Research Article

Qualitative and Quantitative Application of NMR in Rilmenidine Dihydrogen Phosphate API and Its Related Impurity-B and Correlation with Alternate Technique

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ABSTRACT

A rapid, specific and accurate Quantitative Nuclear Magnetic Resonance Spectroscopy (qNMR) method using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) technique was developed to determine Rilmenidine dihydrogen phosphate and its related impurity-B. The method (^{1}H qNMR) is based on the ^{1}H-NMR spectroscopy technique using Tetrachloronitrobenzene (TCNB) as an internal standard and Methanol-d4 as NMR solvent for the assay of Rilmenidine dihydrogen phosphate. The method of quantification of Rilmenidine dihydrogen phosphate was validated for the specificity, linearity, precision, robustness, accuracy and solution stability study. The Rilmenidine related impurity-B was quantified based on the impurity area with respect to Rilmenidine dihydrogen phosphate. The method of quantification of Rilmenidine related impurity-B was validated for the specificity, linearity, precision, robustness, accuracy and solution stability study. Limit of detection (LOD) was obtained as 0.03% and limit of quantification (LOQ) was obtained as 0.10% with respect to test concentration. The advantage of this method is that, no reference standard of the analyte drug is required for the quantitative determinations and also provides a unique spectrum as a confirmatory identification of Rilmenidine dihydrogen phosphate.

Keywords: Quantitative Nuclear magnetic resonance spectroscopy, Rilmenidine dihydrogen phosphate, Rilmenidine related impurity-B.

INTRODUCTION

Rilmenidine is a centrally acting antihypertensive that appears to act through stimulation of central imidazoline receptors and also has alpha$_2$ adrecenoceptor agonist activity. It is chemically N-(Dicyclopropylmethyl)-4, 5-dihydro-2-oxazolamine. The drug has general property similar to those of clonidine, but is reported to cause less sedation and central adverse effects.[1-5] The structural formula of Rilmenidine dihydrogen phosphate (RLM) is shown in Figure 1. The drug reported in the Literature survey reveals that the reported methods were HPLC, LC-MS/MS determination have been developed to analyze the drug and their impurities in pharmaceutical formulation or biological fluids.[6-7] The present study uses the proton Nuclear magnetic resonance Spectroscopy (NMR) to quantify Rilmenidine and its related impurity-B (RLM-IMPB) (Figure 1).

Quantitative Nuclear Magnetic Resonance Spectroscopy (qNMR) method is a quantitative spectroscopy tool as the intensity of resonance line is proportional to the number of resonant nuclei. This principle is employed for the quantitative determination of compounds. The most advantage of qNMR is that the determination does not require reference standard of the analyte. The determination is based on the ratio of the integration of a specific signal of the analyte and the internal reference standard.

The aim of the present study is to determine the assay of RLM by qNMR spectroscopy method using internal standard and quantification of RLM-IMPB in Rilmenidine dihydrogen phosphate by area integral method.[8-28]

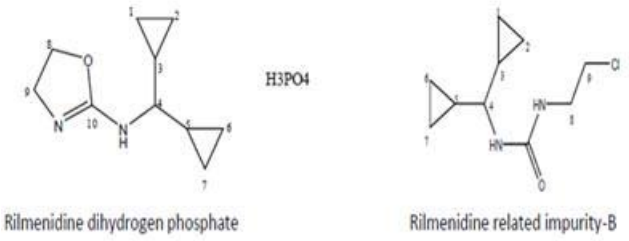


Figure 1: Structure of Rilmenidine dihydrogen phosphate and Rilmenidine related impurity-B

MATERIALS AND METHODS

Chemical and reagents

High purity analytical grade substances were used. Authentic sample of Rilmenidine was obtained from Local pharmaceutical company and used as such. Internal standard used was Tetrachloronitrobenzene (99.9%), Methanol-d4 (99.99%) were purchased from Merck.

Instrumentation

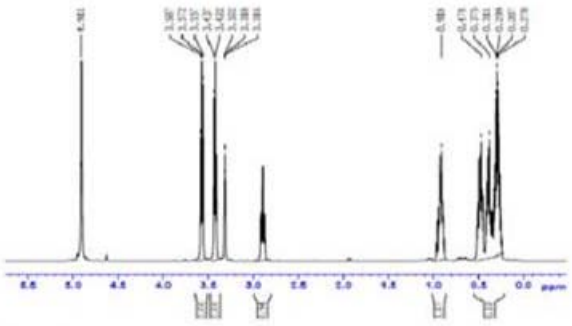
NMR instrument used was Bruker Make Avance-400 operating at 400.23MHz (9.4T) for proton, equipped with a 5mm multinuclear broad band observe (BBO) probehead.

Methodology

NMR is an analytical method based on the magnetic properties of certain atomic nuclei, the magnetic moments of which can be made to process coherently in an applied magnetic field by the application of radio frequency pulses. This gives rise to detectable signals which can be used to produce a spectrum. When NMR spectrums are run under quantitative conditions, protons give rise to responses that are directly proportional to their concentrations. If the areas of the known weights of the sample and reference standards (of known purity) are measured, then the assay or the content of the sample can be calculated from the equation 1. It is important that the absorptions for the both sample and reference standard are properly phased and completely resolved. This method should give content or assay that is accurate to ±2% at 95% confidence level.

NMR analysis was performed on Rilmenidine sample measured at 400MHz Spectrometer. Typically 64 scans were collected for assay of RLM and quantification of RLM-IMPB with 32768 data points using 90°pulse length, spectral width of 9615.385Hz, digital resolution of 0.2934Hz/points, dead time of 6µs and acquisition time of 1.7039 s. A delay time of 20s and 12s respectively were used for assay of RLM and quantification of RLM-IMPB which was sufficient to relax the protons. The FID was apodised with 1Hz exponential line broadening function before Fourier Transformation. Automatic phase correction and baseline correction were employed. Identifications were done for the RLM and RLM-IMPB by ^{1}H NMR spectroscopy. On the another technique, for Quantification of Rilmenidine related impurity-B, an absolute method is used, in which intensity of the signal of the standard is compared with intensity of the signal of test sample for certain atomic nuclei.

Identification of RLM-IMPB by ^{1}H NMR spectroscopy:

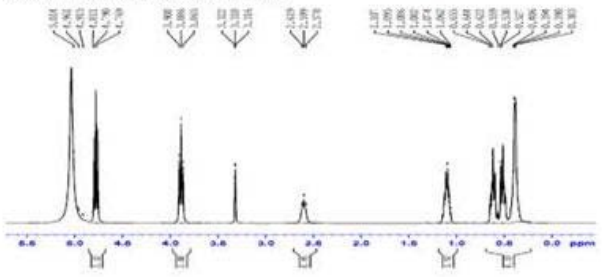


Observation:

Chemical shift, δ, ppm	Multiplicity, proton number	Assignments
0.14 to 0.40	Multiplet, 8H	Ring protons at C-1, C-2, C-6, C-7
0.841	Multiplet, 2H	Methine protons at C-3, C-5
2.860	Quartet, 1H	Methine protons at C-4
3.290	Quartet, 2H	Methylene protons at C-8
3.543	Triplet, 2H	Methylene protons at C-9
5.936, 5.990	Doublet and Triplet , 2H	-NH protons

Conclusion: From the above observation and discussion, it is concluded that the chemical shift in the ^{1}H NMR spectrum of Impurity-B are in accordance with the position and the proton number present in the given molecular structure and hence support the structure of Impurity-B.

Identification of RLM by ^{1}H NMR spectroscopy:



Observation:

Chemical shift, δ, ppm	Multiplicity, proton number	Assignments
0.385 to 0.648	Multiplet, 8H	Ring protons at C-1, C-2, C-6, C-7
1.107	Multiplet, 2H	Methine protons at C-3, C-5
2.603	Triplet, 1H	Methine protons at C-4
3.888	Triplet, 2H	Methylene protons at C-9
4.780	Triplet, 2H	Methylene protons at C-8

Conclusion: From the above data, it is concluded that the chemical shift in the ^{1}H NMR spectrum of Rilmenidine dihydrogen phosphate are in accordance with the position and the protons number present in the given molecular structure and hence support the structure of Rilmenidine dihydrogen phosphate.

Test Method -Determination of assay of Rilmenidine

Method of analysis

NMR spectrometer: Bruker DPX400, 5mm BBO – 1H

Reagent: Methanol-d4

NMR parameters: D1=20sec, NS= 128, LB (Line broadening) is 1Hz

Test solution: Weigh accurately about 15 to 30mg of the RLM and transfer in a clean 10ml glass sample vial, add 1mL Methanol-d4 into each vial and sonicated to dissolve. Weigh accurately about 10 to 15mg of internal standard TCNB into the same vial and mix well.

Procedure: Record 1H NMR spectra using the above acquisition parameters. After the experiment is over, process the spectrum with baseline correction, automatic phase correction and interactive phase. Set the TMS signal to zero. Record the 1H NMR of Methanol-d4 on similar lines.

Determination of relaxation time (t1) in Rilmenidine

For accurate quantification, proper value of relaxation delay is very important. The relaxation delay, D1, depends on the longest longitudinal relaxation time, T_1, of all the signals present in the given molecule. Generally, a delay of five times of the relaxation time, T_1, is sufficient

between the last RF pulse and the application of the next RF pulse. The inversion recovery pulse sequence can be used to measure the T_1 relaxation time as described by the following relation.

$$M_z = M_o [1-e^{-t/T_1}]$$

Where, Mz and Mo are the magnetization along the z-axis after waiting time "t" and at thermal equilibrium, respectively.

Type of proton	Assignment	Chemical shift (δ)	T_1 (second)
Methine proton, triplet, 1H	C-4	2.63ppm	2.989

Since, the T_1=2.989seconds, so relaxation delay of 20seconds is chosen.

Calculation

The amount Px of drug was calculated using the following equations:

$$Px = \frac{Ix}{Istd} \times \frac{Nstd}{Nx} \times \frac{Mx}{Mstd} \times \frac{mstd}{m} \times Pstd \quad .....eq(1)$$

Where,

Px = Assay of the RLM (in %w/w) on as is basis

Ix = Mean Integral value of the analyte 1H signal for test

Istd= Integral value of the 1H signal of TCNB

Nstd = Number of protons for the TCNB

Nx = Number of protons for the analyte 1H in test

Mx = Molar mass of the RLM

Mstd = Molar mass of the TCNB

mstd = Weight of the TCNB. (in mg)

m = Taken weight of the RLM (in mg)

Pstd = Assay of the TCNB (in %)

Test Method -Quantification of Rilmenidine related impurity-B in Rilmenidine dihydrogen phosphate

Method of analysis

NMR spectrometer: Bruker DPX400, 5mm BBO – 1H

Reagent: Methanol-d4

NMR parameters: D1=12sec, NS= 64, LB (Line broadening) is 1Hz

Determination of relaxation time (t1) in Rilmenidine impurity-B

For accurate quantification, proper value of relaxation delay is very important. The relaxation delay, D1, depends on the longest longitudinal relaxation time, T_1, of all the signals present in the given molecule. Generally, a delay of five times of the relaxation time, T_1, is sufficient between the last RF pulse and the application of the next RF pulse. The inversion recovery pulse sequence can be used to measure the T_1 relaxation time as described by the following relation.

$$M_z = M_o [1-e^{-t/T_1}]$$

Where, Mz and Mo are the magnetization along the z-axis after waiting time "t" and at thermal equilibrium, respectively.

Type of proton	Assignment	Chemical shift (δ)	T_1 (second)
Methine proton	C-4	2.88ppm	3.659

Since, the T_1=3.659seconds, so relaxation delay of 12 seconds is chosen.

Preparation of Impurity Standard: Dissolve accurate weigh quantity of impurity-B in solvent and dilute to get 50 ppm of Impurity –B solution.

Preparation of Impurity Standard

Dissolve accurate weigh quantity of Rilmenidine dihydrogen phosphate in solvent and dilute to get 77500 ppm of API test solution.

Procedure: Record 1H NMR spectra using the above acquisition parameters. After the experiment is over, process the spectrum with baseline correction, automatic phase correction and interactive phase. Set the TMS signal to zero. Record the 1H NMR of Methanol-d4 on similar lines. For the total integral calculation, ignore the NMR solvent signals and water integral. After processing, the impurity intensity at the specified resonance is used for determination of content of impurity-B in test API. 1H NMR spectra of Rilmenidine dihydrogen phosphate and its related impurity-B are recorded in Figure-2.

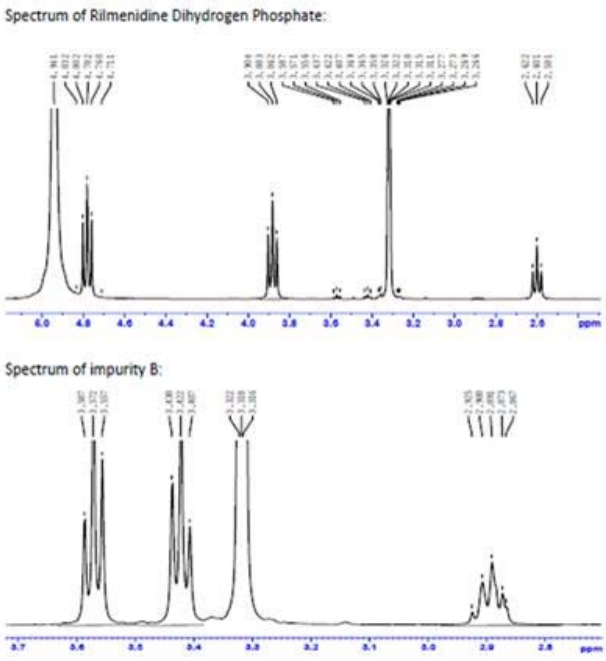


Figure 2: 1H NMR spectra of Rilmenidine dihydrogen phosphate and its related impurity-B

RESULTS AND DISCUSSION

Method validation

The developed ^{1}H qNMR method were validated for the parameters like specificity, system suitability, linearity and range, precision , accuracy, solution stability and robustness. Data obtained during validation study were summarized in Table-1 for test method -Determination of Assay of Rilmenidine and in Table-2 for test method - Quantification of Rilmenidine Related impurity-B in Rilmenidine dihydrogen phosphate.

Test Method -Determination of assay of Rilmenidine

Specificity of method

Specificity study is performed to establish that there is no interference of available related compound or impurity and signal is sufficiently well separated from those of the main compound. When the impurities merge with spinning side bans it becomes important to run the sample using different spinning rates or without spinning. Following table gives the chemical shift values of impurity B and the main compound.

Impurity B: 0.255ppm to 0.966ppm, 2.88ppm, 3.4ppm, 3.6ppm

Main compound: 0.355ppm to 1.145ppm, 2.63ppm, 3.88ppm, 4.7ppm

Thus peak at 2.63ppm, (t), 1H or 3.88ppm, (t), 2H is used for the assay of main compound and 2.88ppm, (t), 1H is used for the impurity-B

Procedure

Approximately 15mg to 20mg of RLM sample is accurately weighed into glass vial. To it is added 1mole equivalent internal standard, which is chosen during method development such that it does not exhibit resonance peaks that overlap those of the sample being analyzed. Add 1mL of suitable deuterated solvent which has good solubility properties and does not exhibit resonance peaks which overlap those of the specimen being analyzed. Normally, TMS or similar is incorporated in the solvent as a chemical shift reference. Ensure that the sample is completely dissolved and transfer the solution to a 5mm NMR tube. Acquire and process the 1H NMR spectrums using sufficiently longer delay time.

Linearity and Accuracy study

The study was performed by taking sample weights from 12mg to 30mg with internal standard weight nearly the same i.e. about 10mg to 12mg. The integral area was plotted against weight of test. The slope, intercept and the correlation coefficient for the sample was determined and summarized in table 1. The slope, intercept and the correlation coefficient for the sample was found to be 9904757, 18155369 and 0.997 respectively. From the results obtained above, it can be concluded that, the system is linear over a range of 11.5mg to 28.5mg sample.

Precision of the system

The precision of the system was determined for the assay by preparing five samples of drug substances with internal standard. The % RSD values are less than 5.0%.

Method precision

The method precision was determined by analyzing two samples in triplicate on two different days. The mean of the assay on each day was determined. The % RSD values are less than 5.0%.

Stability of solution

The Assay test solution was prepared as per the method and sample run for initial test results. The obtained solution was allowed to stand at room temperature and sample were run after 4hours and 12hours interval and test results obtained were reported. The % RSD values are less than 5.0%.

Robustness

Robustness was studied by varying the instrumental parameters like relaxation delay from 25sec to 20sec and found that this does not affect the results. The % RSD values are less than 5.0%.

Comparative study with alternative method

The results obtained with proposed Quantitative Nuclear Magnetic Resonance Spectroscopy (qNMR) method using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) technique were compared with alternate technique (i.e. By titration). The results obtained show the high reliability and reproducibility of the method. The comparative data are presented in Table-1

Table 1: Comparative data of reliability and reproducibility

Linearity and accuracy study for assay of RLM

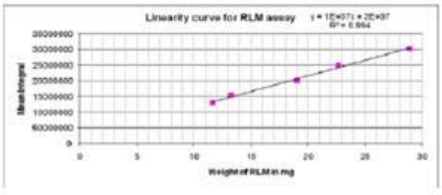
Level	Weight taken in mg		Integral [abs]				
	Test	TCNB	Test	TCNB	Assay	SD	% RSD
50%	11.623	10.105	129795663	59664536	99.8	0.1	0.1
75%	13.231	10.235	194139623	92948739	100.1	0.2	0.2
100%	19.022	10.624	281068597	59400012	99.8	0.3	0.3
125%	22.443	10.481	248701709	80619685	100.2	0.3	0.3
150%	28.888	10.435	302116880	57579288	100.1	0.2	0.2

	% Assay [sample-A]		% Assay[sample-A]
Set 1	100.01	1] Initial	99.14
Set 2	100.08	2] after 4 hours	100.25
Set 3	99.86	3] after 12 hours	100.83
Set 4	100.27	Mean Assay	100.1
Set 5	100.01	SD	0.9
Mean Assay	100.0	% RSD	0.9
SD	0.1		
%RSD	0.1		

	Method precision				Robustness	
	Assay value				Assay value	
	Sample-A	Sample-B	Sample-A	Sample-B	Sample-A, relaxation delay , D1	
	day-1		day-2		25sec	20sec
	100.13	100.20	100.33	100.49	99.14	99.07
	99.88	99.96	100.18	99.91	100.85	99.68
	100.34	100.32	100.05	99.75	99.46	99.31
Mean	100.1	100.2	100.2	100.1	99.8	99.4
SD	0.2	0.1	0.1	0.4	0.9	0.1
%RSD	0.2	0.1	0.1	0.4	0.9	0.1

Comparison table of results for assay by qNMR and Titration (Alternate technique)

	% assay by qNMR	% Assay by Titration
Sample 1	100.1	100.1
Sample 2	100.1	99.88
Sample 3	99.99	100.1



Test Method -Content of Rilmenidine related impurity-B in Rilmenidine dihydrogen phosphate

Specificity

The available related compound or impurity has to be sufficiently well separated from those of the main drug and other impurities to allow quantitative NMR. Sometimes when the impurities merge with spinning side bans it becomes important to run the sample using different spinning rates or without spinning. Following summarized data gives the chemical shift values of impurity B and the Test API.

Impurity B: 0.255ppm to 0.966ppm, 2.88ppm, 3.4ppm, 3.6ppm

Main compound: 0.355ppm to 1.145ppm, 2.60ppm**,** 3.88ppm, 4.7ppm

Thus peak at 3.88ppm, (t), 2H is used for the main compound and 2.88ppm, (t), 1H is used for the impurity-B

Linearity study

Stock Test API preparation (Solution-A): Weigh accurately about 77.5mg of the API in 1ml of Methanol-d4 and sonicated to dissolve. This contains 77,500ppm of API.

Impurity solution (Solution-B): Weigh accurately about 1mg of the Impurity standard in 20ml volumetric flask add about 10mL Methanol-d4 and sonicated to dissolve. Make up to the mark with the same solvent and mix well. This contains 50 ppm of Rilmenidine related impurity-B.

Linearity Solution of impurity-B in API

Transfer 1ml each of solution A and B in NMR tube, mix well, this solution contains 0.06% impurity-B concentration with respect to Test API concentration. Similarly, prepare impurity-B solution of 0.13%, 0.26% and 0.65% concentration with respect to Test API concentration.

Record 1H NMR spectra using acquisition parameters mentioned in method and after processing, the impurity intensity at the specified resonance was plotted against the concentration of impurity-B. The peak area was plotted against the known weight % of the impurity-B. The slope was 1042885.6, intercept was 428555 and the correlation coefficient was 0.9989.

LOD and LOQ Determination

This is determined from the calibration plot of impurity solution B spiked with main drug in Linearity experiment. Limit of quantification and limit of detection was determined as per CH guideline. This method is based on the following formula:

$LOD = 3.3\sigma / s$ & $LOQ = 10\sigma / s$

Where,

σ= Standard error in estimate of Y-axis value

s = Slope of calibration curve

LOD = Limit of detection

LOQ = Limit of quantification

From the linearity graph, we obtained the value of slope as 1042885.6, intercept as 428555 and the correlation coefficient as 0.999. Using the above formula we obtained the LOD and LOQ as

Follow,

LOD obtained from linearity graph is 0.03%

LOQ obtained from linearity graph is 0.10%

System precision

The precision of the system was determined for the impurity by preparing five sample of drug substances spiked with appropriate amount of a stock solution of the impurity-B to give a concentration of 0.13%.

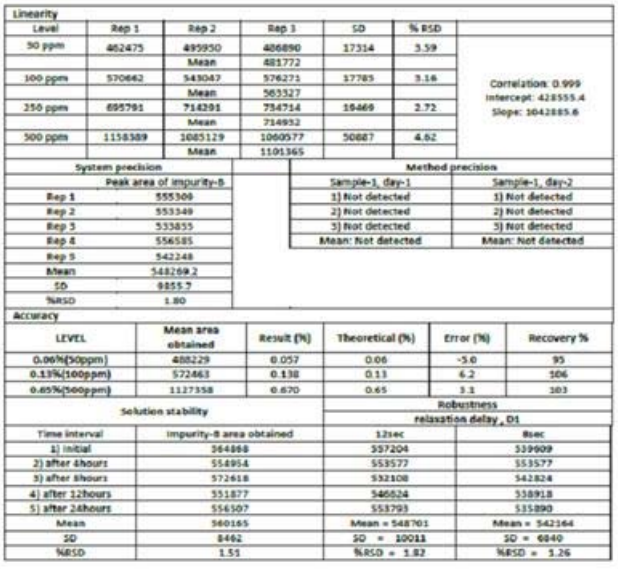
Method precision

The method precision was determined by analyzing two samples (without spiking) in triplicate on two different days. The mean of the impurity level on each day was obtained and recorded in table 2.

Recovery study

The accuracy of an analytical method is the closeness of the test results obtained by the method to be true value. The true value is that result which would be obtained in the absence of error. Accuracy may often be expressed as a percent recovery by the content of known or added amount of the analyte.

Table 2: The mean of the impurity level on each day



Linearity

Level	Rep 1	Rep 2	Rep 3	SD	% RSD	
50 ppm	462475	499950	486890	17314	3.59	
		Mean	481772			
100 ppm	570662	543047	576271	17785	3.16	
		Mean	563327			
250 ppm	695791	714291	734714	19469	2.72	
		Mean	714932			
500 ppm	1158389	1085129	1060577	50887	4.62	
		Mean	1101365			

Correlation: 0.999
Intercept: 428555.4
Slope: 1042885.6

System precision

Peak area of impurity-B	
Rep 1	555309
Rep 2	553349
Rep 3	533855
Rep 4	556585
Rep 5	542248
Mean	548269.2
SD	9855.7
%RSD	1.80

Method precision

Sample-1, day-1	Sample-1, day-2
1) Not detected	1) Not detected
2) Not detected	2) Not detected
3) Not detected	3) Not detected
Mean: Not detected	Mean: Not detected

Accuracy

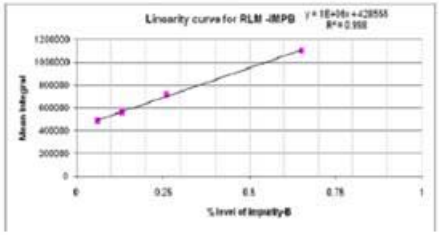
LEVEL	Mean area obtained	Result (%)	Theoretical (%)	Error (%)	Recovery %
0.06%(30ppm)	488229	0.057	0.06	-5.0	95
0.13%(100ppm)	572463	0.138	0.13	6.2	106
0.65%(500ppm)	1127358	0.670	0.65	3.1	103

Solution stability

Time interval	Impurity-B area obtained
1) Initial	564868
2) after 6hours	554954
3) after 9hours	572618
4) after 12hours	551877
5) after 24hours	556507
Mean	560165
SD	8492
%RSD	1.51

Robustness
relaxation delay , D1

12sec	8sec
557204	539009
553577	553577
532108	542824
546624	538918
553793	535890
Mean = 548701	Mean = 542164
SD = 10011	SD = 6840
%RSD = 1.82	%RSD = 1.26



The recovery study was performed by spiking known amount of impurity-B in test sample. The spiking was carried out with impurity concentration of 0.06%, 0.13% and 0.65% with respect to Test concentration. NMR analysis was performed in triplicate for each of the above preparation.

The impurity-B obtained in test solution is tabulated with recovery results in table 2.

Stability of solution

The 0.13% spiked solution of impurity-B with respect to Test concentration is run after 4hours, 8hours, 12hours and 24hours and the impurity-B area values recorded.

Robustness of the method

Robustness was studied by varying the instrumental parameter like relaxation delay from 8 sec to 12 sec and found that this does not affect the results. The 0.13% spiked solution of impurity-B with respect to Test concentration was analyzed as per method except relaxation time was set at 8 sec and 12 sec.

CONCLUSION

Test results obtained using qNMR method and Titration method in the present study shows that, the results obtained by qNMR correlates well with Titration method for the assay of Rilmenidine. Similarly, the qNMR method developed for impurity-B in Rilmenidine shows similar results as the HPLC method proving beyond doubt the usefulness of NMR technique as a powerful quantitative tool. The analysis time of qNMR method and data of validation study support that the method for assay of Rilmenidine dihydrogen phosphate and determination of content of Rilmenidine impurity –B in test API is rapid, precise and accurate. Further as qNMR method for assay test is based on the molar ratio of the components in question it does not require the use of analyte standard and analysis time is shorter.

In summary, good specificity and accuracy of the proposed qNMR method was established for pharmaceutical testing proving beyond doubt the usefulness of NMR technique as a powerful quantitative tool.

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