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^{1}H, ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR

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Summary

A considerable degree of variability exists in the way that ^{1}H, ^{13}C and ^{15}N chemical shifts are reported and referenced for biomolecules. In this article we explore some of the reasons for this situation and propose guidelines for future chemical shift referencing and for conversion from many common ^{1}H, ^{13}C and ^{15}N chemical shift standards, now used in biomolecular NMR, to those proposed here.

Introduction

Recent statistical analyses (Szilagyi and Jardetzky, 1989; Spera and Bax, 1991; Wishart et al., 1991) along with a number of theoretical advances (Ösapay and Case, 1991; Herranz et al., 1992; Williamson et al., 1992; de Dios et al., 1993a,b) have allowed chemical shifts to become a useful adjunct to biomolecular structure determination (Pastore and Saudek, 1990; Reily et al., 1992; Wishart et al., 1992; Ösapay and Case, 1994; Wishart and Sykes, 1994a) and refinement (Gippert et al., 1990; Ösapay and Case, 1991). At the same time, developments in 2D, 3D and 4D heteronuclear NMR techniques (Kay et al., 1990; Clore and Gronenborn, 1991) have led to a rapid increase in the number of ^{1}H, ^{13}C and ^{15}N assignments being reported and to the establishment of the BioMagResBank (Seavey et al., 1991) as a permanent repository for this rapidly growing body of information.

A recent survey of the literature has revealed that nearly a dozen different standards are now being used in the biomolecular NMR community to reference ^{1}H, ^{13}C and ^{15}N chemical shifts (Wishart and Sykes, 1994b). While the differences in most common ^{1}H standards (DSS, TSP and TMS) are small, variations in other standards (H_2O or HDO) can be quite significant (DeMarco, 1977; Wishart and Sykes, 1994b). Similarly, differences in many ^{13}C standards (dioxane, acetone, DSS, TSP and TMS) and ^{15}N standards (NH_3, NH_4Cl, NH_4NO_3, urea, nitromethane) have recently been shown to be quite substantial (Thanabal et al., 1994; Wishart and Sykes, 1994b).

This situation is further complicated by the fact that both internal and external standards are widely used, even though both have their inherent shortcomings. Internal standards (such as TSP and DSS) are almost universally used for ^{1}H NMR, both internal (DSS and TSP) and external (dioxane and TMS) standards are commonly used for ^{13}C NMR, and external standards (such as NH_3 or NH_4Cl) are almost exclusively used in ^{15}N NMR. Internal standards have a wide appeal because of their general convenience. However, they are not without their problems. Sensitivity to pH, salt and temperature variations (DeMarco, 1977), nonspecific interactions with the biomolecule of interest (Lam and Kotowycz, 1977; Shimizu et al., 1994), solvent effects and limited solubility or stability in an aqueous environment – these are all phenomena that can significantly affect the chemical shift of an internal standard.

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Abbreviations: TMS, tetramethylsilane; TSP, 3-(trimethylsilyl)-propionate, sodium salt; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt; TFE, 2,2,2-trifluoroethanol; DMSO, dimethyl sulfoxide.

External standards avoid the problems associated with pH sensitivity, biomolecular interactions, poor solubility or limited stability, but they are affected by bulk susceptibility. Consequently, reference measurements on external standards made with iron core magnets (where the field is perpendicular to the long axis of the sample) differ from reference measurements made in superconducting magnets (where the field is usually parallel to the long axis of the sample). Similarly, because of bulk susceptibility effects, external references measured in coaxial tubes or capillaries will often have different values than those measured in spherical microcells or with magic-angle spinning (Live and Chan, 1969). It has become a common practice to take chemical shift reference measurements reported in the 1970s (performed on iron-core magnets) and to use the same numbers and compounds to indirectly define external chemical shift references on superconducting magnets. This has led to the appearance of so-called virtual or fictitious (Witanowski et al., 1993) standards.

The multitude of ways in which chemical shift standards can be measured is becoming a significant hindrance to both the collection and reporting of data and the comparison of chemical shift assignments. In an attempt to address this situation, we measured a variety of commonly used chemical shift standards under a well-defined set of conditions. By combining these experimental measurements with literature surveys, we have arrived at a set of protocols for reporting and measuring ^{1}H, ^{13}C and ^{15}N chemical shifts of biomolecules in aqueous solutions. These measurements and the accompanying protocols are described in this report.

Materials and Methods

Sample preparation

Several of the common ^{1}H and ^{13}C chemical shift standards were used: DSS (Merck, Aldrich), TSP (Aldrich, MSD Isotopes), TMS (Aldrich), acetone (Fisher), 1,4-dioxane (Fisher), 99.8% D_2O (Sigma, MSD Isotopes) and sodium acetate (Aldrich). For ^{15}N chemical shift standards, the following compounds were used: liquid ammonia, NH_4NO_3 (Aldrich), $^{15}NH_4Cl$ (Cambridge Isotopes), urea (Fisher) and nitromethane (Fisher). Additional solvents (DMSO, $CDCl_3$, HNO_3, etc.) were obtained from local chemical supply houses. Typical concentrations for DSS and TSP were 100 μM to 1 mM (for ^{1}H work) and 1–10 mM (for ^{13}C work). Conditions and concentrations for the other reference compounds are given in Tables 2 and 3 (vide infra). To study the effects of pH and salt (KCl or NaCl) on the HDO resonance, the pH was varied between 2–11 (uncorrected meter reading) and the salt concentration between 0.0 and 1.0 M.

The liquid ammonia sample was prepared as follows: a thick-walled (5 mm o.d., 2.2 mm i.d.) NMR tube was connected to a two-way stopcock. One stopcock port was connected to a vacuum assembly, the other to a small bottle of ammonia gas. The NMR tube was evacuated and then chilled (−195 °C) using liquid N_2. While the tube was still immersed in liquid N_2, ammonia gas was slowly leaked into the NMR tube (where it condensed) until a column height of ~5 cm was reached. With the bottom half of the NMR tube still immersed in liquid nitrogen, an oxy-gas torch was used to seal off the top of the tube. The sample was then placed in an open container to allow it to equilibrate to room temperature. The pressure within the sealed tube was calculated to be approximately 10 atm.

Chemical shift measurements

All ^{1}H, ^{13}C and ^{15}N measurements were performed at 25 °C (unless stated otherwise) on Varian Unity (300 MHz), Bruker MSL (300 MHz) or Bruker AMX (500 MHz) spectrometers with the long axis of the sample parallel to the field (∥). For ^{13}C work, external reference samples were measured using a 1 mm coaxial glass capillary placed inside a standard 5 mm thin-walled NMR tube (Wilmad, NJ) containing 99.8% D_2O. For ^{15}N work, the external reference (the liquid NH_3 sample) was placed coaxially inside a 10 mm thin-walled NMR tube (Wilmad, NJ) containing 99.8% D_2O. In all cases the spectrometer was locked on the ^{2}H resonance of D_2O. For the studies of the thermal dependence of selected chemical shift standards, temperatures were varied from 10 to 55 °C. All NMR spectra were collected with either a 5 mm inverse detection probe (for ^{1}H, ^{13}C and ^{15}N NMR) or a 10 mm broadband direct detection probe (for ^{13}C and ^{15}N NMR). One-dimensional ^{1}H data were acquired with a ^{1}H sweep width of 4000–6250 Hz (depending on the spectrometer frequency) using an acquisition time of 4 s, sufficient to give a digital resolution of < 0.3 Hz/point. The residual HDO signal was suppressed by presaturation. One-dimensional ^{13}C data were acquired directly with a sweep width of 12.5 to 20 KHz (depending on the spectrometer frequency) using broadband WALTZ-16 decoupling during the pulse, acquisition and delay periods. The digital resolution for these ^{13}C spectra was typically less than 0.5 Hz/point. One-dimensional ^{15}N spectra were acquired directly with a sweep width of 15 000 Hz and an acquisition time of 2.1 s, giving a digital resolution of less than 0.5 Hz/point. WALTZ-16 decoupling was used during the acquisition period only. Systematic variations in the decoupling power proved that heating effects were negligible (data not shown). Typical ^{15}N acquisition times were 15 h. Indirect detection of ^{13}C and ^{15}N nuclei was achieved using the INEPT pulse sequence (Morris and Freeman, 1979) with standard experimental parameters. Measurements were conducted independently in at least two different laboratories using two different field strengths (300 and 500 MHz) and two different kinds of spectrometers (Varian and Bruker).

TABLE 1
CHEMICAL SHIFTS (RELATIVE TO DSS) AND ENVIRONMENTAL SENSITIVITY OF COMMON INTERNAL ^1H STANDARDS

Compound (conditions)	Chemical shift (ppm; field ‖)	pH change	T change
DSS (25 °C, D_2O)	0.000	No pH 2–11	No
TSP (25 °C, D_2O, pH 7.0)	−0.015	Yes[a]	No
TSP (25 °C, D_2O, pH 3.0)	0.003	Yes[a]	No
Acetone (25 °C, D_2O)	2.218	No pH 2–11	No
Dioxane (25 °C, D_2O)	3.750	No pH 2–11	No
HDO (25 °C, D_2O)	4.766	Yes −2 ppb/pH	Yes −11.9 ppb/°C

[a] The pH dependence of TSP can be described as: $\delta = \delta_{obs} - 0.019(1 + 10^{(5.0-pH)})^{-1}$.

Ξ ratio determination

The use of Ξ ratios allows the indirect referencing of ^{13}C and ^{15}N chemical shifts through direct referencing to a single, well-determined ^1H standard (Live et al., 1984). Ξ ratios are independent of both the spectrometer design and the sample geometry and, therefore, provide an accurate and consistent way to reference ^{13}C and ^{15}N chemical shifts. For this study, the Ξ ratio for ^{13}C was defined as the ratio of the ^{13}C frequency of the methyl resonances of DSS (in water) divided by the ^1H frequency of the methyl resonances of DSS (in water). The Ξ ratio for ^{15}N was defined as the ratio of the ^{15}N frequency of external anhydrous liquid NH_3 divided by the ^1H frequency of the methyl resonances of DSS (in water). Determination of Ξ ratios was done as follows: the exact (±0.2 Hz) ^1H frequency of DSS (10 mM in D_2O, 25 °C) was determined by measuring its carrier offset frequency and subtracting this value from the exact spectrometer (carrier) frequency. Similar frequency determinations were carried out for the exact ^{13}C frequency of DSS (10 mM in D_2O, 25 °C) and the exact ^{15}N frequency of liquid NH_3 (25 °C). From these three absolute frequencies, Ξ ratios were determined using 10 significant figures throughout the calculations.

Results

^1H, ^{13}C and ^{15}N chemical shifts of common standards

As can be seen from Table 1, the variations in ^1H chemical shifts for the most common standards (DSS, TSP, TMS, dioxane) are trivially small; the ^1H chemical shifts are largely immune to significant pH and temperature effects. An important exception is water (HDO). In addition to its well-known temperature dependence (−11.9 ± 0.3 ppb/°C), HDO is also affected by pH (−2 ppb/pH unit from pH 2 to 7) and by salt concentration (−9 ppb/100 mM salt; the negative sign indicates an upfield shift for HDO with increasing temperature, pH and salt concentration). An earlier study by DeMarco (1977) also indicated a slight pH dependence for the HDO resonance and our studies essentially confirm his results. The fact that HDO is so sensitive to so many commonly varied conditions (pH, temperature, salt) suggests that HDO may be a less than ideal choice as an accurate ^1H chemical shift standard for biomolecular NMR spectroscopy.

The results in Table 2 illustrate the variation in ^{13}C chemical shift values for the most common ^{13}C standards. Unlike the situation for ^1H chemical shifts (where the differences are generally small), DSS, TSP and TMS differ in their ^{13}C chemical shifts by up to 2.8 ppm. For example, depending on how it is measured, 10% dioxane (a common secondary ^{13}C standard) can have a signal at 66.5 ppm (relative to external TMS), 67.5 ppm (relative to internal TMS), 69.3 ppm (relative to DSS) or 69.4 ppm (relative to TSP). It is believed that this variability among these three 'zero-point' standards accounts for most of the observed discrepancies in peptide and protein ^{13}C chemical shift measurements (Wishart et al., 1994b), and likely contributes to similar discrepancies in nucleic acid and carbohydrate ^{13}C NMR measurements as well. In addition to these differences among the primary standards, it is also worth noting that the ^{13}C shift for TSP

TABLE 2
CHEMICAL SHIFTS (RELATIVE TO DSS) AND ENVIRONMENTAL SENSITIVITY OF COMMON INTERNAL ^{13}C STANDARDS

Compound (conditions)	Chemical shift (ppm; field ‖)	pH change	T change
DSS (25 °C, D_2O)	0.00	No	No
TSP (25 °C, D_2O, pH 7.0)	−0.12	Yes[a]	No
TSP (25 °C, D_2O, pH 3.0)	−0.22	Yes[a]	No
TMS (25 °C, MeOD)	1.7	–	Yes −4 ppb/°C
Sodium acetate (30 °C, 0.15 M, pH 7.8)	26.1	No	No
Acetone (25 °C, D_2O)	33.0	No	No
Dioxane (25 °C, 1% in D_2O)	69.3	No	No
Dioxane (25 °C, 10% in D_2O)	69.3	No	No
Dioxane (25 °C, 90% in D_2O)	69.2	No	No

[a] The pH dependence of TSP can be described as: $\delta = \delta_{obs} - 0.10(1 + 10^{(5.0-pH)})^{-1}$.

TABLE 3
CHEMICAL SHIFTS (RELATIVE TO NH_3) AND ENVIRONMENTAL SENSITIVITY OF COMMON EXTERNAL ^{15}N STANDARDS

Compound (conditions)	Chemical shift (ppm)			pH change	T change
	Field ∥	Field ⊥	Field = 54.7°		
NH_3 (25 °C, liquid)	0.0	0.0	0.0	–	Yes 40 ppb/°C
NH_4NO_3 (25 °C, 1 M in 1 M HNO_3)	21.0	21.6	22.8[a]	Yes	Yes 13 ppb/°C
NH_4Cl (25 °C, 2.9 M in 1 M HCl)	23.6	24.9	N/A	Yes	Yes 20 ppb/°C
Urea (25 °C, 1 M in DMSO)	77.0	76.3	79.4[b]	–	No
CH_3NO_2 (25 °C, $CDCl_3$ (1:1))	379.8	379.6	378.1	–	No
CH_3NO_2 (25 °C, neat)	381.7	380.2	381.9	–	No

N/A = not available.
[a] Measured in 2 M HNO_3 (Witanowski et al., 1993).
[b] 10% urea in DMSO (Witanowski et al., 1993).

has a slight pH dependence. This suggests that some caution is warranted in using this standard for ^{13}C work.

Because ^{15}N standards for biomolecular NMR generally have to be measured externally, it is important to be aware of the geometry of both the sample and the magnet when the measurement is made or reported. As can be seen from inspection of Table 3, these geometrical factors can have an important effect on several of the common ^{15}N chemical shift standards. Just as with ^1H and ^{13}C standards, most ^{15}N standards do have some thermal dependence associated with their chemical shift values. This is particularly true for liquid ammonia and for NH_4Cl. On the other hand, 1 M urea in DMSO is a very convenient, easy to prepare ^{15}N standard with good, long-term stability and almost no thermal dependence.

Ξ ratios

Several Ξ ratios for ^{13}C and ^{15}N have been published in the past (Live et al., 1984; Bax and Subramanian, 1986; Edison et al., 1994); however, these were determined quite differently (in the case of ^{15}N) or were set to different zero-point standards (TMS and TSP). To update these earlier values to be consistent with our chosen set of primary (zero-point) standards, we have remeasured the Ξ ratios for ^{13}C-^1H and ^{15}N-^1H using DSS (in water) as the ^1H standard*. Anhydrous liquid ammonia in a thick-walled capillary tube at 25 °C served as the zero-point ^{15}N standard and 10 mM DSS (in water) at 25 °C as the zero-point ^{13}C standard. The corrected Ξ ratios are given in Table 4. As can be seen from this table, all three laboratories produced nearly identical results. In view of the uncertainty in the ninth decimal place for both the $^{13}C/^1H$ and the $^{15}N/^1H$ ratios, we adopted the following 'consensus' ratios: ^{13}C-^1H = 0.251449530 and ^{15}N-^1H = 0.101329118. This ^{13}C-^1H ratio is very close to the Ξ value determined by Bax and Subramanian (1986) for TSP (0.25144954) and the ^{15}N-^1H ratio is only slightly different from the (TMS-referenced) value determined by Live et al. (1984) (0.101329144).

Discussion

Selection of a primary (zero-point) ^1H standard

TMS, TSP and DSS, either as external or internal standards, all resonate within 0.1 ppm of each other – well upfield in a spectral region that is sparsely populated in most biomolecular NMR spectra. Because of the problems of solubility with TMS and pH sensitivity with TSP, and because perdeuterated DSS is now commercially available, we conclude that DSS represents the best current choice as an internal ^1H zero-point standard.

Selection of a primary (zero-point) ^{13}C standard

The silyl-methyl ^{13}C resonances of TMS, TSP and DSS are all found well upfield in a spectral region that is sparsely populated in most biomolecular NMR spectra. Dioxane and acetone resonate too far downfield to be considered as ideal primary standards (however, when correctly referenced, dioxane can serve as an excellent secondary chemical shift standard for both ^1H and ^{13}C NMR – see Tables 1 and 2). Currently, the most popular zero-point (0 ppm) reference is TSP, with TMS and DSS being a distant second and third, respectively. Approximately one-third of protein ^{13}C chemical shifts are reported relative to 'virtual' dioxane, commonly given as 67.8

*During the preparation of the manuscript, one of us (F. Abildgaard) succeeded in preparing a sample in which DSS was dissolved in liquid NH_3. This allowed the measurement of all three (^1H, ^{13}C and ^{15}N) standards in a single homogeneous sample. The $^{13}C/^1H$ Ξ ratio in this sample was found to be 0.251449531 and the $^{15}N/^1H$ Ξ ratio was found to be 0.101329002.

TABLE 4
EXPERIMENTALLY DETERMINED Ξ RATIOS FOR $^{13}C/^{1}H$ AND $^{15}N/^{1}H$ AT 25 °C

Nucleus	Ξ value	Instrument
$^{13}C/^{1}H$	0.251449528	300 MHz (Varian)[a]
$^{13}C/^{1}H$	0.251449537	500 MHz (Bruker)[a]
$^{13}C/^{1}H$	0.251449519	500 MHz (Bruker)[b]
$^{15}N/^{1}H$	0.101329118	300 MHz (Varian)[a]
$^{15}N/^{1}H$	0.101329118	500 MHz (Bruker)[a]
$^{15}N/^{1}H$	0.101329112	500 MHz (Bruker)[b]

[a] Measurements were performed on two samples, one with DSS in D_2O ($^{13}C/^{1}H$) and the other with external liquid ammonia ($^{15}N/^{1}H$) prepared as described in the Methods section. Temperature correction factors were not determined for these samples.

[b] Measurements were made using a single sample containing DSS and 1 M NH_4NO_3 in 1 M HNO_3. The NH_4NO_3 signal at 21.0 ppm was used to determine the ^{15}N zero-point frequency. Temperature correction factors with this sample were determined to be $(T-300\ K)\times 1.04\times 10^{-9}$ (for $^{13}C/^{1}H$) and $(T-300\ K)\times 2.74\times 10^{-10}$ (for $^{15}N/^{1}H$).

ppm (Richarz and Wüthrich, 1978) relative to TMS. Because TSP is slightly pH sensitive and because we selected DSS as the ^{1}H zero-point standard, we concluded that DSS would be the best internal ^{13}C zero-point standard.

Selection of a primary (zero-point) ^{15}N standard

While several early reports of protein ^{15}N NMR used NH_4Cl (LeMaster and Richards, 1985) or NH_4NO_3 (Leighton and Lu, 1987) as zero-point standards, nowadays the most common zero-point reference in biomolecular NMR is liquid ammonia. It is notable, however, that a large number of ^{15}N chemical shifts are reported relative to 'virtual' NH_4Cl, commonly given as 24.9 ppm relative to liquid ammonia (Srinivasan and Lichter, 1977). This value has not been corrected for the fact that it was obtained on a magnet with the field perpendicular to the sample column (Witanowski et al., 1993) and, therefore, is approximately 1.3 ppm higher than the value found when measured on a superconducting magnet (Table 1). As liquid ammonia has become the de facto zero-point standard in many laboratories, we decided that liquid ammonia would be the best ^{15}N (external) zero-point standard.

Indirect referencing for multidimensional heteronuclear NMR (Ξ ratios)

Considerable savings in both time and effort can be gained if frequency ratios (Ξ) are used to calculate chemical shifts when two or more different kinds of nuclei are being measured simultaneously (Live et al., 1984; Edison et al., 1994). This approach requires the measurement of just a single reference compound (DSS) for a single type of nucleus (^{1}H) to indirectly determine the zero-point reference for another (^{13}C or ^{15}N) nucleus. These ratios are universal and can be used on most commercial spectrometers operating under any configuration or field strength. Because of the many advantages of indirect referencing, we propose to use the following consensus Ξ ratios for indirect referencing: $^{13}C/^{1}H = 0.251449530$, $^{15}N/^{1}H = 0.101329118$.

Conclusions

The guidelines resulting from the present study can be summarized as follows:

(1) The primary ^{1}H chemical shift standard for aqueous solutions should be internal DSS at a concentration of 10 mM or less. Conditions concerning its preparation, concentration, pH and temperature should be fully reported.

(2) ^{13}C and ^{15}N chemical shifts should be defined in terms of the frequency ratios (Ξ) $^{13}C/^{1}H = 0.251449530$ and $^{15}N/^{1}H = 0.101329118$, where the ^{1}H frequency is that of DSS in water.

(3) In situations where the methods described above are impractical, so that other internal or external references have to be used, the results should be reported as chemical shifts converted to the reference standards above (points 1 and 2). In such cases, precise details should be given on the referencing and conversion factors employed. Conversion factors for ^{13}C chemical shifts can be derived by comparing the primary reference to the methyl carbon signal of DSS in aqueous solution. Conversion factors for ^{15}N can be derived using liquid NH_3 in a coaxial capillary, as described in the text. If liquid NH_3 is unavailable, 1M urea in DMSO (at 77.0 ppm) can be used as a substitute external reference.

(4) Tables 1–3 may be used to correct or re-adjust previously published results to conform to the suggestions given above.

While the current open-endedness in NMR chemical shift referencing has not generally been much of a problem in the past, it is clear that the lack of rules and clearly defined protocols is now beginning to have an adverse effect on progress in a number of areas of biomolecular NMR spectroscopy. It is hoped that the information contained within this report might offer a framework within which all NMR laboratories can perform consistent chemical shift measurements in biomolecular NMR. This could greatly help both the level of communication and the rate of progress in this fast-growing field.

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