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UPLC METHOD VALIDATION FOR 3-ACETYLPYRIDINE CONTENT IN RISEDRONIC ACID

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ABSTRACT

An Ultra Performance Liquid Chromatography (UPLC) impurity profile method for the Risedronic acid monohydrate was validated in the present work. The validation method utilizes an Inertsil C-8 (100X4.6 mm), 5µ column at 50°C temperature, isocratic elution with aqueous sodium phosphate buffer at pH 7.5±0.05 and methanol as the mobile phase. The mobile-phase flow rate was 0.40 mL min^{-1}. The linearity, method precision, method ruggedness, limit of quantitation and limit of detection of the impurity profile UPLC method are found to be satisfactory. This study showed that 3-acetylpyridine peak was well resolved from the other known impurities and Risedronic acid monohydrate, the purity angle of 3-acetylpyridine is less than the purity threshold and there is no blank interference at the retention time of 3-acetylpyridine peak. Therefore the method is determined to be specific, as judged by resolving 3-acetylpyridine content in Risedronic acid monohydrate by UPLC.

Keywords:Uplc,3-Acetylpyridine,Risedronic acid,Mobile phase

INTRODUCTION

Risedronic acid (**Figure 1**) is a bone resosption inhibitor chemically termed as 1-hydroxy-2-(3-pyridine)ethylidene)-bisphosphonic acid belongs to a group of organic compounds known as bisphosphonates [1]. These bisphosphonates have been used to treat patients with Paget's disease (bone disease), postmenopausal osteoporosis and malignant hypercalcemia [2]. At various stages of production, storage and clinical use of risedronate and their related substances such as phosphite, 2-(3-pyridine)acetic acid, 3-acetylpyridine and phosphate may be present and may cause adverse side effects [3]. The literature survey revealed that there is no method validation that has been reported for 3-acetylpyridine content in Risedronic acid by UPLC [4-11]. Hence, it was considered worthwhile to validate suitable method for an assay of 3-acetylpyridine (**Figure 1**) in risedronic acid in the present work.

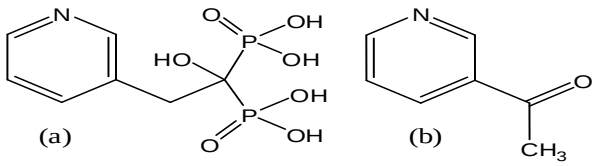

Figure 1. Structure of (a) Risedronic acid (b) 3-Acetylpyridine.

MATERIALS AND METHODS

Chemicals, Reagents and Samples: The chemicals, equipment and samples which were used in the present stduy was given in the following Tables 1-3.

Table 1: List of chemicals.

Name	Grade	Make	Lot No/ B.No.	Assay/Purity (%)
Sodium dihydrogen ortho phosphate	GR	Merck	MH7M572216	99.0
Methanol	HPLC	Rankem	R074K09	99.0
Sodium hydroxide	GR	Merck	MK7M572623	98.0
Titriplex	GR	Merck	MF9M591458	99.0
Water	Milli-Q	-	-	-

Table 2: List of equipments.

Name of the Instrument	I.D.Number	Make	Model
UPLC	PZARD/UPLC/01	Waters	Acquity
Electronic balance	PZARD/BL/01	Stratories	CP224S
pH meter	PZARD/pH/01	Eutech	pH510

Table 3: List of the samples.

Name	Grade	Lot No/B.No	Assay/Purity (%)
Risedronate related compound-B	USP	F01073	100.0
Risedronate related compound-A	USP	F01072	100.0
Deshydroxy related compound-C)	USP	F01082	100.0
3-Pyridyl acetic acid	ALDRICH	LOT09124MH	99.1
3-Acetylpyridine	ALDRICH	LOT 02819PH	99.3

Chromatographic conditions: The chromatographic condition used in the present study were given in the following Table 4.

Table 4: Chromatographic conditions.

Column	Inertsil C-8 (100×4.6 mm), 5µ Make: GL-Sciences, Part No: 2050-01775
Flow	0.40 mL/min
Column oven temperature	50°C
Detector wavelength	UV at 263 nm
Injection volume	8 µL
Run time	15 minutes
Elution	Isocratic
Diluent	Buffer: Methanol (70:30)

Buffer preparation: Weighed and transfered about 1.24 grams of sodium dihydrogen ortho phosphate in to 900 mL of milli-Q water adjusted pH to 7.5±0.05 with sodium hydroxide and added 372 mg of Tris buffer dehydrate salt then filtered the buffer using 0.22µ membrane filter paper.

Mobile phase preparation: Mobile phase was prepared by mixing buffer and methanol in the ratio of 70:30 (v/v).

Preparation of Standard and System Suitability Solution: Weighed and transfered about 10.00 mg of 3-acetylpyridine into a 10.0 mL volumetric flask. Added 5.0 mL of diluent to dissolve and made up to the volume with diluent. Transfered 1.0 mL of above solution in to a 100 mL volumetric flask and made upto mark with diluent. Further dilutions have been used were 1.0 mL to 100.0 mL with diluent.

Blank preparation: Transfered 1.0 mL of 1M NaOH solution into 10.0 ml volumetric flask and made the volume with diluent.

Evaluation of system suitability: Injected blank in duplicate (Table 5), followed by standard solution (five times) in ultra performance liquid chromatography and evaluated the chromatogram. The system is suitable for analysis if and only if, the percentage relative standard deviation of five replicate injections in standard solution for 3-acetylpyridine peak should not be more than 10.00.

Sample preparation: Accurately weighed and transfered about 100.00 mg of Risedronic acid in to 10.0 mL volumetric flask added 1.0 mL of 1M NaOH solution to dissolve and made upto mark with diluent.

Prepared in duplicate (Labeled as Sample preparation-1 and 2).

Procedure: If the system suitability passes, inject the sample preparations 1 and 2 (Order of injections as specified) and record the chromatograms. The approximate retention time for 3-acetylpyridine peak is about 8.0 minutes.

Table 5: Order of Injection.

Name of the preparations	No. of injections	Purpose
Blank	2	Blank
Standard preparation	5	System suitability / Quantification
Sample preparation-1	1	Sample analysis
Sample preparation-2	1	Sample analysis

Calculations

3-Acetylpyridine content was calculated with individual injection and report the average of two injections using the following formulae given below.

3-Acetylpyridine = [(AT/AS) × (WS/10) × (1/100) × (1/100) × (P/100)] × 1000000

AT = Area of 3-Acetylpyridine peak area obtained in sample preparation, AS = Average area of the 3-Acetylpyridine peak area obtained in system suitability solution preparation, WT = Weight of sample in mg, WS = Weight of standard in mg, P = Purity/ assay of 3-acetylpyridine standard and Acceptance criteria: 3-acetylpyridine (Not more than-10 ppm).

RESULTS AND DISCUSSION

System Suitability Data: The system suitability solution (standard solution of 3-acetylpyridine) was prepared and analysed as per the proposed method, the percentage relative standard deviation of 3-acetylpyridine peak in system suitability solution was determined. The % RSD of area for 5 replicate injections was showed in Table 6.

Table 6: Summary of percentage relative standard deviation peak area of 3-acetylpyridine in standard solution.

Experiment	**The percentage relative standard deviation (% RSD NMT 10.00)**
Specificity	0.83
Method precision	0.84
System precision,LOD, LOQ and precision at LOQ	1.16
Linearity	0.97
Accuracy	0.37
Intermediate precision	0.91
Batch analysis data	0.41

Selectivity/Specificity: Each known impurity solution was prepared individually [3-pyridyl acetic acid, 2-pyridynil isomer (USP Risedronate Related compound-A), Dimer impurity (USP Risedronate Related compound-B) & Deshydroxy impurity (Related compound-C) and 3-acetylpyridnine a solution] of all known impurities spiked solution with the Risedronic acid monohydrate at specification level and finally Risedronic acid monohydrate sample was also prepared. All these solutions were analyzed by using the PDA detector.

The acceptance criteria was based on the general recommendations such as 1) Peak should be homogeneous and there should be no coeluting peaks, 2) Peak purity of analyte should pass. For peak purity of analyte, purity angle should be less than the purity threshold and 3) No blank interference should be at the retention time of 3-acetylpyridine. From the data (Table 7) it was showed that 3-acetylpyridine peak was well resolved from the other known impurities and Risedronic acid monohydrate, the purity angle of 3-Acetylpyridine is less than the purity threshold and there is no blank interference at the retention time of 3-acetylpyridine (Figure 2-6). Therefore the method is selective for the determination 3-acetylpyridine content in Risedronic acid monohydrate by UPLC.

Table 7: Summary of retention time (RT), for Risedronate and its related impurities and the peak purity values of 3-acetylpyridine.

Peak name	Retention time (minutes)	Peak purity	
		Purity angle	Purity threshold
(USP Risedronate Related compound-B*) Dimer impurity	2.06	-	-
Deshydroxy impurity * (Related compound-C)	2.22	-	-
2-Pyridynil isomer (USP Risedronate Related compound-A)*	2.26	-	-
Risedronoic acid monohydrate	2.25	-	-
3-pyridyl acetic acid*	3.29	-	-
3-acetylpyridine	8.06	0.234	1.186

* Individual Injection

Limit of Detection (LOD): The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantitated. The limit of detection was determined as the lowest concentration for which the response is approximately two times greater than the baseline noise. The limit of detection is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. The result obtained for 3-acetylpyridine is listed in Table 8. The acceptance criteria is that signal to noise ratio should be ≥ 2:1.

Table 8: Limit of detection for 3-acetylpyridine

Component name	3-Acetylpyridine
Purity (%)	99.3
Weight taken (mg)	10.1
Dilution for LOD solution	Weight taken → 10.0 mL; 1.0mL → 100.0 mL; 1.0 mL → 100.0 mL; 1.0 mL → 10.0 mL with diluent.
Concentration (mg/mL) (with respect to the purity of 3-acetylpyridine)	0.000010
LOD with respect to sample conc. (PPM)	1.00
Signal to Noise ratio	3.1:1
Reported LOD (PPM)	1.0

Limit of Quantitation (LOQ): The Limit of quantitation (LOQ) values was determined from the same experiment as mentioned in the limit of detection section. Based on the limit of detection, roughly three folds of limit of detection solution was prepared and analyzed for the determination of limit of quantitation. The limit of quantitation is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations (approx 3.0 folds to limit of detection) of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably quantified. The result obtained for 3-Acetylpyridine is listed in Table 9. The acceptance criteria is that signal to noise ratio should be ≥ 10:1 and the quantitation limit should be less than level of specification, preferably much less .

Table 9: Limit of quantitation for 3-acetylpyridine

Component name	3-Acetylpyridine
Purity (%)	99.3
Weight taken (mg)	10.1
Dilution for LOD solution	Weight taken → 10.0 mL; 1.0mL → 100.0 mL; 1.0 mL → 100.0 mL; 3.0 mL → 10.0 mL with diluent.
Concentration (mg/mL) (with respect to the purity of 3-acetylpyridine)	0.000010
LOD with respect to sample conc. (PPM)	3.00
Signal to Noise ratio	10.2:1
Reported LOD (PPM)	3.0

Precision at LOQ: The repeatability expresses the precision under the same operating conditions over a short interval of time. It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. The precision at LOQ was performed by analysing six replicate injections of LOQ level solution (concentration). A result of peak area of 3-Acetylpyridine is summarized in Table 10. The acceptance criteria has been given to the percentage relative standard deviation for the peak area of 3-acetylpyridine at LOQ level and it should be less than 15.00. The percentage relative standard deviation for the peak area of 3-acetylpyridine obtained was 1.36 at the LOQ level and it indicates acceptable precision of the limit of quantitation.

Table 10: Summary of peak areas at LOQ level

Injection No.	**3-Acetylpyridine peak area at LOQ level**
1	1616
2	1594
3	1619
4	1648
5	1618
6	1653
Average area	1624.67
SD	22.0877
%RSD	1.36

Linearity: The linearity of the UPLC method was demonstrated for 3-acetylpyridine solutions ranging from LOQ to 250.0% of the specification limit. Results obtained are shown in Table 11 & Figure 8 shows the line of best fit for peak area versus concentration for 3-acetylpyridine. The acceptance criteria is based on the working standards such as 1) No apparent non-linearity should be observed graphically for 3-acetylpyridine, 2) The correlation co-efficient (R) should not be less than 0.9800 and 3) Report the slope and intercept values. As shown in the Figure 8, the linearity results for 3-acetylpyridine in the specified concentration range were found satisfactory, with a correlation coefficient (R) greater than 0.9900.

Table 11: Linearity for 3-acetylpyridine.

Component name	3-Acetylpyridine		
Purity (%)	99.3		
Weight taken (mg)	10.1		
Stock solution : Weight taken (mg)→10.0 mL; 1.0 mL→100.0 mL with diluent.			
Levels	Dilution	Conc.(PPM) (w.r.to purity & sample conc)	Average peak area of 3-acetylpyridine
LOQ level	1.0 mL of stock solution→ 100.0 mL→3.0 mL→10.0 mL with diluent	3.01	1686
50.0 % level	0.50 mL of stock solution→ 100.0 mL with diluent	5.01	2419
100.0% level	1.00 mL of stock solution→ 100.0 mL with diluent	10.03	4633
120.0 % level	1.20 mL of stock solution→ 100.0 mL with diluent	12.04	6519
200.0 % level	2.00 mL of stock solution→ 100.0 mL with diluent	20.06	9452
250.0 % level	2.50 mL of stock solution→ 100.0 mL with diluent	25.07	11939

Regression statistics: Slope (466.8528), Intercept (255.2220), Correlation coeffcient (R) (0.9965) and Coefficient of determination (R^2) (0.9930)

Accuracy: The accuracy was performed on samples spiked with known amounts of 3-acetylpyridine. The inherent amount of the 3-acetylpyridine was taken into account. The results have been calculated as recovery rate: recovered result x 100 / (adherent analyt+spiked analyt). The concentrations considered such as analyt (Risedronic acid monohydrate), analyt (Risedronic acid monohydrate) plus LOQ (3.0 ppm), analyt (Risedronic acid monohydrate) plus 100% of the specified limit (i.e.10.0 ppm), analyt (Risedronic acid monohydrate) plus 120% of the specified limit (i.e.12.0 ppm) and analyt (Risedronic acid monohydrate) plus 250% of the specified limit (i.e.25.0 ppm). The accuracy of the method was

determined using four solutions containing Risedronic acid monohydrate spiked with the 3-acetylpyridine at approximately LOQ, 100.0%, 120.0% and 250.0% of the working concentration. The percentage recovery results obtained for Risedronic acid monohydrate are listed in Table 12. Report percentage recovery and percentage relative standard deviation for each level. The percentage recovery calculated should be in the range of 80.00 to 120.00. The percentage relative standard deviation of the recoveries obtained for 3-acetylpyridine should be less than 15.00. The percentage recovery values obtained for 3-acetylpyridine were in the range of 94.68 to 111.37. The percentage relative standard deviation values of recoveries obtained for 3-acetylpyridine were in the range of 0.29 to 2.79. The acceptance criteria was sucessfully fulfilled.

Table 12: Summary of percentage recoveries for 3-acetylpyridine.

Levels	Sample	Actual conc. Added in sample (PPM)	Area Found in spiked sample	Area (spi sample area -sample area)	Found in spiked sample (PPM)	Recovered (PPM)	% Recovery	Average	STD	%RSD
LOQ Level	Accuracy LOQ- 1	3.009	1580	1580	3.331	3.331	110.70	110.40	0.3166	0.29
	Accuracy LOQ- 2	3.009	1576	1576	3.323	3.323	110.44			
	Accuracy LOQ- 3	3.009	1571	1571	3.312	3.312	110.07			
100% Level	Accuracy 100 %- 1	10.029	4796	4796	10.112	10.112	100.83	101.22	1.3536	1.34
	Accuracy 100 %- 2	10.029	4887	4887	10.303	10.303	102.73			
	Accuracy 100 %- 3	10.029	4762	4762	10.040	10.040	100.11			
120% Level	Accuracy 120 %- 1	12.035	6225	6225	13.124	13.124	109.05	110.16	1.1637	1.06
	Accuracy 120 %- 2	12.035	6282	6282	13.244	13.244	110.05			
	Accuracy 120 %- 3	12.035	6357	6357	13.403	13.403	111.37			
250% Level	Accuracy 250 %- 1	25.073	11259	11259	23.738	23.738	94.68	97.83	2.7282	2.79
	Accuracy 250 %- 2	25.073	11836	11836	24.954	24.954	99.53			
	Accuracy 250 %- 3	25.073	11806	11806	24.891	24.891	99.27			

Range: Range of the method is determined from the linearity and accuracy data. The range of the 3-acetylpyridine was found in between 3.00 PPM (30 %) to 25.073 PPM (251.7%), i.e. LOQ to 250.0 % level. The range should be about LOQ to 250.0 % with respect to the working concentration.

Precision: The repeatability expresses the precision under the same operating conditions over a short interval of time. It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample.

System precision: The system precision was performed by ten replicate injections of a standard solution at 100.0 % of the specified limit with respect to the working concentration. Result of peak area for 3-acetylpyridine for ten replicate injections is summarized in Table 13. The percentage relative standard deviation of peak area of ten replicate injections for peak area of 3-acetylpyridine should not be more than 4.00. The percentage relative standard deviation for the peak area of the 3-acetylpyridine was 1.97 at the working concentration.

Table 13: Summary of peak area for in system precision solution.

Injection No.	Peak area of 3-acetylpyridine
1	4041
2	4124
3	4056
4	4025
5	4114
6	4190
7	4224
8	3973
9	4059
10	4056
Average area	4089.56
SD	80.7142
%RSD	1.97

Method precision: The precision of the method was determined by analyzing a sample of Risedronic acid monohydrate spiked with 3-acetylpyridine at 100% of the specification limit (Six replicate spiked sample preparations). Results obtained are summarized in Table 14. The percentage relative standard deviation for 3-acetylpyridine content (PPM) level in 6 preparations should not be more than 15.00. The percentage relative standard deviation for the 3-acetylpyridine (Risedronic acid monohydrate spiked with 3-acetylpyridine at 10.0 ppm level six times at the specification level) was 0.95 at the working concentration. The acceptance criteria was sucessfully fulfilled.

Table 14: Summary of results for precision of the method.

Preparation No. (Spiked sample preparation)	3-acetylpyridine (PPM)
1	9.82
2	9.86
3	9.78
4	9.67
5	9.79
6	9.62
Average (PPM)	9.76
SD	0.0926
% RSD	0.95

Ruggedness (Intermediate precision): Evaluating the variability of the results obtained for 3-acetylpyridine with the analysis of Risedronic acid monohydrate solution spiked with 3-acetylpyridine six times at the specification limit by different analysts, using different columns on different days and assessed the method ruggedness. Results are summarized in Table 15. The overall percentage relative standard deviation for 3-acetylpyridine content (PPM) level in 12 preparations (method precision and intermediate precision) should not be more than 15.0. The percentage relative standard deviation for 3-acetylpyridine content [Risedronic acid monohydrate spiked with 3-acetylpyridine (method preparation and intermediate precision) at the specification level] obtained were in the range of 0.77 to 0.95 at the working concentration. The overall percentage relative standard deviation for 3-acetylpyridine content (ppm level) in 12 preparations obtained was 0.87. The acceptance criteria was sucessfully fulfilled.

Table 15: Results of ruggedness data.

Sample ID	Analyst(1)/day (1)/column(1) [3-acetylpyridine (ppm)] (method precision)	Analyst (2) /day (2)/column (2) [3-acetylpyridine (ppm)] (intermediate precision)
Sample-1	9.82	9.94
Sample-2	9.86	9.73
Sample-3	9.78	9.85
Sample-4	9.67	9.81
Sample-5	9.79	9.75
Sample-6	9.62	9.80
Average (PPM)	9.76	9.81
SD	0.0926	0.0753
%RSD	0.95	0.77
Over all % RSD (12 preparations)	0.87	

Batch analysis data: The summary of batch analysis data for 3-acetylpyridine content in Risedronic acid monohydrate samples listed in Table 15. The acceptance criteria of 3-acetylpyridine content is NMT 10.0 ppm.

Table 15: Summary of batch analysis data for 3-acetylpyridine content in Risedronic acid monohydrate samples

Batch No.	3-acetylpyridine (ppm)
A159/049	< LOD = < 1.00
A159/057	< LOD = < 1.00
A159/065	< LOD = < 1.00

CONCLUSION

A robust and sensitive UPLC method was validated for the determination of 3-acetylpyridine content in Risedronic acid. The method employs isocratic elution UPLC with PDA detection. The injection precision, linearity, LOQ, LOD, selectivity, accuracy, ruggedness and stability were evaluated and found to be satisfactory. The method can be use routinely to ensure the quality of manufactured Risedronic acid monohydrate.

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