Determination of Analyte Concentration Using the Residual Solvent Resonance in ^{1}H NMR Spectroscopy

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An NMR protocol that uses the residual proton signal from DMSO-d_6 (i.e., DMSO-d_5) to determine the concentration of an analyte in a NMR sample was developed. This technique provides an alternative method for determining the molar concentration of compounds in solution without prior knowledge of their molecular weight. The method is particularly useful when submilligram quantities of compound are to be analyzed and is applicable to a variety of different research areas such as compound management, and natural product, combinatorial, and medicinal chemistry.

For the past 14 years the Eskitis Institute has undertaken high-throughput screening of natural product extracts to discover compounds to treat human diseases. Typically bioassay-guided fractionation has been used as a tool to isolate compounds responsible for specific biological activity. NMR spectroscopy is an integral part of this process since once an active chromatographic fraction has been identified by the bioassay, NMR analysis is conducted to assess the purity and compound structure class present. Since large numbers of bioactive natural product fractions have routinely been analyzed, the potency of these fractions was used as an additional criterion for prioritizing drug discovery projects for further progression. It was therefore necessary to develop a fast and convenient method to measure the concentration of the bioactive constituents in solution in order to determine their potency. We have developed an NMR method to determine the concentration of a dissolved natural product or analyte. This method compares the integrated area of the residual proton signal (DMSO-d_5) in DMSO-d_6 to the protons of the dissolved analyte in order to determine its concentration.

The use of quantitative NMR (qNMR) to measure impurities with moderate accuracy (<1%)[1] has been successfully applied in many research areas including natural products,[2,3] drug analysis,[4] and combinatorial chemistry.[5] This accuracy is attributed to an inherent property of NMR, that the area of an NMR peak from a fully relaxed spectrum is directly proportional to the number of nuclei giving rise to that peak.[6,7] Another application of qNMR has been to determine the concentration of analytes in solution.[8,9] Generally a single well-characterized standard compound has been used to compare the concentrations of analytes present in the NMR sample. Ideally the standard compound was highly pure, was soluble in the chosen solvent, and did not react with the other compounds present in solution. This last requirement is very important if the reactivity of the investigated compounds is unknown. Silanes and methyl- and aromatic-containing compounds have commonly been used as mass standards.[2,10] A disadvantage of using a dissolved standard is that it must be separated from the analytes if the sample is required for further investigations. Alternatively the volatility of some standards has the potential to compromise results.

In the qNMR method discussed below, the concentration of the residual proton signal in DMSO-d_6 was determined using caffeine as a standard. Once the concentration of residual protonated solvent had been determined for each batch, the concentration of compounds dissolved in DMSO-d_6 could be calculated by comparing the integral of the solvent peak (DMSO-d_5) to the integrals of protons from the dissolved compounds. The molar concentration of compounds in solution can be determined without prior knowledge of their molecular weight. This simple quantitative protocol is applicable not only to natural product research but also to other areas of drug discovery or chemical research such as compound management and combinatorial and medicinal chemistry.

Results and Discussion

An important consideration when developing a method to determine the concentration of dissolved analytes was that it should be compatible with liquid-handling robotics. Weighing of individual samples was seen as a bottleneck in the high-throughput screening (HTS) workflow. Many of the bioactive fractions being analyzed contained compounds isolated in less than 1 mg, which limits the use of analytical balances. The industry standard for HTS is dissolution of compounds in DMSO, and for this reason we have used DMSO exclusively in bioassays and DMSO-d_6 as the NMR solvent. All active fractions are routinely analyzed by ^{1}H NMR spectroscopy, so the ability to determine concentration by NMR was an attractive proposition. Dimethylsulfone has been used successfully as an NMR standard,[11] suggesting that DMSO-d_5 would also be suitable. A drawback to using DMSO is its low volatility, making it difficult to remove all traces of this solvent from compounds or fractions after they have been bioassayed. Further spectroscopic and biological analysis of these compounds or fractions necessitated a method to determine compound concentration that did not compromise compound purity. The usual method to obtain the mass of a fraction is by weighing on an analytical balance, a bulk mass measurement. This method does not take into account the purity of the sample and could be inaccurate when weighing small quantities. We have also found that residual water, acids (such as TFA or HCl), DMSO, or other solvents in the sample can contribute significantly to a sample's mass. Residual amounts of solvents can remain even after thorough evaporation, and this is particularly problematic for compounds isolated as gums or oils. These solvents can add to the weight of the sample and as a consequence can lead to an underestimation of the potency of the compound being bioassayed. The contribution of residual solvents to the weight of a sample can be disregarded when using this method, and so the true concentration of an analyte can be used to determine its potency. Even if a compound is 100% pure, determination of its IC_{50} is dependent on knowing the molecular weight of the compound and the amount of the compound dissolved in solution. Thus, a method that determines the concentration of a compound directly was desirable.

The experimental parameters for performing qNMR are well-known.[2,3,8,12] A recent article by Pauli et al. gives a comprehensive

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overview of practical points to consider when acquiring qNMR spectra.[3] For example, Pauli et al. use Garp decoupling to collapse ^{13}C satellites, which could be useful when analyzing complicated NMR spectra. One disadvantage of this ^{13}C NMR decoupling is sample heating during a long relaxation delay, which has the potential for sample degradation. For this reason, and to keep the new method as simple as possible, no Garp decoupling was used. Some important practical aspects for the new qNMR protocol are outlined below.

Spin–Lattice Relaxation Time. When using a 90° pulse to maximize sensitivity, a long interpulse delay is often required to re-establish equilibrium *z*-magnetization before another pulse is applied. This interpulse delay is usually set to ~5 times the longest longitudinal or spin–lattice relaxation time (T_1) in the molecule for complete relaxation. A typical T_1 for a proton in a small molecule ranges between 0.3 and 5.0 s,[2] but T_1 relaxation times greater than 10 s have been observed in some natural products.[13] As a result, knowledge of the T_1 values for protons within a molecule is essential for accurate qNMR. In this investigation 90° pulses (measured on each sample) were used to optimize for sensitivity, and therefore a longer relaxation delay was required. One of the main aims of this method was that it should be generally applicable to most samples. A quick assessment of T_1 relaxation times via the inversion–recovery null point method[7] was performed and indicated that the DMSO-d_5 proton signal always had the longest T_1 compared to any of the protons in the dissolved compounds. The DMSO-d_5 in DMSO-d_6 had a measured T_1 of approximately 12 s, and as a consequence an interpulse delay of 60 s was used in all subsequent experiments. This value for the interpulse delay was in agreement with a general interpulse delay suggested by Shao et al.[14]

NMR Processing. Processing ^1H NMR data to obtain absorption line shape and flat baselines is essential for accurate quantitation. Initially the FID was multiplied by an exponential function (lb = 0.2) and then zero-filled to twice the number of acquired points before the data was Fourier transformed. Manual phasing of NMR signals was performed on each spectrum, as no automated method worked satisfactorily on all data sets. The baseline of a NMR spectrum can also affect the accuracy of integrated signals, and so baselines were fitted to a polynomial function (≤5th order). Integration of isolated and sharp NMR signals is desirable, but if this is not possible, care must be taken when analyzing crowded regions of the NMR spectrum, recognizing the associated errors involved in these measurements.

qNMR Method for Concentration Determination. When using an interpulse delay of 60 s, as a consequence of the long DMSO-d_5 T_1, only protons with T_1 values less than the DMSO-d_5 proton can be used for quantitation. To determine which protons within a sample met this criteria, a two-step protocol was developed and applied to each sample. The first step was an inversion–recovery experiment with a delay of 8.4 s (corresponding to a T_1 of 12 s) between the 180° and 90° pulses. The DMSO-d_5 signal was nulled using this interpulse delay, while proton signals from the compound of interest that were positively phased had T_1values of <12 s and were suitable for quantitative analysis. The second step was acquisition of a normal ^1H NMR spectrum acquired with a 90° pulse and interpulse delay of 60 s, and these data were used for quantitative analysis.

The NMR data were processed as above, and the integral of the DMSO-d_5 was measured over the ~80 Hz region (half-distance to the ^{13}C satellites of both sides on the main DMSO-d_5 peak). It was decided to not include the ^{13}C satellites in the integration to keep the integration region as small as possible. Obviously, if there is overlap of other signals with the DMSO-d_5 peaks, care must be taken. When a single integration is performed over several multiplets, the resulting integral could contain ^{13}C satellites, and therefore the resulting analyte concentration will be slightly larger.

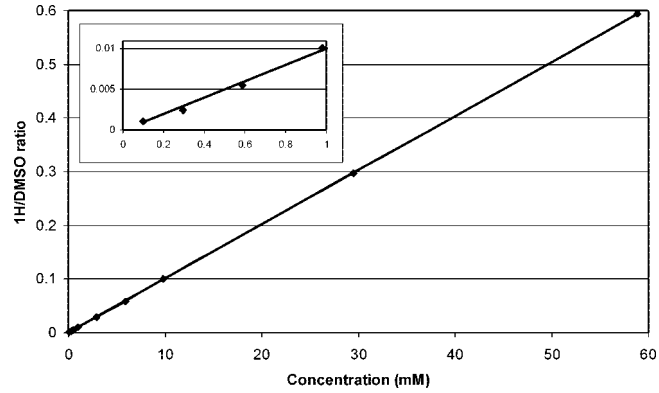

Figure 1. Calibration curve for DMSO-d_5 using different concentrations of caffeine (0.1–58.9 mM). Linear regression: ratio = 0.0101 × (concentration) and $R^2 = 0.999$. The inset is an expansion of the lower concentration region. The concentration for DMSO-d_5 = 99.0 mM.

Calculation of Molar Concentration of DMSO-d_5. The amount of DMSO-d_5 is dependent on the isotopic enrichment of the DMSO-d_6, with only minor changes in the isotopic enrichment having a profound effect on the amount of DMSO-d_5 present. This has meant that there is a need to determine the exact concentration of DMSO-d_5 in each batch of solvent used for qNMR. The concentration of DMSO-d_5 in DMSO-d_6 can be determined by construction of a calibration curve using a known standard. Commercially available caffeine was used in this study and its purity independently determined by C_{18} μPLC analysis to be 98.9%; this was in good agreement with the supplier's purity analysis of 99%. Two signals (δ_H 8.0 [H-8] and δ_H 3.8 [7-CH_3]) in the spectrum of caffeine dissolved in DMSO-d_6 were used for the calibration. Since these signals were in isolated regions of the spectrum, this allowed accurate measurement of their integrals and errors in the integration could be averaged. In all experiments the ratio of H-8 to 7-CH_3 was 1:3, and therefore either peak could be independently used for the calibration. The combined areas of the two peaks were divided by 4 to give the average integral for one proton, and this was compared to the DMSO-d_5 integral. A calibration curve was generated by preparing a serial dilution of caffeine (58.9, 29.45, 9.82, 5.89, 2.95, 0.98, 0.59, 0.29, and 0.10 mM) and comparing the peak integrals for each concentration to the integral of the residual DMSO-d_5 peak. A linear plot of integral ratios versus caffeine concentration was obtained (Figure 1). The concentration of DMSO-d_5 was obtained by extrapolating the curve to a ratio of 1. Since a linear relationship was obtained over this concentration range, subsequent calibrations were performed using a single known concentration of caffeine to determine the concentration of DMSO-d_5. Practically, this meant that an accurate concentration for a compound with a molecular weight of 300 Da obtained in ~0.1 mg yield could be determined experimentally using this qNMR method. The one limitation in using NMR peak integration for quantitation is that the number of protons giving rise to each peak must be known.

Analysis of four batches of DMSO-d_6 using the NMR method demonstrated that there was considerable variation in DMSO-d_5 concentration (Table 1). It was therefore concluded that the concentration of DMSO-d_5 in each batch of DMSO-d_6 should be determined experimentally. DMSO-d_6 is hygroscopic and does not readily undergo D/H exchange; however, it is strongly recommended to store the DMSO-d_6 in a dry environment when not in use.

Use of DMSO-d_5 as a Concentration Standard. The qNMR method was then used to determine the concentration of a series of commercially available crystalline compounds, capsaicin, penicillin G potassium salt, quinine hydrochloride salt dihydrate, Taxol, and caffeine. A weighed amount of each compound was dissolved

Table 1. Comparison of DMSO-d_5 Concentration in Different Batches of DMSO-d_6

lot #	isotopic enrichment (stated on COA)	DMSO-d_5 concentration (mM)
6C-526	99.88	105.3[a]
6K-382	99.89	134.5[b]
6L-488	99.94	59.1[c]
7H-266	99.93	99.0[d]

[a] Calculated from a calibration curve obtained using a single concentration of caffeine (56.9 mM). [b] Calculated from a calibration curve obtained using a single concentration of caffeine (54.6 mM). [c] Calculated from a calibration curve obtained using a single concentration of caffeine (11.3 mM). [d] Calculated from a calibration curve obtained using 9 different concentration of caffeine (58.9, 29.45, 9.82, 5.89, 2.95, 0.98, 0.59, 0.29, and 0.10 mM) as shown in Figure 1.

Table 2. Comparison of Concentration Determinations of Five Commercially Available Compounds (DMSO-d_6, lot # 6K-382)

compound	NMR concentration[a] (mM)	balance concentration[b] (mM)
capsaicin	14.4	14.6
penicillin G potassium salt	25.7	26.8
quinine hydrochloride salt dihydrate	36.5	35.0
Taxol	3.1	3.0
caffeine	42.3	42.7

[a] Calculated by comparing to the concentration of DMSO-d_5 (134.5 mM). [b] Calculated by weighing material on an analytical balance, dissolving in 500 μL of DMSO-d_6, and multiplying by % purity.

in 500 μL of DMSO-d_6. The data were acquired and processed using the two-step protocol detailed above. Integration of as many nonoverlapping multiplets as possible was used from each spectrum and averaged to obtain the integral for one proton. The ratio of the integrals for one proton from each compound to the integral of DMSO-d_5 was used to determine the concentration of the dissolved compound (Table 2). There was excellent agreement between the concentrations derived from weighing and dissolving the compounds and the concentrations derived experimentally using the qNMR method.

The concentration of 18 natural products was determined by weighing and dissolving the compounds in DMSO-d_6. Weights of the natural products ranged from 1.2 to 14.3 mg. These concentrations were compared to the results obtained using the qNMR method (Table 3). The compounds were all isolated as gums and were all >90% pure by NMR analysis, except sample 16, which was >70% pure. These natural products consisted of a range of structural classes including flavonoids,[15] alkaloids,[15] coumarins, lignans,[16] and terpenes. The results showed that in all cases the NMR experiment predicted a lower concentration of compound (24–94%) compared with that determined by weighing the compound after drying. The conclusion from this analysis was that since the compounds appear pure by NMR, the "dried" samples contained considerable residual solvents, water, or other material that was invisible by NMR.

The basic procedures for qNMR have been used to develop a method to estimate the concentration of a compound dissolved in DMSO-d_6 without the need to add an additional calibration standard. This method has been applied to a natural product drug discovery program to estimate the concentration of compounds isolated in quantities less than 5 mg. This simple procedure requires a single calibration per batch (lot number) of DMSO-d_6 and extends the use of the DMSO-d_5 signal from being a chemical shift reference standard to a mass reference standard as well. This methodology can also be applied to other nonvolatile deuterated NMR solvents where the residual solvent peak's concentration remains stable over time and in different sample conditions (e.g., pH and in the presence of different compound classes). The concentration of compounds of unknown molecular weight can be determined, thus allowing an accurate comparison of IC_{50} values to be made early in the drug discovery process. This method is applicable for determining the concentration of compounds obtained in low yield (0.5 μmol) that otherwise would be difficult to weigh on an analytical balance. This new procedure is an alternative method to the use of internal standards, external standards,[17] and electronic signals for quantitation.[9] The method is quick and fits easily into the normal natural product purification or bioassay-guided fractionation workflow.

Table 3. Comparison of Concentration Determinations for 18 Natural Products

sample #[a]	NMR concentration (mM)[b]	balance concentration (mM)[c]
1[d]	12.02	21.21
2[d]	11.36	23.65
3[d]	3.21	7.02
4[d]	15.06	56.23
5[d]	27.40	39.18
6[d]	7.27	29.94
7[d]	8.62	28.69
8[d]	9.36	23.25
9[e]	52.79	84.69
10[e]	37.66	40.18
11[e]	84.26	127.63
12[e]	7.88	13.69
13[e]	29.15	46.80
14[e]	6.69	10.20
15[e]	5.80	9.79
16[e]	5.11	18.39
17[e]	23.43	39.15
18[e]	7.17	11.40

[a] Samples 1–18 were isolated under an AstraZeneca confidentiality agreement. Details of four compounds (11, 12, 15, and 18) are presented in the Supporting Information. [b] Calculated by comparing to the concentration of DMSO-d_5 integrated signal. [c] Calculated by weighing material on an analytical balance, dissolving in a known volume of DMSO-d_6. [d] DMSO-d_6 (lot # 6C-526) concentration of DMSO-d_5 = 105.3 mM. [e] DMSO-d_6 (lot # 6L-488) concentration of DMSO-d_5 = 59.1 mM.

Experimental Section

General Experimental Procedures. The NMR data were acquired on a 600 MHz Varian NMR system (VNMRS) with a cold probe operating at 25 K. DMSO-d_6 was used as the NMR solvent (D 99.9%, lot #: 6C-526, 6K-382, 6L-488, and 7H-266, Cambridge Isotope Laboratories, Inc.). The pulse sequence used was "s2pul" (standard Varian pulse sequence). Two experiments were acquired for all samples. The first experiment was to assess T_1's of the protons within the compound, and an example of the parameters used is pw = 7.2 μs, p1 = 14.4 μs, d2 = 8.4 s, nt = 1, ss = 0, d1 = 58 s, sw = 10 000 Hz. The second experiment was used for the qNMR analysis with the following parameters as an example: pw = 7.2 μs, p1 = 0 μs, d2 = 0 s, nt = 4, ss = 0, d1 = 58 s, sw = 10 000 Hz. For each sample the 90° pulse was measured and then this was used in both experiments for pw (90°) and p1 (180°) pulses with an acquisition time of ~2 s. The data were Fourier transformed after line broadening of 0.2 Hz to ensure minimal truncation artifacts, and the resulting FID was zero-filled to twice the number of points acquired. The spectrum was phased and then baseline corrected with a fifth-order polynomial. The integrals were measured, and the ratio of one proton from the compound of interest to the DMSO-d_5 was calculated.

A Mettler-Toledo AG 245 balance (5 decimal places) was used to weigh all samples. The commercially available compounds were capsaicin (360376, Aldrich lot # 03627DC, purity = 55.84% remainder dihydrocapsaicin), penicillin G potassium salt (P7794. Sigma lot # 062K0842, potency = 1595 units), quinine hydrochloride salt dihydrate (Q1125, Sigma lot # 121K1159, purity = 89.4% with 6.4% dihydroquinine hydrochloride salt), Taxol (1097, Tocris lot # 3A/64349, purity ≥ 99.8%), and caffeine (C53, Aldrich lot # 16004LQ, purity = 99%).

Compound Purity Analysis. Several of the commercial compounds (caffeine, capsaicin, and quinine hydrochloride salt dihydrate) were independently analyzed for purity by C_{18} μPLC. Each compound was made up as a 10 mM solution in 100% DMSO, diluted 1:10 using 100% DMSO (100 μL final), then dispensed into a Greiner 384-well

microtiter plate (Cat. # 781201). All samples were analyzed on a Nanostream CX Microfluidics system using a Brio 24-column μPLC C_{18} cartridge (Grace Vydac GENESIS C_{18} 7 μm, 1.0 × 8.0 mm, cat. # 4209002). Each sample (3 μL) was injected onto one of the 24 Brio cartridge columns. The solvent gradient system consisted of a linear gradient from 10% CH_3CN (0.01% TFA)/90% H_2O (0.01% TFA) to 100% CH_3CN in 12 min, then held at 100% CH_3CN (0.01% TFA) for 3 min at a flow rate of 600 μL/min. All UV-active peaks at 254 nm were integrated and area % was calculated, thus allowing a purity % for each compound to be determined. Retention time/purity % for each compound was as follows: 6.08 min/98.9% (caffeine); 10.59 min/58% (capsaicin); 11.14 min/41.7% (dihydrocapsaicin); 5.70 min/99.5% (quinine hydrochloride salt dihydrate).

Caffeine Standard Solutions. A 58.9 mM caffeine solution was prepared by dissolving caffeine (22.88 mg) in DMSO-d_6 (D 99.9%, 2 mL (2.38 g), lot # 7H-266). The stock solution was serially diluted to generate eight concentrations (29.45, 9.82, 5.89, 2.95, 0.98, 0.59, 0.29, and 0.10 mM). The volume for all NMR samples was 600 μL, and the data were acquired using the defined two-step protocol and processed as above. For the single-point calibration curves, a sample of caffeine was weighed out, then dissolved in 600 μL of DMSO-d_6 (known concentration), and used to acquire the NMR data, which were then processed as above. An example of the calculation used to determine the concentration of DMSO-d_5 from a single-point calibration curve is given in the Supporting Information.

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Supporting Information Available: Tables of experiment and theoretical data are available. This material is provided free of charge via the Internet at http://pubs.acs.org.

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