Asian Journal of Pharmaceutical Education and Research
Vol -11, Issue-3, July- September 2022
ISSN:2278 7496

RESEARCH ARTICLE Impact Factor: 7.014

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CLOMIPHENE CITRATE AND ACETYLCYSTEINE IN THEIR COMBINED TABLET DOSAGE FORM

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Received 17 May 2022; Revised 27 May 2022; Accepted 17 June 2022, Available online 15 July 2022.

Cite this article as: Agawane SS, Tamboli AM, Khan NI, Patil MS, Ghule SS, Mane ST. Development and Validation of RP-HPLC Method for Simultaneous Estimation of Clomiphene Citrate and Acetylcysteine in their combined Tablet Dosage Form. Asian Journal of Pharmaceutical Education and Research. 2022; 11(3): 120-134.

https://dx.doi.org/10.38164/AJPER/11.3.2022.120-134

ABSTRACT

The simple, accurate, rapid, and selective analytical procedures were developed and validated for the simultaneous estimation of Clomiphene citrate and Acetylcysteine using reverse phase High-performance liquid chromatography. The HPLC method employs a C18 Column (250mm x 4.6mm, 5m particle size 10 µL injection volume, column temperature controlled at 30° C, detection at 225nm as a isobestic point, Acetonitrile: Water (70: 30 v/v) is used in the mobile phase with a flow rate of 1ml/min. Parameters consisting of linearity, precision, accuracy, robustness, detection and quantitation are studied. From the investigations, the Absorptive factor of Clomiphene Citrate and Acetylcysteine at both the isosbestic points has been found at 225nm. Acetylcysteine had a 2.43minute retention duration, while Clomiphene Citrate had a 4.26 minute retention period. The detection concentrations for Acetylcysteine and Clomiphene Citrate were linear over the ranges of 240-1200 µg/mL and 20-100 µg/mL, respectively. Acetylcysteine and Clomiphene Citrate have regression equations of y = 147.9x +15846 and y = 187.1x + 6641, respectively with regression coefficients of 0.997 and 0.999. The percentage assay of 99.8% and 101.1% for Clomiphene Citrate and `Acetylcysteine. The recovery of Clomiphene Citrate and Acetylcysteine was found to be 99.6% and 101.3%. The objective of validation of analytical procedures is to demonstrate that it is suitable for its intended purpose.

Keywords: Clomiphenecitrate ; Acetylcysteine ; Simultaneous equation; Validation ; RP-HPLC.

INTRODUCTION

Acetylcysteine, also known as N-acetylcysteine, N-acetyl-L-cysteine, or NAC, is created by combining cysteine with an acetyl group. N-Acetylcysteine is a dietary supplement and active pharmaceutical ingredient used to treat paracetamol overdose and as a mucolytic. Acetylcysteine is an antioxidant in and

of itself, but it can also be deacetylated to cysteine, which is a component of the antioxidant glutathione. Do not take the Acetylcysteine solution if you are allergic to acetylcysteine or any other component.

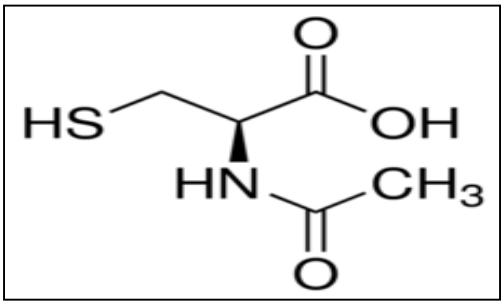


Figure1: Structure of Acetylcysteine

Molecular Formula: $C_5H_9NO_3S$

Molecular Weight: 163.19 g/mg

Clomiphene citrate is also known as 2-[4- (2-chloro-1, 2-diphenyl-ethenyl)-phenoxyl-N, N-diethyl-, 2-hydroxy-1, 2, 3 propane tricarboxylate] and 2-[4- (2-chloro-1, 2-diphenyl-ethenyl)-phenoxyl-N, N-diethyl-, 2-hydroxy-1, 2, 3 propane tricarboxy This substance is steroidal. Since 1962, the drug clomiphene citrate has been used to induce ovulation. So there you have it. This first-line medication for ovulatory infertility in women with naturally oestrogenized illnesses (PCO) has a disproportionately negative impact on women with polycystic ovaries. Clomiphene citrate possesses both oestrogenic and antiestrogenic properties. Oestrogenic and anti-estrogenic characteristics that are deterrent. Clomiphene citrate is thought to displace the oestrogen hypothalamus as well as endogenous oestrogensites in pituitary oestrogen receptors. Insulin-sensitizing medications have been studied for treating the underlying cause of disorders associated to insulin resistance, and the discrepancy may persist with gonadotropin treatment to some extent [1].

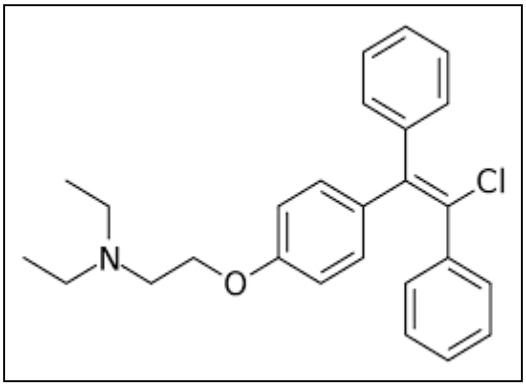


Figure2: Structure of Clomiphene citrate

Molecular Formula: $C_{32}H_{36}ClNO_8$

Molecular Weight: 598.1 g/mg

From literature survey. it was found that no any RP- HPLC method has been reported on this combination respectively. In this present research work, it was proposed to developed and validate a new, simple, and accurate RP- HPLC method for simultaneous estimation of Clomiphene citrate and Acetylcysteine in marketed dosage formulations. Only individual methods for estimating Clomiphene citrate and Acetylcysteine were found in the literature, although methods for simultaneous estimation of Clomiphene citrate and Acetylcysteine were published. both medications' estimations The mobile phase's composition is modified to maintain a high level of precision and specificity results. To obtain good sensitivity for quantitative analysis, a detection wavelength of 225 nm was chosen. Clomiphene citrate and Acetylcysteine in solid dose form were determined [2].

EXPERIMENTAL [3-4]

- **Instrument used:**

  Chromatography was carried out using a Systronic HPLC system that included a Hamilton Syringe, an auto sampler, and a UV detector.

- **Chemical and reagents:**

  The investigation used an analytically pure sample of Clomiphene citrate and Acetylcysteine obtained as a gift sample from Lupin Limited M. I. D. C, Tarapur via Boisar. The pharmaceutical dosage form utilised in this trial was "U MOM," which was described as containing 600 mg of clomiphene citrate and 50 mg of acetylcysteine per tablet. In the preparation of the mobile phase, Acetonitrile and Water (70:30) were utilised as solvents.

- **Preparation of mobile phase:**

  1000ml mobile phase was prepared by mixing 700 ml Acetonitrile and 300ml Water.

- **Degassing the mobile phase:**

  The prepared mobile phase was ultrasonically degassed for 15 minutes to avoid disruptions caused by dissolved gases.

- **Filtration of mobile phase:**

  The degassed mobile phase was filtered through 0.45 µm filters to avoid the column clogging due to smaller particles.

Preparation of Standard stock solution: [5-6]

1. Standard stock solution of Clomiphene citrate:

Prepare a Clomiphene citrate standard stock solution by adding 50 mg to a 50 ml volumetric flask and diluting to 50 ml with diluent. Then pipette out 1 ml, add 10 ml volumetric flask, and dilute with diluent to make the volume 10 ml again. (Clomiphene citrate concentration = 100 g/ml).

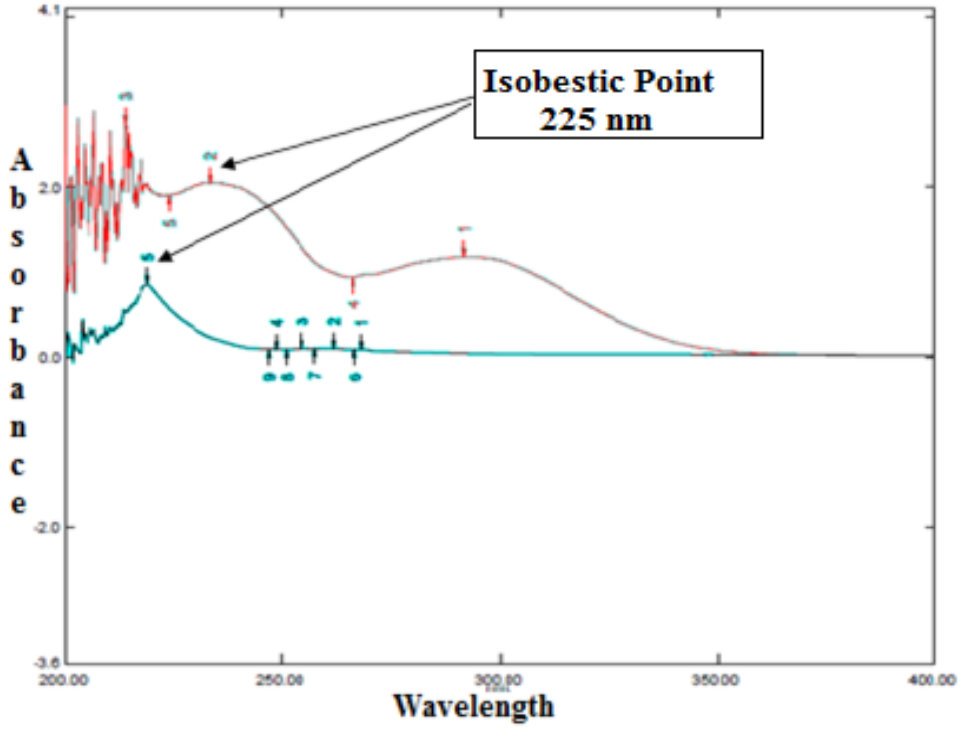
2. Standard stock solution of Acetylcysteine:

Prepare an Acetylcysteine standard stock solution by dissolving 50 mg in a 50 mL volumetric flask and diluting to 50 mL with diluent. Then pipette out 12 ml, add 10 ml volumetric flask, and dilute with diluent to make the volume 10 ml again. (Acetylcysteine concentration = 1200 g/ml).

Selection of detection wavelength:

The material was scanned with a PDA detector from 200 to 400 nm. The wavelength chosen for analysis was 225nm, which was determined based on the proper intensity of Clomiphene Citrate and Acetylcysteine both at the isosbestic sites.

Figure 3: Isobestic point of Clomiphene citrate & Acetylcysteine



Loading of Standard Solution [7]

Clomiphene Citrate and Acetylcysteine were loaded into the HPLC Vial stand, which was placed in the sample tray, and injected.

Table.1 Chromatographic conditions

Coloum temperature	30^0c
Flow rate	1ml/min
Mobile phase	Acetonitrile : Water
Runtime	9 Minutes
Injection volume	10µl
Wavelength	225 nm
Diluent	Mobile phase
Column	Agilent zobrax bonus-RP
Mobile phase ratio	70:30%v/v
Rt of Clomiphene citrate & Acetylcysteine	2 .43min & 4.26 min

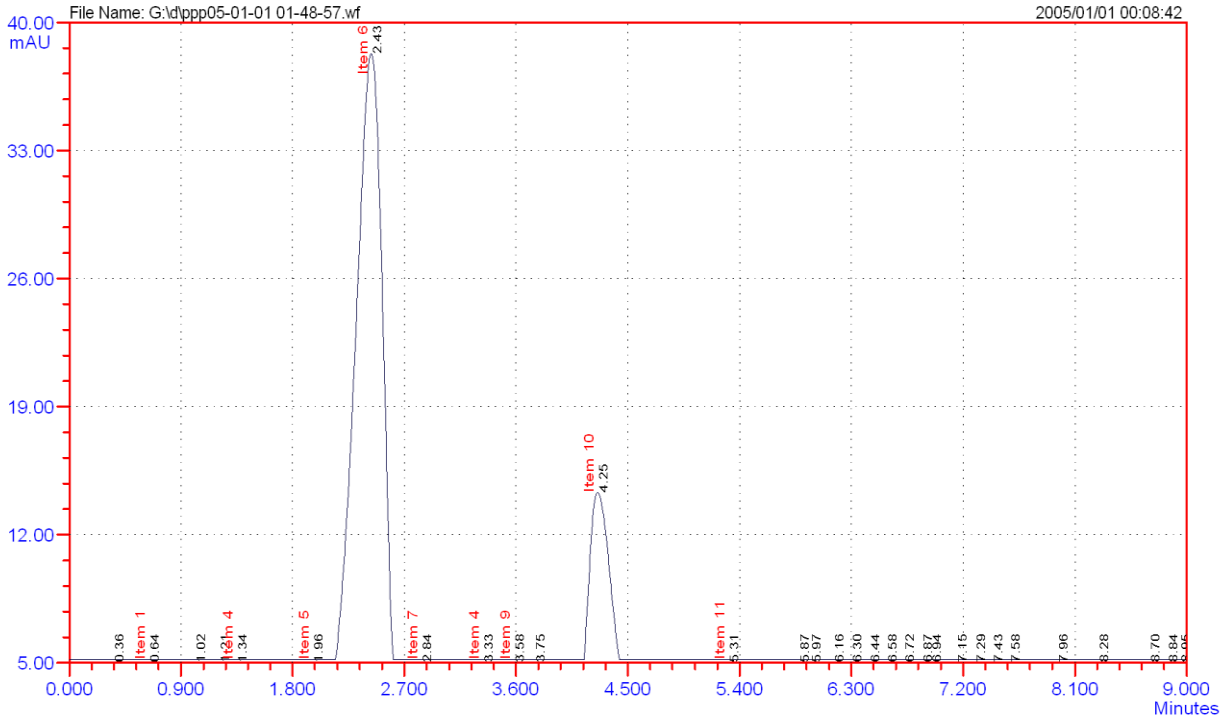


Figure 4: Chromatogram of Sample mixture of Clomiphene Citrate & Acetylcysteine

Preparation of sample solution[8]

Five tablets of brand name "U MOM" were used. From the five tablets accurately weighed the powder equivalent to single tablet (Clomiphene citrate 50mg and Acetylcysteine 600mg) 50 mg Clomiphene

citrate and 50 mg of Acetylcysteine were transferred into a 50 ml volumetric flask and 50 ml solvenet was added and sonicator approximately for 10 min. then filtered through 0.45 µm Nylon Membrane filters and make up volume up to 50 ml from solvent take 1 ml of above filtrate was transferred into 10 ml volumetric flask and the final volume was adjusted upto the mark with same solvent to get the sample solution with the concentration of 100µg/ml Clomiphene citrate and also take 12ml above filtrate was transferred into 10 ml volumetric flask and make the volume Again 10ml with solvent to get the solution with the conc.of Acetylcysteine1200µg/ml respectively.

Table 2: Assay results for Clomiphene Citrate and Acetylcysteine

	Clomiphene Citrate			Acetylcysteine		
Sr. no.	**Peak area**	**Amount recovered (µg/ml)**	**% recovery**	**Peak area**	**Amount recovered (µg/ml))**	**% recovery**
1	16386.4	50	101.9	245446.8	600	101.9
2	16108.9	50	99.0	246989.2	600	99.0
3	16184.1	50	98.8	247523.1	600	98.8
4	16303.1	50	101.1	248671.9	600	101.1
5	16317.7	50	98.2	248781.7	600	98.2

METHOD VALIDATION:

i) Linearity [8]

The linearity of Clomiphene Citrate and Acetylcysteine was determined using a mixed standard solution with concentrations ranging from 20 to 100 g/ml and 240 to 1200 g/ml, respectively. The calibration curves for Clomiphene Citrate and Acetylcysteine had correlation coefficients of 0.997 and 0.999, respectively. For Clomiphene Citrate, the findings are shown in (Table 3 and Fig.5), and for Acetylcysteine, they are shown in (Table 4 and Fig.6). Clomiphene Citrate and Acetylcysteine have regression equations of y = 187.1x + 6641 and y = 147.9x+1584, respectively.

Table 3: Linearity study of Clomiphene citrate

Sr. No	Concentration (µg/ml)	Peak area
1	20	10252.4
2	40	14162.7
3	60	17822.9
4	80	22134.5
5	100	24986.6

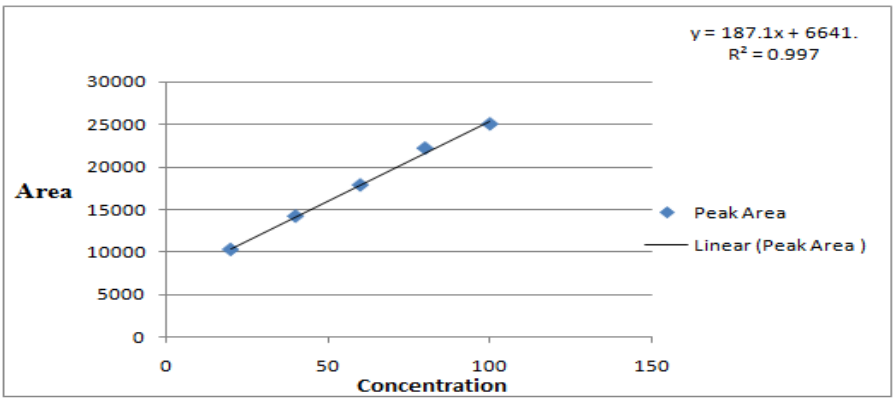


Figure 5: Calibration curve for Clomiphene citrate

Table 4: Linearity study of Acetylcysteine

Sr. No	Concentration (µg/ml)	Peak area
1	**240**	**191786.8**
2	**480**	**231603.3**
3	**720**	**265875.9**
4	**960**	**301263.1**
5	**1200**	**334541.8**

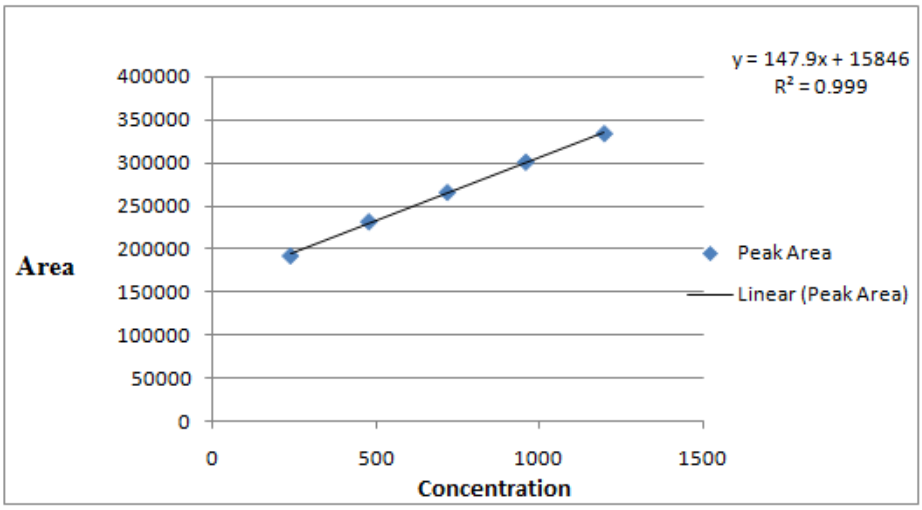


Figure 6:Calibration curve for Acetylcysteine

ii) Precision [9]

Intra-day: The intraday precision for Clomiphene Citrate and Acetylcysteine is shown in table (5 & 6). The % RSD for intraday precision was found to be 1.58 for Clomiphene Citrate and 1.2 for Acetylcysteine.

Table 5: Intra-day precision study of Clomiphene Citrate

Concentration (µg/ml)	Area		Mean	SD	%RSD	Mean %RSD
20	Tri 1	273497	273182	3415.60	1.25	
	Tri 2	273230				
	Tri 3	272819				
40	Tri 1	281309	285088	54469.2	1.91	1.58
	Tri 2	282624				
	Tri 3	291332				

Table 6: Intra-day precision study of Acetylcysteine

Concentration (µg /ml)	Area		Mean	SD	%RSD	Mean %RSD
240	Tri 1	283196.2	28388.1	28226.1	1.0	
	Tri 2	277551.2				
	Tri 3	280416.9				
480	Tri 1	295326.1	292335.6	41105.5	1.4	1.2
	Tri 2	287648.3				
	Tri 3	294032.5				

Inter-day:

The interday precision for Clomiphene Citrate and Acetylcysteine is shown in table (7 & 8). The % RSD for interday precision was found to be 1.04 for Clomiphene Citrate and 1.1 for Acetylcysteine.

Table 7: Inter-day precision study of Clomiphene Citrate

Concentration (µg /ml)	Area		Mean	SD	%RSD	Mean %RSD
20	Tri 1	275341	2752610	6951.13	0.25	
	Tri 2	274529				
	Tri 3	275912				1.04
40	Tri 1	281614	2843028	52306.8	1.83	
	Tri 2	290331				
	Tri 3	280963				

Table 8: Inter-day precision study of Acetylcysteine

Concentration (µg /ml)	Area		Mean	SD	%RSD	Mean %RSD
240	Tri 1	281203.6	2857291	42105.7	1.4	
	Tri 2	285851.3				
	Tri 3	290132.6				1.1
480	Tri 1	291324.5	2882338	26900.9	0.93	
	Tri 2	286419.7				
	Tri 3	286957.2				

iii) Accuracy [10]

The accuracy of the method was confirmed by recovery study from the marketed formulation at three level of standard addition. The results are shown in table (9 & 10).

Table 9: Recovery studies of Clomiphene Citrate

Level	Concentration (µg /ml)		Area	% Recovery	Mean %Recovery
	Sample	Std.			
50%	40	20	10254.4	98.30%	
100%	40	40	14362.7	99%	99.6
150%	40	60	17852.9	101.60%	

Table 10: Recovery studies of Acetylcysteine

Level	Concentration (µg /ml)		Area	% Recovery	Mean %Recovery
	Sample	Std.			
50%	480	240	191786.8	99.4	
100%	480	480	231603.3	101.8	101.3
150%	480	720	265875.9	102.7	

Table 11: System suitability parameters

	Parameter	Clomiphene Citrate	Acetylcysteine
1	Retention Time (min)	4.25	2.43
2	Area	21133.3	66765.9
3	Plates	1595	1395
4	Resolution	1.40	1.14

iv) Limit of detection (LOD) and Limit of Quantification (LOQ): [11-12]

In the present study, the LOD and LOQ were calculated according to the standard deviation of the response and the slope of the calibration curve i.e., 3.3σ / S and 10σ / S criterions, respectively; where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.
The LOD and LOQ of Clomiphene Citrate was found to be 104.39 µg/ml and 316.35µg/ml and for Acetylcysteine was 125.30µg/ml and 379.88µg/ml respectively.

v) Specificity: [13-14]

Specificity of the RP-HPLC method was determined by entire separation of Clomiphene Citrate and Acetylcysteine with parameter like retention time (tR), resolution (Rs), and tailing factor (T). The peak obtained for Clomiphene Citrate and Acetylcysteine were sharp and have clear baseline separation. The results are shown in table 12.

Table 12: Specificity parameters

Parameter	Clomiphene Citrate	Acetylcysteine
Tailing Factor(T)	1.35	0.85
Resolution (RS)	1.40	1.14
Retention time (tR)	4.26	2.43

vi) Robustness [15-16]

This was accomplished by making little changes to the chromatographic conditions, which were found to be unaffected by minor changes such as a 2% change in the volume of the organic solution in the mobile phase.

RESULT AND DISCUSSION [16-20]

In this method 1000 ml of mobile phase was prepared by mixing 700 ml Acetonitrile and 300ml Water. The degassed mobile phase was filtered through 0.45 μm filters to avoid the column clogging due to smaller particles. The flow rate was found to be optimum at 1ml/ min resulting in short retention time, good baseline stability with low noise level. In the present developed RP-HPLC method, the standard and sample preparation required less time and no tedious extraction was involved. By the use of the proposed method the retention time of Acetylcysteine and Clomiphene Citrate was found to be 2.43 minutes and 4.26 minutes respectively. The assay of Clomiphene Citrate and Acetylcysteine in bulk drug and in combined tablet dosage form was found to be 99.8% and 101.1%. A good linear relationship, r^2 =0.997 for Clomiphene Citrate andr^2 =0.999 for Acetylcysteine was observed between the concentration range of 10-100μg/ml and 240 -1200μg/ml respectively. The LOD values were found to be 104.39μg/ml and 125.30μg/ml and LOQ values were found to be 316.35μg/ml 379.88μg/ml for both Clomiphene citrate and Acetylcysteine respectively. Low values of standard deviation of retention time and peak areas indicate high precision of the method. From the recovery studies data, it was found that the mean % recovery was within the limits, indicated high accuracy of the proposed method. There are no additional peaks observed in the chromatogram, which indicates non-interference of the common excipients used in the formulation. No marked changes were observed in % assay of the optimized conditions with that of the altered conditions in the robustness study indicating that the method is robust.

CONCLUSION:

The simultaneous estimate of Clomiphene Citrate and Acetylcysteine in a short analysis time with minimal interference from typical additives included in pharmaceutical formulations was created using a simple and competent method. The proposed method can be used in bulk and tablet dosage forms for routine quality control and analysis.

ACKNOWLEDGEMENT:

I am very much thankful to Sahyadri college of Pharmacy, Methawde (Sangola), Maharashtra, for giving permission to carry out my work.

REFERENCE:

1. Indian Pharmacopoeia Volume III, "Government of India ministry of health and family welfare, published by Indian Pharmacopoeial commission", Government of India Ghaziabad. 2018;1392-1393, 3319-3320.
2. British Pharmacopoeia volume-I The requirement of the 4th edition of the European Pharmacopoeia 2002 as amended by supplement 4.1-4.3 medicinal and Pharmaceutical substances 2004; (A-1): 45.
3. Ghule ss. Analytical method development and validation of RP -HPLC method for simultaneous estimation of Pyridoxamine dihydrochloride and Acetyl cysteine in tablet dosage form, Journal of University of Shanghai for Science and technology, 2021; 23(6) : 992-1000.
4. Chatwal GR. Analytical chemistry,1st ed. Himalaya Publication House, 2008;1-3.
5. United States Pharmacopeial convention: 2013 United States Pharmacopoeia 36; National Formulary 31, US Pharmacopoea Convention, Rockville, MD.
6. Skoog DA, West DM, Fundamental of analytical chemistry. 8thed.Thomson Brooks/Cole, 2007; 1-3.
7. KD Tripathi Essential of Medical Pharmacology, 6thedition, Jaypee Brothers Medical Publishers, 2008;488-489.
8. Elizabeth M Development and Validation of an analytical method for Related substances in N-Acetyl cysteine -L- cysteine Effervescent tablet by RP-HPLC , Indian journal of Pharmaceutical Education and Research , 2007;51(4):626-634.
9. Jadhav N .Validation RP -HPLC Method and development for simultaneous estimation of acetyl cysteine and acebrofyline in Capsule formulation, Journal of Biomedical and Pharmaceutical Research,2014; 3,10-16.
10. Methew E.Development and Validation of an analytical method for Related substances in N-Acetyl cysteine -L- cysteine Effervescent tablet by RP- HPLC, Indian journal of Pharmaceutical Education and Research, 2007; 51, (4): 626-634.
11. Rele R.Simultaneous Spectrophotometric Estimation of Paracetamol and Aceclofenac by second order derivative method in combined dosage form, Journal of Chemical and Pharmaceutical Research, 2015;7(6): 512-517.
12. Athawale R, Nadkar S. Development and Validation of RP-HPLC Method for the Estimation of N-Acetylcysteine in Wet Cough syrup International Journal of Drug Development & Research, 2012; 4(2):284-293.

13. Lalitha K, JadhavN.Development and Validation of Spectroscopic Method for Simultaneous Estimation of Acebrophylline and Acetylcysteinein Capsule Dosage Form. International Journal of Pharmaceutical and Phytopharmacological Research, 2014; 4(2):113-115.
14. SoniA. Goyal S.UV Spectrophotometric analytical method development and validation of Clomiphene citrate in methanol, International Journal of Pharmaceutical and Phytopharmacological Research, 2020;11(5):2229-2237.
15. Kumar C, Method development and validation for simultaneous assessment of clomiphene citrate and n-acetyl cysteine in mixed tablet dosage form by RP-UPLC World journal of pharmacy and pharmaceutical sciences, 2014;3(3):1773-80
16. Mali AD, Bathe R, Tamboli A. Simultaneous determination of Cefiximetrihydrate and Ofloxacin in pharmaceutical dosage form by second order derivative UV spectrophotometry. International Journal of Advances in Pharmaceutical Analysis,2015;5,20-25.
17. Kaur P., Singh B. Analytical Method Development and Validation of Quercetin A Review International Journal of Pharmaceutical Research,2019;1(2) :49-56.
18. Mailvelan M., Mounnissamy M. Development and alidation of Spectrophotometric methods for the Simultaneous Estimation of Ubidecarenone and Clomiphene Citrate in bulk and tablet dosage form, Asian Journal Research Chemistry and Pharmaceutical Science, 2013;23-30.
19. More S, Tamboli A.RP-HPLC Method Development and Validation for the Simultaneous Estimation of Gabapentin and Amitriptyline Hydrochloride in Pharmaceutical Dosage Forms, International Journal of Pharmacy and Pharmaceutical Research, 2019;15(3):15-24.
20. ICH validation of analytical procedures: text and, methodology Q2(R1), 2005.