Journal of Controlled Release 333 (2021) 65–75

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Adaptive perfusion: An in vitro release test (IVRT) for complex drug products

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ARTICLE INFO

Keywords:
Nanoemulsions
Adaptive perfusion
Tangential flow filtration
Globule size
Drug release

ABSTRACT

In this work, adaptive perfusion, a pressure-driven separation method based on the principle of tangential flow filtration (TFF) was developed for investigating the rate and extent of drug release from drug products containing particulates, such as emulsions, suspensions, liposomes, drug-protein complexes. The TFF filters were pre-conditioned with unique conditioning solutions and processes to improve the fiber reproducibility and robustness. The adaptive perfusion method achieved size-based separation of the particulates with simultaneous analysis of the released drug as well as remaining drug. By contrast to conventional dialysis methods, the adaptive perfusion method can be used to measure the rate and extent of the drug release from drug solution, drug loaded micelles and nanoemulsions via adjustment of the filter molecular weight cutoff, feed flow rate or back-pressure. Notably, the adaptive perfusion method provided discriminatory drug release profiles for drug in solution, in micelles, and in small, medium, and large globule size nanoemulsions. The drug release profile obtained using adaptive perfusion method was found significantly faster (e.g., minutes rather than hours) and higher (e.g., >60%) than the release obtained using dialysis method. The IVRT method presented here is free from the constraints of rate-limiting factors, such as diffusion through dialysis membrane, and has potential to be extended further to examine the impact of manufacturing process on drug distribution and release characteristics of other challenging complex drug products.

1. Introduction

In vitro release test (IVRT) has increasingly garnered attention and weight in product development as well as regulatory assessment of complex drug products such as nanoemulsions, suspensions, multivesicular liposomes, and microspheres, as IVRT provides key information about the quality and performance of drug products. An ideal IVRT method should correlate the changes in the critical quality attributes (CQAs) of the drug product directly to the drug's release characteristics, and thus provide valuable information to ensure batch-to-batch consistency in quality, facilitate assessment of post-approval changes, and assist with product comparison to support determination of bioequivalence. However, developing a fit-for-purpose and robust IVRT method for complex drug products has been a challenge [1–4]. IVRT methods have been successfully employed as a quality control test only for a few complex drug products [5]. For a generic drug product, there is the additional complication on how to satisfactorily support bioequivalence with the reference listed drug (RLD), based on in vitro release studies.

Most of the currently available IVRT methods such as microdialysis, (reverse) dialysis, and Franz cell diffusion primarily focus on quantifying the extent of drug release and to a lesser degree on the rate of drug release [6,7]. Furthermore, these commonly used IVRT methods generally rely on the diffusion process, e.g., via a dialysis membrane, to

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https://doi.org/10.1016/j.jconrel.2021.03.024
Received 21 December 2020; Received in revised form 13 February 2021; Accepted 16 March 2021
Available online 23 March 2021
0168-3659/Published by Elsevier B.V.

separate the "released" drug from the "remaining" one, which have inherent limitations. First, the use of a membrane inadvertently introduces a rate-limiting diffusion step and thus prolongs the overall duration of the test. Long experiment duration may not be suitable for formulations (e.g., suspensions) or route of administrations (e.g., ophthalmics) that have rapid drug release characteristics. In addition, rate-limiting diffusion based separation would typically not be sensitive enough to discern minor differences in drug release from complex formulations (e.g., those resulting from manufacturing process changes). Second, with the use of a dialysis membrane in the setup, the user generally focusses on the analysis of the "released" drug, due to the need to maintain sink conditions (i.e., use of larger medium volume to achieve sufficient concentration gradient) across the membrane. The volume needed for sink conditions limits the ability of the user to analyze the percent drug remaining and to achieve mass-balance in drug transport. Third, the trigger for drug release from complex formulation (e.g., emulsions, liposomes) starts with passive diffusion of the free-drug (i.e., drug that is not associated with or contained within a formulation) across the membrane. The passive diffusion process is governed by conditions that are less adjustable from an in vitro drug release method standpoint (e.g., concentration gradient and surface area) and thus limits the options for method development. Therefore, there is an unmet need for developing a fit-for-purpose and robust IVRT method that can detect and discriminate against critical changes in manufacturing process as well as variations in the product quality and performance.

This paper describes the development and optimization of a new adaptive perfusion (AP) method and demonstrates its advantages. The development of the AP method started in view of deeper understanding of the characteristics of complex drug products (e.g., ophthalmic emulsions) and associated factors which may impact the drug release mechanism [8–10]. The knowledge and insight gained through these studies led to the development of the AP method. At the same time, understanding about characteristics of the product also stimulated the demand for a better release method that may: 1) eliminate the need for tedious sample-and-separate technique to isolate the "released" drug; 2) afford flexibility in the setup; 3) eliminate the rate-limiting factors such as dialysis and diffusion; 4) tailor the control over drug transfer according to types of dosage form; and 5) mimic the in-vivo circulation condition. In the current study, we applied the principle of tangential flow filtration (TFF) for separating the complex species within a nanoemulsion and designed a new approach to analyze the drug release. There are three common modes of operation in TFF: concentration (removing solvent while retaining the solute species), diafiltration (removing smaller species while retaining larger ones without changing the volume), and fractionation (separating larger species from the smaller ones). In general, all three modes of operation aim at achieving a higher recovery of the retentate (i.e., the species retained by the TFF filter). In contrast, the newly developed AP method focuses not only on the retained portion (i.e., in the retentate) but also the removed portion (i.e., in the permeate). More importantly, we monitor not only the extent of removal (which reflects the extent of drug release), but also the rate of removal (which provides estimate on the rate of release). Reproducibility of the TFF filter is a critical factor which makes drug release testing possible. However, unit-to-unit TFF filter variation is not uncommon. For this reason, in this study attempts were also made to pretreat the membrane to improve method reproducibility. Optimization and evaluation of AP method were performed using drug in solution, drug containing micelle and drug containing nanoemulsions, where difluprednate was used as a model drug.

2. Materials and methods

2.1. Materials

Difluprednate (>97%) was purchased from RIA International LLC (East Hanover, NJ). Castor oil was purchased from Fisher (Pittsburgh, PA). Polysorbate-80 was purchased from Acros Organics (Morris Plains, NJ). Glycerin, sodium acetate, boric acid, edetate disodium and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (Waltham, MA). Sorbic acid was obtained from MP Biomedicals (Solon, OH). Sodium dihydrogen phosphate and sodium hydroxide (1 N and 2 N) was purchased from Sigma Aldrich (St. Louis, MO). Phosphoric acid was obtained from EMD Millipore Corporation (Burlington, MA). Acetonitrile was obtained from Fisher Scientific (Waltham, MA). Ethanol was procured from Decon Labs (King of Prussia, PA). Deionized water was obtained from Milli-Q Ultrapure Water Systems, EMD Millipore Corporation (Burlington, MA). Unless otherwise specified, all materials were of analytical grade. Spectrum MicroKros® hollow fibers (modified polyether sulfone (mPES) membrane, 30 kD, 100 kD, 300 kD and 500 kD molecular weight cutoff (MWCO), 0.5 mm diameter, 20 cm effective length, 20 cm^2 surface area) and Float-A-Lyzer G2 dialysis tubes (regenerated cellulose, 100 kD MWCO) were purchased from Repligen Corporation (Rancho Dominguez, CA).

2.2. Preparation of drug loaded micelles

Difluprednate micelles were prepared by dispersing and sonicating difluprednate powder in 3.6% *w/v* polysorbate-80 solution in deionized water at room temperature. The final concentration of difluprednate in polysorbate-80 micelles was determined to be 100 μg/mL, by ultra-performance liquid chromatography (UPLC) method (Method 1).

2.3. Preparation of nanoemulsions

Three nanoemulsions with different globule size distributions were manufactured using microfluidization process, while keeping their composition qualitatively (Q1) and quantitatively (Q2) the same as RLD, Durezol. Briefly, primary emulsion was formed by high-shear mixing of the aqueous phase-I (containing a mixture of glycerin, polysorbate-80, and deionized water) with the oil phase (castor oil containing difluprednate) while maintaining the temperature at 65 °C to 70 °C. Primary emulsion was further mixed with the aqueous phase-II (containing sorbic acid, sodium acetate, boric acid and edetate disodium dissolved in deionized water) maintained at 65 °C to 70 °C to obtain a coarse emulsion. pH of the coarse emulsion was then adjusted to 5.2–5.8 at room temperature by using 1 N sodium hydroxide. Thereafter, the coarse emulsion was subjected to microfluidization process, with precise control over the variation of critical process parameters (CPPs) such as pressure and temperature. These variations in CPPs of microfluidization process produced nanoemulsions with small (approximately 80 nm mean size), medium (approximately 120 nm mean size) and large (approximately 150 nm mean size) globule size distributions. The globule size distribution (GSD) of the nanoemulsions was determined by dynamic light scattering method using Zetasizer Nano ZSP instrument (Malvern Panalytical Inc., Westborough, MA). The results for *Z*-average, polydispersity index (PdI) and intensity-weighed distribution (Di10, Di50 and Di90) of each formulation are provided in Table 1.

Table 1
Globule size distribution data of various difluprednate nanoemulsions (Mean ± SD, $n = 3$).

Sample	*Z*-Average (d.nm)	PdI	Di(10) (nm)	Di(50) (nm)	Di(90) (nm)
Large GSD nanoemulsion	152.4 ± 1.3	0.181 ± 0.014	94.1 ± 6.2	171.1 ± 3.9	305.6 ± 18.8
Medium GSD nanoemulsion	121.9 ± 0.9	0.203 ± 0.010	70.3 ± 2.8	140.0 ± 2.9	261.8 ± 13.5
Small GSD nanoemulsion	78.5 ± 0.6	0.206 ± 0.008	44.1 ± 0.9	87.2 ± 2.1	181.0 ± 12.6

2.4. Adaptive perfusion

2.4.1. Setup and procedure

The setup for the AP method (Fig. 1) includes a fresh medium reservoir, a particulate filtering device attached to the inlet of the fresh medium tubing (to prevent particulate contamination), two peristaltic pumps (one for the sample feed and one for the fresh medium), two additional reservoirs (one for the retentate, also called the feed reservoir, and one for the permeate), a hollow fiber TFF filter, a backpressure controlling valve (Flow-rite PV-2, Flow Rite Inc., Mt. Airy, MD), a magnetic stirrer, a stir plate for mixing the sample with the medium, a pressure monitoring system with a minimum of three pressure sensors (inlet, retentate and permeate), a mass flow monitoring system (e.g., flow meter), a data acquisition system (i.e., computer with software), and tubings for providing appropriate connections.

A typical AP study involves three unique stages, i.e., pre-conditioning, run-time and re-conditioning. For all three stages, the tubings for inlet, retentate and permeate were attached to the filter by using one or more of the following configurations: a) *partial-open loop configuration* (Fig. 2A), in which the liquid was pumped from the inlet (①) in to the TFF filter (②) without applying backpressure at the retentate end (③). The retentate was connected to the permeate at one end (④) through a tubing and the liquid was allowed to loop from the retentate into the permeate at that end and flush out through the other open permeate end (⑤); b) *open-loop configuration* (Fig. 2B), in which the liquid was pumped from the inlet (①) in to the TFF filter (②) with or without applying backpressure at the retentate end (③). The liquid was allowed to flush out through the retentate end and through one open end of the permeate (④) with the other permeate end being closed off (⑤); and c) *closed-loop configuration* (Fig. 2C) in which the liquid was pumped from the inlet (①) in to the TFF filter (②) without applying backpressure at the retentate end (③). The retentate was connected to the permeate at one end (④) through a tubing and the liquid was allowed to loop from the retentate into the permeate at that end and flush out through the other permeate end (⑤) into a container from which it was pumped back into the inlet (①) to complete the loop.

In the pre-conditioning stage, the TFF filter was flushed with deionized water (e.g., 2000 mL) and a suitable organic solvent (e.g., 1000 mL of ethanol) to remove the storage media (e.g., glycerin) used by the TFF filter manufacturer. The organic solvent was rinsed out by flushing the TFF filter with deionized water (e.g., 1000 mL). The filter was then conditioned by pumping the conditioning solution (e.g., 1000 mL of 0.07% *w/v* polysorbate-80 solution in deionized water). All the steps in the pre-conditioning stage were carried out by attaching the tubings to the TFF filter in a *partial open-loop configuration* without applying backpressure. This pre-conditioning stage was critical in minimizing the data variation caused by device-to-device variation for TFF filters. In the run-time stage, the conditioning solution was first pumped out of the filter by flushing with deionized water (in a partial open-loop configuration without applying backpressure) followed by flushing with the release medium (in an open-loop configuration by applying backpressure). At the end of this step, all the tubings for inlet, retentate and permeate were emptied to remove any residual liquid which may cause additional dilution of the sample. All the individual components were assembled as shown in Fig. 1. A predetermined volume of the sample (e.g., pure drug solution, drug loaded micelles, and nanoemulsion) was spiked into a fixed volume of the medium in the feed reservoir, which was stirred continuously at 1400 rpm using a magnetic stirrer. After allowing the sample to mix with the medium for 10 s, an aliquot was taken from the feed reservoir to assay for the initial drug concentration (in case of in-situ fiber optic UV–VIS system, sampling was not required as drug concentration was measured in situ and in real time). Sample feed pump was then turned on to pump the sample through the filter at 200 mL/min flow rate in an open-loop configuration with a pre-adjusted backpressure setting of 2.7 (dial mark on the pinch value as shown in Fig. S1 in supplementary material; lower number signifies less opening and greater applied pressure). The sample circulated back to the retentate reservoir to complete the closed-loop

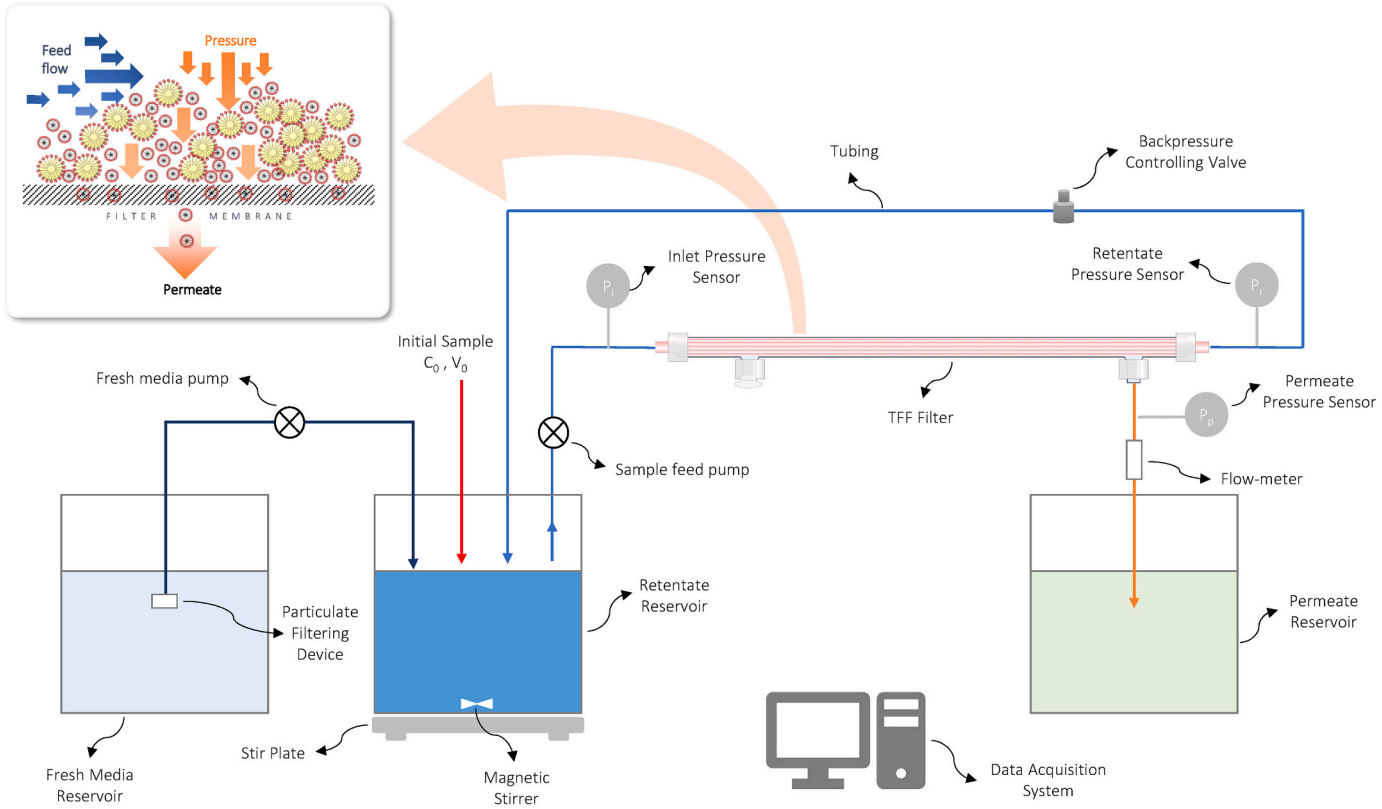

Fig. 1. Schematic representation of adaptive perfusion method using a hollow fiber TFF filter and the principle of size-based separation.

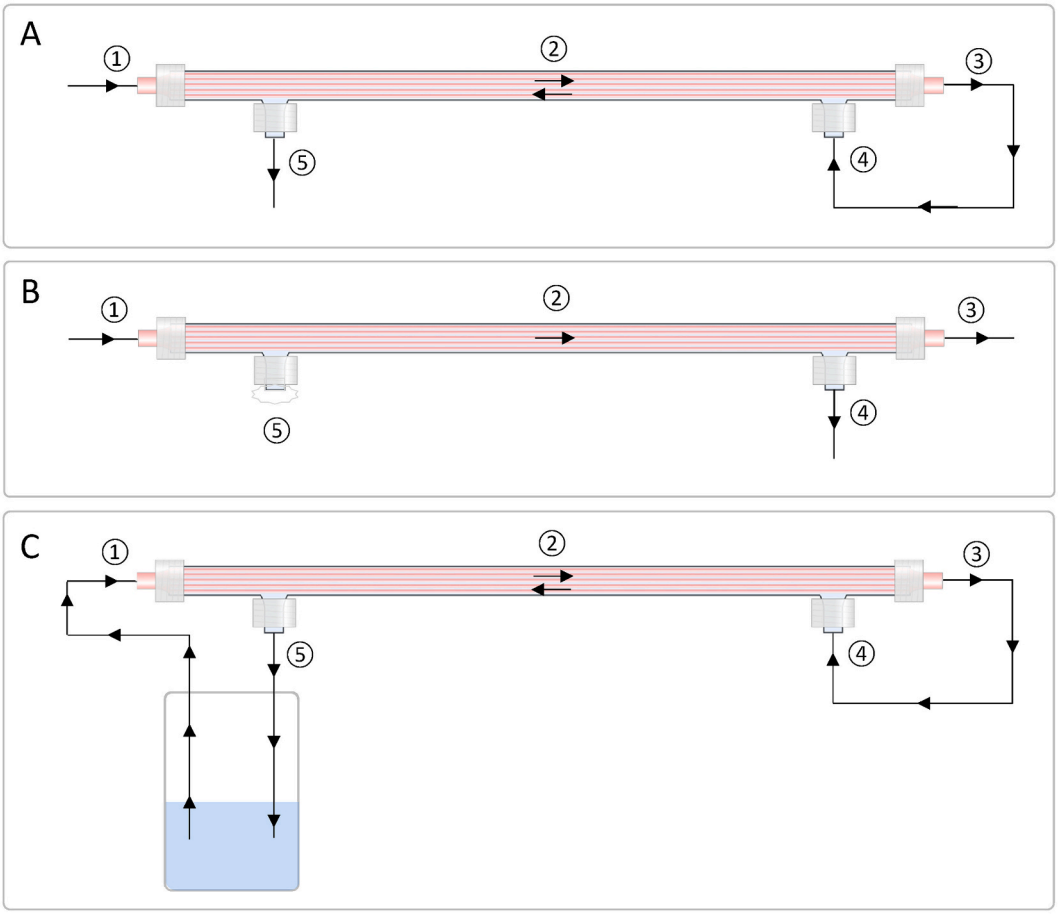

Fig. 2. Schematic representation of the tubing-attachments to the TFF filter in A) partial open-loop configuration (used during pre-conditioning, run-time, and re-conditioning); B) open-loop configuration (used during run-time); and C) closed-loop configuration (used during re-conditioning). The solid lines indicate the tubing-attachments whereas the arrows indicate the direction of the liquid flow.

configuration of the AP process, as shown in Fig. 1. The time interval between the initial dilution of the sample with the medium and the start of the sample feed pump was kept constant for all the experiments (i.e., not more than 30 s). The sample volume in the retentate reservoir was swiftly re-adjusted to the starting volume by turning on the fresh medium pump. This procedure was to account for volume drop in the retentate reservoir due to tubing dead-volume. The moment at which the liquid starts eluting out from the permeate tubing was marked as the beginning of the experiment (i.e., time zero). The fresh medium supply rate was continuously adjusted based on the actual permeate flow rate (e.g., reading from the flow meter) to account for the volume drop due to permeate flow and to maintain constant retentate reservoir volume. At predetermined time intervals, aliquots were taken from the retentate reservoir (i.e., for assay of the remaining drug content) and from the permeate tubing (i.e., for assay of the instantaneous drug transfer). The permeate was also simultaneously collected in the permeate reservoir. At the end of the experiment, an aliquot was also taken from the permeate reservoir and the total volume of permeate was measured to determine the amount of cumulative drug release. Also, during this run-time stage, the inlet, retentate, permeate and transmembrane pressures were continuously monitored by using the pressure monitoring system and the permeate flow rate was simultaneously measured by using the flow meter. In the final re-conditioning stage, the fresh medium supply was replaced with a suitable organic solvent (e.g. ethanol); which was then pumped directly through the TFF filter without applying back-pressure in a partial open-loop configuration. The out-coming fluid was either directed to the permeate reservoir to perform mass recovery or to the waste container for cleaning. Mass recovery can also be performed by increasing the feed flow rate which would lead to higher dilution of the sample in the retentate reservoir and by varying the backpressure to momentarily increase the flux and recover the remaining drug. For thorough cleaning, the TFF filter was further flushed with organic solvent in a closed-loop configuration (without backpressure). The organic solvent left over in TFF filter from the previous step was rinsed out with deionized water in a partial open-loop configuration (without back-pressure). The TFF filter was then re-conditioned with the conditioning solution (e.g., 0.07% *w/v* polysorbate-80 solution in deionized water) in a partial open-loop configuration (without backpressure). After re-conditioning, the filter was stored with the conditioning solution in it until the next experiment (typically the following day). However, if the filter was to be stored for a longer period (e.g., not more than 5 days) without usage, the filter could be flushed with a diluted organic solvent (e.g., 10% ethanol in deionized water), instead of the conditioning solution, using a partial open-loop configuration (without backpressure). After extended storage and prior to use, the filter need to be flushed with deionized water and re-conditioned with the conditioning solution as described earlier.

2.4.2. Drug transfer studies using difluprednate solution and micelles

Difluprednate transfer through the TFF filter in the AP setup was determined using TFF hollow fiber (MicroKros®, mPES membrane, 100kD MWCO, 20 cm^2 surface area). The samples evaluated included pure difluprednate solution and difluprednate polysorbate-80 micelles. The setup and procedure used for performing the drug transfer studies by using AP method was same as described earlier in section 2.4.1. For pure difluprednate solution, 200 μL of difluprednate solution (1 mg/mL

in ethanol) was spiked into 40 mL of release medium (i.e., 10 mM pH 7.4 phosphate buffer mixed with ethanol in the ratio of 80:20 *v*/v) to reach an initial difluprednate concentration of 5 μg/mL. Ethanol was used as a co-solvent to prevent the precipitation of difluprednate in the medium. Difluprednate concentration in the retentate and permeate reservoirs was determined in real time using an in-situ fiber optic UV–Vis system (Pion μDiss Profiler™, Billerica, MA). Zero-intercept-method (ZIM) analysis was performed to remove polysorbate-80 interference by using wavelength within the range of 271 to 274 nm (exact ZIM wavelength varied between probes). For difluprednate polysorbate-80 micelles, 1 mL of difluprednate polysorbate-80 micelle solution (100 μg/mL in 3.6% *w/v* polysorbate-80) was spiked into 40 mL of the release medium (i.e., 10 mM pH 7.4 phosphate buffer) to obtain an initial difluprednate concentration of 2.5 μg/mL. Aliquots of retentate and permeate were collected periodically from the retentate reservoir and from the permeate tubing, respectively, and subjected to UPLC analysis to determine difluprednate concentration. Aliquots of retentate were appropriately diluted by acetonitrile prior to UPLC analysis. The study was conducted at ambient temperature (i.e., 23.5 ± 1.5 °C).

2.4.3. Release studies using nanoemulsions

To demonstrate the discriminatory capability of adaptive perfusion method, in vitro release testing was conducted for nanoemulsions having small, medium and large GSD by using TFF hollow fiber (Micro-Kros®, mPES membrane, 100kD MWCO, 20 cm^2 surface area). The setup and procedure used for performing the release studies by using AP method was same as described earlier in section 2.4.1. A volume of 200 μL of nanoemulsion was spiked directly into 40 mL of medium (10 mM pH 7.4 phosphate buffer) to initiate the adaptive perfusion process with an initial concentration of 2.5 μg/mL for difluprednate. Aliquots of retentate and permeate were collected periodically from the retentate reservoir and from the permeate tubing, respectively, and subjected to UPLC analysis to determine difluprednate concentration. Appropriate dilution was performed for the retentate aliquots by using acetonitrile prior to UPLC analysis. The study was conducted at ambient room temperature (i.e., 23.5 ± 1.5 °C).

2.5. Reverse dialysis

2.5.1. Drug transfer studies using difluprednate solution

Diffusion of difluprednate through dialysis membrane was determined in a USP 2 apparatus setup (Vision Elite 8, Teledyne Hanson Research, with mini-vessels) using commercially available dialysis tubes (Float-A-Lyzer G2, regenerated cellulose, 100kD MWCO). Each dialysis tube was cleaned with 1.0% *w/v* polysorbate-80 solution in deionized water and subsequently washed thoroughly with deionized water. Dialysis tubes, containing about 1 mL of the release medium (i.e., 10 mM pH 7.4 phosphate buffer mixed with ethanol in the ratio of 80:20 *v*/v), were suspended inside the USP 2 mini-vessels, as shown in Fig. 3A. Agitation was controlled at 100 rpm and the temperature was maintained at 34 °C. One milliliter of difluprednate solution (1 mg/mL in ethanol) was spiked directly into the vessel containing 200 mL release medium to achieve an initial drug concentration of 5 μg/mL. Inside the dialysis tube, free drug concentration (i.e., diffused difluprednate) was monitored in real time using the in-situ fiber optic UV–VIS system. The scanning frequency was once every minute for the first 6 h, then switched to once every 5 min for the next 18 h. After first 24 h, the scanning frequency was changed to once every 10 min for the next 40 h. For the last 104 h, the scanning frequency was once every 30 min. The total experimental time was 168 h (7 days). Pure difluprednate solution diluted with release medium at the same concentration of 5 μg/mL was also monitored in parallel as a control to determine any possible change in drug concentration, e.g., due to degradation. Prior to the start of the experiment, UV signals were calibrated using various concentrations of difluprednate standard solutions (0.05, 0.10, 0.50, 1.00, 2.00, 3.00, 4.00, and 6.00 μg/mL). The percentage release was calculated by dividing the measured drug concentration at each time-point by the initial drug concentration (e.g., 5 μg/mL). ZIM analysis was performed to remove polysorbate-80 interference by using wavelength within the range of 271 to 274 nm (exact ZIM wavelength varied between probes). Ethanol was used as a co-solvent to prevent the precipitation of difluprednate in the release medium.

2.5.2. Release studies using nanoemulsions

The IVRT study was also performed by using a reverse dialysis setup inside a USP 2 apparatus equipped with mini-vessels containing 200 mL of release medium at 34 °C and a paddles speed of 100 rpm. Evaluated

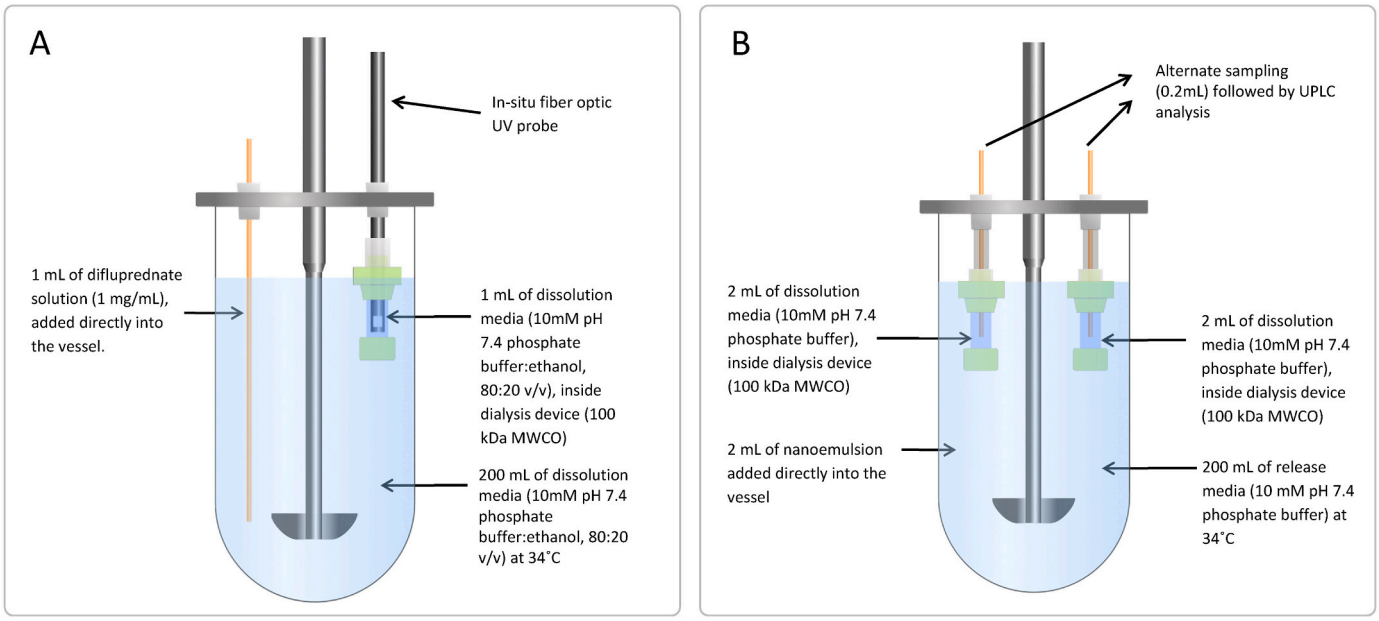

Fig. 3. Setup for in vitro release test using reverse-dialysis method for (A) pure drug solution; (B) nanoemulsions.

samples included nanoemulsions of two different globule size distributions: small and large. The configuration for the reverse dialysis setup is shown in Fig. 3B. Each dialysis tube was cleaned with 1.0% *w/v* polysorbate-80 solution in deionized water and subsequently washed thoroughly with deionized water. Two dialysis tubes (Float-A-Lyzer G2, regenerated cellulose, 100 kD MWCO), each containing 2 mL of fresh release medium (i.e., 10 mM pH 7.4 phosphate buffer), were suspended using an adapter inside each mini-vessel. Two mL of nanoemulsion was added directly into release medium in mini-vessel to achieve an initial concentration of 5 μg/mL for difluprednate. Sampling was performed from alternating dialysis tubes. At each pre-determined sampling time point, 0.2 mL of medium was taken from both inside the dialysis tubes and the outside medium. An equivalent volume of fresh medium was replenished inside the dialysis tube after each sampling. Due to the interference of other formulation components, in-situ UV analysis was not possible, and accordingly difluprednate concentration was determined by UPLC method (i.e., Method 2). The percentage release was calculated by dividing the measured drug concentration at each time-point by the initial drug concentration (e.g., 5 μg/mL).

2.6. Difluprednate analysis by ultra-performance liquid chromatography (UPLC)

The UPLC system consisted of a Waters Acquity UPLC I-Class (Waters Corporation, Milford, MA) equipped with degasser, binary solvent pump, thermostatted autosampler, thermostatted column compartment, and a photo diode array detector. Acquity UPLC BEH C18, 2.1 mm × 150 mm (1.7 μm packing) column (Waters Corporation, Milford, MA) was used along with an Acquity UPLC BEH C18, 2.1 mm × 5 mm (1.7 μm packing) vanguard precolumn (Waters Corporation, Milford, MA). Column temperature was maintained at 50 °C whereas autosampler temperature was kept at 8 °C. A 100 μL extension loop was used. Two separate gradient elution methods (Method 1 in Table 2 and Method 2 in Table 3) were used to analyze difluprednate in different medium, e.g., Method 1 for retentate samples, and Method 2 for permeate samples. Injection volume was 3 μL and 80 μL for Method 1 and Method 2, respectively. The eluted difluprednate was detected at 240 nm. Data collection and analysis were performed using Empower 3 software.

3. Results and discussion

3.1. Development and optimization of Adaptive perfusion method

Development of adaptive perfusion method was based on the principle of tangential flow filtration. Several critical factors, which are important to the TFF operation, were evaluated and optimized.

3.1.1. Selection of filter

(a) Filter module: There are three common forms of TFF filter modules: hollow fibers, spiral wound cartridges and flat plates (also referred as cassettes). Hollow fiber was chosen because it provides a simpler flow path and a lower void volume (typically 0.5 mL). Void volume can impact the initial dilution of the sample. In case of complex formulations like nanoemulsions, in which drug release is highly dependent on the extent of initial dilution [9], a filter module with smaller void volume was considered more ideal.

Table 2
UPLC gradient elution method conditions (Method 1) for the determination of difluprednate concentration.

Time (min)	Flow rate (mL/min)	% Deionized Water, pH 2.5 (pH adjusted with phosphoric acid)	% Acetonitrile
0.0	0.45	50	50
5.0	0.45	50	50
6.0	0.45	0	100
11.0	0.45	0	100
12.0	0.45	50	50
17.0	0.45	50	50

Table 3
UPLC gradient elution method conditions (Method 2) for the determination of difluprednate concentration.

Time (min)	Flow rate (mL/min)	% Deionized Water, pH 2.5 (pH adjusted with phosphoric acid)	% Acetonitrile
0.0	0.50	40	60
2.5	0.50	40	60
3.5	0.50	0	100
10.0	0.50	0	100
11.0	0.50	40	60
15.0	0.50	40	60

(b) Filter membrane chemistry: The physicochemical properties of the drug and membrane plays a vital role in drug adsorption on the membrane, which can be a common cause for low drug recovery and low drug mass transfer. Difluprednate, used in this study, is a highly lipophilic drug (log $P = 3.10$) [8]. Therefore, hydrophilic membranes (such as mixed cellulose ester (ME), polyethersulfone (PES), modified polyethersulfone (mPES)) were considered to be better option than hydrophobic membranes, such as polysulfone (PS), because hydrophilic membranes can reduce lipophilic drug adsorption. mPES membrane was ultimately selected for three reasons: 1) hydrophilic nature of the membrane provides higher flux rates, higher resistance to fouling, and lower chances of drug adsorption, 2) available in multiple surface area options, e.g., from 13 cm^2 to 92 cm^2, and 3) a large range of pore size (from 1 kD to 0.65 μm) allows for more tailored selection of passage of nanoemulsion components.

(c) Filter dimension (diameter, effective length and surface area): Void volume, as mentioned earlier, is affected by several aspects of the hollow fiber, e.g., diameter, effective length and surface area. The selected mPES filters are commercially available in several combinations of these dimensions. Eventually, mPES hollow fiber having a diameter of 0.5 mm, an effective length of 20 cm and a surface area of 20 cm^2 was selected because of its lower void volume.

(d) Filter molecular weight cut-off (MWCO): Filter MWCO is the most critical factor that impacts the rate and extent of drug release, because it governs the size range of particulates that can pass through the membrane. Several mPES hollow fiber filters having different MWCO of 30 kD, 100 kD, 300 kD and 500 kD were screened using commercially available difluprednate nanoemulsion at constant flow rate and backpressure to select the optimum MWCO. As observed in Fig. 4, only 34% drug release was obtained with 30 kD mPES filter in 2 h from difluprednate nanoemulsion; this was 50% lower than that

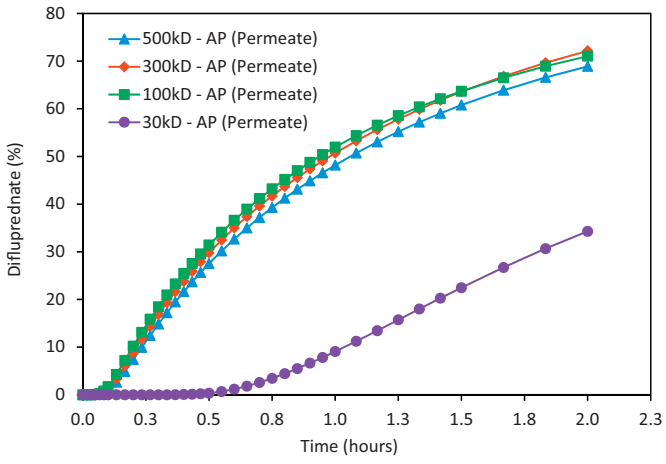

Fig. 4. Effect of four different MWCO mPES hollow fibers ($n = 1$) on drug release (in permeate) from difluprednate nanoemulsions.

observed with the other three MWCO mPES filters. For ophthalmic product, such as difluprednate nanoemulsion which has short residence time in the eye (e.g., approximately 2–5 min [11,12]), slow and low drug diffusion (as in the case 30 kD filter) is undesirable because the diffusion can impose a rate-limiting step for drug release. Negligible difference was observed with 100 kD, 300 kD and 500 kD mPES filters in terms of difluprednate release. Among the three MWCO, 100 kD filter was the least susceptible to membrane fouling (i.e., deposition of particles on the membrane surface). Accordingly, 100 kD mPES filter was selected for further evaluation of different formulations.

3.1.2. Optimization of feed flow rate and transmembrane pressure to maximize permeate flux

In studying release from ophthalmic nanoemulsions, maximum flux in the permeate was desired as facile membrane flux enhances drug removal and contributes to faster drug transfer from oil to aqueous phase. Feed flow rate and transmembrane pressure (TMP) were two key TFF process parameters controlling the permeate flux. At a constant feed flow rate, an increase in TMP (achieved by increasing the backpressure) generally results in a linear increase in permeate flux up to a point and then flattens out as the membrane fouling becomes more severe which reduces permeate flux. The optimal TMP range is just before the curve begins to plateau. Similarly, increase in feed flow rate also leads to higher permeate flux, partly because feed flow rate also increases the TMP (in addition to increase in the mass flow). This complex relationship between feed flow rate, TMP, and flux is well-known and critical process to optimize [13]. Several feed flow rates (ranging 100 mL/min to 200 mL/min) across a wide range of TMP (ranging from 3 psi to 15 psi) were evaluated in a 100 kD, 20 cm^2 mPES hollow fiber setup using commercially available difluprednate nanoemulsion. The TMP was varied by adjusting the backpressure pinch valve as shown in Fig. S1 (in Supplementary Material), a lower number indicated greater applied pressure. Care was taken to ensure that the membrane inlet pressure does not exceed the filter manufacturer recommended pressure limit (e.g., 30 psi) under all experimental conditions. For study of nanoemulsions, maximum flux was achieved with a feed flow rate of 200 mL/min with a backpressure setting of 2.7 (Fig. 5).

3.1.3. Sample dilution

Optimization of the feed flow rate and transmembrane pressure, as described earlier, was carried out by using a sample (i.e., nanoemulsion) to medium dilution ratio of 1:50. Although still operational, membrane fouling worsened at higher feed flow rates and backpressures, as indicated by the decreasing flux after reaching the maximum at higher TMP (Fig. 5). Accordingly, sample dilution was increased (to 1:200), to alleviate membrane fouling and to increase the overall efficiency of the AP process.

3.1.4. Filter conditioning

Filter-to-filter variability is a common problem with the use of TFF filters [14–16]. Membrane fouling is a major factor that has limited the use of TFF processes because it reduces the filtration efficiency and prolongs the filtration run time. Attempts have been made in the past to overcome the membrane fouling issues [14–16]; however, fouling remains a major challenge. In our study, filter conditioning was found to be an essential step in improving filter performance and usage reproducibility. For this purpose, polysorbate-80 was evaluated for conditioning the TFF filter. The advantage of using polysorbate-80 is that the excipient is common in nanoemulsions and increases the compatibility of the conditioning solution with the formulations. Furthermore, higher concentration of polysorbate led to slightly improved flux, e.g., 0.07% *w/v* polysorbate-80 in deionized water yielded higher flux value compared to that of 0.02% w/v concentration. Therefore, 0.07% w/v polysorbate-80 concentration was chosen as the conditioning solution. Another critical factor in the conditioning step was the configuration. Conditioning performed using a partial-open loop configuration (Fig. 2A) provided higher filter-to-filter consistency and data reproducibility compared to open-loop configuration (see Fig. S2). Hence, partial-open loop configuration was selected for conditioning the TFF filters. The optimized process parameters for the AP method have been

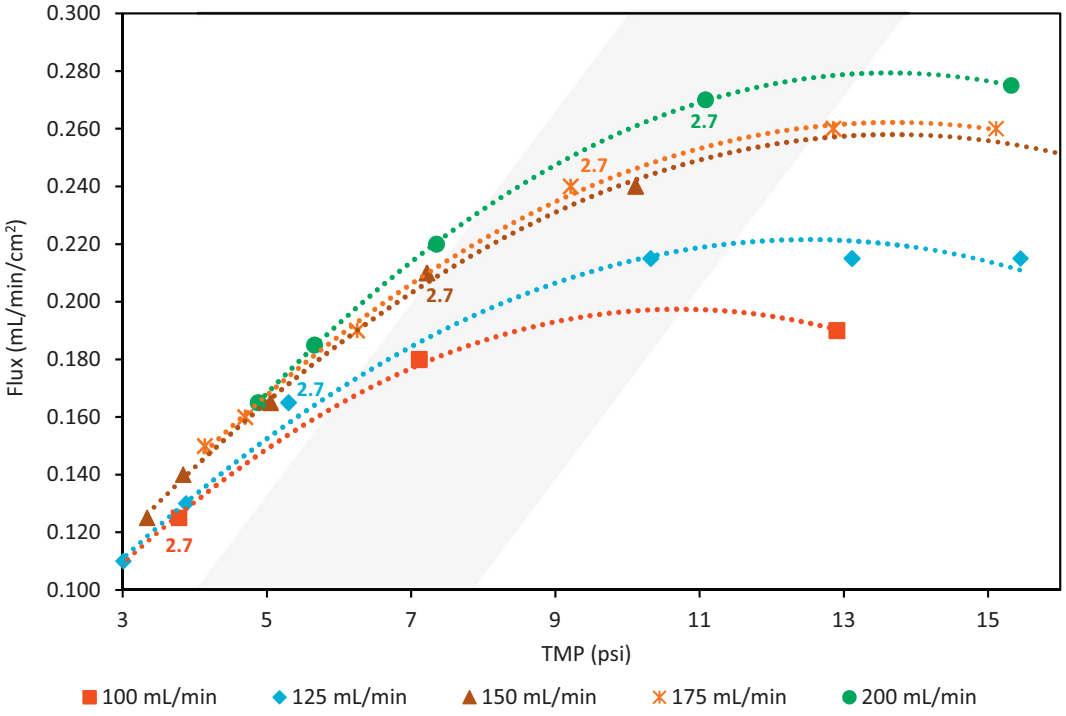

Fig. 5. Effect of changing transmembrane pressure (TMP) on flux at different feed flow rates and backpressures. The grey zone represents optimal TMP range. The backpressure setting of '2.7' has been highlighted for each feed flow rate to demonstrate the impact of a specific backpressure setting on flux and TMP at different feed flow rates. These optimization studies were performed by using a sample to medium dilution ratio of 50×.

summarized in Table S2 (in Supplementary Material).

3.2. In vitro drug release studies using dialysis and adaptive perfusion

3.2.1. Drug release from pure drug solution and nanoemulsions using Reverse-dialysis method

Release of difluprednate from pure drug solution and nanoemulsions was studied using dialysis. More specifically, a reverse configuration was used. The "reverse-dialysis" signifies the direction of the drug transfer is from outside the dialysis device to the inside, as opposed to the normal direction from inside the dialysis device to the outside (i.e., "dialysis"). The main reason for selecting reverse-dialysis over dialysis configuration was to ensure the concentration of free drug (i.e., the drug that was not within the globules) remains low in the immediate vicinity of the globules (for nanoemulsion) to maintain the sink condition, e.g., by dilution using larger medium volume. Furthermore, the drug concentration gradient (i.e., driving force for drug release from emulsion globules) could be maintained throughout the duration of the test. For instance, difluprednate has a partition coefficient (i.e., log *P*) value of 3.10 (n-octanol/water). In a most simplified scenario, assuming difluprednate distributes between the oil (from oil globules in emulsions) and the aqueous phase (from the release medium) based solely on the volume ratio of the aqueous and oil phase, the drug would require an aqueous/oil volume ratio of greater than 12,589-times to achieve at the most 90% drug distribution in water phase. For this reason, the reverse-dialysis configuration provided an advantage in terms of allowing for better control of the dilution and maximum drug concentration gradient across oil/water interface to drive the drug release.

As seen in Fig. 6A, in the case of pure drug diffusion, more than 3 h were needed for 50% of difluprednate to diffuse across the dialysis membrane (100 kD MWCO) and nearly 8 h for the difluprednate concentration to reach plateau (about 75% of the drug diffused). The slow diffusion of difluprednate across the dialysis membrane was attributed to its lipophilicity and potential interaction with the dialysis membrane and very likely the slow membrane diffusion became a rate-limiting step for drug release. Furthermore, a decrease in difluprednate concentration was observed after about 10 h, which was confirmed to be a result of difluprednate degradation in alkaline medium. The degradation of the drug was evident from the control sample data (i.e., direct analysis of difluprednate in solution), where a continuing decay in difluprednate concentration was observed, with only 85% of the pure drug remaining in the bulk medium after 24 h.

The results in Fig. 6B show the overlapping in vitro drug release profiles of nanoemulsions with small and large GSD when tested using reverse-dialysis method. These results clearly show that reverse-dialysis method cannot differentiate between the drug release rates from formulations with significantly different globule sizes (e.g., z-average of 80 nm vs. 150 nm). Furthermore, the drug release from both emulsions was slow and incomplete. The release plateaued off after 48 h with only 18% drug release. To verify the mass balance, SDS was spiked in the external medium at 168 h at a concentration of 0.05% *w/v*. The SDS spike led to an increase in drug concentration inside the dialysis device resulting from drug release from the emulsions. At 192 h, the total drug detected inside the dialysis device was 33.45% and 35.80% for large and small emulsions, respectively, while the outside drug percentage was 49.69% and 47.96% for large and small emulsions, respectively. As such, the total amount of drug recovered by the end of the 192-h experiment was 83.14% and 83.76% for large and small emulsions, respectively. The remaining drug most likely degraded due to prolonged exposure to alkaline medium. In summary, the rate and extent of drug release was found to be slow and incomplete despite all conditions favoring drug release from emulsions such as high dilution ratio (100× dilution by release medium), aggressive agitation (100 rpm), and elevated temperature (34 °C). These results indicate that reverse-dialysis method lacked the capability to discern differences in drug release profiles for small and large globule size nanoemulsions.

3.2.2. Drug release from pure drug solution, drug loaded micelles and nanoemulsions using Adaptive perfusion

Drug transfer from pure difluprednate solution and the difluprednate polysorbate-80 micelles was evaluated by the newly developed AP method. As shown in Fig. 7, more than 90% of the difluprednate transferred across the membrane (in the permeate) in less than 15 min (for pure solution), which was faster compared to the reverse-dialysis method (8 h). Similar to the reverse-dialysis setup, 20% ethanol was used in the release medium for testing pure difluprednate solutions, mainly for two reasons: a) to provide a comparison with the medium used in reverse-dialysis method and b) to prevent drug precipitation due to higher rate of drug transfer achieved with the AP method. With the AP method, the overall rate of drug transfer across the TFF filter was controlled by the filtration process. The results in Fig. 7 show that drug release from micelles was slower as compared to the drug diffusion from pure difluprednate solution and 90% release was achieved in about 25 min. The ability to discern the difference in the release rates of the free drug and drug in micelles is a notable capability of the AP method.

Drug release from difluprednate nanoemulsions consisting of small

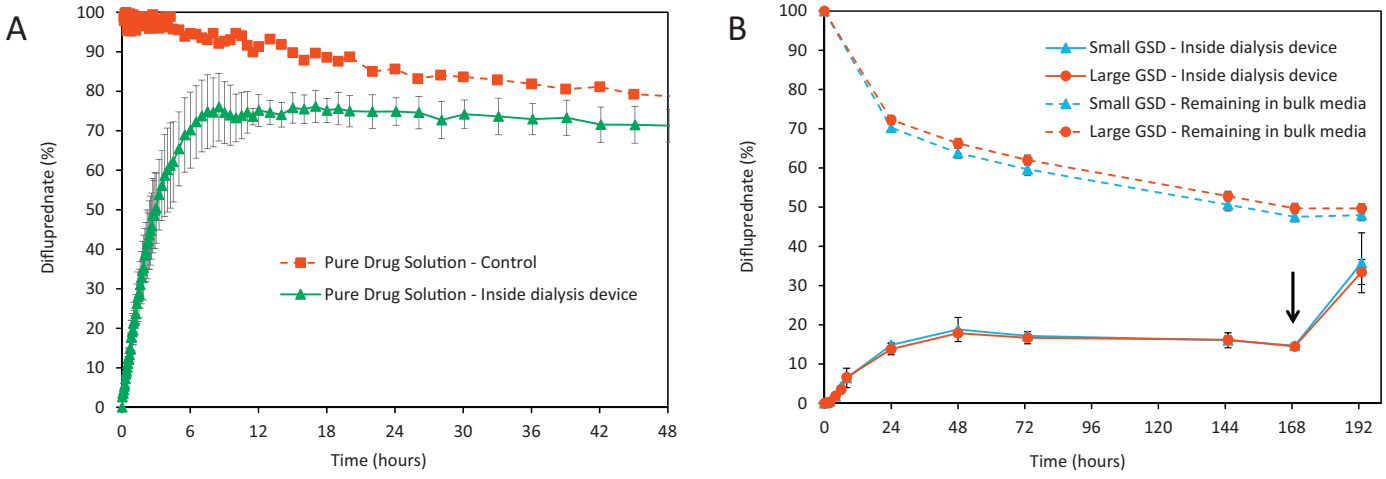

Fig. 6. In vitro release profiles by reverse-dialysis for: (A) pure drug solution (Mean ± SD, $n = 3$ for release test and $n = 1$ for control sample; markers with dashed lines depict the difluprednate degradation in the control sample); (B) nanoemulsions (Mean ± SD, n = 3; markers with dashed lines depict the difluprednate degradation occurring in the bulk medium; downward arrow represents the timepoint at which SDS was spiked in the bulk medium during the in vitro release studies).

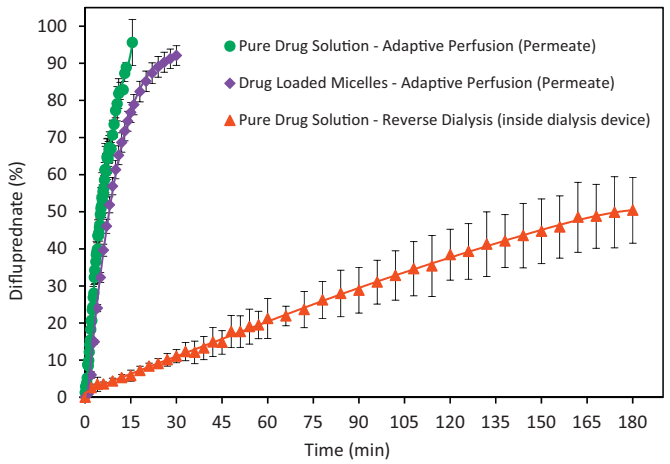

Fig. 7. Overlay of drug transfer profiles for pure drug solution and micelles tested using adaptive perfusion and pure drug solution tested using reverse-dialysis method (Mean ± SD, 3 tests each using a new filter).

or large globules was determined by the AP method (Fig. 8). The results show that percent drug released (in the permeate) from both formulations reached plateau in about 1 h, which was faster than the release rates when determined using reverse-dialysis method (>48 h). Also, the AP method achieved higher drug release as compared to the drug release obtained using reverse-dialysis method. Nearly 80% of drug release was achieved from small GSD nanoemulsion and 68% from large GSD nanoemulsions by using AP method, contrastingly only 18% drug release was obtained when reverse-dialysis method was employed.

Furthermore, the results in Table 4 show that AP method successfully discriminated between different dosage forms of diflupredate, e.g., pure drug solution vs. micelles vs. nanoemulsions. Statistically significant differences were observed when the rates of drug transfer were compared for pure drug solution and drug loaded micelles with the nanoemulsions ($p < 0.05$). The rates of drug transfer for nanoemulsions were 50% lower than those of pure drug solution and drug in micelles. The rate difference was expected due to the additional barrier for drug transfer from oil globules to the release medium. In addition, the lack of ethanol in the release medium used for drug loaded micelles and nanoemulsions (10 mM pH 7.4 phosphate buffer) as compared to the release medium used for pure drug solution (10 mM pH 7.4 phosphate buffer mixed with ethanol in the ratio of 80:20 *v*/v) also explains to some extent the lower drug transfer rate of nanoemulsions. However, the

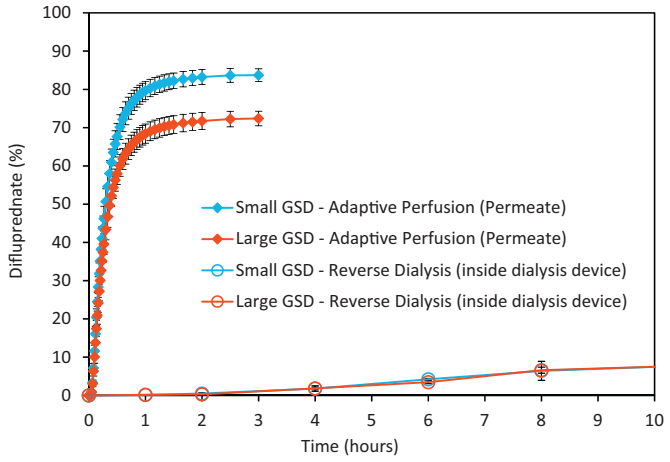

Fig. 8. Overlay of in vitro drug release profiles for small and large globule size nanoemulsions tested using adaptive perfusion and reverse-dialysis methods (Mean ± SD, 3 tests each using a new filter).

Table 4
First-order rate constants, t_{50} and extent of drug transfer for pure drug solution, drug loaded micelles, large, medium and small globule size nanoemulsions for retentate and permeate using the AP method (mean ± sd, $n = 3$).

Sample	Retentate	Permeate		
	k* (min^{-1})	k* (min^{-1})	t_{50} (min)	Maximum Drug Release (%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Pure drug (DFP) solution	0.211 ± 0.056	0.146 ± 0.018	5.3 ± 0.6	93.7 ± 5.5
Drug loaded micelles	0.155 ± 0.016	0.087 ± 0.015	7.7 ± 0.4	96.4 ± 3.1
Large GSD nanoemulsion	0.085 ± 0.002	0.031 ± 0.002	22.4 ± 2.0	72.4 ± 1.9
Medium GSD nanoemulsion	0.085 ± 0.001	0.037 ± 0.001	18.8 ± 0.1	79.6 ± 1.3
Small GSD nanoemulsion	0.083 ± 0.004	0.040 ± 0.003	17.8 ± 1.6	83.7 ± 1.7

*k is the first-order rate constant, determined by equations: $Q = 100*e^{-k*t}$ (retentate) and $Q = 100*(1-e^{-k*t})$ (permeate).

major causative factor for lower drug release rate of nanoemulsions may be attributed to the differences in release processes among solution, micelles, and emulsions.

The change in rate of drug removal, as exhibited by the retentate rate constant values, was subtle when a comparison was made between the different globule size nanoemulsions; however, some level of rate reduction was still discernable moving from larger to smaller globule size nanoemulsions as seen in Table 4 and Fig. 9A.

Statistically significant differences were also observed when the extent of drug release for pure drug solution, drug loaded micelles and nanoemulsions involving different globule sizes were compared (Fig. 9B). AP method provided a clear discrimination between the release profiles of different globule size nanoemulsions with the quickest extent of release coming from smaller globules followed by medium and larger globule size nanoemulsions.

As exhibited in Fig. 9, AP method was able to provide discrimination not only based on the type of dosage forms (i.e., solution, micelles and nanoemulsions) but also based on a difference in a critical quality attribute of the dosage form (e.g., globule size distribution). These studies demonstrated the feasibility of the AP method and its applicability as an important tool for in vitro release testing of drug products containing particulates.

3.3. Other factors that control drug release from nanoemulsions

With the current AP method, the rate and extent of drug release from nanoemulsions were mainly governed by two factors: 1) the surfactant distribution in the nanoemulsions and 2) the flux during the adaptive perfusion process. Consistent with the findings of our previous work [9], surfactant distribution exhibited a direct correlation with the size of the globules, i.e., larger globules (smaller surface area) require smaller amount of surfactant for stabilizing the interface and hence higher percentage of surfactant is available in the bulk aqueous phase to form micelles. Thus, based on the globule size of the nanoemulsion, distribution of surfactant controlled the initial rate of drug removal and the decline of drug concentration in the retentate reservoir (Fig. 9A), i.e., faster decay for larger globule size emulsion. On the other hand, the flux during the AP process governed the extent of drug transfer from the filter to the permeate reservoir. Flux was found to be inversely proportional to the globule size, i.e. smaller the globule size, larger the flux and vice versa (Fig. 10). Hence, there was an increase in the extent of drug release with the decrease in the globule size (Fig. 9B).

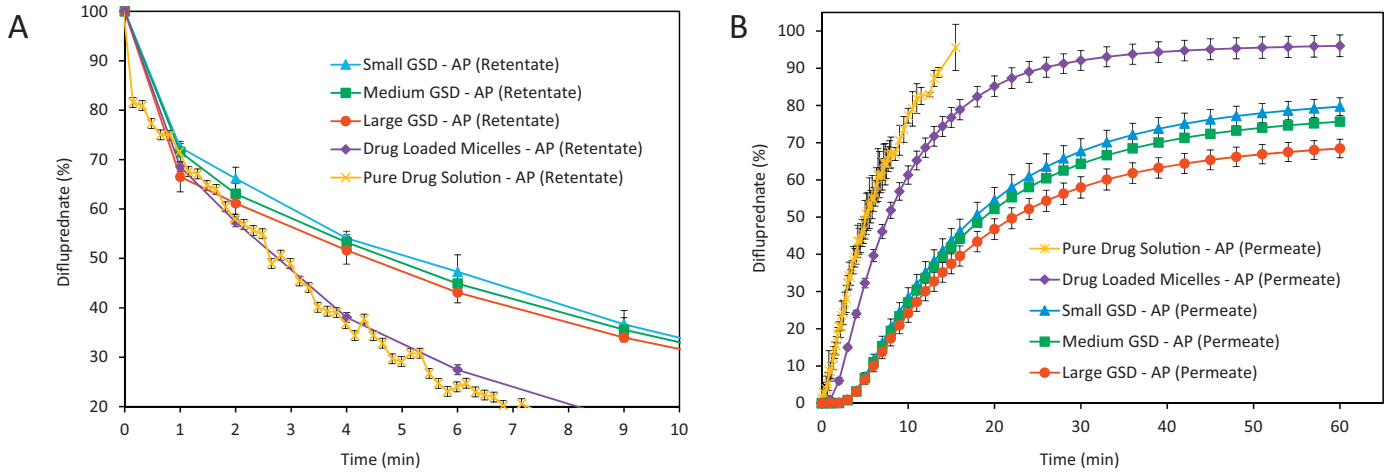

Fig. 9. (A) Initial rate of drug removal and the decline of drug concentration in the retentate reservoir for small, medium and large globule size nanoemulsions, drug loaded micelles and pure drug solution tested using adaptive perfusion method (Mean ± SD, 3 tests each using a new filter); (B) Extent of in vitro drug release in the permeate reservoir for pure drug solution, drug loaded micelles, small, medium and large globule size nanoemulsions tested using adaptive perfusion method (Mean ± SD, 3 tests each using a new filter).

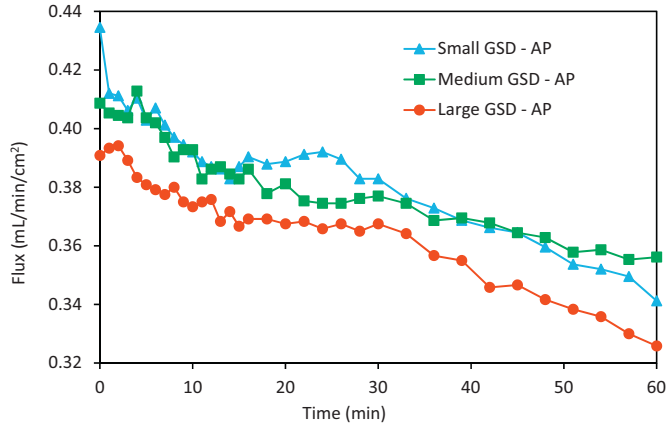

Fig. 10. Flux profiles for small, medium and large globule size nanoemulsions tested using adaptive perfusion method (Mean for 3 tests; each using a new filter).

3.4. On the discriminatory capability of an IVRT method

An IVRT method capable of discriminating among formulations based on changes in critical quality attributes could be useful in new product development, quality control and evaluating the product performance for post approval changes. Changes in critical quality attributes are expected to impact the drug release and hence the product performance and the data provided here show that the AP method can successfully discriminate among formulations having different globule sizes by testing the in vitro drug release. By contrast, the traditional dialysis method was unable to achieve the same level of discrimination.

The AP method described in the current study provides a platform for in vitro release testing of various complex dosage forms. The adaptive nature of the process allows the user to optimize the feed flow rate based on the type of dosage form. For example, higher flow rate may be selected to quickly remove the released drug in case of rapid release from formulations (e.g., micelle phase within emulsions which readily releases the drug within few minutes). Similarly, for formulations with multiphasic release kinetics, flow rates may be adjusted during the experiment to adjust the rate of drug removal. Even though the sample is circulated in a closed loop in the AP method, the simultaneous size-based separation and the concurrent dilution of sample helps in overcoming the limitation due to small medium volume in maintaining continuous sink conditions. Formulation components can be selectively retained based on the MWCO range of membrane. For example, castor oil globules in the nanoemulsions (see Fig. S3) or protein-bound drug containing nanoparticles can be selectively retained and made to continuously circulate in loop (which is being continuously diluted with fresh medium) while allowing smaller species to pass through (see Fig. S4). The selective retention process can effectively help in differentiating the rapid drug release from the extended phase drug release. In addition, AP allows size-based separation while simultaneously analyzing the drug release from the separated components (e.g., in permeate and retentate). Furthermore, there is an increasing need to analyze the role of critical excipients and their impact on drug release. Complex excipients, such as polymers composed of hydrophilic and hydrophobic monomers, can be selectively retained based on their molecular weight by using the AP method. The impact of these polymers on drug release could then be studied, e.g., inducing compositional or manufacturing process changes and evaluating their effect on drug release of resulting product. Of note, that continuous processes in the AP method (i.e., concomitant dilution of the sample and removal of the released drug from the membrane and recirculation of the remaining drug within the AP system) mimics in-vivo conditions such as continuous dilution of the drug on the ocular surface due to tear turnover and continuous absorption of drug after release from the complex formulation. Similarly, drug release testing using the AP method also resembles the drug release condition in parenteral drug delivery, where release of drug into the blood stream occurs during circulation and drug removal occurs due to absorption at the target tissue or organ. Dilution ratio during release testing, which may be highly critical for some dosage forms such as ophthalmics, can be controlled with the AP method; especially for nanosuspensions where dilution can give rise to rapid (instantaneous) dissolution of the nanoparticles and diminish the possibility of differentiating between formulations. Also, in case of nanoemulsions, the initial equilibrium between the oil/aqueous phases present in the formulation governs the drug distribution in each phase; hence very high dilution at the beginning of an in vitro release test may disturb the equilibrium and potentially mask the difference in drug distribution between formulations, especially if the rate of drug removal across the membrane is slow. In case of AP method, dilution rate can be controlled by optimizing the rate of permeate flow so that the sample could be diluted at a lower ratio initially, followed by higher dilution as the test progresses. Because of the controllable rate of drug removal and dilution, the proposed AP method can discern minor differences in drug

release profiles of emulsions with different globule sizes which is an improvement over other available IVRT methods, such as microdialysis, (reverse) dialysis, and Franz cell diffusion. In addition, the AP method also provides the flexibility of tuning the rates of feed flow and fresh medium supply which in turn would govern the rate at which the drug gets diluted as well as the duration required to complete the entire in vitro release test. The capability to tune the conditions provides options for preventing degradation of those drugs which are susceptible to degradation when exposed to large medium volume for a prolonged period as observed in the current study (see Fig. 6A and B). Lastly, the AP method provides the opportunity to expand the application of TFF processes to study in vitro release from complex dosage forms. At present, TFF filters are primarily used in manufacturing processes involving unit operations such as clarification, concentration, purification, or buffer exchange of biological materials as well as pharmaceutical products containing particulates [17–23]. In all these applications, the TFF process is used for the recovery of final product. However, the use of TFF in the AP method described here for testing in vitro drug release from nanoemulsions demonstrates a novel application of TFF process for in vitro performance testing of complex pharmaceutical products. Efforts are currently underway to develop system and setup that would allow use of elevated temperatures (e.g., 37 °C), which may better correlate with in vivo conditions. In addition, the current work is somewhat constrained by the number of options available for hollow fiber chemistry. Future advancement in customizable hollow fibers may further improve the flexibility of the AP method.

4. Conclusion

The adaptive perfusion method provides a new approach to study in vitro drug release from complex formulations. The method overcomes the limitation of the traditional diffusion method (i.e., diffusion rate-limitation by the membrane) and provides a variety of tools that can be modulated to control the rate and extent of drug release depending on the type of drug product, for example, MWCO of the filter, feed flow rate, applied backpressure, controlled rate of dilution and medium volume. The adaptive perfusion method provides an IVRT method that more closely resembles in-vivo physiological conditions compared to other methods such as the dialysis approaches. Different drug release profiles from different types of dosage forms were observed (e.g., solution, micelles and nanoemulsions) and GSD (a critical quality attribute of the product) was directly correlated with drug release characteristics (a performance attribute of the product). AP may serve as a useful tool to support bioequivalence with the brand product as well as serve as a quality control test to ensure batch-to-batch consistency. Such methods can also facilitate new drug product development by providing a better understanding of drug release, especially for complex formulations. Lastly, the method described here has potential to be extended further to analyze the impact of variations in manufacturing process on the drug distribution and release characteristics of other challenging complex drug products.

Disclaimer

This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

Acknowledgements

This project was supported by Critical Path funding from Center for Drug Evaluation and Research (CDER) in FDA, and in part by an appointment to the Research Participation Program at CDER administered by the Oak Ridge Institute for Science and Education through an agreement between the U.S. Department of Energy and FDA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2021.03.024.

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