Drug Development and Industrial Pharmacy, 34:1331–1342, 2008

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040802098177

informa
healthcare

Comparison and Validation of Drug Loading Parameters of PEGylated Nanoparticles Purified by a Diafiltration Centrifugal Device and Tangential Flow Filtration

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This article describes drug loading validation of nanoparticles. Ultracentrifugation was avoided because of problems arising from small-sized particles. Ultrafiltration was adopted in two different modes followed by monitoring of polyvinyl alcohol (PVA), dextran sulfate (DS), and loperamide HCl contents. Diafiltration centrifugation removed all PVA at the fourth cycle and provided significantly (p = .000, .017) higher drug loading values compared with tangential flow filtration (TFF). This was due to residual PVA associated with the nanoparticles. TFF enabled satisfactory dry weight recovery (101.66 ± 4.45%, n = 3) of nanoparticles during extended purification. Indirect drug loading (from the purification curve) was not significantly different (p = .450, .487) to the direct drug loading values. Encapsulation parameters were obtained from the purification curve once quantitative estimation of the all formulation components was established.

Keywords drug loading; ultrafiltration; ultracentrifugation; tangential flow filtration; diafiltration centrifugal devices; nanoparticles; PEGylation

INTRODUCTION

Nanoparticles less than 1 μm in diameter can be prepared from a wide variety of materials not only from synthetic and natural polymers and lipids but also from inorganic materials and metals. Their preparation usually involves high levels of surfactants which solubilize substantial amounts of drug that distributes into the dispersed or external phase. The FDA guidance for liposome drug products recommends assaying for encapsulated and unencapsulated (i.e., free) drug as their relative proportions may exhibit different pharmacokinetic and/or tissue distribution profiles from the same drug substance. It is anticipated that such a requirement may also be relevant to nanoparticulate systems in the future. With the increasing applications of nanoparticles in the drug delivery field, an accurate estimation of free and encapsulated drug has become an essential part of physicochemical characterization. An evaluation of the drug content associated with colloidal systems is complicated owing to the difficulty in separating free from incorporated drug, because of the colloidal structure of the formulation (Boyd, 2005; Heydenreich, Westmeier, Pedersen, Poulsen, & Kristensen, 2003; Michalowski, Guterres, & Costa, 2004).

Ultracentrifugation is the most widely used method to separate free drug from that in the colloid. Free drug is generally determined in the supernatant obtained after ultracentrifugation, and incorporated drug is measured in a settled pellet after dissolving it in a suitable solvent. Total drug content in a formulation is usually measured by dissolving the dispersion in a common solvent, or estimated by a summation of the free and incorporated values. Incorporated drug could also be measured by difference of free drug from the total drug in the formulation that is measured quantitatively or assumed from the initial amount. This approach may generate inaccuracy when ultracentrifugation fails to provide complete separation of free and incorporated drug in nanoparticles (Magneheim & Benita, 1991). Very high speed centrifugation at 50,000–300,000 × g-forces for up to 3 h is used (Govender, Stolnik, Garnett, Illum, & Davis, 1999; Niwa, Takeuchi, Hino, Kunou, & Kawashima, 1993; Riley et al., 1999) to determine drug loading of nanoparticle formulations. This also leads to the formation of pellets, which are generally difficult to redisperse due to aggregate formation (Chiellini, Orisini, & Solaro, 2003). Use of lower g-forces can avoid aggregate formation; however, the use of lower than 50,000 × g force conditions has resulted in incomplete separation of the submicron particles from the supernatant especially for particle size values of less than 100 nm (Allèman, Doelker, & Gruny, 1993).

There are alternative methods; one reported by Illum, Khan, Mak, and Davis (1986) used bathochromic shift and fluorescence spectroscopy that allowed drug quantification without separating free from incorporated drugs. Such a method is specific to certain drug candidates (Illum et al., 1986). The novel analytical technique of field flow fractionation (FFF), specifically sedimentation FFF (Sd FFF) may be utilized to separate

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free drug nanocrystals from incorporated drug. However, such techniques can only process a very small quantity of the sample, thus requiring highly sensitive analytical methods to detect the drug associated with colloids. This has restricted the use of FFF for preparative purposes (Magneheim & Benita, 1991).

Ultrafiltration/centrifugation and microdialysis have also been utilized to determine drug loading, where the free drug content was separated using an ultrafiltration device with a 20K molecular weight cut off (MWCO) membrane (Magneheim & Benita, 1991; Michalowski et al., 2004). This technique uses MWCO ultrafiltration membranes fabricated within the centrifugal devices that operate at relatively lower *g*-forces to provide separation of nanoparticles from the medium within a few minutes. The efficient separation of nanoparticles from soluble components (surfactants and free drug) is dependent on an appropriate membrane MWCO, device design, and optimized concentration and volume of the nanoparticle dispersion, which otherwise could clog the membrane. The total drug content is obtained by completely dissolving the nanoparticle dispersion in a suitable solvent. Thus, an estimated value can be obtained on a sample of the preparation that should be representative of the whole batch. However, there are two limitations of such an approach: first, this device cannot distinguish between nanocrystallized and incorporated drug within nanoparticles; second, the centrifugal devices have limited volume capacity, and therefore a whole batch treatment is generally not possible to obtain recovery for further use of the nanoparticles. Previous report lack details, and cross validation between the estimated and actual incorporated drug contents (Ammoury et al., 1990; Ammoury, Fessi, Devissaguet, Dubrasquent, & Benita, 1991; Fessi, Puisieux, Devissaguet, Ammoury, & Benita, 1989) are obtained using such methods. Although ultrafiltration can potentially separate the soluble fraction from nanoparticle dispersions, the ultracentrifugation method is still being used to date to determine drug content because of the ability to recover nanoparticles for further use (Govender, Stolnik, Garnett, Illum, & Davis, 1999; Niwa et al., 1993; Riley et al., 1999). However, the disadvantage or inability to separate nanocrystallized free drug and incorporated drug using these methods remains a limitation in both methods. Thus, the use of ultrafiltration/centrifugation is not as widely acknowledged as ultracentrifugation, and its usefulness to determine the drug content is rarely reported.

In our experience, previously reported ultracentrifugation conditions did not work satisfactorily for accurate drug loading determination and efficient recovery. Physical destabilization and poor recovery with ultracentrifugation were mainly encountered. As an alternative, diafiltration centrifugal devices (DCDs) were extensively evaluated and resulted in the use of Nanosep™ 300K. This device was further optimized for nanoparticle concentration and sample volume to avoid the general problem of membrane fouling and possible erroneous interpretations of drug loading. However, using small DCDs deemed suitable for drug loading determination required an efficient recovery of the drug loaded nanoparticles devoid of free drug and surplus surfactant, which still remained a challenge on a laboratory as well as a large-scale operation. As in our previous work, we demonstrated purification of drug loaded nanoparticles; in this work, we additionally detail the accuracy of drug loading parameters, total recovery, and mass balance during the TFF purification process. These parameters were compared statistically with the drug loading parameters obtained using a DCD approach to confirm the accuracy and mass balance of the whole approach.

MATERIALS AND METHODS

Materials

Polyvinyl alcohol (PVA) (80–89% hydrolyzed, MW 9,000–10,000) was purchased from Sigma Aldrich, Ann Arbor, MI, USA, and Minimate™ capsule with Omega™ 300K membrane (Lot # 3086E002) was purchased from PALL Scientific, Ann Arbor, MI, USA. The synthesis and characterization of monomethoxy poly(ethylene glycol)–poly (D,L-lactide-co-glycolide) (mPEG–PLGA) copolymer were carried out as described previously (Dalwadi & Sunderland, 2007). The copolymer with a glass transition of 24.58°C and 35,974 average MW was used in this study. Loperamide HCl (99% pure, Lot # 103K0611, Sigma Aldrich, Sydney, Austraila), dalargin (>95% pure, batch no # P20645, Auspep Ltd, Australia), and polyethylene glycol (monomethyl ether) (99% pure, Lot # 44931/1, Fluka, Munich, Germany) were used. Glycolide monomer (PurasorbG, 99.9% pure, Lot # 0305000324) and D,L-lactide monomer (PurasorbD, 99.9% pure Lot # 0310000164) were purchased from PURAC International, Gorinchem, The Netherlands. Stannous octoate (95% pure, Lot # 102K0104, Sigma Aldrich, Sydney, Australia) was also used. Ultrapure water (<6 μS) prepared from a Milli-Q purification system was used in all experiments.

Preparation and Characterization of Nanoparticles

Preparation of Drug Loaded Nanoparticles

For the preparation, 200 mg of mPEG–PLGA copolymer was dissolved in 6 mL of 2 mg/mL loperamide HCl solution in acetone, and a further 4 mL of acetone was added for solubilization followed by the addition of 0.025 mL of dichloromethane (DCM). To this organic phase, 0.3 mL of 20 mg/mL solution of dextran sulfate (DS) (MW 5,000) was added. The entire organic phase was added dropwise to a 0.6% 60 mL solution of PVA. The solution was stirred at 200 rpm for 7.5 h at ambient conditions (temperature 18–22°C, and relative humidity 30–40%).

Preparation of Empty Nanoparticles

Double strength (400 mg copolymer in 60 mL of PVA solution) empty nanoparticles were prepared excluding drug from the previous preparation. After preparation, 30 mL of nanoparticle suspension was mixed with 30 mL of 0.25 mg/mL loperamide HCl in 0.6% PVA solution. This provided an equivalent concentration to a single strength (200 mg in 60 mL) nanoparticle with

almost 3.75 mg loperamide HCl in solubilized form (6 mg was not achieved due to solubility limitations); this was done to imitate the existence of free drug in the nanoparticle dispersion.

The particle size was measured using photon correlation spectroscopy (PCS) at 25°C with a detection angle of 90°. The mean size *Z* was derived from the raw data using a Zetasizer 3000HS (Malvern, UK) in cumulative analysis mode. The zeta potential measurements were performed by laser Doppler anemometry. The nanoparticle preparations were diluted fivefold with distilled water before these measurements.

Quantification of Surfactant and Drugs

Quantification of PVA

A colorimetric assay for PVA was adapted from Finley, (Finely, 1961) and performed as described previously (Dalwadi & Sunderland, 2007). From a 2 mg/mL PVA stock solution, specific volumes of the fractions were transferred separately to 10 mL volumetric flasks, and then 5 mL of water, 3 mL of 4% boric acid, and 0.6 mL of 0.1 N iodine solution were added in each flask and finally made up to 10 mL with water. This generated a complex between boric acid and PVA which had a green color in the presence of iodine solution that was measured for absorbance at 690 nm using UV–VIS spectrophotometry. This method was found to be linear over 0–35 μg/mL concentration range ($y = 0.0221x + 0.0364$, $R^2 = 0.9993$) with an accuracy of 100.30 ± 4.37%, $n = 3$, and was also specific to quantify PVA in the nanoparticle matrix.

To determine the amount of PVA in the filtrate, a known volume of filtrate was firstly diluted with water, then mixed with boric acid and iodine solution, and adjusted to volume with water as described above, and then absorbance value was obtained at 690 nm, which was then converted into concentration using the above calibration curve.

Quantification of Dextran Sulfate Using Aqueous Gel Permeation Chromatography

Quantification of DS was performed on a GPC system comprised of Biosep™ 2000, a size exclusion chromatography (SEC) column (300 × 7.5 mm, Phenomenax, Sydney, Australia) attached to photo diode array (PDA, model 2996, Waters, Australia) and refractive index (RI) detectors (model 2414, Waters, Australia) in series. The sample and mobile phase were injected onto the column via a separation module (model 2965 Alliance™, Waters). Also, 20 mM phosphate buffer of pH 6.85 was used as mobile phase at 1.0 mL/min flow rate. The samples were analyzed using Empower™ software. The calibration was performed over a concentration range of 0.1 to 1 mg/mL in the presence of 0.6% PVA ($y = 229{,}189x + 2{,}425$, $r^2 = 0.9996$). DS was monitored at 261 nm on the PDA. This method was adopted to determine the amount of free DS within the nanoparticle formulation. This was done using 0.1 mL of suspension and the Nanosep™ 300K centrifuged at 4000 × *g* for 20 min. The clear centrate was injected directly onto the aqueous GPC setup mentioned above to quantify the free DS.

High-Performance Liquid Chromatograph Assays of Loperamide HCl and Dalargin

An high-performance liquid chromatograph (HPLC) method was developed using a reversed phase C18 column and a Waters HPLC system which comprised of dual wavelength UV detector, dual piston pump, and autosampler controlled by Breeze™ software. Dalargin was quantified over a concentration range 0.2–300 μg/mL ($y = 14{,}853x - 6{,}884$, $r^2 = 0.9999$) with an accuracy of 92.6 ± 5.4%, $n = 3$, using 22% acetonitrile and containing 0.1% TFA in water as a mobile phase at 1 mL/min flow rate with detection at 215 nm.

On the same HPLC system, loperamide HCl was also quantified as described in previous work (Dalwadi & Sunderland, 2007). This assay was also applied to dried recovered mass. Briefly, 100 mg, accurately weighed dried nanoparticle mass was digested in 10 mL of methanolic KOH (2%, wt/vol) using sonication for 5 min; a clear fraction was obtained by centrifugation of this sample (9000 × *g* for 5 min) and diluted 10-fold with mobile phase and subjected to loperamide HCl quantification using HPLC analysis.

Drug Loading on Nanosep™ 300K

Nanosep™ 300K, an ultrafiltration centrifugal device fabricated with Omega™ membrane, was used to determine the free drug concentration within the nanoparticle dispersion. A sample of 0.1 mL was taken into the reservoir of the device, immediately after preparation of the nanoparticle batch, and centrifuged for 20 min at 4000 × *g*. After the centrifugation cycle, 0.1 mL of centrate was obtained, 0.05 mL of which was diluted with an equal volume of PBS buffer and analyzed by HPLC. The remaining 0.05 mL was subjected to PVA analysis. The retentate was again filled with 0.1 mL of water, and the cycle was repeated five times for dalargin and six times for loperamide HCl loaded nanoparticles, where PVA was only monitored for a total four cycles.

To evaluate any adsorption and the recovery of the drug onto the device membrane, 0.1 mL of the solubilized portion of the drug was filled into the Nanosep™ and centrifuged under similar conditions followed by HPLC analysis of the centrate obtained after each cycle. A similar procedure was used for the standard PVA solution (0.6%) to evaluate PVA adsorption on the membrane.

To evaluate the impact of a nanoparticles layer on free drug removal from the device, a 0.1 mL dispersion mix of empty nanoparticles and free loperamide HCl solution was placed in the Nanosep™ and treated similarly as mentioned above. In the case of dalargin, single strength empty nanoparticles were prepared, 6 mg of dalargin was dissolved externally at the end of the nanoparticle preparation to imitate the existence of free drug, and 0.1 mL was treated by Nanosep™ similarly as mentioned above.

A plot of % drug released from the Nanosep(device versus number of centrifugal cycles was prepared for all centrifugal

TABLE 1
Initial Formulation Contents

Materials	Theoretical Level (mg)	
	Amounts Added to Starting Volume (70 mL)[a]	Calculated Content in TFF Purification Sample (50 mL)[b]
Copolymer	200	181.82
PVA	360	327.27
Drug (Loperamide HCl)	12	10.90
Dextran sulfate	24	21.82
Total	596	541.82

PVA, polyvinyl alcohol.

[a]Starting volume is volume of solvent system (10 mL) + dispersed system (60 mL).

[b]Purification sample volume is the volume left after 7.5 h stirring time (55 mL) – (5 mL) taken for particle size and zeta potential measurement.

treatments mentioned above. These plots were employed to estimate drug loading parameters. The percentage of drug release difference between empty nanoparticle plus free drug mix and loaded nanoparticle samples provided the percentage encapsulation efficiency (Table 2). Using this, the encapsulated drug amount was derived from the total amount of the drug in the starting volume of the preparation (Table 1), which was converted into percentage loading using the following equation

$$\text{Percentage loading} = \frac{\text{Amount of drug encasulted}}{\text{Amount of estimated recovery}} \times 100$$

The estimated recovery is the sum of each ingredient left in the retentate after centrifugation treatment using Nanosep™. This included the amounts of copolymer, DS, and residual PVA along with encapsulated drug residual PVA along with encapsulated drug. The amount of copolymer is equal to its level in the starting volume of the preparation (Table 1). The amount of the DS retained was (9.4 mg out of 24 mg) estimated using the aqueous GPC method mentioned before. The residual amount of PVA associated with nanoparticles was calculated as below, and values are shown in Table 2.

$$\text{Residual amount of PVA} = \frac{\text{Residual percentage of PVA} \times \text{total amount of PVA}}{100}$$

Residual percentage of PVA values are mentioned in Table 2, and total amount of the PVA in the preparation can be obtained from Table 1.

Drug Loading on TFF

The TFF system was set up as illustrated in Figure 1 and adopted for this work (Dalwadi, Benson, & Chen, 2005). The nanoparticle suspension was purified at 20 psi transmembrane pressure (TMP) on a TFF system comprised of Minimate™ fabricated with Omega™ 300K membrane (PALL Scientific). Concentrated mode was adopted for purification, where 50 mL of the drug loaded nanoparticle dispersion was continuously circulated back to the same reservoir under stated conditions until the retentate volume reached approximately 10 mL. The short purification process was restricted to one additional 30 mL purified water wash after the starting dispersion volume was concentrated to a minimal volume during purification. For extended purification, an additional three washes, each of 30 mL, followed the first purification cycle. In both cases, the filtrate fractions were collected from the filtrate port during the entire process and analyzed for PVA and loperamide HCl using the stated methods.

Nanoparticle Recovery and Loading from the Purification Curve

From HPLC analysis of the filtrate fractions, the purification curve of cumulative percentage of loperamide HCl and PVA removed versus cumulative time (h) was plotted for both cases (short and extended purification) to estimate nanoparticle recovery. It was calculated by subtracting the total loss of PVA, free drug, and DS in the TFF filtrate (Table 5) from their initial values in the samples (50 mL) before purification (Table 1). The net amounts of each component were expected to be recovered along with total amount of the copolymer (Tables 1 and 5) in recovery.

From the percentage difference between free drug losses in the filtrate and its starting amount (100%) in the preparation (Table 1), the percentage encapsulated efficiency was calculated (Table 2). The equivalent amount of encapsulated drug (mg) was calculated using the initial level of loperamide HCl in the purification sample (Tables 1 and 5). This amount was converted to the percentage loading (Table 6) using the nanoparticle recovery obtained from the purification curve (Table 5) as mentioned below

$$\text{Percentage loading} = \frac{\text{Encapsulated amount of drug}}{\text{Amount of recovery}} \times 100$$

Nanoparticle Recovery and Loading on a Dry Weight Basis

At the end of purification, the contents were recovered when concentrated to a minimal volume the second time in the case of the short purification and to a minimal volume the fourth time in the case of the extended purification. This occurred in a preweighed empty container by pushing air into the TFF device, followed by their lyophilization. The dry-weight-based recovery was calculated from the difference in weights after drying the contents and corrected to a percentage (Table 5).

TABLE 2
Estimation of the Loading Parameters Using Nanosep™ 300K

	Percentage Free Surfactant and Drug in Centrate after First Centrifugation Cycle				
	Initial Level in Loaded NPs (a)	From Loaded NPs (b)	From *SD* Solution (0.6% PVA) (c)	Residual Amount Associated with NPs	
				% (a – b)	mg[a]
Surfactant (PVA)					
1st Cycle	98.49	72.85	103.25	25.64	92.30
	104.50	82.15	98.45	21.30	76.68
	95.77	79.16	106.23	16.61	59.80
Mean	99.24	78.05	102.64	21.95	79.02
SD	3.89	4.75	3.93	4.52	16.29
	Drug Loaded NPs (a)	Empty NPs + Drug Solution (b)	Encapsulations Efficiency (b – a)	Percentage Loading[b]	
Loperamide HCl					
1st Cycle	18.58	96.12	77.54	3.12	
	16.52	92.24	79.60	3.05	
	23.57	100.00	72.55	3.08	
Mean	19.56	96.12	76.56	3.08	
SD	3.63	3.88	3.63	0.04	
Dalargin					
1st Cycle	13.65	92.28	78.63	2.89	
	21.85	100.02	70.43	2.73	
	17.74	84.56	74.53	3.05	
Mean	17.75	92.28	74.53	2.89	
SD	4.10	7.72	4.10	0.16	

[a]Residual amount of PVA (mg) calculated as per Equation 2 (refer material method).
[b]Percentage loading calculated as per Equation 1.

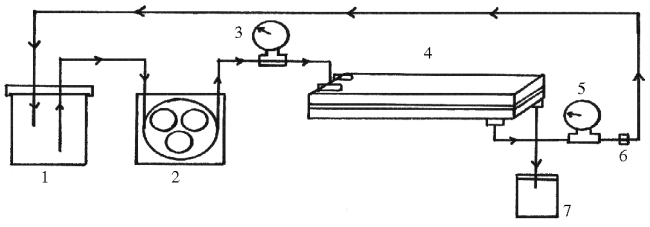

FIGURE 1. Components of the schematics of TFF (Adopted from Dalwadi, 2005). (1) sample reservoir; (2), peristaltic pump-1; 3, feed pressure gauge; (4) Minimate™ 300K; 5, transmembrane pressure gauge; (6) screw clamp valve; 7, filtrate collector.

A 100 mg sample of dry recovered mass was analyzed for its loperamide HCl content (Table 5) as discussed before and corrected to a percentage after considering a moisture content of 3.2 mg/100 mg, determined using thermo gravimetric analysis (TGA), briefly 15.79 mg of sample was heated to 700°C on a Thermal Advantage (TA) Instrument, Q50 model, and loss on drying was decided at 180°C (Table 6).

Comparisons of Loading Parameters

The nanoparticle recoveries estimated from the TFF purification curves were compared with their dry weight recoveries. At the same time loperamide HCl loading calculated from respective recoveries were compared to evaluate the accuracy of this methodology. The drug loading value and PVA removal efficiency obtained using Nanosep™ (300K) was statistically compared with TFF purifications outcomes using (p) values obtained by one-way ANOVA or a paired sample t test.

RESULTS AND DISCUSSION

Nanoparticle Preparation

The drug loading concept was adapted from previous work (David, Eric, Hatem, & Erie, 1997; Falk, Randolph, Meyer, Kelly, & Manning, 1997; Meyer et al., 1995; Powers, Matsuura, Brassell, Manning, & Shefter, 1993). The use of polyelectrolytes to entrap an oppositely charged compound is well known as coacervation (Morawetz & Hughes, 1951). Grafting of positively and negatively charged entities on a PLGA backbone also trapped oppositely charged drug molecules (Dailey & Kissel, 2005). A similar strategy was adopted here to trap cationic drug molecules. Negatively charged DS (5,000 MW) was selected to enhance the drug loading of two cationic drugs loperamide HCl (lipophilic) and dalargin (hydrophilic, a custom synthesized hexapeptide). Despite using PEGylated PLGA in the preparation of the nanoparticles, use of PVA was still required, although less was used than in non-PEGgylated PLGA nanoparticle formation (Niwa, Takeuchi, Hino, Kunou, & Kawashima, 1993, 1994). The level was still sufficient to cause membrane fouling during treatment with the ultrafiltration devices. Use of 0.6% PVA and control of DCM levels in the preparation provided control of the particle size of the nanodispersion (Table 6), which effectively remained larger than the pore size (<40 nm) of the Nanosep(membrane. Due to this size difference, the nanoparticles were able to be completely separated from excess surfactants using Nanosep(300K.

Loperamide HCl solubility in 0.6% PVA during the process time (7:5 h) was 0.250 mg/mL, which suggested that a 12 mg dose can be dissolved in 60 mL of 0.6% PVA. Hence, it was unlikely that loperamide HCl would crystallize in the dispersion phase (0.6% PVA). The solubilized portion that remained free was likely to come out in the filtrate in TFF purification and in the centrate in the case of Nanosep™ 300K operations. Above the solubility level, loperamide HCl was observed to crystallize, producing greater than 1,000 nm crystals (data not presented). But at the ratio of drug and dispersed phase used in this study, no such crystallization was observed and was also confirmed from the nanoparticle average size (Table 6) and distribution profile that was unimodal with no particle size greater than 150 nm. Therefore, interpretation obtained from Nanosep™ and TFF devices was based on a nanoparticle suspension rather than including any possibility of nanocrystals.

Drug Loading Using Nanosep™ 300K

Previous studies have utilized 20K MWCO centrifugal devices for drug content determination. Use of the same MWCO caused membrane fouling; a 100K MWCO also failed to provide sufficient flux due to fouling caused by a PVA interaction with the membrane (Dalwadi et al., 2005). Using a higher MWCO (300K) successfully removed PVA without membrane fouling. After centrifugation at 4000 × *g* for 20 min, 100% flux was obtained as a clear centrate, with complete retention of the nanoparticles. This was confirmed by testing clear centrate for the absence of nanostructure using PCS at 25°C. Analysis of the centrate showed that 19.56 ± 3.63%, *n* = 3, of free dissolved loperamide HCl was removed after the first cycle (Table 2). From the mixture of empty nanoparticles and free drug solution, all of the loperamide HCl was released in the centrate (Table 2), which indicated unencapsulated drug or the layered nanoparticles on the membrane did not impair free drug removal. From the difference in these values it was estimated that 76.56 ± 3.63%, *n* = 3, of drug was encapsulated (Table 2). The first cycle of centrate was also analyzed for free DS using aqueous GPC, a total of 14.60 ± 0.60 mg, *n* = 3, out of 24 mg per 55 mL remained free, and the remainder was considered to be within the nanoparticles possibly as an ion pair with the drug. At the same time, only 78.05 ± 4.75%, *n* = 3, of PVA was removed in the centrate from the loaded nanoparticles (Table 2). The remainder was postulated to be associated with nanoparticles, because the standard PVA solution treatment on the Nanosep™ suggested minimal adsorption of PVA on the membrane at the proposed concentration. This associated PVA was removed gradually in three subsequent cycles (Figure 2). The amounts of DS and PVA associated with the nanoparticles after the first cycle were used in Equations 1 and 2 to calculate the percentage drug loading values (Table 2). Because PVA was removed gradually from the drug loaded nanoparticles during Nanosep™ treatment, at every centrifugal cycle a finite amount of PVA was removed. Therefore, the percentage drug loading is different at each stage. After the first centrifugation cycle, the loading of loperamide HCl was found to be 3.08 ± 0.04%, *n* = 3, (Table 2) compared with the fourth

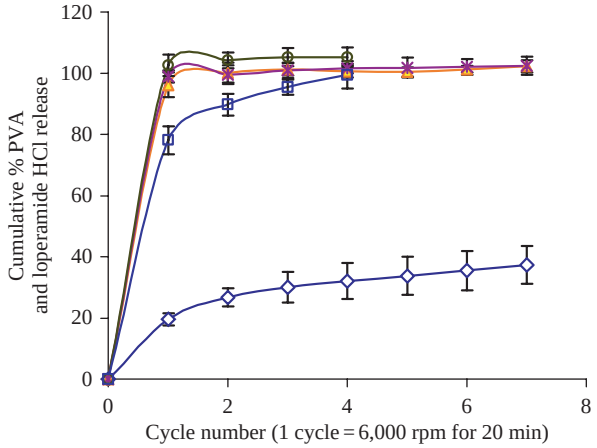

FIGURE 2. Determination of drug loading of loperamide HCl loaded nanoparticles using Nanosep™ 300K (an ultrafiltration centrifugal device). Δ, empty nanoparticles (no drug with dextran sulfate) and free drug solution;◊, loperamide HCl loaded nanoparticles; □, % PVA released from the loperamide HCl loaded nanoparticles; ○, % PVA release from standard PVA solution (0.6%); ×, standard loperamide HCl solution in 0.6% PVA. Results are mean ± standard deviation of (*n* = 3) samples.

cycle, where drug loading was 4.20 ± 0.05%, n = 3, as almost all PVA was removed from the dispersion.

From the treatment of standard drug solutions on Nanosep™ 300K, it was confirmed that no drug adsorption was evident on the Omega™ membrane (Table 2). This supported the approach of considering the percentage of drug released from the Nanosep™ after the first cycle in the loading calculations without any correction factors for membrane adsorption of the drug candidates.

Nanoparticles loaded with dalargin when treated similarly showed 17.75 ± 4.10%, n = 3, (Table 2) free drug in the centrate after the first cycle. From the mixture of empty nanoparticles and free drug solution, all of the dalargin was released in the centrate, and hence no drug was in the encapsulated condition (Figure 3). From the difference of these values, it was estimated that 74.53 ± 4.10%, n = 3, of drug was encapsulated (Table 2). In the case of dalargin encapsulation, no free DS was found in the centrate, which indicated that the whole amount was ion paired with drug. Therefore, it would be expected to be recovered along with the nanoparticles, and was considered in the recovery (Equation 1). This provided 2.89 ± 0.16%, n = 3, loading of dalargin (Table 2). The difference between loperamide HCl and dalargin ion pairing tendencies could be attributed to different magnitudes of charge on the drugs. Dalargin is a cationic hexa-peptide having multiple charges and could coacervate greater amounts of DS (Morawetz & Hughes, 1951).

Due to the limited volume handling capacity of the DCD (Nanosep(), total recovery was not possible to give a substantial amount of the dry mass. Therefore, the drug loading parameters were estimated indirectly by treating the dispersed suspension using DCD. This limitation was overcome using TFF that allowed the treatment of a large sample volume followed by its recovery in a concentrated form. To confirm the validity of all estimated values obtained by Nanosep(300K and TFF, the dry mass of encapsulated drug after purification was subjected to HPLC analysis. In this case, loperamide HCl was studied extensively using TFF purification, due to its lower cost, whereas dalargin was restricted only to a Nanosep™ 300K processing due to its limited availability and cost.

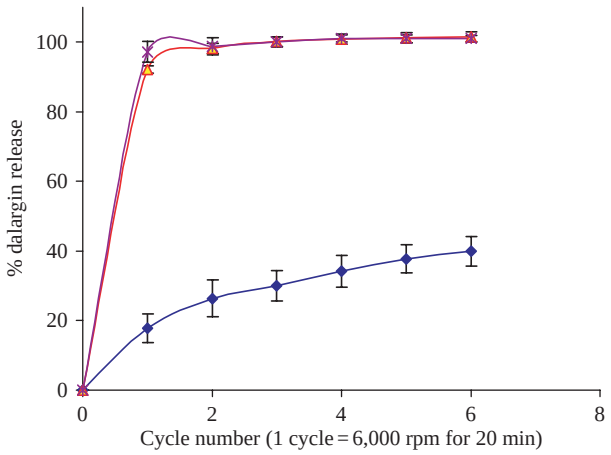

FIGURE 3. Determination of drug loading of dalargin loaded nanoparticles using Nanosep™ 300K (an ultrafiltration centrifugal device). Δ, Empty nanoparticles (no drug and dextran sulphate) and free drug solution; ♦, dalargin loaded nanoparticles; ×, free drug solution. Results are mean ± standard deviation of (n = 3) samples.

Drug Loading Using TFF

Percentage Encapsulation Efficiency from TFF Purification Curves (Short vs. Extended)

In our previous work, the empty nanoparticles (without DS) were purified completely from PVA within 1 h (Dalwadi & Sunderland, 2007). However, in this study, drug loaded nanoparticles (with DS) treated similarly did not satisfactorily remove PVA within 1 h. Only 31.33 ± 2.72%, n = 5, of the PVA was removed in the filtrate within an hour, the majority being found on the nanoparticles, whereas 16.60 ± 6.67%, n = 5, was estimated to have been adsorbed on the membrane (Table 4; Figure 4). This slow purification behavior was largely caused by the presence of DS in the preparation (Dalwadi & Sunderland, 2007). Therefore, extended purification was performed that removed additional PVA in the filtrate. This also effectively reduced membrane adsorption of PVA and the percentage of PVA left on the nanoparticle (Table 4).

During the short purification, 22.49 ± 0.83%, n = 5, of loperamide HCl was also removed in the filtrate along with the PVA (Table 3); this value reached a plateau level which indicated that the free drug was removed quickly compared with that encapsulated (Figure 4). Within that time period, PVA was not removed to the desired extent; therefore, an additional three washing steps were required to remove additional PVA. However, it was possible that dilution of the formulation could release more drug. Therefore, drug release was also monitored along with PVA removal during the extended purification process (Dalwadi & Sunderland, 2007). During the extended purification time, significantly more (p = .037) loperamide HCl was removed than during the short purification (Table 3). Notably, 72.93 ± 2.26%, n = 3, of loperamide HCl remained encapsulated after extended purification, which was marginally (p = .052) lower compared with that encapsulated after the short purification process (Table 3).

Recovery and Drug Loading from Purification Curves (Short vs. Extended)

A higher recovery was anticipated for the short purification process; however, this was only marginal and insignificant (p = .055) compared with the recovery after extended purification (Table 5). This was attributed to the higher amounts of PVA associated with the nanoparticles during the short process (Table 4). This further reflected on the percentage loading values obtained from the short purification curve, which was significantly (p = .014) lower than the value derived from the extended purification curve (Table 6). This was the reversed

TABLE 3
Distribution of Loperamide HCl (%) of the Formulations Purified by the TFF System

Purification	Percentage Loperamide HCl Distribution				
	Removed in the Filtrate[a]	Left in Nanoparticles[b]	Left in Nanoparticles[c]	Adsorbed on the Membrane[d]	Total[e]
Short					
1	22.00	78.00	69.65	8.35	91.65
2	23.72	76.28	62.59	13.69	86.31
3	22.90	77.10	67.60	9.50	90.50
4	22.23	77.77	70.55	7.22	92.78
5	21.61	78.39	71.32	7.07	92.93
Mean	22.49	77.50	68.34	9.17	90.83
SD	0.83	0.86	3.50	2.71	2.71
Extended					
1	28.43	71.57	79.12	0	107.55
2	24.46	75.54	73.51	2.03	97.97
3	28.30	71.70	70.26	1.44	98.56
*Mean	27.06	72.93	74.29	1.55	101.36
SD	2.26	2.26	4.48	1.15	5.37

[a]Assayed.
[b]Estimated (100 – a).
[c]Assayed.
[d]Estimated 100 – (a + c).
[e]Estimated (a + c).
*Mean and *SD* extracted from Dalwadi, 2007.

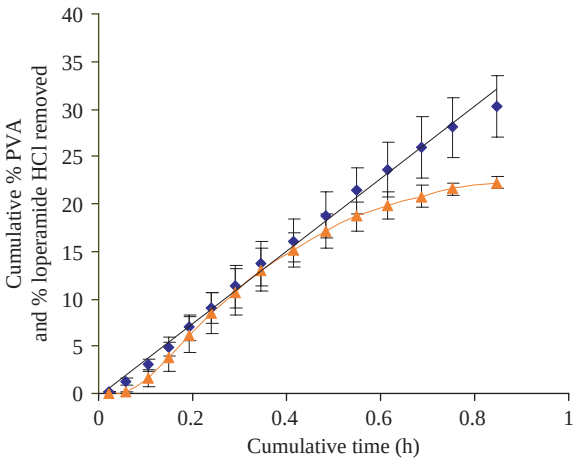

FIGURE 4. Short purification of loperamide HCl loaded nanoparticles on TFF at 20 psi TMP, comparison of drug release during purification period, TMP 20 psi, (n = 5) sample. ♦, cumulative % PVA removed, ▲, cumulative % loperamide HCl removed in the filtrate.

order of the relevant encapsulation efficiencies obtained from TFF purification curves and suggested that the percentage loading values were largely dependent on the residual amounts of PVA. Encapsulation efficiencies from TFF curves were dependent on the percentage of free drug lost in the filtrate (Table 6) where residual PVA was not accounted for in the calculation.

Recovery and Drug Loading from Recovered Dry Weights (Short vs. Extended)

The encapsulation efficiencies obtained from the recovered dry mass after shorter purification was insignificantly (p = .092) lower than that obtained from extended purification (Table 6). This was opposite in trend to that observed when encapsulation efficiencies were calculated from the purification curves. This was largely attributed to the amount of residual PVA associated with nanoparticles and its impact on sample preparation for assay. The dried mass of nanoparticles digested in 2% KOH in methanol only dissolved the mPEG–PLGA polymer and drug, followed by a separation of PVA as a gel, which potentially entrapped some of the drug and reduced the amount to be quantified. This therefore showed a lower encapsulation efficiency for the short purification process. The extent of the gelation was less in the dry mass recovered after extended purification due to relatively less residual PVA, hence improved assay recovery and higher encapsulation efficiency values. Residual PVA also affected the percentage loading in a similar way, with a significantly (p = .024) lower (2.33 ± 0.18%, n = 5) loading obtained after short purification compared with that from the extended purification process (2.76 ± 0.23%, n = 3) (Table 6).

The percentage drug loading values derived from purification curves and recovered as dry mass (short and extended) were not significantly different (p = .450, .487) and suggested that purification curves were able to provide estimated loading

TABLE 4
Distribution of PVA (%) in the Formulations Purified on the TFF System

	PVA Distribution (%)			
Purification	Removed in the Filtrate[a]	Left on Nanoparticles[b]	Adsorbed on the Membrane[c]	Total Removed[d]
Short				
1	27.68	49.15	23.16	50.84
2	32.59	60.54	6.82	39.42
3	29.24	57.96	12.79	42.03
4	33.21	46.27	20.50	53.73
5	33.94	46.30	19.77	53.72
Mean	31.33	52.03	16.60	47.94
SD	2.72	6.74	6.67	6.76
Long				
1	64.50	30.85	4.65	69.15
2	68.38	33.04	3.61	71.95
3	60.66	28.05	6.3	66.96
*Mean	64.50	30.64	4.85	69.35
SD	3.84	2.50	1.36	2.50

[a]Assayed.
[b]Assayed.
[c]Estimated (b – a).
[d]Estimated (a + c)
Assay limit for PVA is 100.30 ± 4.60, (n = 3).
*Mean and *SD* extracted from Dalwadi, 2007.

values once quantitative estimation all of components were included (Table 6).

Comparison of Loading Parameters from TFF Purification Curves and Dry Weight Recovery

From the short purification curve, 369.13 ± 22.04 mg, n = 5, of total mass of nanoparticles was estimated to be recovered; however, 90.13 ± 10.50% was recovered as a dry weight which was not significantly different (p = .101) to that expected (Table 5). However, substantial deviation was largely due to reversible adsorption of PVA on the polyether sulfone membrane of the TFF cassette (Dalwadi et al., 2005). During the short purification process, substantial amounts of PVA remain adsorbed on the membrane. It was also possible that this adsorbed PVA entrapped some nanoparticles and impaired recovery.

From the extended purification curve, 298.58 ± 7.97 mg, n = 3, of total mass of nanoparticles was estimated to be recovered, and 101.66 ± 4.45% was recovered on a dry weight basis (Table 5), which was not significantly (p = .590) different from that expected from the extended purification curve. Additionally, lower deviation in recovered amounts during extended purification was largely due to the additional washing steps that removed the adsorbed PVA and allowed better recovery of the nanoparticles. Thus recovery values met the expected values and indicated the reliability of the recovery approach.

Comparisons of Drug Loading Nanosep™ vs. TFF

The Nanosep™ device provided a significantly higher (p = .000, .017) percentage of loperamide HCl loading compared with values obtained after short and extended purification (Table 6). This can be explained by the association of residual PVA with the nanoparticles (Equations 1 and 2) at each step of centrifugation. The amount of PVA removed after the first centrifugation cycle was 78.05 ± 4.75%, n = 3; the residual PVA was most likely associated with the nanoparticles (Table 2). This was significantly less (p = .026, .048) than the amount of associated PVA following short and extended purification processes, respectively, by TFF (Tables 2 and 4) which achieved higher percentage loading values. The residual PVA was removed slowly in subsequent centrifugal cycles due to its entrapment within the core of the nanoparticles; by the fourth centrifugation cycle most of the PVA was removed from the nanoparticles (Figure 2). This was due to significantly higher percentage loading values.

TABLE 5
Calculation of Recovery After TFF Purification Processes

Formulation Contents	PVA (mg)		Loperamide HCl (mg)			Copolymer (mg)		Dextran Sulfate (mg)		Recovery From		
	Loss[a]	Remained[b]	Loss[c]	Remained[d]	Remained[e]	Loss[f]	Remained[f]	Loss[g]	Remained[g]	Purification Curve[h] (mg)	Dry Mass[i] (mg)	Percentage[j] of Dry Mass Recovered
Purification Short												
1	166.38	160.89	2.40	8.50	7.59	0	181.81	13.29	8.52	359.72	318.30	88.49
2	129.01	198.26	2.59	8.31	6.82	0	181.81	13.29	8.52	396.90	326.50	82.26
3	137.55	189.72	2.50	8.40	7.37	0	181.81	13.29	8.52	388.45	320.10	82.40
4	175.84	151.43	2.42	8.48	7.69	0	181.81	13.29	8.52	350.24	313.70	89.57
5	175.81	151.46	2.36	8.54	7.77	0	181.81	13.29	8.52	350.33	378.10	107.93
Mean		170.35		8.446	7.45					369.13	331.34	90.13
SD		22.13		0.09	0.38					22.04	26.54	10.50
Extended												
1	226.31	100.96	3.10	7.80	8.62	0	181.81	13.29	8.52	299.09	295.00	98.63
2	235.47	91.80	2.67	8.23	8.01	0	181.81	13.29	8.52	290.36	310.00	106.76
3	219.14	108.13	3.08	7.82	7.66	0	181.81	13.29	8.52	306.28	305.00	99.58
Mean		100.30		7.95	8.09					298.58	303.33	101.66
SD		8.19		0.24	0.70					7.97	7.64	4.45

[a]PVA (mg) lost assayed (filtrate + membrane).

[b]PVA (mg) remained (assayed in dispersion recovered after purification).

[c]Loperamide HCl (mg) loss (assayed filtrate only).

[d]Loperamide HCl (mg) remained, estimated (10.90 mg – c).

[e]Loperamide HCl (mg) remained assayed from the obtained recovery *k*.

[f]Copolymer (mg) loss—nil due to insolubility of copolymer in disperse medium forms nanoparticle that cannot be infiltered through TFF membrane, hence all amount should be within retentate.

[g]Dextran sulfate (mg) lost in the filtrate is 13.29 ± 0.6 mg from 21.81 mg, based on aqueous GPC in Materials and Methods.

[h]Recovery from purification curve (mg) = (*b* + *d* + *g* + *i*).

[i]Dry mass recovered after purification.

[j]Percentage of dry mass recovered.

TABLE 6
A Comparison of Loading Parameters (TFF vs. DCD)

		Loading Parameters of Nanopaticles Suspension			
		% Encapsulated		% Loading	
Treatment Device	% Free Drug (Lost in Filtrate)	From TFF Purification Curve	From Dry Weight Recovery	From TFT Purification Curve	From Dry Weight Recovery
Loperamide HCl					
TFF Short Purification (n = 5)	22.49 ± 0.83	77.50 ± 0.86	68.34 ± 3.50	2.30 ± 0.16	2.33 ± 0.18
TFF Purification Extended (n = 3)	27.06 ± 2.26	72.93 ± 2.26	74.29 ± 4.48	2.67 ± 0.15	2.76 ± 0.23
Nanosep™ (n = 3)	19.56 ± 3.63	76.56 ± 3.62	N/A	3.08 ± 0.04	N/A
Dalargin					
Nanosep™ (n = 3)	17.75 ± 4.10	74.53 ± 4.10	N/A	2.89 ± 0.16	N/A

N/A means not applicable because Nanosep™ was not capable of providing recovery nanoparticles.

CONCLUSION

The use of Nanosep™ effectively removed most of the PVA from nanoparticles at the end of four washing cycles. This provided accurate drug loading values when all of the surfactant was removed from the formulation. Ideally nanoparticles should be recovered; however, a limited volume handling capacity was a hurdle in achieving a dry weight based recovery of the purified nanoparticle suspension. The use of TFF overcame this, but proved relatively inefficient for the removal of all PVA within a short time period in comparison with Nanosep™. An extended operation time was required to remove further amounts of PVA, where it could potentially release the encapsulated drugs; however, within that timeframe, free drug losses reached a plateau level and PVA removal was therefore continued. The drug loading parameters estimated at an incomplete purification point gave relatively lower values when compared with those measured on Nanosep™ for the same batch of nanoparticles. However, due to a quantitative analysis of all formulation components, these differences were resolved using mass balance calculations. This allowed TFF purification to be continued to achieve the ideal situation using Nanosep™. A comparison of loading parameters showed that quantitative considerations of the residual PVA and other excipients was essential in the determination of accurate drug loading values using any mode of ultrafiltration.

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