Anal. Chem. **2005,** *77,* 3123−3131

Quantitative ^{1}H NMR with External Standards: Use in Preparation of Calibration Solutions for Algal Toxins and Other Natural Products

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We examine the use of external standards for quantitative measurement by ^{1}H NMR of solution concentrations of natural products and other low molecular weight, hydrogen-containing compounds and show that precision and accuracy ca. 1% is obtainable with a commercial 11.7 T spectrometer when standards and analytes are contained in separate but identical sealed precision glass NMR tubes. Numerous factors contributing to the intensity of the NMR signals are evaluated. Precise measurements of 360° pulse lengths for each sample provide direct corrections for variations in probe Q-factor that enable samples in different solvents to be compared, provided single-coil excitation and detection is used throughout. Samples need not be prepared in deuterated solvents if the ^{1}H spectra of the solvents are simple enough for peak suppression by presaturation. The approach is particularly suitable for hazardous materials kept in sealed tubes and for the preparation of certified calibration solution reference materials for use with LC-MS and other techniques where deuterated solvents should be avoided.

The lack of accurate calibration standards for natural products such as algal toxins (Figure 1) is a major impediment to the development, validation, and routine implementation of analytical methods, as illustrated by poor reproducibility in a recent interlaboratory study of microcystins in algal and drinking water samples.[1] Preparation of such standards is hampered because of difficulties in quantifying the compounds, many of which are produced by unidentified sources, obtainable only in minute amounts of unknown purity, hygroscopic, and difficult to isolate or crystallize in known anhydrous salt forms. Incomplete knowledge of the composition of extracts precludes quantitation by weighing, and use of spectroscopic absorbances requires previous calibration by some other means. Such information is not available for new compounds or for new structural variants of known compounds. Some natural products such as algal toxins are hazardous, and quantitation methods should allow safe handling. Here, we evaluate the utility of ^{1}H NMR spectroscopy on the basis of external standards as a means of overcoming these difficulties.

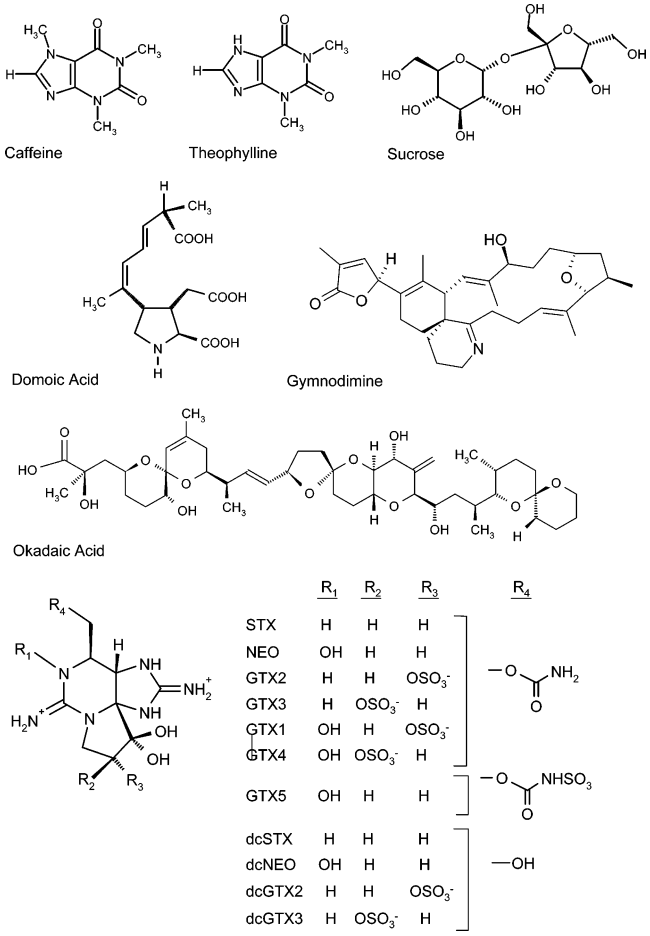

Figure 1. Structures of compounds used for concentration determinations by NMR, including the shellfish toxins domoic acid, gymnodimine, okadaic acid, and some saxitoxin analogues.

Quantitative uses of NMR spectroscopy[2−9] have included analysis of mixtures,[2,3,10] contaminants in drinking water,[11] agri-

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10.1021/ac048385h CCC: $30.25 Published 2005 Am. Chem. Soc.
Published on Web 03/26/2005

cultural chemicals,[12] gasolines,[13] metabolites in biomedical studies,[6,14,15] military nerve agents,[16] pharmaceuticals,[8,17] and validation of natural product reference compounds.[18] A general discussion[3] from 1980, covering most of the basic concepts of quantitative NMR, states that accuracy with external standards rarely exceeds ±5%, and their subsequent use has been infrequent. Most published methods for quantitative analysis by ^{1}H NMR use internal standards,[5,7,8,10] although there are examples where external standards have been employed in concentric tubes[2,10,11,16] or separate precision tubes.[19−21] To our knowledge, there has been no attempt to validate the potential advantages of external standards in quantitative ^{1}H NMR except for the "ERETIC" method which uses electronic signals.[22,23]

The ^{1}H NMR procedures described here use separate but identical tubes for analytes and for readily prepared external standards. They apply to solutions of low-molecular-weight proton-containing stoichiometric compounds including most natural products and are geared to the production of phycotoxin certified reference materials (CRMs) by our institute's Certified Reference Materials Program (CRMP). As CRMs are often used in regulatory monitoring for the calibration of liquid chromatography−mass spectrometry (LC−MS) equipment, we usually avoid deuterated solvents that could cause isotopic exchange and internal standards that would adulterate the purified toxins. The NMR spectra are acquired using simple single-pulse sequences, with presaturation if necessary for solvent suppression. We consider aspects of NMR quantitation that are a particular concern with external standards and evaluate the fundamental quantitative performance of the spectrometer by comparing single-pulse spectra from neat solvents (H_2O, CH_3OH, $CHCl_3$) of known density. The procedures have been validated by cross-comparison of gravimetrically prepared solutions of USP-certified standard compounds (caffeine, theophylline, arginine, and sucrose) and applied to phycotoxins such as okadaic acid, domoic acid, gymnodimine, and various saxitoxin analogues (Figure 1). Phycotoxin concentrations measured by NMR have also been checked by gravimetry (where possible) or by liquid chromatography with a chemiluminesence nitrogen detector (LC−CLND).

EXPERIMENTAL SECTION

Preparation of Standards and Samples. USP-certified caffeine, theophylline, arginine, and sucrose were chosen for inter-comparison and use as standards. All contain some nonexchanging hydrogens, have certified composition, high stability, and no water of crystallization, permitting preparation of solutions having accurate concentrations by weighing after drying. Three are nitrogen-containing compounds, allowing for the independent checking of concentration by LC−CLND. ^{1}H NMR assignments were known for all compounds.

Standards of USP and other certified materials were prepared by weighing all components using three balances (Mettler models AE240, AT250, and AT20, which display, respectively, to 0.01, 0.01, and 0.001 mg) maintained in our Certified Reference Materials Laboratory and calibrated annually. Materials were dried prior to weighing according to instructions supplied and protected from light: caffeine USP (A&C Chemical, Montreal, Cat. No. 08500, Lot I; also Sigma Anhydrous USP, Cat. No. C-7731, Lot No. 68H0018) at 80 °C for 4 h; theophylline USP (A&C Chemical, Montreal, Cat. No. 65300, Lot I) at 105 °C for 4 h; L-arginine (A&C Chemical, Montreal, Cat. No. 04250 Lot G-1) at 105 °C for 3 h; sucrose (USP Cat No. 62363, Lot No. G-1; also Anachemia Cat. No. AC8688, Lot 390526) at 105 °C for 3 h.

Solutions were prepared with weighed quantities of solvents in volumetric flasks, ensuring complete dissolution, uniform mixing, and noting solvent and air temperatures. Solvents were deionized H_2O prepared with Barnstead EASYpure LF or Milli-Q gradient A10 water purification systems; D_2O from Cambridge Isotope Laboratories Cat. No. DLM-4 (99.9% D); CD_3OH (Isotec Cat. No. T82-00035 Lot no. SN1486-3, 99.8% D); and CH_3OH and $CHCl_3$ (both Burdick and Jackson, high-purity grade). Sucrose solutions contained 0.02% NaN_3 to prevent bacterial degradation.

For all NMR spectroscopy, 0.7 mL of each solution was transferred to a dry Wilmad 535pp precision glass 5-mm NMR tube and sealed. Solutions were well-mixed and free from bubbles, particulates, macromolecules, and adsorbents.

Solvents used for phycotoxin experiments are given in Table 1. Solutions of saxitoxin analogues were prepared in deionized H_2O (as above) containing 0.1 M acetic acid (Caledon glacial, assay by GC 99.7%). LC−CLND analyses were performed using an Agilent (Palo Alto, CA) model 1100 liquid chromatograph interfaced with an Antek (Houston, TX) 8060R chemiluminescence nitrogen detector. The column was a Zorbax SB-C8 (2.1 × 150 mm, 5 μm) from Agilent, and the mobile phases used were either aqueous methanol or tetrahydrofuran, with heptanesulfonic acid as an ion-pairing agent. Details of the LC−CLND procedures will be published separately.

NMR Spectroscopy. Quantitative ^{1}H NMR experiments were performed with a Bruker DRX-500 spectrometer operating at 500.13 MHz with sample temperature equilibrated at 20.0 ± 0.5 °C. To minimize probe Q-damping and radiation damping effects (see Results and Discussion), many experiments used a 5-mm Bruker "broad band X-nucleus" probe (probe X) in which the ^{1}H coil is outside the coil used for "X"-nuclei (^{31}P, ^{13}C, ^{15}N, etc.). To explore the effects of increased Q-damping, further experiments with salt solutions used a Bruker 5-mm "Triple-band Inverse" triple-axis gradient probe (probe T) having the ^{1}H coil closer to the sample. Unless otherwise stated, all samples, which were of the same volume (0.7 mL) and identically positioned in the probe without spinning, were individually tuned and matched precisely to 50 Ω resistive impedance with the built-in RF bridge and swept

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Table 1. Concentrations Measured in "Stock" Solutions, μM ± sd

analyte	reference method	μM	sd	analyte solution	calibrant	NMR measurements μM	sd	% rsd	% deviation
sucrose	gravimetric	48730		H_2O	caffeine in H_2O (calibration curve)	48776	248	0.5	0.1
		19740				20017	116	0.6	1.4
		9680				9721	199	2.0	0.4
		3940				3894	105	2.7	−1.2
		1930				1875	45	2.4	−2.8
theophylline	gravimetric	991		H_2O	caffeine in H_2O (single calibrant)	1022	22	2.2	3.1
		1015				1040	22	2.1	2.5
		1019				1043	22	2.1	2.4
		1983				1986	42	2.1	0.2
		2030				2016	43	2.1	−0.7
		2035				2040	43	2.1	0.2
		3950				3975	84	2.1	0.6
		4050				4045	86	2.1	−0.1
		4060				4043	85	2.1	−0.4
domoic acid	LC and gravimetric	4865		D_2O	sucrose in D_2O	4833	79	1.6	−0.7
		5894				5856	104	1.8	−0.6
		4385				4332	68	1.6	−1.2
okadaic acid	gravimetric	2421		CD_3OH	caffeine in H_2O	2364	41	1.7	−2.4
STX	LC-CLND	4144	77	H_2O	caffeine in H_2O	4300	140	3.3	3.8
					theo in H_2O	4280	140	3.3	3.3
NEO	LC-CLND	2325	17	H_2O	theo in H_2O	2258	63	2.8	−2.9
					caffeine in H_2O	2250	32	1.4	−3.2
dcSTX	LC-CLND	3024	22	H_2O	caffeine in H_2O	3005	29	1.0	−0.6
GTX5	LC-CLND	865	7	H_2O	caffeine in H_2O	866	17	2.0	0.2
GTX1	LC-CLND	849	15	H_2O	caffeine in H_2O	843	16	1.9	−0.7
GTX4	LC-CLND	264	12			269	3	1.2	1.8
	sum	1113	19			1112	16	1.5	−0.1
GTX2	LC-CLND	1262	11	H_2O	caffeine in H_2O	1290	75	5.8	2.2
GTX3	LC-CLND	419	4			389	23	5.9	−7.2
	sum	1681	12			1679	78	4.7	−0.1
dcGTX2	LC-CLND	1409	38	H_2O	caffeine in H_2O	1414	45	3.2	0.4
dcGTX3	LC-CLND	407	21			382	13	3.4	−6.1
	sum	1816	43			1796	47	2.6	−1.1
gymnodimine	no independent measurement			CD_3O H	caffeine in H_2O	5130	190	3.7	
						5140	240	4.7[a]	

[a] Measurement performed with probe T. All others performed with probe X.

frequency display, using the Bruker XWINNMR "wobb" command. Solutions extended well past the sensitive area of the ^{1}H coils. When nondeuterated solvents were used, the magnetic field was shimmed initially using a tube containing the same volume of the corresponding deuterated solvent, and each nondeuterated sample was shimmed further by maximizing the area of the ^{1}H free-induction decay (FID) following repeated short pulses (flip angle ca. 10°). Field-frequency lock was used if a sample contained deuterated solvent, otherwise spectra were obtained at constant field taking advantage of the low drift rate of our magnet (<1 Hz/h at 500 MHz, which may not be obtainable with all systems). Spectra of standards and analytes were recorded under the same conditions with a repeated one-pulse sequence, using digital quadrature detection. Flip angles were 90° (ca. 5−6 μs for probe T; 11 μs or 19 μs for X) in most cases, although some experiments used 47° pulses. A known number of FIDs (normally a power of 2) were summed and Fourier transformed to produce a spectrum of adequate S/N for each sample. The number was typically 1 for solvent signals or highly concentrated solutions, 16 for standards, 32 for typical analyte samples ca. 1 mM, and 1024 for dilute solutions <50 μM.

If presaturation was necessary, the solvent resonance was irradiated immediately preceding each pulse at a power level 50−65 dB lower than the typical power used for excitation pulses, insufficient to cause detectable heating. Typical conditions were spectral width SW (7507.5 Hz, 15.0 ppm), acquisition time (2.18 s), flip angle (90°), presaturation time (4.0 s), delay (11.0 s) after each acquisition and before presaturation, temperature (20.0 °C), and 16 "dummy" (unrecorded) scans. The receiver gain (RG) setting for a given pulse sequence was adjusted manually in preliminary experiments to ensure that the largest FID amplitude from a series of analytes A and standards S was less than 1/2 the level needed for onset of digitizer saturation. RG was held constant for the series. Where the number of scans N differed between A and S, integrated intensities were corrected by multiplying those for A by N_S/N_A. For experiments comparing the intensities of solvent resonances, no presaturation or dummy scans were used and the spectrum was recorded with a single 90° pulse, usually at minimum receiver gain, and with >60 s between experiments. Pulse lengths p_{90} for 90° pulses were set to 1/4 of the 360° pulse length, which was determined precisely (usually within ca. 30 ns) as the time at which the residual dispersive signal of the solvent

peak had zero integral. The natural abundance of ^{1}H was assumed constant at 99.985% (effectively 100% within the accuracy obtainable by these procedures).

Data Processing. Data were processed using Bruker XWinNMR software (version 2.6). Prior to Fourier transformation (FT), the FIDs were apodized using a decreasing exponential with a line broadening (LB) parameter of 0.1 Hz. Following FT, spectra were phased for pure absorption mode, and baseline corrections were applied, using the Bruker "basl" command to subtract the simplest polynomial that would produce a straight baseline at zero intensity between resonances, correcting over small ranges of the spectrum individually where necessary. This command subtracts a polynomial of the form $A + B\nu + C\nu^2 + D\nu^3 + E\nu^4$ where ν is frequency, A to E are varied by the operator, and the origin is at the high-frequency end of the chosen range. Typically, A and B terms were sufficient (linear correction). Terms C−E were only needed in regions of high baseline curvature, usually resulting from solvent saturation residual signals, and inflections in baseline corrections were avoided. Digital integration of resonances was performed with further "bias and slope" (linear) corrections for each integrated range. The criterion for a correct integral was a horizontal region of zero curvature (within the limits determined by the noise level) in the integral trace on either side of the peak. Where this was impossible to achieve for individual resonances, groups of peaks with clear baseline on each side were integrated together, keeping account of the number of proton resonances in the group and the overlap of ^{1}H − ^{13}C satellites. Integral values were entered into Microsoft Excel spreadsheets for further processing and statistical comparisons.

^{1}H NMR responses were compared as integrated signal areas per proton, from spectra obtained under identical conditions and processed with a constant (initially arbitrary) integral scale. ^{1}H resonance assignments were known. Where concentrations were also measured by other methods, the integral per proton per mole (relative molar response, RMR) was calculated.

RESULTS AND DISCUSSION

Factors Influencing NMR Signal Intensity. We will restrict discussion to Fourier transformed spectra integrated in the frequency domain. The strength I_A of the ^{1}H NMR signal (per proton in a given chemical environment) produced by an analyte A, compared to that (I_S) of a "standard" S recorded under identical conditions, is proportional to the ratio N_A/N_S, where N_A and N_S are the respective numbers of ^{1}H nuclei in A and S, assumed to have the same geometrical distribution within the sensitive area of the probe. Where protons are in more than one chemically distinct environment, independent measurements of I may be obtainable from one spectrum. As N_A and N_S are directly proportional to the concentrations C_A and C_S, respectively, $I_A = k_A C_A$ and $I_S = k_S C_S$ and the molar ratio C_A/C_S may be determined directly from the intensity ratio if $k_A = k_S$.

The following experimental tests and discussion cover the steps necessary to ensure $k_A = k_S$ when standards and analytes are in separate tubes. The Supporting Information contains tables and figures denoted "S" and expanded discussion "SD" of some topics.

RF Pulse Calibration and Relaxation Effects. Compared to shorter pulses, the use of 90° pulses with long intervening delays lessens the dependence of signal strength on precise setting of the pulse length. Errors in calibration of p_{90} by an angle θ would reduce the resonance intensity from the maximum value by a factor cos θ. Thus, an 8° error would produce a 1% decrease in intensity, and a 1° error a 0.015% decrease. For comparison, a 1° error in a 45° pulse would result in a 1.7% intensity change. 90° pulses should be short enough to provide uniform excitation over the full spectral width SW (i.e., $1/p_{90} \gg$ SW).

The null corresponding to a 360° pulse is more clearly defined for calibration purposes than that for a 180° pulse,[24] particularly when radiation damping is prominent. RF pulses on our instrument were rectangular when displayed on an RF oscilloscope with a 50 Ω load (apart from a 5% initial overshoot which decays within 2.5 μs), showing <0.08% intensity reduction with time from 2.5 μs to 100 μs and 90% rise and fall times <50 ns, so the assumption $p_{90} = p_{360}/4$ should apply with a precision better than 1°, and signal intensity errors due to missetting of p_{90} should be negligible.

Relaxation to within 0.7% of the maximum occurs after a time $5T_1$, where T_1 is the spin−lattice relaxation time for a given nucleus. Factors affecting choice of pulse intervals have been evaluated[25] (see Supporting Information SD2). Relaxation times may vary considerably making it impractical to use delays long enough to completely relax all protons. Those having large T_1 may be left out of calculations. The long time between pulses (17 s) used in our standard procedure allows relaxation to within 0.7% of equilibrium for protons with $T_1 < 3$ s and within 0.1% for $T_1 < 2$ s. Nevertheless, some high-purity standard samples of 40 mM caffeine in D_2O showed incomplete relaxation of CH_3 resonances used for quantitation (97.5% recovery after a 17-s delay compared to a 120-s delay). Similar samples in H_2O had faster relaxation (recovery 99.6% ± 0.4% SD at 17 s) attributable to intermolecular dipolar relaxation via solvent protons and were preferred for use as standards.

Instrumental Factors. Timing and amplitude of RF pulses must be precise and probe arcing avoided. Nonlinearities in digitization of NMR signals are recognizable by distortions in the baselines of transformed spectra. Analogue filter "ripple" is circumvented in newer instruments by digital filters. Overloading of the digitizer must be avoided by setting the receiver gain to accommodate the strongest signals produced by either analyte or standard, including residual partially saturated solvent resonances. With this proviso, gain should be as large as possible to optimize digitizer resolution (see Experimental section). Problems resulting from digitization have been reviewed in detail,[26] but subsequent improvements in instrumentation have greatly reduced most of the artifacts discussed. When comparing samples and standards, the same receiver gain and acquisition conditions should be used to avoid reliance on the accuracy of attenuators, unless these are calibrated. Temperature must also be constant (see SD3).

Uniformity of Pulse Excitation and Signal Filtering. Effects of audio filter bandwidth and transmitter offset have been evaluated for single-pulse spectra.[25] We have checked the performance of our instrument using a 40 mM solution of caffeine in D_2O with a trace of added $GdCl_3$, using a >60-s delay between

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pulses and varying the transmitter frequency across a 15 ppm (7508 Hz) spectral width, for probe T (p_{90} = 5.85 μs). The response, which includes all contributions from nonuniform irradiation, detection, and digital filtering, was constant within 1.0% rsd to within 265 Hz from the edge of the spectral window (Table S1).

Variation of Probe Q Factor and Effect on 90° Pulse Width. Probe tuning and matching at the observing frequency, without sample rotation, is essential for consistency of subsequent signal amplification. Highly conducting samples (salt solutions) dampen the probe quality factor Q, to which NMR signal intensity is proportional,[27] but the principle of reciprocity[28] indicates (see SD4) that the 90° and 360° pulse lengths (p_{90} and p_{360}, respectively) should be inversely proportional to Q when the same coil provides both RF irradiation and detection. This prediction was tested with probe T using solutions in H_2O containing caffeine (4 mM) and NaCl (1−100 mM). Linear regressions versus NaCl concentration (Table S2, Figure S2) showed that p_{90} increased by a factor of 1.2335 from 1 mM to 100 mM NaCl, while RMR_{90} for the H_2O resonance (single pulse) decreased by 0.8135, equal within error to the reciprocal of the p_{90} increase. The corresponding average caffeine methyl resonance intensities (multiple 90° pulses, with presaturation, 16 s between pulses) decreased by 0.83. The product $RMR_{90} \times p_{90}$ was constant to 0.52% rsd for the H_2O resonance of the different solutions and to 1.3% rsd for the caffeine resonances. The same samples in probe X showed less reduction in intensity (measured factor 0.985 at 100 mM NaCl) and less increase in p_{90} (measured factor 1.016 = 1/0.985).

Further comparisons, using probe T and single 90° pulses, of neat $CHCl_3$ (100%), CH_3OH (>99.8%), and the H_2O signal from 1 mM NaCl/4 mM caffeine, showed that the product [$RMR_{90} \times p_{90}$] was constant to 0.45% rsd across the different solvents (Table S3). Experimental integrated intensity ratios, $I_{SOLVENT}/I_{H2O}$ = 0.112 for $CHCl_3/H_2O$, 0.603 for CH_3OH/H_2O, and 0.200 for CH_3OH/H_2O, differed from predicted ratios (respectively, 0.1126, 0.6673, and 0.2224) on the basis of known solvent densities,[29] whereas the corrected product ratios [$I_{SOLVENT} \times p_{90SOLVENT}$]/[$I_{H2O} \times p_{90H2O}$] (0.113 ± 0.001, 0.660 ± 0.007, and 0.219 ± 0.002) agreed closely.

These results show that a direct measurement of p_{90} (or p_{360}) may be used to correct for intensity changes arising from Q-damping, provided samples are tuned and matched and the same coil is used for pulse excitation and detection. This removes one of the major obstacles to quantitative use of NMR with separate external standards. The effects of highly Q-damping solutions on the ERETIC method[22,23] do not appear to have been evaluated experimentally.

Radiation Damping. Radiation damping[30] increases the rate of decay of the FID from a high concentration of nuclei in a tightly coupled probe (e.g., H_2O in probe T) but does not degrade the integral of the resulting transformed peak, as the amplitude of the first point of the FID is unaffected (assuming the decay time is large compared to the receiver switching delay). This is illustrated experimentally by the comparisons of neat solvents above (Table S3), for which the concentrations of protons vary by a factor of 9, but the [$I_{SOLVENT} \times p_{90SOLVENT}$] product ratios are proportional to the concentrations.

Effects of Mistuning and Mismatching. Compared to the 50 Ω tuned and matched state, $p_{90}I$ was altered appreciably by gross mistuning, mismatching, or both (Tables S3 and S4). This effect is attributable to differing impedance characteristics between the pulse power amplifier and the signal preamplifier. The precise tuning and matching display readily allowed reproducibility of $p_{90}I$ to within <1% among different samples (Tables S2 and S3).

Precision of NMR Tube Diameter. When comparing samples in different tubes, variations in cross-sectional area contribute to error. The Wilmad 535 pp NMR tubes used for this study are stated by the manufacturers to be totally within the stated ID tolerance, 4.200−4.213 mm, corresponding to an extreme variation in cross-sectional area $\Delta A/A$ of 0.62%. The manufacturers also report (private communication) that measurements of a sample of 20 such tubes showed that all IDs were between 4.211 and 4.214 mm ($\Delta A/A$ 0.14%). These uncertainties are small to negligible compared to other error sources in our experiments.

An independent measure of the variability in tube diameter was obtainable from the integrated area $I(H_2O)$ of the fully relaxed H_2O resonance following a single 90° pulse without presaturation, using the lowest setting of the receiver gain (RG = 1). At low concentrations of solute, the H_2O concentration is effectively constant. A series of 19 solutions of theophylline (three each at 1, 2, and 4 mM) and caffeine (4 mM containing from 0 to 100 mM NaCl), each in its own individual tube (Table S5), showed the product $I(H_2O) \times p_{90}$ was 1865 ± 7 with a maximum difference from the mean of 14 units (0.8%). Inclusion of similar results from arginine and saxitoxin (STX) solutions gave $I(H_2O) \times p_{90}$ = 1864 ± 11 (0.59% rsd).

As this experimental variance also includes errors from all other sources including signal amplification and integration, errors arising from tube diameter fluctuations are certainly <±1%. Constancy to within 0.45% rsd is also shown in the above molar comparison of [$I_{SOLVENT} \times p_{90SOLVENT}$] for neat solvents ($H_2O$, $CHCl_3$, CH_3OH, Table S3).

Data Processing and Integration of Spectra. These are potentially the most subjective aspects of NMR quantitation, particularly where large baseline distortions are introduced by incomplete suppression of the solvent signal or impurities are present. Further details of our data processing methods are in the Supporting Information (SD5). We compared integrals from two different operators (A and B) following the criteria specified in the Experimental section, using unprocessed FIDs from the 2 to 49 mM solutions of caffeine (Figure S3), and sucrose, in H_2O. Phasing, baseline correction, and ranges of integration were at the operator's discretion. The ratio of average integrals over all solutions obtained by A, to those obtained by B, was 0.9963 ± 0.0039 (five caffeine solutions) and 0.991 ± 0.011 (five sucrose solutions). Thus, error introduced by subjectivity in data processing by different operators in favorable cases may be less than 1%, although it is likely to increase with more complex spectra.

Resonances overlapping from impurities must be dealt with on a case-by-case basis: their presence may be inferred from area

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ratios of analyte peaks and from 2D spectra (TOCSY or HSQC). Residual lipid is a common cause of interference in the region 0.5−4 ppm.

Factors affecting the accuracy and precision in data processing of quantitative NMR spectra have been discussed in general[3,7,9,26] and in the context of natural products.[18] Published studies of integration reproducibility with ^{1}H spectra of a drug[31] and a pure natural product[18] showed integration errors of 0.5−2%, repeatability within one laboratory being better than reproducibility between different laboratories.[31]

Solvent Suppression and Effects of Presaturation. Dilute solutions in protonated solvents such as H_2O require solvent suppression for adequate digitizer resolution of analyte signals. The simplest method is selective presaturation of the solvent signal using a low-power single-frequency irradiation during the latter part of the relaxation delay. We avoid multipulse sequences for solvent peak suppression in quantitative applications as there is less control over the uniformity of the excitation profile across the spectrum and use low-average RF power dissipation to prevent sample heating. For consistency of experimental conditions, when comparing standards in deuterated solvents with analytes in protonated solvents, the same presaturation sequence should be used initially for both, to check for differences due to changes in RF paths through the preamplifier.

The bandwidth of the presaturating irradiation (maximum 10 Hz) used in our experiments was small compared to the frequency separation between the solvent resonance and the nearest resonances used for measuring RMR. Presaturation of strong solvent resonances will also cause partial saturation of signals from all nuclei exchanging with the solvent, which could cause unreliability in cases where the exchanging signals underlie resonances from nonexchanging protons used for RMR measurements. Such resonances should not be used for calculation of concentration. Of the samples used in our experiments, caffeine and theophylline have no exchanging hydrogens, and both arginine and neosaxitoxin (NEO) contain exchangeable hydrogens resonating at high frequency that are not included in calculations for quantitation. The two hydrogens resonating at lowest frequency in saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), and NEO (Table 1) are exchangeable,[20] but the rate is sufficiently slow that the intensity per proton is the same as for signals of nonexchanging protons and is not measurably affected by presaturation.

Intermolecular nuclear Overhauser enhancements (NOEs) of solute resonances, resulting from presaturation of the solvent resonance, are a potential source of error if ^{1}H−^{1}H dipolar interactions with the solvent contribute to spin−lattice relaxation of the solute. This is unlikely to be appreciable where intramolecular ^{1}H−^{1}H distances are short (e.g., for vicinal H) or where other contributions to relaxation are large (e.g., spin rotation in methyl groups, presence of dissolved oxygen, or trace paramagnetics); however, it may be noticeable in highly purified samples at isolated solvent-exposed protons, leading to increased intensity relative to other protons in the same molecule. In addition, intramolecular NOEs may arise if any solute resonances overlap the irradiated solvent peak. Resonances exhibiting enhancements relative to others from the same compound should not be used for quantitation.

While the likelihood of intramolecular NOEs can be determined from spectra of a compound in deuterated solvent, intermolecular NOEs depend on the solvent contribution to individual solute proton relaxation, which is impractical to measure directly except for highly concentrated solutions. We measured an intermolecular NOE (ca. 5−8%) at the CH of some caffeine (and theophylline) standards in H_2O, so these resonances were not used for quantitation; however, comparisons of 40 mM solutions with and without presaturation showed that the CH_3 resonances were not affected. Similar measurements on concentrated (11 mM) domoic acid solutions also showed no intermolecular NOE with irradiation of H_2O.

Repeatability of Individual Measurements and Short-Term Stability. Repeated runs with similar samples on different days showed small variations in p_{90} (ca. 1%) and similar but opposite variations in the H_2O integral (Tables S2 and S3), which may be attributable to minor changes in probe Q produced by factors such as atmospheric humidity. For a series of 30 spectra of caffeine, caffeine with up to 100 mM NaCl, theophylline, arginine, and saxitoxin solutions, obtained with probe X at intervals over a 3-week period (Table S5), the product $[I(H_2O) \times p_{90}]$ was virtually constant with time, having a 0.57% standard deviation and showing no consistent trend to increase or decrease. The small variation is attributable to error in measurement of integrals and pulse widths.

Linearity of Response and Comparison of Pure USP-Certified Standards. The linearity of the NMR response was tested with probe X using accurate gravimetrically prepared caffeine and sucrose solutions in H_2O at ca. 49, 20, 10, 4, and 2 mM (Figures S3, S4; Tables S6 and S7). Linear regressions of signal (average integral per proton, I) versus gravimetric concentration (C_G in mM) showed excellent linearity and relatively small intercepts:

$$I = 429.5C_G - 32.7 \ (R^2 = 0.99997) \text{ for caffeine}$$

$$I = 430.5C_G - 27.0 \ (R^2 = 0.99996) \text{ for sucrose}$$

The essentially equal slopes indicate clearly the equimolar response of the NMR system between different compounds. The use of caffeine as a calibrant was tested by interpolation of sucrose responses into the caffeine calibration curve. As shown in Table 1, the maximum deviation of NMR results from gravimetric values for sucrose was 2.8%. Linear regression of the resulting sucrose concentration C_S determined by NMR versus gravimetric value C_G gave

$$C_S = 0.9976C_G - 0.0126 \ (R^2 = 0.99996)$$

Such a high degree of linearity and near-zero intercept suggested that a single external standard could be used for quantitation. To test this, concentrations of the sucrose solutions were determined using each of the individual caffeine standards (Figure S4, Table S7) and were constant to ca. 0.8% rsd. Agreement of average NMR concentrations with gravimetric concentrations was within 0.64, 1.7, 0.3, −2.5, and −6.2% for the

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respective sucrose solutions from 49 mM to 2 mM. Peaks showing anomalous intensity attributable to presaturation were not included in calculations. Linear regressions of sucrose concentration (mM) by NMR versus gravimetric concentration (mM) showed gradients 1.0027, 1.0026, 1.0077, 1.018, and 1.0026, respectively, for 2−49 mM caffeine standards, all with $R^2 = 0.99996$ and intercepts in the range −0.0628 to −0.0637 mM. These spectra were obtained prior to a preamplifier failure (see below).

Similarly, theophylline samples prepared gravimetrically in H_2O at 1, 2, and 4 mM (Figure S5), run with a new preamplifier of slightly higher gain, yielded a linear regression:

$$I = 456.4C_G - 4.61 \text{ with } R^2 = 0.9998$$

When compared with a single 4 mM caffeine solution of known gravimetric concentration, at the same p_{90}, the regression (Figure S5) of NMR-determined theophylline concentration C_T versus theophylline gravimetric concentration was

$$C_T = 1.0012C_G \ (R^2 = 0.99976) \text{ for regression forced through the origin or}$$

$$C_T = 0.9926C_G + 0.0261 \ (R^2 = 0.99986) \text{ for regression through the data points only}$$

The maximum deviation of NMR results for theophylline from the gravimetric values was 3.1% (Table 1).

Comparable results were obtained from theophylline and caffeine spectra recorded with a flip angle of 47° using probe X (Figure S5, RMR_{47} ca. 336). Regressions for NMR-determined versus gravimetric concentrations were

$$C_T = 0.9969C_G \ (R^2 = 0.9992, \text{ origin included})$$

$$C_T = 1.0034C_G - 0.0171 \ (R^2 = 0.9993, \text{ data points only})$$

Further spectra obtained with probe X (constant measured p_{90}) from 4 mM solutions of USP-certified pure arginine, caffeine, and theophylline prepared gravimetrically in H_2O, yielded respective RMR_{90}'s (±propagated sd) of 453 ± 10, 455 ± 22, and 455 ± 12. On the same day, a 0.400 mM caffeine sample gave an RMR_{90} of 455 ± 12. One peak (CH) in each spectrum showed anomalous intensity attributable to presaturation and was excluded.

In view of the results obtained with weighed pure standards, it was concluded that it is sufficient to compare three tubes of an analyte solution with three tubes of one external standard solution. The standard concentration should be higher than that of the analyte, as we have established that the NMR method is highly linear, and stronger signals allow for better integration. Nevertheless, the standard should show no signs of aggregation or precipitation, and its NMR signal should be accommodated by a receiver gain setting appropriate to the analyte.

Long-Term Stability of Instrument Calibration. Long-term stability of the RF irradiation and detection circuitry of the Bruker DRX-500 instrument has been tested with both sucrose and caffeine solutions over different periods. Spectra of a 17.77 mM sucrose standard solution in D_2O were obtained under the standard conditions over a 9-month period, with probes X and T. No other hardware components differed. RMR_{90}'s after 6 months were 0.989 ± 0.023 (probe T) and 0.975 ± 0.030 (probe X) of their initial average values. During the final 3 months of this series, a slight decline in RMR_{90} to 0.95 of the initial value coincided with noticeably increased p_{90} traced to problems with the ^1H preamplifier, which subsequently failed. Replacement led to a discontinuous 6% increase in RMR values attributable to a slight gain change. Subsequent comparison with probe X of the methyl resonances of two 4 mM caffeine solutions over a 4-month period showed high consistency:

$$RMR_{90} \text{ (end)} = (1.0006 \pm 0.0065) \times RMR_{90} \text{ (start)}$$

Care is necessary to ensure that data being compared are obtained without any change in instrument hardware, particularly RF filters, amplifiers, detection, and digitization circuitry. Standards and analytes must be compared with the same pulse sequence to avoid differences in the paths of RF signals in the preamplifier.

Limits of Quantitation, Dynamic Range, and Quantitation against Solvent Signal. Very dilute solutions (<100 μM) present problems for quantitation by our method. Data accumulation time is limited by instrumental drift when the instrument is operated without a field-frequency lock. Trace impurities and background resonances are more likely to influence integration. Methyl resonances from a 4 μM caffeine sample yielded S/N (peak-to-peak) ca. 2.5 after 2048 scans, but two peaks could not be integrated accurately as they overlapped a broad background peak and a trace methanol contaminant, and complete relaxation was compromised by use of a 4-s delay between pulses. The integral of the one distinct methyl resonance was determined with ca. 20% error. As sharp methyl peaks are particularly favorable for quantitation, we estimate that ca. 40 μM represents a lower limit for practical quantitation of most natural products by this method, and at that level we would not expect the precision to be better than ca. 10%. Where the application is the preparation of certified standards, more accurate results are obtainable by quantifying a more concentrated solution by NMR, followed by precise dilution. If long runs without deuterated solvents are necessary, the procedure could be modified using a 2-mm coaxial tube (e.g., Wilmad WGS-5BL) of D_2O for a lock signal, provided that the same tube is inserted in both analyte and standard solutions for reproducibility of sample volume. This modification has not been tested and would preclude sealed tubes.

Dynamic range was tested by comparing the concentrations of caffeine (40 mM), domoic acid (11 mM), and decarbomyl neosaxitoxin (dcNEO, 2.2 mM) determined against the signal from the H_2O solvent using single pulses at low receiver gain with those determined gravimetrically or against caffeine standards in H_2O (Table S8). The results indicate that an instrument with a 16 bit digitizer and oversampling can yield results accurate to <2% over a dynamic range of ca. 10^4 and 10^5, respectively. Quantitative studies of reaction mixtures have been reported in nondeuterated solvents at concentrations ca. 0.1−1 M.[32]

Application to Phycotoxin Solutions. Concentrations of phycotoxin (Figure 1) stock solutions have been determined by

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Table 2. Estimated Relative Contributions of Potential Sources of Error in Quantitative NMR with External Standards

error source	contribution, % rsd estimated	measured
sample tube volume	0.3 range	
temperature (for ±0.5 °C control)[a]	0.34 range	
probe Q × p_{90}	0.16	
deviation from 90° pulse (extreme case, probe T)	0.3	
electronics (pulse amplitude, amplification, digitization)	<0.1	
integration (best case, single solvent peak, no impurities)	0.3	
overall % rsd from sum of above	1.2	0.6
concentration of calibration standard	0.5	0.5
presaturation and integration	sample dependent	

[a] Estimate based on Boltzmann distribution of spin level populations, see SD3.

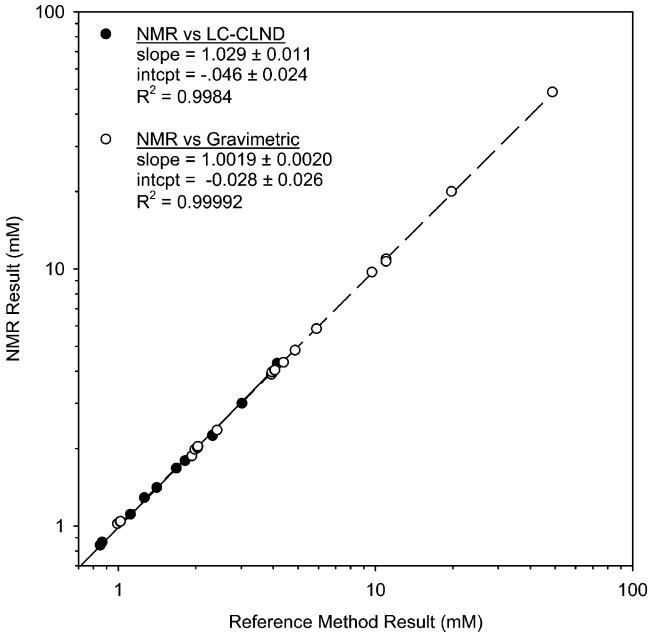

Figure 2. Concentrations of all solutions determined by NMR (in Table 1) versus concentrations of the same solutions from gravimetry or LC−CLND.

NMR and compared with gravimetric or LC−CLND results, prior to dilution for issue as Certified Reference Materials (CRMs). NMR concentrations (Table 1) are slightly lower than gravimetric (by up to 2.4%), which may be partly attributable to residual sample impurities (salts, moisture, column material). Differences compared to CLND determinations are of either sign, the largest being 3.8% except for individual members of enantiomeric pairs (GTX1/GTX4, etc.). The latter differences are attributable to perturbation of enantiomeric equilibrium from the NMR to the LC−CLND measurements, as the sums for each pair show good agreement between the techniques.

Figure 2 shows linear regressions of the Table 1 NMR results versus gravimetric or LC−CLND determinations of concentration (μM), respectively:

$$C_{NMR} = 0.9999C_G - 9.81\ \mu M\ (R^2 = 0.9998)$$

$$C_{NMR} = 1.026C_{CLND} - 33.5\ \mu M\ (R^2 = 0.9986)$$

Overall, the agreement is highly encouraging and the NMR measurements proved invaluable for the preparation of several calibration solution CRMs that are now available to the analytical community.

CONCLUSIONS

Quantitative NMR using precision tubes and external standards enables the direct measurement of analyte concentrations without contaminating the sample with internal standards or compromising subsequent use of the solutions with other techniques, such as LC−MS. The method is also advantageous for use with hazardous materials as samples may be kept in sealed tubes throughout. Expensive deuterated solvents can be avoided, overlap of standard with analyte resonances is not a concern, impurities in the standard are physically separated from the analyte, and standards may be prepared with greater accuracy. The ^{1}H spectrum provides in addition a measure of the analyte purity and integrity. Analytes and standards in different solvents or in salt solutions may be compared by quantitative corrections of Q-damping effects via ratios of analyte/standard p_{360} values or by measurements of solvent resonance integrals following a single 90° pulse.

The factors affecting the accuracy and precision of quantitative NMR using external standards have been explored and their relative influence is summarized in Table 2. Provided that all samples are accurately tuned and matched at the observing frequency, that sample temperatures are constant, and that adequate relaxation delays are used, the major contributions to error arise from the accuracy and purity of the gravimetrically prepared external standards, the purity of the sample (particularly freedom from proton-containing impurities producing resonances that overlap with the analyte, polymerization of the analyte, and macromolecules), subjectivity in the integration of spectra, and intermolecular NOEs if presaturation is used to suppress resonances of nondeuterated solvents.

Integration subjectivity is highly sample-dependent. With pure analytes having sharp well-separated resonances (e.g., caffeine), different experienced operators processing and integrating the same data set can agree to better than 0.3%. Results with natural products that have more complex spectra and which may contain residual protonated impurities are influenced by the choice of peaks to integrate and baseline correction, and the only option may be to average the results obtained by two or more operators on the same data set. Spectral overlaps with proton-containing

impurities may sometimes be detected by 2D TOCSY spectra. Impurities that adsorb the analyte will lead to broadening or indetectability of resonances and must be avoided. These problems also occur to the same extent when internal standards are used and may be further compounded by overlap of analyte and standard resonances.

Intermolecular NOEs are not accurately predictable, although as illustrated by the agreement between NMR concentration measurements and other methods (Table 1, Figure 2), they are likely to be negligible within error for most ^{1}H resonances in natural products. A practical strategy is to disregard peaks for quantitation if their intensity is significantly enhanced relative to others and to employ structural knowledge of the molecule to decide which H atoms are likely candidates for this effect.

The potential problems peculiar to NMR with external standards contribute little to the overall accuracy and precision. With single-coil RF irradiation and detection, differences in damping between analyte and standard may be corrected from the ratio of 360° pulse lengths. A series of measurements with two probes and a variety of solvents and solutes showed that the product of the molar response of the solvent resonance and the 360° (or 90°) pulse was constant, for a given probe, to better than 1%, the standard deviation being typically less than 0.5%. This error also includes any variation in sample tube diameter and is consistent with the tolerances claimed by the manufacturer. Other possible sources of variation included within this figure are temperature effects on level populations, drift of total amplifier gain, digitizer response, and integration. Constancy of the total response over a period of months has been demonstrated so it is possible to use the technique with confidence using periodic calibrations against standards.

Although we have worked with samples in nondeuterated solvents owing to the ultimate uses of the reference materials, the methods are more readily utilized with deuterated solvents as presaturation is unnecessary and field-frequency lock can be used.

We have also shown that dilute solutions (mM range) of analytes in otherwise pure nondeuterated solvents may be quantified with a spectrometer having highly linear amplification stages and accurately calibrated receiver attenuation by recording the solvent-suppressed analyte signal with multiple 90° pulses at high gain against the solvent signal from the same solution recorded from a single 90° pulse at low gain. This method is faster as it does not require external standards, exact tuning, precisely matched sample tubes, damping corrections, or multiple samples, and it could be used with probes having separate irradiation and detection coils. Necessary precautions are to ensure dryness of solvents and the same signal path in the instrument for measurements of solvent and analyte signals.

ACKNOWLEDGMENT

The authors thank Kelly Boutilier, Krista Thomas, and Sheila Crain for preparation of calibration solutions; Maurice Laycock, Shawna MacKinnon, Cheryl Craft, and Patricia LeBlanc for preparation of pure toxins; Hughie Fraser and Dominik Wechsler for LC-CLND analyses; Robert Boyd and Don Leek for helpful comments; and the Asia-Pacific Economic Cooperation (APEC) TILF fund for financial assistance.

SUPPORTING INFORMATION AVAILABLE

Additional discussions, tables, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review November 1, 2004. Accepted February 22, 2005.

AC048385H