

# Development of Analytical Method for In Vitro Release Testing of Dexamethasone Nanosuspensions

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## ABSTRACT

The aim of this study was to develop and compare analytical methods for in vitro release testing (IVRT) of an active substance, dexamethasone, from nanosuspension using three apparatuses for IVRT of topical formulations (immersion cell, vertical diffusion cell, and dialysis bag membrane methods), taking into account the physiological conditions at the point of application of these therapeutic systems. Sensitive and reproducible methods to assess in vitro release rates were developed, and immersion cell method was chosen as most appropriate for evaluation of dexamethasone release.

**KEYWORDS:** Dexamethasone, nanosuspension, in vitro release testing (IVRT), immersion cell, vertical diffusion cell, dialysis bag membrane method

## INTRODUCTION

There has been an increasing focus on evaluation and research of new routes for dexamethasone administration to provide targeting of the drug for local action, and consequently reduce systemic side effects and avoid the first pass metabolism through the liver. For topical administration of dexamethasone, it is necessary to develop an appropriate therapeutic system that would enable sustained and controlled release of the drug.

Topical drug delivery systems serve as carriers for drugs that are delivered by skin and mucosa. Because of their peculiar rheological behavior, topical formulations can adhere to the tissue surface for sufficiently long periods, which helps prolong drug delivery at the application site. A topical drug delivery system is advantageous in terms of its easy application, simple formulation, and ability to deliver a wide variety of drug molecules (1).

In the last decades, a great attention has been paid to the development of nanoscale systems, new drug delivery systems, based on nanoparticles that would overcome the deficiencies of existing conventional delivery systems. Some of the most promising drug delivery nanoscale systems are pharmaceutical nanosuspensions. A pharmaceutical nanosuspension is defined as very

finely colloid, biphasic, dispersed solid drug particles in an aqueous vehicle, size below 1  $\mu\text{m}$ , stabilized by surfactants and polymers, and prepared by suitable methods for drug delivery applications. Nanosuspensions provide efficient delivery of hydrophobic drugs and increases the bioavailability (2).

High-pressure homogenization is the relatively simple, most widely used method for the preparation of nanosuspensions of many poorly water-soluble drugs (3). Microfluidizer technology, a type of high-pressure homogenization that relies on pumping a drug through micron-sized orifices under the high pressure, was used to prepare dexamethasone nanosuspensions in the present study.

The purpose of a performance test for a dosage form is to predict and monitor the consistency in manufacturing and quality of that dosage form. In vitro release testing (IVRT) represents a useful tool during the development of a topical dosage form. Monitoring the release of a drug from its dosage form during clinical trials can be critical in understanding the efficacy of the formulation and can be successful in establishing an in vitro–in vivo correlation for topical products (4).

IVRT methods should be reproducible and sensitive to small changes in physicochemical properties of the

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dosage form (5). Development of IVRT tests for the nanosuspension delivery systems is especially challenging due to difficulties with sample filtration and/or separation.

In the present study, IVRT methods have been developed for evaluation of in vitro release of dexamethasone from a nanoparticle-based therapeutic system using three different apparatuses for testing of topical formulations (immersion cell, vertical diffusion cell, and dialysis bag membrane method). Discriminatory power of the methods related to differences in drug loading and particle size was tested, as well as repeatability of the measurements. Pros and cons for each method were recognized, and the most appropriate IVRT method for evaluation of dexamethasone release was identified.

## MATERIALS AND METHODS

### Materials

Citric acid, disodium phosphate, ethanol, and potassium dihydrogen phosphate were purchased from Kemika d.d., Croatia. Micronized dexamethasone (particle size,  $d_{90} = 10 \mu\text{m}$ ) was obtained from Pfizer, Inc., USA. MONTANOX Polysorbate 20 was purchased from SEPPIC S.A., France, tyloxapol from Albany Molecular Research, Inc., USA, and Blanose 7MF sodium carboxymethyl cellulose (CMC) from Ashland Global Holdings, Inc., USA. Sodium chloride, hydrochloric acid, sodium hydroxide, and acetonitrile were obtained from Merck, Germany.

### Preparation of Nanosuspension

Micronized dexamethasone was homogeneously suspended in an aqueous solution of a surfactant, tyloxapol, using an T 25 ULTRA-TURRAX disperser (IKA-Werke GmbH & Co. KG, Germany). The obtained concentrate was homogenized using a Biopharmaceutical Microfluidizer Processor M-110EH-30 (Microfluidics Corp., USA) at pressure of  $1.8 \times 10^5$  kPa. As a part of formulation and process optimization, the number of homogenization cycles (30, 40, and 50) was varied, as was the concentration of dexamethasone (1%, 5%) and tyloxapol-surfactant (0.5%, 2.5%). After homogenization, dexamethasone concentrates were diluted with a CMC/sodium chloride aqueous solution to obtain the final formulation (0.1% dexamethasone, 0.5% CMC, and 0.9% sodium chloride). Hydrochloric acid and sodium hydroxide aqueous solutions were used for pH adjustment to 7.4. Particle size distribution of formulations was analyzed by low-angle laser light scattering (LALLS). Based on the obtained particle size results, 40 homogenization cycles of 1% dexamethasone concentrate containing 0.5% tyloxapol were selected for the preparation of the nanosuspension.

### Equilibrium Solubility of Dexamethasone in McIlvaine Buffer

The equilibrium solubility of dexamethasone was determined by the shake flask method. As thermodynamic solubility was measured, particle size of tested substance is not relevant parameter. Therefore, micronized dexamethasone was tested in this experiment ( $d_{90} = 10 \mu\text{m}$ ). Solubility in McIlvaine buffer (buffer solution composed of citric acid and disodium hydrogen phosphate) with addition of different amounts of ethanol (EtOH) (0%, 10%, and 20%; v/v) was determined. The analysis was carried out in triplicate for each combination of active pharmaceutical ingredient (API) and medium. Dexamethasone was added in surplus to medium and shaken under predetermined conditions: 24 h, 75 rpm, and 32 °C. The saturation was confirmed by observation of the presence of undissolved material. A 0.45- $\mu\text{m}$  filter was used for separation. After filtration, the samples were taken for analysis and diluted to prevent recrystallization. The amount of solute contained in the samples was determined by high-performance liquid chromatography (HPLC).

### HPLC Analysis

Quantification of dexamethasone in the samples was determined by HPLC (Agilent 1100/1200 Infinity Diode Array Detector, Agilent Technologies, Inc., USA). Separation was performed on a Kinetex C18 4.6  $\times$  50 mm column packed with 2.6- $\mu\text{m}$  particles and a pore size of 100 Å (Phenomenex, Inc., USA), maintained at 25 °C. The injector temperature was set at 20 °C. Injection volume was 100  $\mu\text{L}$  and chromatograms were recorded at 241 nm. Mobile phase consisted of 70% phosphate buffer pH 2.0 and 30% acetonitrile with an isocratic flow rate of 1.5 mL/min.

### Evaluation of the Suitability of the Membranes

In the present study, three membranes were tested: a 0.1- $\mu\text{m}$  polyethersulfone (PES) membrane (Pall Corp., USA) and 50- and 300-kDa cellulose ester (CE) dialysis membranes. The membranes were soaked in a solution of the API in the receptor medium: McIlvaine buffer with 10% EtOH and McIlvaine buffer with 0.3% tyloxapol (surfactant present in the formulation). Membrane-binding studies were performed to determine whether the API binds to a specific membrane. API binding to membrane (recovery) was determined by HPLC analysis in comparison to API solutions in which membranes were not soaked.

## In Vitro Testing

### Immersion Cells

In vitro dissolution was carried out via USP apparatus 2 (paddle) with the small spin-paddles at a speed of 100 rpm in 75 mL of receptor medium (McIlvaine buffer pH 7.4/ethanol; 90/10 (v/v)) maintained at 32 °C. Analysis of each sample was performed in sextuplicate. PES membranes (previously soaked in the receptor medium for at least 30 min) with a pore size of 0.1 µm were selected as the barrier. Dexamethasone nanosuspension samples in volumes of 1 mL were applied in the donor chamber of the immersion cell. Membranes were carefully placed over the top of the sample compartment to minimize the possibility of air bubble formation between the surface of the sample and the membrane. After the immersion cell components were assembled, the completed assembly was carefully placed into the bottom of the dissolution vessel with the membrane facing up. Sampling was performed manually at 20, 40, 60, 80, 100, 120, 140, 160, and 180 min, in a volume of 1 mL without replacement with fresh medium. Drug concentrations were quantified using HPLC.

### Dialysis Bag Method

A magnetic stirrer (IKA-Werke GmbH & Co. KG, Germany) with the ability to regulate temperature was used to control rotation and heating of the system. Analysis of each sample was performed in sextuplicate. McIlvaine buffer pH 7.4 was used as a receptor medium in a volume of 200 mL per each glass and it was maintained at 32 °C.

CE dialysis membranes (300-kDa) were previously soaked in the receptor medium for at least 30 min. A volume of 1 mL of dexamethasone nanosuspension was put in a dialysis bag (total surface area of 960 cm<sup>2</sup>). Both ends were tied. Dialysis bags with samples were carefully placed in beakers filled with thermostated medium. The speed of the magnetic stirrer was 400 rpm. Sampling was performed manually at 5, 10, 15, 20, 30, 40, 50, 60, 75, and 90 min, in a volume of 1 mL without replacement of fresh medium. Drug concentrations were quantified using HPLC.

### Vertical Diffusion Cells

An automatic Franz cell system (Vision Microette, Teledyne Hanson Research, Inc., USA) with six vertical diffusion cells of 12 mL (with an effective diffusion area of 1.7 cm<sup>2</sup>) was built using a PES membrane (previously soaked in the receptor medium for at least 30 min) with a pore size of 0.1 µm, which was placed on the upper donor chamber of the diffusion cell, separating this compartment from the receptor chamber. Dexamethasone nanosuspension samples in volumes of 1 mL were applied in the donor

chamber of the cell. The receptor medium was 80/20 (v/v) McIlvaine buffer pH 7.4/ethanol. The temperature of the diffusion cells was maintained at 32 °C. Analysis of each sample was performed in sextuplicate. The magnetic stirrer speed was 400 rpm. The tubes were rinsed with 1.5 mL of fresh receptor medium prior to sampling. Every hour for 13 hours a 1-mL sample was withdrawn automatically and replaced with fresh, prewarmed medium. Drug concentrations were determined using HPLC.

### Calculation of Rate and Amount of Drug Released

Denoted as  $AR_n$ , the amount released (µg/cm<sup>2</sup>) at a given time ( $t_1$ ,  $t_2$ , etc.) is calculated for each sample as follows:

Amount released at

$$t_1 AR_1 = \left( \frac{AU_1}{A_S} \right) \times C_S \times 1000 \times \left( \frac{V_C}{A_0} \right) \quad \text{Eq. (1)}$$

Amount released at

$$t_2 AR_2 = \left( \frac{AU_2}{A_S} \right) \times C_S \times 1000 \times \left( \frac{V_C}{A_0} \right) + \left[ AR_1 \times \left( \frac{V_S}{V_C} \right) \right] \quad \text{Eq. (2)}$$

$$AR_n = \left( \frac{AU_n}{A_S} \right) \times C_S \times 1000 \times \left( \frac{V_C}{A_0} \right) + \left[ \left( \frac{V_S}{V_C} \right) \times \sum_{i=1}^{n-1} \left( \frac{AU_{(n-1)}}{A_S} \right) \times C_S \times 1000 \times \frac{V_C}{A_0} \right] \quad \text{Eq. (3)}$$

where  $AR$  = amount of drug released per unit area of the membrane (µg/cm<sup>2</sup>);

$A_U$  = response (e.g., peak area, or peak height or absorbance) from the sample solution;

$A_S$  = average response (e.g., peak area, or peak height or absorbance) from the standard solution;  $C_S$  = concentration of the standard solution (mg/mL);

$V_C$  = volume of the receptor medium (mL);

$A_0$  = area of the membrane (cm<sup>2</sup>); and

$V_S$  = sample volume (mL).

For each cell, the individual amount of drug released per unit area of the membrane (µg/cm<sup>2</sup>) is plotted versus the square root of time ( $t$ ) according to the Higuchi model for drug release.

The cumulative amount of dexamethasone released was calculated and plotted against the square root of time. The slope of the resulting line represents the rate of drug release.

Each sample was analyzed six times. The average value for each sampling time and the standard deviation (SD)

was calculated as a measure of dispersion of the results around the average value and relative standard deviation (RSD) was determined as the ratio of the standard deviation and average value (6).

To determine the repeatability of all selected methods, the average values of slopes (estimated in vitro release rates) for targeted 100% nanosuspensions, which were obtained by sensitivity testing on changes in concentration and particle size of the API, were used. The percentage difference was calculated for the average values of slopes (estimated in vitro release rates) according to the following formula:

$$\frac{ABS (Value 1 - Value 2)}{AVG (Value 1, Value 2)} \times 100 \quad \text{Eq. (4)}$$

where ABS = absolute value; AVG = average value; Value 1 = average value of slopes for targeted 100% nanosuspension, which were obtained by sensitivity testing on changes in concentration of the API; and Value 2 = average value of slopes for targeted 100% nanosuspension, which were obtained by sensitivity testing on changes of API particle size.

## RESULTS AND DISCUSSION

### Preparation of Nanosuspension

Nanosuspensions were effectively produced by high pressure homogenization. After 40 homogenization cycles, particles sizes for milled sample (d10 = 0.36 µm; d50 = 0.61 µm; d90 = 1.03 µm) were approximately 6 times smaller compared to non-milled sample (d10 = 0.79 µm; d50 = 2.66 µm; d90 = 6.65 µm). To evaluate discriminatory power of the methods for the differences in particle size, untreated and homogenized sample suspensions were used.

### Equilibrium Solubility and Determination of Release Medium

The equilibrium solubility of dexamethasone was determined in the following medium: Mcllvaine buffer with different percentage compositions of ethanol (0%, 10%, and 20%, v/v). The equilibrium solubility of dexamethasone in Mcllvaine buffer was 0.084 mg/mL, in Mcllvaine buffer with 10% ethanol it was 0.167 mg/mL, and in Mcllvaine buffer with 20% ethanol it was 0.324 mg/mL.

Data shown in Table 1 indicate a comparison of concentrations in case of completely released API and theoretical concentrations of solute (API) required to achieve sink conditions for the selected methods.

With regards to the results of the equilibrium solubility testing and sink conditions, Mcllvaine buffer with 20%

ethanol was used as receptor medium for IVRT with vertical diffusion cell, Mcllvaine buffer with 10% ethanol was used as receptor medium for IVRT with immersion cell, and pure Mcllvaine buffer was selected as receptor medium for the dialysis bag method.

Table 1. Comparison of Concentrations of Solute (API) Required to Achieve Sink Conditions for the Selected IVRT Methods

IVRT Method	Maximum Concentration (mg/mL)	Theoretical Concentrations of API (mg/mL)
Immersion Cell Method	0.013	0.040
Dialysis Bag Method	0.005	0.015
Vertical Diffusion Cell Method	0.083	0.250

API, active pharmaceutical ingredient; IVRT, in vitro release testing.

### Suitability of the Membranes

The membrane keeps the product and the receptor medium separate and distinct and represents the critical factor in development of the IVRT methods for nanosuspension delivery systems. Membranes are chosen to offer the least possible diffusional resistance and not to be rate controlling.

Membrane-binding studies were performed to determine whether the API binds to a specific membrane. Buffer with 10% of EtOH (middle alcohol amount) served as a representative of all three media used in the IVRT experiments. Recovery for the CE 300-kDa membrane was 98.6% in the 10% EtOH medium and 98.2% in the 0.3% tyloxapol medium. For the CE 50-kDa membrane, recovery was 101.8% and 99.7% in 10% EtOH and 0.3% tyloxapol, respectively. The result of API binding (recovery) to the PES 0.1-µm membrane was 98.7% and 100.5% in 10% EtOH and 0.3% tyloxapol. Based on these results, all the membranes tested were within the set requirements (recovery: 98.0–102.0%) and were considered suitable for use in IVRT of dexamethasone.

PES 0.1-µm and CE 300-kDa membranes, with pore sizes smaller than the milled dexamethasone particle size in tested samples, were selected for further use. The CE 50-kDa membrane was excluded from further use due to diffusional resistance as a consequence of small pore size.

### Discriminatory Power of the Methods for Differences in Dosage Strength

The IVRT method must be discriminatory, i.e., it should detect differences in release rates which may occur due to formulation changes or manufacturing process that could influence the product efficacy in vivo. In accordance

with predetermined conditions, IVRT with immersion cell, vertical diffusion cell, and dialysis bag method was performed for three formulations with different dosage strengths of the dexamethasone nanosuspensions: 50%, 100%, and 150% API nanosuspensions. Data on the amount of drug released at predefined time intervals were obtained.

Figures 1, 2, and 3 depict the in vitro release profiles of the three formulations with different dosage strengths of the API nanosuspension (50%, 100%, and 150%) that were obtained using immersion cells, vertical diffusion cells, and the dialysis bag membrane method. The dexamethasone release rate from the nanosuspension is represented by the slope of the line obtained from plotting amount of dexamethasone released per unit area of the membrane ( $\mu\text{g}/\text{cm}^2$ ) versus the square root of time ( $t$ ) (5). This release rate measure is formulation-specific and can be used to monitor product quality.

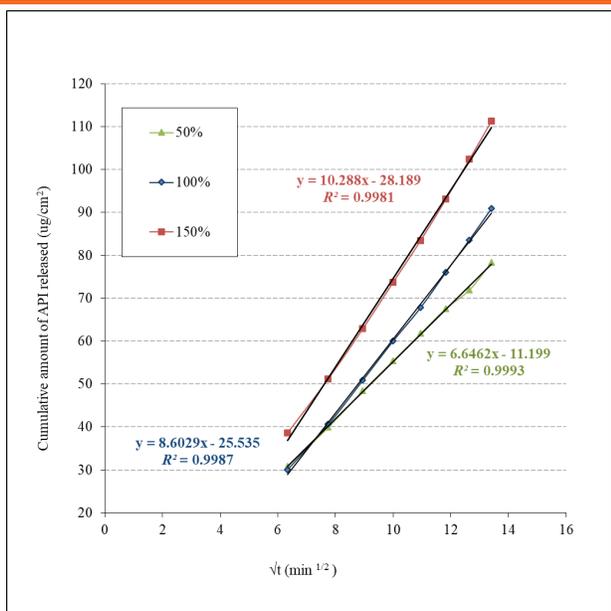


Figure 1. Drug release profiles from formulations at three dosage strengths that were obtained using immersion cells ( $n = 6$ ). Black lines represent the slope. API, active pharmaceutical ingredient.

The slope in Figure 1 shows the difference in the release rate due to different dosage strength. The release rate of dexamethasone increases with increasing drug concentration in the formulation. This is expected because in the formulation with higher concentration of dexamethasone a greater amount of dexamethasone, which can diffuse through the membrane into the receptor medium, is available.

Six individual slopes (estimated in vitro release rates) obtained by testing a 100% API sample were compared

with six individual release slopes (estimated in vitro release rates) of the 50% API sample, and then with six individual slopes (estimated in vitro release rates) of the 150% API sample.

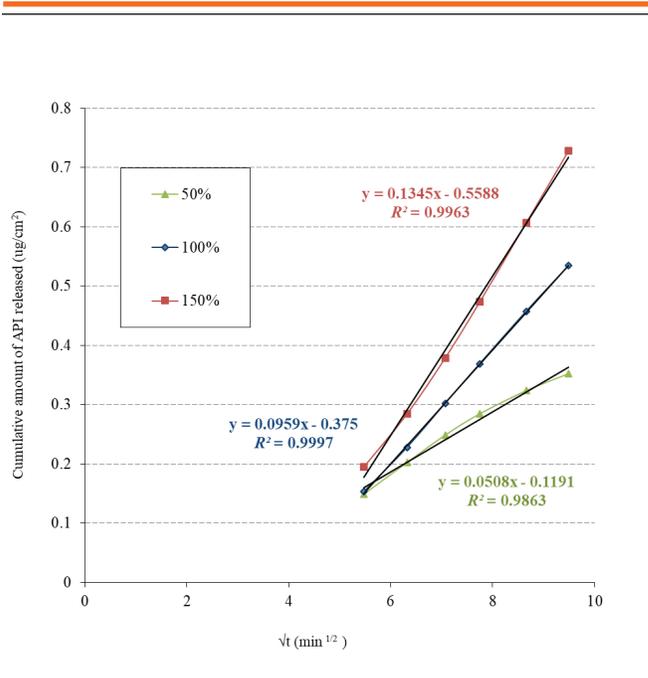


Figure 2. Drug release profiles from formulations at three dosage strengths that were obtained using dialysis bag method ( $n = 6$ ). Black lines represent the slope. API, active pharmaceutical ingredient.

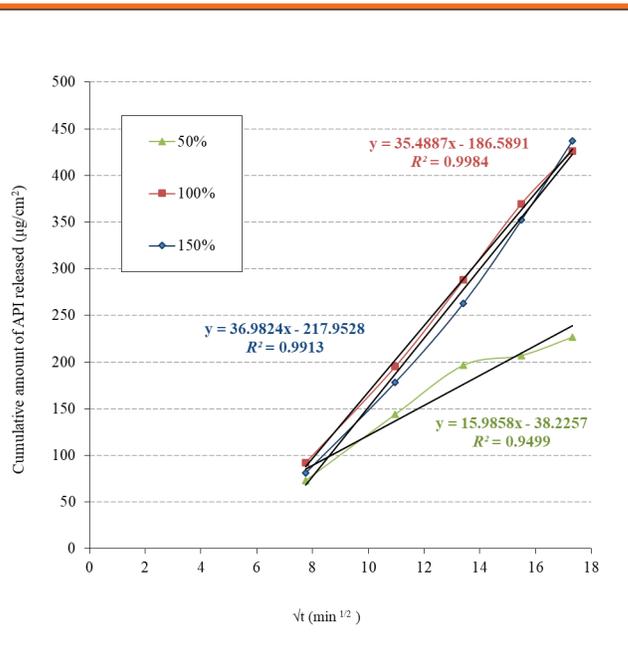


Figure 3. Drug release profiles from formulations at three dosage strengths that were obtained using vertical diffusion cells ( $n = 6$ ). Black lines represent the slope. API, active pharmaceutical ingredient.

According to the Scale-Up and Post-Approval Change Semisolid (SUPAC-SS) guideline, a 90% confidence interval (CI) of IVRT for two tested formulations should fall within the limits of 75% to 133.33% to declare two tested samples as similar, i.e., to have the same in vitro release rate of the API. If the 90% CI falls within the limits of 75% to 133.33%, no further in vitro testing is necessary (7). Ninety percent CI levels of IVRT with immersion cell, vertical diffusion cell, and dialysis bag method for three formulations with different dosage strengths of the API in nanosuspensions are shown in Table 2. Method with vertical diffusion cell was shown to be sufficiently sensitive to detect changes in concentration of the API only in the case of lower concentration (50% vs 100%). When samples with high concentration were tested, no differences in release rates were observed. This behavior can be explained by the small surface of the membrane across which the diffusion of the therapeutic agent occurs. In case of product with higher drug concentration, membrane surface area represents limitation factor for drug release. The dialysis bag membrane method revealed sufficiently sensitive to the tested parameter, but great variability in the results was observed due to more consecutive steps in method performance and nonstandard set up of the method (e.g., unstandardized precut dialysis bag lengths and closures). Immersion cell method was shown as discriminative for changes in drug concentration, with low variability within the results.

### Release Rate Dependence on Particle Size

In accordance with predetermined conditions, IVRT was performed using immersion cell, vertical diffusion cell, and dialysis bag methods for two formulations with different API particle size – milled (d90 ~ 1.0 μm) and non-milled (d90 ~ 6.65 μm) samples. Data on the amount of the drug released at predefined time intervals were obtained (Fig. 4).

Six individual slopes (estimated in vitro release rates) obtained by testing formulation with larger particle size was compared with six individual slopes (estimated in

vitro release rates) of the formulation with smaller particle size. A 90% CI for comparison of these formulations was 70.25%–78.87% for immersion cell method and 73.60%–112.35% for dialysis bag method. Therefore, it can be concluded that these methods are sensitive to changes in API particle size. The first step of similarity requirement between two tested formulations was met in the computation of the CI for method with vertical diffusion cell. As the 90% CI was 85.18%–102.66%, it was concluded that vertical diffusion cell method is not sensitive to changes in API particle size from the tested formulations.

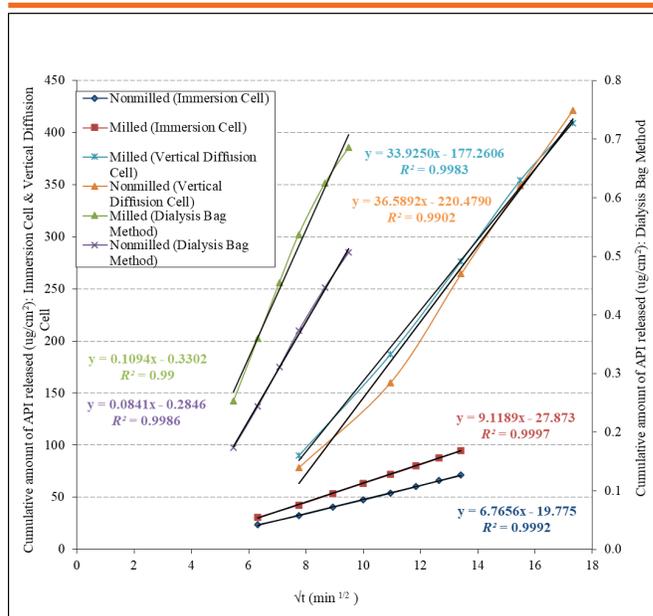


Figure 4. Drug release profiles from formulations with different particle size that were obtained using immersion cell, vertical diffusion cell, and dialysis bag method (n = 6). Black lines represent the slope. API, active pharmaceutical ingredient.

### The Repeatability of the Methods

Repeatability of the methods was analyzed by comparing the average values of slopes (estimated in vitro release rates) for targeted 100% nanosuspension that were obtained by sensitivity testing on changes in concentration and particle size of the API. The percentage difference was

Table 2. Results of Selected IVRT Methods for Three Dexamethasone Nanosuspension Formulations with Different Dosage Strengths (50%, 100%, and 150%)

IVRT Method	90% CI		Average Slope		Difference (%)*
	50% vs 100%	100% vs 150%	Concentration Sensitivity	Particle Size Sensitivity	
Immersion Cell Method	68.60%–83.16%	74.63%–93.22%	8.6029	9.1189	5.82
Dialysis Bag Method	49.43%–58.50%	59.58%–89.89%	0.0959	0.1094	13.15
Vertical Diffusion Cell Method	41.49%–47.19%	86.98%–103.84%	35.489	33.925	4.51

CI, confidence interval; IVRT, in vitro release testing.

\*Percent difference was calculated for the average slopes (estimated in vitro release rates) to determine repeatability of the methods.

calculated for the average values of slopes (estimated in vitro release rates). The results, shown in Table 2, indicate acceptable repeatability of all selected methods.

## CONCLUSION

In the present study, three IVRT methods were developed for dexamethasone nanosuspensions. Immersion cell, dialysis bag methodology, and vertical diffusion cell methods were utilized to evaluate critical parameters of dexamethasone release from nanosuspension.

Diffusion of the drug across the PES membrane and CE dialysis membrane demonstrated low drug adsorption and low diffusional resistance. After an initial lag phase, the amount of drug released became proportional to the square root of time. The slope in the linear portion of the release curve was used as a measure of release rate. Although the dialysis bag membrane method revealed sufficient sensitivity to the tested parameters, higher variability in the results was observed.

In the last decade, IVRT using vertical diffusion cells has been a promising tool to assess the release of API from topical products; however, in this study, this method did not show sufficient sensitivity for dexamethasone released from nanosuspension due to the small surface of the membrane across which diffusion of the therapeutic agent occurs.

The immersion cell method had the best discriminatory power and repeatability for IVRT of dexamethasone from nanosuspension. Therefore, this method is the most appropriate for evaluation of dexamethasone release from nanoparticle-based therapeutic systems.

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## CONFLICT OF INTEREST

The authors disclosed no conflicts of interest related to this article.

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