance the resolution and also to achieve acceptable retention times. Finally Acetonitrile + water (10+90) with isocractic elution were selected because it results in a greater response of active pharmaceutical ingredient. During the optimization of the method various stationary phases such as C_8, C_{18} and amino, phenyl columns were tested. From these trials the peak shapes were relatively good with Luna C_{18} 250x4.6mm, 5µ with a PDA detector. The mobile phase flow rate has been done at 245 nm in order to obtain enough sensitivity. By using above conditions we get retention times of Cyproheptadine and Tricholine Citrate were about 2.877 min and 6.465 min with a tailing factor of 1.45 & 1.21. The number of theoretical plates for Cyproheptadine and Tricholine Citrate were 2412, 8927 which indicate the column's successful output the % RSD for six replicate injections was around 0.5% and 0.11%, the proposed approach suggests that it is extremely precise. According to ICH guidelines, the method established was validated.
Till today there are no HPLC methods were reported in the literature, but only few methods are developed in individual analysis of Cyproheptadine and Tricholine Citrate. Hence we developed method for the simultaneous quantification of Cyproheptadine and Tricholine Citrate. The developed HPLC method was utilized for the estimation of the combined drugs by *in vitro* method.

Validation procedure:
The analytical parameters such as system suitability, precision, specificity, accuracy, linearity, robustness, LOD, LOQ were validated according to ICH Q2 (R1) guidelines.

Chromatographic conditions:
The HPLC analysis was performed on reverse phase HPLC system with isocratic elution mode using a mobile phase of Hexane + THF and 0.1% Formic Acid (80+20) and Chiral Cell ODH 150x4.6mm, 5µ column with a flow rate of 1 ml /min.

Preparation of the standard solution

Preparation of stock solution-A:

Add 70ml of diluents to 20mg of Cyproheptadine taken in a 100 ml volumetric flask and sonicate for 10 minutes to fully dissolve the contents and then make up to the mark with diluent. Further dilute 1 ml of the above solution to 10 ml with diluents. This is the Cyproheptadine stock solution.

Preparation of stock solution-B:
Add 70ml of diluents to 275mg of Tricholine Citrate taken in a 100 ml volumetric flask and sonicate for 10 minutes to fully dissolve the contents and then make up to the mark with diluent.

Preparation of Standard solution:
5 ml of stock solution-A and 5 ml of the stock solution-B were drawn and transferred into a 50ml volumetric flask and diluted up to the level.

Sample Solution Preparation:
Take 5 ml of the sample into a 100mL clean dry volumetric flask add diluents, sonicate it up to 30 min to dissolve, and centrifuge for 30min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron syringe filter (Stock solution). Further pipette 5 ml of the above stock solutions into a 50ml volumetric flask and dilute up to the mark with diluent (275ppm of Tricholine Citrate, 20pm of Cyproheptadine).

RESULTS:

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The main analytical challenge during development of a new method was to separate active Pharma ingredients. In order to provide a good performance the chromatographic conditions were optimized.

System suitability

Device suitability was performed by injecting standard solution containing 2 µg/ml of Cyproheptadine and 275 µg/ml of Tricholine Citrate in six replicates. The results show that the machine fitness parameter is within the limit provided by ICH. In System suitability injecting standard solution and reported USP tailing and plate count values are tabulated in table 1 and the standard chromatogram was shown in figure 1.

Specificity:

There was no interference from blank at the retention time of Tricholine Citrate and Cyproheptadine. This proves the technique is specific. Figure 2 shows the blank chromatogram.

Linearity:

The Linearity was calculated by plotting a calibration curve of the peak area against its respective concentration, linearity was determined. From this calibration curve, it was noticed that the curve was linear between the range of 0.5-3µg/ml of Cyproheptadine and 68.75-412.5µg/ml of Tricholine Citrate. The regression equations for calibration curve was Y=14445.43x+28228.75 (R^2=0.9998) for Tricholine Citrate and Y= 661344.1x+23228.07 (R^2=0.9996) for Cyproheptadine respectively. The results are given in table 3.

Intraday precision:

The precision of the analytical technique is the degree of proximity of the sequence of measurements obtained from multiple homogeneous mixture samplings. The accuracy of the process of the drugs were calculated by injection of six individual determinations of Cyproheptadine (2 µg/ml) and Tricholine Citrate (275µg/ml). Method precision results were shown in table 4 and sample chromatogram was shown in figure 5 and figure 4 represents method precision chromatogram.

Inter-day precision:

Six replicates of the sample solution were analyzed by different researchers and different tools were checked on separate days. The peak regions used to assess the average percent of RSD values have been determined. The findings are shown in the table below. The results are given in table 5.

LOD and LOQ:

LOD and LOQ were determined separately using the calibration curve technique. The LOD and LOQ of the compound were measured using the developed RP-HPLC method by injecting lower and lower concentrations of the standard solution. The LOD and LOQ concentrations and their s/n values of Cyproheptadine and Tricholine Citrate were represented in the table 6. Table 6 gives the LOD and LOQ concentrations.

Robustness:

The conditions of the experiment were designed to measure the robustness of the intentionally changed conditions such as flow rate, organic percentage in mobile phase and results were tabulated in Table 7.

Table 1: Results of system suitability

System suitability parameter	Acceptance criteria	Drug name	
		Cyproheptadine	Tricholine Citrate
USP Plate Count	NLT 2000	2412	8927
USP Tailing	NMT 2.0	1.45	1.21
USP Resolution	NLT 2.0	----	13.86
% RSD	NMT 2.0	0.502	0.112

Table 2: Linearity of Cyproheptadine and Tricholine Citrate

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S.No	Cyproheptadine		Tricholine Citrate	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	0.50	380900	68.75	1015947
2	1.00	678518	137.50	2004579
3	1.50	1034872	206.25	3061723
4	2.00	1338064	275.00	4026983
5	2.50	1661996	343.75	5014944
6	3.00	2012360	412.50	5929021
Regression equation	y = 661344.1x +23228.07		y =14445.43x + 28228.75	
Slope	661344.14		14445.43	
Intercept	23228.07		28228.75	
R^2	0.9996		0.9998	

R^2 - Correlation coefficient

Table 3: Results of accuracy

S. No	% Level	Cyproheptadine % Recovery	Tricholine Citrate % Recovery
1	50	101.3	99.7
2	100	101.5	100.5
3	150	98.3	100.6

Table 4: Intraday precision results of Cyproheptadine and Tricholine Citrate

S. No.	Area for Cyproheptadine	Area for Tricholine Citrate
1	1407683	4093146
2	1391885	4033781
3	1371892	4048617
4	1400573	4071718
5	1407434	4036021
6	1409585	4029747
Average	1398175	4052171
Standard Deviation	14439.04	25201.25
%RSD	1.03	0.62

Table 5: Inter-day accuracy of outcomes of Cyproheptadine and Tricholine Citrate

S. No.	Area for Cyproheptadine		Area for Tricholine Citrate	
	Day-1	Day-2	Day-1	Day-2
1	1388108	1375648	4013080	4021648
2	1391885	1352350	4034136	4019075
3	1370942	1329413	4083705	4057332
4	1375216	1363849	4058990	4044256
5	1382396	1349211	4062443	4036879
6	1375229	1317757	4064982	4063794
Average	1380629	1348038	4052889	4040497
Standard Deviation	8222.57	21429.41	25142.78	18262.38
%RSD	0.59	1.58	0.62	0.45

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Table 6: LOD and LOQ for Cyproheptadine and Tricholine Citrate

Name of drug	LOD(µg/ml)	LOQ(µg/ml)
Cyproheptadine	0.002	0.006
Tricholine citrate	0.275	0.825

Table 7 : Robustness data Cyproheptadine and Tricholin Citrate

Parameter name	% RSD	
	Cyproheptadine	Tricholine Citrate
Flow minus (0.8 ml/min	1.01	0.75
Flow plus (1.2 ml/min)	0.34	1.16
Organic minus (-10%)	0.71	0.96
Organic plus (+10%)	0.84	1.53

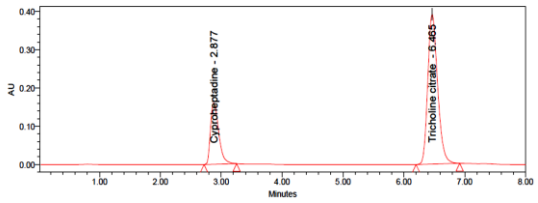


Fig. 1: Chromatogram of standard

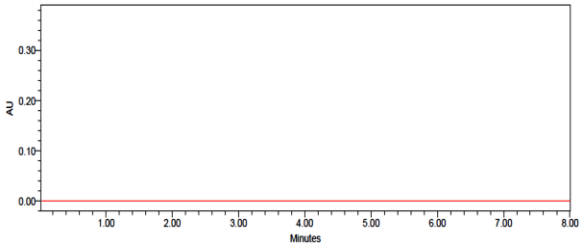
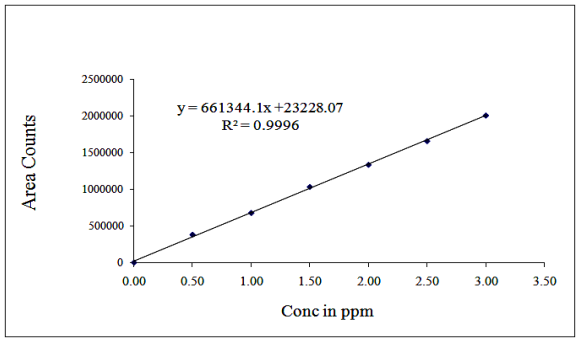
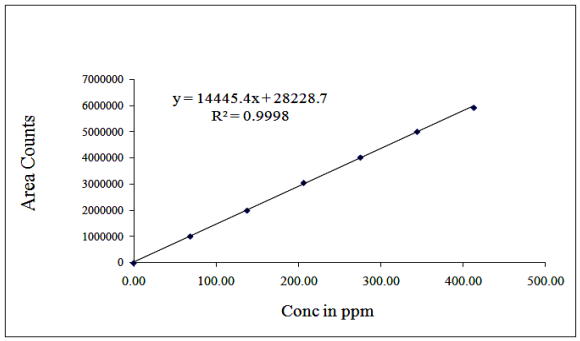


Fig.2: Chromatogram of blank



(A) Cyproheptadine



(B) Tricholine Citrate

Fig. 3: Calibration plots of (A) Cyproheptadine (B) Tricholine Citrate

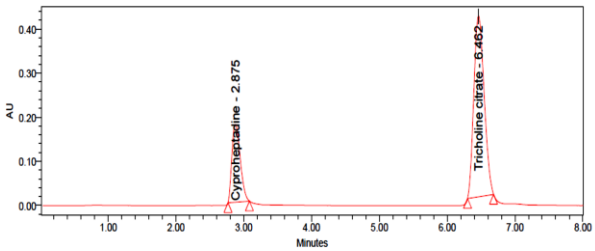


Fig. 4: Chromatogram of method precision

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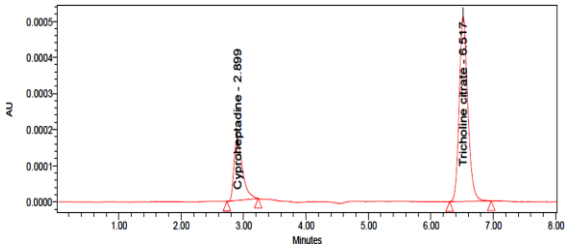
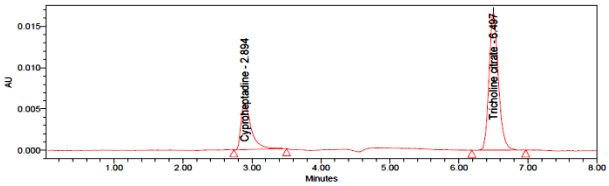


Fig. 5: Chromatogram of (A) LOD and (B) LOQ **(A) LOD**



(B)LOQ

Chemometric Analysis:

In this chemometrics assisted HPLC study ,PCA,PLS calibrations were used to analyse the drugs of Tricholine citrate and collagen type II at 221 nm by using PDA detector. The dataobtained from analysed drugs were stored in computer having required software to perform chemometric analysis.

Acquisition software: In present study we are using following chemometric techniques.

- Principal component analysis(PCA)
- Partial least squares technique(PLS)

We are download the unscrambler (camo software),it facilitates the PCA,PLS analysis morerobust, accessible.

PLS Approach:

PLS calibration using the orthogonalized PLS algorithm involves, simultaneously, independent and dependent variables on the data compression and decomposition operations. In the HPLC data analysis, HPLC-PLS calibration was obtained by decomposition of both the drugs of concentration , peak area matrix into latent variables. PLS calibration was obtained using the relationship between the decomposed peak area data and concentration set.

Table 8: PLS Accuracy numerical data of Tricholine citrate and Cyroheptadine

S.NO	Y REFERENCE	Y PREDICTED TCC	Y PREDICTED CH
	1	2	2
1	50	50.2227135	49.6584244
2	50	49.939949	49.8927803
3	50	50.7262688	49.5737305
4	100	100.300949	101.712364
5	100	98.9490051	100.40934
6	100	99.3553467	99.4655609
7	150	150.095764	147.337311
8	150	151.235016	151.181519
9	150	149.504822	150.458633

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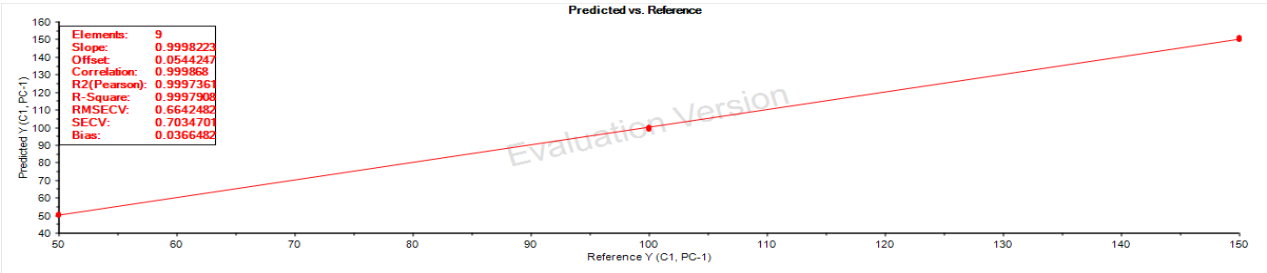

Figure 6: PLS of accuracy spectral data of Tricholine citrate

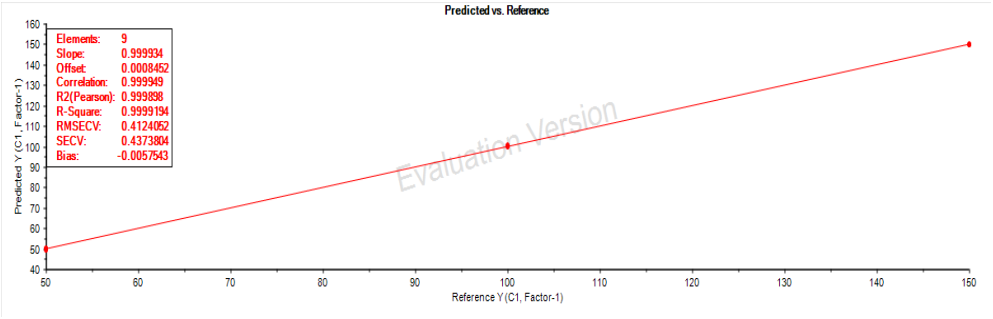

Figure 7: PLS of accuracy spectral data of Cyroheptadine

Table 9: PLS Linearity numerical data of Tricholine citrate and Cyroheptadine

S.NO	Y REFERENCE	Y PREDICTED TCC	Y PREDICTED CH
	1	2	2
1	0.000	-0.0649180412	-0.16383934
2	1.2500	1.2415266	1.39277053
3	2.5000	2.48528671	2.47310305
4	3.7500	3.82946229	3.83651876
5	5.000	5.03985786	4.96297503
6	6.2500	6.28625965	6.17124653
7	7.5000	7.36492062	7.53159428

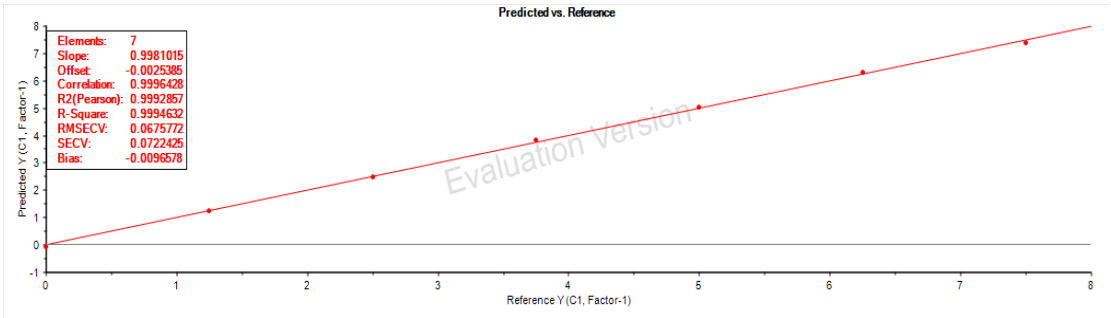

Figure 8: PLS of Linearity spectral data of Tricholine citrate (AFLAPIN)

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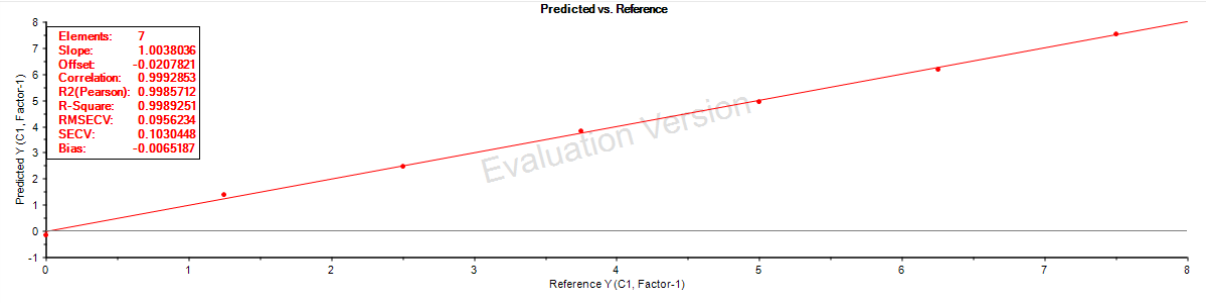

Figure 9: PLS of Linearity spectral data of Cyroheptadine

PCA approach:

In PCA technique it gives relevant information from data set, and it can be used express the data on the basis of their similarity and differences. It is used to develop correlation structure between variables, and examine the changes. In PCA data transferred to describe the amount of same variability. In these HPLC data analysis the data of both drugs of Tricholine citrate and Cyroheptadine peak area we get the Bio-plot.

Table 10: PCA Accuracy Numerical Data of Tricholine citrate and Cyroheptadine

PC-1	PC-2
-2159108.75	5529.875
-2166353.75	699.25
-2145101.75	11054.1875
16970.8398	-16242.3809
-34857.4102	-16801.8047
-24382.8184	-1265.46387
2158614.5	25973.875
2202457.25	229.75
2151761.25	-9177.5625
-2159108.75	5529.875

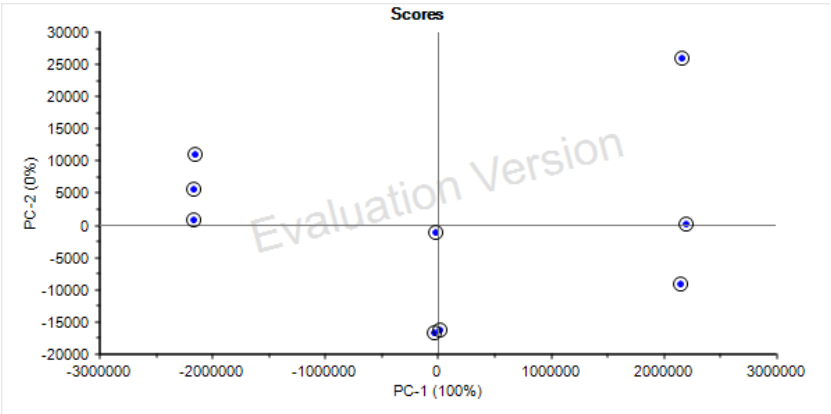

Figure 10: PCA Accuracy Spectral Data of Tricholine citrate and Cyroheptadine

Table 11: PCA Linearity Numerical Data of Tricholine citrate and Cyroheptadine

PC-1	PC-2
-3174303.5	13249.8125
-2090055.38	-27239.8125
-1058030.25	2660.65625
57552.3164	-1526.69922
1069162.75	15702.5625
2108863	20424.3125

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3086811.25	-23270.5625

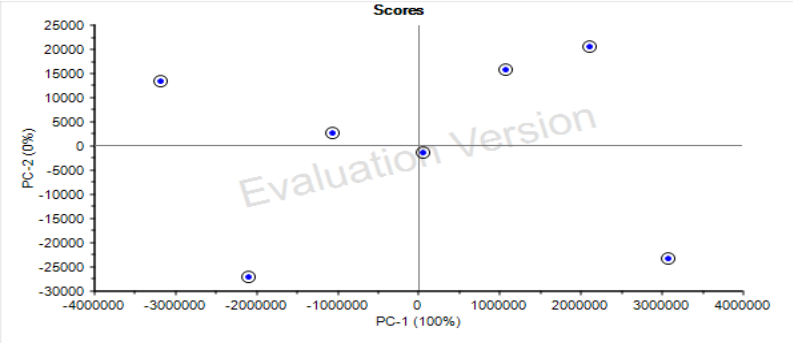

Figure 11: PCA Linearity Spectral Data of Tricholine citrate and Cyroheptadine

PCA analysis produces several types of outputs which must be taken into account when drawing conclusions. It gives some guidance on interpretation but it is not intended to be an exhaustive list (please note some of the outputs mentioned will not be available in all software packages and the terminology also varies slightly across packages).

CONCLUSION:

This method described the quantification of Cyproheptadine and Tricholine Citrate in bulk and pharmaceutical formulation as per ICH guidelines. The evolved technique was found to be accurate, precise, linear and reliable. The advantage lies in the simplicity of sample preparation and reproducibility data are satisfactory. The evolved chromatographic method can be effectively applied for regular investigation in drug research.

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