ORIGINAL ARTICLE

Estimation of Cyproheptadine Hydrochloride and Tricholine Citrate Simultaneously in Syrup Dose by Applying Stability Indicating HPLC Methodology

Kancharla Vijayalakshmi[*1], Bethapudi Samuel Anand Andrews[2]

[1]Department of Quality Control, Divis laboratories limited, Hyderabad – 508252, Telangana, India
[2]Department of Chemistry, Gitam Institute of Technology, GITAM University, Visakhapatnam - 530045, Andhra Pradesh, India

ABSTRACT

An stability indicating HPLC methodology for the concurrent estimation of Tricholine citrate (TRC) and Cyproheptadine hydrochloride (CYH) in syrup dose and bulk using Waters column reverse phase C18 (5 μm, 250 mm and 4.6 mm) as stationary phase and 0.1M Na_2HPO_4 of pH 4.5 and acetonitrile in proportion of 60:40 (*v/v*) at flow of 1.0 ml/min rate as mobile phase was reported. The linear scales were 275-825 μg/ml for TRC and 2-6 μg/ml for CYH with correlation coefficients of 0.9999 for TRC and 0.9997 for CYH. Followed ICH Q2(R1) strategies for validating the suggested method for precision, sensitivity, robustness, specificity, selectivity and accuracy. The measures of LOD and LOQ are 0.023 μg/ml and 0.079 μg/ml for CYH, while for TRC it was 0.565 μg/ml and 1.885 μg/ml, respectively. The precision measures for CYH and TRC were 0.073 and 0.212 relative measured deviation percentage, respectively. The accuracy measures for CYH and TRC were 99.40% and 99.09% mean assay percentiles, respectively. Recovery percentiles measures of CYH and TRC were ranged between 99.48% to 100.35% and 100.38% and 100.41%, respectively. While in degradation investigation, peaks of degraded products are very well differentiated from TRC and CYH peaks suggesting the specificity and stability of suggested methodology. The results permit the application of the proposed stability indicating HPLC methodology in syrup dose forms.

*Corresponding Author

Name: Kancharla Vijayalakshmi
Phone: +91-8886765050
Email: vijayalakshmikancharla.msc@gmail.com

INTRODUCTION

Cyproheptadine hydrochloride (CYH) is an antiserotonergic, antihistaminic and histamine H1 blocking agent (Kapur *et al.*, 1997; Feng *et al.*, 2015). CYH relieves allergy symptoms like redness in eye, itching in eyes, watery eyes, itching in nose, runny nose, sneezing, hives caused because of cold temperature exposure, and itching because of allergy in skin. CYH acts by preventing a certain natural substance termed histamine which is made by the body in an allergic response. This CYH also prevents one other natural substance named, serotonin, in the body. Tricholine citrate (TRC) is a lipotropic representative (Columbo and Rohr, 2016; Mehedint and Zeisel, 2013; NCBI, 2020). TRC is utilized in the therapy of

elevated cholesterol levels. TRC is a binding agent of bile acid. Bile acids were expelled from the body by TRC. The liver at that point produce more bile acids utilizing cholesterol, accordingly, the cholesterol levels are brought down in the body.

CYH and TRC combination increases appetite, gives effective hepato-protective act, enhances protein production and assures weight gain (Kiran *et al.*, 2018). CYH and TRC combination also used in anorexia-related hepatobiliary complications that may lead to weight losing. The CYH and TRC combination is accessible as formulation of syrup (Anuj, 2020), accessible in the markets of pharmacy as Cyprosal, Cyprohar, Cyprolac -T, Cypro -T and Clopex TC. The labelled claim of CYH and TRC in all syrup formulations was 2 mg of CYH and 275 mg of TRC per five ml.

Analysis of pharmaceutically important compounds by reverse-phase liquid chromatography provides larger sensitivity, specificity and consumes less time. It's the first time, to our information from internet sources, that a stability indicating RP-HPLC dependent method for analysing the combination of CYH and TRC has been developed for syrup formulation.

MATERIALS AND METHODS

Systems and Conditions

The HPLC system employed for CYH and TRC combined analysis comprised of Waters 2695 chromatography system, Waters 2998 photodiode detector and Waters column reverse phase C18 (5 μm, 250 mm and 4.6 mm). System management and processing of chromatography data of CYH and TRC were executed using version 2 of the Empower program. Mobile phase contained 0.1M Na_2HPO_4 (Sd Fine Chemicals Ltd, India) of pH 4.5 and acetonitrile (Merck India Ltd, India) in proportion of 60:40 (*v/v*) at flow of 1.0 ml/min rate. Injector sample volume, column's temperature and quantification wavelength was optimized at 10 μl, 25 $^{\circ}$C and 265 nm, respectively.

CYH and TRC Solutions

Stock CYH and TRC solution of concentration 40 μg/ml CYH and 5500 μg/ml TRC was made ready through dissolving reference CYH (4 mg, obtained from Rainbow pharma training labs, India) and reference TRC (550 mg, obtained from Rainbow pharma training labs, India) in 100 ml volume of 0.1M Na_2HPO_4 (pH 4.5) and acetonitrile (60:40, v/v) solvent combination. From stock CYH and TRC solution, serial dilutions were made using 0.1M Na_2HPO_4 (pH 4.5) and acetonitrile (60:40, v/v) sol-

vent combination to obtain solutions for calibration in range of 2-6 μg/ml CYH and 275-825 μg/ml TRC. Working CYH and TRC solution of concentration 4 μg/ml CYH and 550 μg/ml TRC was made ready through diluting stock CYH and TRC solution with 0.1M Na_2HPO_4 (pH 4.5) and acetonitrile (60:40, v/v) solvent combination.

CYH and TRC Calibration Curves

The linear correlation connecting area response of CYH and TRC with concentration of CYH and TRC was appraised by making measurements using proposed method at 5 concentration levels covering the range of 275-825 μg/ml for TRC and 2-6 μg/ml for CYH.

CYH and TRC Content Assay in Cypro -T Syrup

Volume of Cypro -T syrup (Nexkem Pharmaceutical, Indore, India; labelled strength 2 mg of CYH and 275 mg of TRC per five ml) equal to 2 mg of CYH and 275 mg of TRC was mixed through 20 min sonication with 25 ml of 0.1M Na_2HPO_4 (pH 4.5) and acetonitrile (60:40, *v/v*) solvent combination. Filtered the solution by 0.45 μm filters and diluted to 50 ml volume with 0.1M Na_2HPO_4 (pH 4.5) and acetonitrile (60:40, *v/v*) solvent combination. This is stock syrup solution of concentration 40 μg/ml CYH and 5500 μg/ml TRC. Working syrup solution of concentration 4 μg/ml CYH and 550 μg/ml TRC was made ready through diluting stock syrup solution with 0.1M Na_2HPO_4 (pH 4.5) and acetonitrile (60:40, *v/v*) solvent combination. The area response of CYH and TRC peaks in working syrup solution were evaluated using recommended method. The contents of CYH and TRC in Cypro -T syrup was assessed from obtained area response of CYH and TRC peaks using their matching calibration graphs.

Degradation Studies on CYH and TRC

In this study, intended degradation was made by exposing a syrup solution (10 ml, 40 μg/ml CYH and 5500 μg/ml TRC) to conditions of stress (ICH, 2003): alkaline (0.1 N NaOH, 10 ml), acidic (0.1 N HCl, 10 ml), oxidation (30% H_2O_2, 10 ml), thermal (60°C, 30 min) and photo (24 hr). In alkaline, acidic and oxidation, flask contents were 30 min sonicated at ambient temperature. After respective time periods, degraded syrup solution of concentration 4 μg/ml CYH and 550 μg/ml TRC was made ready through diluting with 0.1M Na_2HPO_4 (pH 4.5) and acetonitrile (60:40, *v/v*) solvent combination. The degraded syrup solutions were then analysed in the same way as explained in the Cypro-T syrup analysis.
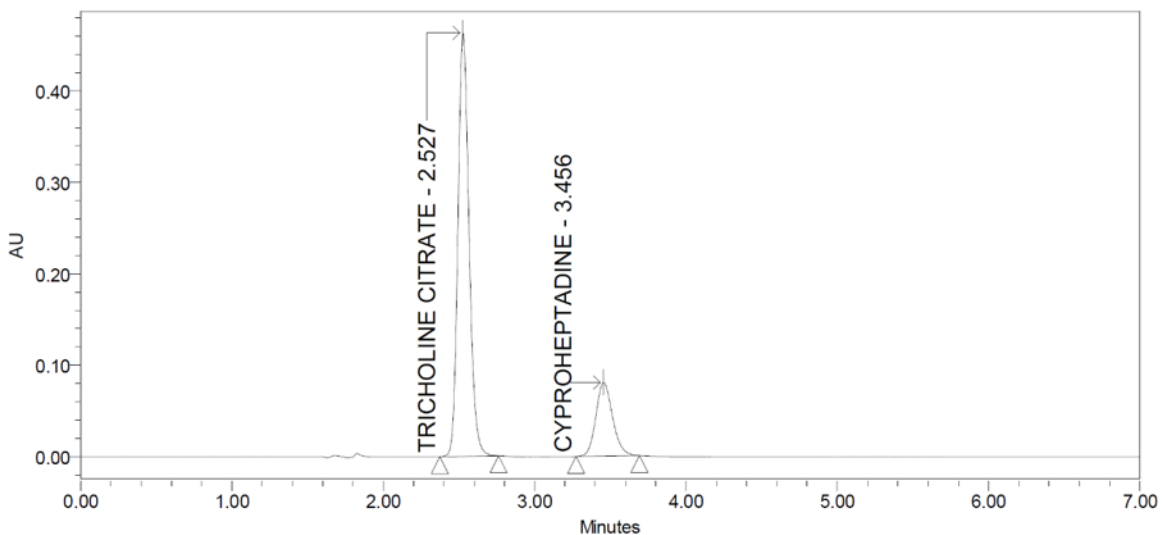
Peak Name	RT	Area (µV*sec)	USP Resolution	USP Plate Count	USP Tailing	s/n
CYPROHEPTADINE	3.456	618238	5.46	4748	1.15	506.3

Peak Name	RT	Area (µV*sec)	USP Plate Count	USP Tailing	s/n
TRICHOLINE CITRATE	2.527	2361285	5789	1.17	2918.0

Figure 1: Chromatogram and system appropriateness parameters values for CYH and TRC with optimum chromatography conditions

Table 1: TRC and CYH recoveries

| Spiked	"μg/ml" Added	"μg/ml" Determined	"%" Recovery	"%" Mean
	TRC			
Level: 50 %	272.250	269.706	99.07	
	272.250	271.598	99.76	99.48
	272.250	271.187	99.61	
Level: 100%	544.500	545.849	100.25	
	544.500	543.882	99.89	100.03
	544.500	544.291	99.96	
Level: 150%	816.750	819.016	100.28	
	816.750	818.502	100.21	100.35
	816.750	821.213	100.55	
	CYH			
Level: 50 %	1.980	1.983	100.16	
	1.980	1.990	100.51	100.38
	1.980	1.989	100.46	
Level: 100%	3.960	3.976	100.40	
	3.960	3.980	100.50	100.40
	3.960	3.972	100.31	
Level: 150%	5.940	5.962	100.36	
	5.940	5.970	100.51	100.41
	5.940	5.961	100.36	
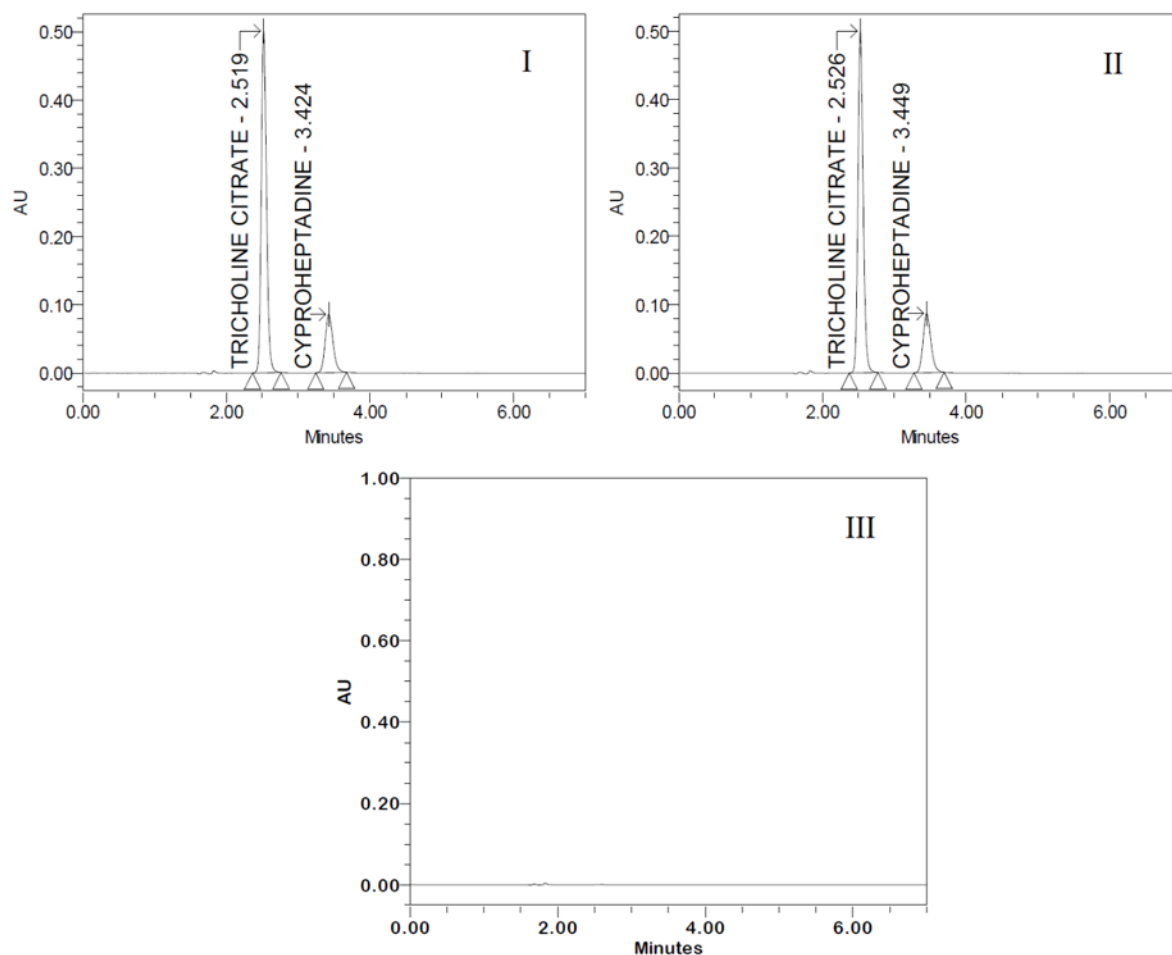
Figure 2: [I] Working syrup sample [II] Working CYH and TRC solution [III] Blank solvent combination

RESULTS AND DISCUSSION

The intent of this research was to create a stability indicating RP-HPLC approach for the measurement of CYH and TRC in the Cypro-T syrup dose type.

Chromatography was tested on C18 stationary phase columns like YMC, Aligent and Waters. The wavelength assignment was based on the highest area response for optimal sensitivity. Several trials were made with distinct solvents (K_2HPO_4: Methanol, K_2HPO_4: Methanol, Na_2HPO_4: Acetonitrile) with varying buffer type and polarity in different proportions to get sharp peaks with best resolution.

Finally, Waters C18 stationary phase columns and mobile phase contained 0.1M Na_2HPO_4 of pH 4.5 and acetonitrile in proportion of 60:40 (*v/v*) at flow of 1.0 ml/min rate was opted. Column's temperature and quantification wavelength was optimized at 25 ^{o}C and 265 nm, respectively. Figure 1 shows the chromatogram for CYH and TRC with optimum chromatography conditions. The system appropri-

ateness parameters (tailing factor, resolution and counts of plate) determined with optimum chromatography conditions are also acceptable.

Validation

Followed ICH Q2(R1) strategies for validating the suggested method (ICH, 2005; Vidushi and Meenakshi, 2017).

Linearity

Linearity was found over the scope of 275-825 μg/ml for TRC and 2-6 μg/ml for CYH. The area response of CYH and TRC peaks were calculated for every concentration level. The graph of calibration was plotted for CYH and TRC concentrations against the area response of CYH and TRC peaks. The straight-line regression formulas with correlations calculated were:

For TRC: y = 4596.9 *x* - 16377.2 (R^2 = 0.9999)

For CYH: y = 165179.3 *x* - 1861.4 (R^2 = 0.9997)

Selectivity
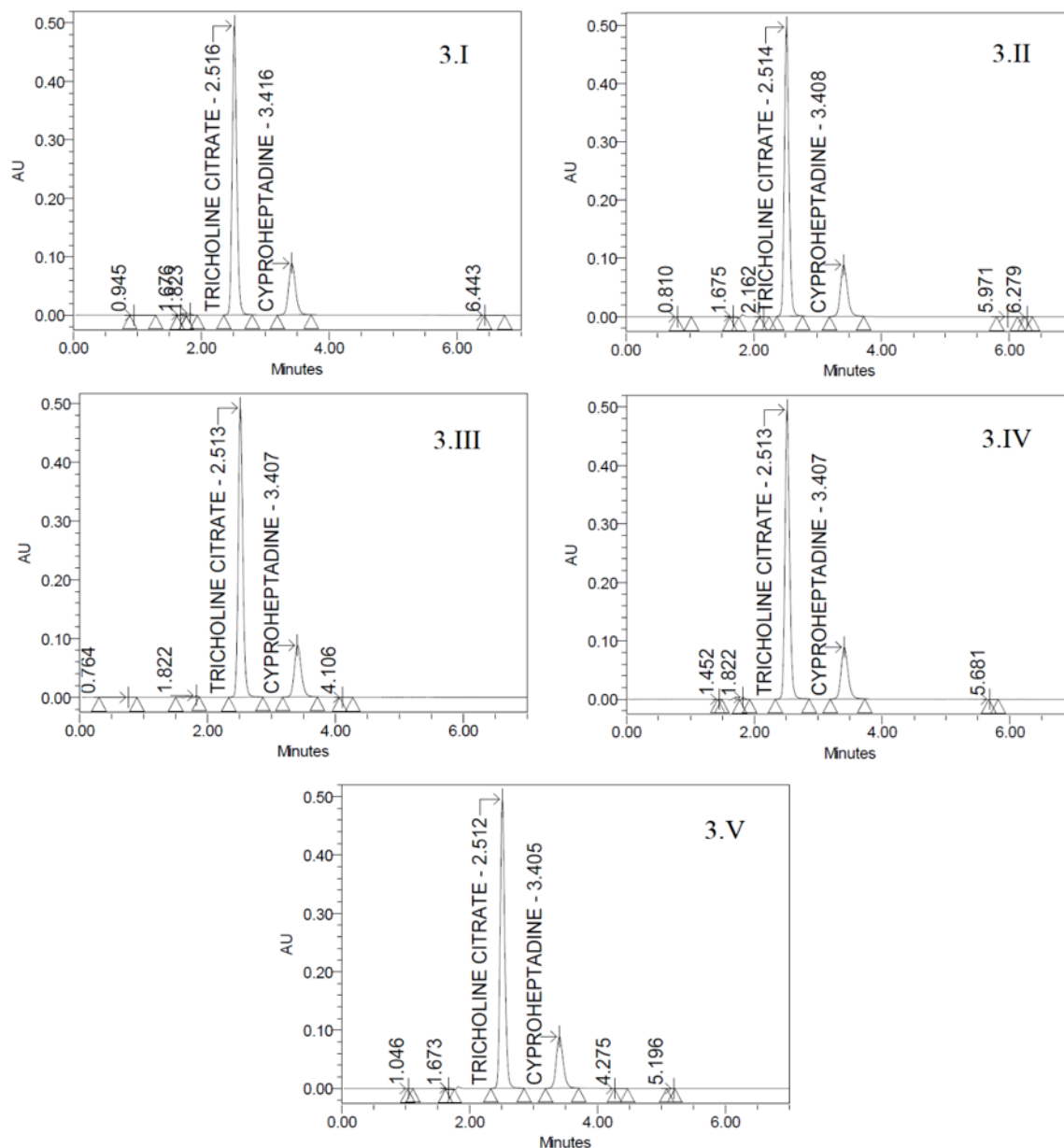
Selectivity valuation was conducted by infusing

Figure 3: Syrup solution chromatograms after treating with [I] 0.1 N HCl [II] 60°C [III] Sunlight [IV] 0.1N NaOH [V] 30% Peroxide

working CYH and TRC solution (CYH 4 μg/ml and TRC 550 μg/ml), working syrup sample (CYH 4 μg/ml and TRC 550 μg/ml) and blank solvent combination (0.1M Na2HPO4, pH 4.5 and acetonitrile in 60:40 v/v proportion solvent combination) into the Waters C18 column one by one, and analysed by recommended methodology. For chromatograms see Figure 2.I, Figure 2.II and Figure 2.III. No peaks are getting in the way that interferes with peaks of CYH and TRC.

LOD & LOQ

The values of LOD & LOQ for CYH and TRC were accomplished dependent on the area response standard deviation and calibration graph slope. The LOD was expressed as: 3 × area response standard deviation/calibration graph slope and LOQ was expressed as: 10 × area response standard deviation/calibration graph slope. The value of LOD for CYH and TRC were 0.023 μg/ml and 0.565 μg/ml, respectively. The value of LOQ for CYH and TRC were 0.079 μg/ml and 1.885 μg/ml, respectively.

Precision

The precision valuation was conducted by analysing six independent replicates of the working CYH and TRC solution (CYH 4 μg/ml and TRC 550 μg/ml). Precision was stated as relative measured deviation

Table 2: Reports of robustness

Variation parameter	Varied value	Analyte	Count of plat	Resolution	Tailing factor
Acetonitrile volume	35%	TRC	5670	-	1.14
		CYH	4451	5.24	1.14
	45%	TRC	6162	-	1.17
		CYH	5039	5.42	1.16
Temperature at column	23 °C	TRC	5810	-	1.15
		CYH	4612	5.32	1.14
	27 °C	TRC	6162	-	1.17
		CYH	5039	5.42	1.16
Flow rate	0.9 ml/min	TRC	5670	-	1.14
		CYH	4451	5.24	1.14
	1.1 ml/min	TRC	6413	-	1.18
		CYH	5137	5.38	1.19
pH	4.4 units	TRC	5900	-	1.17
		CYH	4816	5.45	1.16
	4.6 units	TRC	5933	-	1.16
		CYH	4771	5.43	1.15
Wavelength	263	TRC	5796	-	1.17
		CYH	4735	5.45	1.16
	267	TRC	5793	-	1.17
		CYH	4774	5.44	1.16

percentage for CYH and TRC area responses. Precision was 0.073 and 0.212 relative measured deviation percentage for CYH and TRC, respectively.

Accuracy

The accuracy valuation was conducted by analysing six independent replicates of the working CYH and TRC solution (CYH 4 μg/ml and TRC 550 μg/ml). Accuracy was stated as mean assay percentile for CYH and TRC content. Accuracy was 99.40% and 99.09% mean assay percentiles for CYH and TRC, respectively.

Recovery

The accuracy valuation was also through recoveries, computed after spiking solution of syrup (CYH 4 μg/ml and TRC 550 μg/ml) with CYH and TRC references at distinct three levels: 50% (CYH 1.98 μg/ml and TRC 272.250 μg/ml), 100% (CYH 3.96 μg/ml and TRC 544.50 μg/ml) and 150% (CYH 5.94 μg/ml and TRC 816.750 μg/ml). Recovery percentiles of CYH and TRC were calculated (Table 1). The recovery percentiles values signify the applicability of recommended methodology for CYH and TRC analysis in formulation of syrup.

Degradation Studies on CYH and TRC

The chromatogram for syrup solution degraded with 0.1N HCl showed 4 additional peaks at retention periods 0.945 min, 1.676 min, 1.823 min and 6.443 min (Figure 3.I). 10.98% of TRC and 11.34% of CYH are degraded with 0.1N HCl. Syrup solution degraded at 60°C showed the occurrence of 5 additional peaks at retention periods 0.810 min, 1.675 min, 2.162 min, 5.971 min and 6.279 min in the chromatogram (Figure 3.II). In thermal heat induced stress, degradation of TRC and CYH are 12.38% and 9.71%, respectively. In degradation using sunlight, TRC and CYH degradation was evidenced at 6.55% and 8.36%, respectively.

In response to degradation using sunlight, three additional peaks at 0.764 min, 1.822 min and 4.106 min (Figure 3.III) were observed. 0.1 N NaOH induced stress degraded 6.27% of TRC and 5.25% of CYH, and respective chromatogram presented three additional peaks at 1.452 min, 1.822 min and 5.681 min (Figure 3.IV). Under 30% peroxide stress, 4.46% of TRC and 7.96% of CYH are degraded. The chromatogram obtained displayed 4 additional peaks at retention periods 1.046 min, 1.673 min, 4.275 min and 5.196 min (Figure 3.V). The extra peaks in stress studies denotes the development of deterioration products. These experiments have shown the specificity/stability of suggested methodology, because the peaks of degraded products are very well differentiated from the peaks of TRC and CYH (Figure 3.I, Figure 3.II, Figure 3.III, Figure 3.IV and Figure 3.V).

Robustness

To highlight robustness five altered parameters were investigated:

1. Changed proportion of acetonitrile - $\pm$ 5.0% volume

2. Changed pH - $\pm$ 0.1 unit

3. Changed flow rate - $\pm$ 0.1 ml/min

4. Changed wavelength at detector - $\pm$ 2 nm

5. Changed temperature at column - $\pm$ 2.0°C.

The system appropriateness parameters (tailing factor, resolution and counts of plate) are determined with variation parameters for TRC and CYH (Table 2). We got acceptable results.

CONCLUSION

An HPLC methodology for the estimation of TRC and CYH in formulations of syrup was herein reported. Validation specifications were evaluated as per ICH guidance. This HPLC methodology enabled selective, sensitive, robust, precise, specific, accurate and high throughput quantification of TRC and CYH simultaneously in formulations of syrup and also in bulk materials.

ACKNOWLEDGEMENT

Conflict of Interest

The authors declare that there is no conflict of interest for this study.

REFERENCES

Anuj, S. 2020. Cyproheptadine + Tricholine Citrate. 1mg.com, Accessed on: 30 December 2020.

Columbo, M., Rohr, A. S. 2016. Asthma in the elderly: the effect of choline supplementation. *Allergy, Asthma & Clinical Immunology*, 12(1):1–5.

Feng, Y. M., Feng, C. W., Chen, S. Y., Hsieh, H. Y., Chen, Y. H., Hsu, C. D. 2015. Cyproheptadine, an antihistaminic drug, inhibits proliferation of hepatocellular carcinoma cells by blocking cell cycle progression through the activation of P38 MAP kinase. *BMC Cancer*, 15:134.

ICH 2003. ICH Stability testing of new drug substances and products Q1A (R2). International conference on the harmonization.

ICH 2005. ICH harmonized tripartite guideline. International conference on the harmonization, Validation of analytical procedures: Text and methodology Q2(R1).

Kapur, S., Zipursky, R. B., Jones, C., Wilson, A. A., Dasilva, J. D., Houle, S. 1997. Cyproheptadine: a potent in vivo serotonin antagonist. *American Journal of Psychiatry*, 154(6):884.

Kiran, M. D., Sheikh, S. N., Pawaskar, L. J. 2018. Safety and Efficacy of a Combination of Cyproheptadine and Tricholine Citrate: Phase IV Study. *Annals of International medical and Dental Research*, 4(2):1–5.

Mehedint, M. G., Zeisel, S. H. 2013. Choline's role in maintaining liver function. *Current Opinion in Clinical Nutrition and Metabolic Care*, 16(3):339–345.

NCBI 2020. Tricholine citrate. PubChem Compound Summary for CID 11024. National Center for Biotechnology Information (NCBI), Accessed on: 25 July 2020.

Vidushi, Y., Meenakshi, B. 2017. A Review on HPLC Method Development and Validation. *Bioinformatics, Pharm and Chemical Sci*, 2(6):166–178.