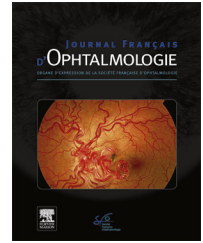




Disponible en ligne sur

ScienceDirect
www.sciencedirect.com

Elsevier Masson France

EM|consulte
www.em-consulte.com


ORIGINAL ARTICLE

Physicochemical and microbiological stability of insulin eye drops in an artificial tear vehicle used in the treatment of refractory neurotrophic keratopathy[☆]



Stabilité physico-chimique et microbiologique d'un collyre à l'insuline utilisé dans le traitement de la kératopathie neurotrophique réfractaire

M.H. Le Nguyen^b, M.S. Naoum^a, C. Andre^c, L. Lethier^c,
 S. Limat^{b,d}, C. Fagnoni-Legat^b, Y. Guillaume^{b,c},
 A.S. Gauthier^{a,*,e,d}

^a Service d'ophtalmologie, CHU Jean Minjoz, 2 boulevard Fleming, 25030 Besançon, France

^b Pôle pharmaceutique, CHU Jean Minjoz, 2 boulevard Fleming, 25030 Besançon, France

^c Pôle chimie analytique et physique, EA 481 « Neurosciences Intégratives et Cliniques », UFR Santé, université Bourgogne Franche-Comté, 25030 Besançon, France

^d Inserm, EFS BFC, UMR 1098, université Bourgogne Franche-Comté, 25030 Besançon, France

^e Faculté de médecine, université Bourgogne Franche-Comté, 25030 Besançon, France

Received 31 March 2022; accepted 21 April 2022

Available online 29 July 2022

KEYWORDS

Neurotrophic keratopathy;
 Insulin;
 Ophthalmic solution;
 Physicochemical
 microbiological
 stability;
 Container-content
 interaction

Summary Neurotrophic keratopathy (NK) is a degenerative corneal disease with a loss of corneal sensitivity and impairment of corneal healing. Low dose insulin eyedrops have been shown to be a simple and effective treatment for refractory NK when the response to the usual treatment is incomplete. At present, there are no commercially available forms, and there is no data regarding the stability of these products as prepared by compounding pharmacies. In this work, we studied the physicochemical and microbiological stability of an insulin ophthalmic formulation obtained by mixing insulin lispro in artificial tears with a polyethylene and propylene glycol base. The stability of this 1 IU/mL insulin ophthalmic formulation was analysed

[☆] This study was presented at the "Convergences Santé Hôpital 2020" conference: poster PT138.

* Corresponding author.

E-mail address: asgauthier@chu-besancon.fr (A.S. Gauthier).

for 12 months in low-density polyethylene (LDPE) multidose eye droppers at 4°C. The studied parameters of physicochemical stability were: visual inspection, turbidity, UV spectral absorption, osmolality and pH. In addition, insulin and m-cresol concentrations and quantification of impurities (insulin covalent aggregates and insulin fragments) were studied thanks to the development of a new Size Exclusion Chromatographic method. For unopened eye droppers, all tested physicochemical parameters remained stable for 12 months at 4°C, and excellent microbiological stability was obtained. In conditions of simulated use, these parameters also remained stable for one month at 4°C, and no impact of potential temperature rises on the insulin and m-cresol concentrations in the insulin eyedropper was observed.

© 2022 Elsevier Masson SAS. All rights reserved.

MOTS CLÉS

Kératite
neurotrophique ;
Collyre à insuline ;
Solution
ophtalmique ;
Stabilité
physico-chimique et
microbiologique ;
Interaction
contenant-contenu

Résumé La kératite neurotrophique (KN) est une maladie dégénérative avec diminution de la sensibilité et altération de la cicatrisation cornéenne. Les collyres à l'insuline à faible dose se sont révélées être un traitement simple et efficace pour la KN réfractaire lorsque la réponse au traitement habituel est incomplète. À l'heure actuelle, aucune forme commerciale n'est disponible et il n'existe aucune donnée concernant la stabilité de ces préparations. Dans cette étude, nous avons évalué la stabilité physico-chimique et microbiologique d'une formulation d'un collyre à l'insuline obtenue en mélangeant de l'insuline lispro dans des larmes artificielles à base de polyéthylène et de propylène glycol. La stabilité de cette formulation ophtalmique d'insuline à 1 UI/mL, conservée dans des compte-gouttes multidoses en polyéthylène basse densité (PEBD) à +4°C, a été analysée pendant 12 mois. Les paramètres de stabilité physico-chimique concernaient l'inspection visuelle, la turbidité, le spectres UV, l'osmolalité et le pH. La stabilité du collyre (concentration de l'insuline et du m-crésol, recherche d'impuretés: agrégats covalents d'insuline et fragments d'insuline) a été étudiée par une nouvelle méthode chromatographique d'exclusion de taille. Tous les paramètres physico-chimiques testés sont restés stables pendant une période de 12 mois à +4°C et une excellente stabilité microbiologique a été obtenue. Dans des conditions d'utilisation simulées, ces paramètres sont également restés stables pendant 1 mois à +4°C et aucun impact d'une éventuelle augmentation de température sur la concentration d'insuline et de m-crésol dans le compte-gouttes d'insuline n'a été observé.

© 2022 Elsevier Masson SAS. Tous droits réservés.

Introduction

Neurotrophic keratitis is a degenerative corneal disease due to the damage to the trigeminal nerve leading to a corneal loss of corneal sensation and breakdown of the corneal epithelium [1,2]. The etiology of NK is very large such as herpes simplex, varicella zoster, neurosurgery for trigeminal neuralgia, diabetes or central nervous system disease [1,2]. Different treatments were developed to restore corneal integrity and to prevent its progression. Among them there were the nerve grafts, amniotic membrane transplantation [3–5] or therapeutic bandage contact lens (BCL) [6]. Nerve growth factor (NGF) is a neurotrophic factor released by corneal epithelial cells and supports corneal integrity through multiple mechanisms [7,8]. NGF stimulated cell proliferation and survival of the sensory nerves that innervate the cornea. At present time the recombinant human NGF cenegermin ophthalmic solution 0.002% is the main treatment specifically indicated for the treatment of NK [8]. Plasma rich in growth factors (PRGF) eye drops could be also a safe and effective therapeutic option for patients with NK [9]. Insulin receptor (INSR) and insulin growth factor type

1 (IGF-1R) played a major role in the regulation of molecular processes including differentiation and survival [10,11]. Due to their strong similarity of structure the formation of insulin and IGF-1 hybrid receptors (Hybrid-R) is possible [12]. It was showed an accumulation of Hybrid-R in the corneal epithelial cell nucleus in response to stress induced by growth factor deprivation [13]. This nuclear accumulation is associated with partial cell cycle arrest and a reduction in mitochondrial respiration. Thus, in the cornea, Hybrid R expression is mediated by the presence of insulin and serve to regulate key functions required for cell growth and survival [14]. Studies have shown that supra-physiological levels of insulin applied topically to the eyes promotes corneal wound healing in animals with diabetes [15,16].

Despite all this recent and favorable clinical data, there is still currently no commercially insulin delivery system in the treatment of ulcer cornea or NK. Indeed, at present time, insulin delivery devices have been developed to deliver bioactive insulin in order to maintained normo – glycemic blood glucose levels for the treatment in diabetes melitus [17,18]. The major insulin delivery devices that are currently in use for the treatment of diabetes were insulin syringes [19,20],

insulin pens [21], insulin jet injectors [22,23], insulin infusion pumps [24,25], insulin inhalers [26–28]. Expanding the effort to expand the delivery routes have focused on the development of nasal [29,30], buccal, oral [31,32], and transdermal routes [33]. For the ocular route, for the treatment of diabetes, it suffered from low bioavailability caused by blinking and tearing [34].

Concerning treatment of corneal ulcer, it was demonstrated that topical insulin drops at 1 UI/mL could be a simple and effective treatment for refractory neurotrophic corneal ulcers [35]. A complete corneal re-epithelialization within 7 to 25 days was shown for patients with neurotrophic corneal ulcers refractory to a range of medical and surgical treatments [35]. A topical insulin up to 2 UI per drop is safe for human ocular usage for diabetic patients on postoperative corneal epithelial wound healing after vitreoretinal surgery [36]. A 50 UI/mL topical insulin might improve the rate of epithelial wound healing [37]. No adverse events were noted with the use of topical insulin at concentration up to 100 UI/mL of the published cases [38].

For insulin ocular administration for corneal ulcers, it is important to increase the precorneal residence time of the eye drop formulation. Therefore, the new generations of artificial tears integrated in their formulation natural polymer (methylcellulose derivatives) and synthetic polymers (polyethylene glycol, hydroxypropyl Guar). These polymers added a higher viscosity, prolonging the drug residence time on the cornea at physiological pH tear (pH = 7.4).

Despite all these favorable clinical data, there is currently no commercially available formulation of insulin eyedrops. As well, no studies have been published concerning the stability of insulin eyedrops, and generally the analysis where either lacking several tests or suffered from shortcomings concerning impurities products research.

The aim of this study was to assess the physicochemical stability and to control the sterility of a new ophthalmic insulin eye drops formulation. This one was obtained by diluting the commercial Humalog insulin lispro solution (100 UI/mL) with artificial tear lubricant eye drops to a final concentration of 1 UI/mL in a low-density polyethylene (LDPE) multidose eyedropper, the most use container in hospital pharmacy.

The 1 UI/mL insulin concentration was used because it was previously demonstrated that this dose was non-toxic and was the optimal dose for the treatment of refractory corneal ulcer for diabetics and non-diabetics patient [35].

The stability of this ophthalmic insulin formulation was studied at 4°C for 12 months in unopened eyedroppers and in simulated use conditions at 4°C and 25°C for one month.

Material and methods

Preparation and storage of insulin solution formulations

The formulation of the 1 UI/mL insulin solution was prepared using two commercial solutions:

- the commercial artificial tears Systane Ultra solution (Alcon Laboratories, Rueil-Malmaison, France) with the following composition: Polyquad (polidronium chloride) 0.001% m/v; aminomethylpropanol, boric acid, hydroxy-

propyl guar, polyethylene glycol 400, propylene glycol, sodium chloride, potassium chloride, sorbitol, purified water;

- the commercial Humalog solution (Eli Lilly Laboratories, Fegersheim, France) with the following: insulin lispro (100 UI/mL (i.e., 3.5 mg/mL in a hexameric form (data sheet))), m-cresol (3.15 mg/mL), glycerol, disodium monohydrogenophosphate heptahydrate, zinc oxide, chlorhydric acid and sodium hydroxide for adjusted pH (7.0–7.8), sterile water for injection.

The formulation was prepared by mixing 0.1 mL of the Humalog solution with 9.9 mL of Systane at room temperature under gentle agitation inside the bioquell Qube sterility isolator (This Qube is an aseptic processing workstation with an integrated Hydrogen Peroxide Vapor (HPV) decontamination system). The obtained insulin solution was thus sterilely distributed (8 mL per unit, for a maximum filling capacity of 10 mL for eye droppers) into the white Squeezable Eye Dropper Low-Density Polyethylene (LDPE) Bottles (Gravis, Trelaze, France) which dispense one uniform drop at a time. The dropper tips and caps were made in polyethylene terephthalate (PET). The dropper tips have an accurate opening to guarantee consistent and uniform drops (drop volume averages 40 µL per drop).

Study design

The stability of the 1 UI/mL insulin solutions was studied in unopened eyedroppers stored vertically in the dark for 12 months at 4°C. A simulated use study was also performed during 30 days at 4°C and at 25°C in a climate chamber.

Stability of 1 UI/mL insulin in unopened multidose eyedroppers

The eyedroppers containing insulin were stored vertically at 4°C in the dark. Immediately after the preparation, (day 0), 8, 15, 20, 29, 60, 90, 120, 150, 180, 240, 270, 360 days, four units ($n=4$) were submitted to several analyses: visual inspection, insulin and m-cresol quantification, impurities research (resulting from insulin covalent aggregates or insulin degradation products), osmolarity, pH, UV absorption spectra acquisition and turbidity. Sterility was also assessed using four units immediately after preparation and after 60, 90, 120, 180, 240 and 360 days of storage.

Stability of insulin in opened multidose eyedroppers (simulated use study)

The eyedroppers were subjected to a simulation of patient use. Immediately after the preparation and every day for one month, one drop was manually emitted out of each eye dropper morning and evening. As well, day 0, 8, 15, 20 and 29, for four units ($n=4$), few drops of each eye dropper was manually emitted out of the bottle, collected and mixed for analysis. After analysis, the units were stored at 4°C. As well, during this period, pH and osmolarity measurements were carried out. Similar experiments were carried out at 25°C.

Analyses performed on the insulin solutions

Visual inspection

The insulin solutions were emptied into glass test tubes, and these solutions were visually inspected under suitable lighting on a white and black background. Aspect, colour and a screening of visible macroparticles, haziness, or gas development were performed.

Spectrophotometric analysis

By using an UV spectrophotometer (UV1800, Shimadzu, France) the UV absorption spectra of the insulin solutions were acquired. As well, the turbidity of the insulin solutions was measured at 600 nm. The optical density at 600 nm was used as a quantitative indicator of the turbidity resulting from insoluble insulin particles. An elevation of the absorbance measured at 600 nm over 0.1 indicated a precipitate was generated in the solution [39].

Insulin quantification and IPs research

Size Exclusion Chromatography (SEC) material

The High Performance Liquid Chromatography (HPLC) system consisted of an Agilent 1260 Quaternary pump VL (G1311C), an Agilent 1260 DAD VL (G1315D), an Agilent 1260 Infinity High Performance Autosampler (G1329B) and an Agilent 1260 Infinity Thermostated Column Compartment (TTC) (G1316A) (Paris, France). The AdvanceBio SEC 300 Å 7.8 × 300 mm, 2.7 µm (p/nPL1180-5301) column was furnished by Agilent (Paris, France). The mobile phase consisted of a mixture of (200 mL of anhydrous acetic acid (glacial) + 300 mL of acetonitrile (ACN) + 400 mL of water) adjusted to pH = 3.00 with concentrated ammonia and then diluted to 1000 mL with water. The column was thermostated at a temperature of 25 °C and the mobile phase flow-rate was optimized so as to obtain an efficient separation of the molecules of interest on the studied chromatogram. The detection wavelength was fixed at 276 nm. All the products used in this work were of analytical grade [40].

Forced degradation studies of insulin: thermal stress

In order to exclude potential interferences of insulin impurities (insulin covalent aggregates and insulin fragments) with insulin quantification, the commercial 100 UI/mL insulin HUMALOG solution was subjected to the following forced degradation conditions. All experiments were performed using a 4 mL amber glass vial with a PTFE cap (Thermo-scientific, USA). For the heat stress, 3.0 mL of the HUMALOG solution was placed in the vial and heated in a water bath at 70 °C for 24 h. The same forced degradation procedure was carried out with 3 mL of the commercial Systane Ultra solution. The stress conditions were performed in triplicate.

Method validation

The method linearity was studied by the preparation of one calibration curve daily for four days using five concentrations of insulin in µg/mL (17.50; 28; 35; 42; 70 µg/mL). These concentrations were obtained by an appropriate dilution of the 100 UI/mL Humalog solution with the Systane solution base.

The calibration curve should have a determination coefficient r^2 equal or higher than 0.999. The homogeneity of the curve was determined by the use of a Cochran – Test. ANOVA tests were applied to determine applicability. Each day for three days, 6 solutions of insulin 1 UI/mL were prepared, analysed and quantified using a calibration curve prepared the same day. The repeatability of the method was estimated by the calculation of the relative standard deviation (RSD) of intraday analysis and intermediate precision was calculated using an RSD of inter-days analysis. Both RSDs were considered acceptable if they were lower than 5%. Method accuracy was estimated by the evaluation of the recovery of five theoretical concentrations to experimental values found using mean square equation, and results should be found within the range 95–105%. The overall accuracy profile was constructed following the instructions from reference [41].

m-cresol was quantified using the same method as for insulin quantification, using a calibration curve ranging from 16 to 70 µg/mL validated using the same methodology as previously described for the validation of the insulin quantification method.

Osmolality, pH measurements

For each unit, pH measurements were carried out using a MA235Advanced pH – Meter (Mettler Toledo, Bèthune, France). Before measurements, a validation procedure was carried out by the use of a series of four standard buffer solutions of pH between 1 and 8. The Osmolality was determined on 20 µL samples using a freezing point osmometer Loeser TYP15 (Löser Messtechnik, Berlin, Germany). Before measurements, the osmometer was calibrated using a calibrated osmolality standard.

Sterility assay

Following the sterility assay [42], each eyedropper was opened under a laminar air flow of a microbiological safety cabinet. A fraction of the insulin solution was inoculated on the microbiological media, fluid thioglycolate medium with resazurin, used for the growth of aerobic and anaerobic bacteria incubated at 37 °C for 15 days. A second fraction of the insulin solution was inoculated on the soy-bean casein digest medium, used for the growth of aerobic bacteria and fungi incubated at 25 °C for 15 days. Each culture medium was then visually studied for any signs of microbial growth.

Data analysis and stability criteria

The visual aspect of the solution, UV spectra acquisition, turbidity, pH, osmolality, insulin concentration and presence or absence of impurities (insulin covalent aggregates and insulin fragments) were analysed throughout this work. This study was carried out following the recommendations of the international conference on harmonisation for stability study [43], the French society of clinical pharmacy (SFPC) and the Evaluation and Research Group on Protection in Controlled Atmosphere (GERPAC) [44]. A variation of concentration outside the 90–100% range initial concentration (with limits of a 95% confidence interval of the measures), the presence of impurities products and the variation of the physicochemical parameters were not

acceptable. As usual, osmolality data were studied considering clinical tolerance of the insulin formulation and pH measurements must not vary by more than one pH unit from the initial value [44].

Results

Research of the optimal chromatographic conditions for the SEC separation of insulin with the other compounds in the insulin eyedropper

A total of 10 μ L of the new insulin ophthalmic solution (1 UI/mL) freshly prepared was eluted isocratically with the mobile phase (200 mL of anhydrous acetic acid (glacial) + 300 mL of acetonitrile (ACN) + 400 mL of water) adjusted to pH = 3.00 with concentrated ammonia and then diluted to 1000 mL with water) at a column temperature equal to 25 °C. So as to obtain an analysis time less than 25 min and a resolution at least equal to 1.5 for the worst separated pair of peaks on the chromatogram the flow-rate was fixed at 0.8 mL/min. The corresponding chromatogram was given in Fig. 1A. Three peaks were clearly distinguished at retention times of 10.82 min, 16.56 min, 19.07 min with the addition of a negative peak (NP) (13.72 min). This NP can be attributed to the absorbance of solute less than that of mobile phase and from the end of the entropy-dominated size separation range on the size exclusion chromatography (SEC) column [45].

To find which compound corresponded to each of these peaks, m-cresol and the 100 UI/mL Humalog and Systane solutions were successively injected into the chromatographic system in the same chromatographic conditions. The retention times of m-cresol (16.20 min) was found on the chromatogram of the Humalog solution (Fig. 1B). The negative peak (NP) and the positive peak provided from the Systane solution injection inside the chromatographic system (Fig. 1C).

Therefore, the peak at 10.82 min corresponded to insulin in its monomeric form due to the dilution and disassembly of its hexameric form upon injection onto the SEC column [46].

A summary of the m-cresol, Positive Systane Peak (PSP) retention times and relative retention times (relative to insulin monomer) was given in Table 1.

Insulin quantification and impurities products (IPs) research

For six replicates of the 1 UI/mL ophthalmic solution, the RT and area RSDs for the insulin peak (RT = 10.826 min; Area = 24.2294) were within the acceptable limit of $\pm 3\%$ demonstrating the excellent reproducibility and precision of our SEC technique for the insulin quantification.

This analytical method for the quantification of insulin in the Systane solution base was linear for concentration ranging from 17 μ g/mL to 70 μ g/mL including the insulin concentration target of 1 UI/mL = 35 μ g/mL in eye dropper. The mean linear regression was equal to $A = 0.7821 \times x - 3.2089$. In this equation A was the surface

area of the insulin peak and x (μ g/mL) the concentration of insulin. The intercept was not significantly different from zero and the mean determination coefficient r^2 of three calibration curves was 0.9997. This last result indicated an excellent dose-dependent correlation between peak area and insulin concentration. The relative mean relative bias coefficients were less than 3.0% for the calibration points. The mean repeatability RSD coefficient and mean intermediate precision RSD coefficient were less than 5%. The accuracy profile constructed with the data showed that the limits of 95% interval coefficients were all within 3% of the accepted value. The limit of quantification was equal to 10 μ g/mL.

Concerning m-cresol quantification, similar results were obtained with the following mean linear regression $A = 9.1102 \times x - 4.6148$ with an excellent determination coefficient r^2 of three calibration curves (0.9999) for concentration ranging from 17 μ g/mL to 70 μ g/mL including the m-cresol concentration target of 31.5 μ g/mL in the insulin eye dropper.

Size exclusion chromatography separation methods are integral components of quality control strategies for biomolecules. SEC is the long-standing standard for detection and quantification of proteins impurities particularly covalent aggregates (CAs) and fragments (Fs). Insulin covalent aggregates (InCAs) elutes earlier than the main peak of insulin monomer (In) while the insulin fragments (InFs) elutes later.

Fig. 2A showed the change of size profile of the insulin lispro (Humalog) under thermal stress conditions compared to the initial chromatogram of this Humalog solution at Day0 (Fig. 1B). The peak of insulin monomer (In) at 10.95 min disappeared. Two peaks appeared on the chromatogram at retention times of 5.65 min and 12.52 min corresponding respectively to InCAs and InFs. The peak of m-cresol remained unchanged (16.21 min). The resolution between two adjacent peaks on the chromatogram was always higher than 1.5.

The chromatogram of the Systane solution under these thermal stress conditions showed no peak of degradation products or aggregates (Fig. 2B) compared to the initial chromatogram of Systane (Fig. 1C). The NP and the PSP remained unchanged on these two chromatograms.

A summary of the impurity (InCAs and InFs) retention times and relative retention times (relative to insulin monomer) was given in Table 1.

Stability of 1 UI/mL Insulin in unopened multidose eyedroppers at 4 °C for 12 months

Physical stability

All samples stayed limpid and uncolored. There was no appearance of any visible particle matter, haziness or gas development. No elevation of the absorbance value measured at 600 nm was over 0.1 (Table 2).

Chemical stability

Evolution of pH and osmolality throughout the study was presented in Tables 3 and 4. Throughout the study, osmolality did not vary by more than 2% of the initial osmolality (279 mOsm/kg) after 12 months of storage at 4 °C. Moreover, pH

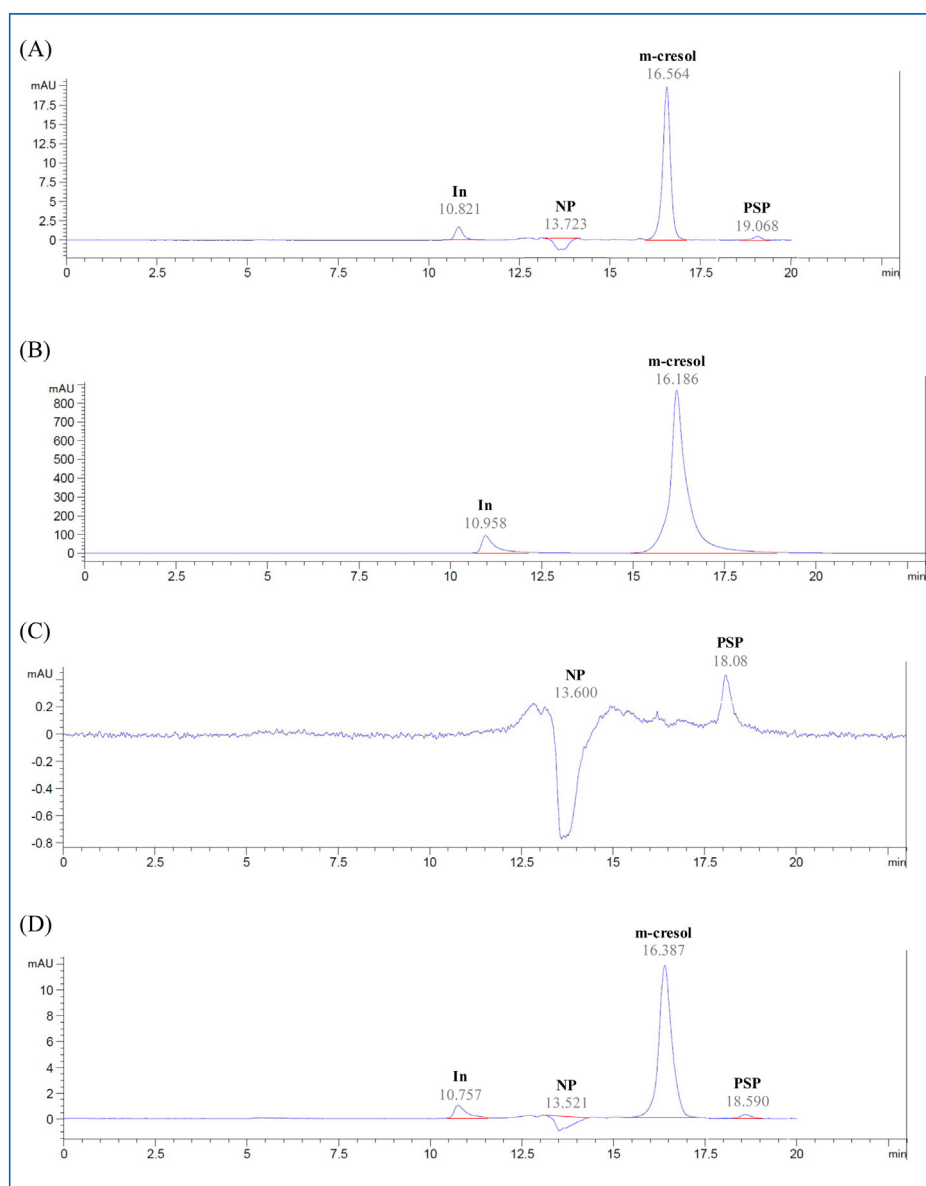


Figure 1. Reference chromatogram at 276 nm of (A) the 1.0 UI/mL insulin ophthalmic formulation (Day0) (B) the 100 UI/mL insulin Humalog commercial Solution (C) the Systane commercial solution and (D) the 1.0 UI/mL ophthalmic formulation (Day 360).

Table 1 Insulin monomer (In), insulin covalent aggregates (InCAs), insulin fragments (InFs), m-cresol and Positive Systane Peak (PSP) retention times (RT) and relative retention times (rRT) (relative to insulin monomer).

Peak of	RT (min)	rRT
In	10.9	1
InCAs	5.6	0.51
InFs	12.5	1.15
m-cresol	16.2	1.49
PSP	18.1	1.66

did not vary by more than 0.5% of the initial pH value (7.83) after 12 months of storage at 4 °C.

Throughout the study, insulin and m-cresol concentrations remained well within the 90–110% concentration range when the formulations were stored at 4 °C for 12 months as presented in Figs. 3A and 4A.

The chromatograms showed no sign of the presence of insulin covalent aggregates (InCAs) and insulin fragments (InFs) after 12 months of storage at 4 °C (Fig. 1D). As well, the Negative Peak (NP) and the Positive Systane Peak (PSP) of the Systane solution observed on the SEC column remained unchanged after 12 months of storage at 4 °C (Fig. 1D).

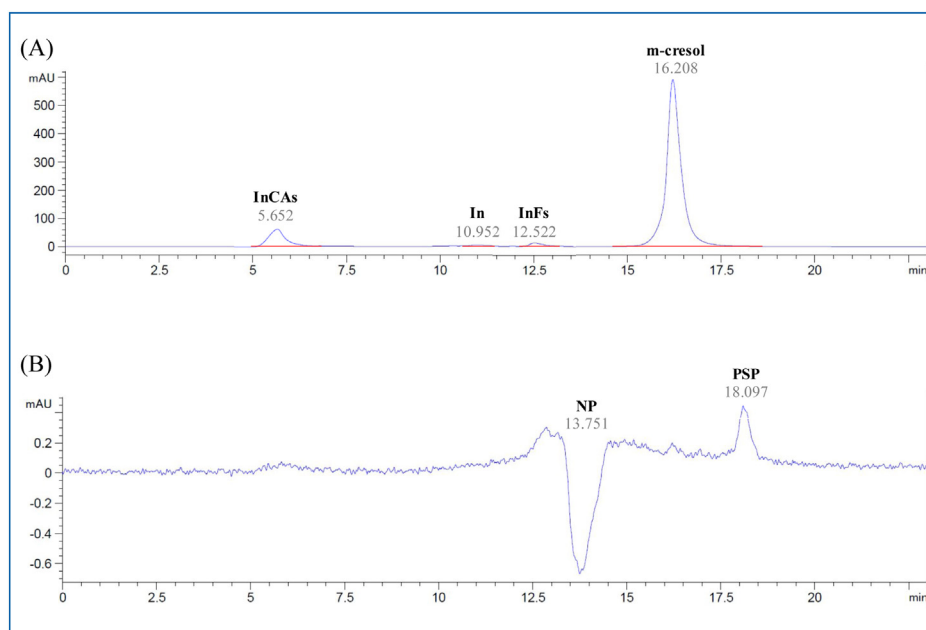


Figure 2. Reference chromatogram at 276 nm under thermal stress conditions of (A) the 100 UI/mL insulin Humalog commercial Solution and (B) the Systane commercial solution.

Table 2 Evolution of turbidity over time ($n=4$, mean \pm 95% confidence interval): *in the unopened eyedroppers for 12 months for a temperature storage of 4 °C (A). *In a patient simulated use for one month for a temperature storage of the eyedroppers of 4 °C (B) and 25 °C (C).

Day	0	8	15	20	30	60	90	120	150	180	240	270	360
A	0.01	0.02	0.01	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.02
B	0.01	0.02	0.02	0.01	0.02	*	*	*	*	*	*	*	*
C	0.02	0.01	0.02	0.03	0.02	*	*	*	*	*	*	*	*

Table 3 Evolution of pH over time ($n=4$, mean \pm 95% confidence interval): *in the unopened eyedroppers for 12 months for a temperature storage of 4 °C (A). *In a patient simulated use for one month for a temperature storage of the eyedroppers of 4 °C (B) and 25 °C (C).

Day	0	8	15	20	30	60	90	120	150	180	240	270	360
A	7.83 ± 0.04	7.85 ± 0.01	7.85 ± 0.01	7.84 ± 0.02	7.87 ± 0.01	7.86 ± 0.02	7.86 ± 0.02	7.85 ± 0.01	7.86 ± 0.01	7.85 ± 0.02	7.86 ± 0.01	7.86 ± 0.01	7.86 ± 0.01
B	7.84 ± 0.03	7.86 ± 0.01	7.85 ± 0.02	7.85 ± 0.01	7.85 ± 0.01	*	*	*	*	*	*	*	*
C	7.84 ± 0.02	7.87 ± 0.02	7.84 ± 0.02	7.85 ± 0.02	7.86 ± 0.02	*	*	*	*	*	*	*	*

Table 4 Evolution of osmolality (mOsm/kg) over time ($n=4$, mean \pm 95% confidence interval). *in the unopened eyedroppers for 12 months for a temperature storage of 4 °C (A). *In a patient simulated use for one month for a temperature storage of the eyedroppers of 4 °C (B) and 25 °C (C).

Day	0	8	15	20	30	60	90	120	150	180	240	270	360
A	279 \pm 3	278 \pm 1	275 \pm 3	282 \pm 1	275 \pm 4	276 \pm 5	274 \pm 3	280 \pm 2	280 \pm 2	281 \pm 4	280 \pm 4	281 \pm 2	283 \pm 2
B	279 \pm 2	273 \pm 3	274 \pm 4	280 \pm 2	275 \pm 5	*	*	*	*	*	*	*	*
C	281 \pm 2	281 \pm 3	276 \pm 3	282 \pm 2	278 \pm 2								

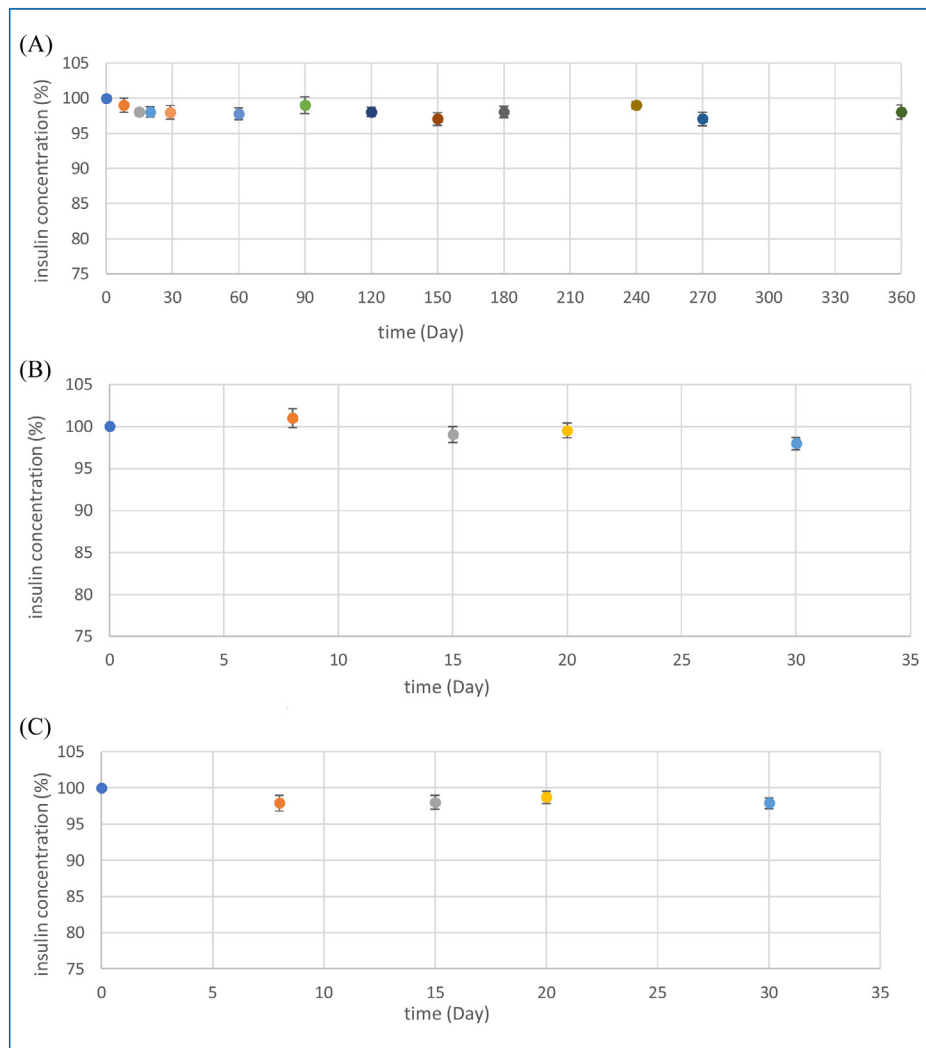


Figure 3. Evolution of insulin concentration (expressed in percentage of the initial concentration over time): *in the unopened eyedroppers for 12 months for a temperature storage of 4 °C (A). *In a patient simulated use for one month for a temperature storage of the eyedroppers of 4 °C (B) and 25 °C (C).

The UV spectra of the insulin solutions remained virtually unchanged over these 12 months.

The UV spectra at Day360 compared to that at Day0 showed that the shift in maxima and minima's wavelengths were inferior to 2 nm which was considered acceptable (Fig. 5). As well, the shift in maxima and minima's absorbance were inferior to 3% (Fig. 5).

Sterility assay

None of the seven analyzed solutions conserved in unopened bottles at day 0, day 60, day 90, day 120, day 180 day 240 and day 360 showed any signs of microbial growth.

Insulin and m-cresol concentrations in eye drop simulated use at 4 °C and 25 °C

The simulated use test was performed on formulations for 30 days at 4 °C. The insulin and m-cresol concentrations remained well within the 90–110% concentration range as

seen in Figs. 3B–4B. Turbidity, pH and osmolality remained unchanged (Tables 2–4).

To study the impact of potential temperature rise during storage on insulin and m-cresol concentrations, the same experiments were carried out for a storage temperature of 25 °C. The insulin and m-cresol concentrations remained well within the 90–110% concentration range as seen in Figs. 3C–4C. Turbidity, pH and osmolality values remained unchanged (Tables 2–4).

Discussion

This work described data on the physicochemical stability of a new formulation of a 1 UI/mL insulin solution conditioned in sterilized LDPE eye dropper which can be used for the treatment of refractory neurotrophic keratopathy.

Eye drops are the most commonly used solutions in the treatment and diagnosis of eye disease. To avoid adverse reactions, eye drops should have characteristics such as

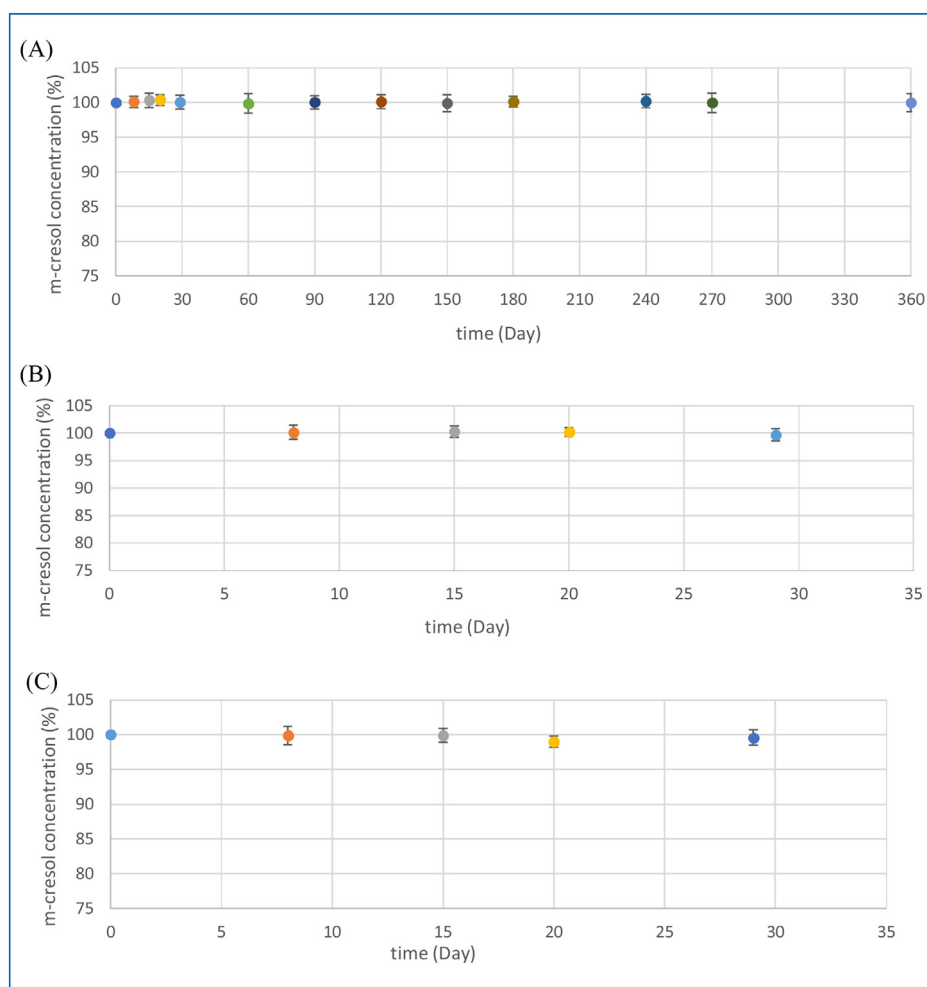


Figure 4. Evolution of m-cresol concentration (expressed in percentage of the initial concentration over time): *in the unopened eye-droppers for 12 months for a temperature storage of 4°C (A). *In a patient simulated use for one month for a temperature storage of the eyedroppers of 4°C (B) and 25°C (C).

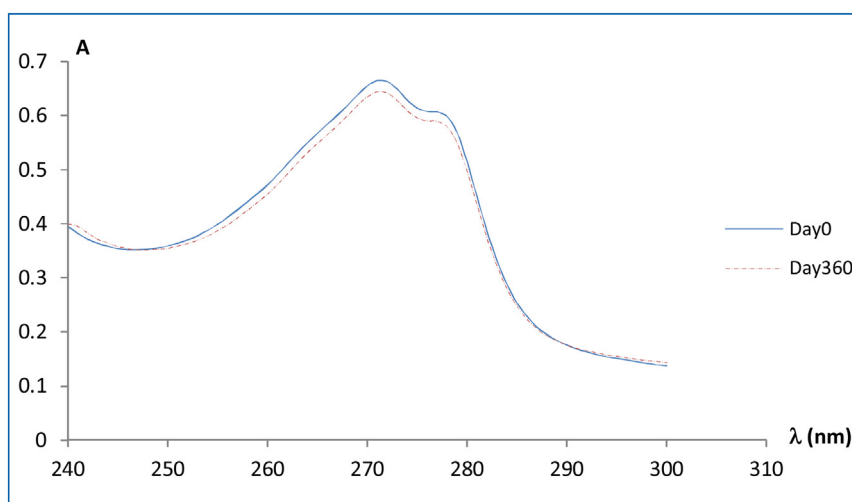


Figure 5. Evolution of the UV spectrum (Absorbance A versus wavelength λ (nm)) of the 1UI/mL insulin eye drops formulation at Day0 and Day360.

being clear, isotonic or sterile that adapt to the physiological conditions of the eye.

Tears have a normal pH of 7.4 to 7.7, although different diseases can modify their pH [47,48]. The buffer capacity of tears can neutralize solutions with a wide range of pH values (3.5–10.5) providing the solutions are not buffered. In our formulation the pH eye drop was maintained in a pH around 7.85 which was an excellent point for clinical tolerance. Indeed, if the pH values get outside the range 4–8, there may be irritation and the drug bioavailability can decrease because of increasing tearing. The pH of our eye drops stored at 4 °C for 12 months remained within this range over the study period.

Another key aspect of the eye drop production process is that they must be isotonic with natural tears. The physiological osmolality of human tears ranges from 302 mOsm/L to 318 mOsm/L [49,50].

Healthy eyes can tolerate solutions with an osmotic pressure of up to 425 mOsm/L without pain or excessive tear production [51]. The results show that the osmolality (around 279 mOsm/kg) of our insulin eye drops that had been conserved at 4 °C for 12 months was strongly appropriate for ophthalmic administration.

Insulin was composed of 51 amino acids in two peptide chains (A and B) linked by two disulfide bonds. Insulin existed as a monomer at a very low concentration (0.6 mg/mL). At a higher concentration such as 1 UI/mL (i.e., 35 µg/mL) the hydrophobic association of the B23 to B28 regions on insulin monomers could result to the formation of a non-covalent dimer [52]. In the presence of zinc ions and in the pH range 4.0–8.0, three dimers assembled primarily to form a non-covalent hexamer. In the lilly pharmaceutical formulation, insulin lispro was a non-covalent hexamer stabilized by zinc and m-cresol [53,54]. During the topical administration m-cresol diffused out, weakening dimer interactions in this insulin form and leads to hexamer disassembly into insulin monomers. Monomer thus bind to insulin receptor on the ocular surface, which in turn promotes the therapeutic effect.

Problem relating to aggregation of insulin results from the formation of covalent-dimer or higher covalent oligomer leading to covalent aggregates (CAs) on long-term storage of insulin preparation. The presence of such CAs may result to immunological side effect.

For this novel formulation, all parameters were in favor of a physical stability for 12 months at 4 °C. No modifications of visual aspects were detected. The solutions remained colorless, limpid through the study. Turbidity analysis did not reveal the formation of any additional particles and the pH and osmolality values also remained within specifications of any additional particles such as insoluble precipitates of insulin due to the formation of insoluble covalent aggregates. This result was reinforced by the fact that our SEC experiments did not revealed the appearance of Insulin covalent aggregates, Insulin fragments or the formation of degradation products of the Systane solution. As well, the UV spectrum of this insulin formulation remained unchanged.

Insulin and m-cresol concentration remained within specifications after 12 months of storage thus conserving good therapeutic efficiency during the conservation period. No degradation rate was observed during this period for m-cresol and insulin. The fact that the concentration of the

preservative m-cresol was practically constant during this conservation period maintained an excellent stability for insulin. Indeed, at the insulin lispro interface, close to the tetrahedral Zinc, there is a hydrophobic pocket where m-cresol can bind and thus stabilized the hexamer structure and prevented thus the appearance of InCAs (or InFs) during the storage of insulin eye droppers.

The sterility assay did not reveal any microbial contaminations in units stored for 12 months at 4 °C.

During the simulated use study, insulin and m-cresol were quantified in the emitted drops to evaluate any potential loss of Active Pharmaceutical Ingredient (API) by sorption for example on the dropper tips in PTE during the emission of drops.

For a storage at 4 °C, during the 30 days study, insulin and m-cresol concentrations in the emitted drops remained stable. There was thus no significant loss of insulin and m-cresol during the direct topical administration by the patient. A similar result was observed for a storage at 25 °C during the 30 days study demonstrating clearly no impact of potential temperature excursions during storage on insulin eye droppers.

Conclusion

Our work brought important information about the stability of 1 UI/mL insulin eye droppers. By the development of a new Stability Indicating Size Exclusion chromatographic method it was demonstrated that 1 UI/mL insulin solutions were physicochemical and microbiological stable for 12 months when stored in LDPE bottles at 4 °C. It was showed an absence of significant interaction between the LDPE container and the PTE dropper tip and cap with the studied insulin ophthalmic formulation. In simulated use conditions, for one month of storage, no impact of potential temperature excursion on the insulin and m-cresol concentration in the insulin eyedropper was observed. This shelf life of 12 months, with no commercial alternative, allows the preparation to be computed in advance, stored, dispensed and used by the patient.

Disclosure of interest

The authors declare that they have no competing interest.

References

- [1] Mastropasqua L, Massaro-Giordano G, Nubile M, Sachetti M. Understanding the pathogenesis of neurotrophic keratitis: the role of corneal nerves. *J Cell Physiol* 2017;232:717–24.
- [2] Dua HS, Said DG, Messmer EM, et al. Neurotrophic keratopathy. *Prog Retin Eye Res* 2018;66:107–31.
- [3] Malhotra R, Elalfy MS, Kannan R, Nduka C, Hamada S. Update on corneal neurotization. *Br J Ophthalmol* 2019;103:26–35.
- [4] Terzis KK, Dryer MM, Bodner BI. Corneal Neurotization. A novel solution to neurotrophic keratopathy. *Plast Reconstr Surg* 2009;123:112–20.
- [5] Murri MS, Moshirfar M, Birdsong OC, Ronquillo YC, Ding Y, Hoopes PC. Amniotic membrane extract and eye drops: a

- review of literature and clinical application. *Clin Ophthalmol* 2018;12:1105–12.
- [6] Grey F, Carley F, Biswas S, Troman C. Scleral contact lens management of bilateral exposure and neurotrophic keratopathy. *Cont Lens Anterior Eye* 2012;35:288–91.
 - [7] Bonini S, Lambiasi A, Rama P, Caprioglio G, Aloe L. Topical treatment with nerve growth factor for neurotrophic keratitis. *Ophthalmology* 2000;107:1347–51.
 - [8] Sheha H, Tighe S, Hashem O, Hayashida Y. Update on cenegermin eye drops in the treatment of neurotrophic keratitis. *Clin Ophthalmol* 2019;13:1973–80.
 - [9] Sanchez Avilla RM, Merayo Lloves J, Riestra AC, Fernandez-Vega Cueto L, Anitua E, Begona L, et al. Treatment of patients with neurotrophic keratitis stages 2 and 3 with plasma rich in growth factors (PRGF - Endoret) eye drops. *Int Ophthalmol* 2018;38:1193–204.
 - [10] King ER, Wong KK. Insulin like growth factor: current concepts and new developments in cancer therapy. Recent patents on anti-cancer drug discovery. *Recent Pat Anticancer Drug Discov* 2012;7:14–30.
 - [11] Sarfstein R, Werner H. Minireview: nuclear insulin and insulin-like growth factor – 1 receptors: a novel paradigm in signal transduction. *Endocrinology* 2013;154:1672–9.
 - [12] Bailyes EM, et al. Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptors species by selective immunoprecipitation and immunoblotting. *Biochem J* 1997;327:209–15.
 - [13] Wu YC, Zhu M, Robertson DM. Novel nuclear localization and potential function of insulin like growth factor receptor/insulin receptor hybrid in corneal epithelial cells. *PLoS One* 2012;7(8):e42483, <http://dx.doi.org/10.1371/Journal.pone.0042483>.
 - [14] Cruz-Cazarim ELC, Cazarim MS, Ogunjimi AT, Petrilli R, Rocha EM, Lopez RFV. Prospective insulin based ophthalmic delivery system for the treatment of dry eye syndrome and corneal injuries. *Eur J Pharm Biopharm* 2019;140:1–10.
 - [15] Friend J, Snip RC, Kiorpes TC, Thoft RA. Insulin sensitivity and sorbitol production of the normal rabbit corneal epithelium. *In vitro. Invest Ophthalmol Vis Sci* 1980;19:913–9.
 - [16] Zagon IS, Kloczek MS, Sassani JW, McLaughlin PJ. Use of topical insulin to normalize corneal epithelial healing in diabetes mellitus. *Arch Ophthalmol* 2007;125:1082–8.
 - [17] Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 2001;358:221–9.
 - [18] Stumvoll M, et al. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* 2005;365:1333–46.
 - [19] Chu JP, et al. Non-stick syringe needles: Beneficial effects on thin film metallic glass coating. *Sci Rep* 2016;6:31847.
 - [20] Khan AM, Alswat KA. Benefits of using the i-port system on insulin treated patients. *Diabetes Spectr* 2019;32: 30–55.
 - [21] Bailey TS, Stone JY. A novel pen-based Bluetooth-enabled insulin delivery system with insulin dose tracking and advice. *Expert Opin Drug Deliv* 2017;14:697–703.
 - [22] Barolet D, Benohanian A. Current trends in needle-free jet injection: an update. *Clin Cosmet Investig Dermatol* 2018;11:231–8.
 - [23] Engwerda EE, et al. Needle free jet injection of rapid-acting insulin improves early postprandial glucose control in patients with diabetes. *Diabetes Care* 2013;36:3436–41.
 - [24] McAdams BH, Rizvi AA. An overview of insulin pumps and glucose sensors for the generalist. *J Clin Med* 2016;5:5.
 - [25] El-Khalib FH, et al. Home use of a bi-hormonal bionic pancreas versus insulin pump therapy in adults with Type 1 diabetes/A multicenter randomized crossover trial. *Lancet* 2017; 389:368.
 - [26] Selam JL. Inhaled insulin for the treatment of diabetes: projects and devices. *Expert Opin Pharmacother* 2005;4:1373–7.
 - [27] Oleck J, et al. Commentary: Why was inhaled insulin a failure in the market? *Diabetes Spectr* 2016;29:180–4.
 - [28] Son YJ, Mcconville JT. Advancements in dry powder delivery to the lung. *Drug Dev Ind Pharm* 2008;34:948–59.
 - [29] Nazar H, Tsibouklis J. Towards the nasal delivery of insulin. *Ther Deliv* 2012;3:1241–3.
 - [30] Djupesland PG. Nasal drug delivery devices: characteristics and performance in a clinical perspective-a review. *Drug Delivery Transl Res* 2013;3:42–62.
 - [31] Banerjee A, et al. Ionic Liquids for oral Insulin delivery. *Proc Natl Acad Sci USA* 2018;115:7296–301.
 - [32] Abramson A, et al. An ingestible self-orienting system for oral delivery of macromolecules. *Science* 2019;363:611–5.
 - [33] Lee H, et al. Device-assisted transdermal drug delivery. *Adv Drug Delivery Rev* 2018;127:35–45.
 - [34] Xuan B, et al. Alternative delivery of insulin via eye drops. *Diabetes Technol Ther* 2005;7:695–8.
 - [35] Wang AL, Weinlander E, Metcalf BM, Barney NP, Gamm DM, Nehls SM, Struck MC. The use of topical insulin to treat refractory neurotrophic corneal ulcers. *Cornea* 2017;36:1426–8.
 - [36] Fai S, Ahmen A, Mustapha M, Mohd Noh UK, Bastion MC. Randomized controlled trial of topical insulin for healing corneal epithelial defects induced during vitreoretinal surgery in diabetics. *Asia Pac J Ophthalmol (Phila)* 2017;6:418–24.
 - [37] Bastion ML, Ling KP. Topical insulin for healing of diabetic epithelial defects? A retrospective review of corneal debridement during vitreoretinal surgery in Malaysian patients. *Med J Malaysia* 2013;68:208–16.
 - [38] Bartlett JD, Turner Henson A, Atchison JA, Wooley TW, Pillion DJ. Insulin administration to the eyes of normoglycemic human volunteers. *J Ocul Pharmacol* 1994;10:683–90.
 - [39] Woods RJ, Alarcon J, McVey E, Pettis RJ. Intrinsic Fibrillation of fast acting insulin analogs. *J Diabet Sci Technol* 2012;6:265–76.
 - [40] Choudhuri AKR. Using instruments to quantify colour: in principles of colour and appearance measurement. Cham Switzerland: Elsevier; 2014. p. 270–317.
 - [41] Hubert P, Boulanger B, Nguyen-Huu JJ, Chapuzet E. Validation des procédures analytiques quantitatives. Harmonisation des démarches. *STP Pharma Pratiques* 2003;13:101–38.
 - [42] European Pharmacopeia. Monograph 2.6.1 Sterility 10. 2 2020. Strasbourg, France: European Directorate for the Quality of Medicines and Healthcare; 2020.
 - [43] International Conference of Harmonization (ICH) Quality Guidelines: Guidelines for stability Q1A to Q1F ((Accessed on 18 june 2020)). Available on line: <http://www.ich.org/products/guidelines/%20quality/article/quality-guidelines.html>.
 - [44] French Society of Clinical Pharmacy (SFPC), Evaluation and Research Group on Protection in Controlled Atmosphere (GER-PAC) Methodological Guidelines for stability studies of Hospital Pharmaceutical Preparations. Société Française de Pharmacie Clinique, Groupe d'Evaluation et de Recherche sur la Protection en Atmosphère Contrôlée. Paris, France: ICH Official web site; 2013.
 - [45] Held D, Gores F. Tips and tricks GPC/SEC: System peaks or ghost peaks in GPC/SEC. LC/GC the column. *GPC_column_test.pdf* (pss-polymer.com) 2019;15:17–21.
 - [46] Teska BM, Kumar A, Carpenter JF, Wempe MF. Analyzing insulin samples by size exclusion chromatography: a column degradation study. *J Pharm Sci* 2015;104:1555–60.
 - [47] Coles WH, Jaros PA. Dynamics of ocular surface pH. *Br J Ophthalmol* 1984;68:549–52.
 - [48] Itendra J, Sharma PK, Banik A, Dixit S. A new trend ocular drug delivery system. *Int J Pharm Sci* 2011;2:720–4.

- [49] Gilbard JP, Farris RL. Tear osmolarity and ocular surface disease in keratoconjunctivitis sicca. *Arch Ophthalmol* 1979;97:1642–6.
- [50] Tomlinson A, Khanal S, Ramaesh K, Diaper C, McFadyen A. Tear film osmolarity: determination of a referent for dry eye diagnosis. *Invest Ophthalmol Vis Sci* 2006;47:4309–15.
- [51] Liu H, Begley C, Chen M, Bradley A, Bonanno J, McNamara NA, et al. A link between tear instability and hyperosmolarity in dry eye. *Invest Ophthalmol Vis Sci* 2009;50:3671–9.
- [52] Brange J, Langkjoer L. Insulin structure and stability. *Pharm Biotechnol* 1993;5:315–50.
- [53] Whittingham JL, Edwards DJ, Antson AA, Clarkson JM, Dodson GG. Interaction of phenol and m-cresol in the insulin hexamer and their effect on the association properties of B28Pro fleche Asp insulin analogues. *Biochemistry* 1998;37:11516–23.
- [54] Teska BM, Alarcon J, Pettis RJ, Randolph TW, Carpenter JF. Effects of phenol and meta cresol depletion on insulin analog stability at physiological temperature. *J Pharm Sci* 2014;103:2055–267.