Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Magnetic Resonance 181 (2006) 327–330
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

Communication

An improved method for suppressing protein background in PFG NMR experiments to determine ligand diffusion coefficients in the presence of receptor

Bridget A. Becker [a], Kevin F. Morris [b], Cynthia K. Larive [a,*]

[a] University of California, Department of Chemistry, 328 Physical Sciences Building 1, Riverside, CA 92521-0403, USA
[b] Carthage College, Department of Chemistry, 2001 Alford Park Drive, Kenosha, WI 53140, USA

Received 4 October 2005; revised 23 February 2006
Available online 15 May 2006

Abstract

In NMR diffusion experiments to study ligand-protein binding equilibria, the spectral background due to broad protein resonances can contribute significantly to the measured ligand signal intensity resulting in erroneous binding affinities. One method to suppress the protein spectral background involves coupling a CPMG pulse train before or after the BPPSTE pulse sequence to allow for differential T_2 relaxation of the broad protein resonances. Here, we present an improved method, the Gradient Phase Encoded Spin-lock (GraPES) experiment that integrates the relaxation filter into the diffusion period. Compared with sequential CPMG-BPPSTE pulse sequences, GraPES offers effective suppression of the protein background with improved signal-to-noise ratios and shorter experiment times.

Keywords: Diffusion; PFG NMR; PGSE NMR

1. Introduction

Pulsed field gradient (PFG) NMR diffusion experiments are powerful techniques in drug discovery for identifying, quantifying, and mapping ligand-protein interactions [1–4]. For ligand-protein solutions, suppression of the protein background is of utmost importance or the power of diffusion techniques may be thwarted leading to misleading or incorrect conclusions [1,2,5]. Ligand signal intensities in NMR diffusion measurements can be significantly affected by overlap with protein resonances, even when the ligand is present at a large molar excess. Because ligand diffusion coefficients are much greater than those of the protein, the protein spectral background is especially problematic in experiments with the highest gradient areas [2,5]. Protein background suppression has been accomplished previously by spectral subtraction [5] or by combining the diffusion experiment with a CPMG pulse train to eliminate the broad protein background by differential T_2 relaxation [6,7].

Recently, IDOSY experiments were introduced that combine internal diffusion encoding with elements of other experiments such as 2D-*J*-resolved spectroscopy, HMQC, and INEPT [8,9]. The main advantages of the IDOSY pulse sequences over the corresponding sequential pulse sequences are reduced experiment time and increased signal-to-noise ratios [8].

Building on the IDOSY concept, we developed a new pulse sequence, the Gradient Phase Encoded Spin-lock (GraPES), which combines a continuous wave spin-lock for protein background suppression with bipolar gradient pulse pair sandwiches for diffusion encoding and decoding. The spin-lock retains the diffusion and chemical shift information for small molecules while acting as a filter to selectively eliminate the broad protein background. Fig. 1 illustrates the pulse sequences for the three experiments used in this work, BPPSTE [10], BPPSTE-CPMG [6,7], and GraPES. Results obtained with these pulse sequences are compared for the ligand-protein system of

* Corresponding author. Fax: +1 951 827 4713.
E-mail address: clarive@ucr.edu (C.K. Larive).

1090-7807/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.jmr.2006.04.010

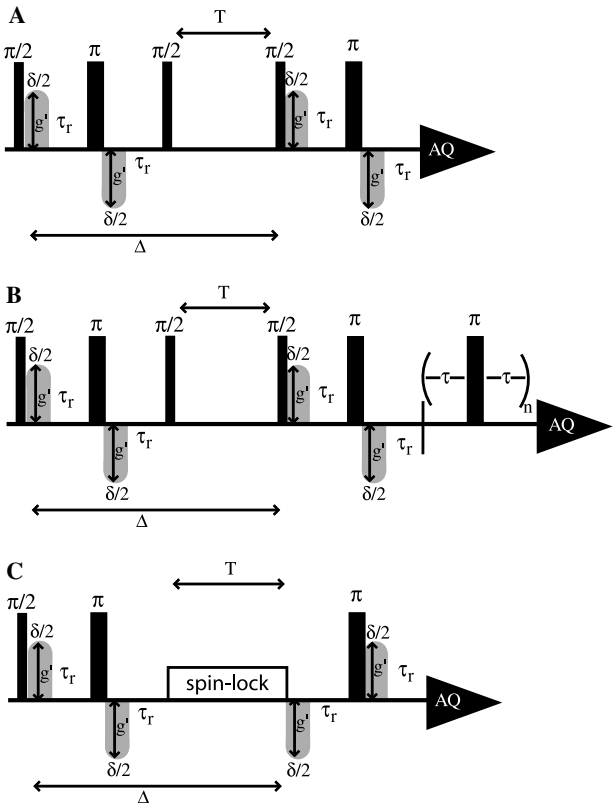

Fig. 1. (A) The pulse sequence for the bipolar pulsed pair stimulated echo (BPPSTE) experiment, (B) BPPSTE pulse sequence with appended Carr Purcell Meiboom Gill pulse train for T_2 editing (BPPSTE-CPMG), and (C) GraPES pulse sequence. Black bars represent rf pulses and gray bullets represent gradient pulses. The gradient amplitude is denoted by g′ and the gradient duration by $\delta/2$. The effective diffusion time is represented by Δ, the diffusion delay by T, the gradient recovery delay by τ_r, and the delay in the CPMG pulse train by τ.

R-propranolol (Fig. 2A) and human α_1-acid glycoprotein (AGP). AGP is the major serum binding protein for a number of basic drugs, including the anti-hypertension drug propranolol [11]. The dissociation constant (K_d) for R-propranolol and AGP is 2.65 μM [12].

2. Results and discussion

The spectra resulting from the BPPSTE, BPPSTE-CPMG, and GraPES experiments with a gradient amplitude of 18.34 G/cm are shown in Figs. 2B–D. The presence of the protein spectral background in Fig. 2B and difference in signal-to-noise ratio (S/N) between spectra 2C and D can be easily discerned by visual inspection. In our quantitative comparison, we selected the propranolol resonance at 8.20 ppm for the S/N calculation because the intensity of this peak will be least affected by the protein background. The S/N calculated for the spectra shown in Fig. 2 are 144, 43, and 106 for the BPPSTE, BPPSTE-CPMG, and GraPES experiments, respectively. Although the BPPSTE experiment yields the spectrum with the highest overall S/N, the protein background makes it ill-suited

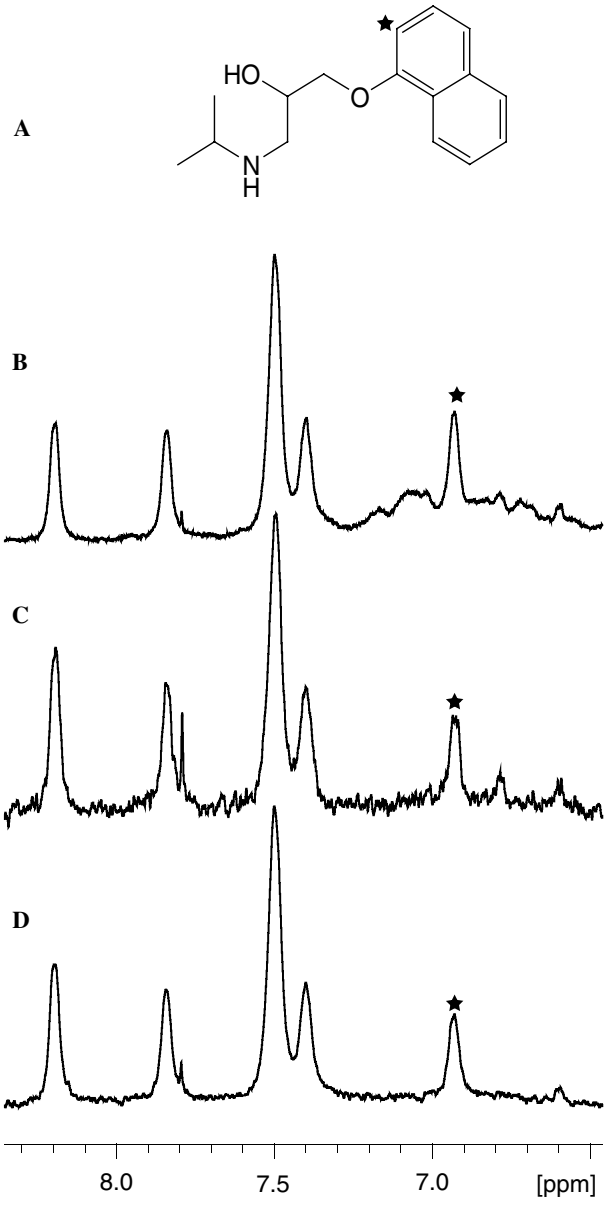

Fig. 2. (A) Propranolol structure. The ★ indicates the hydrogen responsible for the resonance at 6.93 ppm used to obtain the diffusion decays plotted in Fig. 3. (B) BPPSTE spectrum (C) BPPSTE-CPMG spectrum (D) GraPES spectrum.

for quantification of ligand intensity. As shown in Fig. 2C, the protein background is suppressed by the BPPSTE-CPMG experiment however, the S/N is significantly reduced relative to that obtained with the GraPES pulse sequence (2D). Greater signal losses are expected in the BPPSTE-CPMG experiment because of the sequential application of the diffusion period and the CPMG pulse train. Because the GraPES spin-lock is integrated into the diffusion period, intensity losses are minimized [8]. Depending on the particular behavior of the ligand-protein system and the extent to which ligand resonances are broadened by chemical exchange, additional sensitivity gains are expected from GraPES compared with the

analogous pulse sequence incorporating an internal CPMG relaxation period. Comparisons of GraPES, the pulse sequence with an internal CPMG pulse train, and the sequential BPPSTE-CPMG experiment for another R-propranolol and AGP solution gave signal-to-noise ratios of 34, 21, and 12, respectively, for the resonance at 8.20 ppm.

Careful inspection of Fig. 2 reveals a protein resonance near 6.6 ppm present in both the CPMG-BPPSTE and GraPES experiment that is not completely suppressed by either relaxation filter. Both CPMG and spin-lock relaxation filters rely on differences between the relaxation rates of the ligand and protein to preferentially eliminate the protein resonances. Large proteins have much longer rotational correlation times than small molecule ligands. Therefore, the nuclei of large proteins generally have faster rates of T_2 and $T_{1\rho}$ relaxation than do smaller proteins or ligands. However, NMR relaxation rates depend also on local correlation times, therefore nuclei in regions of high local mobility often have slower relaxation rates compared with more rigid segments of the protein and are not as effectively suppressed by relaxation filters.

The protein spectral background can distort ligand resonance intensities in diffusion experiments, especially at higher gradient amplitudes, leading to erroneous diffusion results. The diffusion decays for the propranolol resonance at 6.93 ppm are plotted in Fig. 3. The diffusion plot for the BPPSTE data is curved due to greater contribution of the protein background at higher gradient amplitudes. This problem is eliminated by both the BPPSTE-CPMG and GraPES experiments as evidenced by the good linear fits to the diffusion results in Fig. 3. Diffusion coefficients calculated from these fits, $5.65 \pm 0.11 \times 10^{-6}$ cm^2/s (BPPSTE-CPMG) and $5.60 \pm 0.13 \times 10^{-6}$ cm^2/s (GraPES) are in good agreement. However, these diffusion coefficients are quite different from that calculated from the BPPSTE data, $4.88 \pm 0.22 \times 10^{-6}$ cm^2/s. The higher error in the diffusion coefficient determined from the BPPSTE data results from the poorer linear fit due to the greater contribution of the protein spectral background at higher gradient amplitudes. In a binding affinity measurement, this BPPSTE result would lead to an overestimation of the fraction of propranolol bound to AGP. For this case, the fraction bound propranolol calculated from the diffusion coefficient measured using the BPPSTE experiment is 29%, while the fraction bound calculated using the BPPSTE-CPMG and GraPES experiments are 16% and 17%, respectively.

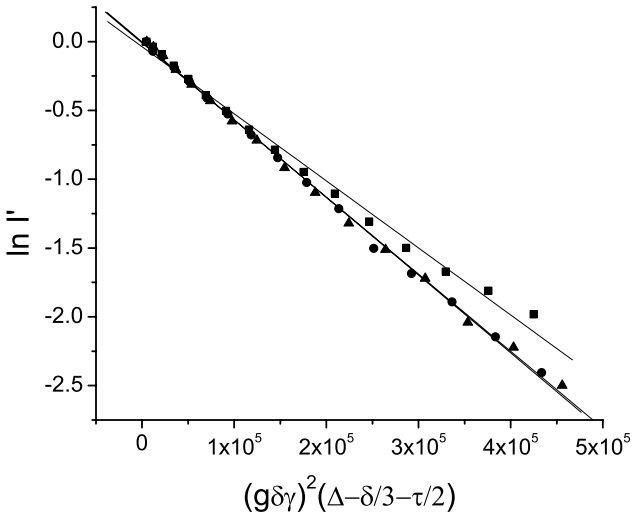

Fig. 3. Normalized diffusion decays for the propranolol resonance indicated in Fig. 2A for the BPPSTE experiment (■), the BPPSTE-CPMG experiment (●), and the GraPES experiment (▲).

3. Conclusion

By combining the diffusion and relaxation periods, the GraPES pulse sequence effectively suppresses the protein background while minimizing signal losses for the ligand resonances. As a result, this approach facilitates the accurate measurements of ligand diffusion coefficients in ligand-protein affinity experiments. Regardless of the specific diffusion experiment used care must be exercised in the selection of the ligand-protein concentration ratio. Depending on the binding affinity and exchange properties of the ligand-protein system, extensive broadening of ligand resonances may hinder efforts to measure the ligand diffusion coefficient, especially at low ligand-protein ratios, which minimize non-specific binding. These limitations are not specific to NMR diffusion measurements, but as pointed out by Fielding et al. [13] are a more general limitation of NMR measurements that rely on the properties of the free ligand.

4. Experimental

R-propranolol was from Tocris, human AGP was purchased from Sigma, and 99.99% D_2O was purchased from Cambridge Isotope Labs. All reagents were used as received.

All spectra were acquired with a Bruker Avance 600 MHz NMR spectrometer. The spectra shown in Figs. 2 B–D were acquired with a gradient amplitude of 18.34 G/cm for a solution of 2 mM (R)-propranolol and 0.1 mM AGP in D_2O, pD 7.42 in a limited volume Shigemi tube. The spectra used to produce the diffusion decays (Fig. 3) were acquired with the same solution for gradient amplitudes ranging from 3.32 to 31.50 G/cm. Each spectrum in the diffusion data sets was signal averaged for 512 scans, acquired with 23362 complex points during the 1.5 s acquisition time, and processed with 2 Hz of line broadening. The filter time for BPPSTE-CPMG and GraPES experiments was 50 ms. The repetition rate for the CPMG pulse train was 1.7178 ms and the GraPES spin-lock field strength was 3.64 kHz. Diffusion encoding gradient pulses of 1.75 ms were followed by 50 μs gradient recovery delays for each experiment. The diffusion delay time for the BPPSTE experiment was 46.3 and 50 ms for BPPSTE-CPMG. A 1 ms homospoil during the longitudinal storage period of the BPPSTE and BPPSTE-CPMG experiments were employed, however, dur-

ing the GraPES experiment magnetization remains transverse during the diffusion period and a homospoil cannot be used.

Although the spectra presented here were acquired without solvent suppression, standard solvent suppression methods (such as WET) should be compatible with all experiments. However, solvent suppression by selective saturation is not recommended for protein binding experiments to avoid transfer of saturation to ligand resonances. For solutions with a significant solvent resonance, we recommend the use of water suppression with the GraPES experiment because the lack of a homospoil gradient in this experiment can produce a phase twist due to residual transverse solvent magnetization [14].

Sixteen points make up each of the diffusion decays which were fit with Origin v.7. Errors of the diffusion coefficients were calculated by multiplying the standard fitting error calculated within Origin by the t value for the 95% confidence level.

References

[1] L.H. Lucas, J. Yan, C.K. Larive, E.R. Zartler, M.J. Shapiro, Transferred nuclear Overhauser effect in nuclear magnetic resonance diffusion measurements of ligand-protein binding, Anal. Chem. 75 (2003) 627–634.

[2] L.H. Lucas, C.K. Larive, Measuring ligand-protein binding using NMR diffusion experiments, Concepts Magn. Reson., Part A 20A (2004) 24–41.

[3] J. Yan, A.D. Kline, H. Mo, E.R. Zartler, M.J. Shapiro, Epitope mapping of ligand-receptor interactions by diffusion NMR, J. Am. Chem. Soc. 124 (2002) 9984–9985.

[4] A. Chen, M. Shapiro, Nuclear Overhauser effect on diffusion measurements, J. Am. Chem. Soc. 121 (1999) 5338–5339.

[5] T.S. Derrick, E.F. McCord, C.K. Larive, Analysis of protein/ligand interactions with NMR diffusion measurements: the importance of eliminating the protein background, J. Magn. Reson. 155 (2002) 217–225.

[6] J.A. Chin, A. Chen, M.J. Shapiro, SPEEDY: spin-echo enhanced diffusion filtered spectroscopy. A new tool for high resolution MAS NMR, J. Comb. Chem. 2 (2000) 293–296.

[7] W.H. Otto, C.K. Larive, Improved spin-echo-edited NMR diffusion measurements, J. Magn. Reson. 153 (2001) 273–276.

[8] M. Nilsson, A.M. Gil, I. Delgadillo, G.A. Morris, Improving pulse sequences for 3D diffusion-ordered NMR spectroscopy: 2DJ-IDOSY, Anal. Chem. 76 (2004) 5418–5422.

[9] M.J. Stchedroff, A.M. Kenwright, G.A. Morris, M. Nilsson, R.K. Harris, 2D and 3D DOSY methods for studying mixtures of oligomeric dimethylsiloxanes, Phys. Chem. Chem. Phys. 6 (2004) 3221–3227.

[10] D.H. Wu, A.D. Chen, C.S. Johnson, An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses, J. Magn. Reson. A 115 (1995) 260–264.

[11] R. Kaliszan, A. Nasal, M. Turowski, Quantitative structure-retention relationships in the examination of the topography of the binding site of antihistamine drugs on alpha(1)-acid glycoprotein, J. Chromatogr. A 722 (1996) 25–32.

[12] K. Hanada, T. Ohta, M. Hirai, M. Arai, H. Ogata, Enantioselective binding of propranolol, disopyramide, and verapamil to human alpha(1)-acid glycoprotein, J. Pharm. Sci. 89 (2000) 751–757.

[13] L. Fielding, S. Rutherford, D. Fletcher, Determination of protein-ligand binding affinity by NMR: observations from serum albumin model systems, Magn. Reson. Chem. 43 (2005) 463–470.

[14] A.R. Waldeck, A.J. Lennon, B.E. Chapman, P.W. Kuchel, ^{4}Li and ^{23}Na nuclear magnetic resonance studies of transport and diffusion in liposomes: Comparison of transport rate constants estimated using pulsed field gradient and magnetization-transfer procedures, J. Chem. Soc. Faraday Trans. 89 (1993) 2807–2814.