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Emulsion of Chloramphenicol: an Overwhelming Approach for Ocular Delivery

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Background: Ophthalmic formulations of chloramphenicol have poor bioavailability of chloramphenicol in the ocular cavity.

Aim: The present study aimed at exploring the impact of different oil mixtures in the form of emulsion on the permeability of chloramphenicol after ocular application.

Materials and methods: Selection of oil mixture and ratio of the components was made by an equilibrium solubility method. An emulsifier was chosen according to its emulsification properties. A constrained simplex centroid design was used for the assessment of the emulsion development. Emulsions were evaluated for physicochemical properties; zone of inhibition, *in-vitro* diffusion and *ex-vivo* local accumulation of chloramphenicol. Validation of the design using check-point batch and reduced polynomial equations were also developed. Optimization of the emulsion was developed by software Design® expert 6.0.8. Assessment of the osmolarity, ocular irritation, sterility testing and isotonicity of optimized batch were also made.

Results: Parker Neem®, olive and peppermint oils were selected as an oil phase in the ratio 63.64:20.2:16.16. PEG-400 was selected as an emulsifier according to a pseudo-ternary phase diagram. Constrained simplex-centroid design was applied in the range of 25-39% water, 55-69% PEG-400, 5-19% optimized oil mixture, and 1% chloramphenicol. Unpaired Student's t-test showed for *in-vitro* and *ex-vivo* studies that there was a significant difference between the optimized batch of emulsion and Chloramphenicol eye caps (a commercial product) according to both were equally safe.

Conclusion: The optimized batch of an emulsion of chloramphenicol was found to be as safe as and more effective than Chloramphenicol eye caps.

BACKGROUND

Conjunctivitis as a polymicrobial infection is susceptible to chloramphenicol, ampicillin and streptomycin.[1] *Staphylococcus epidermidis* is a common pathogen of conjunctiva and it could cause endophthalmitis, however its presence in cul-de-sac region. Healthy conjunctiva isolates are more resistant to antibiotics than those from ocular infection. It has been found that ocular infection isolates are not resistant to chloramphenicol so it has been recommended in treatment of ocular infections.[2] Chloramphenicol is a broad spectrum antibiotic effective against gram-positive, gram-negative, anaerobic bacteria, extracted from *Streptomyces venezuelae*. The mechanism of its action is based on inhibition of protein synthesis at prokaryotic ribosomal level by binding with 50S part of ribosomal subunit and thus preventing association of aminoacyl-tRNA to ribosome. Chloramphenicol can provide bacteriostatic effect when used in high concentration and/or against highly sensitive organisms.[3] Well known ophthalmic formulations OTC-brands of market like Chloramphenicol eye caps and ointments (these are products registered in India) have many drawbacks, which result in poor bioavailability of chloramphenicol in the ocular cavity. This may be overcome by the use of emulsion of chloramphenicol.[4] The emulsions are mixtures of two or more immiscible liquids. Generally, pharmaceutical emulsions are divided into macro-emulsions (O/W, W/O), multiple emulsions (O/W/O, W/O/W) and micro-emulsions. The use of an emulsifier is of

utmost importance to ensure the physical stability of the emulsion. Various factors could affect the process of emulsification, such as the nature of oil, emulsifier, the emulsifier concentration used, as well as the temperature, rpm etc.[5] Neem oil have a strong antibacterial effect. MIC value of Neem oil for *Staphylococcus epidermis* is also remarkable. Olive oil is penetration enhancer and peppermint oil is good solubilizer, penetration enhancer and flavouring agent. All three are non-toxic and non-irritant in ophthalmic preparation.[6] Chloramphenicol is characterized by molecular weight 323.13 (□500 Dal), BCS Class III drug (high solubility and low permeability), lipophilicity 1.14[7] and MIC value for *Staphylococcus epidermis* 8 μg/ml.[8]

AIM

The aim of this study was to explore the impact of different oil mixtures in the form of emulsion on the permeability of chloramphenicol after ocular application.

MATERIALS AND METHODS

Parker Neem® Oil was purchased from Parker Biotech Pvt. Ltd. Chennai, olive and Peppermint oil were purchased from Astron Chemicals Ltd, PEG-400 was purchased from Seva Fine Chemicals Ahmadabad, Chloramphenicol eye caps were purchased from Jyoti capsules, Kanpur, sodium chloride was purchased from Oxford laboratory, Mumbai, fluid thioglycolate medium and soya bean-casein digest medium were purchased from Hi-media, Mumbai.

Preliminary studies

A standard stock solution of chloramphenicol was prepared with water and methanolate phosphate buffer pH 7.4, respectively. UV-scan was taken between wavelength ranges of 200-400 nm by Double Beam UV-visible Spectrophotometer (LT-2900, Labtronics (I) Pvt. Ltd, Ambala, India). A wavelength at which it showed maximum absorbance was selected as λ_{max} for further analytical work. From the stock solution, appropriate aliquots were taken into 10 ml different volumetric flasks and made up to 10 ml with respective solvent. The absorbance of these solutions was measured at selected λ_{max}.[9] Among available oils, those oils that were found to have no absorbance in between 200-400 nm were shortlisted for screening purpose.[10] Screening of oils was made by equilibrium solubility study by orbital shaking incubator (1HB-164, Remi Equipment Ltd. Vasai, India),[11,12] ratio of oils were optimized by applying constrained simplex-centroid design and design® expert 6.0.8 with maximum desirability.[12,13]

Pre-formulation and formulation

Selection of emulsifier was done by pre-formulation study on the basis of emulsification properties. Preparation of pseudo-ternary phase diagram was done using software state ease Statistica® 13.0.159.7 (State soft India).[14] There was application of constrained simplex-centroid design in the development of emulsion. One check-point batch was also made for validation. Chloramphenicol was added to water and shaken vigorously until it solubilized in it. PEG-400 was added, and finely the optimized oil mixture with constant stirring at 300 rpm on the magnetic plate (2MLH, Remi Equipment Ltd. Mumbai, India). These were subjected to evaluation and preparation of polynomial equation in Excel (version Professional Plus 2010, Microsoft Corporation, Redmond, USA).

Evaluation

Organoleptic tests

Emulsions were inspected for odour, visual inspection for colour, homogeneity, optical clarity by clarity chamber and fluidity.[15]

Physicochemical parameters

pH was measured by the digital pH meter of Shimadzu, Japan. Oswald's viscometer of Borosil® was used to measure viscosity at room temperature.[12] The following equation was used for the determination of the emulsion viscosity:

$$\acute{\eta}_1 = \frac{d_1 t_1}{d_2 t_2} \acute{\eta}_2 \qquad (1)$$

Where η_1=viscosity of emulsion; η_2=viscosity of water=0.845 cp; d_1=density of emulsion; d_2=density of water=1.004 gm/ml; t_1=time required reaching from A to B in Viscometer for emulsion; t_2= time required reaching from A to B in Viscometer for water.[16]

Density was determined, at ambient conditions, using a 10 ml capacity specific gravity bottle of Borosil®. Emulsion was subjected to extract chloramphenicol from it by methanol. Suitable dilution was made with methanol and concentration was measured by the double beam UV-visible spectroscopic method at 274 nm by keeping methanol as the reagent blank. Centrifugation parameter was measured to evaluate physical stability.[17] The emulsion was centrifuged at ambient temperature and

4000 rpm by clinical centrifuge (Remi Equipment Ltd. Mumbai, India) for 15 minutes to evaluate the system for creaming or phase separation. The system was observed visually for appearance.[5] Dye test was performed using Sudan III (oil soluble) dye, it was added to the emulsion and small drop was taken onto the glass slide and observed under the microscope (2165, Olamus Ltd, India). The globule size for emulsion was determined by using the microscopic method with the help of stage micrometer.[18] Emulsions were subjected to examination under the light microscope (L-3276, Olamus Ltd, India).

Diffusion studies

In-vitro and *ex-vivo* chloramphenicol diffusion studies were performed by Franz diffusion cell (Durasil® (I) Pvt. Ltd.) with an effective diffusional area of 3.14 cm^2 and 20 ml of receiver chamber capacity using Cellophane® membrane (Merck (I) Pvt. Ltd) and ocular goat membrane. Freshly excised goat ocular membrane was procured from local goat slaughterhouse for human feeding to laboratory in cold (2-4°C) 0.9% w/v saline within 3 h of slaughtering. No goat was separately killed for the study, however approval prior to conducting the ocular study from the Institutional Animal Ethics Committee (IAEC) New Delhi, India was obtained, under the reference number of RKCP/COL/RP/16/74. Cellophane® membrane was heated in 0.1 N NaON for half an hour to make it semipermeable having the pore size of 80 µm. The membrane was mounted between the donor and receiver compartments of the Franz diffusion cell.[19] Initially, the donor compartment was empty and the receiver chamber was filled with methanolic phosphate buffer pH 7.4[20] (30:70% V/V) and covered with aluminium foil to prevent drying out. The receiver fluid was stirred with a magnetic stirrer with a hot plate at a speed of 100 rpm where the temperature was maintained at 37 ± 1°C.[21] Samples were withdrawn at regular intervals and analysed for chloramphenicol content by UV at 274 nm,[12] then the time required to release 50% of chloramphenicol release, more than 90% of chloramphenicol release and to achieve MIC value were noted, % chloramphenicol unabsorbed, % chloramphenicol retained within ocular membrane and % chloramphenicol penetrated across ocular membrane after 6 h[4] were also found. The local accumulation efficacy (LAC) values were derived by the ratio of chloramphenicol retained into the ocular membrane to that delivered across ocular membrane after 6 h. Normalized local accumulation efficacy (NLAC) i.e. LAC of emulsion to LAC of chloramphenicol eye caps were also derived.[22] The cumulative amount of chloramphenicol permeated through the membrane (mg/cm^2) was plotted as a function of time for each formulation. Chloramphenicol flux (permeation rate) at steady state (J_{ss}) was calculated by dividing the slope of the graph linear portion with the diffusion cell area (mg/cm^2h) Permeability coefficient (K_p) was calculated by dividing J_{ss} by the initial concentration of the chloramphenicol in the donor cell. Enhancement ratio (E_r) was calculated by dividing J_{ss} of the emulsion by J_{ss} of the Chloramphenicol eye caps.[12] The lag time (T_{lag}) was determined by extrapolating the linear portion of the cumulative amount permeated versus time curve to the abscissa[23] Since there was a possibility of unpredictable alteration in diffusion studies due to penetration property of oils, therefore, in order for better approximation of diffusivity of chloramphenicol diffusion parameter (D/h^2) was also derived by D/h^2 equation[22]:

$$D/h^2 = \frac{1}{6 \times T_{leg}}$$

Partition coefficient (K) values were calculated by the following equation:

$$K = \frac{\text{concentration of Chloramphenicol in receiver chamber}}{\text{concentration of Chloramphenicol remained in the emulsion at the end of partitioning study}} \quad (2)$$

Partition ratio (P_R) i.e. partition coefficient of an emulsion to partition coefficient of Chloramphenicol eye caps, Diffusivity ratio (D_R) i.e. diffusion coefficient of an emulsion to diffusion coefficient of Chloramphenicol eye caps were also derived.

Microbial assay of emulsion

Ditch plate technique was used for evaluation of bacteriostatic activity of chloramphenicol by way of measuring % Zone of inhibition as per equation 3.[24]

$$\%\ \text{inhibition} = \frac{\text{length of inhibition}}{\text{total length of the streaked culture}} \times 100 \quad (3)$$

Calculation of constrained simplex-centroid design equation

Polynomial equations were derived and validated by extra-design checkpoint batch response for each parameter of the emulsion.[13]

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Optimization of emulsion

Optimization was done within an interior of triangle region of constrained simplex centroid design. Now applying Design expert® 6.0.8 Portable version optimized batch of the emulsion was selected with maximum desirability for prospective studies. All above parameters of emulsions were also evaluated for optimized batch of emulsion.

Evaluation of optimized batch of emulsion

Osmolarity of optimized batch was measured by following equation[25]:

$$\text{mOsm/L} = \frac{\text{concentration}(\frac{g}{L})}{\text{molecular weight}} \times 100 \quad (4)$$

A hypotonic solution can be made isotonic by adding an adjusting substance, usually sodium chloride (NaCl); the exact amount of NaCl can be calculated by equation 5[26]:

$$\text{weight of Sodium Chloride required} = \frac{0.52 - 0.08}{\text{freezing point depression of 1\% solution of chloramphenicol}} \quad (5)$$

Optimized batch of emulsion was autoclaved (Vertical autoclave, Remi Equipment Ltd. Mumbai, India) at 15 psi; 120°C for 90 min. Two ml of the optimized batch was removed with a sterile syringe and aseptically transferred to fluid thioglycolate medium (25 ml) and soya bean-casein digest medium (25 ml) separately. The media were incubated for 2 weeks at 30°C to 35°C in the case of fluid thioglycolate medium and 20°C to 25°C in the case of soya bean-casein digest medium.[4] Chloramphenicol eye caps and optimized batch of emulsion were subjected to the test for ocular irritation on goat cul-de-sac region. The test was carried out at local goat slaughterhouse for human feeding. Approval prior to conducting the ocular irritation study from the Institutional Animal Ethics Committee (IAEC), New Delhi, India was obtained, under the reference number RKCP/COL/RP/16/74. The ocular irritation and inflammation were scored as follows: 0 for none, 1 (slight pink) for slight, 2 (dark pink) for well-defined, 3 (light red) for moderate and 4 (dark red) for severe ocular irritation and inflammation.[27] 0.5 ml of optimized batch of emulsion was applied into cul-de-sac region of goat then after 30 min, goat cul-de-sac region was observed for appearance of ocular irritation. Experiment was made in both eyes of two goats.

Statistical analysis

Student's unpaired t-test with equal variance was used to find any statistically significant difference in the *in-vitro* and *ex-vivo* chloramphenicol transport profile between optimized batch of emulsions and Chloramphenicol eye caps through Cellophane® membrane and goat ocular membrane at 5% level of significance.[28] Data were presented as mean ± standard deviation from five independent experiments.

RESULTS

All standard curves showed maximum absorbance at 274 nm in methanol as well as in methanol/phosphate buffer 7.4. 274 nm was selected as λ_{max} for chloramphenicol in methanol and methanol/phosphate buffer 7.4 for further study of analytical work. The calibration curve for chloramphenicol showed the linear relationship in 10-25 µg/mL. The solubility values of chloramphenicol in various oils showed that Parker Neem® Oil (0.5 ± 0.005 mg/g), Olive Oil (0.45 ± 0.004 mg/g) and Peppermint Oil (0.6 ± 0.048 mg/g) should be selected for further study in a ratio of 63.64% : 20.2% : 16.16%, respectively. Constrained simplex-centroid design was applied in the range of 25-39% water, 55-69% PEG-400, 5-19% oil mixtures, and 1% chloramphenicol. Emulsions had the pleasant odour of peppermint and faint yellow colour; they are opaque, optically clear, viscous and able to flow; viscosity around 100 ± 1 cP at room temperature, pH around 7.4 ± 0.01, and specific gravity of about 1.1 ± 0.01 g⁄mL. Uniform dispersion of the red color was observed after the addition of Sudan III dye to the emulsion indicating a W/O type emulsion and globule size in the range of 15±0.1 to 55±1 µM. There was no clear straight line in Er for Cellophane® membrane against log p of Cellophane® membrane/emulsion (R^2=0.048) and there was clear straight line in Er for Cellophane® membrane against D_R (R^2=0.9875; strong positive monotonic co-relation) (**Fig. 1**). The time required to achieve MIC value was between 25±1 and 1300±21 sec; Er was between 2.56±0.25 and 31.82±0.06; P_R values were between 0.82±0.01 and 42±2; D_R values were between 24±1 and 2398±10; LAC values were between 15.5±0.5 and 48.5±1. For *in-vitro* study pooled degree of freedom was found to be 10 (5+7-2=10). Tabulated t value at 5% level of significance was 2.23. Calculated t value was higher than tabulated ($P<0.05$). It can occur less than 5 times in 100 i.e. a very low

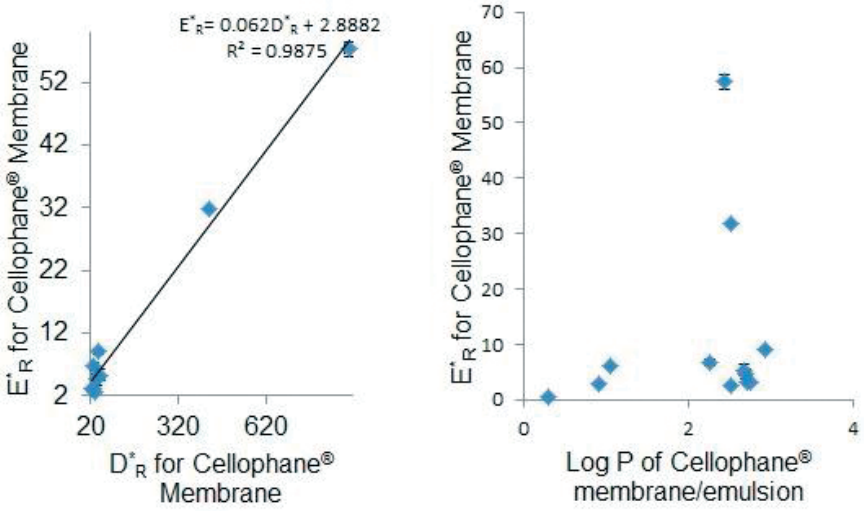

Figure 1. Relationship between E^*_R vs D^*_R (strong positive monotonic co-relation) and E^*_R vs Log p of chloramphenicol for Cellophane® membrane/emulsion (very weak positive monotonic co-relation); n=5; mean±SD; *respect to Chloramphenicol eye caps.

frequency, hence more significant (t=2.55; P<0.05 significant at 5%). For *ex-vivo* studies pooled degree of freedom was found to be 18 (10+10-2=18) and tabulated t value at 5% level of significance was 2.1. Calculated t value was lower than tabulated (P>0.05). It can occur more than 5 times in 100 i.e. very frequent, hence insignificant (t=0.04, P>0.05 not significant at 5%). Percentage zone of inhibition was between 33-50% for different emulsions.

DISCUSSION

Among various emulsifiers used for pseudo ternary phase diagram PEG-20 Sorbitan monooleate, GELUCIRE® 44/14 and PEG-400 were selected for prospective study of chloramphenicol loading. PEG-20 Sorbitan monooleate and GELUCIRE® 44/14 were found to be eye irritant in high concentration; therefore, they were rejected as emulsifiers. PEG-400 was selected for prospective study of chloramphenicol loading in pseudo ternary phase diagram. No creaming or phase separation by centrifugation were observed, which infers that these emulsions were kinetically stable.[5] Prediction for penetration due to the effect of prominent improvement in diffusivity and less improvement in partitioning value of chloramphenicol from emulsion to Cellophane® membrane and as chloramphenicol is BCS class III drug with molecular weight of 323.13, emulsion provides both intracellular and intercellular penetration of chloramphenicol, but more prominently

$$\text{Viscosity} = 100.66X1 + 105.66X2 + 89.29X3 + \varepsilon;\ (R^2 = 0.9998) \quad (6)$$

$$\%\ \text{Zone of Inhibition} = 35.30X1 + 36.53X2 + 39.76X3 + \varepsilon;\ (R^2 = 0.995) \quad (7)$$

$$K_{\text{Cellophane}^{®}\text{ membrane}} = 846.49X2 + \varepsilon;\ (R^2 = 0.9447) \quad (8)$$

$$\text{Log p}_{\text{Cellophane}^{®}\text{ membrane}} = 1.21X1 + 2.91X2 + 2.43X3 + \varepsilon;\ (R^2 = 0.9933) \quad (9)$$

$$\%\ \text{Chloramphenicol retained for ocular membrane of goat} = 82X1 + 82X2 + 73X3 + \varepsilon;\ (R^2 = 0.977) \quad (10)$$

where, X1 = water, X2 = PEG - 400, X3 = optimized ratio of oils mixture, ε = practical error.

Software product Design expert 6.0.8 version provided one optimized batch with maximum desirability of 0.446 and ratio of water: PEG-400: optimized oil mixture = 34.02:58.67:7.31. This optimized batch of emulsion was evaluated for all parameters and it was found a lower % of deviation. Its osmolarity was 3.09 mOsm/L and after calculation 0.799 g/100 mL NaCl was added in the water phase to make it isotonic with lachrymal fluid. There was no turbidity and no microbial growth observed in the sterility test. The optimized batch of emulsion showed score 0 for ocular irritation.

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intracellular diffusion. Chloramphenicol presents in solubilized form in the emulsion but it was also found in the water phase more than in the oil phase. That leads to penetration of chloramphenicol mostly by a way of diffusion. *In-vitro* diffusion profile of the optimized batch of emulsion was superior to Chloramphenicol eye caps in all parameters. *Ex-vivo* release studies across goat ocular membrane were performed to check retention of the chloramphenicol from different emulsion within ocular membrane. Goat cornea eye does not exactly mimic human eye model but is so applicable to demonstrate superiority of the developed formulation.[29] NLAC values were more than 1 indicating that more chloramphenicol accumulated in the cul-de-sac region than Chloramphenicol eye caps. K of emulsions were higher and log p-value, more than 4, because a very low percentage of chloramphenicol remained unabsorbed.[22] Here oil and water, both portions of the emulsion are helpful in the penetration of chloramphenicol as the oil portion leads to improvement in lipid-protein partitioning of the drug whereas water leads to improvement in the drug diffusivity. After six hours not much higher concentration of chloramphenicol penetrated in each of the emulsions, which could not produce a systemic effect. The emulsion was safe for ophthalmic application of 2-3 times in a day. Biostatistical study concluded that penetration of chloramphenicol from optimized batch of emulsion and Chloramphenicol eye caps across ocular goat membrane after 6 h was the same or both were equally safe.[30] Unlike Chloramphenicol eye caps examination under microscope showed water globules in oil background indicating no change in isotropic character and no crystals of the chloramphenicol. This indicated that the chloramphenicol was completely dissolved in the emulsion. Microbial assay proved that there was a diffusion of chloramphenicol from the emulsion. pH was 7.4 for all formulations. Globule size and globule size distribution was due to temperature and rpm so except water all factors were insignificant over them. Zone of inhibitions (%) were due to chloramphenicol itself so almost all factors were insignificant. For J_{ss}, K_p, E_r and T_{leg} all parameters were insignificant because permeability is due to the oil phase and chloramphenicol is soluble in the water phase. Predicted value based on an equation of the extra-design checkpoint had a response of all parameters and was much closer to the observed value. This was confirmation of adequacy of an equation as a predictor of all parameters of the emulsion. Like Chloramphenicol eye caps, optimized batch of emulsion recorded a score of 0, confirming the absence of ocular irritation.[31] Optimized batch passed a sterility test.

CONCLUSION

It was concluded that the present investigation of chloramphenicol emulsion has successfully increased the drug permeability across Cellophane® and the local accumulation across goat ocular membrane in respect to Chloramphenicol eye caps. Emulsions were characterized and optimized. They were found to be safe and more effective than currently available well-known OTC-brands of the market, Chloramphenicol eye caps, by biostatistical means. It had the advantages of the Neem oil which is strongly antibacterial, and the additive effect of peppermint oil and olive oil which are penetration enhancers. Here penetration depends upon diffusivity as well as upon partition theory.

ABBREVIATIONS

O/W: oil-in-water; W/O: water-in-oil; rpm: revolution per minute; mg: milligram; g: gram; PEG: polyethylene glycol; log P: log of partition co-efficient; µg: microgram; mL: millilitre; UV: Ultra violet; nm: nanometre; vs: version; °C: degrees Centigrade; cP: centipoise; R^2= co-relation co-efficient; MIC: minimum inhibitory concentration.

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AUTHORS' CONTRIBUTIONS

Mr. Kalpesh C. Ashara carried out the study and analyzed data. Dr. Ketan V. Shah participated in the design of the study and the research guide. All authors read and approved the final manuscript.

AUTHORS' DISCLOSURES

Authors disclosed that there was neither conflict of interest nor ethical issues and no funding source as well. The literature review that leads to this research

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DISCLAIMER

Any opposing opinions, findings, and conclusions or recommendations expressed in this material are those of the corresponding author only.

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Эмульсия хлорамфеникола: изумительный подход применения в офтальмологии

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Введение: Офтальмологические лекарственные препараты обладают низкой биодоступностью хлорамфеникола в глазном яблоке.

Цель: Целью настоящей работы является исследование степени влияния различных масляных смесей в виде эмульсий на проницаемость хлорамфеникола в ходе офтальмологического применения.

Материал и методы: Селекция масляных смесей и соотношение компонентов была осуществлена методом равновесия растворимости. Был выбран эмульгатор в соответствии с эмульгирующими свойствами. Ограниченный симплекс-центроидный план (constrained simplex centroid design) был применен для мониторинга разработки эмульсии. Эмульсии прошли мониторинг физико-химических свойств, зоны ингибирования, in-vitro диффузии и ex-vivo локального накопления хлорамфеникола. Валидация модели была осуществлена в контрольной партии (check-point batch) и были разработаны редуцированные полиномиальные уравнения. Оптимизация эмульсии была доработана с использованием программного обеспечения Design® expert 6.0.8. Были осуществлены мониторинг осмолярности, раздражения глаз, испытание на стерильность и изотоничность оптимизированной партии.

Результаты: Оливковое и мятное масла Parker Neem®, были селектированы для масляной фазы в соотношении 63.64:20.2:16.16. PEG-400 был выбран в качестве эмульгатора в соответствии с псевдо-трехкомпонентной фазовой диаграммой. Ограниченный симплекс-центроидный план был применен в диапазоне 25-39% воды, 55-69% PEG-400, 5-19% оптимизированной масляной смеси, и 1% хлорамфеникола. Непарный t-тест Стьюдента показал, что при in-vitro и ex-vivo исследованиях устанавливается значительная разница между оптимизированной партией эмульсии и глазными каплями Хлорамфеникол (коммерческий продукт), по результатам данного теста обе пробы являются одинаково безопасными.

Заключение: Установлено, что оптимизированная партия эмульсии хлорамфеникола является столь же безопасной и даже более эффективной по сравнению с глазными каплями Хлорамфеникол.