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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF PYRIDOXAMINE DIHYDROCHLORIDE AND ACETYL CYSTEINE IN TABLET DOSAGE FORM

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ABSTRACT

A simple, very selective, linear, precise and accurate RP-HPLC method was developed and validated as per ICH guidelines for assay of Pyridoxamine dihydrochloride and Acetyl cystenine in bulk and in tablet dosage forms. An isocratic elution at a flow rate of 1.0 ml/ min was employed on a C_{18} (phenomenon, cremini 250*4.6mm, 5 micron) column at room temperature. The mobile phase consisted of potassium dihydrogen phosphate buffer and methanol in the ratio of 90:10 at pH 2.2 with ortho phosphoric acid. The detection wavelength was 210nm and 20µl of sample was injected. This method obeys Beer's law and the correlation coefficient value was above 0.999. The method was successfully applied to commercial formulation of this combination and validated as per standard analytical procedures. The proposed method applicable for routine analysis of Pyridoxamine dihydrochloride and Acetyl cystenine in bulk and in tablet dosage forms.

Keywords: Pyridoxamine dihydrochloride, Glycated proteins, Determination, Acetyl cystenine.

INTRODUCTION

Pyridoxamine dihydrochloride [1] is used for diabetic nephropathy which is approved by FDA. Pyridoxamine blocks the formation of Advanced Glycation End products (AGE) which is responsible for the diabetes. It traps the intermediates from glycated proteins. Acetyl cysteine acts as a mucolytic agent [2] which is official in the united states of pharmacopeia. Acetyl cysteine exhibits its action by breaking the disulfide linkages in the mucus to liquefy it. Together with the glutathione binds to the toxic metabolites to protect the liver from acetaminophen poisoning (NAPQI toxicity). There is no RP-HPLC method available for the determination of Pyridoxamine dihydrochloride and Acetyl cystenine in bulk and in tablet dosage forms. Fewer method available for Acetyl cysteine [3], combination of Acetyl cystenine with other drugs [4, 5] and few HPLC method also available for Pyridoxine dihydrochloride [6, 7]. The aim of the present work was to develop and validate a simple and reliable isocratic RP-HPLC method determination of Pyridoxamine dihydrochloride and Acetyl cystenine in bulk and in tablet dosage forms. The method validated as per ICH guidelines [8, 9].

EXPERIMENTAL MATERIALS

The Reference standards namely pyridoxamine dihydrochloride and acetyl cysteine were kindly provided as gift sample by fourrts (India) laboratories private limited Chennai. A placebo for the validation study was prepared with its declared excipients. All the chemicals used were of HPLC grade.

Equipments

The chromatographic system used to develop this technique, is a HPLC –Agilent 1100 series with chemstation featuring a column oven, a quaternary pump, an automatic injector and PDA detector.

Chromatographic Conditions

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Chromatographic separation of pyridoxamine dihydrochloride and its related substance was performed using C_{18} (phenomenon, cremini 250*4.6mm, 5 micron) made of stainless steel. The mobile phase consists of potassium dihydrogen phosphate buffer and methanol in the ratio of 90:10 at pH 2.2 with ortho phosphoric acid. The mobile phase filtered through a 0.45µm membrane filter and pumped through the column at the flow rate of 1.0ml/min. The injection volume to carry out the chromatography was set at 20µl. The wavelength was fixed at 210nm.

RP-HPLC METHOD VALIDATION

Solutions

Mixed standard solution was prepared by dissolving 150mg of acetyl cysteine and 25mg of pyridoxamine dihydrochloride into a 100ml volumetric flask. Add 20ml of mobile phase dissolve and dilute to 100ml. Then dilute 2.5ml of above solution with mobile phase to 50ml.

Stock solution was prepared by mixing average weight of 20 tablets about 350mg into 100ml volumetric flask. Add 20ml of mobile phase and sonicate for 10 minutes and make up the volume to 50ml with mobile phase.

Linearity and Range

Linearity of method was checked using fine different concentrations 0.0125mg/ml to 0.0375mg/ml (Pyridoxamine dihydrochloride) and 0.075mg/ml to 0.225mg/ml (Acetyl cysteine).

Accuracy and Precision

The accuracy of the method was determined by recovery experiment. The recovery studies were carried out at five different levels of 50-150% and the average percentage recovery was observed.

The precision of the method was done by system precision and method precision. The percentage RSD value was found to be within the limit.

Robustness and Ruggedness

Robustness is checked by making slight deliberate change in the experimental procedure. It was determined by carrying out the analysis under the condition during which flow rate, composition ratio, pH was altered and the changes on RT values and peak areas were noted. The ruggedness was also noted.

RESULT AND DISCUSSION

Selection of the detection wavelength

The overlain UVspectra of Pyridoxamine dihydrochloride and Acetyl cystenine in the mobile phase consists of potassium dihydrogen phosphate buffer and methanol in the ratio of 90:10 at pH 2.2 with ortho phosphoric acid, scanned in the region of 200 and 400nm, and 210nm was selected as the detection wavelength.

Optimization of the chromatographic conditions

The selection of the mobile phase depends upon the nature of the sample, molecular weight and solubility of the drug. Among C8 and C18, C18 column was selected. The mobile phase consists of potassium dihydrogen phosphate buffer and methanol in the ratio of 90:10 at pH 2.2 with ortho phosphoric acid to give symmetric peaks with short run time. The optimized chromatogram shown in Figure 2.

Validation ofmethods

Linearity

Six serial dilution concentrations of the drug solutions used for calibration graphs, linear relationships between the ratios of peak area to that of drug concentration were observed, as shown by the results presented in Table 1 - 4. The standard deviations of the slope and intercept values were low. The determination coefficient (r2) exceeded 0.999. The linearity graph were shown in figure 3 – 4.

System suitability

The resolution factor between pyridoxamine dihydrochloride and acetyl cysteine, in the method, was above 2. The system suitability parameters were shown in table 5.

Precision

The repeatability study (n=6) carried out showed in table 6 & 7. The percentage RSD values less than 2 for the peak area ratio of Pyridoxamine dihydrochloride and Acetyl cystenine obtained, thus the results showing that the equipment used for the work. For the intermediate precision a study carried out by the same analyst working on 3 consecutive days (n = 3) indicated a R.S.D. of 0.1911 and 0.329399, and indicated a good method precision.

Accuracy

The accuracy were expressed in terms of percentage recoveries of Pyridoxamine dihydrochloride and Acetyl cystenine. The results were shown in table 8 & 9. The recoveries of Pyridoxamine dihydrochloride and Acetyl cystenine were within the range of 99.82 – 99.99% and 98.41 – 99.94%.

Ruggedness and Robustness

Ruggedness indicates that analysis of an homogeneous sample in different laboratories, by different analysts, under prevalent environmental conditions using the specified parameters. Robustness can be described as the ability to reproduce the method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained results. The Ruggedness and Robustness %RSD less than 2, and values presented in table 10 – 12.

Table 1. Linearity dilution

Sl.No.	Pyridoxamine dihydrochloride (gm/ml)	Acetyl cysteine (gm/ml)	Volume of Pyridoxamine dihydrochloride stock solution to be taken (ml)	Volume of Acetyl cysteine stock solution to be taken (ml)	Diluted to volume (ml)
1	0.125	0.075	2.5	2.5	50
2	0.0175	0.105	3.5	3.5	50
3	0.0225	0.135	4.5	4.5	50
4	0.0275	0.165	5.5	5.5	50
5	0.0325	0.195	6.5	6.5	50
6	0.0375	0.225	7.5	7.5	50

Table 2. Optical Characteristics Of Pyridoxamine Dihydrochloride And Acetyl Cysteine By Rp - HPLC

Parameters	Pyridoxamine dihydrochloride	Acetyl cysteine
λ_{max} (nm)	210	210
Beers law limit (mg/ml)	0.0125-0.0375	0.075 – 0.225
Correlation coefficient (r)	0.99981	0.99974
Regression equation (y=mx+c)	y= 8.136x + 11.89	y= 40.23 x + 1.032
Slope (m)	8.136	40.23
Intercept (c)	11.89	1.032
LOD (µg/ml)	0.273	0.424
LOQ (µg/ml)	0.0930	0.472

Table 3. Linearity data of Pyridoxamine Dihydrochloride

Linearity level	Conc. (mg/ml)	Peak Area
1	0.0125	1123174
2	0.0175	1572557
3	0.0225	2021842
4	0.0275	2471114
5	0.0325	2920484
6	0.0375	3369682

Table 4. Linearity data of Acetyl cysteine

Linearity level	Conc. (mg/ml)	Peak Area
1	0.075	28033111
2	0.105	39246074
3	0.135	50459624
4	0.165	61672922
5	0.195	72886011
6	0.225	84099418

The following parameters are to be evaluated during this study.

(1) System suitability
(2) Precision
(3) Linearity
(4) Accuracy
(5) Range
(6) Robustness

System suitability

Preparation of Standard solutions

Prepare and analyse standard solutions of Pyridoxamine dihydrochloride and Acetyl cysteine. Calculated the % RSD.

Table 5. System suitability Acceptance criteria

S. No	Standard peak Area of Pyridoxamine dihydrochloride	Standard peak Area of Acetyl cysteine
1	1123755	28033210
2	1127232	27955877
3	1130886	27992667
4	1126954	27964985
5	1122468	27930643
6	1129149	27999087
Avg. peak area	**1126740**	**27979412**
%RSD	**0.3**	**0.5**

The Percentage relative standard deviation (%RSD) should be not more than 2.0

Table 6. Precision data of Pyridoxamine dihydrochloride

S. No	Weight of sample (mg)	Sample Area	% Drug Released
1	439.2	1123787	99.40999
2	441.2	1127292	99.43919
3	438.9	1130834	99.74774
4	441.4	1126954	99.82698
5	442.3	1122468	99.04269
6	446.7	1129154	99.00979
Mean			99.41273
SD			0.189754
%RSD			0.191106

Table 7. Precision data of Acetyl cysteine

S. No	Weight of sample (mg)	Sample peak(nm)	% Drug Released
1	439.2	28033221	99.58895
2	441.2	27955879	100.0809
3	438.9	27992990	100.1466
4	441.4	27964985	100.12753
5	442.3	27930653	99.75761
6	439.7	27999972	100.1263
Mean			99.971315
SD			0.329008
%RSD			0.329399

Table 8. Accuracy Data For Pyridoxamine Dihydrochloride by RP - HPLC

S.No	Spiked level	Amount of drug (mg/ml)	Amount added (mg/ml)	%Recovery Avg ± S.D	%R.S.D
1	50%	0.00625	0.010	99.91 ± 0.293	0.290
2	100%	0.0125	0.010	99.82 ± 0.273	0.282
3	150%	0.01875	0.010	99.99 ± 0.139	0.141

(n=3)

Table 9. Accuracy Data For Acetyl Cysteine by RP - HPLC

S.No	Spiked level	Amount of drug (mg/ml)	Amount added (mg/ml)	%Recovery Avg ± S.D	%R.S.D
1	50%	0.0375	0.010	99.94 ± 0.180	0.192
2	100%	0.075	0.010	99.73 ± 0.285	0.292
3	150%	0.1125	0.010	98.41 ± 0.569	0.578

(n=3)

Table 10. Ruggedness of Pyridoxamine Dihydrochloride and Acetyl Cysteine

Drug	Analyst 1 (area)	Analyst 2 (area)	SD	%RSD (Limit NMT 2.0%)
Pyridoxamine dihydrochloride	1123792	1123784	0.792	0.091
Acetylcysteine	28033274	28033248	0.484	0.252

Table 11. Robustness of Pyridoxamine Dihydrochloride

S.No	1	2	3
Parameter	**Rt**	**Area**	**Tailing factor**
Initial Escitalopram oxalate	2.610	1123781	1.7
Flow (-0.1ml/min)	2.712	1123854	1.69
Flow (+0.1ml/min)	2.492	1123689	1.83
wavelength(-2nm)	2.613	1123559	1.78
wavelength(+2nm)	2.622	1123872	1.78
S.D	0.153		0.0368
R.S.D	0.183		0.0242

Table 12. Robustness of Acetyl Cysteine

S.No	1	2	3
Parameter	**Rt**	**Area**	**Tailing factor**
Initial clonazepam	9.24	28033221	1.785
Flow (-0.1ml/min)	10.58	28033472	1.348
Flow (+0.1ml/min)	8.16	28033119	1.543
wavelength(-2nm)	9.27	28033864	1.687
wavelength(+2nm)	9.33	28033784	1.147
S.D	0.283		0.0222
R.S.D	0.072		0.0184

Fig 1. Chemical structures of (a): Pyridoxamine dihydrochloride (b): Acetyl cystenine

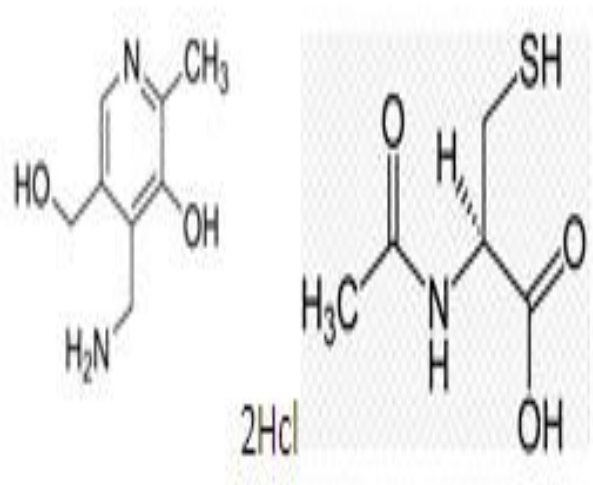


Fig 2. Chromatogram for Pyridoxamine dihydrochloride and Acetyl cysteine

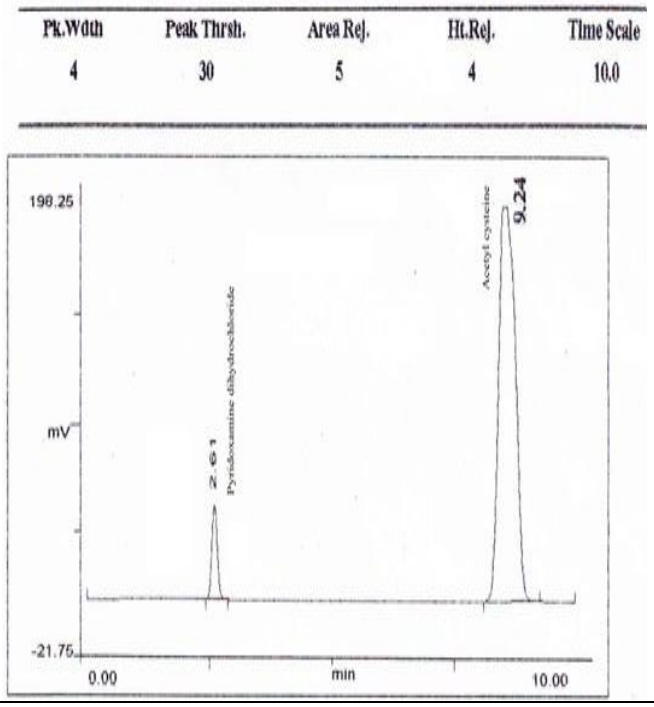


Fig 3. Graph for Linearity data of Pyridoxamine dihydrochloride

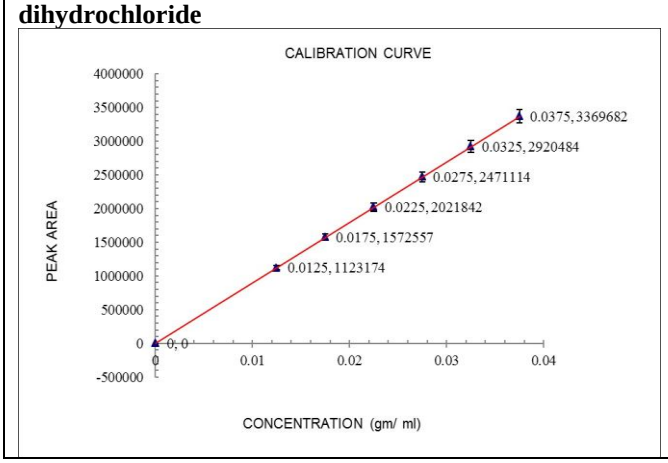
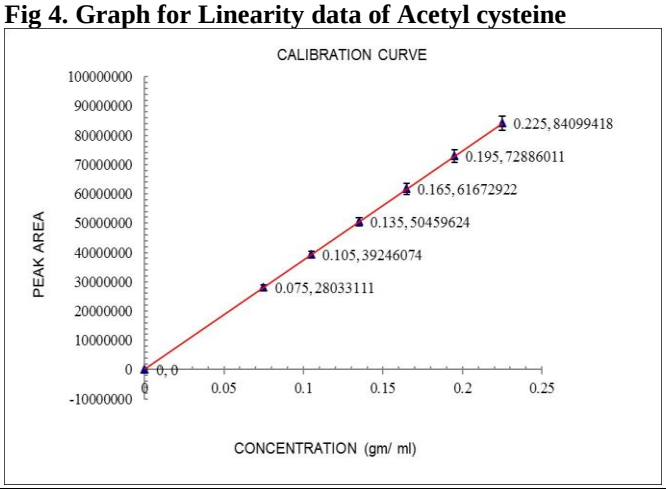


Fig 4. Graph for Linearity data of Acetyl cysteine



CONCLUSION

The validated HPLC methods employed here proved to be simple, fact, reliable, selective and sensitive. Since none of the method is reported for simultaneous estimation of pyridoxamine dihydrochloride and acetyl cysteine from combined dosage form. These can be used for routine analysis of two components without prior separation. The proposed method for simultaneous estimation of pyridoxamine dihydrochloride and acetyl cysteine in combined dosage form was validated as per ICH guidelines.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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