In vitro release testing method development for ophthalmic ointments

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A B S T R A C T

It is essential as well as challenging to develop a reliable *in vitro* release testing method for determining whether differences in release profiles exist between qualitatively and quantitatively equivalent ophthalmic ointment formulations. There is a lack of regulatory guidance on *in vitro* release testing methods for ophthalmic formulations. Three different *in vitro* release testing methods 1) USP apparatus 4 with semisolid adapters; 2) USP apparatus 2 with enhancer cells; and 3) Franz diffusion cells were investigated. Qualitatively and quantitatively equivalent ointments were prepared *via* hot melting and simple mixing methods using four different sources of excipients (*i.e.* white petrolatum). The ointment formulations were characterized for content uniformity, particle size, and rheological parameters. All the formulations showed adequate content uniformity and similar particle size. The ointments prepared *via* the hot melting processes showed higher rheological parameters, as did the ointments prepared using 'white' petrolatum that exhibited a yellowish color. The three *in vitro* release testing methods were compared and evaluated for reproducibility, discriminatory capability, and correlation with the rheological parameters. Compared with the compendial methods, the non-compendial method (Franz diffusion cells) showed poorer reproducibility. All three methods possessed the ability to discriminate between the ophthalmic ointments with manufacturing differences. However, the USP apparatus 4 method displayed the largest margin of discrimination between the release profiles of the different ophthalmic ointments. In addition, the *in vitro* release rate obtained using the USP apparatus 4 method showed the strongest logarithmic linear correlation with the rheological parameters (Power law consistency index (K value) and crossover modulus) compared to the other two methods.

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1. Introduction

One of the major challenges for topical ocular drug delivery is the limited precorneal retention time of active pharmaceutical ingredients (APIs), which results in low bioavailability. The impermeability of human corneas as well as the biological barriers of the other parts of the human eye limits drug absorption. In conventional topical ocular drug delivery, aqueous solutions (i.e. eye drops) are the most convenient and patient compliant dosage form. However, ophthalmic solutions have particularly poor bioavailability due to their transient retention time on the eye surface. A plethora of strategies including ointments (Greaves et al., 1993), gels (Kushwaha et al., 2012; Patel et al., 2013), liposomes (Monem et al., 2000; Agarwal et al., 2016), nanoparticles (Diebold et al., 2007; Calvo et al., 1996; Gupta et al., 2010; Seyfoddin et al., 2016), and mucoadhesive formulations (Snejdrova et al., 2016) have been utilized to increase drug retention time at the corneal surface. Compared to eye drops, ophthalmic ointments possess higher viscosity and therefore can prolong drug-ocular contact time and reduce systemic toxicity (Greaves et al., 1993; Robin and Ellis, 1978). There are four types of ointment bases listed in the USP 36 ⟨711⟩: hydrocarbon, absorption, water-removable

and water-soluble. To date, most of the ophthalmic ointment formulations available on the market (Bao et al., 2017) are hydrocarbon based. Even though ophthalmic ointments are a conventional dosage form, there is a paucity of literature reports regarding formulation development and characterization (such as physicochemical properties, *in vitro* drug release testing, and *ex vivo* and *in vivo* performance).

In vitro release testing is a fundamental tool to ensure consistent performance and quality of generic products. Release testing of ophthalmic ointments is an effective approach to monitor post-approval changes, scale-up, lot-to-lot changes and stability studies in the pharmaceutical industry (Shah et al., 1999). In generic product development, formulations that possess qualitative (Q1) and quantitative (Q2) sameness may present different physicochemical properties and *in vitro* and *in vivo* performance due to different manufacturing processes. Therefore, a discriminating release testing method is pivotal to identify all the possible changes to product performance generated from manufacturing to the final formulations. There is no standard *in vitro* release testing method suggested in the US pharmacopeia regarding semisolid ophthalmic ointments. Although the FDA's guidance for scale-up and post approval changes for non-sterile semisolids (SUPAC-SS) that are Q1/Q2 equivalent recommends the Franz diffusion cell method for *in vitro* release testing (FDA, 1997), this method may or may not be appropriate for ophthalmic semisolid ointments. In the past several decades, the Franz diffusion cell method and modifications thereof have been commonly utilized for *in vitro* release testing of most topical formulations (Shah and Elkins, 1995; Valenta et al., 2000; El Gendy et al., 2002; Yoshida et al., 2004; Özsoy et al., 2004; Ishii et al., 2009). There have also been a few literature reports of using USP apparatus 2 with different sample loading cells to perform *in vitro* release testing of topical formulations (Chattaraj et al., 1998; Ahmed et al., 2011; Xu et al., 2015). In addition, there has been one report of using USP apparatus 4 with an 'insertion cell' for *in vitro* release testing of semisolid formulations (Chattaraj and Kanfer, 1996). However, this method with the 'insertion cell' showed poor reproducibility. To date, there have been no reports published regarding the evaluation of the reproducibility and discriminatory capability of different *in vitro* release testing methods for semisolid ophthalmic ointments.

Three different *in vitro* release testing methods (Franz diffusion cells, USP apparatus 2 with enhancer cells, and USP apparatus 4 with semisolid adapters) were utilized and evaluated for their reproducibility and ability to discriminate among Q1/Q2 ophthalmic ointment formulations with manufacturing differences. Loteprednol etabonate, a corticosteroid for treatment of ophthalmic inflammatory conditions (Howes, 2000), was used as a model drug molecule and the commercial product Lotemax® was used as the reference listed drug (RLD). Three different manufacturing processes and four different sources of white petrolatum were utilized to prepare the Q1/Q2 equivalent loteprednol etabonate ointments. These formulations were characterized for drug content uniformity, particle size and rheological parameters. Correlation between the critical rheological parameters (crossover modulus and K value) and the *in vitro* drug release profiles was evaluated based on a previously reported relationship (Bao et al., 2017).

2. Material and methods

2.1. Materials

Loteprednol etabonate (particle size: 19 μm) was purchased from Pure Chemistry Scientific Inc. Four different sources of white petrolatum (OWP (laboratory grade), NWP (USP grade), VWP (USP grade) and PWP (USP grade)) were purchased from Fisher®, Fougera Pharmaceutical Inc., Vaseline®, and Penreco, respectively. Mineral oil USP, sodium chloride, calcium chloride, sodium dodecyl sulfate (SDS), was purchased from Sigma-Aldrich. Sodium bicarbonate was purchased from Fisher®. Unless otherwise specified, all materials were of analytical grade.

2.2. Preparation of loteprednol etabonate ointments

Loteprednol etabonate ointments that have Q1/Q2 sameness to the commercial product Lotemax® ointment were prepared as previously reported (Bao et al., 2017). In brief, a mixture (batch size: 50 g) of white petrolatum, API and mineral oil was added in a plastic jar (Unguator®). The mixture was processed with three different manufacturing methods including: 1) simple mixing at room temperature (SRT); 2) hot melting at 65 °C and mixing with cooling at room temperature (HMRT); and 3) hot melting at 65 °C and mixing with immediate cooling in a −20 °C freezer (HMIC). The stirring speed of mixing (Unguator® e/s mixer, GAKO® International GmbH) was 1450 rpm and the mixing time for the simple mixing and hot melting methods were 6 and 5 min, respectively. Four different sources of white petrolatum (Fisher® (OWP), Fougera® (NWP), Vaseline® (VWP) and Penreco (PWP)) and a mean particle size of 19 μm of the API were used to prepare the loteprednol etabonate ophthalmic ointment formulations.

2.3. HPLC analysis of loteprednol etabonate

The concentration of loteprednol etabonate was determined using a PerkinElmer Flexar HPLC system with a UV detector set at 244 nm. The mobile phase was a mixture of acetonitrile, water, and acetic acid (65/34.5/0.5, v/v/v). Zorbax® Eclipse XDB-Phenyl C18 (250 × 4.6 mm, 5 μm; Agilent Technologies, USA) column was used with a flow rate of 1 ml/min and the column temperature was set at 30 °C. Fifty microliters of the samples were injected into the HPLC. The chromatographs were analyzed using the Chromera software kit V3.0. Adequate linearity was shown in the concentration ranges of 0.02 to 1.00 μg/ml ($r^2 = 0.99$) and 0.10 to 5.00 μg/ml ($r^2 = 0.99$). Both concentration ranges showed adequate inter- and intra-day precision (RSD (%) <2.0).

2.4. Drug loading and content uniformity

The drug was extracted from the ointment using melting and the addition of acetonitrile. 100 mg of the ointments (3 replicates from different regions of the jar containing the formulations) were weighed and 1.0 ml of acetonitrile was added into a vial and tightly sealed. The vials were put into a water bath at 65 °C for 1 min and then vortexed immediately for 2 min. This heating-vortex cycle was repeated three times to ensure complete drug extraction. The extracted solution was diluted with mobile phase and centrifuged at 14,000g for 5 min. The samples were filtered (Millex® HV, PVDF 0.45 μm syringe filter) and further diluted with the mobile phase. The loteprednol etabonate concentration in the solution was determined *via* HPLC.

2.5. Particle size analysis

The particle size and distribution of loteprednol etabonate in the ointments were analyzed using an Olympus BX51 polarized light microscopy (PLM) (Olympus America Inc. New York). Aliquots of ointments were spread on a glass slide and dispersed with one drop of mineral oil. Cover slips were placed on top of the dispersed ointment samples. At least three microscopy images were acquired at 20× magnification while maintaining constant camera parameters (*e.g.* image capture time, contrast and tone) for each sample.

2.6. Rheological characterization

The rheological properties of the loteprednol etabonate ointments were characterized using a Rheometer (ARES-G2, TA Instruments, USA) equipped with a step-peltier stage and a 20 mm AL ST plate. For each test, approximately 0.3 g of the ointment was placed on the lower plate. Initially, the upper plate was set at 1050 μm to trim the excess sample from its edge and then the gap was set at 1000 μm. The following procedures were performed in sequence to characterize the rheological behavior of the samples: 1) a conditioning step to set the testing temperature at 37 °C; 2) a time sweep step was maintained for 45 min to allow the material to fully recover from the shear applied during sample preparation (monitored at oscillatory stress 0.1 Pa and 0.1 Hz oscillation frequency); 3) a stress sweep step was utilized to determine the onset point and crossover point of the sample (briefly, the oscillatory stress was changed from 0.1 to 25 Pa while maintaining the temperature (37 °C) and frequency (0.1 Hz) constant); 4) a time sweep step (as described in Step 2); and 5) a steady state flow step was used to characterize the flow properties of the sample. In this step, the shear rate ($\dot{Y}$, 1/s) was changed from 10^{-4} to $10^{3}\,s^{-1}$ while maintaining the temperature at 37 °C. The viscosity of the sample was measured in log mode (2 points per decade were collected). During the measurement, the% tolerance in each point was set to 5.0%. All samples were performed in triplicates.

2.7. In vitro release testing of the ointments

Three release testing methods (USP apparatus 4, USP apparatus 2 and Franz diffusion cells) were used to investigate the in vitro release of the loteprednol etabonate ointments. The release testing was performed in pH 7.4 artificial tear fluid (containing 0.67% (w/v) of NaCl, 0.2% (w/v) of NaHCO$_3$, and 0.008% (w/v) of CaCl$_2$·2H$_2$O) with 0.5% SDS (w/v) at 37 °C. Cellulose acetate membranes (Sartorius®, 0.45 μm average pore size) were used as the artificial membrane and maintained in Millipore water for 30 min prior to ointment loading. At predetermined time intervals (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 h), a certain volume of sample was withdrawn and replenished with fresh media. Fifty microliters of the samples were injected into HPLC system for analysis.

2.7.1. USP apparatus 4 with semisolid adapters

Semisolid adapters (exposure area of 1.54 cm^2, Sotax Corporation, USA) were used with USP apparatus 4 (Sotax CE7 smart with CY 7 piston pump, Sotax Corporation, USA) to determine the in vitro release profiles of the loteprednol etabonate ointments (Fig. 1). The reservoirs of the adapter cells (depth: 2.6 mm) were filled completely with the ointments (~330 mg) and the surface was flattened with a thin plastic tool to avoid air entrapment between the ointment surface and the membrane. Cellulose
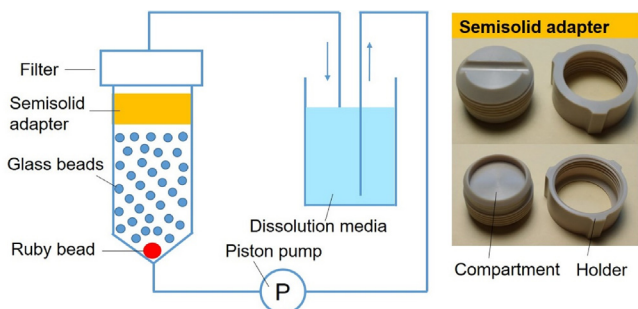
acetate membranes were placed over the surface of the sample compartments and the adapters were assembled as per the manufacturer's instructions. The adapters with the membrane facing down were loaded into flow-through cells (22.6 mm in diameter) prefilled with 14 g of glass beads (1 mm in diameter). 50 ml of release media was circulated through the flow-through cells at a flow rate of 8 ml/min at 37 °C. At pre-determined time intervals, 1 ml of the release medium was withdrawn and replenished with fresh media.

2.7.2. USP apparatus 2 with enhancer cells

Enhancer cells (surface area: 4 cm^2, Agilent Technologies, USA) were used with USP apparatus 2 equipped with 200 ml flat bottom dissolution vessels to determine the in vitro release profiles of the loteprednol etabonate ointments (Fig. 2). Fifty milligrams of the ointment samples were filled into the compartment (depth: 0.4 mm) of the enhancer cells. To prevent bulge or air entrapment between the ointment surface and the membrane, the ointment surface was flattened using a thin plastic tool. Cellulose acetate membranes were placed on the surface of the ointment samples and the cells were assembled as per the manufacturer's instructions. The assembled enhancer cells were placed at the bottom of the dissolution vessels with the membrane facing up and the pre-heated (37.0 ± 0.5 °C) release medium (40 ml) was then added to start the test. The mini paddles were used and the agitation speed was set at 150 rpm. At pre-determined time intervals, 1 ml samples of the release medium were withdrawn and replenished with fresh media.

2.7.3. Franz diffusion cells

Vertical Franz diffusion cells with a volume of 12 ml (contact area: 1.77 cm^2, PermeGear Inc.) were used to determine the in vitro drug release of the loteprednol etabonate ointments (Fig. 3). Cellulose acetate membranes were placed on top of the receptor chambers of the cells following the addition of the release media. Then the donor chambers were mounted on the membranes and clamped tightly. 150 mg samples of the ointments were loaded into the donor chambers and 250 μl of the release medium were added to the top of the ointment to simulate the small amount of tear secreted on the eye surface. The stirring speed of the Franz diffusion cells was set at 600 rpm. At pre-determined time intervals, 0.15 ml of the media were withdrawn from the receptor chambers and replenished with fresh media.

2.8. Reproducibility study of the three release methods using Lotemax®

The commercial loteprednol etabonate ointment formulation (Lotemax®) was used to determine the reproducibility of the three
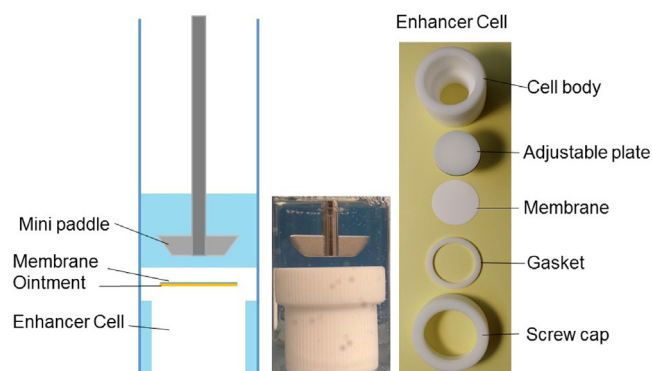

Fig. 1. Graphic demonstration of USP apparatus 4 with semisolid adapters.

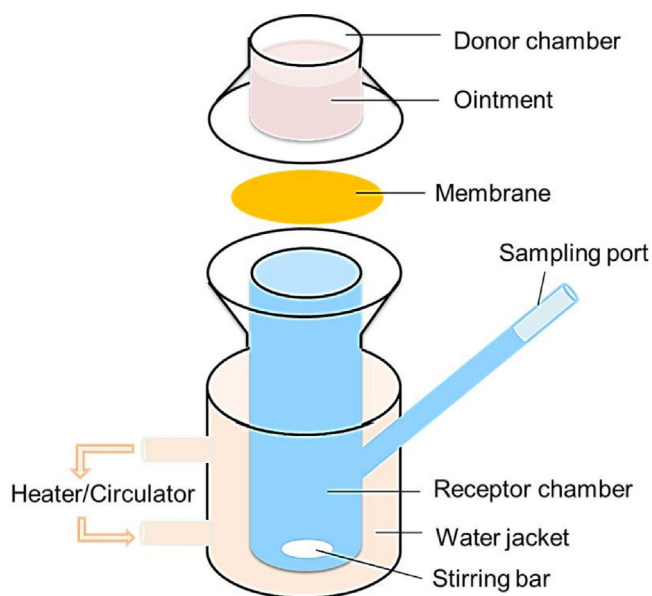**Fig. 2.** Graphic demonstration of USP apparatus 2 with enhancer cells.

Fig. 3. Graphic demonstration of Franz diffusion cells.

release methods. The release tests were repeated on three separate days (three runs) with six replicates per run for the purpose of method validation. The relative standard deviation (RSD) or coefficient of variance (CV) of the cumulative drug release amount for each time point among the three runs and between each run were calculated. In addition, the CV of the drug release rate among the three runs was calculated for comparison.

2.9. Validation of discriminatory ability of three release methods

The discriminatory ability of the three release methods was also evaluated using ointment formulations with 50% more drug and with 50% less drug. These ointments were prepared using the SRT method with OWP, and their *in vitro* release profiles were determined using the three different release methods. The drug release rate values of the ointments were calculated using the Higuchi model.

2.10. Comparison of the three release methods with ointments prepared using different manufacturing processes

Based on the release data, three formulations (HMICOWP19, HMRTNWP19 and SRTNWP19) with manufacturing differences were selected to compare the discriminatory capability of the three release methods. The drug release profiles and rate values of the three formulations were compared using the *t*-test and the Wilcoxon Rank Sum/Mann-Whitney rank test.

2.11. Correlation between rheological parameters and in vitro release rate

Log-log linear regression of the two critical rheological parameters (CM and K value) against the *in vitro* release rate obtained using USP apparatus 4 and USP apparatus 2 were performed for all nine ointment formulations. The goodness of fit (R^2) were compared between the two compendial release testing methods.

2.12. Statistical analysis

ANOVA analysis with Bonferroni test was utilized to compare the mean difference of the parameters. $p < 0.05$ was considered to have significant difference. The linear regression and fitting were performed using OriginPro2017 software (OriginLab Corporation).

3. Results and discussion

3.1. Drug content and uniformity of the ophthalmic ointments

Due to the low drug content for each Q1/Q2 formulation (target drug loading 0.5% w/w), uniform formulations were difficult to prepare. The drug content uniformity directly impacts the reproducibility of the *in vitro* release as well as the *in vivo* performance. Accordingly, it was very essential to monitor the drug loading and uniformity of the ointment formulations. The drug content uniformity was determined by testing the drug concentration at different regions of the ointment base in the jar. The relative standard deviation (RSD) of the drug content is a good indication of the uniformity of the ointments. As shown in Table 1, all the prepared ointments had approximately 0.5% (w/w) drug loading. In addition, the RSD of the drug concentration calculated based on samples taken from different regions of the ointments was less than 3.5%, indicating adequate uniformity of the drug particles in the ointments.

3.2. Drug particle size and distribution

The drug particle size of the ointments was analyzed using Image J software (National Institutes of Health, USA). As shown in Fig. 4, the drug particle size in all the final formulations was significantly ($p < 0.05$) reduced from 19 μm to 10 μm following the different manufacturing processes. This reduction in particle size may be due to the high shearing force during the mixing processes. The drug particles in the ointment formulations remained in the crystalline state.

3.3. Rheological characterization

Based on our previous research (Bao et al., 2017), four rheological parameters of the ophthalmic ointments were investigated: *1*) storage modulus (G') in the linear viscoelastic region (SM); 2) onset point (OP) of oscillatory stress when G' began to drop from the linear viscoelastic region (where both G' and G"(loss modulus)) were constant; 3) crossover modulus (CM) where the G' = G"; and 4) Power law consistency index (K value). The K values were extrapolated from the Power law equation *via* linear regression of the log of the apparent viscosity versus the log

Table 1
The drug content uniformity of the loteprednol etabonate ointment formulations.

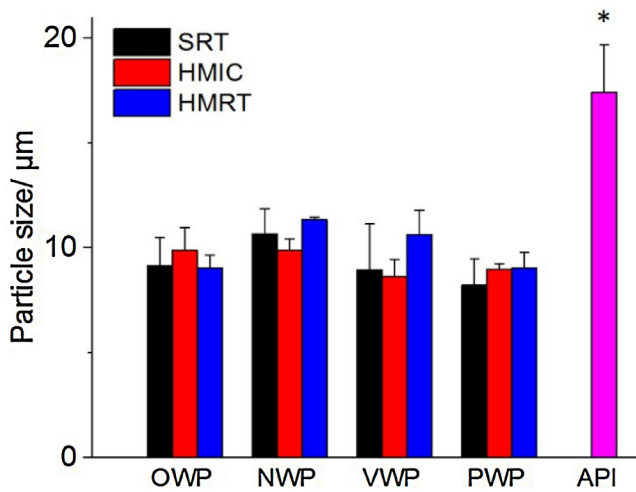| Formulations	Average Drug Loading ± SD (%, w/w)	RSD (%)
SRTOWP19	0.48 ± 0.01	2.87
SRTNWP19	0.49 ± 0.01	1.60
SRTVWP19	0.54 ± 0.02	3.00
SRTPWP19	0.49 ± 0.02	3.47
HMICOWP19	0.49 ± 0.01	1.22
HMICNWP19	0.47 ± 0.00	0.91
HMICVWP19	0.52 ± 0.01	1.94
HMICPWP19	0.51 ± 0.01	2.62
HMRTOWP19	0.51 ± 0.02	3.27
HMRTNWP19	0.48 ± 0.01	1.05
HMRTVWP19	0.50 ± 0.01	2.43
HMRTPWP19	0.50 ± 0.01	1.16

Fig. 4. Particle size of the drug inside the loteprednol etabonate ointments and API ($n = 3$) (SRT: simple mixing process; HMIC: hot melting with immediate cooling process; HMRT: hot melting with gradual cooling at room temperature; OWP: white petrolatum from Fisher®; NWP: white petrolatum from Fougera®; VWP: white petrolatum from Vaseline® and PWP: white petrolatum from Penreco).
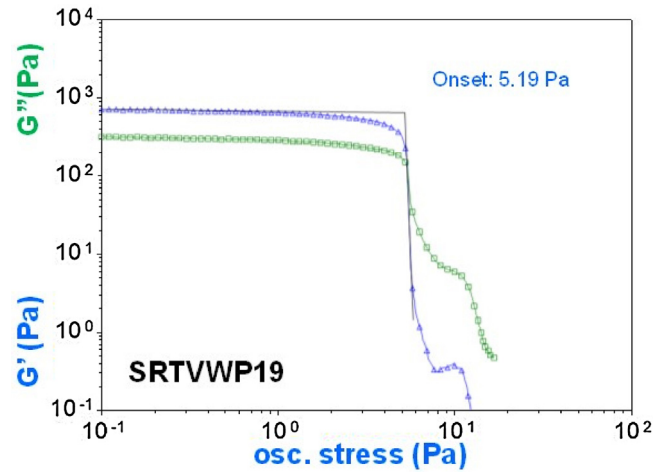

Fig. 6. A representative graph of plotting the log of G' versus the log of oscillatory stress and the log of G" versus the log of oscillatory stress. (Formulation: SRTVWP19) (SRT: simple mixing process; and VWP: white petrolatum from Vaseline®.).
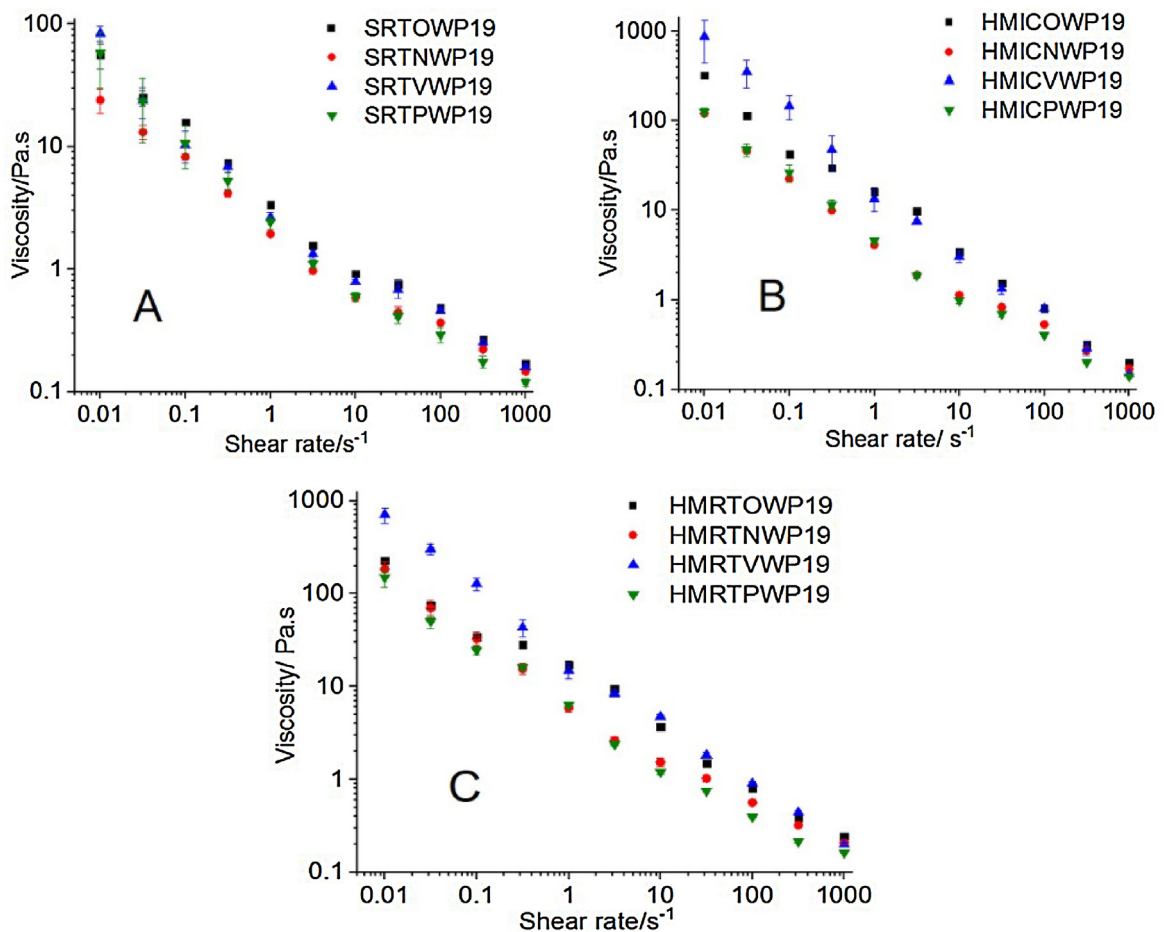

Fig. 5. The rheograms of loteprednol etabonate ophthalmic ointments prepared using: **A**) SRT; **B**) HMIC; and **C**) HMRT with different sources of white petrolatum. (SRT: simple mixing process; HMIC: hot melting with immediate cooling process; HMRT: hot melting with gradual cooling at room temperature; OWP: white petrolatum from Fisher®; NWP: white petrolatum from Fougera®; VWP: white petrolatum from Vaseline® and PWP: white petrolatum from Penreco).

Fig. 7. Rheological parameters: **A**) onset point; **B**) crossover modulus; **C**) storage modulus; and **D**) Power law consistency index (K value) of loteprednol etabonate ophthalmic ointments prepared with different manufacturing processes. (SRT: simple mixing process; HMIC: hot melting with immediate cooling process; HMRT: hot melting with gradual cooling at room temperature; OWP: white petrolatum from Fisher®; NWP: white petrolatum from Fougera®; VWP: white petrolatum from Vaseline® and PWP: white petrolatum from Penreco).
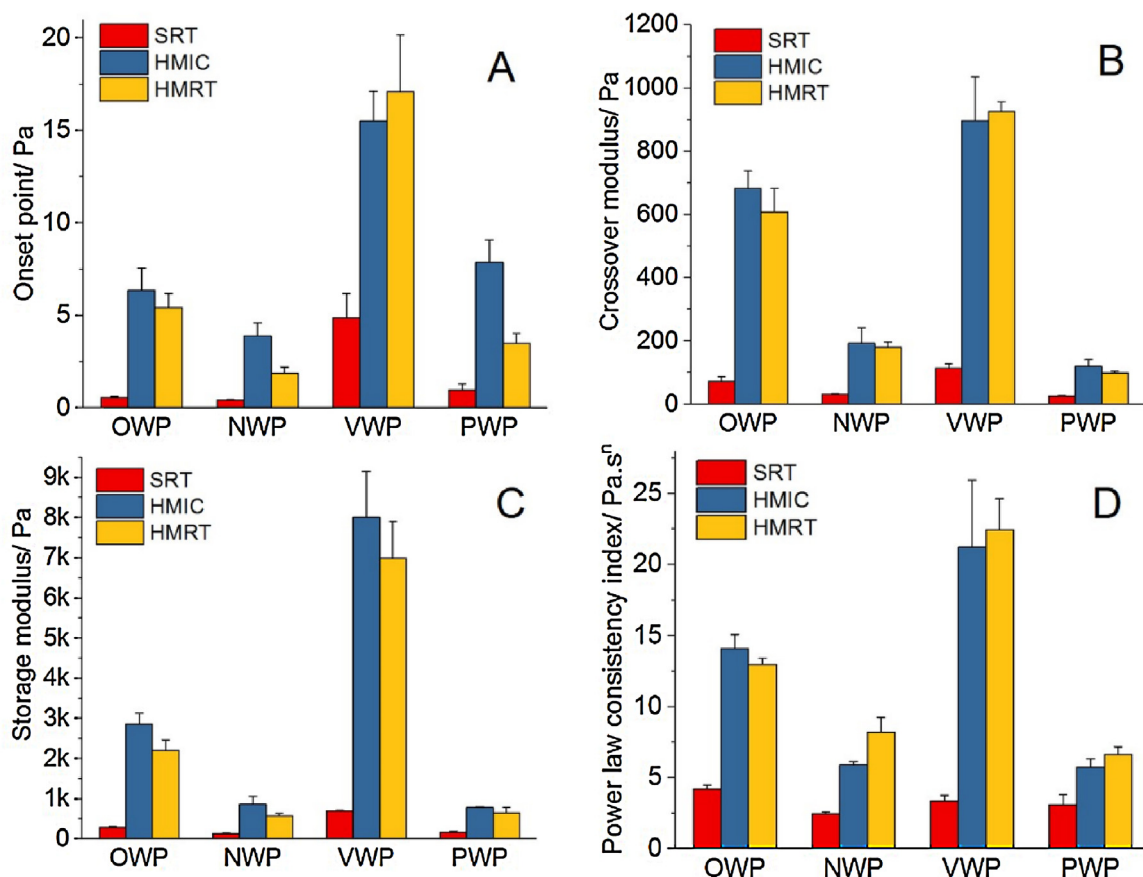
of the shear rate plot (Fig. 5). The OP and CM values were obtained by plotting the log of G' versus the log of oscillatory stress and the log of G" versus the log of the oscillatory stress, respectively (Fig. 6). All of the rheological parameters (OP, CM, SM and K value) are shown in Fig. 7.

The simple mixing processing method (SRT) showed significantly lower ($p < 0.05$) rheological parameters (OP, CM, SM and K value) compared with the hot melting processing methods (HMIC or HMRT), regardless of the source of white petrolatum used. However, the rheological parameters of the ointment formulations prepared using the HMIC and HMRT processing methods were not significantly different. These results are in agreement with previously reported data (Bao et al., 2017).

The rheological parameters of the ointments prepared with four sources of white petrolatum showed the following order: VWP > OWP > NWP ≈ PWP. Among the four sources of white petrolatum, OWP is non-USP grade and the other three (NWP, VWP and PWP) are USP grade. The rheological parameters of the ointments prepared using VWP and NWP were significantly ($p < 0.05$) different even though these two excipients were manufactured according to the same USP standard. The four white petrolatum excipients differed in color, NWP and PWP were white whereas VWP and OWP were yellowish. The USP grade VWP was similar in color to the non-USP grade OWP. Interestingly, both of the yellowish colored 'white' petrolatum exhibited higher rheological parameters than lighter colored petrolatum (NWP and PWP). As discussed in a previous report (Bao et al., 2017), this is believed to be due to different degrees of refinement of the petrolatum, where the yellowish colored petrolatum contains more sulfur, nitrogen

and hydrogen groups and accordingly, there is higher intermolecular interaction. In addition, the yellowish colored petrolatum has more double bonds, which imparts increased rigidity.

3.4. Reproducibility of three release testing methods using Lotemax®

In the development of *in vitro* release testing methods, reproducibility is essential to obtain reliable release data of the formulations. The release tests were repeated on three separate days (three runs) with six replicates per run for the purpose of method validation (Fig. 8). The percent coefficient of variance (CV %) of the cumulative amount released ($\mu g/cm^2$) within each run and among runs, as well as the drug release rate among runs were evaluated for all three release methods using Lotemax®. The CV% within each run at each time point using the Franz diffusion cell method ($<19\%$) is slightly higher compared to USP apparatus 2 ($<16\%$) and USP apparatus 4 methods ($<13\%$). However, the CV% of the drug release rate among the three runs were 5.56, 6.24 and 5.16 for the USP apparatus 4, USP apparatus 2 and Franz diffusion cell methods, respectively, indicating no significant difference among the three methods (Table 2).

There are several factors that may affect the accuracy of drug release data when using the Franz diffusion cell method. It has been reported that air is often entrapped between the ointment and membrane while loading the Franz cells and this can lead to inaccuracy in the amount of sample loaded (Chattaraj and Kanfer, 1996). In addition, it is difficult to load exactly the same amount of the ointment into the Franz cells each time due the thickness of the ointments and the fact that the Franz cells have a high capacity and
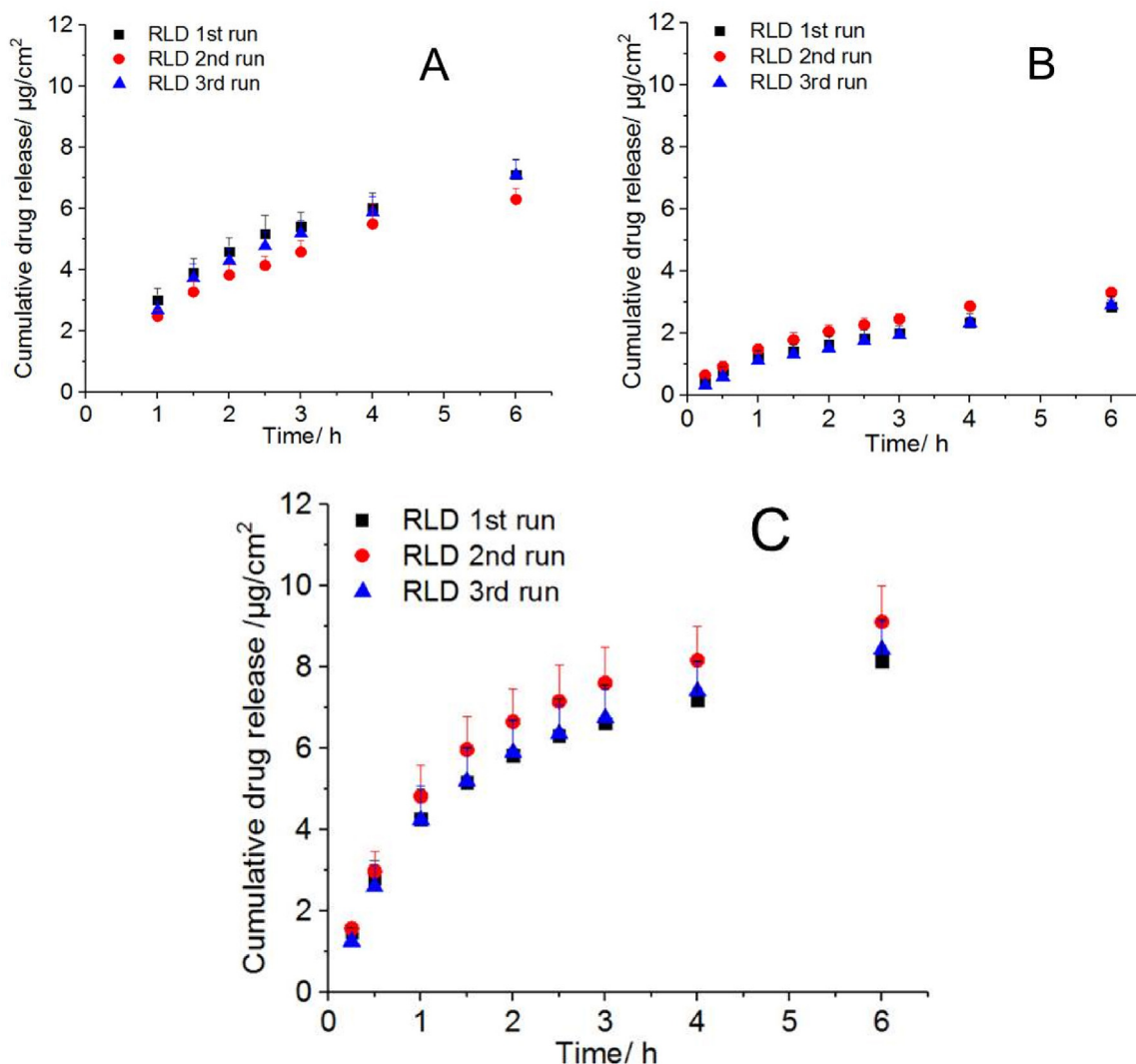
Fig. 8. *In vitro* drug release profiles of Lotemax® (RLD) obtained using different release testing methods: **A**) USP apparatus 4 with semisolid adapters; **B**) USP apparatus 2 with enhancer cells; and **C**) Franz diffusion cells (three runs, $n = 6$).

Table 2
In vitro drug release rate (the Higuchi model) of Lotemax® obtained using different methods.

	Average Release Rate ± SD ($\mu g/cm^2/min^{1/2}$)	CV%
USP apparatus 4	0.36 ± 0.02	5.56
USP apparatus 2	0.16 ± 0.01	6.24
Franz diffusion cells	0.36 ± 0.02	5.16

therefore the ointment samples do not completely fill the cells. Whereas, in the case of the compendial USP apparatus, the ointment samples can be completely filled into the enhancer cells and the semisolid adapters. The surfaces of the enhancer cells and semisolid adapters are flattened and any excess ointment and any bubbles are removed. Therefore, both compendial release testing methods showed better reproducibility of the release data.

3.5. Validation of release testing methods

To determine the discriminatory capability of the three release testing methods, SRTOWP19 (0.5% (w/w)) with 50% more drug (0.75% (w/w)) and 50% less drug (0.25% (w/w)) were tested using the three release methods. The higher the drug loading, the greater

the release rate (Fig. 9 and Table 3). All three release testing methods investigated showed adequate discriminatory capability, differentiating the ointments with higher and lower drug loading. In addition, the *in vitro* release data obtained using all three release testing methods showed a good fit to the Higuchi model ($R^2 > 0.99$). The two compendial methods exhibited a better fit compared to the Franz diffusion methods.

3.6. Evaluation of the three release testing methods via wilcoxon rank sum/Mann-Whitney rank test

In vitro release testing of ointments prepared with white petrolatum from four different sources and prepared using the three manufacturing processes were performed using the three release testing methods (Fig. 10). Three of the formulations (HMICOWP19, HMRTNWP19 and SRTNWP19) exhibited a significant difference ($p < 0.05$) in the *in vitro* drug release rate for all three testing methods. Compared with the compendial release testing methods, the Franz diffusion cells method was less able to discriminate between HMICOWP19 and HMRTNWP19 (somewhat overlapped by each other). However, the ANOVA test showed a significant difference ($p < 0.05$) in the release rate of HMICOWP19 and HMRTNWP19. The USP apparatus 4 with semisolid adapters
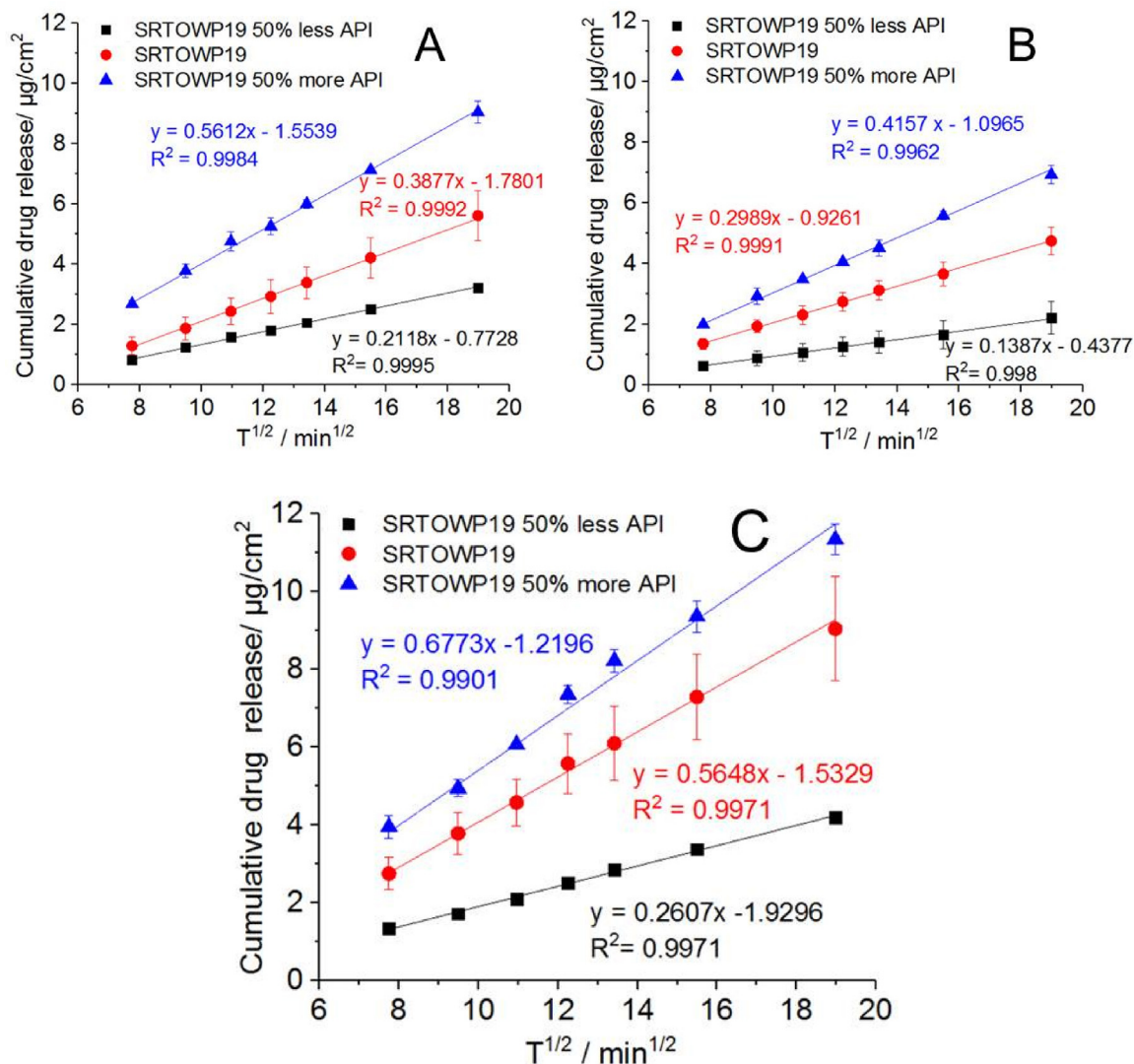
Fig. 9. Linear regression of the *in vitro* release profiles of SRTOWP19, and SRTOWP19 with 50% less drug and with 50% more drug obtained using: **A**) USP apparatus 4 with semisolid adapters; **B**) USP apparatus 2 with enhancer cells; and **C**) Franz diffusion cells ($n = 3$). (SRT: simple mixing process; OWP: white petrolatum from Fisher®.).

Table 3
Release rate of SRTOWP19 and SRTOWP19 with 50% less drug and with 50% more drug obtained using different release testing methods ($n = 3$).

Release testing method	Formulations	Average rate ± SD ($\mu g/cm^2/min^{1/2}$)	Ratio
USP apparatus 4	SRTOWP19 (0.25% loading)	0.21 ± 0.01	1.0
	SRTOWP19 (0.5% loading)	0.39 ± 0.05	1.8
	SRTOWP19 (0.75% loading)	0.56 ± 0.01	2.7
USP apparatus 2	SRTOWP19 (0.25% loading)	0.14 ± 0.04	1.0
	SRTOWP19 (0.5% loading)	0.30 ± 0.03	2.2
	SRTOWP19 (0.75% loading)	0.42 ± 0.08	3.0
Franz diffusion cells	SRTOWP19 (0.25% loading)	0.26 ± 0.01	1.0
	SRTOWP19 (0.5% loading)	0.57 ± 0.09	2.2
	SRTOWP19 (0.75% loading)	0.68 ± 0.02	2.6

demonstrated the best discriminatory capability among the three testing methods investigated. For comparison, the individual release rate data are summarized in Table 4.

To further evaluate the discriminatory capability of the three release testing methods for the loteprednol etabonate ointments, the Wilcoxon Rank Sum/Mann-Whitney rank test was performed according to the FDA SUPAC-SS guidance (FDA, 1997). For this, one formulation is regarded as the reference (R) and the other as the test formulation (T). The six release rate values ($n = 6$) of the test formulations were divided by the six release rate values of the reference formulation resulting in a total of 36 T/R individual percentages. The 36 T/R individual percentages were rank ordered from the lowest to the highest and the 8th and 29th ordered individual percentages were used in the analysis. At the 90% confidence interval, if the 8th and 29th ordered individual percentages of the two formulations (reference and test) fall
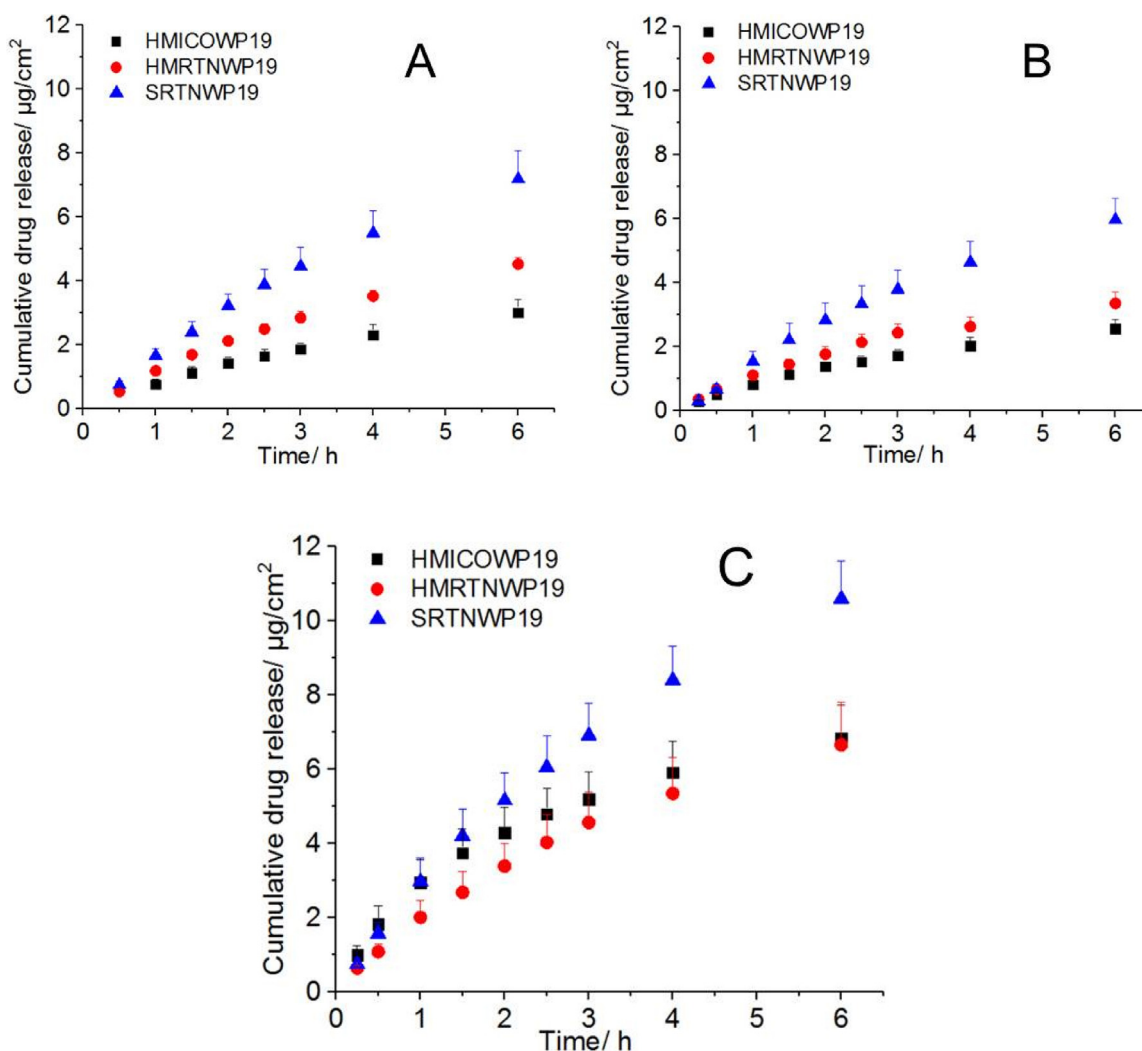
Fig. 10. *In vitro* release profiles of formulations HMICOWP19, HMRTNWP19 and SRTNWP19 obtained using: **A**) USP apparatus 4 with semisolid adapters; **B**) USP apparatus 2 with enhancer cells; and **C**) Franz diffusion cells method ($n=6$). (SRT: simple mixing process; HMIC: hot melting with immediate cooling process; HMRT: hot melting with gradual cooling at room temperature; OWP: white petrolatum from Fisher®; NWP: white petrolatum from Fougera®; and VWP: white petrolatum from Vaseline®.).

Table 4
Release rate of each cell of the three selected ointment formulations obtained using the different release testing methods ($n=6$).

Formulations	Release rate from USP apparatus 4 ($\mu g/cm^2/min^{1/2}$) cell 1 cell 2 cell 3 cell 4 cell 5 cell 6						Average ± SD
HMICOWP19	0.21	0.19	0.19	0.24	0.18	0.19	0.20 ± 0.02
HMRTNWP19	0.32	0.30	0.30	0.31	0.30	0.28	0.30 ± 0.01
SRTNWP19	0.49	0.43	0.58	0.42	0.55	0.51	0.50 ± 0.06
	Release rate from USP apparatus 2 ($\mu g/cm^2/min^{1/2}$)						Average ± SD
HMICOWP19	0.14	0.16	0.13	0.18	0.17	0.14	0.15 ± 0.02
HMRTNWP19	0.18	0.19	0.18	0.23	0.22	0.21	0.20 ± 0.02
SRTNWP19	0.39	0.41	0.43	0.38	0.43	0.39	0.40 ± 0.02
	Release rate from Franz diffusion cells ($\mu g/cm^2/min^{1/2}$)						Average ± SD
HMICOWP19	0.30	0.32	0.31	0.39	0.39	0.35	0.34 ± 0.04
HMRTNWP19	0.50	0.34	0.40	0.35	0.51	0.44	0.42 ± 0.07
SRTNWP19	0.80	0.70	0.64	0.64	0.70	0.62	0.68 ± 0.07

between 75% and 133.33%, the two formulations are regarded as not significantly different. From Table 5, for the USP apparatus 4, all the T/R percentages of the three formulations do not fall within the range of 75% to 133.33%, indicating significant differences between the formulations and showing the good discriminatory capability of this method. For the Franz diffusion cell and USP apparatus 2 with enhancer cells methods, the 29th ordered individual percentages for HMICOWP19 and HMRTNWP19 were out of range, suggesting these two release methods can also discriminate the three formulations. However, the 8th ordered individual percentages between these two formulations fell within the range mentioned above. Accordingly, the USP apparatus 4 with semisolid adapters method exhibited the best discriminatory capability among the three release testing methods.

Table 5
In vitro release profile comparison of the three formulations using release rate ratio rank order based on the Wilcoxon Rank Sum/Mann-Whitney rank test.

Ratio Order	Test: Reference:	HMRTNWP19 HMICOWP19	SRTNWP19 HMICOWP19	SRTNWP19 HMRTNWP19
		USP apparatus 4		
8th (%)		143	224	144
29th (%)		162	286	185
		USP apparatus 2		
8th (%)		116	236	181
29th (%)		151	300	222
		Franz diffusion cells		
8th (%)		102	178	138
29th (%)		143	224	188

3.7. In vitro drug release rates of the ointment formulations using three different release testing methods

To compare the *in vitro* drug release rate of the formulations obtained from different release testing methods, all the release profiles of the ointments were fitted using the Higuchi model. The Franz diffusion cell method showed poorer reproducibility and discriminatory capability compared to the USP compendial methods, Fig. 11 shows the release data for six formulations (prepared with OWP and NWP). The *in vitro* release rates of the ointment formulations showed almost the same rank order for the three different methods. The hot melting process exhibited significantly ($p < 0.05$) lower drug release rates compared to non-hot melting process.

The impact of the sources of 'white' petrolatum on drug release rate of the Q1/Q2 equivalent ointment formulations prepared *via* the SRT process was not significant compared to the formulations prepared *via* hot melting. Ointments prepared using yellowish colored petrolatum (OWP (non-USP grade) and VWP (USP grade)) demonstrated similar release rates, and likewise, the formulations prepared using lighter colored petrolatum (NWP and PWP (both USP grade)) showed similar release rates. The release rates of the formulations prepared using yellowish petrolatum were lower than those of the formulations prepared using lighter colored petrolatum. The results indicated that color difference of the white petrolatum may indicate the significant differences in properties and resultant variations in *in vitro* drug release. Since the color of the USP grade petrolatum varies from yellowish to almost transparent, it is very crucial to carefully evaluate excipient sources to ensure reproducible ointment products.

3.8. Comparison of three release testing methods regarding correlation between critical rheological parameters and in vitro release rate

It has been reported that a strong correlation was established between the rheological parameters (crossover modulus and K value) and the *in vitro* release rate using a logarithmic model (Bao et al., 2017). To a certain extent, the goodness of fit (R^2) of the model appears to be an indicator of the discriminatory capability
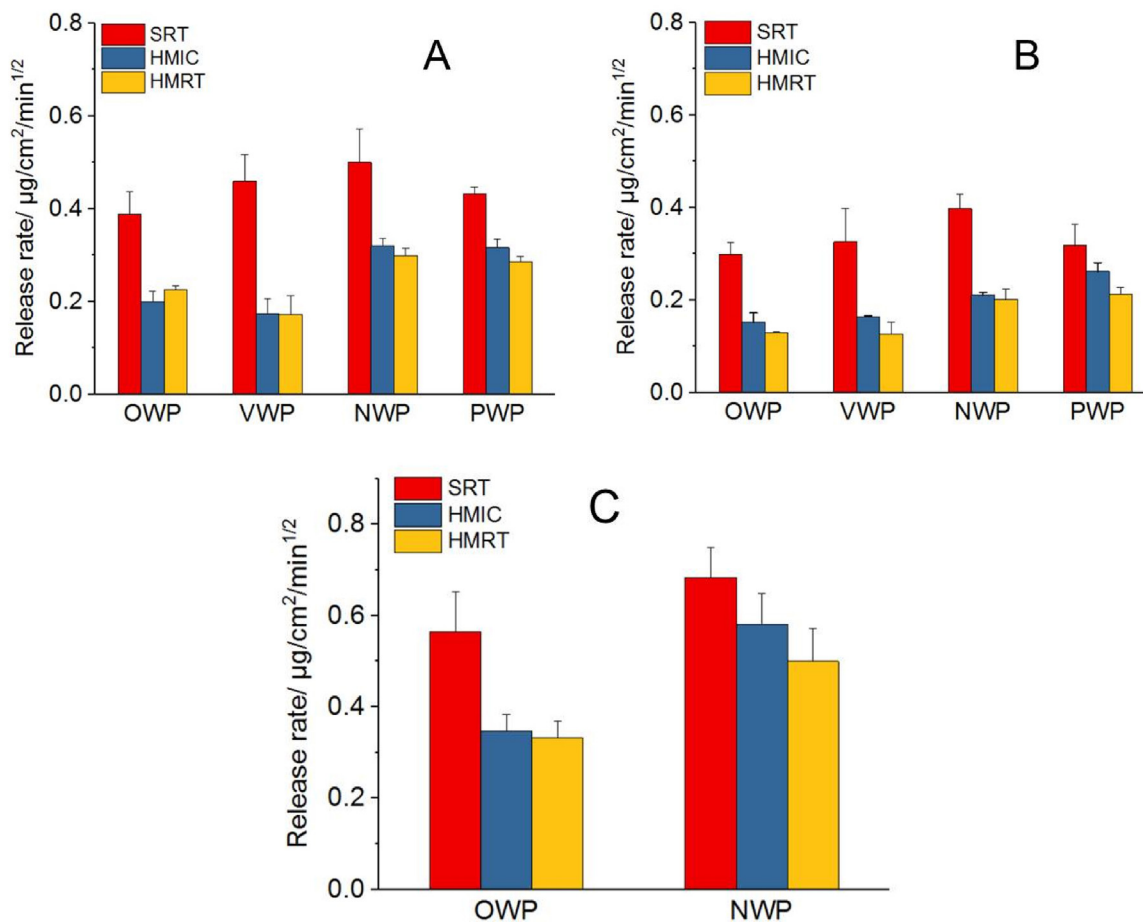

Fig. 11. *In vitro* release rate of loteprednol etabonate ointment formulations obtained using: **A**) USP apparatus 4 with semisolid adapters; **B**) USP apparatus 2 with enhancer cells; and **C**) Franz diffusion cells method ($n = 3$). (SRT: simple mixing process; HMIC: hot melting with immediate cooling process; HMRT: hot melting with gradual cooling at room temperature; OWP: white petrolatum from Fisher®; NWP: white petrolatum from Fougera®; VWP: white petrolatum from Vaseline®; and PWP: white petrolatum from Penreco.).
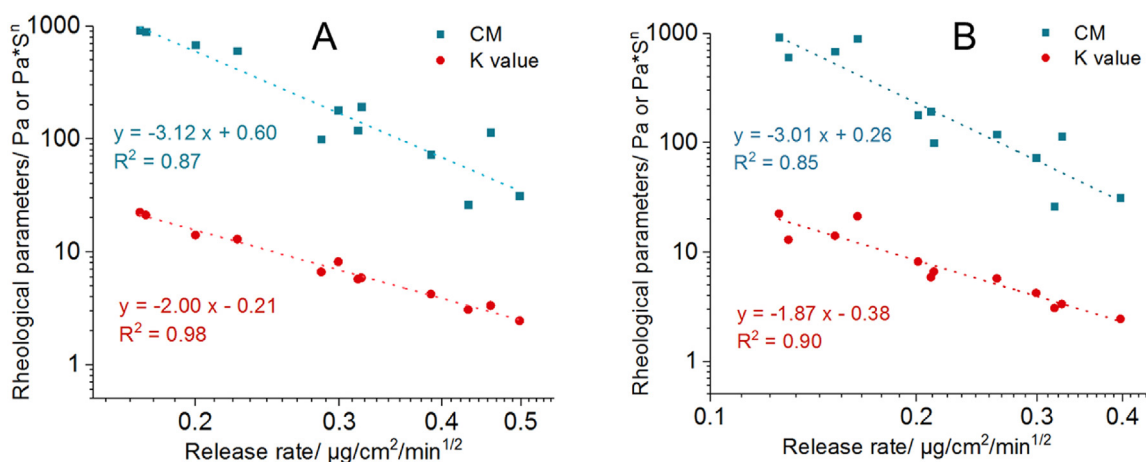
Fig. 12. Profiles of the log–log linear regression of the CM or K values versus the drug release rate obtained using: **A**) USP apparatus 4 with semisolid adapters; and **B**) USP apparatus 2 with enhancer cells methods.

and reproducibility of the release testing method. The log–log linear regression of the rheological parameters (CM and K values) versus the release rate of the nine ointments were carried out on the data obtained using the USP apparatus 4 with semisolid adapters and the USP apparatus 2 with enhancer cells methods. A strong logarithmic correlation ($R^2 > 0.85$) between the rheological parameters (CM and K values) and the release rate was demonstrated for both compendial release testing methods. USP apparatus 4 showed a higher goodness of fit for both the CM and K values (Fig. 12), compared to USP apparatus 2 (CM: 0.87 *vs.* 0.85; K value: 0.98 *vs.* 0.90), which indicates the superiority of USP apparatus 4 to USP apparatus 2 in predicting the *in vitro* drug release based on the rheological parameters. The correlation between the *in vitro* drug release and the critical rheological parameters was not strong for Franz diffusion cell method (the R^2 is less than 0.90, data not shown here).

4. Conclusions

The excipient source is an important factor in the development of compositionally equivalent semisolid ophthalmic formulations. Even USP grade 'white' petrolatum from different sources resulted in ointment formulations with significantly different physico-chemical properties (rheological parameters and *in vitro* release). According to the present study, the compendial *in vitro* release testing methods (USP apparatus 2 and USP apparatus 4) are more suitable than the Franz cell method for loteprednol etabonate ophthalmic formulations in terms of the reproducibility and discriminatory capability. One potential reason for the superiority of these compendial methods is the high-quality design of the sample loading cells or adapters (enhancer cells and semisolid adapters), which ensure good reproducibility of sample loading. In addition, these compendial methods are standardized and therefore facilitate inter-laboratory data comparison. This is the first report of using USP apparatus 4 with semisolid adapters for ophthalmic ointment formulations. Although the *t*-test is normally used to identify the significant differences among variables, it was shown that the *t*-test is not sufficiently sensitive to compare the discriminatory capability of the *in vitro* release testing methods investigated. The Wilcoxon Rank Sum/Mann-Whitney rank test appears to be a better method of evaluating the discriminatory capability of these *in vitro* release testing methods. The USP apparatus 4 method displayed the largest margin of discrimination between the release profiles of the different ophthalmic ointments. The results reported here confirmed that a correlation can be made

between the *in vitro* release rate values and the rheological parameters (K value and crossover modulus). In addition, the *in vitro* release rate obtained using the USP apparatus 4 method showed the strongest logarithmic linear correlation with the rheological parameters.

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