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A Simple and Noninvasive DOSY NMR Method for Droplet Size Measurement of Intact Oil-In-Water Emulsion Drug Products

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Abstract

In a typical oil-in-water emulsion drug product, oil droplets with varied sizes are dispersed in a water phase and stabilized by surfactant molecules. The size and polydispersity of oil droplets are critical quality attributes of the emulsion drug product that can potentially affect drug bioavailability. More critically, to ensure accuracy in characterization of the finished drug product, analytical methods should introduce minimal physical perturbation (e.g., temperature variation or dilution) before the analysis. The classical methods of dynamic light scattering or electron microscopy can be used but they generally require sample dilution or harsh preparation conditions, respectively. By contrast, the size distribution of emulsion formulations can be assessed with a simple and noninvasive solution nuclear magnetic resonance method, namely, two-dimensional Diffusion Ordered Spectroscopy. The two-dimensional Diffusion Ordered Spectroscopy method probed signal decay of methyl resonances from oil and sorbate molecules and was applied to 3 types of U.S.-marketed emulsion drug products, that is, difluprednate, cyclosporine, and propofol, yielding measured droplet sizes of 40–280 nm in diameter. The high precision of ± 6 nm of the new nuclear magnetic resonance method allows analytical differentiation of lot-to-lot and brand-to-brand droplet size differences in emulsion drug products, critical for drug-quality development, control, and surveillance.

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Keywords

NMR spectroscopy; particle sizing; diffusion; surfactants; emulsion/microemulsion

Introduction

Advances in pharmaceutical sciences and manufacturing methods have given rise to an increase in the development and approval of complex drug products. Complex drug products can have molecular, compositional, or drug distribution heterogeneity, pertaining to differences in higher order structure, particle size distribution (PSD),^{1,2} chemical composition,^{3,4} or multiphase dosage forms.⁵ These quality attributes can affect drug bioavailability, efficacy, safety, immunogenicity, and PK/PD properties, and present challenges for drug manufacturers and regulatory agencies to define robust and sensitive methods for measuring these critical quality attributes.

One class of complex drug products is emulsion formulations, which are composed of oil droplets dispersed in a continuous water phase. The emulsion drug products usually contain hydrophobic molecules as active pharmaceutical ingredients that are formulated in oil-in-water dosage forms for ease of application, safety, and efficacy.⁶ In an emulsion formulation, oil globules (~diameter in 30–300 nm) are dispersed within an aqueous phase with the aid of surfactants, which serve as emulsifiers and stabilizers by lowering interfacial tension.⁷ Depending on the formulation composition and manufacture process,⁸ the particle size and activity of an emulsion drug product can vary.^{9–11} Therefore, a facile measurement of the emulsion droplet size distribution is a critical measure to ensure the consistent quality of an emulsion drug product.

The classical methods of light scattering (LS),¹² dynamic light scattering (DLS),¹³ electron microscopy (EM),¹⁴ and acoustic spectroscopy^{15,16} have been applied to analyze droplet sizes of emulsion formulations. These methods often require preparation of the emulsion sample either through dilution (for LS and DLS) or sample alteration (for EM). Thus, LS, DLS, and EM sample preparation protocols may affect the accuracy of the measured emulsion droplet sizes. In addition, the classical methods often are not able to distinguish particles with different chemical compositions.^{17,18} In addition, for regulatory purposes, analytical methods that can measure the drug formulation in conditions that are as close as possible to the final drug product are desired. Presently, there are few simple and robust measurement techniques that can measure the droplet size of undiluted emulsion products that have an oil volume fraction greater than a few percent without drug sample manipulation.

In the present study, two-dimensional (2D) Diffusion Ordered Spectroscopy (DOSY) nuclear magnetic resonance (NMR) using 4-mm diameter NMR tubes (instead of the more common 5 mm tubes) and a room temperature probe was demonstrated to achieve the necessary DOSY signal decay while avoiding heat-driven convection within the viscous oil emulsion samples. The new method was applied to 3 types of U.S.-marketed emulsion products, yielding measured droplet sizes of 40–280 nm in diameter with a precision of ± 6 nm. These data show that DOSY NMR can be applied to the analysis of intact emulsion

drug products, yield precise measurements of droplet size distributions, and assure the chemistry of the measured particles.

Materials and Method

Samples/Materials

Studies were performed on commercially available emulsion drug products purchased from U.S. market. A total of 8 lots of difluprednate (DFPN) ophthalmic emulsion, 3 lots of cyclosporine A (CsA) ophthalmic emulsion, and 4 lots, 2 lots each from 2 different manufacturers, of propofol injectable emulsion products were analyzed (Supplementary Table S1). Per label claim, DFPN and CsA ophthalmic emulsion products, which are marketed under the trade name Durezol® and Restasis®, respectively, contain castor oil and polysorbate 80 (PS-80) as the major oil-droplet components. Parenteral emulsions of propofol (Diprivan® and Propofol Injectable Emulsion) contain soybean oil and egg lecithin as the major oil droplet components. All drug formulations appeared as white opaque, to slightly translucent, stable emulsions.

NMR Spectroscopy

Each NMR sample was composed of 0.33 mL of emulsion drug product and 0.02 mL of D₂O (Cambridge Isotope Laboratories, Tewksbury, MA) with 3-(trimethylsilyl)-2,2,3,3-tetradeutero propionic acid (Sigma Aldrich, St. Louis, MO). The total of 0.35 mL sample was loaded to a 4 mm NMR tube (Wilmad, NJ) (Supplementary Fig. S1). It could also be possible to use 5 mm NMR tube with a glass insert containing D₂O to avoid sample dilution. ¹⁹F NMR experiments were performed on a Bruker 850-MHz NMR instrument equipped with a triple-gradient TXI room temperature probe capable of generating gradient field strength of 54 G/cm. The modified DOSY pulse sequence (ledbpgp2s) with 3919 water suppression was used to measure diffusion coefficients.¹ The DOSY diffusion time interval (d20) and gradient pulse length (p30) were set at 1500 ms and 2 ms (2000 ms and 2.8 ms for propofol emulsions), respectively, with a recycle delay (d1) of 3 s. Each 1D free induction decay had 4K complex points with 64 scans averaged. The diffusion gradients were ramped from 2% to 98% at linear increments to generate 64 increments in the diffusion dimension. About 75%–85% signal attenuation was achieved for the above 2D DOSY experimental parameters. All experiments were performed at 25°C temperature and each experiment took about 5.5 h.

DOSY NMR data processing was performed using MestReNova 11.0 software (Mestrelab Research S.L.). Each free induction decay in the 2D DOSY data sets were apodized using a Gaussian 2 Hz line broadening function, zero filling to 16-K data points, and baseline correction with a first-order polynomial fit. Manual phase correction was performed to further fine tune the first 1D spectrum and then was applied to the entire data set. The 2D DOSY data were processed along the second dimension using the Bayesian Transform algorithm in MestReNova. The DOSY processing parameters were set at 1×10^{-8} and 1×10^{-5} cm² s⁻¹ for minimum and maximum diffusion coefficient limits, respectively; 1 for resolution factor, 5 for repetition number, 512 for the number of points in diffusion dimension, and 1 for reduce size factor. The 2D DOSY NMR spectra peak picking was performed using the Mnova automatic peak picking function for all emulsion formulations.

The standard method of automatic peak picking in Mnova picked all the peaks over the sensitivity threshold limit set to 100. Peaks between 0.84 and 0.95 ppm corresponding to fatty acid terminal methyl groups (H18) were selected for analysis as the representative peaks for oil droplet diffusion measurements.

Data Analysis

The exponential decay of DOSY NMR peak intensity $I(x)$ can be described by Stejskal-Tanner²⁰ equation Equation 1,

$$I(x) = I_0 \cdot e^{(-Dx)} \quad (1)$$

where, $I(x)$ is the observed peak intensity, I_0 is the reference (un-attenuated) peak intensity, and D is the diffusion coefficient in $\text{cm}^2 \text{s}^{-1}$. The parameter x can be calculated by Equation 2 below,

$$x = \gamma^2 G^2 \delta^2 (\Delta - \delta/3) \quad (2)$$

where, γ is the gyromagnetic ratio (^1H) of observed nucleus ($2.675 \times 10^4 \text{ s}^{-1} \text{ gauss}^{-1}$), G (gauss cm^{-1}) is the corrected gradient strength obtained from the “difflist” file in NMR data set, δ is the-gradient pulse length ($p30 \times 2$, 0.004 s) for coherence echo, and Δ is the diffusion time (d20, 1.5 s) between the 2 gradient echo pulses.

Glycerol, a commonly found excipient in emulsion products, served as internal calibration molecule. The experimental glycerol diffusion coefficient $D_{gly(meas-f)}$ value was obtained by curve fitting of the integrals of the glycerol peak between 3.52 ppm and 3.62 ppm. The peak area (I) and gradient-specific data series x , defined in Equation 2, were fitted to triple exponential fitting Equation 3 within the Grace-5.1.25 software package,

$$I = I_0 + I_1 \cdot e^{(-x D_{gly(meas-f)})} + I_2 \cdot e^{(-x D_{gly(meas-s)})} + I_3 \cdot e^{(-x D_{gly(meas-s3)})} \quad (3)$$

where, $D_{gly(meas-f)}$ and $D_{gly(meas-s)}$ represent the diffusion coefficients of faster and slower diffusing glycerol species and I_1 , I_2 , and I_3 were the spin intensities of respective molecular species. I_0 is the baseline intensity. The faster diffusing glycerol was designated as water-soluble free glycerol, and the value of $D_{gly(meas-f)}$ was used for viscosity-corrected diffusion coefficient calculations (Supplementary Table S1).²¹

The experimental values of oil diffusion coefficient $D_{oil(meas)}$ obtained from 2D DOSY NMR spectra were corrected to the oil droplet diffusion coefficient $D_{oil(cor)}$ at 25°C in pure water solvent through Equation 4, where the measured $D_{gly(meas-f)}$ was obtained from Equation 3, and the standard $D_{gly(25^\circ\text{C})}$ glycerol diffusion coefficient ($1.025 \times 10^{-5} \text{ cm}^2 \text{s}^{-1}$ at 25°C) in pure water was used.²²

$$D_{oil(cor)} = D_{oil(meas)} \cdot D_{gly(25^\circ\text{C})} / D_{gly(meas-f)} \quad (4)$$

The corrected oil diffusion coefficients $D_{oil(cor)}$ were then used for calculating respective oil droplet hydrodynamic diameter using the Stokes-Einstein Equation 5.

$$D_h = kT / (3\pi\eta D_{oil(cor)}) \quad (5)$$

where, D_h is hydrodynamic diameter ($D_h = 2 \times$ hydrodynamic radius R_h), k is Boltzmann constant ($1.38 \times 10^{-16} \text{ cm}^2 \text{ g s}^{-2} \text{ K}^{-1}$), T is temperature (298.15 K), η is the viscosity of water ($0.00910 \text{ g cm}^{-1} \text{ s}^{-1}$) at 25°C. The hydrodynamic diameter calculation assumes spherical shape for oil droplets.

DOSY peak integrals were plotted against the corresponding oil droplet sizes. The DOSY data for DFPN samples had a larger heterogeneity and broader peak distribution; therefore, D10, D50, and D90 values were used to report the droplet size distribution. The DOSY derived D10/D50/D90 values are the first-pass intercepts for 10%, 50%, and 90% of the cumulative 2D DOSY NMR peak integration area.

For DOSY NMR spectra of CsA and Propofol emulsions, diffusion coefficients from the automatic detected methyl peaks were designated to the individual droplet diameters (D_i). The corresponding peak integrals were recorded as a pseudopopulation or volume representation. The peak integral area and the corresponding D_i were weight averaged to yield mean droplet size $\langle D_h \rangle$ using Equation 6, where “ n ” is the number of methyl peaks identified in an individual 2D DOSY NMR spectrum.

$$\langle D_h \rangle = \sum_{i=1}^n \% \text{ normalized peak integral} \cdot D_i \quad (6)$$

Results

Chemical Nature of DOSY NMR–Measured Particle

For the emulsion products studied here, oil and surfactant were the major components of oil droplets and produced strong NMR signals due to the presence of flexible aliphatic chains (Supplementary Fig. S2). The 2D ^1H - ^{13}C heteronuclear single quantum coherence NMR spectral analysis of drug products and literature results^{23–26} confirmed that most of the strong DOSY NMR peaks belong to excipients such as glycerol, surfactants, and castor oil/soybean oil. The terminal methyl groups of the fatty acid chains are suitable markers for measuring the diffusion properties of the oil droplets because of their strong intensity. The assignments of these key resonances (Supplementary Table S2 and Figs. S3 and S4) were used to identify respective molecules and measure diffusion coefficients of associated components using 2D DOSY NMR spectra.²⁷ Although similar 18-carbon aliphatic chains were present in castor oil and PS80, due to the unique hydroxyl group at C12 in ricinoleic acid (Table S2 and Fig. 1a), the oil-specific peaks can be identified at chemical shifts 2.18 ppm and 1.44 ppm for ricinolein H11 and H13, respectively. These ricinolein oil signature peaks were observed at slower diffusion coefficient region of 2D DOSY spectra and were associated with oil droplet sizes of 50 nm and above (Fig.1a). The oil signature peaks were

not detectable in 2D DOSY peaks smaller than 50 nm, probably due to the less oil content in such particle. Nevertheless, the size of pure PS-80 micelle is about 8–12 nm,^{28–30} so it was believed that all the identified DOSY H18-methyl peaks (>15 nm) belong to oil droplets with PS-80 as the surfactant. However, when the 2D DOSY spectrum plotting threshold was significantly lowered, the possible pure PS-80 micelle diffusion peaks were also observed at relatively faster diffusion coefficient region of the DOSY spectrum (Supplementary Fig. S5). The total peak area for these micelle diffusion peaks was about 0.03% of the smallest area peak for an oil droplet, suggesting the number of pure micelles to be very low.

DOSY Method Reproducibility

The 2D DOSY NMR experiment measures diffusion coefficient along the y -axis and the ^1H chemical shift along the x -axis (Figs. 1a and 1b).³¹ The H18 methyl diffusion peaks around 0.9 ppm from either castor oil or PS-80 fall in the range between $1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ and $3 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for DFPN emulsion (Fig. 1b). The droplet size distributions were represented by cumulative size distribution values D10, D50, and D90 (Fig. 1c). Five technical replicates for a single lot of DFPN emulsion were used to test DOSY method reproducibility (Table 1). The droplet size observed for D10 was $56 \pm 2 \text{ nm}$. The average size of D50 species was $97 \pm 6 \text{ nm}$ and that of D90 was $119 \pm 6 \text{ nm}$. Because the 2D DOSY presents data based on the Bayesian fitting processing, a larger method variation is expected for complex formulations such as polydisperse emulsions. The integral normalized average droplet size was $93 \pm 5 \text{ nm}$ for this sample. The overall method variation in droplet sizes for the tested DFPN sample lot was about 5%, demonstrating a high method reproducibility in analyzing complex emulsion formulations.

Droplet Size Distributions for DFPN Emulsions

DFPN oil droplet sizes were measured for 8 lots. The interlot-averaged droplet sizes were $51 \pm 7 \text{ nm}$, $84 \pm 13 \text{ nm}$, and $103 \pm 16 \text{ nm}$ for D10, D50, and D90, respectively. The interlot variation of about 14%–16% observed in droplet sizes was much larger than the method reproducibility variation of 5%. The interlot size variation for integral normalized diameter was about 15% and was also higher than the values obtained for the method variation of 5% (Tables 1 and 2). Similar covariance of 14%–16% was observed for individual D10, D50, and D90 droplet sizes. The integral normalized average droplet size indicates that most oil is distributed around $81 \pm 12 \text{ nm}$, which is closer to the D50 size droplet species (Table 2). These data suggest that most of the oil was formulated in the range between 70 and 90 nm. The fact that interlot variations were larger than the method variations demonstrated that the 2D DOSY method is sufficiently sensitive to evaluate differences across multiple lots.

Droplet Size Distribution for CsA Emulsions

The measured DOSY methyl diffusion peaks for the CsA emulsions were closely spaced, and fewer DOSY peaks were observed compared to the DFPN products. Because of the narrow distribution of particle sizes, no cumulative distribution analysis was necessary for CsA samples. The diffusion-derived droplet sizes were simply integral averaged (Eq. 6) to obtain the mean oil droplet diameter of $102 \pm 1 \text{ nm}$ for the 3 different lots (Table 3).

Droplet Size Distribution for Propofol Emulsions

The droplet size variation of propofol emulsions from 2 different manufacturers were measured and compared. Similar to CsA, a narrow droplet size distribution was observed for all propofol products tested. In addition, the automatic peak-picking protocol identified multiple diffusion peaks that had a tight distribution. Therefore, D-value cumulative distribution analysis was not required. The interlot average droplet size for the Diprivan® product was 259 ± 10 nm, and the approved generic propofol product sized closely at 273 ± 15 nm (Table 3). The droplet sizes observed for propofol formulations were much larger than the DFPN and CsA ophthalmic emulsion products and demonstrated that the 2D DOSY method can be applied to a wide range of particle sizes.

Discussion and Conclusion

In this work, a simple and robust technique using 4-mm NMR tubes and a 2D DOSY NMR method was developed to measure emulsion droplet size in an intact oil-in-water emulsion formulation with minimal sample preparation (i.e., the addition of 5% D₂O for a lock signal). By selecting NMR peaks with chemical shifts specific for oil droplet components from the DOSY spectra, a wide range of oil droplet diffusion coefficients can be identified in various emulsion products with little interference from nonoil droplet molecules. The NMR chemical shift data for Durezol® also indicated that nearly all the surfactant molecules were associated with oil droplet. Therefore, micelles composed of only PS-80 surfactant in these oil-in-water emulsion products might be minimal (Supplementary Fig. S5). The method presented here was demonstrated to be fit-for-purpose for measuring the oil droplet size in various oil-in-water emulsion drug products. Because only ¹H NMR on a regular room temperature probe was collected in this work, the DOSY method should be adaptable using widely available solution state NMR instruments (including lower field) equipped with a z-gradient amplifier. Furthermore, the approach was shown to yield meaningful interlot and inter-brand differences with a precision of $\pm 5\%$. The sensitivity and reproducibility of the method are sufficient to detect differences in emulsion formulations caused by differences in the manufacturing processes, materials, or the source of the excipients used in drug products.

In the current method of 2D DOSY NMR, the peak integrals (spin intensity) contributed by H18 protons of ricinoleic acid and PS80 were considered as the population measure of each diffusion species, assuming no difference in spin relaxation rates among differently sized oil droplets. Therefore, the DOSY NMR-measured droplet size results should be comparable to volume (mass)-averaged droplet size from other methods. The DOSY NMR-measured integral average size of oil droplets in Restasis® was 102 ± 1 nm. Recently reported data for the DLS-based volume-averaged droplet size distribution for 10x diluted Restasis® sample had 2 populations centered around 35 nm (~85% volume between 20 and 140 nm range) and 240 nm (~15% volume between 160 and 600 nm range).¹⁴ Similar volume averagebased size distribution has been observed for Restasis® using asymmetric flow field flow fractionation (AF4) coupled with refractive index (RI) detector, where a majority of oil concentration can be seen between 35 and 80 nm (~90% of total volume) and minor species sizing between 100 and 300 nm size.¹⁴ It is not clear if any correction factors like viscosity or RI needs to be adjusted in DLS and AF4. Nevertheless, the DOSY NMR results of 102

± 1 nm for intact in Restasis® converge between the 2 populations reported by DLS and AF4, but closer to the primary species, suggesting DOSY-NMR measures an ensemble average for all the droplet species. In another comparison, propofol emulsion products reported a volume percent size range between 40 nm and 500 nm with a mean volume percent size peak around 200 nm.^{13,32} The DOSY NMR measured integral average size of oil droplets in Diprivan® was 259 ± 10 nm. Therefore, the particle sizes determined by 2D DOSY NMR in the present study are on par with the previously reported sizes.

The DOSY NMR method described here is a sensitive and noninvasive analytical method for volume-averaged particle size and chemical component analysis and is a new analytical tool for the analysis of complex oil-in-water emulsion drug products. Although we are not sure about the upper size detection limit of DOSY NMR method, currently 283 nm for one intravenous product (Table 3), we estimate the DOSY sensitivity is up to micron range. Nevertheless, the proposed 2D DOSY-NMR method provides an improved quality measure for formulation consistency across different lots and brands, enabling comparisons among complex emulsion products with minimal sample preparation. The designed analytical technique can be a potential method for comparative analysis in developing complex generic products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

2D DOSY NMR analysis of one lot of emulsion drug product Durezol®. (a) 2D DOSY NMR spectrum. RA indicated ricinoleic acid, acid part of the castor oil component ricinolein. PS indicated polysorbate 80. The terminal H18 methyl peaks of RA and PS overlapped and were read out for diffusion coefficients and peak areas for oil droplet size calculation. (b) The expanded DOSY spectrum showing H18 methyl diffusion peaks corresponding to oil droplets with varied size [black rectangle at around 0.9 ppm in A] (c) The normalized H18 methyl peak integral area corresponding to the respective droplet size.

Table 1

2D DOSY NMR Measured Oil Droplet Sizes for a Single Lot of Durezol®

Replicates	Droplet Size (nm)			Integral Normalized D_h (nm)
	D10	D50	D90	
1	52.58	97.36	111.22	88.52
2	54.84	95.04	119.52	92.10
3	57.42	95.02	121.14	93.34
4	58.86	107.22	127.78	101.41
5	54.56	91.82	117.1	89.81
Average	56 ± 2	97 ± 6	119 ± 6	93 ± 5
% CV	4%	6%	5%	5%

Table 2

Interlot Emulsion Droplet Size Distribution for Durezol®

Lot No.	Droplet Size (nm)			Integral Normalized D_h (nm)
	D10	D50	D90	
1	37.91	69.68	90.06	67.50
2	45.76	66.86	78.56	64.35
3	56.50	88.2	111.08	85.60
4	56.19	93.94	105.88	85.86
5	49.73	77.80	96.54	75.44
6	50.28	78.56	97.50	77.27
7	58.30	102.82	129.26	99.31
8	54.56	91.82	117.10	89.81
Average D_h	51 ± 7	84 ± 13	103 ± 16	81 ± 12
% CV	14%	15%	16%	15%

Table 3

Interlot Oil Droplet Size Distribution for Restasis® and Two Propofol Drug Products Diprivan-® and Propofol-Injectable Emulsions

Drug Product	Lot No.	Droplet Peaks Identified	Hydrodynamic D _h (nm)	Integral Normalized D _h (nm)	Interlot Averaged D _h (nm)
Restasis®	1	1	102.62	102.62	102 ± 1
		2	102.62		
	2	1	101.48	101.48	
		2	101.48		
		3	101.48		
		4	102.83		
		5	102.83		
	3	1	101.62	102.39	
		2	102.99		
	1	1	266.14	266.14	259 ± 10
Diprivan®	2	1	248.58	252.52	
		2	251.99		
		3	257.27		
	1	1	283.54	283.54	
		2	283.54		273 ± 15
Propofol Injectable Emulsion	2	1	248.87	262.38	
		2	260.09		
		3	266.10		