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BIOPHARMACEUTICAL ASSESSMENT OF EYE DROPS CONTAINING ALOE (*ALOE ARBORESCENS* MILL.) AND NEOMYCIN SULPHATE

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Abstract: The subject of the studies was eye drops made of aloe, containing the group of aloe chemical substances of anti-inflammatory use and neomycin sulphate. The aim of the studies was to evaluate the permeability of biologically active aloe substances, determined as aloenin, through synthetic lipophilic and hydrophilic membranes in a standard perfussion apparatus and *in vitro* verification of the transport possibilities of these substances through the isolated cornea of pig's eye. The permeability process of biologically active aloe substances determined as aloenin, through synthetic lipophilic and hydrophilic membranes, was analyzed using the first-order kinetics. Estimated quotas of permeability rate constant show that the investigated chemical compounds of aloe, included in the eye drops, diffused through the applied membranes. The studies of permeability through isolated pig's cornea proved that biologically active aloe substances could not overcome this biological barrier. On the basis of biopharmaceutical studies it can be concluded that the eye drops containing aloe and neomycin sulphate, due to the lack of permeating abilities through the eye cornea, should be particularly useful in the treatment of inflammations and infections of external parts of the eye, such as conjuctiva, eyelid edges, lacrimal sac and cornea.

Keywords: *Aloe arborescens*, aloe containing neomycin sulphate, eye drops containing aloe.

Aloe eye drops are the sterile aqueous extract of fresh *Aloe arborescens* Mill., supplemented with neomycin sulphate and necessary additives. The anti-inflammatory activity of eye drops is based on the content of such biologically active substances as glycoproteins (lectins) (1–4), polysaccharides (1–5), aloenin and aloin (6–8), aloesin and its esters: 2''-O-coumaroylaloesin, 2''-O-feruloylaloesin (4, 8) and magnesium lactate (8, 9). The anti-infectious properties of eye drops depend on the content of aminoglycoside antibiotic of anti-bacterial activity.

Neomycin as a polar compound of cationic character is slowly resorbed by the conjunctiva and cornea, which can enable the efficient concentration of the antibiotic in the place of application. It justifies the use of neomycin preparations in the topical treatment of the infections of external parts of the eye e.g. conjunctiva, eyelid edges, lacrimal sac and cornea ulcerations caused by Gram-positive cocci, especially *Staphylococcus sp.* and Gram-negative bacilli, especially *Klebsiella sp.*, *Enterobacter sp.* and indol-positive *Proteus*.

The eye drops containing aloe and neomycin sulphate are the new conception of the ophtalmic form of drug, whose procedure of production was submitted to the Patent Office of Poland in 2001. Considering the studies which were carried out, the eye drops met the requirements of Polish Pharmacopoeia V (PPhV), mentioned in the monograph *Guttae ophthalmicae*; they were sterile, clear, their osmotic pressure was approximate to that of the lacrimal fluid and they had the required pH.

The aim of the studies was the assessment of the permeability of the group of the biologically active aloe substances determined as aloenin, through the synthetic lipophilic and hydrophilic membranes in the standard perfussion apparatus and the verification of transport possibilities of these substances through the isolated cornea of pig's eye *in vitro*.

EXPERIMENTAL

Material under investigation

The eye drops made of aloe and neomycin sulphate containing: aqueous extract of fresh leaves of *Aloe arborescens* Mill., neomycin sulphate, boric acid, sodium pyrosulphite, disodium EDTA, thiomersal, β-phenylethyl alcohol.

The preparation of the drops was performed in the following stages: washing and crushing the leaves, the extraction of the leaves pulp with the distilled water at 95–98°C, straining out of the extract and filtration through the Schott G-1 and G-2 filters. Then, the additives and neomycin sulphate were dissolved in the extract, the drops were filtered through G-4 filters, they were poured

into infusion bottles and sterilised at the temperature of 100°C for 30 min.

Reagents

Glutathione, oxidized form (Roanal Hungary); synthetic hydrophilic dialysis membranes of 2.5 μm declared pores' diameter (VEB Filmfabrik Wolfen, Germany).

Reference substances: aloenin, aloin, aloesin, coumaroylaloesin, feruloylaloesin, aloe–emodine – isolated from the dry extract of fresh leaves of *Aloe arborescens* Mill. (4).
Double distilled water of quartz apparatus.
The other reagents were pure p.a..

Apparatus

pH–meter (Cyber 500, Singapore); osmometer (Trident 800cl); Höppler viscosimeter KF10 (Prüfgeräte–Werk Medingen – Dresden); the apparatus for membrane filtration (Sartorius); Specord UV–VIS M–40 (Carl Zeiss Jena, Germany), UV lamp Emita VP–60 (UV_{365}), densitometer – Mettler Toledo DA–110M; water bath with shaking, type 357 (Elpan, Lubawa); a dialysis perfussion apparatus (constructed in the laboratory).

The qualitative assessment of eye drops

1. The assessment of the appearance – clarity and colour were evaluated.
2. The pH was determined by the pH–meter.
3. Osmotic pressure was determined by the osmometer.
4. TLC analysis.

The drops were condensed at the decreased pressure at the temperature of 70°C. The remains were extracted with ethyl acetate and the filtered extract was applied linearly at the starting point of chromatographic plates Kieselgel 60 Merck (20×5 cm). The chromatograms were developed in the system: ethyl acetate–methanol–water (100:16.5:13.5) (10). Simultaneously, the chromatograms of reference samples were prepared: *viz.* of aloenin, aloin, aloesin, coumaroylaloesin, feruloylaloesin, and aloe–emodine. After drying, both the undeveloped plates and those developed with the fumes of concentrated ammonia were visualised in UV_{356} (Table 1).

5. The determination of the aloenin and aloin content in the drops.

The drops were condensed under the reduced pressure at 70°C and the residue were extracted with ethyl acetate. The separation of aloenin and aloin from the accompanying chemical compounds was performed using TLC. In the UV_{356} light on the chromatograms the studied compounds were localised, the gel layers containing them were transferred to the separate tubes and eluted with ethanol at the concentration of 760 g/l, the gel was separated by centrifugation. The absorbance of the sample solutions was measured with the spectrophotometer at 1 cm cuvette, at wavelength $\lambda = 310$ nm (aloenin), 300 nm (aloin), against distilled water as a blank sample. The concentration of aloenin and aloin in the drops (%) was established on the basis of the extinction coefficients, determined for the chromatographic–spectrophotometric method (11):

$$A^{1\%}_{1cm}\ \text{(aloenin)} = 166\ (100\ cm^2g^{-1}),$$
$$A^{1\%}_{1cm}\ \text{(aloin)} = 155\ (100\ cm^2g^{-1}).$$

6. The determination of the content of biologically active substances determined as aloenin.

1.5 ml of drops was measured exact to a 0.02 ml and then put to a 100 ml volumetric flask. Afterwards, the volume of the flask was supplemented with distilled water. The absorbance of the solution was measured with the spectrophotometer in a 1 cm cuvette at $\lambda = 310$ nm against distilled water as a blank sample. For the determination of the percentage content of the complex of biologically active substances determined as aloenin, the aloenin absorbance coefficient: $A^{1\%}_{1cm} = 221\ (100\ cm^2g^{-1})$, established for the direct spectrophotometric method, was used (12). Results are shown in Table 1.

7. The anti–microbial activity of neomycin in the drops.

The activity of neomycin was determined by cylinder – plate method according to the PPhV with the use of PA4 medium and the test microorganism *Bacillus pumilus* NCTC 8241. Basic agar medium (PA 4) was poured on the Petri dishes 10 cm in diameter. At the same time, the infected medium was prepared by the inoculation of PA4 medium at 50°C with the suspension of the test strain. The equal layer of the inoculated medium was spread on the plates with congealed basic medium (PA 4). On the inoculated PA 4 medium standardised cylinders were put and the standard solution of neomycin and appropriately diluted drops were introduced into them. The plates were left for 2 hours at 20°C to allow the diffusion of the antibiotic into the medium, and then transferred into thermostat at 37°C for 18 hours. Afterwards, the diameter (mm) of the areas of inhibited growth of the test bacterium strain was measured on the plates. The results are presented in Table 1.

8. The density of drops was measured by densitometer and the viscosity was determined by Höppler viscometer (Table 1).

Table 1. The qualitative assessment of the drops assigned to biopharmaceutical studies

No.	The studies of drops	Results
1	The appearance of drops	The drops were clear of light yellow colour
2	pH	4.89 ± 0.01
3	Osmotic pressure (mOsm/l)	299.0 ± 5.0
4	TLC analysis	The presence of spots of main biologically active substances on chromatograms: aloenin (Rf 0.46), aloin (Rf 0.51), aloesin (Rf 0.40), coumaroylaloesin + ferulolylaloesin (Rf 0.58), aloe – emodine (Rf 0.97)
5	The content of aloenin (%) The content of aloin (%)	0.0186 ± 0.0011 0.0168 ± 0.0009
6	The content of aloe substances converted to aloenin (5)	0.0645 ± 0.0010
7	The areas of inhibited growth of test bacterium (mm) The antimicrobial activity in relation to the reference (%)	Reference sample: 17.8 ± 0.1 Studied sample: 18.2 ± 0.1 102.25
8	Density (g/ml) Viscosity (mPas)	1.0107 ± 0.0004 1.5812 ± 0.0011
9	The sterility studies of drops according to PPhV	drops were sterile

9. The sterility of drops

The sterility of drops was determined by PPhV method taking into account the membrane filtration, with the use of liquid thioglycollate medium (PD1) and the medium containing casein hydrolysate and soya (PB2).

The studies of permeability of biologically active aloe substances through synthetic membranes determined as aloenin

The preparation of synthetic lipophilic membranes

The lipophilic membranes were prepared on the basis of dodecanol and koloxylin. The prepared solution of dodecanol in collodion and ether was poured on glass plates. After the evaporation of the solvent, the membranes were soaked in distilled water and then, they were withdrawn and stored in the desiccator. The assessment of the thickness of the membranes was carried out on the basis of the spectrophotometry using the infra – red radiation. The average thickness of the lipophilic membranes was 9.5 μm.

Conditions of biopharmaceutical studies

To determine the permeability rate constant of the biologically active substances through the synthetic membranes, the described dialysis perfussion apparatus, with the area of active exchange between compartments = 1.33 cm^2, was used (15, 16). Before the experiment, the membranes were preserved in the studied drops for 24 hours. After mounting the synthetic lipophilic or hydrophilic membrane in the chamber, a 1 ml of drops was put in the donor compartment, while the receiving compartment was filled with 5 ml portion of Ringer's solution, pH = 7.15 and osmotic pressure 300 mOsm/l. The apparatus prepared in this way was put into a thermostatic water bath at the temperature of 37°C. During the experiment, the solutions were mixed by letting compressed nitrogen through the chambers of both compartments. At given intervals, 0.2 samples were withdrawn from the compartment with the automatic pipette and 3 ml portion of Ringer's solution was added

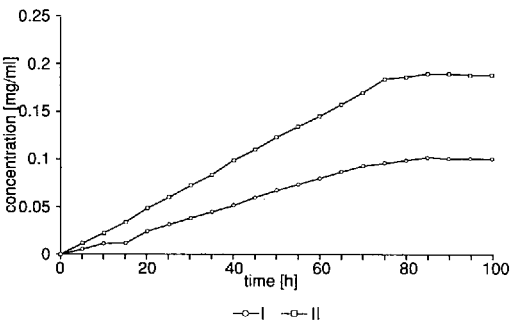

Figure 1. The dependence of concentration changes as a function of time in the studies of the permeability rate of biologically active substances through synthetic lipophilic (I) and hydrophilic (II) membranes at pH = 7.15.

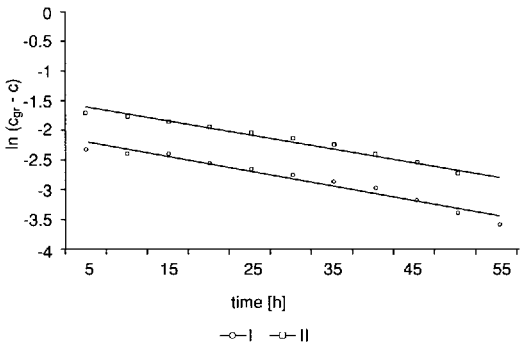

Figure 2. Semi – logarithmic dependence of concentration differences (c_{gr}–c) as a function of time in the studies of the permeability rate of biologically active substances through synthetic lipophilic (I) and hydrophilic (II) membranes.

into them. The withdrawn sample was replaced with the equal volume of Ringer's solution. The absorbance of the samples was measured spectrophotometrically at the wavelength λ = 310 nm against the Ringer's solution serving as a reference. The measurements were carried out until the system reached equilibrium state. The studies which followed the accepted standard, were repeated three times. The changes of the concentration of the biologically active aloe substances determined as aloenin, submitted to the dialysis through the synthetic lipophilic or hydrophilic membranes of pH = 7.15 as the function of time, are presented in the Figure 1.

The obtained results were interpreted on the basis of the first–order equation transport kinetics:

$$\ln(c_{gr}-c)=\ln(c_{gr}-c_o)-kt$$

where:

c_{gr} – concentration of aloenin after time t_{gr}

c – concentration at a given time t

c_o – start concentration at time t_o

The linear semi – logarithmic dependence c_{gr} – c as a function of time (Figure 2) enabled to calculate the first–order rate constants of transport of biologically active aloe substances through synthetic lipophilic and hydrophilic membranes at pH = 7.15. The obtained results with their statistical assessment are presented in Table 2.

The studies of permeability of biologically active substances determined as aloenin through the pig's cornea *in vitro*

The isolated eyeballs, which came from eight – month pigs and did not have any visible epithelial damages, were transported on ice to the laboratory and used for experiments during the first hour after the killing. The eyeball together with the fascia

Table 2. First – order rate constants [k] of transport of biologically active aloe substances converted to aloenin at a temperature of 37°C and pH = 7.15 through synthetic lipophilic (1) and hydrophilic (2) membranes

The type of a synthetic membrane	10^2k[h^{-1}]	[r]	Wz [%]
1	2.4424 ± 0.1695	0.980	1.60
2	2.3104 ±0.1322	0.988	1.41

where: r – correlation coefficient
Wz – coefficient of variation

remains was mounted onto an autopsical table. Afterwards, the cornea with a 1–2 mm ring of sclera were isolated with a scalpel and scissors. Until the cornea was put into the apparatus, the epithelium of the cornea was moistened with Ringer's solution of pH = 7.15 containing oxidized glutathione at the concentration of 0.18 g/l. Both solutions were additionally saturated with a mixture of oxygen and carbon dioxide. The isolated corneas were submitted to a fluorescein test classifying them for a further experiment (14). After the isolated cornea was transferred into the chamber and the tightness between chambers was tested, 1 ml of the studied drops was put into a donor compartment while a 5 ml portion of Ringer's solution of pH = 7.15 was put into the receiving compartment. The procedures of withdrawing samples and their analysis were identical to the ones described earlier. The changes of the concentration in the receiving compartment were analyzed during four hours of the experiment. On the basis of the studies of three different corneas, which were carried out three times, it can be concluded that in the applied experimental standard *in vitro*, the compounds of the studied eye drops did not show the permeability through this biological barrier.

DISCUSSION AND CONCLUSIONS

The evaluation of penetration abilities of therapeutic substances through the eye cornea is based mainly on the observation of stimulated myotic and mydriatic effects or the measurement of the concentration, possibly the amount of medicine which is transported to the liquid eye compartments e.g. aqueous humour or vitreous body (17). Considering the low precision on the former methods and the high invasive propensity of the latter, connected with the necessity of frequent puncturing of testing animals, it is advisible to use the standard research

method based on synthetic membranes of the character similar to the physiological biological barriers, as well as isolated live pig's corneas in applied experimental conditions. On certain assumptions, those methods enable the correlation of the *in vitro/in vivo* results. The subject of the studies were the aloe eye drops containing biologically active substances, composed of anti – inflammatory chemical substances, characterized by the absorbance at the wavelength λ = 310 nm, e.g. aloenin, aloin, aloesin and its esters: 2''–O–*p*–coumaroylaloesin, 2''–O–feruloylaloesin and aloe – emodine (4). It was found (Table 1) that the main compounds of the group are aloesin (29.83%) and aloin (26.04%). The studied eye drops met the requirements of the Polish Pharmacopeia V, mentioned in the monography *Guttae ophthalmicae* (Table 1).

The antimicrobial activity of neomycin in the drops (Table 1) was higher than that of the solution of neomycin sulphate in phosphate buffer, which served as reference. It can be explained by the presence of disodium EDTA and β–phenylethyl alcohol in the drops. The additive substances, mentioned above, can increase the permeability of bacteria's membrane, which could result in easier neomycin permeability into a bacterial cell, changing its antimicrobial activity (19).

A cornea has a lipophilic – hydrophilic character, its application in the studies of the synthetic membranes of the properties similar to the cornea's is fully justified.

To determine the permeability rate constants of the biologically active substances by the synthetic membranes of the lipophilic and hydrophilic character, the described dialysis perfussion apparatus was applied (15, 16). The lipohilic membranes were obtained by the modified Fürst *et al.* method. Their physicochemical characterization was carried out earlier on the basis of the measurement of thickness, resistance and electrical conductivity (20). The obtained values of concentration changes of biologically active substances determined as aloenin (Figure 1) as a function of time were interpreted according to the first – order kinetics. The linear semi – logarithmic dependence $c_{gr} - c = f(t)$ (Figure 2) enabled to calculate the first – order rate constants of permeability through synthetic lipophilic and hydrophilic membranes at pH = 7.15 (Table 2). Their values show that this process takes place with a relatively low kinetics in comparison to the permeability rate constants of 5–fluorouracil, sulfadicramide or indomethacin, which were determined earlier in analogous conditions (15, 16, 21).

Taking into account the hydrophilic and lipophilic character of the eye cornea and the results of the experiments which were carried out, it was decided to test the penetration possiblity of the studied eye drops by this biological barrier. The experiment was carried out using isolated live pig's corneas in *in vitro* conditions. It was found that the permeability process of most therapeutic substances through anatomical layers of the cornea is limited first of all by the molecular mass, polarity and the solubility degree in water and lipids (17).

It was shown that in the applied experimental conditions *in vitro* the compounds of drops did not permeate through the animal's eye cornea, which can be the result of a large molar mass. Moreover, it was very difficult to determine the low content of the biologically active substances in the receiving compartment as a result of the insufficient sensitivity of the spectrophotometric method in the research.

In the treatment of eyeball infections and coexisting inflammations of its external structures, ready – made eye drops are often used, in which neomycin sulphate is mixed with corticosteroids e.g. fluorohydrocortisone or dexamethasone. However, side effects of cortisosteroids limit the application of those drops. They impede specific immunology mechanism of animal's eye, especially if an inflammation is accompanied by a virus infection or mycotic one. The topical application of corticosteroids is also contraindicated if the structure of front epithelium of the cornea is injured because corticosteroids make the regeneration of tissues and healing of wounds more difficult; the application of corticosteroids in such cases make an infection more probable. Therefore, the suggested and biopharmaceutically evaluated eye drops containing aloe should show anti – inflammatory and anti – infectious effect in relation to external anatomical structures of the eye, not penetrating aqueous humour of the front and back chamber of the eyeball.

REFERENCES

1. Yagi: Fukuyama Daigaku Yakugakubu Kenkyu Nenpo 11, 27 (1993).
2. Kodym A.: Farm. Pol. 54, 887 (1998).
3. Suzuki I., Saito H., Inoue Sh., Migita T., Takahasi T.: J. Biochem. 85, 163 (1979).
4. Kodym A.: Pharmazie 46, 217 (1991).
5. Kodym A., Broda H., Dragunowicz I.: Herba Polon. 25, 193 (1979).
6. Nakagomi K., Yamamoto M., Tanaka H., Tomizuka N., Masui T., Nakazawa H.: Agric. Biol. Chem. 51, 1723 (1987).

7. Kodym A., Kowalkiewicz A.: Farm. Pol. 43, 516 (1987).
8. Hirata T., Suga T.: Z. Naturforsch. 32c, 731 (1977).
9. Kodym A.: Farm. Pol. 44, 71 (1988).
10. Hörhammer L., Wagner H., Bittner S., Graf E.: Deutsche Apotheker Zeitung 105, 827 (1965).
11. Kodym A., Bujak T., Kaczyńska E.: Farm. Pol. 54, 1073 (1998).
12. Kodym A.: Farm. Pol. 47, 11 (1991).
13. Fürst W., Neubert R., Reppel L.: Pharmazie 35, 105 (1980).
14. Richter H., Morbitzer B., Fürst W.: Pharmazie 33, 523 (1978).
15. Grześkowiak E.: J. Pharm. Bel. 52, 190 (1997).
16. Grześkowiak E.: Eur. J. Pharm. Sci. 6, 247 (1998).
17. Orłowski J., (ed.) Okulistyka współczesna. PZWL, Warszawa 1992.
18. Hostyn P., Villain F., Malek–Chehire N., Kuhne F., Takesue Y., Parrish R.K., Parel J.–M.: J. Fr. Ophtalmol. 19, 133 (1996).
19. Kodym A., Marcinkowski A., Kukuła H., Olejnik P.: Farm. Pol. 57, 979 (2001).
20. Grześkowiak E., Simon M., Partyka D., Wiśniewska A.: Acta Polon. Pharm. – Drug. Res. (in press).
21. Kodym A., Grześkowiak E., Malinowska M.: Farm. Pol. 50, 685 (1994).

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