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Application of Ultra-Centrifugation and Bench-Top ^{19}F NMR for Measuring Drug Phase Partitioning for the Ophthalmic Oil-in-Water Emulsion Products

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Abstract

Generic drug products are expected to have the same active pharmaceutical ingredient (API) (Q1) with the same content (Q2) and microstructure arrangement (Q3) as the innovator product. In complex oil-in-water emulsion drugs, the hydrophobic API is mainly formulated in oil droplets stabilized by surfactant and micelles composed of extra surfactant molecules. The API phase partition in oil and water (mainly micelle) is a critical quality attribute (CQA) of emulsion product in demonstrating physicochemical equivalence using difluprednate (DFPN) emulsion product Durezol® as a model, we developed a novel low-field benchtop NMR method to demonstrate its applicability in measuring DFPN phase partition for ophthalmic oil-in-water emulsion products. Low-field ^{19}F spectra were collected for DFPN in formulation, in water phase and oil phase after separation from ultra-centrifugation. The NMR data showed the mass balance of DFPN before and after phase separation. The average water phase content of different Durezol® lots was $32 \pm 3\%$ with 1% variation from method reproducibility test. The partition results were $52 \pm 2\%$ for the in-house control products prepared in Q1/Q2 equivalence to Durezol® but by a different process. The significant difference in DFPN-phase partition between Durezol® and the in-house formulation

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demonstrated manufacture difference readily changed the API partition. The newly developed ultra-centrifugation and ^{19}F NMR by benchtop instrument is a simple, robust, and sensitive analytical method for ophthalmic emulsion drug product development and control.

Keywords

durezol; API; distribution; phase; NMR

INTRODUCTION

Difluprednate (DFPN), a potent corticosteroid, is an active pharmaceutical ingredient (API) used in ophthalmic emulsion drug products, prescribed for the treatment of postoperative ocular inflammation and pain. The originator product Durezol®, containing 0.5 mg/mL of DFPN, was approved by the U.S. FDA in 2008 (1–3). The drug is an oil-in-water emulsion where oil globules are dispersed within an aqueous phase with the aid of a surfactant which serves to cover the oil/water interface and lower the interfacial tension. In the emulsion formulation, excess surfactants (beyond those required to stabilize the oil/water interface) are commonly used and may form micelles. Depending on the formulation composition and/or manufacture process, the drug may distribute in various phases, *i.e.*, oil, micelle and aqueous phase (4). Therefore, measurement of the distribution of the API in each phase is important as the phase distribution may impact drug delivery in ocular tissues (4,5). DFPN (Fig. 1) is not soluble in water (9.7 $\mu\text{g/mL}$ at 25 °C) and its water phase partition is expected to be predominantly in micelle and surfactant layer on the oil droplet surface. Presently, there is no simple measurement technique that measures drug-phase distribution in emulsion products.

In the current study, ultra-centrifugation was selected to separate the oil and aqueous phases of emulsion products (6,7). Once separated, a method for detection of the API in each hydrophobic phase was necessary. Previously, low-field Nuclear Magnetic Resonance (NMR) spectroscopy had been used for emulsion globule size measurements (8,9), and, for DFPN, ^{19}F NMR was selected for quantitative analysis of the fluorinated drug (10). ^{19}F NMR has unique advantages in measuring a clean API spectrum because excipients usually do not have ^{19}F atoms. In addition, proton decoupled ^{19}F NMR can sharpen the ^{19}F peaks further by removing ^1H - ^{19}F scalar coupling if present. Notably, at low field (< 100 MHz), the large chemical shift dispersion nuclei like ^{19}F is disbursed across a smaller frequency range which simplifies data analysis. Overall, the low-field bench-top NMR has practical value as a simple and inexpensive method compared to traditional high-field NMR (> 500 MHz) (11). Here, originator and in-house made oil-in-water emulsions were separated by ultra-centrifugation then tested with a 43-MHz bench-top NMR. The method reproducibly quantified DFPN phase partitioning in oil-in-water emulsions.

MATERIALS AND METHODS

Drug Samples

A total of nine lots of Durezol® oil emulsion products (A1–A9) and 2 in-house formulations (B1 and B2) (as control products made by a different process) were used for analysis (Table S1 and Fig. S1). Each milliliter of ophthalmic emulsion contains difluprednate 0.5 mg (0.05%), boric acid, castor oil, glycerin, polysorbate 80, water for injection, sodium acetate, sodium EDTA, and sodium hydroxide (to adjust the pH to 5.2 to 5.8). The in-house formulations were made qualitatively (Q1) and quantitatively (Q2) equivalent to the Durezol® but differed in manufacturing processes and physicochemical properties from the reference listed drug (RLD), Durezol®.

NMR Samples

Ultra-centrifugation was performed using an Optima™ TLX ultracentrifuge (Beckman Coulter, Brea, CA) to separate the water phase from the oil phase (Fig. S2). Each 1.00 mL aliquot of the emulsion formulation was loaded into a 1.5-mL ultracentrifuge tube (Beckman Instruments, Palo Alto, CA) and spun at 55,000 rpm for 6 h at 25 °C using rotor model #TLS-55 (equivalent to 2.6×10^5 g). The higher speed allowed a cleaner separation of oil phase from water phase. Longer spin time might be needed for other viscous drug samples like Restasis®. About 0.9 mL of the water phase was extracted out immediately after ultracentrifugation using a 1-mL needle-syringe (Becton Dickinson and Company, Franklin Lakes, NJ) punched through the lower sidewall of the ultracentrifuge tube. The water phase samples were lyophilized overnight and dissolved in 0.5-mL co-solvent of DMSO-*d*₆/CDCl₃ (2:1, v/v) (Cambridge Isotope Laboratories, Andover, MA). The remaining oil phase was about 60 mg in a semi-solid state, which was left in the open ultracentrifuge tube for about 20 h at room temperature to liquefy before being dissolved in the same 0.5-mL co-solvent of DMSO-*d*₆/CDCl₃. Another two 1-mL intact emulsion formulations (A3 and B1) were lyophilized overnight as well to remove all the water, and then the remaining liquid was dissolved in 0.5-mL of the co-solvent for the total assay. For standard curve, pure DFPN (Sigma, St. Louis, MO) was prepared at a range of concentration from 0.01 mg/mL to 5 mg/mL in the same co-solvent (Fig. S3). Each dissolved sample was transferred to a 5-mm NMR tube (Wilmad LabGlass, Vineland, NJ) for analysis. The sample volume was determined by comparing sample height in the NMR tubes to a series of separately prepared blank co-solvent samples in similar NMR tubes.

Low-Field ¹⁹F NMR Spectroscopy

High-field ¹⁹F NMR spectra can have a huge baseline hump from Teflon probe materials. Low-field bench-top NMR with a built-in ¹⁹F probe free of fluorinated materials is an alternative and economic choice to obtain ¹⁹F spectra. A 43-MHz (¹H frequency) bench-top NMR instrument Magritek Spinsolve™ 43 (Magritek, San Diego, CA) with a custom ¹⁹F probe was used for collecting the 1D proton decoupled ¹⁹F NMR data. The acquisition parameters were optimized for the best signal to noise ratio (S/N) within realistic experimental time (Table S2). Each spectrum was collected with 8 k complex points and 32 k scans averaged. The ¹⁹F carrier frequency was set at –175 ppm and spectral width of 5000 Hz was used. The acquisition time was 819 ms and repetition time was 1 s. The spectrometer

temperature was 28.5 °C. The total experimental time was about 9 h. Data was collected in Spinsolve Test® 1.7.0 software.

NMR Data Processing

The NMR data were processed and analyzed using MestReNova 11.0.4 NMR software. The raw free induction decay data points were apodized with a Gaussian function of 1 Hz line-broadening and zero-filled to 16 k points. The first point was scaled 0.5. The spectra were manually phase corrected and the baseline correction was performed using the third order Bernstein polynomial fit. Integrations were calculated by sum-up method from – 162 to – 170 ppm for the F28 peak, and from – 185 to – 189 ppm for the F29 peak. S/N is calculated by taking the region of – 210 to – 190 ppm as noise level, and the region of – 190 to – 155 ppm as signal.

DFPN Quantification

$$\text{DFPN quantity} = \text{peak area(a.u.)} \times \text{NMR sample volume}(\mu\text{L}) \quad (1)$$

$$\text{WaterPhase \%} = (\text{quantity in water})/(\text{quantity in water} + \text{quantity in oil}) \quad (2)$$

The NMR peak integration and sample volume can be used to quantify the API using Eq. (1). The range of the linearity was determined through the NMR measurements over a series of standard DFPN samples with varied concentrations (Fig. S3). The DFPN water phase content was calculated using Eq. (2). The DFPN phase partitioning difference between Durezol® and the in-house formulation was evaluated using *T*test. The *P* value was derived from an unpaired two-tail distribution *T*test function in Excel 2016.

RESULTS AND DISCUSSION

¹⁹F Spectra and Mass Balance

Ultra-centrifugation can lead to coalescence of oil globules and promote oil-water-phase separation. To ensure the separation step did not cause any loss of DFPN, a mass balance was confirmed on one lot of each from Durezol® and in-house formulations. Shown in Fig. 2 are low-field ¹⁹F spectra for DFPN extracted from the whole formulation (the total assay), water phase or oil phase after separation. The absolute chemical shift values of nuclei F28 and F29 were slightly different among spectra of different samples, *e.g.*, the largest 0.25 ppm difference was observed in peak F29 (Fig. 2a), which was attributed to the differences in excipient chemicals from sample to sample (12) and no reference correction to the field drifting. In this study, because F28 and F29 peaks were well-resolved with a consistent chemical shift separation of 21.63 ± 0.05 ppm, the small peak drifts (*e.g.*, 0.25 ppm) do not affect the peak integration and quantification. The peak area of F29 was selected to determine assay since it had a higher S/N than peak F28 (Table I). The limit of detection (LOD) and limit of quantification (LOQ) on peak F29 were determined to be 0.025 and 0.05 mg/mL, respectively (Fig. S3). The current DFPN drug NMR samples were about 0.5 mg/mL, within the range of linearity of the NMR method.

For mass balance evaluation, in formulations A3 and B1, the phase combined API quantities were within 5 and 3%, respectively, to the quantity (*i.e.*, Peak Area $\times$ Volume) of the whole formulation (Table I, bolded numbers). The NMR peak integration results showed the amount of DFPN molecules in the original formulation was conserved after phase separation, therefore, mass balanced. In addition, the API quantities measured from the whole formulation spectra were 5.88×10^4 and $5.85 \times 10^4 \mu\text{l} \times \text{a.u.}$ for A3 and B1, respectively, which were within 0.5% of the difference. The results indicate NMR is inherently quantitative and can be an effective assay method across the brands.

Method Variation

The method variation was evaluated with five replicates on the same lot of innovator A3 and the in-house lot B1. The resulting water phase partition, including DFPN in micelle and surfactant bound forms, of A3 or B1 were $35 \pm 1\%$ or $51 \pm 1\%$, respectively (Table S3 and Fig. 3), which indicated high reproducibility of the centrifugation and phase separation process.

Inter-Formulation and Inter-Lot Comparison

The DFPN water phase content of each formulation was measured (Table S4) and plotted (Fig. 4). The average water phase content from different Durezol® lots and in-house formulations were $32 \pm 3\%$ and $52 \pm 2\%$, respectively. The 2–3% inter-sample variation is larger than the method reproducibility of 1%, meaning the measured inter-lot variation of Durezol® is real.

The phase partitioning between Durezol® ($32 \pm 3\%$) and the in-house formulation (52%) is significant with a *P* value of 0.002, less than the typical *P* value threshold of 0.05. The results demonstrated that the method was sufficiently sensitive to evaluate differences across different manufacturing processes.

CONCLUSION

In this work a simple method combining ultra-centrifugation and low-field bench-top ^{19}F NMR was developed and demonstrated to be fit-for-purpose for measuring API DFPN phase partition in oil-in-water emulsion drug products. The method is highly reproducible and yielded meaningful inter-lot and inter-formulation differences. The measured DFPN water phase content includes free DFPN in water, DFPN in micelle and DFPN in surfactant around the oil droplets. The approach can be applied to other oil-in-water emulsion products as well (*e.g.*, cyclosporine emulsions). The sensitivity and reproducibility of the method allows the correlation of API phase partitioning with differences in the manufacturing processes or, possibly the source of the oil used in the process. In summary, the new centrifuge-NMR method provided an improved measure for drug partitioning across different phases and enabled rapid comparisons between oil-in-water emulsion drug products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Structure of difluprednate (DFPN). It has fluorine atoms at positions 28 and 29

Fig. 2. Low-field ^{19}F NMR spectra of DFPN extracted from the whole emulsion drug products (black), water phase (blue), and oil phase (red). Spectra from Durezol® lot A3 (A) and in-house lot B1 (B) are shown. DFPN chemical shift assignments were taken from the literature (13). The absolute chemical shifts values were slightly different due to the differences in residual chemicals including oil (12) and the field drifting in the bench-top NMR spectrometer

Fig. 3.
The DFPN water phase partition for five replicates on drug lots A3 and B1

Fig. 4.
The DFPN water phase partition for all the 11 tested formulations

Table I.

NMR Peak Integration Results

Sample	Volume (μL)	Peak F28			Peak F29			API Quantity ^a (μL × a.u.)	API Quantity ^a (μL × a.u.)
		S/N	Peak	Area (a.u.)	Quantity ^a (μL × a.u.)	S/N	Peak	Area (a.u.)	
Durezol® A3	Oil phase	66	69.3		3.60×10^4	78	68.2		3.55×10^4
	Water phase	35	35.1		1.79×10^4	39	35.8		1.82×10^4
	Oil + water phase	-	-		5.39×10^4	-	-		5.37×10^4
	Whole emulsion	84	103.5		6.00×10^4	102	101.4		5.88×10^4
In-house B1	Oil phase	51	51.1		2.71×10^4	59	51.2		2.71×10^4
	Water phase	46	51.0		2.81×10^4	50	51.2		2.82×10^4
	Oil + water phase	-	-		5.52×10^4	-	-		5.53×10^4
	Whole emulsion	75	97.1		6.02×10^4	90	94.4		5.85×10^4

Bolded numbers are showing mass balance results

^a API quantity was calculated by the product of the peak area and NMR sample volume (Eq. (1))