Title: PREPARATION FOR STABILIZING THE PROTECTIVE LAYER ON THE EPITHELIUM OF THE CORNEA OR THE ALVEOLI

Abstract: The invention relates to a preparation for stabilizing the protective layer on the epithelium of the cornea or the alveoli, comprising an aqueous solution of at least one divalent or polyvalent cation and at least one divalent or polyvalent anion, wherein the preparation is preferably an aqueous solution of Ca⁺⁺ ions and borogluconate⁻⁻ ions, to be obtained by bringing together calcium D-gluconate monohydrate and boric acid in an aqueous solvent, such as water.
PREPARATION FOR STABILIZING THE PROTECTIVE LAYER ON THE EPITHELIUM OF THE CORNEA OR THE ALVEOLI

The invention relates to a preparation for stabilizing the protective layer on the epithelium of the cornea or the alveoli which is intended for treatment and prophylaxis of dry eye and “Respiratory Distress Syndrome” (RDS).

“Dry eye” is a non-specific term for a complex of symptoms, such as redness, a burning sensation, itching, light-sensitivity, tearing. The cause of these symptoms is drying out of the cornea due to an unstable tear film. The dry eye syndrome, also known as “ocular surface disease” is a disorder which occurs in approximately one in five people.

The tear film is a protective layer which lies over the epithelium of the cornea and the conjunctiva and which can be up to 40 μm thick and consists of 98% water. The outer layer of the tear film, roughly 200 nm thick, contains lipids and the rest of the aqueous layer contains a quantity of glycoproteins increasing toward the epithelium.

The layer of lipids behaves independently of the aqueous layer underneath and is, as it were, anchored to the openings of the meibomian glands. The lipids are released by means of cytolysis of the gland cells. Their molecular composition comprises virtually the whole range of fatty substances known in cell biology.

The thin layer forming the boundary between the epithelium of the alveoli and the outside world is referred to as “lung surfactant”. This layer comprises phospholipids with saturated fatty acids, such as dipalmitoylphosphatidylcholine. The non-functioning or poor functioning of the layer sometimes occurs in premature infants, whereby the expansion of the lungs as well as the gas exchange proceed with difficulty. This
syndrome is designated Respiratory Distress Syndrome (RDS) in prematures.

The invention is based on the fact that on the air-water boundary surface of the tear film there forms certain a monolayer, and possibly also a multilayer, of phospholipids, which are probably the most important barrier against evaporation of water from the tear film.

The most common lipids and the most significant for the "dry eye" problem are the phosphatidylcholines (PC) and the phosphatidylethanolamines (PE) with saturated fatty acids. These are molecules which consist of an apolar part with 2 fatty acid tails and a polar part, the phosphate group of which carries a negative charge and the choline respectively ethanolamine group carries a positive electrical charge. Such molecules order themselves in water as bimolecular layers with the polar groups directed toward the water and the apolar groups directed toward each other. The distances between the fat molecules, and therewith the stability of the layers, are determined by the molecular heat movement which drives them apart, and on the other hand by London-Van der Waals attraction between the hydrocarbon chains of the fatty acids and the electrostatic attraction between the polar atomic groups of the molecules.

The invention is now based on the insight that the integrity of the lipid layer must be ensured so that no or less evaporation of tear-water can occur. The phenomenon of 'dry eye' can hereby be reduced or prevented.

It is known that maximal stacking of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) occurs in the presence of straight (saturated) hydrocarbon chains and charge compensation of the negative phosphate groups by divalent cations and of the positive choline or ethanolamine groups by divalent
anions, also including the negative charge carriers of proteins.

The invention now provides a preparation, comprising an aqueous solution of at least one divalent or polyvalent cation and at least one divalent or polyvalent anion. The preparation can not only be used to stabilize the protective layer on the epithelium of the cornea, but also to stabilize the protective layer on the epithelium of the alveoli, and thus improve the function of the so-called "lung surfactant" by compensating the deficiency of lung surfactant. The compensation takes place by giving the quantity of surfactant which is present a better cohesion.

It is recommended that both positive charges on the divalent cations are spatially concentrated. This means that, seen spatially, they lie so closely together that they can hold together the negative phosphate groups of the lipids.

The divalent cations are preferably chosen from the group consisting of Ca\(^{++}\), Ba\(^{++}\) and Mg\(^{++}\).

The divalent anions are inorganic or organic anions, wherein the organic anions are advantageously the coupling product of boric acid and a water-soluble organic acid with at least two OH-groups, which are located substantially adjacent to each other and in substantially the same direction so as to enter into the coupling with boric acid.

It is recommended that the organic acid is an acid derived from a hexose, for instance gluconic acid, since such acids occur in the body normally. The monovalent, rather weak gluconic acid is converted by the addition of boric acid into the divalent, quite strong boro-gluconic acid. The dissociated boro-gluconic acid provides the required divalent anions in the correct ratio relative to the divalent cations.

Different alternatives to boro-gluconic acid can be envisaged. Which other anions are suitable can be
determined by the skilled person by means of a simple test, known as tricomplex flocculation test, which consists of 1 ml of a slightly milky suspension of egg-lecithin in water being added to 10 ml of the solution of cations and anions for testing. The intensity of the flocculation is determined relative to flocculation of 1 ml of egg-lecithin in 10 ml of 5 mM calcium boro-glucunate. This egg-lecithin model is a rough model, but very well usable for a first selection. More refined methods comprise of determining the compression behaviour and/or contraction behaviour in a Langmuir trough of a monolayer of egg-lecithin or, better still, of lipids from the meibomian glands of test animals, for instance rabbits (see Greiner et al., Current Eye Research 15(4), 371-375, 1996).

By performing the above stated test it was found that as alternatives to boro-glucunate acid for instance micro-anions of divalent or polyvalent acids can be used, and particularly those of sulphuric acid, citric acid and maleic acid.

Another alternative to boro-glucunate is formed by the micro-anions of monovalent polyhydroxy acids which have been converted by boric acid into trivalent acids. Suitable polyhydroxy acids which can be converted by reaction with boric acid into a suitable anion are mucinic acid and galacturonic acid.

A final group of anions are the colloidal anions. It was found that a solution of the sodium salt of chondroitin-6-sulphate in boric acid with addition of calcium acetate gives a rapid and strong tricomplex flocculation with egg-lecithin. The same result was obtained with carrageenan. With glycogen there still occurs a light flocculation.

The intensity of tricomplex flocculation increases with decreasing polarizability of the anion in the series phosphate-carboxyl-water-sulphate.
It can be generally stated that a preparation according to the invention can be prepared using all divalent or polyvalent carboxyl acids, all polyhydroxy acids and carbohydrates reacting with boric acid, sulphuric acid and all water-soluble compounds with two or more sulphate groups. All substances mentioned here in both monomer and polymer form. For all substances it is the case that the acid groups must be in a position such that they can enter into electrostatic interaction with two or more tertiary-amino groups of phospholipids in aqueous dispersion.

For the use of these substances in eye drops it is recommended that the acid groups are dissociated close to a neutral pH, that the solubility product of their calcium salt allows a concentration in aqueous solution of at least 5 meq/L and that they are permissible from a pharmaceutical viewpoint.

In a preferred embodiment the preparation according to the invention consists of 0.45 to 4.5 g/L, preferably about 2.25 g/L calcium D-gluconate monohydrate and 2.5 to 25 g/L, preferably about 12.5 g/L boric acid, in an aqueous solvent, in particular water. Eye drops with this preparation give a great and clinically relevant extension of the "breaking-up time" (BUT) of the tear film.

The preparation according to the invention can occur in different forms of administration. It can thus be used as eye drop and as a formulation which can be inhaled. In the latter case the formulation is advantageously an aerosol.

The invention further relates to the use of a preparation as described above for manufacturing a therapeutic mixture for the treatment and/or prophylaxis of dry eye or for the treatment and/or prophylaxis of disorders wherein the "lung surfactant" does not function optimally, such as RDS.
The invention further relates to a method for manufacturing a preparation for stabilizing the tear film or the lung surfactant, comprising of combining boric acid and an aqueous solution of calcium gluconate. It has been found that in this way calcium-borogluconate is formed which exists in water in a balance with the dissociation products thereof, Ca\(^{++}\) and borogluconate\(^{-}\) ions.

The present invention will be further illustrated with reference to the examples following hereinbelow.

**EXAMPLES**

**EXAMPLE 1**

*Manufacture of a preparation according to the invention*

In 1 litre of demineralized water 2.25 g calcium gluconate is dissolved under light heating. 12.5 g boric acid is added to this solution. The thus obtained preparation is ready for use and can furthermore withstand sterilization by means of for instance boiling.

Treatment and/or prophylaxis of dry eye takes place by instillation of the eye surface with 1 drop in the manner usual for eye drops using an eye dropper.

**EXAMPLE 2**

*Testing the activity of the preparation according to the invention*

**Test 1**

The inventor has applied the preparation to himself for more than a year. The beneficial effect was achieved by instillation of 1 drop in the morning. Occasionally 1 drop was again used at night.

The positive effect can be measured subjectively through the observation of the inventor himself. The activity was further also determined objectively by means of usual tests, which were carried out by an ophthalmologist once a week for approximately 6 months.
The preparation remained free of opalescence for the whole test-year, which indicates that a negligible development of micro-organisms has taken place.

Test 2

After test 1 the preparation was applied by the ophthalmologist to about fifty patients with dry-eye symptoms such as tearing, a burning sensation etc., by instilling 1 drop at a time during a normal eye examination. All patients except one showed signs of perceived improvement.

In 9 of these patients the subjective observation was further supported by determining the "Breaking-up time" (BUT) in per se known manner before and after administration. The results hereof are shown in the table below.

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<th>Gender</th>
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<th>BUT in seconds after treatment</th>
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<td>F</td>
<td>4-5</td>
<td>10</td>
</tr>
</tbody>
</table>

EXAMPLE 3

Testing alternative anions

1. Methods

1a. Titration

pH-determinations and potentiometric titrations of the anions were performed using the Handylab mV-meter and the Blueline combination vitreous electrode (Schott). A self-made micro-burette was used in the
tiritations. The droplet volume of the 0.5 M NaOH solution is determined by weighing 20 droplets while taking into account the density of NaOH.

The average volume of 1 droplet was 0.016 ml. The quality of the titration method is determined by titrating 25 ml of 0.1 M HCl with 0.5 M NaOH.

1b. Tricomplex flocculation test

In a so-called tricomplex flocculation test a number of anions were tested for suitability for use in the preparation according to the invention. In this manner it is also possible to determine for other anions whether they can be applied in the preparation.

A slightly milky suspension of egg-lecithin in water was prepared by adding 2 g to 100 ml water.

Added to 1 ml of this suspension was 10 ml of the solution of cations and anions for testing. The intensity of the flocculation is determined relative to flocculation of 1 ml of egg-lecithin in 10 ml of 5mM calcium boro-gluconate.

2a. Results with micro-anions of divalent or polyvalent acids

Sulphuric acid

The sulphate ion can be used because the solubility product of CaSO₄ (6x10⁻³) corresponds to a salt concentration of 8 mM. In this concentration CaSO₄ with egg-lecithin produces after a long time a very light flocculation which can be easily disturbed by stirring.

Citric acid

This is a trivalent acid, wherein the pK values of the carboxyl groups do not vary much and lie well below 7. Ca-citrate very slowly produces a light flocculation in the concentration of 10 mM.
Maleic acid

This is a divalent acid wherein the pK values of the carboxyl group vary considerably, the highest being just below 7. Ca-maleate very slowly produces a finer flocculation than Ca-citrate in the concentration of 10 mM with the egg-lecithin.

2b. Results with micro-anions of monovalent polyhydroxy acids which have been converted into trivalent acids by boric acid

Gluconic acid

Gluconic acid was already used in example 1. Gluconic acid is further examined here. An equimolar quantity of gluconic acid added to 0.2 M boric acid causes the pH of this solution to fall from 4.7 to 1.7. The gluconic acid, which is supplied in the lactone form, converts only very slowly into the free dissociated acid toward the end of the titration with lye. The acid formation was checked by adding 0.5x, 1x and 2x the equimolar quantity of Ca-gluconate to 0.06 M boric acid. These solutions are titrated with NaOH just as those with boric acid only and Ca-gluconate only. Ca-gluconate alone produces the titration curve of the salt of a strong acid and a strong base. Boric acid alone produces the curve of a weak acid with pK-value of ± 9. The two gluconic acid molecules of Ca-gluconate are converted into a trivalent gluconate-borate complex, 2 acid groups of which are already compensated by Ca-ions. The third group is titrated as an acid with pK of ± 5.5.

With 1x the equimolar quantity of Ca-gluconate the conversion to boro-gluconate is practically complete. With 2x the equimolar quantity the acid formation increases by only a few percent. With 0.5x the equimolar quantity the titration curve of the boric acid is too predominant to enable proper determination of the end point.
Mucinic acid
This acid is derived from galactose and comprise 2 terminal carboxyl groups. The saturated solution is 0.016 M and is titrated as 0.014 M monovalent acid.

After addition of boric acid to a concentration of 0.16 M the pH decreases, from the final value 10.9 during the titration, to 6.6. A saturated solution of mucinic acid neutralized with Ca(OH)$_2$ and supplemented with boric acid to 0.2 M, produces with egg-lecithin a tricomplex flocculation about as strong and rapid as the eye drops from Example 1. No flocculation occurs without addition of boric acid.

Galacturonic acid
This aldehyde carboxylic acid is about as strong as gluconic acid (pK: ± 3.5). Due to OH groups in the cis-position it could also react with boric acid. A solution of 0.03 M galacturonic acid, 0.2 M boric acid and 0.05 M Ca-acetate, with NaOH brought to pH 7, produces after hours a very light flocculation in the egg-lecithin sol.

glycerophosphoric acid
The two remaining OH-groups of this glycerol ester could still react with boric acid to form an anionic group in addition to the already present phosphate group. A 0.03 M solution of glycerophosphate disodium has a pH of 9.26. Addition of an equivalent quantity of boric acid reduces the pH to about 7.6. However, this solution, supplemented with the equivalent quantity of Ca-acetate, produces no flocculation whatsoever with egg-lecithin.

2c. Results with colloid anions
dextran
This is a readily soluble polyglucose with straight chains. A solution of 0.36 g dextran in 25 ml of 0.2 M boric acid having added thereto 2.5 ml of 0.5 M Ca-
acetate, final pH 6.3, produces no tricomplex flocculation with egg-lecithin.

Glycogen

This is a readily soluble polyglucose with shorter and more branched chains than in the case of starch. A solution of 0.32 g of glycogen in 25 ml of 0.2 M boric acid having added thereto 2.5 ml of 0.5 M Ca-acetate, final pH 6.3, slowly produces a light, easy to disturb flocculation with egg-lecithin.

Chondroitin-6 sulphate (sodium salt)

This is a polymer of 2-aminogalactose acetate and glucuronic acid, esterified with sulphuric acid. A solution of 0.25 g chondroitin sulphate in 25 ml of 0.2 M boric acid having added thereto 6 ml of 0.1 M Ca-acetate produces a rapid and strong tricomplex flocculation with egg-lecithin.

Carrageenan

Iota-carrageenan is a polygalactose sulphate with 1 sulphate group per galactose monomer. A solution of 0.34% carrageenan contains about 13 mgeq/L galactose sulphate. This solution supplemented with the equimolar quantity of Ca-acetate and 0.2 M boric acid has a pH of 5.7 and causes a rapid, coarse and complete flocculation of the egg-lecithin sol.

Pectic acid

This polymer of galactonic acid is poorly soluble in water. A saturated solution contains about 9 mgeq/L of galacturonic acid and has a pH of 3. This solution supplemented with 0.2 M boric acid and brought to pH 7 with Ca(OH)₂ produces practically no flocculation with the egg-lecithin sol.
3. **Conclusion**

The above tests show that the egg-lecithin model is particularly suitable for determining the possible usefulness of determined anions in the preparation according to the invention.
CLAIMS

1. Preparation for stabilizing the protective layer on the epithelium of the cornea or the alveoli, comprising an aqueous solution of at least one divalent or polyvalent cation and at least one divalent or polyvalent anion.

2. Preparation as claimed in claim 1, characterized in that both positive charges on the divalent cations are spatially concentrated.

3. Preparation as claimed in claim 2, comprising divalent cations chosen from the group consisting of Ca++, Ba++ and Mg++.

4. Preparation as claimed in claims 1-3, characterized in that the anions are the coupling product of boric acid and a water-soluble monovalent or polyvalent organic acid with at least two OH-groups, which are located substantially adjacently of each other and in substantially the same direction so as to enter into the coupling with boric acid.

5. Preparation as claimed in claim 4, characterized in that the organic acid is an acid derived from a hexose.

6. Preparation as claimed in claim 5, characterized in that the organic acid is gluconic acid.

7. Preparation as claimed in claims 1-6, characterized in that the preparation is an aqueous solution of Ca+++ ions and borogluconate-- ions.

8. Preparation as claimed in claims 1-7, at least comprising 0.45 to 4.5 g/L, preferably about 2.25 g/L calcium D-gluconate monohydrate and 2.5 to 25 g/L, preferably about 12.5 g/L boric acid, in an aqueous solvent, in particular water.

9. Preparation as claimed in claims 1-3, characterized in that the anions are chosen from the group consisting of divalent or polyvalent carboxyl
acids, polyhydroxy acids and carbohydrates reacting with boric acid, sulphuric acid and water-soluble compounds with two or more sulphate groups.

10. Preparation as claimed in claim 9,

5 characterized in that the anions are micro-anions of divalent or polyvalent acids.

11. Preparation as claimed in claim 10,
characterized in that the anions are derived from sulphuric acid, citric acid or maleic acid.

12. Preparation as claimed in claim 9,
characterized in that the anions are micro-anions of monovalent polyhydroxy acids which are converted by boric acid into trivalent acids.

13. Preparation as claimed in claim 12,
characterized in that the anions are derived from mucinic acid or galacturonic acid by reaction with boric acid.

14. Preparation as claimed in claim 9,
characterized in that the anions are colloidal anions.

15. Preparation as claimed in claim 14,
characterized in that the anions are derived from glycogen by reaction with boric acid, or are derived from carrageenan or chondroitin-6-sulphate with or without reaction with boric acid.

16. Preparation as claimed in claims 1-15, for use as eye drop.

17. Preparation as claimed in claims 1-15, for use as preparation which can be inhaled.

18. Preparation as claimed in claim 17,
characterized in that the preparation which can be inhaled is an aerosol.

19. Use of a preparation as claimed in claims 1-15 to manufacture a therapeutic mixture for the treatment of dry eye.

20. Use of a preparation as claimed in claims 1-15 to manufacture a therapeutic mixture for the treatment
of disorders wherein the "lung surfactant" does not function optimally.

21. Method for manufacturing a preparation as claimed in claims 1-8, comprising of combining boric acid and an aqueous solution of calcium gluconate.