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Quantification of drugs encapsulated in liposomes by ^{1}H NMR

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ABSTRACT

Liposomes are one of the most important and extensively studied drug delivery system due to their ability to encapsulate different kinds of drugs. Exploiting the advantages of ^{1}H Nuclear Magnetic Resonance (NMR) spectrometry, we established a rapid and easy method for quantification of drugs encapsulated in liposomes. An internal standard, pyridine, was used for quantitative determination of drug concentration. Two different drugs were involved in this work, one hydrophilic, methotrexate disodium salt, and another hydrophobic, tamoxifen. The specificity and selectivity of the suggested method were evaluated by the absence of overlapping of at least one signal of each drug with pyridine in the NMR spectrum. The accuracy and precision of the method were assessed by adding a known amount of each drug to unloaded liposomes. Results obtained by quantitative NMR (qNMR) were validated and confirmed by comparing with two other traditional techniques, Ultraviolet-Visible (UV–vis) spectrophotometry and High-Performance Liquid Chromatography (HPLC). It was found that the results were consistent with the ones obtained from our proposed qNMR method. Considering all the experiments conducted in this study, we deliberate that qNMR can be a suitable tool for the determination of drugs encapsulated in liposomes.

1. Introduction

Liposomes have been considered the most suitable drug delivery system for a range of pharmaceutical and biomedical applications [1]. Due to their versatility, they can incorporate drugs with distinct solubilities. Hydrophobic drugs have affinity to the lipid bilayer and hydrophilic drugs are entrapped in their aqueous compartment. The delivery of drugs by liposomes enhances their therapeutic index and alters their biodistribution profile [2].

The immediate result of production of liposome encapsulating drugs is a mixture of encapsulated and free drug. After applying the separation process, several techniques are used for drug quantification, including spectrophotometry, fluorescence spectroscopy, enzyme-based methods, electrochemical techniques and chromatographic methods [3]. The assessment of the drug concentration encapsulated in liposomes by traditional methods such as Ultraviolet-Visible (UV–vis) spectroscopy and High-Performance Liquid Chromatography (HPLC) can be a challenge for investigators. Although UV − vis methodology allows a rapid and simple measurement of the drugs, some difficulties may appear. An inefficient drug quantification may occur due to possible interactions among components and when the maximum absorbance of the drug is close to the maximum absorbance of an eventual component of the formulation. Regarding the HPLC, the time of analysis is a principal concern. Before starting the experiments is necessary spend time with, for example, column equilibration [4], extensive preparation of samples and buffers, being also of high cost. To minimize some of these problems, Nuclear Magnetic Resonance (NMR) spectrometry can be a suitable solution. NMR is a powerful technique for structure determination, that has also emerged as an important analytical tool in the biomedical and pharmaceutical field for quantitative determination of drugs in different matrices, providing high specificity and sensitivity [5–7]. Quantitative NMR (qNMR) have several advantages such as great reproducibility, automation, quantification without identical standard material, and total detection permitting an unbiased overview of the sample composition [8,9].

The principle of qNMR analysis reveals that integrated peak area of each ^{1}H NMR signal provided corresponds directly to the equal number of equivalent nuclei responsible for that signal. Therefore, adding an internal standard is possible to quantify the amount of test drug. The most significant conditions for an internal standard are its solubility and its chemical interaction with the drug to be quantified [10]. Numerous studies confirm that qNMR is a valid technique for pharmaceutical analysis [11,12].

In the scope of the current study, we outline a rapid and easy method based on ^{1}H NMR spectroscopy with an internal standard (pyridine) to determine the concentration of drugs encapsulated in

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liposomes. The concentration of two different drugs was determined, one hydrophilic, methotrexate (MTX) disodium salt, and another hydrophobic, tamoxifen (TAM). A comparative study was performed based on the results obtained by ^{1}H NMR spectroscopy with two other techniques: UV–vis spectrophotometry and HPLC/UV-vis.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE–mPEG) were achieved from Lipoid GmbH (Germany). Deuterium oxide (D_2O) and deuterated chloroform ($CDCl_3$) were obtained from Cortecnet (France). All the other chemicals using in this work were purchased from Sigma-Aldrich (USA), except the MTX that was acquired from Huzhou Zhanwang Pharmaceutical (China).

2.2. Liposome preparation

Liposomes composed of DOPE/Cholesterol/DSPE-mPEG (54:36:10, molar ratio) [13] were produced by ethanol injection method [14]. Firstly, an amount of DOPE, Cholesterol and DSPE-mPEG was dissolved in ethanol and secondly injected under vigorous magnetic stirring to phosphate-buffered saline (PBS, pH 7.4), at 70 °C. Encapsulation of drugs was done by their mixture during the formation of the liposomes. MTX disodium salt, as hydrophilic drug, was added in aqueous phase (PBS) and TAM, as hydrophobic drug, was included in organic phase (ethanol).

2.3. Physicochemical characterization of liposomes

The physicochemical characterization of liposomes was evaluated using dynamic light scattering (DLS) technique in terms of size distribution and ζ-potential. The analysis was determined at pH 7.4 ± 0.02 (PBS buffer) and at 25.0 °C, using a Malvern Zetasizer Nano ZS (Malvern Instruments) by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively. The values for viscosity and refractive index of dispersant were taken as 0.8616 cP and 1.332, respectively (values automatically calculated by the software, considering the components of PBS aqueous buffer). Each sample was measured in triplicate and results are presented as mean value ± standard deviation (SD).

2.4. Determination of drug concentration

The non-encapsulated drugs were removed from the liposomes after passage through a gel filtration chromatography column (GE Healthcare, UK), with 5 kDa cut-off (PD-10 Desalting Columns containing 8.3 mL of Sephadex™ G-25 Medium). After separation of the free drug from liposomal formulation, the concentration of each drug encapsulated in liposomes was determined using three different techniques: ^{1}H NMR, UV–vis spectrophotometry and HPLC/UV-vis.

2.4.1. ^{1}H NMR

^{1}H NMR experiments were performed using a Bruker Avance III Instrument, operating at 400 MHz. After freeze-drying of liposomes (remove water molecules to not interfere in NMR analysis), the ones contain MTX were dissolved in deuterium oxide and the ones with TAM dissolved in deuterated chloroform. For the quantitative analysis, a known amount of pyridine of high purity was used as internal standard. The calculations were assessed by comparing the integration of one peak of the drug with the integration of one peak of pyridine. NMR signal multiplicity is given as: s (singlet), d (doublet), t (triplet), q (quadruplet), dd (doublet of doublets), dt (doublet of triplets), tt (triplet of triplets) and m (multiplet).

2.4.2. UV–vis spectrophotometry

Quantification of MTX and TAM was evaluated by measuring the absorbance at the maximum wavelength of each drug (MTX at 303 nm in PBS, and TAM at 280 nm in methanol). When necessary, dilutions of liposomes encapsulated drugs were performed, in order to be in the range of calibration curve. UV–vis spectra of liposomes encapsulated drugs were recorded on spectrophotometer BioTek Synergy™ HT using a quartz microplate. The final drug concentration was determined based on the respectively calibration curve.

2.4.3. HPLC/UV-vis detector

The HPLC/UV-vis analysis were performed using an ultra HPLC Nexera, SHIMADZU (Kyoto, Japan). To both drugs, the separation was achieved using a KNAUER C18 column maintained at 35 °C. The mobile phases were filtered and degassed prior to use. To MTX, the mobile phase A was 0.1% trifluoroacetic acid (TFA) in ultra-pure H_2O and mobile phase B was 0.1% TFA in acetonitrile. The flow rate of mobile phase B consisted in a linear gradient from 20 to 40%, flowing at a rate of 1.2 mL/min. The sample injection volume was 20 µL and MTX detected at 303 nm. Standard MTX solutions were prepared in a concentration range of 0.1 to 0.005 mg/mL, diluting the stock solution in PBS. To TAM, the mobile phase consisted of methanol / ammonium acetate buffer solution 0.5 M (75:25 v/v) at a flow rate of 1 mL/min, monitored at 280 nm. The injection volume was 40 µL. Standard TAM solutions were prepared in a concentration range of 0.5 to 0.015 mg/mL, diluting the stock solution in methanol.

2.5. Method validation

The accuracy of the method was determined by the standard addition method (recovery experiments), in which dispersions containing the liposomal formulation, were added to different amounts of drug standard solution to attain six different drug concentrations in a range of 0.1–5 mg/mL. After drug quantification by ^{1}H NMR, the obtained values were compared with theoretical values and reported as % recovery, following the equation:

$$\text{Recovery}\,(\%) = \frac{\text{Obtained value (mean value)}}{\text{Theoretical value}} \text{x}\,100$$

The precision of the method was determined by replicate analysis of each calibration standards. The relative standard deviation (RSD) values were calculated from the ratios of the SD to the mean and expressed as percentage by the following equation:

$$\text{Relative Standard Deviation}\,(\%) = \frac{\text{Standard deviation (SD)}}{\text{Obtained value (mean value)}} \text{x}\,100$$

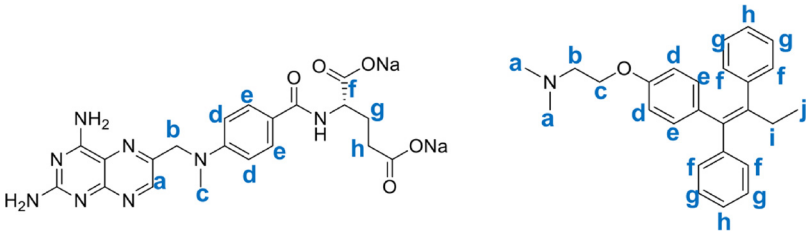
3. Results and discussion

3.1. Assignment of ^{1}H NMR signals

Prior to quantification of the drugs encapsulated in the liposome, the ^{1}H NMR analysis of the drugs structure was performed. Is possible to observe in Table 1 the complete analysis of the ^{1}H NMR spectra of MTX and TAM. Both compounds have aliphatic protons that can be observed between δ_H 0.93–4.79 ppm and aromatic protons between δ_H 6.57–8.59 ppm.

Our liposomes are constituted by a mixture of DOPE, Cholesterol and DSPE-mPEG which are based on aliphatic chains. The protons of these type of compounds are observed in the NMR spectra below δ_H 4.0 ppm (data not shown), leaving the aromatic area empty. Most constituents of liposomes found in the literature are based on the same

Table 1
^{1}H NMR characterization of **A**) MTX in D_2O and **B**) TAM in CDCl3.



	Protons (ppm)									
	a	b	c	d	e	f	g	h	i	j
MTX (A) in D_2O	8.59 (s, 1 H)	4.79 (s, 2 H)	3.17 (s, 3 H)	6.89 (d, J = 8.8 Hz, 2 H)	7.72 (d, J = 9.2 Hz, 2 H)	4.32 (dd, J = 8.8, 4.4 Hz, 1 H)	1.29-2.08 (m, 1 H), 2.12-2.21 (m, 1 H)	2.29-2.34 (m, 2 H)	—	—
TAM (B) in $CDCl_3$	2.29 (s, 6 H)	2.65 (t, J = 6 Hz, 2 H)	3.93 (t, J = 6 Hz, 2 H)	6.57 (d, J = 8.8 Hz, 2 H)	6.77 (d, J = 8.8 Hz, 2 H)	7.09-7.20 (m, 5 H), 7.24-7.29 (m, 4 H), 7.35 (t, J = 8.4 Hz, 1 H)			2.47 (q, J = 7.2 Hz, 2 H)	0.93 (t, J = 7.2 Hz, 3 H)

compounds, or equivalents in terms of type of chemical character [15].

Based on our results, the signal of the liposome constituents does not interfere with the aromatic signals of our tested drugs. Considering that a broad range of drugs possess in their constitutions aromatic rings, or allyl protons [16], the following methodology presented can be widely applied for drug quantification.

3.2. Quantification of drugs by ^{1}H NMR

The quantification of drugs by ^{1}H NMR is based on the addition of an internal standard. This standard is chosen centered on the tested drug structure, and its crucial for an accurate quantification. In this way, a suitable internal standard must consider certain criteria, (i) have signals (chemical shifts) that don't interfere with other signals, (ii) be accessible in pure form, (iii) have solubility in different NMR solvents, (iv) be easily measured, (v) nonreactive, (vi) nonvolatile, (vii) stable at long-term, and (viii) ideal molecular weight [17].

After analysis of the drug structure, the next step is the identification of the possible signals that can be used for quantification without interference of the internal standard (signal overlap) [18]. This statement means that we must guaranty that the signals of the drug and the internal standard are integrated separately [4]. The concentration of the drugs is calculated based on the integration of one peak of the internal standard compared with the integration of one peak of the drug.

In the present study, pyridine was used as internal standard, since it supplies a well-separated signal without any interference with the tested drugs signal in the NMR spectra. Additionally, pyridine is soluble in both solvents used in this work, deuterium oxide and deuterated chloroform.

As can be observed in Fig. 1A and B, pyridine is a compound that shows three distinct peaks, independently of the deuterated solvent used. In deuterium oxide, the first peak is observed at δ_H 7.49 ppm, corresponding to protons **2** (Fig. 1 in red), and should appear as a triplet. However, besides being possible to identify the triplet in the spectrum (J = 8 Hz), this signal unfolds being observed as a multiplet. The second peak, corresponding to proton **3,** appears at δ_H 7.91 ppm as a triplet of triplets (tt), with coupling constants of J = 8 Hz and J = 2 Hz. The protons in the *ortho* position of the nitrogen atom are observed at δ_H 8.56 ppm, corresponding to protons **1**, as a doublet of triplets (dt), with coupling constants of J = 4.4 Hz and J = 2 Hz.

From these three peaks of pyridine, at least two of them are well separated from the peaks of the tested drugs, as can be observed in Fig. 1.

The analysis of ^{1}H NMR spectra revealed that both aromatic protons **d** and **e** of MTX in D_2O and TAM in $CDCl_3$ were well separated from the other aromatic signals. For quantitative purposes, these signals were selected since they were not overlapped with any other signals, including the peaks of the internal standard.

The liposomal formulation used in this study previously proved to be an efficient delivery system for the encapsulation and delivery of both hydrophilic and hydrophobic drugs [13]. The results support their use as therapeutic delivery systems as demonstrated by the biological effect of several drugs *in vitro* as well as *in vivo* [13,19]. Furthermore, pharmacokinetics studies demonstrated that to contrast to free MTX, the liposomes encapsulated MTX are selectively retained in plasma and are not subject to immediate filtering and absorption by the main organs [20]. As a hydrophilic drug, the MTX disodium salt is incorporated in the internal aqueous core of the liposome, and the hydrophobic TAM in the lipid bilayer [2]. The Table 2 summarizes the physicochemical characterization of unloaded liposomes and liposomes encapsulating MTX and TAM. Since the extrusion process was not performed to promote the size reduction and homogeneity between the samples, all liposomal formulations were in a higher size range, 120–150 nm. The zeta-potential of all produced liposomes was close to zero, underlining the neutral charge of the DOPE-derived neutral liposomes [21]. The liposomal formulations used in this work were stable at least six months, without any significant size and polydispersity index (PDI) change and drug leakage (data not shown).

In the liposomal formulation, comparing the integration of a proton signal of the drug with pyridine, the concentration of the drugs can be easily calculated (Fig. 2). To remark, a shift of proton **3** occurred between analyses of both drugs since different deuterated solvents were used. Based on the ratio of pyridine/drug given by the integration of one peak of both compounds, is possible to determine the molar concentration, based on a known amount of internal standard used. Founded on these experiments, we consider qNMR an appropriate and fast technique to quantify drugs in the presence of liposomes.

3.3. ^{1}H NMR validation for quantification of drug encapsulated in liposomes

Accuracy and precision are the most important validation parameters. In this study, unloaded liposomes were added to the analytical drug solutions and the accuracy was investigated at six drug concentration levels, in the range of 0.1–5 mg/mL for both drugs. The mixture to be quantified is in the range of interest and the matrix

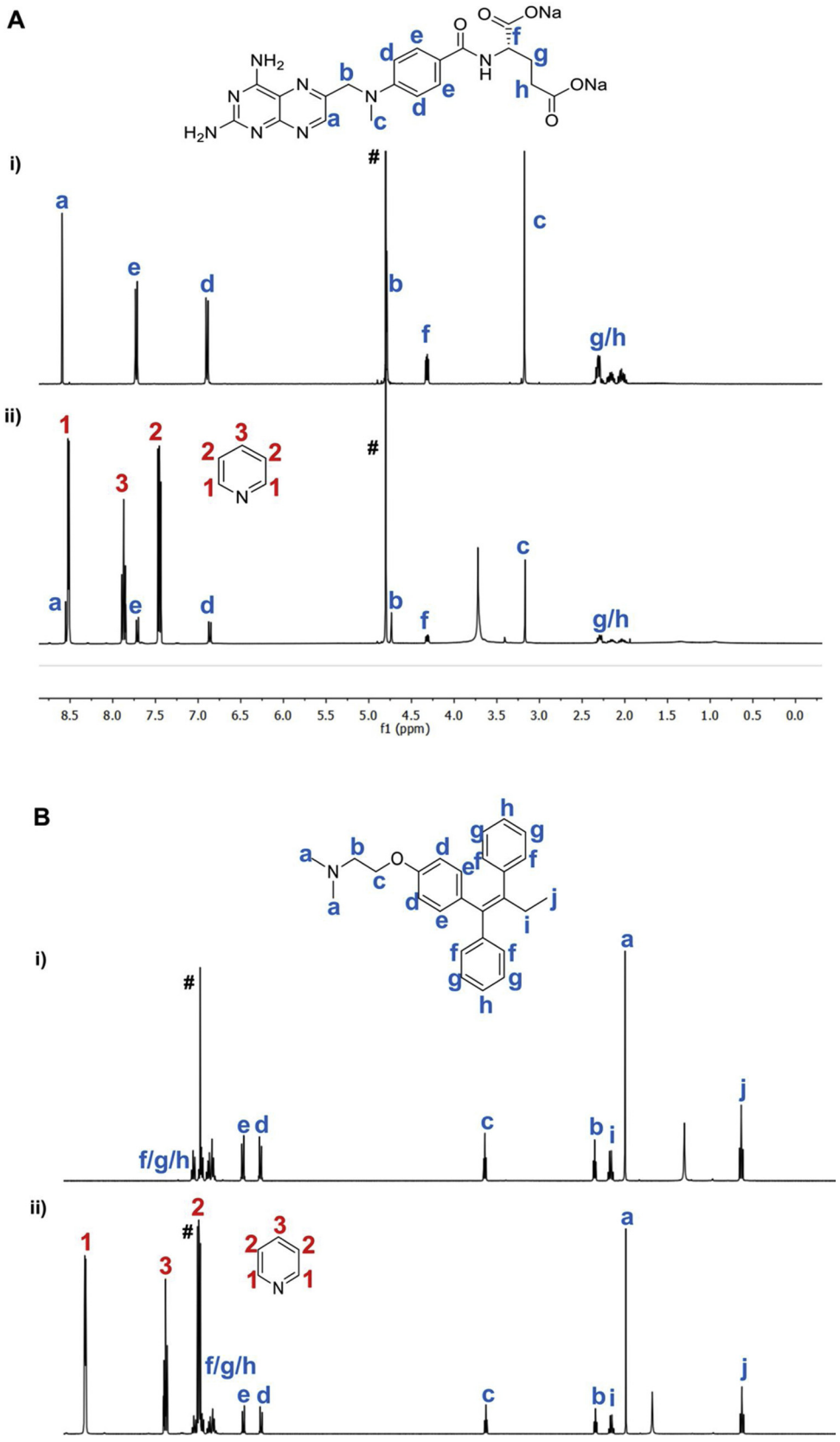


Fig. 1. ^{1}H NMR spectra of **A)** i: MTX in D_2O and ii: MTX in D_2O with pyridine. **B)** i: TAM in $CDCl_3$ and ii: TAM in $CDCl_3$ with pyridine. The blue letters represent the protons of each drug and the red numbers the protons of pyridine. # Peaks related to the solvent residual signal. Pyridine was used at the molar concentration of 0.124 M (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Physicochemical characterization of liposomes evaluated by DLS.

Sample	Z-average (d.nm)	PDI	Zeta-potential (mV)
Liposomes	147.7 ± 2.631	0.084 ± 0.023	−0.591 ± 0.326
Liposomes + MTX	128.8 ± 1.833	0.091 ± 0.011	−0.675 ± 0.370
Liposomes + TAM	123.3 ± 0.849	0.097 ± 0.001	−2.930 ± 1.110

composition like the test sample [10].

The accuracies were expressed as the closeness to the true value and are calculated as the percent recovery related to the theoretical values. Tables 3 and 4 present the percentage of drug recovered relative to the theoretical values for MTX and TAM, respectively. The determined values were close to the true value, ranging the % recoveries from 99.4 to 101.9 for MTX and from 97.8 to 102.7 for TAM. These high values of the % drug recovered reflect the accuracy of the assay method. To remark that, the minimum theoretical value was taken as the lowest drug concentration (0.1 mg/mL), since that at lower concentrations the peaks intensity is very low for both drugs resulting in an inefficient quantification of the drugs.

The method precision was measured by the RSD expressed as percentage over the concentration range of drug through validation. The

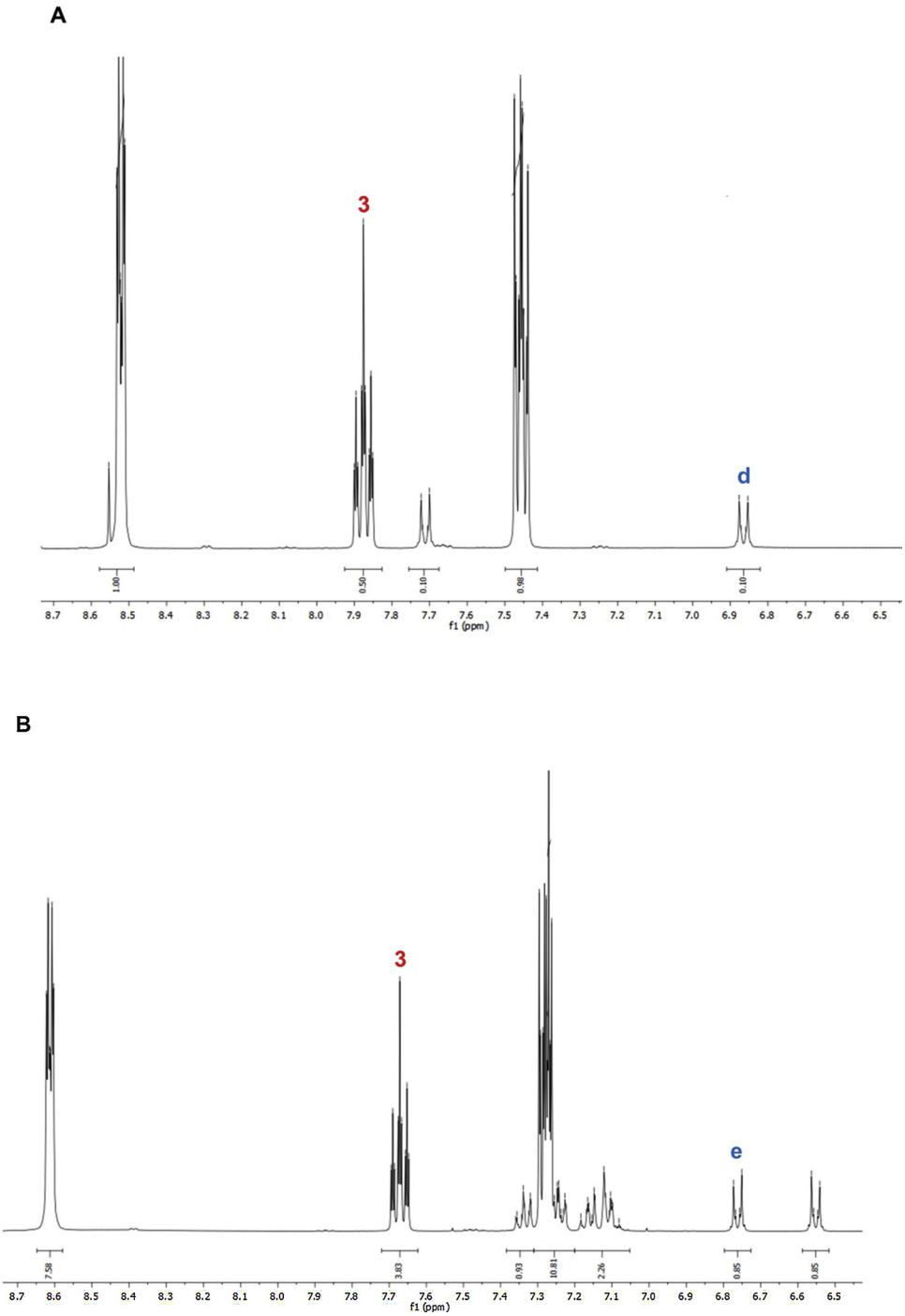


Fig. 2. [1]H NMR spectra of liposomes encapsulating **A)** MTX and **B)** TAM. The blue letters, **d** and **e,** represent the protons used of each drug, respectively. The red number **3,** represent the proton used of pyridine for the quantification of both drugs. Pyridine was used at the molar concentration of 0.124 M (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Validation parameters calculated for MTX. Values represent the mean ± SD of 3 independent experiments. Differences were tested for statistical significance by two-way analysis of variance, being not significant.

Theoretical value (mg/mL)	Obtained value (mg/mL)	Recovery (%)	Relative Standard Deviation (%)
0.100	0.100 ± 0.001	100.9	0.25
0.250	0.250 ± 0.010	99.9	3.93
0.500	0.509 ± 0.013	101.9	2.73
1.000	0.994 ± 0.022	99.4	2.23
2.500	2.540 ± 0.049	101.6	1.94
5.000	5.004 ± 0.188	100.1	3.76

Table 4
Validation parameters calculated for TAM. Values represent the mean ± SD of 3 independent experiments. Differences were tested for statistical significance by two-way analysis of variance, being not significant.

Theoretical value (mg/mL)	Obtained value (mg/mL)	Recovery (%)	Relative Standard Deviation (%)
0.100	0.100 ± 0.002	100.3	1.9
0.250	0.245 ± 0.004	97.8	1.48
0.500	0.502 ± 0.004	100.4	0.82
1.000	1.010 ± 0.018	101.1	1.86
2.500	2.486 ± 0.005	99.4	0.22
5.000	5.028 ± 0.195	100.6	3.87

Table 5
Comparison of different techniques for drugs quantification in liposomes. Values represent the mean ± SD of 2 independent experiments.

Technique	Standard	[MTX] mg/mL	[TAM] mg/mL
^{1}H NMR	Pyridine	3.495 ± 0.130	0.551 ± 0.001
UV-Vis	Calibration curve	3.507 ± 0.255	0.555 ± 0.019
HPLC/UV-vis	Calibration curve	3.480 ± 0.113	0.556 ± 0.017

RSD% values are presented in Table 3 and 4 for MTX and TAM, respectively. The RSD% of MTX ranged from 0.25 to 3.93 and of TAM ranged from 0.22 to 3.87. The low values of RSD% prove the precision of the NMR method for quantification of these drugs.

3.4. Comparison of ^{1}H NMR method with HPLC/UV-vis and UV–vis spectrophotometry

Mass-Spectrometry (MS) can be considered a highly specific and sensitive technique that is used for the quantification of MTX [22] and TAM [23]. In a previous work (data not shown), we validate the results obtained with NMR analysis with that one obtained with MS. However, several studies also report the use of HPLC/UV-vis and UV–vis spectrophotometry for determination of MTX and TAM concentration [24–26], highlighting the easily and practicality of the methods [27]. The results obtained by qNMR were then validated in comparison with these two techniques (Table 5).

No significant differences were observed between the concentration values of each drug encapsulated in liposomes determined by the qNMR method and the other two techniques involved in the study. Taken together, the results indicated that the proposed qNMR method is effective for drug quantification as the HPLC/UV-vis and UV–vis spectrophotometry, despite the differences in the techniques' basic principle. HPLC/UV-vis and UV–vis spectrophotometry is based on light absorption, requiring a previous matrix effect evaluation to use the calibration curve approach. Otherwise, ^{1}H NMR signal provided corresponds directly to the equal number of equivalent nuclei responsible for that signal. The concentration of the drugs is then calculated based on the relation between the peak of the internal standard and the one of the drug. Thus, the suggested qNMR is an accessible method and can be considered an alternative and reliable method for quantification of drugs encapsulated in liposomes.

4. Conclusion

The present study demonstrates that ^{1}H NMR should be used for quantification of drugs encapsulated in liposomes, independently of their hydrophilic or hydrophobic character. The internal standard used, pyridine, appears to be a versatile compound for quantification of drugs even encapsulated in nanoparticles such as liposomes.

Comparing the assay results obtained by qNMR with other two different techniques, UV-vis spectrophotometry and HPLC/UV-vis, no significantly differences in drug concentration were observed. qNMR is an absolute quantification method that proves to be an excellent choice over previously described methods for quantification of drug concentration in liposomes. Furthermore, the determination of drug concentration by qNMR proves to be a precise and an accurate methodology. The implementation of this methodology for drug quantification in liposomes showed also to be also inexpensive and fast, since is only necessary a simple step of sample preparation and a brief experiment time. Moreover, the present approach can be extended to other delivery systems. In summary, the present study offerings a simple, fast, reproducible, and relatively sensitive qNMR analysis method for quantitation of drugs encapsulated in liposomes.

Declaration of Interest

The authors report no conflicts of interest.

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