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A new approach to quantitative NMR: Fluoroquinolones analysis by evaluating the chemical shift displacements

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Abstract

Quantitative NMR spectroscopy is always an attractive goal as the identity and quantity could be simultaneously determined. Although significant advancements have been achieved in this field it is common that all reported quantitative NMR methods perform the analysis by utilizing the average integral intensities of selected signals. During the calculation of the area under NMR peaks several response problems can occur which should always be treated carefully to overcome inaccuracies. In the method proposed in this work the quantitative information is obtained utilizing the measurement of selected protons chemical shift displacements which is a quite straightforward and highly reproducible process. The ^{1}H NMR spectra of multiple fluoroquinolone (FQ) solutions revealed that the chemical shifts of protons, especially the aromatic ones, were concentration dependent for all tested compounds, as a result of extensive self-association phenomena. In the present work a novel methodology is described for the quantitation of several FQs based on this dependence. The proposed method was applied to Ciprofloxacin solutions over a wide range of concentrations. Evaluation of the obtained data presented acceptable characteristics regarding accuracy, precision, and robustness. The applicability limitations of this method were found to be posed by current instrumentation, mainly by the magnetic field frequency e.g. the slope of the response function achieved with a 400 MHz instrument was twice the one achieved at 200 MHz. The pH effect was negligible from pD 2.5 to 5.5. The phenomenon appeared in a pattern that can be applied for a plethora of drug categories revealing self-association phenomena in a range of concentration determined by the magnet strength of the instrument.

Keywords: Fluoroquinolones; Quantitative ^{1}H NMR; Ciprofloxacin; Norfloxacin; Enoxacin; Self-association; Chemical shift

1. Introduction

The interest for quantitative NMR spectroscopy is continuously increasing as the simultaneous determination of both identity and quantity is always an attractive goal. Several studies have been published concerning application of NMR for many analytical purposes [1,2] including the quantitation of drug molecules [3–8].

New FT-NMR instruments show increased sensitivity and signal resolution, thus improving remarkably the limit of quantitation due to their stronger magnetic flux density and their ability to accumulate pulses [9]. Nevertheless from analytical point of view ^{1}H NMR techniques still remain quite insensitive, when compared to the current UV or MS techniques.

All up to date described methodologies make use of the average integral intensities of selected NMR signals comparing them to those of external or internal standards. Successful evaluation of the above data requires careful treatment of the resulting response problems.

A novel approach is proposed in this work obtaining quantitative information from the measurement of chemical shift displacements of selected protons which is a quite straightforward and highly reproducible process. Chemical shifts provide information about the chemical surroundings of atoms and their changes have been extensively evaluated to monitor inter- and intra-molecular interactions occurring through covalent or non-covalent binding. They have been studied to characterize aggregation [10], hetero [11] or self-association [12], or molecular recognition phenomena [13,14] and to calculate the association constants [15,16]. Unprecedented concentration dependent chemical shift variations in ^{1}H NMR spectra have been reported for quinolines [17]. Whatsoever and to the best of our knowledge there is no report utilizing them for quantitative purposes.

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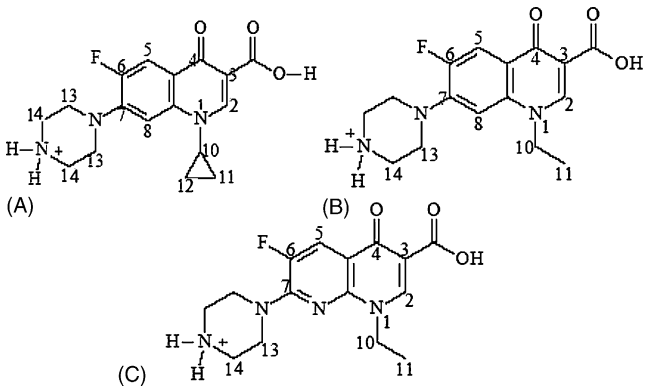

Fig. 1. Chemical structure and numbering of Ciprofloxacin (A), Norfloxacin (B) and Enoxacin (C).

Table 1
Slopes, intercepts and correlation coefficients of calibration lines calculated for Norfloxacin and Enoxacin hydrochloride solutions

Proton	Slope (ppm)	Intercept (ppm)	r
Norfloxacin			
H-2	0.3862	7.8239	0.9955
H-5	0.6313	6.2271	0.9966
H-8	0.3599	6.3029	0.9956
Enoxacin			
H-2	0.2244	8.2646	0.9910
H-5	0.4737	6.3812	0.9927

FQ antibacterials, also known as DNA gyrase inhibitors, are prescribed predominantly in the treatment of respiratory, urinary tract, enteric, human skin and soft tissues infections, as well as against sexually transmitted diseases [18]. Among the NMR spectroscopy studies of FQs few relevant articles deal with their quantitative determination [19].

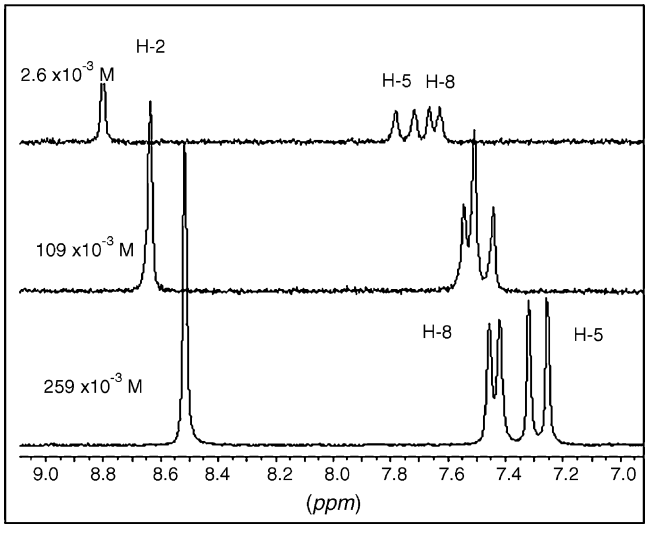

Fig. 2. Partial ^{1}H NMR spectra of Ciprofloxacin aromatic protons signals, at different concentrations ranging from 2.6×10^{-3} to 2.6×10^{-2} M. The crossover of peaks corresponding to H-5 and H-8 is apparent, while corresponding J constants remain unchanged.

Initially in this work the ^{1}H NMR spectra of three antibacterial FQs Ciprofloxacin, Enoxacin and Norfloxacin (Fig. 1) in D_2O solutions have been studied. The results presented in Table 1 reveal strong concentration dependence of the chemical shift values, for all tested compounds. A novel methodology based on the proportionality of the aforesaid dependence (Fig. 2) was then developed to achieve the quantitation of Ciprofloxacin in aqueous solutions.

The proposed method was evaluated regarding the accuracy, precision, and robustness characteristics. The pH and temperature effect as well as the applicability limitations of the method posed by current instrumentation are being discussed.

2. Experimental

2.1. Materials

Ciprofloxacin hydrochloride was kindly offered by ELPEN Pharm. Co. Inc. (Pikermi, Attica, Greece) both as chemical reference standard and laboratory powder of known potency (total impurities less than 0.5%) and stored under light protection. Ciprofloxacin spiked with impurities B, C, D, E was obtained from European Pharmacopoeia as batch 1. Norfloxacin and Enoxacin were purchased by Sigma–Aldrich Co. (St. Louis, USA).

Deuterium oxide (99.9% D), deuterium chloride solution (99 atom% D, 35 wt.% in deuterium oxide) and sodium deuteroxide (99 atom% D, 40 wt.% in D_2O) were used for the preparation of acidic and alkaline solutions. The peak of DSS [3-(trimethylsilyl)-1-propanesulfonic acid–sodium salt 1% (w/w) in D_2O 99.9 atom% D] at 0 ppm was used as the chemical shift external reference. All deuterated solvents and reagents were purchased from Sigma–Aldrich Co. and from euriso-top (Gif-Sur-Yvette France).

2.2. Preparation of solutions

Three stock standard solutions of Ciprofloxacin hydrochloride (25.9, 10.4 and 2.6×10^{-3} M) in deuterated water were used to prepare the calibrator standard solutions. At least eight levels of concentration ranging from (1.5 to 25.9) $\times 10^{-3}$ M were used for the calibration of the chemical shift scale with pD levels ranging from 4.6 to 3.9, respectively. Stock solutions for Norfloxacin hydrochloride (26.3, 10.5 and 3.9×10^{-3} M) and Enoxacin hydrochloride (28.1, 11.3 and 2.8×10^{-3} M) were prepared in D_2O using equimolar quantities of DCl 0.1 M solution. A separate batch of solutions was also prepared as above and was used to evaluate the quality characteristics of the proposed method.

For the evaluation of method robustness samples with pD 5.3, 4.5, 3.7, 3.2, 2.6 were prepared by addition of appropriate amounts of 0.01 M DCl and 0.01 M NaOD in aliquots of a 7.77×10^{-3} M Ciprofloxacin solution. Additionally, in aliquots of a 6.26×10^{-3} M standard solution increasing amounts of KCl were added to prepare solutions with salinity of 0.9, 2.0, 4.0, 6.0, 10.0% (w/v).

All pD measurements were performed using an ISFET pH Meter IQ120, IQ Scientific Instruments Inc. (California, USA).

2.3. NMR measurements

^{1}H NMR experiments were performed on a Bruker 200 and Bruker DRX-Avance 400 NMR spectrometers with the operating frequency 200.13 and 400.13 MHz. One-dimensional spectra were acquired using 32k data points and zero-filled to 64k data points before Fourier transformation. Probe temperature was maintained using a BVT-3000 Bruker control unit. Chemical shifts are relative to DSS.

Aliquots of 0.7 ml from each calibrator solution were transferred in 5 mm tubes and each ^{1}H NMR spectra was recorded in triplicate under constant temperature control. It is self evident that thoroughness during the preparation of the solutions and temperature control in order to minimize systematic and random errors is a prerequisite.

The effect of temperature was further evaluated by recording the ^{1}H NMR spectra of 1.04, 1.56, 2.6, 3.9, 5.2, 7.8 and 10.4×10^{-3} M Ciprofloxacin standard solutions at three different temperatures 278, 293, and 308 K.

The use of either external or internal standard has its drawbacks. The capillary that contains the former can disturb the homogeneity in the magnetic field that broadens the bands in such an extent that the small shifts due to solute–solute interactions cannot be measured with sufficient precision. The latter may influence the weak solute–solute interactions.

3. Results and discussion

FQs studied in the present work appear in cationic, zwitterionic, non-ionic or anionic forms depending on medium pH and individual dissociation constants (K_a), as they bear two protonation sites: the carboxylic group and the secondary piperazine nitrogen. All FQs quantitation experiments were performed in their hydrochloride form [20].

The dramatic variations of FQ chemical shifts observed in the current study (Table 1 and Fig. 2) could be consequence of homo-association phenomena. The non-covalent weak forces that stabilize assemblies are suggested to be intermolecular hydrogen bonds, π–π aromatic stacking and electrostatic or cation–π interactions, leading to "head to head" or "head to tail" dimers. In such assemblies the number of molecules, their mutual interactions and tightness of association should vary as a function of concentration which in turn should appear in the altered chemical shifts.

3.1. Detector's response

The chemical shifts assigned to Ciprofloxacin aromatic protons were considered as the detector's response in order to develop a quantitative NMR methodology. Comparison of spectra obtained from samples of different concentration levels showed that all proton resonances moved downfield upon dilution. These chemical shift displacements were more intense for the aromatic protons especially for H-5. It was found that signals attributed to H-5 were displaced from 7.28 to 7.81 ppm when the concentration changed from (25.9 to 1.5) $\times 10^{-3}$ M. The responses from H-5 were finally selected to derive the response function.

3.2. Response function and linearity

The signal displacements induced by concentration changes follow a specific pattern. This pattern was further elucidated by investigation of spectral data from calibration curve solutions. Chemical shift values of the selected protons were plotted against the concentration. A linear graph was obtained when these chemical shift values were correlated to the negative decimal logarithm of concentration (pC):

$$\delta = A - S \log C = A + S\,\mathrm{p}C \quad (1)$$

where δ is the chemical shift (ppm or Hz); C the sample concentration (M); S the slope (ppm); A the intercept (ppm).

The proposed quantitative method utilizes Eq. (1) which was derived by the above-described correlation and can be applied to any Ciprofloxacin aromatic proton as well.

The above-mentioned equation is empirical and provided good fit with experimental data from other FQs as well (Table 1). However generalization to other aggregating drugs requires further verification.

3.3. Assessment of method accuracy and sensitivity

The accuracy of the method was evaluated by assaying samples with known added amount of analyte at five concentration levels. Results are summarized in Table 2.

The sensitivity of the method appears to be ultimately influenced by the measurement of the chemical shift changes (the band maximum frequency) provided by the instrument utilized. The minimum measurable change of concentration (ΔC_{min}) corresponds to the minimum chemical shift displacement ($\Delta\delta_{min}$) which can be accurately measured.

Assuming that $\Delta\delta_{min} = \delta_2 - \delta_1$, where δ_1 and δ_2 are the chemical shift values measured for C_1 and C_2, the corresponding ΔC_{min} will be equal to the difference $C_2 - C_1$.

The response function (Eq. (1)) can be used to calculate $\Delta\delta_{min}$ and ΔC_{min} (method sensitivity parameters) using the following set of transformations:

$$\delta_1 = A - S \log C_1 \Rightarrow C_1 = 10^{A-\delta_1/S} \quad (2)$$

$$\delta_2 = A - S \log C_2 \Rightarrow C_2 = 10^{A-\delta_2/S} \quad (3)$$

Table 2
Bias measurements using chemical shift data from Ciprofloxacin H-5 as determined using 400 MHz spectrometer

Nominal C ($\times 10^{-3}$ M)	Determined C ($\times 10^{-3}$ M)	% bias
1.560	1.561	0.2
2.590	2.523	2.6
3.890	3.832	1.5
5.180	5.045	2.6
7.780	7.991	2.7

Table 3
Response functions, correlation coefficients and the minimum percentage of concentration accurately measured as determined using 400 and 200 MHz spectrometers

Instrument	Proton	Response function[a]	r	$\%(\Delta C_{min}/C_1)$
400 MHz	H-2	$\delta = 79.97pC + 3298.3$	0.992	1.74
	H-5	$\delta = 153.84pC + 2699.6$	0.999	0.90
	H-8	$\delta = 64.04pC + 2884.6$	0.998	2.18
200 MHz	H-2	$\delta = 41.773pC + 1650.1$	0.996	3.36
	H-5	$\delta = 76.601pC + 1360.9$	0.998	1.82
	H-8	$\delta = 29.585pC + 1453.9$	0.997	4.78

[a] Values expressed in Hz.

Combining the above we obtain the following equation:

$$\frac{C_2}{C_1} = 10^{((A-\delta_1)-(A-\delta_2))/S} = 10^{\Delta\delta_{min}/S} \quad (4)$$

Finally, ΔC_{min} can be illustrated as a function of slope (S) and $\Delta\delta_{min}$ values as follows:

$$\frac{C_2 - C_1}{C_1} = \frac{\Delta C_{min}}{C_1} = [10^{\Delta\delta_{min}/S} - 1] \quad (5)$$

$$\frac{C_2 - C_1}{C_1} \times 100 = \% \frac{\Delta C_{min}}{C_1} = [10^{\Delta\delta_{min}/S} - 1] \times 100 \quad (6)$$

The term $\%(\Delta C_{min}/C_1)$ represents the minimum percentage of concentration C_1, which can accurately be quantitated and could be used to characterize the sensitivity of the method. Accordingly, for a typical experiment with a 400 MHz NMR spectrometer, the $\Delta\delta_{min}$ value can be estimated at 0.6 Hz level. Inserting this value to Eq. (6) we obtain:

$$\% \frac{\Delta C_{min}}{C_1} = [10^{0.6/S} - 1] \times 100 \quad (7)$$

In Fig. 3, $\%(\Delta C_{min}/C_1)$ is plotted against the slope for standard $\Delta\delta_{min}$ values.

The percentage of concentration which can be accurately measured $\%(\Delta C_{min}/C_1)$ by the proposed method is always a function of the slope of the straight line and the digital resolution of the spectrometer used.

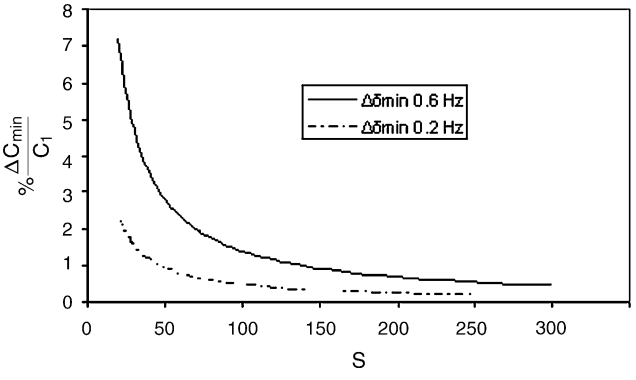

Fig. 3. Minimum percentage of concentration C_1, which is accurately measured, is plotted against the response function slope using standard $\Delta\delta_{min}$ values 0.6 and 0.2 Hz for a 400 and a 200 MHz NMR spectrometer, respectively.

3.3.1. The slope

The slope of the straight line (Eq. (1)) is mainly related to structural features of the analyte, favoring or not the association phenomena. Among the tested FQ molecules the phenomenon appeared more pronounced at Ciprofloxacin. The plots of chemical shifts corresponding to Ciprofloxacin aromatic protons (H-5, H-2, H-8) at 293 K against concentration provide three different calibration curves. Since the results obtained from H-5 signals were the optimum (Table 3), this proton was selected for the calculations.

It is obvious that the slope of the line is proportional to the measurable chemical shift displacements and it determines the method accuracy. These displacements as a function of concentration create a measurement scale. The divisions of the scale can be correlated to concentration levels through the response function (Eq. (1)).

The digital resolution of the instrument $\Delta\delta_{min}$ determines the minimum detectable change of concentration ΔC_{min}, which defines the gradient of the measurement scale. The longitudinal relaxation time (t_2) is determining $\Delta\delta_{min}$ parameter. The slope calculated by linear regression, as can be seen in Table 3, was different for each instrument/magnetic field used. Better results are obtained at 400 MHz, as the values calculated for $\%(\Delta C_{min}/C_1)$ are roughly half the values obtained at 200 MHz.

Taking into account that the new NMR instruments bear superconducting magnets ranging up to 800 MHz the perspective sensitivity level can be suggested much below.

3.4. Measurement of precision

The repeatability of the proposed method was assessed by performing all within-day measurements in a continuous session and by using statistical averaging techniques. Five levels of concentration with three replicates at the same day provided a mean standard deviation of 0.12%. Intermediate precision was also evaluated providing a mean standard deviation of 0.2%.

3.5. Limits of detection and quantitation

Both limits of detection and limit of quantitation are functions of the magnetic field, as the shape and the sharpness of the peaks imminently affect the quality of the chemical shift measurements. The higher the resolution of the spectrometer, the lower the detection and quantitation limits are.

For the described method using a 400 MHz instrument the concentration level which provides accuracy of 0.6 Hz in the

Table 4
Chemical shift values (ppm) at different temperatures and Ciprofloxacin concentration levels as determined using 400 MHz spectrometer

Temperature (K)	Concentration ($\times 1.04 \times 10^{-2}$ M)			Concentration ($\times 1.04 \times 10^{-3}$ M)			$\%(\Delta C_{min}/C_1)$[a]	r[a]
	H-2	H-5	H-8	H-2	H-5	H-8		
278	8.3674	7.2106	7.2885	8.5827	7.5935	7.4482	0.88	0.9998
293	8.6327	7.5046	7.5252	8.8324	7.8867	7.6857	0.90	0.9996
308	8.9298	7.8653	7.7953	9.0371	8.1624	7.905	1.2	0.998

[a] Calculated from H-5 data.

measurement of chemical shift values can be accepted as quantification limit of the method and was found to be 5×10^{-4} M.

The detection limit of the method was assessed based on the signal to noise ratio and was found to be 10^{-4} M which provides a signal to noise ratio of about 3.

3.6. Specificity

In order to discuss specificity of the proposed method the possible effect of the presence of related substances to the equilibrium influencing the reading δ values was also investigated. A series of experiments were conducted with high purity Ciprofloxacin reference standard and Ciprofloxacin reference standard spiked with less than 0.1% impurities B, C, D, and E. The results were perfectly comparable in all cases with those obtained from bulk Ciprofloxacin containing total impurities less than 0.5%.

3.7. Robustness

The experiments were repeated in solutions of intermediate concentration with different acidic pD ranging from 2.5 to 5.5. The results obtained from all tested solutions were almost identical. In Fig. 4 it is evident that chemical shift values were not influenced significantly within the selected pD range and therefore the method remains applicable. Furthermore, the changes of the chemical shift values cannot be induced by the slight pD changes caused by dilution of Ciprofloxacin solutions.

The effect of salinity was also investigated in an additional experiment. The measured chemical shifts of all protons were not significantly affected by the presence of KCl added in a range of 0.9–10% (w/v). The average shift changes were found to be 0.15% (±0.13) and 0.27% (±0.12) at the low and high salinity levels, respectively.

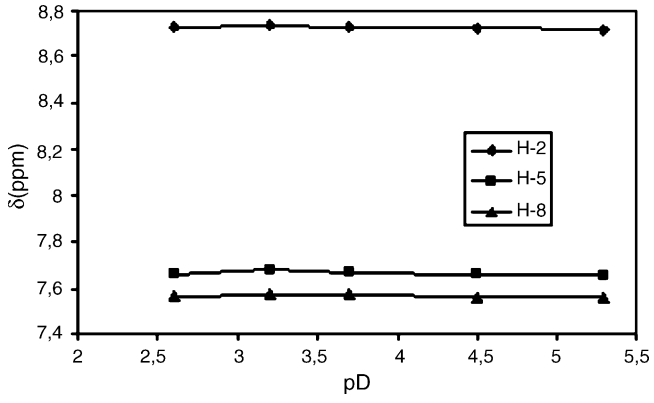

Fig. 4. The effect of pH on the chemical shift values of Ciprofloxacin aromatic protons in acidic media.

3.8. The magnetic field effect

The higher-field spectrometers give sharper bands with a favorable signal to noise ratio and provide more accurate measurements. A 400 MHz instrument would provide a 0.6 Hz change in frequency for a displacement of 0.001 ppm.

Separate calibration lines were constructed utilizing a 200 and a 400 MHz instrument using the same calibrators in order to evaluate the effect of the magnetic field frequency. Evaluating the results obtained from both instruments (summarized in Table 3) can easily be concluded that the increase of the magnet strength from 200 to 400 MHz improves 50% the accuracy of the method, since the new calculated $\%(\Delta C_{min}/C_1)$ value is approximately the half of the previous one.

3.9. The temperature effect

Temperature control during the analysis is important because it can affect the extent of self-association phenomena and as a consequence the reliability of the results. Calibration lines were constructed at 278, 293 and 308 K and the corresponding equations were derived by linear regression.

Results are summarized in Table 4. It is clear that at low temperature the displacement of the chemical shift values are greater in all cases providing a larger measuring scale. At lower temperatures the peaks get sharper providing less uncertainty in the reading of the ppm scale. This fact also reflects to the improved linearity of the method. Finally the slope of the response function is significantly reduced at 308 K which results to increased $\%(\Delta C_{min}/C_1)$ values. These observations can be attributed to weakening of aggregation at higher temperature.

4. Conclusion

A novel quantitative NMR methodology is proposed to achieve FQ analysis in aqueous solutions based on chemical shift displacements as a function of concentration. The new method presented acceptable characteristics regarding accuracy, precision and robustness. The applicability limitations of this method depend mainly on the extent of the association phenomenon of the investigated molecule and the current NMR instrumentation, particularly the magnetic field frequency. The accuracy achieved with a 400 MHz instrument was twice the one achieved with a 200 MHz instrument.

The methodology proposed and the quality characteristics presented claim attention for future development of quantitative NMR applications. The theoretical and experimental results of this work hold obvious significance because of its possible application on several fluoroquinolones as well as the increasing interest on the molecular aggregation phenomena which are evident for a plethora of molecules. However generalization to other aggregating drugs requires further verification.

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