Quantitative [1]HNMR Spectroscopy: Analysis of Zinc Gluconate in Utozinc[R] tablets, a Mixture of Zinc Gluconate and Vitamin C

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Abstract:

Background: Zinc is an essential metal for humans and plays key roles in several biological events such as immunity, allergy, growth, and inflammation. The deficiency in zinc causes an increased infection rate with pathogens. Organo-zincates such as zinc gluconate are known for better absorption compared with their inorganic zinc salts. Its role in enhancing the immune system has driven a huge demand for organo-zinc supplements and in the treatment protocol of coronavirus disease, the causative agent of the COVID-19 pandemic.

Objective: Herein, we report on a quantitative analysis of zinc gluconate in the authentic form in presence of vitamin C and the method was applied to their dosage form (UtozincR tablets). The method is simple, accurate, and validated according to ICH guidelines.

Methods: Quantification of zinc gluconate formulated with vitamin C (UtozincR tablets) using Q-^{1}HNMR. Maleic acid and deuterium oxide were used as internal .standards and solvents, respectively

Results: The linearity range, the limit of detection and quantification, stability, precision, and accuracy, were validated. The validation of the method within five concentration levels (from 10 to 50 mg/0.5 mL D2O) afforded a limit of detection of 4.58 mg/mL, a quantification limit of 15.27 mg/mL, and excellent linearity.

Conclusion: The method proposed in the present study is simple, fast, non-destructive, and accurate. Zinc gluconate quantification values obtained by the Q-^{1}HNMR method were found to show an acceptable correlation with those obtained by the thin-layer chromatographic technique.

Highlights: The method was successfully applied on UtozincR tablets, and the results were compared with the reported reference pharmacopeial method. The salt exchange between maleic acid (IS) and zinc gluconate was tested by noticing the change in the chemical shift of IS and zinc gluconate.

Keywords: Q-^{1}HNMR, Zinc gluconate, Maleic acid, D_2O, UtoZinc^{R}, Vitamin C.

Introduction

Zinc is a molecular signal for immune and neuronal cells (1). Zinc is required for DNA synthesis, RNA transcription, and cell division (2). Zinc deficiency induces cell-mediated immune dysfunction and leads to increased rate of infections of human subjects with foreign pathogens such as bacteria and viruses. Zinc is essential for serum thymulin activity, a thymic hormone which is required for T helper cell (Th) differentiation and proliferation. The zinc-dependent transcription factors, T-bet and STAT4 along with interferon-γ (Inf-γ) are required for the differentiation of Th 1 cells. The process of generating interleukin-2 m-RNA (IL-2 m-RNA) from Th 1 cells requires the zinc-dependent transcription factors, nuclear factor kappa B (NF-κB), activator protein-1(AP1), and specialty protein-1(SP-1). Thus, a down-regulation of IL-2 is associated with zinc deficiency in humans (3). IL-2 is required for activation of Natural Killing (NK) and T cytolytic cells which are involved in killing foreign pathogen as well as cancer cells. Zinc deficiency adversely affects Th1 cells with no impact on Th 2 cells, leading to in a shift from Th1 to Th2 functions and results in cell-mediated immune dysfunction. Also, zinc deficiency activates monocytes-macrophages, which generate free radicals leading to oxidative stress and upregulate generation of inflammatory cytokines such as the Tumor necrosis factor-α (TNF-α), IL-1β, and IL-6. (4) It become evident that proper zinc supplementation would be useful in strengthening subjects' immune system and combating microbial infections. COVID-19 mortality/morbidity risk increases with age and for those chronic disease co-morbidities, both of which are associated with lower zinc status, as is the risk of infection (5). Zinc is formulated in several pharmaceutical formulations such as ZincTron^{R} (zinc amino acid chelate), Octozinc^{R} (zinc sulfate), and UtoZinc^{R} (zinc gluconate). Quantitative proton nuclear magnetic resonance spectroscopy (Q-^{1}HNMR) has been used to assay the concentration and the purity of small molecules, biopolymers, for example proteins or nucleic acids (6). The structural information provided by Q-^{1}HNMR, the proportionality of the signal intensity to the number of contributing nuclei enables Q-^{1}HNMR to be used as a nondestructive means of the analysis of the contents of individual analytes in a complex matrix without the need for external reference, and a short analysis time compared with conventional chromatographic methods. These inherent advantages made Q-^{1}HNMR a powerful tool for quantification, gaining increasing popularity. Several Q-^{1}HNMR methods have

been reported for analysis of drugs (7), natural products (8), food analysis (9), and analysis of drug metabolites (10). Several methods have been developed for zinc analysis, including spectrophotometric methods (11), high-performance liquid chromatography (HPLC) (12) and capillary zone electrophoresis methods (13). Herein, we wish to report a rapid analysis of zinc gluconate in authentic and UtoZinc^{R} tablets that contains vitamin C as an additive using Q-^{1}HNMR with maleic acid as an internal standard (Figure 1).

Experimental

Materials and reagents

Chemicals, deuterated solvents; D_2O (99.9%), DMSO-d_6 (99%) were purchased from Merck. Authentic zinc gluconate was provided as a gift from Utopia Pharmaceuticals Company, Cairo, Egypt. Utozinc^{R} tablets (76.56 mg of zinc gluconate and 90 mg vitamin C was purchased from the local Egyptian market (manufactured by Utopia Pharmaceuticals company).

Instrumentation

^{1}H-NMR spectra were recorded on Bruker AVANCE (400 MHz) spectrometer with maleic acid as an internal standard. Chemical shifts are reported in parts per million, and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or (broad).

Mixtures and solutions

For quantitative analysis, stock solutions of standard zinc gluconate was prepared by dissolving 100 mg of zinc gluconate, added to 1 mL of D_2O in stoppered glass vials, then the solutions were sonicated to confirm complete dissolution. Accurately measured aliquots of the drug stock solutions covering the concentration range 10–50 mg/mL were transferred separately to NMR tubes with adding an appropriate volume of the internal standard in concentration of 10 mg/0.5mL of D_2O, after that the data acquisition was analyzed. All concentrations were assessed in triplicate way to ensure the optimum accuracy of results.

Preparation of Utozinc^{R} tablet solution: Ten commercially obtained Utozinc^{R} tablets were accurately weighed and powdered homogenously by means of a mortar and a pestle. The amount of one tablet (equal to 76.56 mg zinc gluconate and 92.3 vitamin C) was dissolved in a volume of 1 mL of D_2O to produce the concentration range 10-50

mg/mL zinc gluconate, then the standard assessment procedures were accurately followed for the triplicate measurement of each concentration.

Procedure for Q-^{1}HNMR spectroscopy

The selectivity of Q-^{1}HNMR depends on using maleic acid as IS whose signal does not interfere with zinc gluconate signals. Integrated analyte ^{1}H signal (doublet) was obtained at 4.57 ppm with respect to ^{1}H signal (singlet) of maleic acid IS at 6.51 ppm. The amount (Wx) and potency (Px) of drug was calculated using the following Eq. (14):

$$Wx = (IX \setminus Istd) * (Nstd \setminus Nx) * (Mx \setminus Mstd) * (mstd)$$

$$Px = (IX \setminus Istd) * (Nstd \setminus Nx) * (Mx \setminus Mstd) * (mstd \setminus m) * Pstd$$

Where, Wx = Weight of zinc gluconate (in mg), Px = potency of zinc gluconate (in %w/w) on as such basis, Ix = Mean integral value of the analyte ^{1}H signal obtained at 4.57 ppm, Istd = Integral value of the ^{1}H signal of maleic acid IS obtained at 6.51 ppm, Nstd = Number of protons for the maleic acid IS (2.0), Nx = Number of protons for the analyte ^{1}H in drug (12), Mx = Molar mass of zinc gluconate (455.685 g/mole), Mstd = Molar mass of the maleic acid IS (116.07 g/mole), mstd = Weight of the maleic acid IS (in mg), m = Taken weight of the analyte drug (in mg), Pstd = Potency of the maleic acid IS (99.90%).

Results and discussion

Confirmation ^{1}HNMR structure for zinc gluconate and IS

^{1}HNMR of zinc gluconate was measured in D_2O and DMSO-d_6 for structural characterization. When analysis was performed in D_2O, all OH protons present in zinc gluconate were exchanged with deuterium and disappeared. Analysis in DMSO-d_6 showed all protons in the drug as shown in (Figure 2). DMSO-d6 was primarily used for determining the chemical structure of the molecule but not for quantitative purpose.

Quantitative ^{1}HNMR method

Maleic acid was used as the internal standard because its signal did not interfere with the signals of zinc gluconate. Experimental trials proved that maleic acid was very suitable to the method with respect to its solubility and chemical shifts. The singlet of maleic acid chosen for Q-^{1}HNMR was assigned as an integration value of 1.00 in each NMR spectra. For zinc gluconate, the doubled was at 4.57 ppm, originating from two

protons attached to the two carbons that are neighbor to carboxylate groups in zinc gluconate. This peak appears well separated from other signals. The ^{1}HNMR spectrum of zinc gluconate bulk in D_2O shows a well-separated doublet of each analyte proton away from the IS.

^{1}HNMR characterization of the active ingredients of Utozinc^{R}

For characterization purposes, ^{1}HNMR of zinc gluconate, vitamin C, maleic and a mixture of the three components were measured in D_2O (Figure 3).

Results and Discussion:

Optimization of different NMR parameters.

A Q-^{1}HNMR technique need to an internal standard that must give limited well-defined signals matched with high organic purity and it should show high stability in solvent solution, absence of residual water to avoid line broadening or baseline distortion. The drug solid state (zinc gluconate) should not be hygroscopic, and the liquid state (D_2O) should not be volatile to maintain accurate weight and accurate concentration measurements. The selected quantitative signals of zinc gluconate and the internal standard (maleic acid) should not display any overlap between each other or with any of other present signals for an accurate quantitative result to be obtained, in addition to optimization of spectral acquisition parameters as shown in (Figure 2). In this method, maleic acid is used as an internal standard as its singlet quantitative signal was chosen at 6.51 ppm without interference with the region of the integration signal of zinc gluconate at 4.57 ppm. For determination of zinc gluconate in Utozinc^{R} tablets, there is not any interference from signals of vitamin C with the signals of zinc gluconate or maleic acid, so this indicates the possibility of application this method on pharmaceutical dosage forms.

Several important factors were optimized before the application of quantitative drug analysis, including the following: Selection of Deuterated Solvent Scanning of the available deuterated, suitable number of scan and relaxation delay. Highly volatile solvents as acetone-d6 and chloroform-d1 were excluded as they alter the volume of the solution and consequently its concentration, which assumes impossible accurate quantifications. Deuterium oxide was preferred as a perfect solvent since it ensured an excellent solubility of the two investigated compounds and not volatile at room

temperature. Whereas the selected internal standard was maleic acid since, it is stable and soluble with the investigated drug in D_2O as shown in (Figure 5).

Method validation

The developed method was validated according to international conference on harmonization guidelines (ICH) (15).

System suitability

Selecting flip angles smaller than 90° results in a smaller signal to noise ratio S/N during each acquisition cycle, but full spin relaxation is reached faster, and the acquisition cycle can be repeated more often (16). Determination of signal to noise ratio (S/N) of the analyte was used to elicit the system suitability of the method (more than 150 ppm), and difference of the shift of analyte signal (more than 0.2 ppm).

Specificity and selectivity

A study of specificity was applied by analyzing the internal standard, pure standard solution of zinc gluconate, vitamin C and the tablet solution with maleic acid separately. ^{1}HNMR spectra are presented in (Figures 5). It was obvious that the solvent and excipients did not affect zinc gluconate signals at 4.57. Furthermore, the selected quantifiable signals of zinc gluconate, maleic acid and vitamin C did not overlap. Spectral data revealed a good selectivity and specificity of the Q-^{1}HNMR determination of zinc gluconate. Stability of maleic acid and zinc gluconate in D_2O was examined. To examine whether a salt exchange between maleic acid and zinc gluconate occurs, ^{1}HNMR of the mixture (1:1 molar ratio, in D_2O) was measured over a span of three days (Figure 5). It was observed that there is not any change in the chemical shift of the internal standard and zinc gluconate.

Precision and intermediate precision

The precision of an analytical method expresses the closeness of agreement between series of measurements obtained from multiple sampling of the same homogenous sample. According to the ICH guidelines the precision will be acquired by six repeated determinations (n=6) and intermediate precision will be evaluated by different analyst on different day and/or different NMR prob and/or different NMR spectrometer with different magnetic field strength. The precision was assessed by six separate sample

preparations and Intermediate precision was determined by performing measurements on three different occasions as shown in table (1).

Accuracy

The accuracy of an analytical method expresses the closeness of agreement between an accepted reference value and the value found. The accuracy of an analytical procedure should be established across its range. Nine determinations over three concentration levels covering the specified range were measured and determined. The accuracy was studied at 80, 100 and 120 % levels with respect to the sample by preparing the solutions in triplicate at each level. The results confirm the accuracy of the developed technique as in table 1

Linearity

Q-^{1}HNMR as a method itself is linear because the intensity of the response signal is directly proportional to the number of nuclei contributing to this signal. Linearity was checked by preparing standard solutions at five different concentration levels according to the content of analyte in test sample. Linearity curve was drawn for taken drug amount (in mg) vs. The ratio between the integration value of analyte to the integration value of internal standard. The equation for curve was (y = 0.039/2 x +0.05/2) The correlation coefficient was found 0.99, indicating good linearity as shown in (Figure 6).

LOD and LOQ

In the case of NMR, the LOD and LOQ must be calculated by the standard deviation of the response (S.D) and the slope (S) of the calibration curve obtained in Linearity study. The LOD and LOQ were very suitable for determination of zinc gluconate as shown in table (2).

Analyte stability in the solution *(17)*

Authentic powder of zinc gluconate or its tablets were analyzed at ambient room temperature at different time intervals 0 h (Initial), 6 h, 12 h, 18 h and 24 h intervals and calculated % assay for each interval. The difference between the determined percentage for each preparation at different time intervals with respect to the corresponding start value. It was found that no major change exit. Results are tabulated in table (3).

Robustness

The capacity of the method to remain unaffected by small experimental variations was tested. The number of scans (64 scans±16) and the internal standard amount (10 % variation) (10±1.0 mg) were used to evaluate robustness of the method as in table (4).

Comparison with other reported methods

The Pharmaceutical formulation containing zinc gluconate (Uotozinc^{R}) have been successfully analyzed by the proposed method. Results obtained were compared to those obtained by applying reported reference pharmacopeial method (18). The pharmacopeial method is a thin-layer chromatographic method by using mobile phase concentrated ammonia R, ethyl acetate R, water R, ethanol (96 %) R (10:10:30:50). Student's t test and F-test were performed for comparison. Results was shown in table (5). where the calculated t and F values were less than tabulated values which in turn indicate that there is no significant difference between proposed method and reference method relative to accuracy and precision. Also, ANOVA analysis was carried out to test the null hypothesis between the results of the proposed method and the reference one (18) as shown in table (6).

Conclusions

The present study purposes, for the first time, a straightforward equation for the quantification of zinc gluconate based on Q-^{1}HNMR technique. No significant difference in the zinc gluconate quantification values were obtained when compared with reported reference pharmacopeial method. The method proposed in present study is simple, fast, non-destructive, and accurate. Zinc gluconate quantification values obtained by Q-^{1}HNMR method were found to show the acceptable correlation with those obtained by thin-layer chromatographic technique. Therefore, Q-^{1}HNMR could be employed as a quick, non-destructive, and cost-effective alternative for the quantification of zinc gluconate in authentic form or pharmaceutical dosage form.

CRediT Author Statement

Marwa H. Hasan*[1] (corresponding author): Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing - Review & Editing, Project administration

Abdalla E. A. Hassan[2]: Writing - Review & Editing, Supervision, Project administration, Resources

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Conflict of Interest: All authors declare no conflict of interest.

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Table 1. Results of precision, intermediate precision, and accuracy for Q-^{1}HNMR analysis of zinc gluconate.

	AV ±SD %	Accuracy & recovery %
Precision	100±0.31	100.39±0.88
		100.026±1.53
Intermediate precision	100.07±0.73	99.86±0.89

Table 2. Results of validation parameters for Q-^{1}HNMR analysis of zinc gluconate in authentic form.

[a]**Taken concentration mg/0.5 mL D_2O**	[a]**Recovery %**	[a]**Found concentration mg/0.5 mL D_2O**	**Mean%**	100.13	**Slope**	0.0195
10	100	10	**S D±**	0.7	**LOD mg/mL**	4.58
20	101.28	20.26	**RSD%**	0.7	**LOQ mg/mL**	15.27
30	100	30	**SE±**	0.31	**S.S.**	0.023
40	99.36	39.74	**Variance**	0.49		
50	100	50				

[a]Average of three independent procedures.

Table 3. Stability of analyte in solution test results

For standard preparation			**For sample preparation**		
Time interval	**%Assay**	**% Diff.**	**Time interval**	**%Assay**	**% Diff.**
Initial	100.05	---------	Initial	99. 34	--------
After 6	100.2	0.15	After 6	99.59	0.25
After 12	100.3	0.25	After 12	99.79	0.45
After 18	100.24	0.19	After 18	99.45	0.11

After 24	100.36	0.31	After 24	99.52	0.18

Table 4. Robustness for Q-^{1}HNMR analysis of zinc gluconate

Parameter[a]	Change	Recovery, % ± SD
Number of scan (64 scans ± 16)	48	99.43±0.76
	64	100.09±1.11
	80	100.12±1.32
Internal std, mg (10±1.0 mg)	9	100.32±0.77
	10	100.09±0.54
	11	100.26±1.42

[a]Average of three **analysis**.

Table 5. Statistical analysis of results obtained by the proposed method applied on zinc gluconate in the form of Utozinc^R tablets compared with reference method

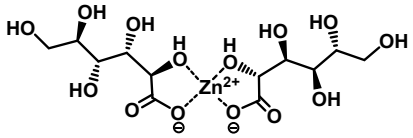
No.	**Proposed method** Average of % recovery of five results	**Reference method (18)** Average of % recovery of five results	**Calculated t-values**[a]	**Calculated F-values**[a]
5	100.74±0.70	101.07±1.35	0.54	2.38

[a] Tabulated t-values and F -ratios at p = 0.05 are 2.57 and 5.

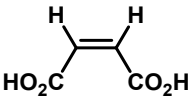
Table 6. Statistical analysis of results obtained by applying ANOVA study for make comparison between the proposed method and the reference one (18).

	Sum of squares	**Mean squares**	**Degrees of freedom**	**F**	**P**
Between	2.5	2.5	1	2.55	0.05
Within	3.4	0.735	3		
Total	6.2		4		

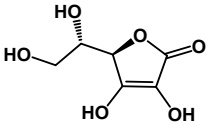
Where the critical F -ratios at p = 0.05 are 10.13



(A)



(B)



(C)

Figure 1. Structures of zinc gluconate (A), maleic acid(B), and vitamin C (C).

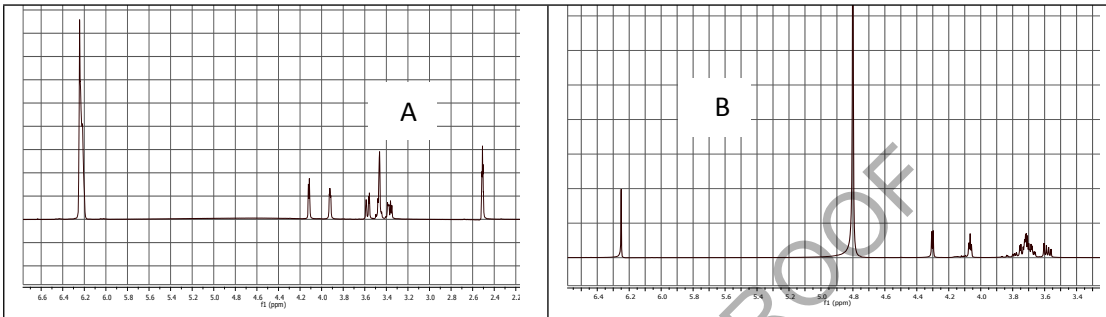


Figure 2. [A] ^{1}H NMR spectrum of 50 mg zinc gluconate and 10 mg IS (maleic acid) in DMSO-d_6. [B] ^{1}H NMR spectrum of 50 mg zinc gluconate and 10 mg IS (maleic acid) in D_2O.

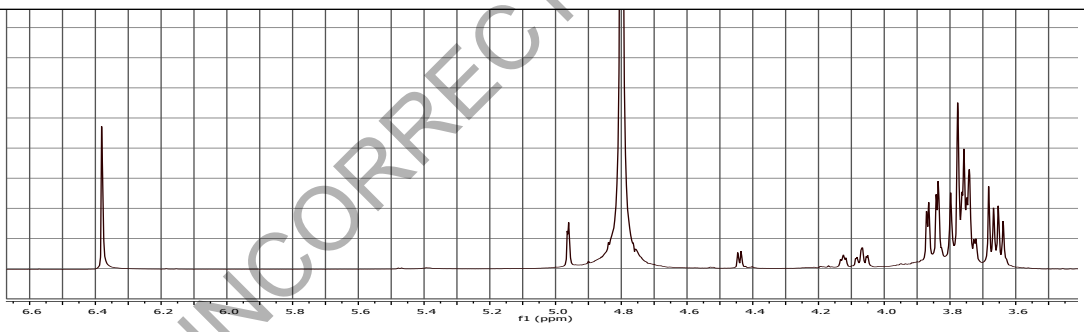


Figure 3. ^{1}H NMR spectrum of 10 mg zinc gluconate, 13 mg vitamin C and 10 mg IS (maleic acid) in D_2O.

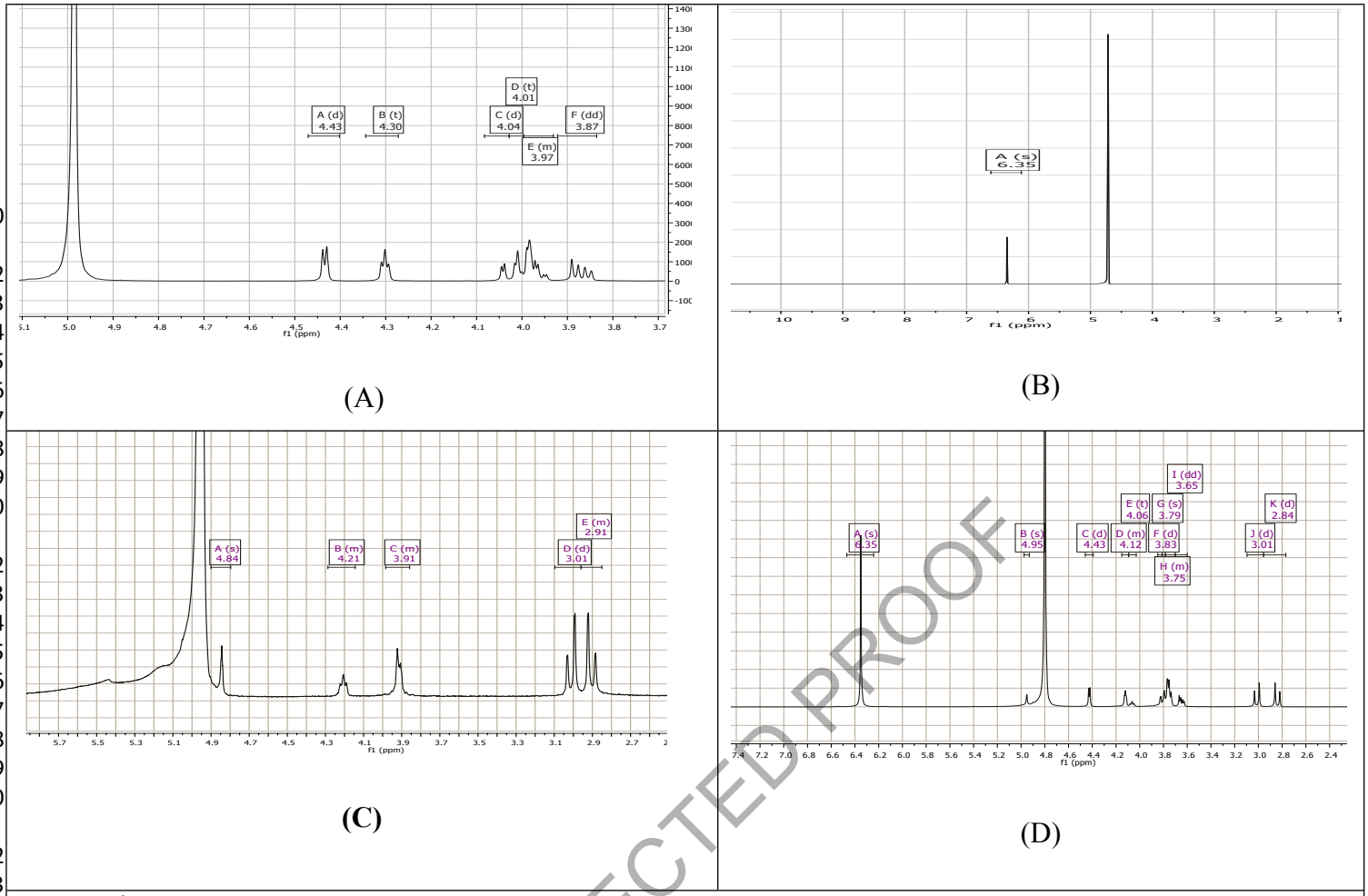


Figure. 4. ^{1}H NMR spectrum of [A] 5 mg zinc gluconate, [B] 5 mg IS maleic acid , [C] 5 mg vitamin C and [D] mixture of zinc gluconate, vitamin C and maleic acid.

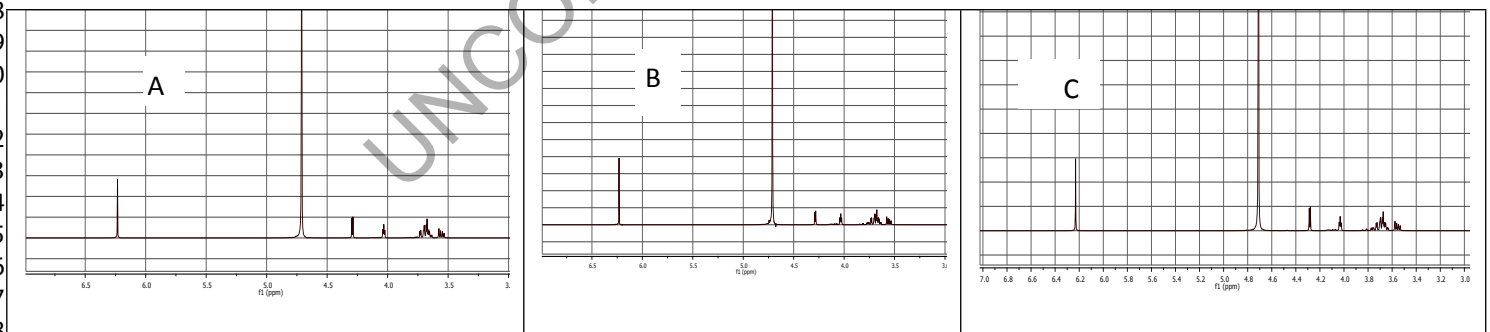


Figure 5. [A] ^{1}H NMR spectrum of a mixture of maleic acid (5 mg) and zinc gluconate (19.6 mg) after one day, [B] after two days and [C] after three days

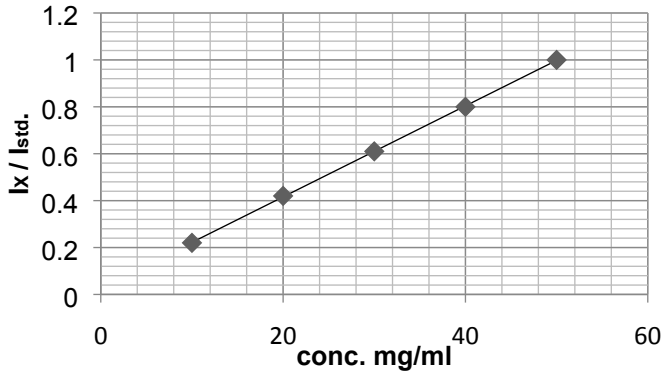


Figure 6. Linearity curve of taken drug in mg vs. peak intensity for Q-^{1}HNMR analysis of zinc gluconate