Ultra-clean pure shift NMR with optimal water suppression for analysis of aqueous pharmaceutical samples

Guilherme Dal Poggetto*[a], Adam DiCaprio[b], Mikhail Reibarkh[a] and Ryan D. Cohen[a]

a. Merck & Co., Inc., 126 East Lincoln Avenue, 07065, Rahway, NJ – USA

b. Merck & Co., Inc., 770 Sumneytown Pike, 19846, West Point, PA – USA

* Email: guilherme.dal.poggetto@merck.com

Abstract: Pure shift NMR experiments greatly enhance spectral resolution by collapsing multiplet structures into singlets and, with water suppression, can be used for aqueous samples. Here, we combine ultra-clean pure-shift NMR (SAPPHIRE) with two different internally encoded water suppression schemes to achieve optimal performance for small molecule and macrocyclic peptide pharmaceuticals in water and acetonitrile-water mixtures.

Nuclear Magnetic Resonance (NMR) is one of the most widely employed analytical tools for pharmaceutical research and quality control. For small to medium-sized molecules (< 10 kDa), NMR is routinely used for compound identification and structure elucidation. It is also commonly applied for fragment-based drug discovery, quantification, reaction monitoring, and metabolite profiling [1] in a non-destructive manner. For large molecules, such as therapeutic proteins and monoclonal antibodies, NMR is used to examine interactions with excipients and evaluate higher-order structures.

One common limitation of ^{1}H NMR spectroscopy is the use of protonated solvents. Signals arising from these solvents can overwhelm and obscure analyte signals and often preclude sample analysis.[2] Solvent suppression and water signal suppression in particular has been an active research field for over forty years. Many NMR experiments have been developed for this purpose, which include presaturation,[3] Water suppression EnhancemenT (WET),[4]and diffusion filters[5]. The use of water peak selective defocusing, by either Excitation Sculpting (ES)[6] or WATER-suppression by GrAdient-Tailored Excitation (WATERGATE) [7] are most frequently used as they do not suppress signals from exchangeable protons.[8] This is particularly important for maintaining observation of the exchangeable protons from NH residues in proteins and peptides, where mixtures of water/organic protonated solvents are commonly used due to solubility limitations and to preserve native conformations. [9]

Another significant challenge, particularly for the analysis of mixtures and molecules exhibiting congested spectra, is signal overlap caused by homonuclear scalar couplings (J_{HH}). Such couplings cause the splitting of ^{1}H NMR peaks into multiple smaller peaks, resulting in a fine structure that extends the frequency range of the peaks. The so-called “pure (chemical) shift”, or homonuclear decoupling, experiments have been developed to effectively suppress the scalar coupling effects, collapsing all resonances into singlets.[10] Pure shift NMR experiments can be roughly divided into band selective or broadband. While the former focuses only on a narrow frequency range, the latter is applied to the entire 1H spectrum. Amongst all broadband pure shift experiments reported to date, PSYCHE (Pure Shift Yielded

by CHirp Excitation)[11] has been the most widely utilized in a variety of applications[12] due to its high sensitivity, spectral purity and ease of acquisition and automation. Moreover, the combination of PSYCHE with excitation sculpting [11] or, more recently, with NOESY-pre-saturation,[13] has demonstrated a need for robust water suppression with pure shift NMR in the analysis of complex aqueous samples.

The main disadvantage of PSYCHE is that it must be acquired as a pseudo-2D experiment via interferogram acquisition.[14] A series of Free Induction Decays (FIDs) are acquired, and only the first "chunk" of each FID, where the homonuclear *J* is refocused at its center, is stored. Each chunk is concatenated to form a new "pure shift" FID. The periodic discontinuity of the interferogram FID causes pronounced artifacts, especially in the case of intense signals such as the water peak in aqueous samples. PSYCHE-SAPPHIRE (Sideband Average with Periodic PHase Incrementation of the Residual *J* Evolution) can be used to suppress these chunking artifacts,[15] including those arising from the incompletely suppressed residual water peak. This was recently demonstrated by Chen et al., combining SAPPHIRE and NOESY-pre-saturation.[16]

Here, we illustrate two new experiments that combine PSYCHE-SAPPHIRE and water suppression by internally encoding either Excitation Sculpting (iES) or WATERGATE-5 (iW5)[17] elements into the original SAPPHIRE pulse sequence. This simple approach eliminates the need for long recycle delays used for the pre-saturation and increases signal-to-noise per time unit relative to experiments based on pre-saturation. Figure 1 shows a comparison between these new methods with others from the literature for a sample of bupivacaine hydrochloride in 9:1 H_2O/D_2O (v/v). Analyte signals were obscured in conventional (Fig. 1A), and TSE-PSYCHE-ES (Fig. 1B) experiments due to the intense water peak at 4.68 ppm. In the PSYCHE experiment, periodic chunking artifacts heavily distorted the baseline and significantly attenuated the signal at 4.17 ppm, proximate to the residual water resonance. The recently introduced PSYCHE-NOESY-pre-saturation (Fig. 1C) experiment exhibited good performance for water suppression and homonuclear decoupling. Still, a significant reduction in signal-to-noise ratio (SNR) per unit time was observed due to the long pre-saturation recovery times. Both SAPPHIRE-iES (Fig. 1D) and SAPPHIRE-iW5 (Fig. 1E) preserved signals near the water resonance, with the latter yielding nearly complete solvent suppression with minimal baseline distortion. Both also provided a sensitivity improvement (1.4x and 2.6x, respectively) compared to the PSYCHE-NOESY-pre-saturation (Fig. 1).

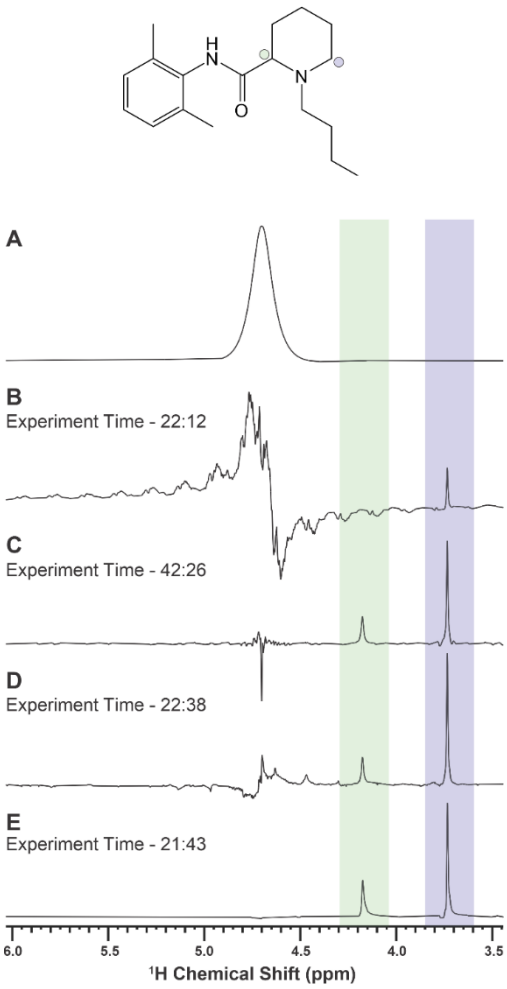

Fig 1. Comparison of solvent suppression pulse sequences for bupivacaine HCl D_2O/H_2O 10%/90% (v/v). Standard ^1H NMR spectrum (A) is shown for comparison. Peaks highlighted in green, or purple are proximate to the water peak and are used to indicate non-selective suppression. TSE-PSYCHE-ES ^1H NMR (B) suppressed most of the HOD peak, albeit with significant baseline distortions and suppression of nearby signals. Pure shift ^1H NMR spectral quality was improved significantly with 1D NOE-PSYCHE (C) and SAPPHIRE-iES solvent suppression (D) experiments, though residual solvent signal and slight baseline distortions were still observed. Applying the SAPPHIRE-iW5 pulse sequence (E) led to near-complete solvent suppression and maximum intensity of analyte peaks neighboring the residual solvent peak. Non-SAPPHIRE experiments were acquired with four times more scans than SAPPHIRE experiments to keep experiments comparable. The average signal-to-noise ratio per time unit in (E) is about 2.6 times larger than (C) due to the reduction in recycle delay and a slight increase in signal-to-noise ratio due to smaller loss by relaxation. The average signal-to-noise ratio per time unit in (D) is about 1.4 times larger than (C) due to the reduction in recycle delay and a small decrease in signal-to-noise ratio due to more significant loss by relaxation and diffusion. All spectra were adjusted for comparable intensities. Experimental duration (in minutes and seconds) is shown along with their respective spectra.

For these novel internally encoded water suppression PSYCHE-SAPPHIRE experiments (Figure 2), hard 180° (π) pulses which evolve the homonuclear *J* couplings are replaced with either excitation sculpting or WATERGATE-5 elements during τ_1 and τ_3. Both operate in a similar fashion: while a 180° pulse inverts all signals of the spectrum, the water spins are flipped by 360°. Pulse field gradients flanking either iES or iW5 element (G_1 and G_3) defocus any magnetization that was not inverted 180°. In iES, this is achieved by combining hard and selective (soft) 180° pulses, while in iW5, this is achieved with a series of composite pulses separated by equal delays. In the conventional WATERGATE experiment, only three pulses (90°x, 180°x, 90°-x) are used for this suppression, while in WATERGATE-5, each 90° pulse is replaced by five composite pulses, which provides a narrower inversion profile. Data acquisition and processing of SAPPHIRE data follow the same steps as described by Moutzouri et al. [15a]

It is important to note that pure shift methods like those described here work by refocusing the effects of homonuclear couplings at the cost of SNR. The SNR reduction is because only active spins are observed while passive spins are used for refocusing couplings. However, the use of modern cryogenically cooled probes can alleviate this penalty.

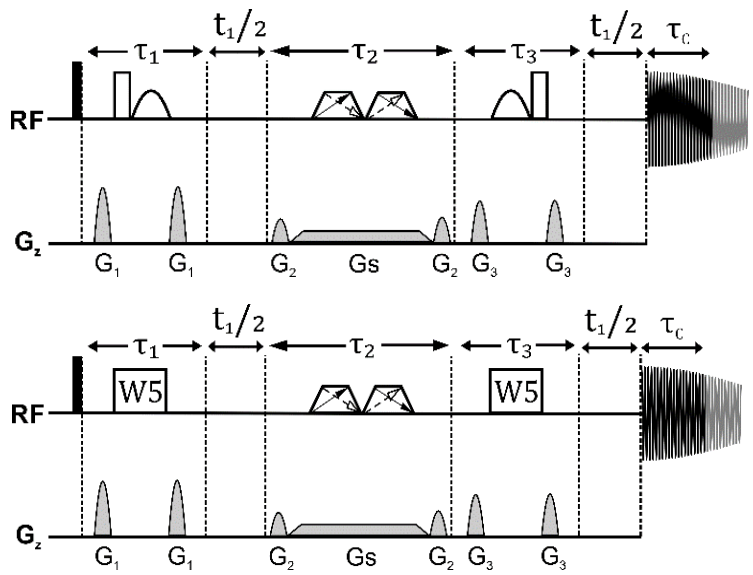

Fig 2. Pulse sequences for SAPPHIRE-iES (top) and SAPPHIRE-iW5 (bottom) experiments. Narrow-filled and wide white rectangles represent hard 90° and 180° pulses, respectively. White-shaped RF pulses are selective 180° refocusing pulses used to flip the solvent magnetization. Either RSNOB or REBURP pulses are optimal for solvent-selective pulses. W5 represents the WATERGATE-5 pulse block, with bandwidth and delay for binomial water suppression set to 20 kHz and 125 µs, respectively. Trapezoids with cross-diagonal arrows are low-power chirp pulses of small flip angle β, set to 15° in all experiments for improving signal-to-artifact ratio due to strong coupling. G_1, G_2, G_3, and G_S are set to 41.2, 26.2, 33.7, and 1.2 G cm^{-1}, respectively. All gradient pulses, represented by grey-shaped pulses, with 1 ms duration, were followed by a recovery delay of 1 ms. t1 is incremented at every chunk for the pure shift dimension, reconstructed from the acquisition of a series of 50 chunks of 10 ms (τ_C) each. τ_1 and τ_3 are used to modulate the point of J refocusing during each chunk used for the interferogram, keeping a constant duration in comparison to each SAPPHIRE increment. Four SAPPHIRE increments were used in all experiments, but good chunk artifact suppression can also be achieved with only two increments. Full experimental details are provided in the ESI. ‡

Figure 3 shows a comparison of three SAPPHIRE water suppression methods for a sample of the macrocyclic peptide Aureobasidin A (AbA, Figure 3),[18] dissolved in 10% H_2O / 90% CD_3CN mixture: NOESY-pre-saturation (Fig. 3A), iW5 (Fig. 3B), and iES (Fig. 3C). Aureobasidin A, a cyclic depsipeptide antifungal antibiotic, was chosen as a stringent test case, since in solution it exists as a mixture of trans and cis-proline (Pro) amide bond conformers,[18] greatly complicating NMR spectra as most protons yield two sets of resonances. Several aliphatic protons of AbA are near the water peak, and it is immediately noticeable that both optimized NOESY-pre-saturation and iW5 strongly attenuated these proton resonances. The pre-saturation pulse in the former was optimized for slightly stronger power, as a low power saturation tends to leave unwanted water magnetization, which causes severe baseline distortion. Although iW5 completely eliminated the water peak, SAPPHIRE-iES was the only method to retain nearby desirable resonances as close as 0.1ppm from the water peak while adequately suppressing it. No chunking artifacts were observed from any of these methods, yielding clean spectra. Because pre-saturation requires a long water saturation period, the overall experimental duration is approximately twice as long as for the internally encoded water suppression experiments.

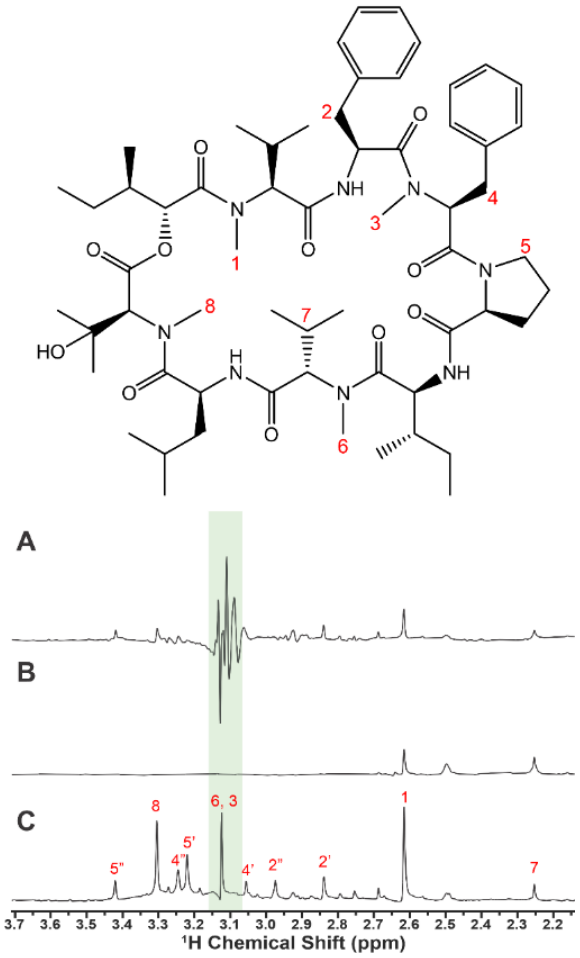

Fig. 3. Aliphatic region of 1H pure shift NMR spectra of aureobasidin A in 10% H2O / 90% CD_3CN mixture. Application of SAPPHIRE-NOESY-pre-saturation (A) generated a spectrum with a large residual solvent peak (green box) and significantly reduced the intensity of nearby analyte signals. SAPPHIRE-iW5 (B) led to complete suppression of the residual water peak but also suppressed neighboring analyte signals. SAPPHIRE-iES (C) demonstrated the best H_2O suppression selectivity by preserving the observation of analyte signals in immediate proximity to the suppressed frequency. Note that peak assignments are for the trans-Pro rotamer of AbA, which is the major conformer under these solvent conditions. Unlabelled minor peaks correspond to the cis-Pro rotamer of AbA.

Screening of water / organic solvent mixtures with varying solvent ratios is common for macrocyclic peptides. This is done to improve solubility, optimize NMR lineshape, attenuate the presence of minor conformers, and resolve overlapped signals. [9a, 18c] Changing water/organic solvent ratios can drastically change the frequency of the water peak, necessitating the frequency flexibility of the water suppression pulse sequence. SAPPHIRE-iES was found to possess the requisite flexibility, as demonstrated on AbA samples in CD_3CN/H_2O mixtures in Figure 4. Changing water amount from 10% to 30% by volume moves its chemical shift downfield significantly, by almost one ppm. A typical solvent screen is aimed at finding conditions providing the best compromise between solubility, lineshape, and minimal interference of the water peak with sample resonances. The optimal solvent mixture would then be utilized for more advanced NMR experiments (as an example, see ESI Figure 4). In this context, SAPPHIRE-iES was the most useful pure shift experiment because of its ability to selectively suppress the solvent peak without interfering with nearby proton signals of interest. The flexible settings of the selective pulse duration in SAPPHIRE-iES allow facile optimization of solvent suppression versus signal retention for a variety of aqueous/organic solvent mixtures.

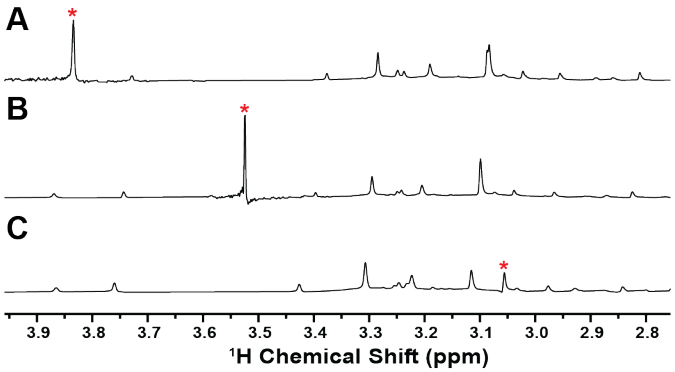

Fig 4. N-methyl and alpha proton regions of ^{1}H pure shift NMR spectra of aureobasidin A in 3 different H_2O/CD_3CN solvent mixtures using SAPPHIRE-iES: 30% (A), 20% (B) and 10% (C). Water resonances were selectively suppressed without a suppression of nearby analyte peaks. Residual water signals are marked with red asterisks in all 3 spectra.

The new class of ultra-clean pure shift NMR methods with internally encoded water suppression presented here offers a marked improvement for the analysis of aqueous samples. Overall, SAPPHIRE-iES has demonstrated superior, frequency-agnostic performance and we recommend that it should be used as the standard pure shift experiment for both aqueous samples and water/organic solvent mixtures. Although SAPPHIRE-iW5 showed near complete suppression of water resonances even in samples comprised almost entirely of protic water (i.e., 90% H_2O), its poorer selectivity can also non-selectively suppress desired analyte peaks close to the solvent resonance. One shared limitation observed for all three SAPPHIRE experiments was their inability to suppress strong coupling artifacts (Fig. ESI 2). iW5 was found to be the least favorable experiment for the observation of strongly coupled systems. Additionally, we expect that the iES approach can easily be implemented to suppress multiple solvent resonances simultaneously in cases of non-deuterated solvent mixtures.

Conflicts of interest

We thank Emma Gates, Dr. Jonathan Bradley, Dr. Daniel Berry, Prof. Mathias Nilsson, Prof. Gareth Morris, Dr. Ralph Adams, and Dr. Laura Castañar for sharing a preprint (ChemArxiv) describing related work, carried out independently.

Notes and references

‡ The interferogram FIDs were reconstructed with TopSpin macros available at https://www.nmr.chemistry.manchester.ac.uk/.The reconstruction for SAPPHIRE data is a two-step process: (i) the FID chunks are arrayed to form new FID interferograms, one per SAPPHIRE increment; (ii) each SAPPHIRE increment is added for the final averaged pure shift FID.

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