Analytical Method Development And Validation Of Rp-Hplc Method For Simultaneous Estimation Of PyridoxamineDihydrochloride And Acetylcysteine In Tablet Dosage Form.

SNEHA S. GHULE [1]*, ASHPAK M. TAMBOLI[1] , SNEHAL D. PATIL[1]

[1]Department of pharmaceutical Chemistry ,Sahyadri College of Pharmacy, Methwade, Sangola, 413307, Solapur, Maharashtra, India

Email Id : snehaghule02@gmail.com

ABSTRACT :

A reverse phase high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Pyridoxamine dihydrochloride and Acetylcysteine in marketed formulation is developed. Chromatography carried out at 30^{o}c temperature on Agilent Zorbax Bonus-RP (250 x 4.6 mm, 5 µ) coloum. Coloum using a mobile phase 0.1% trifluroacetic acid in water: acetonitrile (80:20v/v) with flow rate 1ml/min (DAD scan at 210nm). Validation parameter such as system suitability, linearity, precision, accuracy are considered as reported International Conference on Harmonization guidelines. The retention times for Pyridoxamine dihydrochloride and Acetylcysteine are 2 min and 3.4 min. The linearity range for Pyridoxamine dihydrochloride and Acetylcysteine is 30-70 µg/ml and 180-420 µg/ml. The %RSD for accuracy were found to be less than 2%. Hence the proposed method was found to be accurate, precise, reproducible and specific and can be used for simultaneous analysis of these drugs in tablet formulation.

KEY WORDS : Pyridoxaminedihydrochloride, Acetylcysteine, RP-HPLC

INTRODUCTION :

Pyridoxaminedihydrochloride chemical name is 4-(aminomethyl)-5-(hydroxymethyl)-2-methylpyridin-3-ol dihydrochloride.It is small molecule derivative of pyaridoxal phosphate (vitamin B_6) with the distinct chemical structure that inhibit the formation of advanced glycation end-products (AGE).Pyridoxaminedihydrochloride is used for the treatment of vitamin deficiency. It also used in the Diabetic neuropathy. It is block the pathogenic oxidative pathways in progression of diabetic neuropathy. Pyridoxamine inhibits a broad range of pathogenic oxidative chemistries that lead to AGE formation, including activity against toxic carbonyls, reactive oxygen species, and the conversion of glycosylated proteins to AGEs.

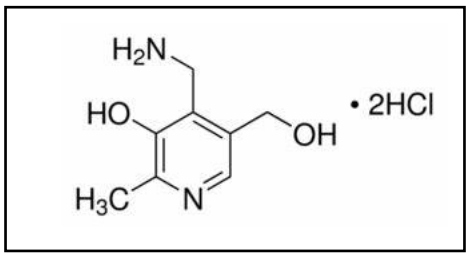

Figure 1: Structure of Pyridoxamine dihydrochloride

Acetylcysteine is also known as (N-Acetylcysteine or N-acetyl-L-cysteine or NAC) is primarily used as a mucolytic agent. It is used as an antidote for acetaminophen overdose to prevent hepatic injury.Acetylcysteine can be also used as a general antioxidant which can help mitigate symptoms for a variety of diseases exacerbated by reactive oxygen species. Acetylcysteine is in a class of medications called mucolytic agents. Intravenous and oral formulations of Acetylcysteine are available for the treatment of paracetamol overdose.

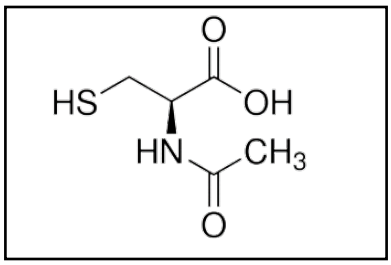

Figure 2: Structure of Acetylcysteine

Literature review reveals only individual methods for estimation of Pyridoxamine dihydrochlorideand Acetylcysteine but methods were reported for simultaneous estimation of Pyridoxamine dihydrochloride and Acetylcysteine. So method was developed method more superior than previously published method of individual estimation of both drugs. The composition of mobile phase is adjusted to maintain highly accurate and specific results. The detection wavelength of 210nm was chosen in order to achieve a good sensitivity for quantitative determination of Pyridoxamine dihydrochloride and Acetylcysteine in solid dosage form.

MATERIAL AND METHODS:

Chemical and reagents:

Analytical pure sample of Pyridoxamine dihydrochloride and Acetylcysteine were received as a gift sample from Cipla Private Limited were used in the study. The pharmaceutical dosage form used in this study was NEFROSAVE FORTE labeled to contain Acetylcysteine and Pyridoxamine dihydrochloride 300/50 mg per tablet. The solvent used were of HPLC 0.1% TFA water and Acetonitrile used in preparation of mobile phase.

Preparation of mobile phase:

1000ml mobile phase was prepared by mixing 800ml 0.1% trifluroacetic acid in water and 200ml Acetonitrile.

Apparatus and chromatographic conditions:

Chromatographic separation Agilent zorbax bonus-RP (250 × 4.6 mm, 5µ) coloum was used for separation. The elution was carried out gradient at flow rate of 1ml/min using 0.1% trifluroaceticacid : acetonitrile (80:20 v/v) mobile phase.

Preparation of Standardstock solution :

Standard stock solution of Acetylcysteine :

Initially prepare a standard stock solution (SSS-1) of by adding 30mg of Acetylcysteine in 10ml volumetric flask & add 5ml diluent, mix for 2 minutes and make the volume to 10ml with diluent (conc.ofAcetylcysteine = 3000 µg/ml).

Standard stock solution of Pyridoxamine dihydrochloride :

Then prepare a standard stock solution (SSS-2) of pyrodoxaminedihydrochloride by adding 5mg in 10 ml volumetric flask & add 5 ml diluent, mix for 2 min. & make the volume to 10 ml with diluent.(conc. Of Pyridoxamine dihydrochloride = 500 µg/ml).

Then add 1.0 ml of SSS-1 & 1.0 ml SSS-2 in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent.(conc.ofAcetylcysteine = 3000 µg/ml & conc. Of Pyridoxaminedihydrochloride = 500 µg/ml).

Selection of wavelength:

The sample was scanned from 200-400 nm with PDA detector. The wavelength selected for analysis chosen was 210 nm on basis of appropriate intensity of Acetylcysteine and Pyridoxaminedihydrochloride.

Table.1 Chromatographic conditions:

Coloum temperature	30^0c
Flow rate	1ml/min
Mobile phase	0.1%TFA Water : Acetonitrile
Runtime	8 Minutes
Injection volume	10µl
Wavelength	210 nm
Diluent	Mobile phase
Column	Agilent zobrax bonus- RP
Mobile phase ratio	80:20%v/v
Rt of Pyridoxaminedihydrochloride& Acetylcysteine	2 min & 3.4 min

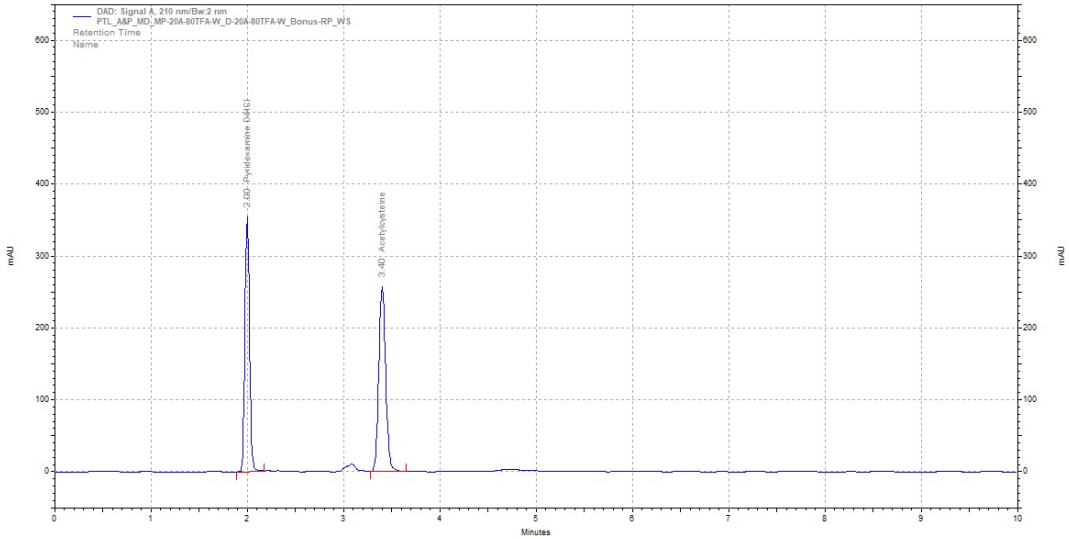

Figure. 3: Chromatogram of standard mixture of Pyridoxaminedihydrochloride&Acetylcysteine

Preparation of sample solution :

Tablet sample solution (TSS) :

10 tablets were weighed and average weight was calculated and tablets were crushed in mortar and pestle.Powder weight equivalent to 3000 µg Acetylcysteine and 500 µg Pyridoxamine dihydrochloride were weighed into 10 ml volumetric flask & add 5 ml diluent, sonicate for 10 minutes and make the volume to 10 ml with diluent. (conc.ofAcetylcysteine = 3000 µg/ml, Pyridoxaminedihydrochloride = 500 µg/ml).

Assay :

Individual samples of Acetylcysteine and Pyridoxamine dihydrochloride were prepared of 300 µg/ml and 50 µg/ml, respectively and peaks were for identified from Retention Time. Blank was injected to ensure there is no blank peak interfering with the main analyte peaks.

METHOD VALIDATION :

Linearity :

A series of standard solution 30-70 µg/ml of Pyridoxamine dihydrochloride and 180-420 µg/ml of Acetylcysteine were prepared. An aliquot of 10 µl of each solution was injected 5 times for each standard solutions and peak area was observed. 5 samples of varying concentrations ranging from 60-140% were made. The results obtained are shown in table (Table 4) for Pyridoxamine dihydrochlorideand in (Table 5) for Acetylcysteine.

Table 2: Linearity dilutions :

Sr.No	Pyridoxamine dihydrochloride (µg/ml)	Acetylcysteine (µg/ml)	Volume of Pyridoxamine dihydrochloride stock solution to be taken(ml)	Volume of Acetylcysteine stock solution to be taken (ml)	Diluted to volume (ml)
1	30	180	0.6	0.6	10
2	40	240	0.8	0.8	10
3	50	300	1	1	10
4	60	360	1.2	1.2	10
5	70	420	1.4	1.4	10

Precision :

The precision of the method was done by system precision and method precision.The percentage RSD value was found to be within the limit below 2. The percentage RSD values less than 2 for peak area ratioof Pyridoxamine dihydrochlorideand Acetylcysteine obtained, thus the results showing that equipment used for the work.

Accuracy :

The accuracy of the method was determined by calculating recovery values of Pyridoxamine dihydrochloride and Acetylcysteine by standard addition method. The recovery studies were carried out at different levels of 80-120% and average % recovery was observed. Samples were prepared of 80%, 100% and 120% concentration by spiking the same amount of concentration given below in table for both Acetylcysteine (table 9) and Pyridoxamine dihydrochloride (table 8). Samples were injected in duplicate to calculate % RSD. % recovery was also calculated.

System suitability :

A single sample was prepared as described and 5 injections were made from same sample and checked for system suitability.

Limit of detection (LOD) and Limit of Quantification (LOQ) :

The LOD and LOQ were found to be 0.56 µg/ml and 1.70 µg/ml for Pyridoxamine dihydrochloride the LOD and LOQ were found to be 14.08 µg/ml and 42.65 µg/ml for Acetylcysteine,respectively.

RESULT AND DISCUSSION :

Table 3: Assay data of Pyridoxamine dihydrochloride and Acetylcysteine

The % assay was found to be 99.61% for Pyridoxamine dihydrochloride and 100.25 % for Acetylcysteine. Assay result shown in below table 3.

	Pyridoxamine dihydrochloride		**Acetylcysteine**	
Sample	Working Standard	Drug Product	Working Standard	Drug Product
Area	2735672.2	2725104	2801012.2	2808113
Assay	--	99.61	--	100.25

Table 4:Linearity data of Pyridoxamine dihydrochloride

Linearity was studied by plotting a graph of area v/s concentration. A series of standard solution of Pyridoxamine dihydrochloride were prepared in the concentration range of about 30µg/ml to 70 µg/ml is shown in belowtable**.**Linearitygraph of Pyridoxaminedihydrochloride shown in Figure.no.4.

Linearity level %	**Concentration (µg/ml)**	**Peak area**
60	30	1660163
80	40	2205780
100	50	2734970
120	60	3262741
140	70	3798616

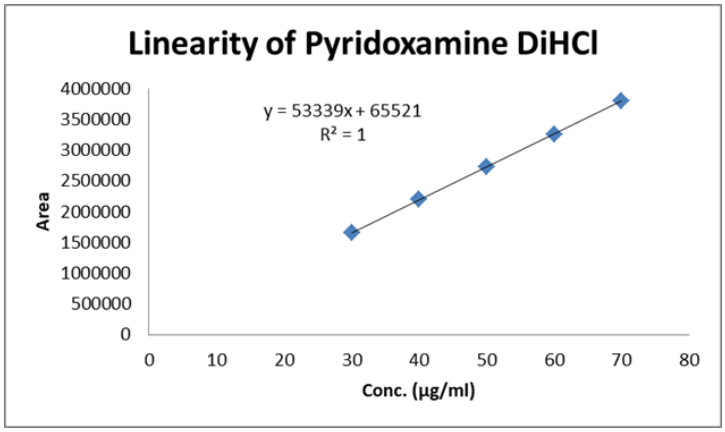

Figure.4:Linearity graph of Pyridoxaminedihydrochloride

Table 5:Linearity data of Acetylcysteine

Linearity was studied by plotting a graph of area v/s concentration. A series of standard solution of Acetrylcysteine were prepared in the concentration range of about 180µg/ml to 420µg/ml is shown in below table.Linearity graph of Acetylcysteine shown in Figure.no.5

Linearity level %	Concentration (µg/ml)	Peak area
60	180	1704234
80	240	2262575
100	300	2775515
120	360	3353136
140	420	3939717

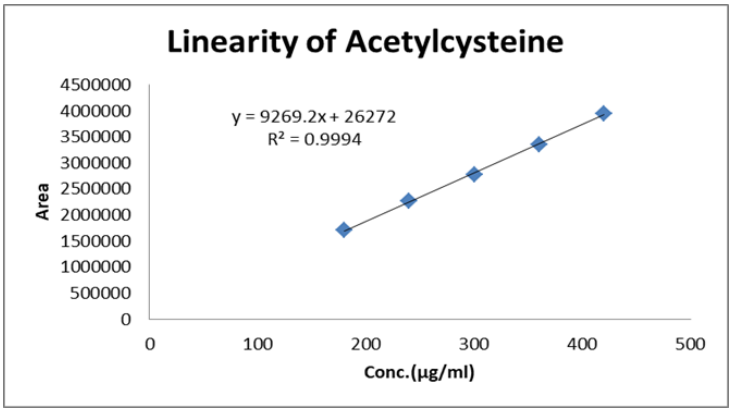

Figure.5:Linearity graph of Acetylcysteine

Table 6: Precision data of Pyridoxamine dihydrochloride

The precision of the Pyridoxamine dihydrochloridemethod was found tobe good with % RSD less than 2, indicate that method was precise and the results presented below table. In this concentration of sample is 50µg/ml.

Pyridoxaminedihydrochloride		
Conc of sample	**Sample ID**	**Area**
50	Rep 1	2734970
50	Rep 2	2728190
50	Rep 3	2732303
50	Rep 4	2751647
50	Rep 5	2731251
Average		2735672.2
STDEV		9254.311898
RSD		0.34

Table 7: Precision data of Acetylcysteine

The precision of theAcetylcysteinemethod was found to be good with % RSD less than 2, indicate that method was precise and the results presented below table. In this concentration of sample is 300µg/ml.

Acetylcystiene		
Conc. of sample	**Sample ID**	**Area**
300	Rep 1	2775515
300	Rep 2	2831962
300	Rep 3	2785807
300	Rep 4	2804169
300	Rep 5	2807608
Average		2801012
STDEV		21752.39
RSD		0.78

Table 8: Accuracy data for Pyridoxaminedihydrochloride by RP-HPLC:

In accuracy study percentage recovery range of Pyridoxamine dihydrochloride is 100.97% to 100.28%. The range of % RSD is 0.41% to 0.36%.

Sample ID	**Reps**	**Spiked Conc. (ug/ml)**	**Area**	**Amt Recovered (ug/ml)**	**% Recovery**	**Average**	**STDEV**	**RSD**
80%	Rep 1	39.988	2262575	40.38	100.97	100.68	0.4172	0.41
	Rep 2	39.988	2249354	40.14	100.38			
100%	Rep 1	49.985	2775515	49.53	99.09	100.10	1.424987	1.42
	Rep 2	49.985	2831962	50.54	101.10			
120%	Rep 1	59.982	3353136	59.84	99.76	100.02	0.36466	0.36
	Rep 2	59.982	3370470	60.15	100.28			

Table 9: Accuracy data for Acetylcysteine by RP-HPLC:

In accuracy study percentage recovery range of Acetylcysteine is 100.79% to 99.57%. The range of % RSD is 0.35% to 0.13%.

Sample ID	Reps	Spiked Conc. (ug/ml)	Area	Amt Recovered (ug/ml)	% Recovery	Average	STDEV	RSD
80%	Rep 1	239.928	2205780	241.82	100.79	101.04	0.35211	0.35
	Rep 2	239.928	2216678	243.01	101.29			
100%	Rep 1	299.91	2734970	299.83	99.97	99.85	0.17524	0.18
	Rep 2	299.91	2728190	299.09	99.73			
120%	Rep 1	359.892	3262741	357.69	99.39	99.48	0.12951	0.13
	Rep 2	359.892	3268754	358.35	99.57			

Table 10: system suitability parameter

Parameter of system suitability is Retention time,Theroticalplates,Asymmetry(Tailing factor),Resolutionis shown in table 10.

Parameter	Pyridoxamine dihydrochloride	Acetylcysteine
Retention time	2	3.4
Therotical plates	7971	10424
Asymmetry (Tailing factor)	1.03	1.06
Resolution	0.00	12.42

Table 11:LOD& LOQ Data

LOD and LOQ of Pyridoxamine dihydrochloride is 0.56µg/ml and 1.70µg/ml and Acetylcysteine is 14.08µg/ml and 42.65µg/ml.

Drugs	LOD µg/ml	LOQ µg/ml
Pyridoxamine dihydrochloride	0.56	1.70
Acetylcysteine	14.08	42.65

CONCLUSION :

It concludes that the developed method is simple, accurate and precise and suitable for the routine analysis. The developed methods were validated as per ICH guidelines and were found to be within limit.

ACKNOWLEDGEMENT :

I am very much thankful to sahyadri college of pharmacy, Methwade (Sangola), Maharashtra, for giving permission to carry out my work.

REFERENCES :

[1] United States Pharmacopeial convention: United States Pharmacopoeia 36; National Formulary 31, US Pharmacopoea Convention, Rockville, MD, 2013.

[2] KD TripathiEssential of Medical Pharmacology, 6th edition, Jaypee Brothers Medical Publishers,2008.

[3] BharathiD.,Development and validation of RP-HPLC method for simultaneous estimation of pyrodoxaminedihydrochloride and actylcysteine in tablet dosage form,International journal of medicinal chemistry and analysis.Vol.6, 94-99, 2016.

[4] British Pharmacopoeia vol-I Introduction General notice Monograph, Medicinal and Pharmaceutical substances (A1),45,2004.

[5] Nalluri N. J, Development and validation of a new RP-HPLC method for simultaneous estimation of N-Acetylcysteine and L-arginine in combined dosage form,Original journal of chemistry,2014, 3:1372-1378.

[6] SudheerM., Analytical method development and validation of RP-HPLC method for simultaneous estimation of N-Acetylcysteine and cefexime from its fixed dose combination. Research journal of pharmacy and technology. Vol. 9, 835-842, 2016.

[7] Vedang K. , A novel stability indicating RP-HPLC method for the simultaneous estimation of N-Acetylcysteine and ambroxolin combined tablet dosage form. International journal of pharmaceutical sciences and research, vol. 8,2161-2167.

[8] K. SrinivasaRao, RP-HPLC Method for the Determination of Losartan Potassium and Ramipril in Combined Dosage Form. Indian Journal of Pharmaceutical Sciences, 108-111,2010

[9] Patel H. , RP-HPLC Method Development and Validation for Simultaneous Estimation of Cilnidipine and BisoprololFumarate in Tablet Dosage Form. International Journal of ChemTech Research. Vol. 12, 269-276, 2019.

[10] Patil S. , Development and validation of RP-HPLC method for the simultaneous estimation bisoprololfumarate and telmisartan from pharmaceutical formulations. International journal of pharmacy and analytical research. Vol. 9, 129-136,2020.

[11] More S. ,Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Pregabalin and Amitriptyline Hydrochloride From Pharmaceutical Formulations. International Journal of Research and Analytical Reviews (IJRAR). Vol. 6, 667-678, 2019.

[12] Sohan S C. , RP-HPLC method for simultaneous estimation of amlodipine and metoprolol in tablet formulation. Asian Journal of Pharmaceutics.232-234, 2008.

[13] N. Mukuntha Kumar, Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Telmisartan and Hydrochlorothiazide in Bulk and Pharmaceutical Dosage Form. International Journal of Pharma Sciences and Research (IJPSR).Vol. 5,646-654, 2014.

[14] M. PrasadaRao, Simultaneous estimation of Ramipril and OlmesartanMedoximil by RP-HPLC method. International Journal of Pharmaceutical Chemistry and Analysis, vol. 4, 106-111.

[15] NuranErcal, High-performance liquid chromatography assay for N-acetylcysteine in biological samples following derivatization with N-(1-pyrenyl)maleimide. Journal Of Chromatography B:Biomedical Applications, 229-234, 1996.

[16] ICH guidelines, validation of analytical procedures: text and methodology,Q2A(R1) Nov;2005.

[17] Snyder L. R.. Practical HPLC Method Development, John Wiley & Sons Inc., Edition 2.