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Quantitative Application of NMR In Ropivacaine Hydrochloride And Its Related Impurity-A With Correlation By Alternate Techniques

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Abstract: The present work is application of quantitative NMR is increasingly used as a practical tool with respect to elucidation of structure and quantitative analysis. In the present study, we show the ability of ^{1}H NMR to determine the content of drug and its related impurity-A in Ropivacaine hydrochloride. Rapid, specific and accurate proton NMR method developed for the drug Ropivacaine hydrochloride using Internal standard. For the drug quantification, proton signal at 7.14ppm and 8.2ppm for Tetrachloronitrobenzene (TCNB) were used. Assay method for proton NMR were validated for specificity, precision and intermediate precision, linearity, solution stability and Robustness. Impurity profiling of impurity-A (2,6 dimethyl aniline) were also developed and validated for specificity and precision. LOD and LOQ were also established.
Keywords: NMR, Ropivacaine Hydrochloride,Related Impurity Impurity-A of Ropivacaine hydrochloride, Quantitative Application .

Introduction

Ropivacaine hydrochloride is (2S)-N-(2,6-dimethyl-phenyl)-1-propyl-2-piperinecarboxamide is an anesthetic drug. The drug act by blocking the conduction of impulses in the target nerve structures, primarily located within the subarachinoid space [1-3]. The drug reported in the Literature survey reveals that the reported methods were HPLC determination in USP[2]. Several methods using GC [4], HPLC [5, 6] and HPLC-MS [7] or amperometric detection [8] and capillary electrophoresis [9] have been developed to analyse the drug and their impurities in pharmaceutical formulation or biological fluids. The present study uses the proton Nuclear Magnetic resonance Spectroscopy (NMR) to quantify Ropivacaine and its related impurity i.e. 2, 6-dimethylaniline NMR 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 Q-NMR is that the determination does not require standard of the analytic. 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 Ropivacaine by quantitative NMR spectroscopy using internal standard method and related impurity 2,6-dimethylaniline , a genotoxic impurity by area integral method [10-13].

Experimental

High purity analytical grade substances were used. Authentic sample of Ropivacaine 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 (9.38T) for proton equipped with a 5mm multinuclear broad band observe (BBO) probehead.

Structure interpretation of drug and Impurity by NMR, IR spectroscopy and Mass spectrometry

NMR Parameters

NMR analysis was performed on Ropivacaine sample measured at 400MHz Spectrometer. Typically 16 scans were collected for assay and 64 scans for related impurity into 32768 data points using 90°pulse length, spectral width of 8803.817Hz, digital resolution of 0.268641Hz/points, dead time of 6µs and acquisition time of 1.8613 s. A delay time of 20s was used for Ropivacaine determination and impurity determination which was sufficient to relax the protons. The FID were apodised with 1Hz exponential line broadening function before Fourier Transformation. Automatic phase correction and baseline correction were employed.

***Procedure for determination (assay) of drug* :**

Weigh and transfer accurately about 10mg to 15mg each of of Ropivacaine hydrochloride sample and TCNB into a 10ml glass vial, to it is added 0.6mL of MeOD and sonicated to dissolve. The solvent is chosen during method development such that it does not exhibit resonance peaks that overlap those of the sample being analyzed. The solvent should have 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.

Formula of assay / content is

$$\% \text{ assay , w/w} = \frac{It \times Ns \times Mt \times Ws \times P}{Is \times Nt \times Ms \times Wt}$$

Where,

It	Integration of the test sample
Is	Integration of the standard sample
Ws	Weight of the standard sample
Wt	Weight of the test sample
Mt	Molecular weight of the Test sample
Ms	Molecular weight of the Standard sample
Ns	Number of Standard sample protons
Nt	Number of test sample protons
P	Strength / assay of the standard sample

***Specificity of NMR Method* :**

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 bands it becomes important to run the sample using different spinning rates or without spinning. Following table gives the chemical shift values of impurity A and the main compound.

First the identification were done for the Ropivacaine and impurity A by proton NMR, Carbon 13 NMR, FTIR and Mass spectrometry.

Chemical name

1-propylpiperidine-2-carboxylic acid (2,6-dimethyl phenyl)amide hydrochloride monohydrate

Chemical Formula $C_{17}H_{27}ClN_2O$

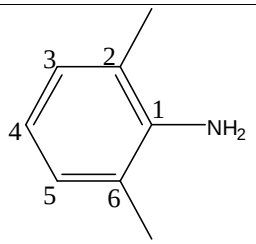
Molecular weight 328.89 g/mole

^{1}H NMR analysis

^1H NMR spectral analysis of impurity-A, was performed on NMR spectrometer of Bruker 400 UltraShield, model-Avance-II, in Methanol-d6, using Tetramethylsilane as an internal standard.

Table 1 Observation Impurity-A of Ropivacaine hydrochloride by ^{1}H NMR spectroscopy

Chemical shift, δ, ppm	Multiplicity, proton number	Assignments
2.09	singlet, 6H	Methyl protons at C-2, C-6
6.88, 6.86	Doublet, 2H	Aromatic protons at C-3, C-5
6.61	Triplet, 1H	Aromatic protons at C-4



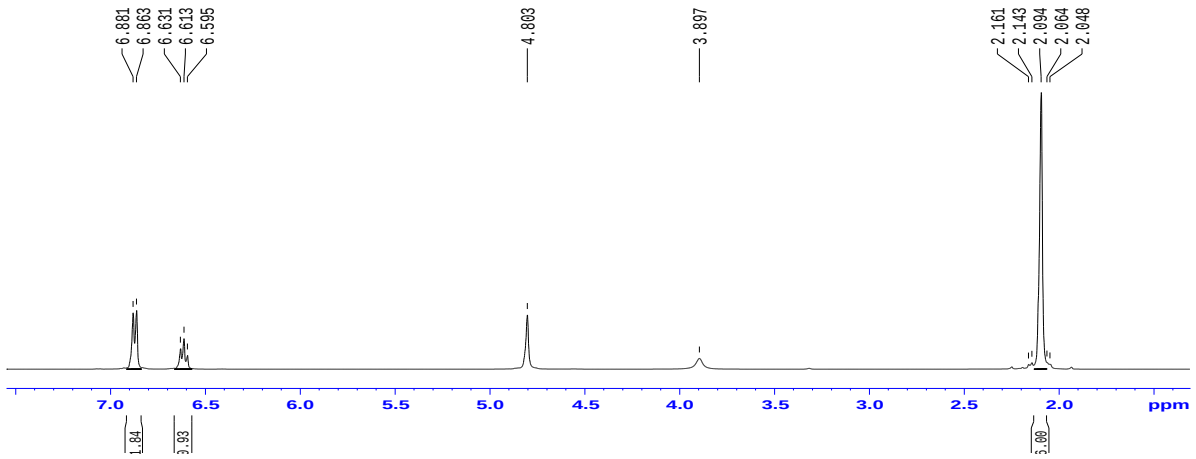


Fig. 1 Identification Impurity-A of Ropivacaine hydrochloride by ^{1}H NMR Spectroscopy

Remark

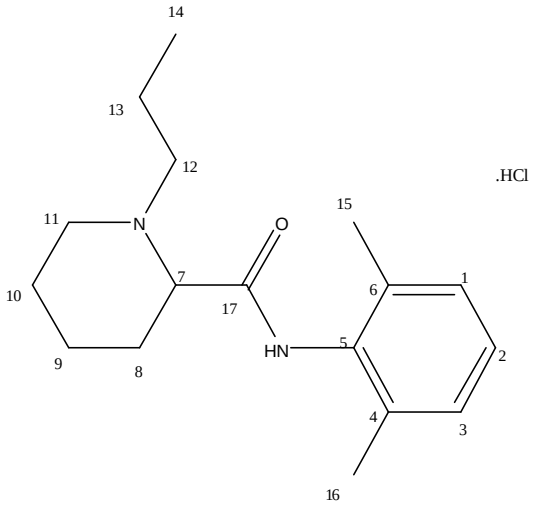
From the Table and Figure 1 observation and discussion, it is concluded that the chemical shift in the ^{1}H NMR spectrum of Impurity-A are in accordance with the position and the proton number present in the given molecular structure and hence support the structure of Impurity-A.

Result and Discussion

The small broad singlet at 10.251ppm is due exchangeable protons –NH and HCl. Aromatic protons at C-1, C-2 and C-3 appear as multiple at 7.135ppm. Methine protons C-7 appear at 4.360ppm, while methylene protons at C-12, C-11, C-13, C-10, C-8, C-9 appear at 3.655ppm, 3.159ppm, 2.398ppm and 1.935ppm respectively.

Table 2 Observation API by ^{1}H NMR spectroscopy

Chemical shift, δ , ppm	Multiplicity, proton number	Assignments
10.251	Small signal, exchangeable , 2H	-NH and HCl
7.135	Multiplet, 3H	Aromatic protons at C-1, C-2, C-3
4.360	Doublet, 1H	Methine at C-7
3.655, 3.159 2.398, 1.935	Multiplets, 12H	Methylene protons at C-12, C-11, C-13, C-10, C-8, C-9
2.239	Singlet, 6H	Methyl protons at C-15, C-16
0.994	Triplet, 3H	Methyl protons at C-14



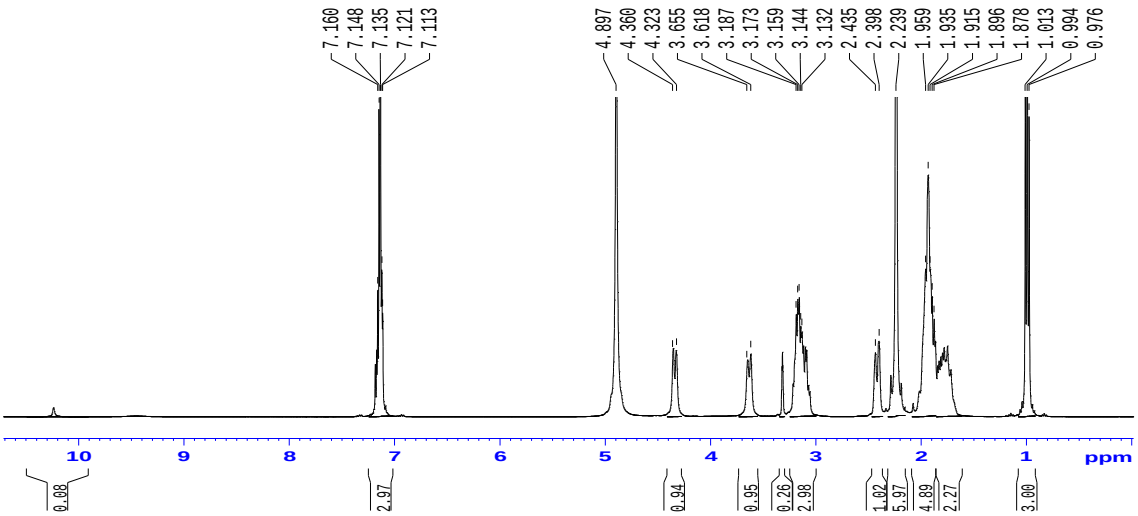


Fig. 2 Identification API by ^{1}H NMR Spectroscopy

Remark

From the Table and Figure 2 it is concluded that the chemical shift in the ^{1}H NMR spectrum of Ropivacaine hydrochloride are in accordance with the position and the proton numbers present in the given molecular structure and hence support the structure of Ropivacaine hydrochloride.

^{13}C NMR analysis

^{13}C NMR spectral analysis of Impurity-A, was performed on NMR spectrometer of Bruker 400 UltraShield, model-Avance-II, in Methanol-d4, measured at 100.63MHz, using Tetramethylsilane as an internal standard and data are respectively in Table 3.

Table 3 Observation API by ^{1}H NMR spectroscopy

Chemical shift, δ, ppm	Assignments
17.14	Methyl protons at C-2, C-6
117.97	Aromatic protons at C-7, C-8
121.80	Aromatic protons at C-4
128.31	Aromatic protons at C-3, C-5
143.11	Aromatic carbon at C-1

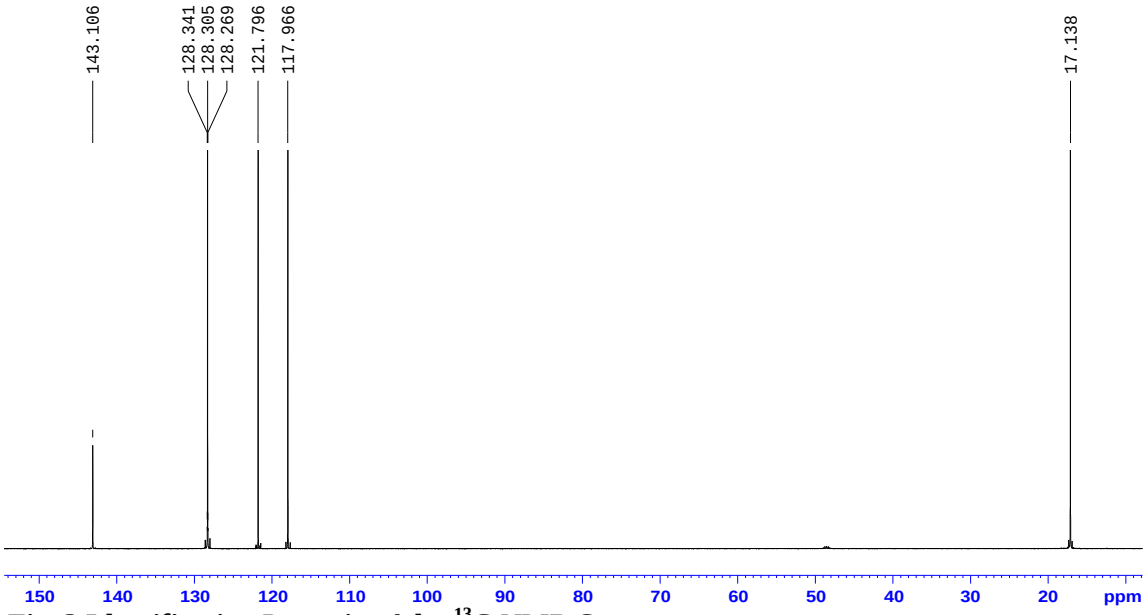


Fig. 3 Identification Impurity-A by ^{13}C NMR Spectroscopy

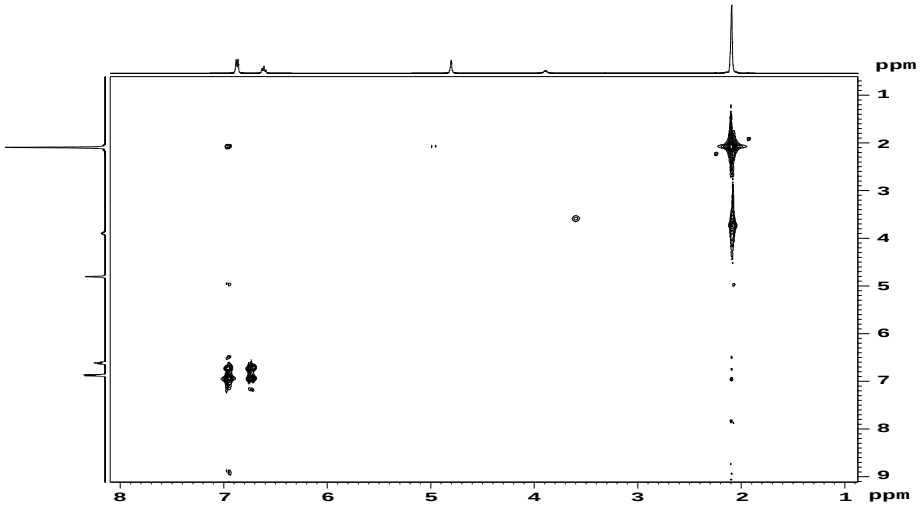


Fig. 4 Identification of Impurity-A by 2-dimensional NMR: Homo nuclear 2D, i.e. HH-cosy.

Interpretation of 2D HH Cosy of Impurity-A

Aromatic protons at C-3 , C-4 and C-5 are observed in off-diagonal region of the 2-D HH COSY spectrum which is correlating whereas protons at C-2, C-6 and amine protons are not correlating and data are respectively in figure 4.

Procedure for assay of ropivacaine hydrochloride and its method validation

NMR spectrometer : Bruker DPX400,5mm BBO – 1H
Reagent : Methanol-d4 (MeOD)
Internal standard as Tetrachloronitrobenzene, TCNB
NMR parameters : D1=20sec, NS= 16, LB (Line broadening) is 1Hz
Impurity A 2.09ppm , 6.61ppm, 6.88ppm

***Ropivacaine HCl* (Main compound)**

0.99ppm, 1.88ppm, 1.96pm, 2.23ppm, 2.40ppm, 3.16ppm, 3.66pm, 4.32ppm, 7.14ppm
Thus peak at 7.14ppm, (m), 3H is used for the assay of main compound and 2.09ppm, (s), 6H is used for the impurity-A quantification purpose.

Determination of relaxation time (T1) in ropivacaine hydrochloride API :

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)
Aromatic	C-1, C-2, C-3	7.14	2.77

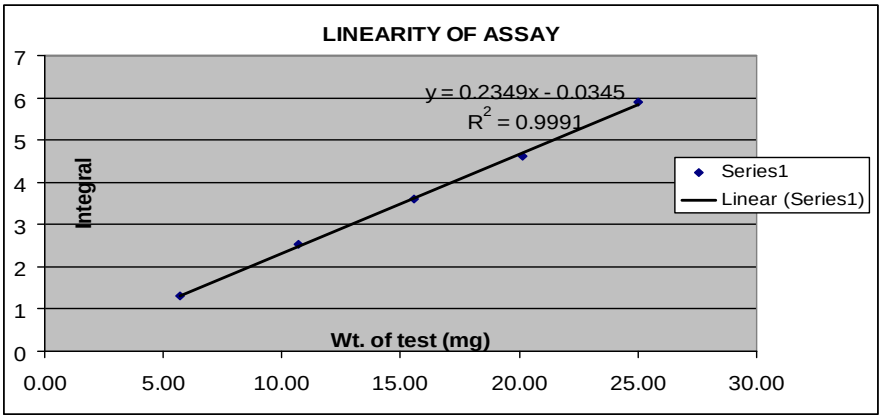
Since, the T_1=2.77, so relaxation delay of 20 seconds is chosen.

Linearity of Assay

The assay was performed by taking sample weights from 5mg to 25mg with internal standard weight nearly the same i.e. about 10mg. The integral was plotted against weight of test. The slope, intercept and the correlation coefficient for the sample was determined Table 4.

Table 4 Linearity of assay

	Wt. of test (mg)	Integral [abs] obtained on 3 determinations	Wt. of internal standard (TCNB) (mg)
Assay-1 (37%)-Level	5.70	1.2985	10.46
Assay-2 (69%)-Level	10.71	2.5355	10.10
Assay-3 (100%)-Level	15.58	3.5897	10.39
Assay-4 (129%)-Level	20.12	4.6277	10.65
Assay-5 (161%)-Level	25.03	5.8980	10.15



Remark

From the results obtained above the system is linear over a range of 5.7mg to 25.03mg. The limit of detection (LOD) and limit of quantification (LOQ) obtained were 0.86mg and 2.6mg per 0.6ml solution.

Precision of The System For Assay

The precision of the system were determined for the assay by preparing five samples of drug substance with Internal standard and measured by NMR.

Method Precision For Assay

The method precision was determined by analyzing two samples in triplicate on two different days. The mean of the assay on each day was obtained by NMR.

Stability of Solution For Assay

The Assay test solution (say sample-A) is run after 4hours and 24hours and the assay determined in Table 5.

Table 5 Stability of Solution For Assay

Sample- A	Assay %
1) Initial	99.80
2) after 4hours	100.06
3) after 24 hours	99.94
MEAN value of Assay	99.93
SD	0.13
% RSD	0.13

Robustness For Assay

Robustness were studied by varying the instrumental parameters like relaxation delay from 25sec to 20sec and assay were determined on one sample.

Remark of assay validation by qNMR

Since the method showed relative standard deviation of less than 5.0% for precision of the system, method precision, stability of solution and robustness suggesting that the method meets the requirement for assay determination.

Remark

From the above analysis and results obtained, we conclude that analysis by Titration (alternate method) correlates with that of quantitative NMR spectroscopy analysis for the above product.

Table 6 Comparison table of results for assay by qNMR and alternate technique (using Titration)

Sr. No.	Batch number	% assay by qNMR	% Assay by Titration
1	Sample 1	99.75	99.81
2	Sample 2	99.64	99.22
3	Sample 3	99.69	99.88

Table 7 Summary of validation results obtained are tabulated in summary

Sr. No.	Validation parameters for assay	Results	Acceptance criteria
1.0	**Specificity**		
	No interference from other signals at 7.14ppm for analyte	No interference is observed	No interference should be observed
2.0	**Linearity of assay**		
	Correlation coefficient for calibration	R= 0.9995	R should not be less than 0.99
3.0	**System precision/ Method precision :**		
	RSD of replicate measurement of test sample for System precision	0.20%	Not less than 5.0 %
	RSD of replicate measurement of test sample-A , day 1 and day-2 for method precision	0.22% and 0.22%	Not less than 5.0 %
	RSD of replicate measurement of test sample-B , day 1 and day-2 for method precision	0.15% and 0.36%	Not less than 5.0 %
4.0	**Solution stability for assay**	0.10%	Not less than 5.0 %
5.0	**Robustness for assay**	0.22%	Not less than 5.0 %

Procedure for impurity-a determination in ropivacaine hydrochloride and its method validation

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

Reagent : MeOD-d4

NMR parameters : D1=20sec, NS= 32 , LB (Line broadening) is 1Hz

Stock API preparation

Weight accurately about 10mg of the API in duplicate and transfer in a clean 10ml glass sample vial, add 1mL MeOD into each vial and sonicate to dissolve. Make up to the mark with same solvent and mix well. (0.10% API solution stock).

Impurity solution

Weight accurately about 10mg of impurity-A i.e. 2.6-dimethylaniline in 50ml volumetric flask, sonicate to dissolve and make up to mark with methanol. Dilute 10ml to 100ml with methanol. This is 200ppm solution of impurity-A. Dilute 10ml to 25ml to get a solution of 80ppm. Make a solution of this impurity by half dilution to obtain 40ppm, 20ppm, 10ppm , 5ppm and 2.5ppm by diluting 80ppm solution in methanol. To 1ml each of the above solution add 1ml of 0.10% Ropivacaine hydrochloride solution, so as to obtain 100ppm, 40ppm, 20ppm, 10ppm, 5ppm and 2.5ppm solution of impurity-A in 0.10% Ropivacaine hydrochloride solution.

Specificity of NMR Method

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 band it becomes important to run the sample using different spinning rates or without spinning. Following table gives the chemical shift values of impurity-A and the main compound i.e. Ropivacaine hydrochloride.

Impurity A 2.09ppm, 6.61ppm, 6.88ppm

***Ropivacaine HCl* (Main compound)**

0.99ppm, 1.88ppm, 1.96pm, 2.23ppm, 2.40ppm, 3.16ppm, 3.66pm, 4.32ppm, 7.14ppm

Thus peak at 7.14ppm, (m), 3H is used for the assay of main compound and 2.09ppm, (s), 6H is used for the impurity-A quantification purpose.

Determination of relaxation time (T1) in Impurity-A in hydrochloride API:

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)
Methyls	**C-2, C-6**	**2.09**	**1.72**

Since, the T_1=1.72, so relaxation delay of 20seconds is chosen.

Linearity for Impurity-A by NMR

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 MeOD on similar lines. For the total integral calculation, ignore the NMR solvent signals and water integral. After processing, the normalized impurity intensity or impurity-A ratio at the specified resonance (Hm) was obtained using the following formula: Hm = [Hi / (Hi + Hd)]

Where, Hi is the intensity of the impurity-A i.e at 2.17ppm at the specified resonance and Hd is the intensity of the drug substance i.e. Ropivacaine hydrochloride API at 2.24ppm.

Linearity For Impurity-A

The normalized intensity (Mean of three determinations) was plotted against the known weight % of the impurity-A. The slope was 30.378, intercept was 0.00842 and the correlation coefficient was 0.9989 for the impurity was determined.

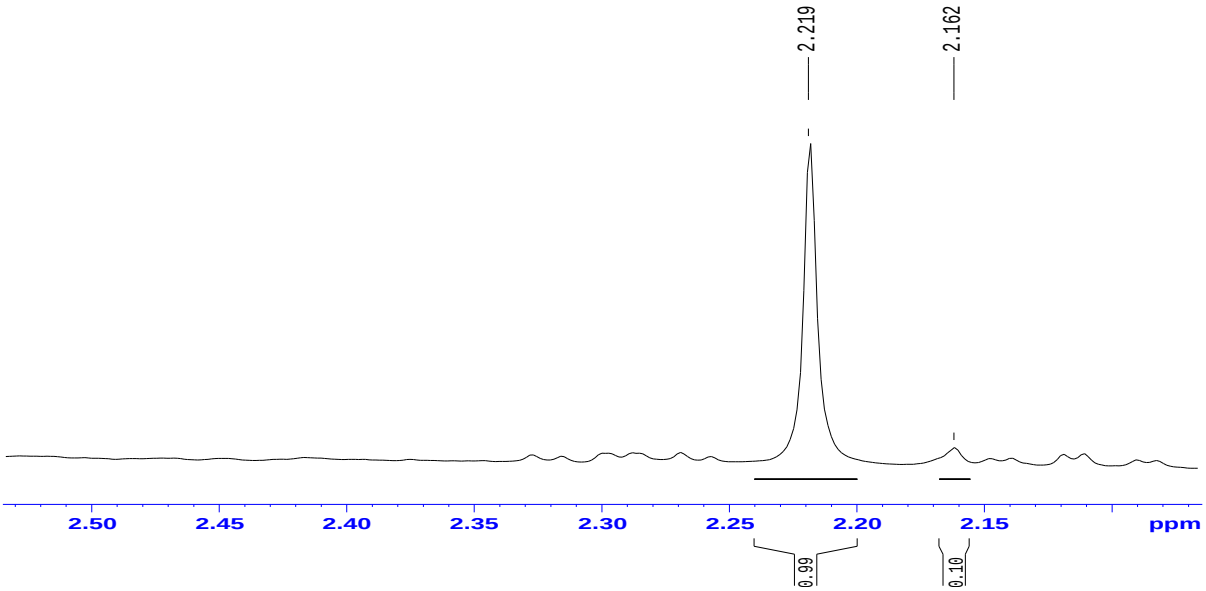


Fig. 5 NMR spectrum of (2.5ppm) of impurity-A spiked with 0.1% Ropivacaine hydrochloride API in MeOD.

Table 8 Linearity Of Impurity-A Spiked In 0.10% API Solution

Impurity-A, %	Hi	Hd	Hi + Hd	Impurity-A ratio, Hi/ (Hi + Hd)
0.00025	47558	470072	517630	0.0919
0.0005	44820	412156	456975	0.0981
0.001	61807	467524	529331	0.1168
0.002	85469	510632	596101	0.1434
0.004	104655	403312	507967	0.2060

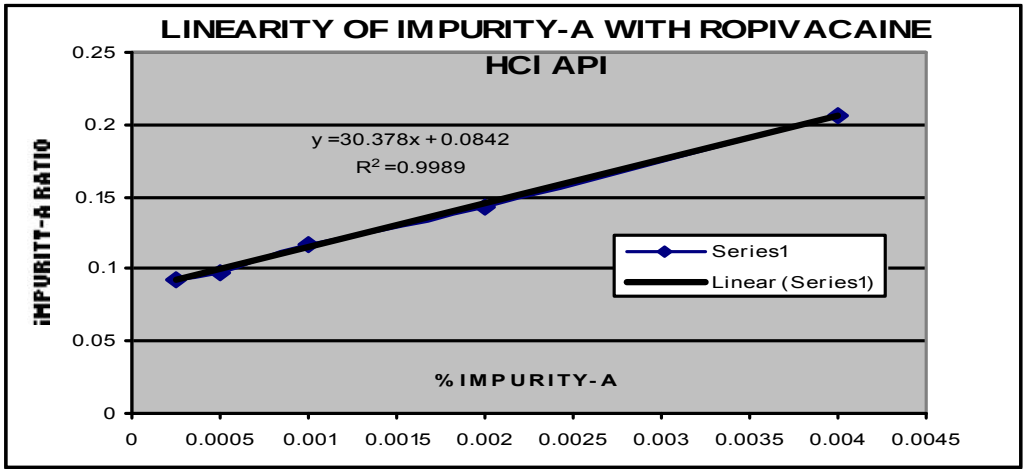


Fig. 6 LOD obtained from linearity graph is 0.0002% (2ppm) and LOQ obtained from linearity graph is 0.0005% (5ppm) .

Limit of Detection And Limit Of Quantitation For Impurity-A In Ropivacaine Hydrchloride

This is determined from the calibration plot of impurity solution A spiked with main drug in Linearity experiment based on the standard deviation of the peak area and the slope of calibration curve.

System Precision For Impurity

The system precision was determined for the impurity-A by preparing five sample of drug substance spiked with appropriate amount of a stock solution of the impurity-A to give a concentration of 0.0010%.

Method Precision For Impurity

The method precision was determined by analyzing two samples in triplicate on two different days.

Recovery Study For Impurity-A In Ropivacaine HCl API

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.

In the present case since impurity is not detected so the sample is spiked with known concentration of impurity say 5ppm, 10ppm, 20ppm and 40ppm and measured by NMR, the recovery was calculated based on the amount recovered against amount added.

Since the method showed recovery of impurity-A well within the range of 80% to 120% suggesting good recovery of the method.

Stability of Solution For Impurity-A

The test solution (say 0.0010% impurity solution) were run after 4hours , 8hours, 10hours and 12hours and the impurity-A ratio values were recorded.

Robustness Of The Method For Impurity-A

Robustness was studied by measuring the NMR signal for test solution (say 0.0010% impurity solution)varying the instrumental parameter like relaxation delay from 20sec to 15sec.

Remark of Impurity A Validation By qNMR

Since the method showed relative standard deviation of less than 5.0% for precision of the system, method precision, stability of solution and robustness suggesting that the method meets the requirement for impurity determination by qNMR.

Table 9 Recovery study for impurity A in Ropivacaine HCL API

Recovery samples	Impurity-A ratio, Hi/ (Hi + Hd)	Impurity-A obtained	% Recovery
5ppm RECOVERY	0.0970	4.2ppm	84%
10ppm RECOVERY	0.1167	10.7ppm	107%
20ppm RECOVERY	0.1494	21.5ppm	108%
40ppm RECOVERY	0.2073	40.6ppm	102%

Table 10 Summary of results for validation of related impurity-A of Ropivacaine Hydrochloride API by qNMR

Sr. No.	Validation parameters for assay		Results	Acceptance criteria
1.0	**Specificity**			
	No interference from other signals at 7.14ppm for analyte		No interference is observed	No interference should be observed
2.0	**Linearity of assay**			
	Correlation coefficient for calibration		R= 0.9995	R should not be less than 0.99
3.0	**System precision/ Method precision :**			
	RSD of replicate measurement of test sample for System precision		0.20%	Not less than 5.0 %
	RSD of replicate measurement of test sample-A , day 1 and day-2 for method precision		0.22% and 0.22%	Not less than 5.0 %
	RSD of replicate measurement of test sample-B , day 1 and day-2 for method precision		0.15% and 0.36%	Not less than 5.0 %
4.0	**Solution stability for assay**		0.10%	Not less than 5.0 %
5.0	**Robustness for assay**		0.22%	Not less than 5.0 %
6.0	**Accuracy :**			
	1	At 50 %	84%	70%-130 %
	2	At 100 %	107%	70%-130 %
	3	At 150 %	108%	70%-130 %

Conclusion

qNMR and Titration results obtained in the present study shows that the results obtained by qNMR correlates well with titration method for the assay of Ropivacaine HCl drug. Similarly the NMR method developed for impurity-A in Ropivacaine HCl showed similar results as the titration method proving beyond doubt the usefulness of NMR technique as a powerful quantitative tool. The advantage of NMR method is that it does not require the use of reference standard of drug to be quantified and also it is a non-destructive technique. The study shows good correlation between NMR assay method and titration method for Ropivacaine HCl drug.

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