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Ocular Drug Metabolism of the Bioactivating Antioxidant N-acetylcarnosine for Vision in Ophthalmic Prodrug and Codrug Design and Delivery

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The basic idea in this study relates to the interesting research problem to employ with the knowledgeable pharmacy staff *N*-acetylcarnosine (NAC) in the developed suitable compounded prodrug ophthalmic preparations, which are currently used for the treatment of cataract and have antioxidant effect, in order to provide the molecular support to one of the most *popular beliefs* of the growing market for the treatment of senile cataract in patients and animals with efficacious NAC drug formulations worldwide patented by the author.

This work presents the progress in ocular NAC prodrug and codrug design and delivery in light of revealed ocular metabolic activities. There is a considerable interest in the ophthalmic codrug design including NAC prodrug based on the strategies to improve ophthalmic drug delivery of the active peptide principal L-carnosine through the sustained intraocular metabolic activation of a dipeptide while making it resistant to enzymatic hydrolysis. Novel approaches to ocular NAC drug delivery, developed by Innovative Vision Products, Inc. (IVP), aim at enhancing the drug bioavailability by ensuring a prolonged retention of the medication in the eye, and/or by facilitating transcorneal penetration.

IVP team studied the effects of lubricant eye drops designed as 1% NAC prodrug of L-carnosine containing a mucoadhesive cellulose-based and corneal absorption promoters in a drug delivery system. The predicted responses of the corneal and conjunctival penetrations to the synergistic promoters are useful in controlling the extent and pathway of the ocular and systemic absorptions of instilled NAC prodrug in designed ophthalmic formulations thereof. Utility of peptidase enzyme inhibitors in the codrug formulation to modulate the transport and metabolism of NAC prodrug appears to be a promising strategy for enhancing dipeptide drug transport across the cornea.

The developed and officially CE mark registered by IVP NAC prodrug and codrug lubricating eye drop systems (including principal regulatory registered eye drops design and lubricating eye drops marketed under numerous brand labels), increase the intraocular uptake of the active principle L-carnosine from its ophthalmic carrier NAC in the aqueous humor and the permeability of a drug into the eye, and so enhance the ocular bioavailability, bioactivating universal antioxidant, and anti-cataract efficacy (in human and in canine eyes) of the developed NAC eye drops.

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INTRODUCTION

The incidence of cataract increases with aging with almost 50% of people suffering cataract by 75 years of age. This is associated with significant functional impairment because of reduced visual acuity, contrast sensitivity, and debilitating glare (Robertson, 1990). With an aging population and increasing longevity, cataract is set to have significant socioeconomic demand on healthcare resources. There is an urgent need for a method of cataract prevention or medical drug treatment that could slow the progression of cataract. One might consider the eye an ideal, easily accessible target organ for drug delivery. However, the eye is, in fact, well protected against absorption of foreign materials (i.e., drugs and xenobiotics), first by the eyelids and tear-flow, and then by the cornea and conjunctival epithelial barriers of the eye) (Robertson, 1990). Furthermore, although the mammalian eye is seldom considered an organ of drug metabolism, in this respect, biotransformation activities have been detected in various ocular structures (Al-Ghananeem & Crooks, 2007). These metabolizing capabilities of the mammalian eye are unique in providing protection from xenobiotics and foreign compounds. A drug applied to the surface of the eye may cross ocular–blood barriers where it may encounter metabolizing enzymes and cellular transporters before it distributes to the site of action, such as the crystalline lens.

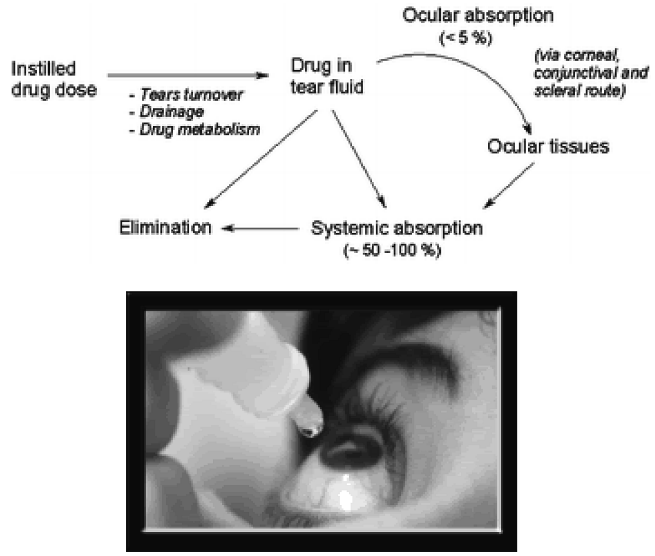


FIGURE 1. Absorption and dissipation pathway of a topically administered ophthalmic drug.

Other problems of drug delivery to the eye include poor bioavailability of ophthalmic solutions, clearance and drainage from the eye (ophthalmic drugs typically achieve <10% ocular bioavailability), side effects resulting from ophthalmic drugs reaching the systemic circulation, decreased visual acuity, and patient compliance. Topical drug delivery to the eye is often impaired by removal mechanisms (blinking, tears) and by the barriers of the pre-corneal area (Figure 1) (Barbu, Verestiuk, Nevell, & Tsibouklis, 2006). Therapeutic advances in the ocular peptide delivery are hindered by the difficulties of increasing bioavailability and of delivering actives to the site of action, such as to crystalline lens in the attempt to manage cataract, a focus of our intensive studies conducted during recent decade (Figure 2A and B) (Babizhayev, 1989a; Babizhayev, 1996; Babizhayev & Deyev, 1989). Novel approaches to ocular drug delivery, commercialized in recent times or still under evaluation, aim at enhancing the drug bioavailability by ensuring a prolonged retention of the medication in the eye and/or by facilitating transcorneal penetration. New, more efficient medications for dry eye conditions are actively investigated. Some approaches to ocular drug delivery are briefly considered with following techniques:

1. In situ activated gel-forming systems;
2. mucoadhesive polymers;
3. absorption promoters;
4. vesicular/colloidal systems;
5. inserts and collagen shields.

Mucoadhesive polymers, i.e., macromolecules capable of retaining the medication in the precorneal area by establishing physico–chemical interactions with the mucin layer covering the corneal epithelium, are a relatively recent discovery. The following synthetic, semi-synthetic, and naturally occurring polymers have been found at Innovative Vision Products, Inc., (IVP) laboratories to possess mucoadhesive properties: carboxymethylcellulose, hydroxypropylcellulose, polyvinyl alcohol, polyacrylic acid (Carbomer), high molecular weight (>200,000) polyethylene glycols, chitosans, hyaluronic acid, polygalacturonic acid, xyloglucan, etc. . The use of corneal absorption promoters, i.e., of substances facilitating drug penetration through the corneal tissues, is a potentially interesting,

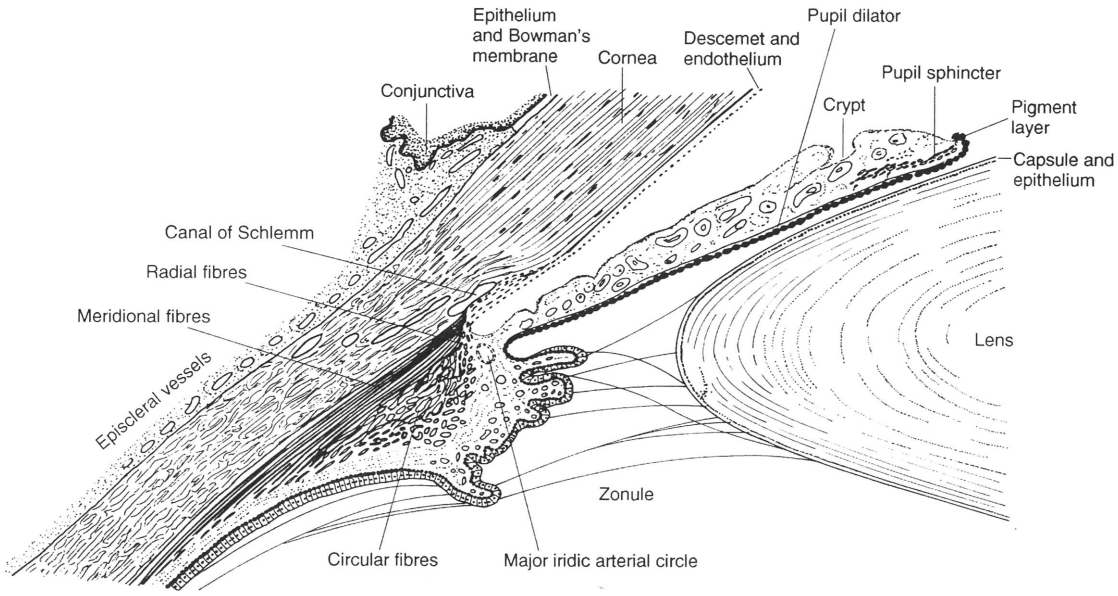


FIGURE 2. (A) Human crystalline lens in the anterior chamber of the eye and its metabolism. The interaction of tissues of the eye influences the overall viability of the lens organ through the aqueous humor. (B) The appearance of excised human crystalline lenses.

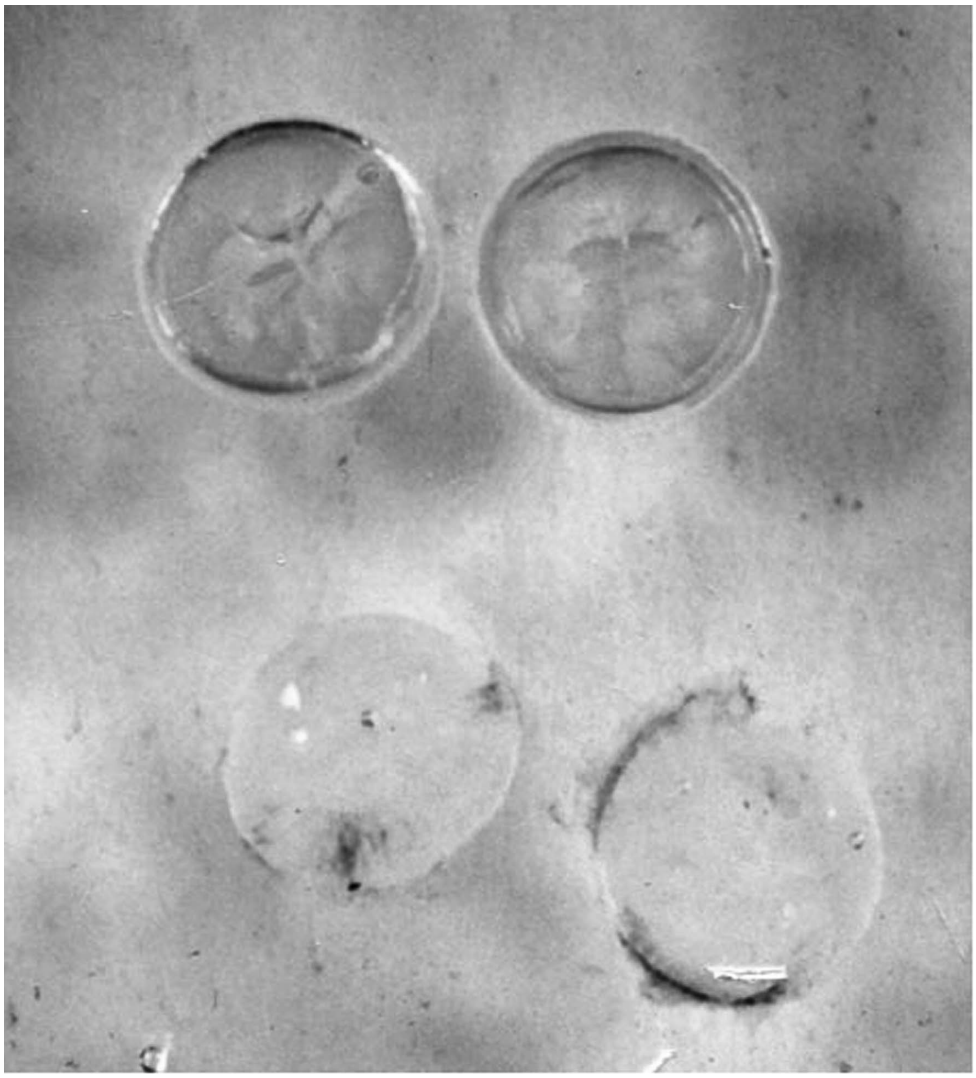


FIGURE 2. (Continued).

still little-exploited approach to improving ophthalmic bioavailability. The effect of these substances on the cornea in IVP studies is to enhance the permeability of corneal epithelium by altering the cell membranes and loosening the tight junctions between superficial cells.

Currently, there is considerable interest in ophthalmic drug design based on strategies to improve ophthalmic peptide drug delivery through metabolic activation. As ocular enzymes and transport systems are better characterized, their properties became integral consideration in peptide drug design and development. This study has addressed ocular metabolic activity of *N*-acetylcarnosine (NAC) and its utility in metabolism-focused, ophthalmic-specific drug design worldwide patented by Innovative Vision Products, Inc. (IVP). This specific area of NAC drug development is the focus of the present research article.

Utilizing the specific purity NAC ingredient manufactured at the cGMP facility according to specification developed by IVP (Table 1), as a source of pharmacological principal L-carnosine, we have created the ophthalmic formulations, which contain varying amounts of the actives tailoring the enhanced intraocular absorption of the beneficial ingredient, naturally occurring dipeptide L-carnosine, to optimize its specific effect and purpose in producing the basic bioactivating antioxidant activity in vivo and reducing toxic effects of lipid peroxides to the crystalline lens.

MATERIALS AND METHODS

This project was approved by the ethic commission of Helmholtz Research Institute for Eye Diseases (no. 008/136/30).

Lenses

Rabbit transparent lenses were obtained from freshly enucleated eyes of the Chinchilla race animals. Normal mouse lenses were extracted from the eyeballs by the posterior

TABLE 1
Specification of cGMP Manufactured N-Acetyl-L-Carnosine Used in IVP Drug Development and Clinical Studies

No.	Test Name	Specification
1	Appearance	White powder
2	Identification	Positive
3	Optical rotation	$[\alpha]_D^{20} + 25.2^\circ + 27.5^\circ$
4	pH	4.5 – 5.5
5	Heavy metals	NMT 10 ppm
6	Related substances	L-Carnosine: NMT 0.3% Others: NMT 0.2 %
7	Residual solvent	2-propanol: NMT 500 ppm
8	Water	NMT 5.0%
9	Residue on ignition	NMT 0.10%
10	Assay	NLT 99.5% (HPLC area)

NMT: not more than; NLT: not less than.

approach from mice of the strain C57BL or hybrids F_1 (CBA × C57BL) resistant to cataract formation. In all cases, the integrity of the lens capsule was preserved. Quantitative assessment of the degree of opacity of the lens was undertaken by determination of relative areas of zones with maximal optical density during investigation of the lens by quantitative morphometry on the Leitz (Germany) television image analyzer (Figure 3) (Babizhayev, Deyev, & Derevyagin, 1989). Capsule defects were defined, classified according to the location, size, shape, and tension of the capsule. The shape of the capsule defects included fissure-like, triangle, round, irregular, and fan-like. All lenses with capsule defects have been sorted into one of these types and excluded from the study. After extraction, normally whole lenses were briefly rinsed in Hanks' medium for 20–30 s and then immediately placed into a moist chamber. They were handled with the greatest care with a pair of curved forceps when being placed inside and being removed from their individual containers for measurements. The lenses were used either directly after the extraction procedure or surgery, or at least 1–3 h were allowed to elapse between the dissection from the eye and the start of the measurement. The findings evidenced that this delay did not influence the results.

Lipid Extraction and Quantification in the Aqueous Humor

Aliquots of fresh aqueous humor from each patient or animal were immediately extracted into 40 vol of chloroform–methanol (2:1, vol/vol) with 4-methyl-2,6-ditert-butyl-phenol (BHT) antioxidant addition (0.5 mg/100 ml) during 10 min. The obtained extract was washed according to the modified Folch procedure, and the chloroform layer was recovered and evaporated under argon (Babizhayev, Deyev, & Linberg, 1988). Aliquots of the lipid residue were analyzed for total phospholipid phosphorus and fractioned to discriminate cholesterol, free fatty acids, and the individual spots of the polar lipids as described previously (Babizhayev, 1996; Babizhayev, & Bozzo Costa, 1994).

Lipid Extraction from the Lens

Immediately after the lens material had been obtained, lipids were extracted from the lens by Folch method (Folch, Lee, & Sloane-Stanely, 1957). The extraction was carried out by tissue homogenization in 20 vols of chloroform/methanol mixture (2:1 by vol) with BHT antioxidant addition (0.5 mg/100 ml) for

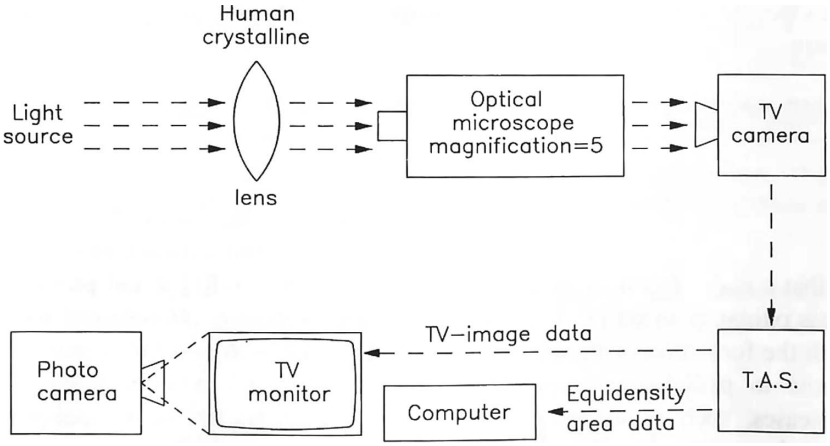


FIGURE 3. Image analysis procedure, which makes it possible to obtain an image of the lens, to determine values of the optical density in different parts of the lens, to divide the lens surface into zones of defined density range, to measure the areas of equidensities, and to evaluate the precise topography of every zone. All parameters are estimated quantitatively by use of a computer. The lens is illuminated in a frontal projection, adjusting the intensity of the illuminating light to make the transmission through each lens constant. An electronically produced measuring field is determined by a circular mask whose position is under the control of the analyser. To control its position, the coordinates of the central point of the mask are automatically digitally defined. The image is then scanned and the density at each coordinate stored in the computer.

10 min. After filtration, the sample obtained was put into a separating funnel for 5–8 h to stratify. Water was added in 7:1 ratio to promote the stratification. Temperature was maintained at 0°C for all the operations. After separation of the phases and removal of the aqueous-methanol layer, the lower chloroform fraction was evaporated. Phospholipid content was assessed by the results of organic phosphorus evaluation (Chen, Toribara, & Warner, 1956). Total lipid amount in the extract was determined gravimetrically, as well as by characteristic absorption in 206–210 nm area of the lipid sample after dissolution in 4 ml of methanol/heptane mixture (5:1 by vol).

Determination of Lipid Peroxidation Products

Accumulation of the lipid peroxidation (LPO) primary molecular products was estimated spectrophotometrically from characteristic absorbents of diene conjugates in the UV-region at 232 nm characterizing the level of hydroperoxides of polyunsaturated fatty acids, as well as by LPO secondary molecular product absorbance at 274 nm, corresponding to the concentration of conjugated trienes and cetodienes (Bolland & Koch, 1945) on "HITACHI-557" spectrophotometer (Japan). The absorption spectra were recorded after the chloroform–methanol (2:1, vol/vol) extraction as described above and the dissolution of a dry lipid residue in 2.5 ml of methanol–heptane (5:1, vol/vol) mixture. This protocol removes any water-soluble secondary oxidation products, leaving them in the methanol-aqueous phase. The equalization of the extracted lipid concentrations to the measured phosphorus was done using the characteristic absorption in the 206- to 210-nm area of the lipid sample. The content of the source material in the samples was also equalized by amount of phospholipids. An average molecular weight of phospholipid was assumed as approximately 730 kDa. The content of end molecular fluorescent LPO products was determined from the fluorescence intensity of the lipid extract at 365 nm excitation and 420–440 nm emission wavelengths (Chio & Tappel, 1969), measured on a "HITACHI-MPF-4" spectrofluorometer. The spectrofluorometer was calibrated at the beginning of every working day against a solution of quinine sulfate (1 μg/ml in 0.1 N H_2SO_4) standard, at 435 nm fluorescence emission and 365 nm excitation wavelengths.

Pharmacokinetics of Topical NAC Application in Ophthalmic Formulations

Formulations and Animals

Grey Chinchilla rabbits (male) aged 3–4 months weighing 2–3 kg were used. Animal experiments conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research. Thirty minutes before the ocular incision, the right eyes of rabbits were instilled with 80 μl of formulation A containing 1% NAC and the control right eyes of the separate rabbits were similarly instilled with their vehicle (placebo) solutions. Preferably, each composition is in the form of an isotonic solution. Because the composition is applied to the eye, the composition should be sterile. The isotonicity of different prepared ophthalmic preparations (A, B, C, D, E, and F) was measured and adjusted as calculated correctly by addition of small concentrations of sodium chloride. Formulation A (Can-C™) (Figure 4) contained the following ingredients:

Deionized water	970 g
Glycerine (1.0%)	13 g
NAC (1.0%)	10 g
Carboxymethylcellulose (0.3%)	3 g
Benzyl alcohol (0.3%)	3 g
Potassium borate	7.91 g*
Potassium bicarbonate	3.47 g*

*Or what is necessary to bring the solution up to around a pH of 6.3–6.5.

FIGURE 4. Can-C™ brand of *N*-acetylcarnosine lubricant eye drops developed by Innovative Vision Products, Inc.

The specification of NAC is presented in Table 1. Formulation A was presented in the final ophthalmic tubes (per volume of 2.5 ml) and in the moiety of the plastic bottles. Placebo (Formulation B) solution contained the same ingredients without NAC. The solution of NAC in the phosphate buffer was also administered in the right eyes of the separate rabbits. The following formulations were used:

Formulation C: just NAC dissolved in sodium phosphates solution pH 6.3. This was the research 1% NAC formula with the preservative Thimerosol added at 0.004 g/100 ml.

Formulation D (NAC, 1% is added).
Benzyl alcohol dissolved in the sodium phosphate buffers, pH 6.3 and at the same dissolution rate.

Formulation E (NAC, 1% is added).
Phenyl ethyl alcohol dissolved in the sodium phosphate buffers, pH 6.3 and at the same dissolution rate.

Formulation F (CE marked registered eye drops) is presented in Appendix 1.

Surgical Procedure

Topical anaesthesia of the rabbit eyes was performed after 25 min of instillation of the formula ophthalmic solutions with instillations of 4% lidocaine hydrochloride solution eye drops (three times with 1 drop at 1.5–2.0 min intervals). The eye drops of 4% lidocaine hydrochloride contain benzaltonium chloride preservative. When ocular anaesthesia was achieved, the lids were extended and fixed with the lid-holder and the ocular bulb was fixed by tweezers in the area of the inferior rectus muscle. A stab incision was performed transcorneally 1.0–2.0 mm from the limbus in the temporal superior quadrant. Aqueous humor (0.1–0.2 ml) was aspirated from the anterior chamber of a rabbit eye with 25-gauge needle connected to an insulin syringe and immediately introduced into an Eppendorf tube with addition of ethanol (0.2 ml), keeping the sample on ice before extraction.

Extraction of Imidazoles from Aqueous Humor

Extractions of imidazole-containing compounds from the aqueous humor aliquots were performed according to Babizhayev et al. (1996). Portions of aqueous humor were added to ethanol as above and thoroughly mixed (20°C, 15 min).

Extracts were centrifuged ($2000 \times g$, 15 min) and the supernatants removed .Samples were frozen in the gradient of temperatures to –70°C and lyophilized using the apparatus JOAN (France). The lyophilized residue was dissolved in 1 ml of 0.1 M Na_2HPO_4 (pH 2.1 adjusted with 85% phosphoric acid) and filtrated through the membrane filter with the dimensions of pores 0.22 μm directly before the analysis.

Analytical High-Performance Liquid Chromatography

Reverse-phase analytical high-performance liquid chromatography (HPLC) was performed using a Breeze chromatography system (USA), detector Waters 2487 Dual λ Absorbance Detector, column (250 × 4.6 mm) Symmetry 300 C_{18} 5 μm (Waters), loop 20 μl. The column was eluted isocratically at 30°C with the cited phosphate buffer 0.1 M Na_2 HPO_4 (pH 2.1) over 25 min at a flow rate of 1.0 ml/min. Eluates were monitored for absorbance at 210 nm. The standards of L-carnosine and NAC were prepared by weighing the dry material using the analytical balance Mettler Toledo (accuracy 0.00004) and were further dissolved in the phosphate buffer 0.1 M Na_2 HPO_4 (pH 2.1). The quantitative determination of L-carnosine and NAC in the samples was undertaken using the technique of external standard according to the area of the peak and linear extrapolation. The standards of eye drops were prepared by dissolution of initial solutions of eye drops by 100-fold using the phosphate buffer 0.1 M Na_2 HPO_4 (pH 2.1). Statistical significance was evaluated by the unpaired Student's *t*-test and $p = .05$ was taken as the upper limit of significance.

Peroxidation Reaction System

The techniques for phospholipid extraction, purification, and preparation of liposomes (reverse-phase evaporation technique) have been described previously (Babizhayev, 1996; Babizhayev & Bozzo Costa, 1994). Peroxidation of phosphatidylcholine (PC, derived from egg yolks) was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbic acid to the suspension of liposomes (1 mg/ml) in 0.1 M Tris–HCl buffer (pH 7.4). The incubations were performed at 37°C. The tested compounds, NAC and L-carnosine, were added at 10–20 mM concentration to the system of iron-ascorbate-induced liposome PC peroxidation. The kinetics of accumulation of LPO products in the oxidized liposomes were measured by reaction with thiobarbituric acid (TBA). The peroxidation reaction was arrested by adding EDTA to a final concentration of 50 μM or by the addition of 2.0 ml of ice-cold 0.25 M HCl containing 15% (wt/vol) tri-chloroacetic acid (TCA). TBA (0.125% wt/vol) was then added to the mixture and followed by boiling for 15 min. The TBA assay was described previously (Babizhayev & Bozzo Costa, 1994). The differential absorbance of the condensation product, malonyl dialdehyde (MDA), at 535 and 600 nm was measured spectrophotometrically ($\varepsilon_{535} = 1.56 \times 10^5\ M^{-1}.cm^{-1}$). The TBA reaction itself was not affected by the components of the radical generators or scavengers used in the study. To determine conjugated dienes, the lipid residue of the samples was partitioned through chloroform during the extraction procedure (Babizhayev & Bozzo Costa, 1994).

Correlation of the extracted lipid concentrations to the measured phosphorus was done by means of characteristic absorption at 206–210 nm of the lipid sample (redissolved in 2–3 ml of methanol/heptane mixture 5:1, vol/vol). Accumulation of net diene conjugates corresponding to the level of lipid hydroperoxides was assessed from characteristic absorbance of diene conjugates at ~230 nm ($\eta CD = 2.8 \times 10^4\ M^{-1}.cm^{-1}$), in a Shimadzu UV-260 spectrophotometer (Japan) (Babizhayev & Bozzo Costa, 1994). Absorbance of the secondary LPO products

at ~274 nm, corresponding to the concentration of conjugated trienes and ketodienes, was also measured spectrophotometrically from the lipid spectra (Babizhayev & Bozzo Costa, 1994).

Statistical significance was evaluated by the unpaired Student's *t*-test, and $p = 0.01$ was taken as the upper limit of significance.

Uptake of l-Carnosine by the Lens

In a separate series of experiments, the kinetics of L-carnosine penetration into the isolated rabbit lenses were evaluated (five lenses studied). To assess the ability of the lens to accumulate L-carnosine, it was placed in 5 mM (or higher concentration) L-carnosine solution in Hanks' salt medium (without bicarbonate, pH 7.4), containing 7 mM glucose (Babizhayev, 1989b) and incubated at room temperature (20°C). After 1 h, the extraction procedure was utilized (Babizhayev, 1989b) to isolate L-carnosine from the nonprotein fraction of the lens. The nonprotein fraction removed was stained with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride) reagent and then applied for Thin Layer Chromatography (TLC) in aqueous ethanol ($EtOH/H_2O$, 77:23). The concentration of L-carnosine in the sample was estimated spectrophotometrically from its characteristic absorbance at 420/600 nm in a Hitachi-557 double-beam spectrophotometer (Japan). Standard L-carnosine samples were used to evaluate the quantitative recording of the absorbance. TLC was applied earlier (Babizhayev, 1989b) to evaluate the L-carnosine level in eye lenses.

Incubation of Lenses with Liposomes

To control effects of metal ions or potential $OH^{\bullet}$ scavengers (such as glucose present at scavenging concentrations in standard culture media), the lens culture medium was composed of 10 mM Tris–HCl, 140 mM NaCl (pH 7.4), phosphate and glucose-free (Medium A) or glucose-containing medium nutritious to the lens comprising 50 mM Tris–HCl, 5.5 mM glucose, 4.0 mM KCl, 102.5 mM NaCl, 1.0 mM K_2HPO_4 (pH 7.2–7.4) (Medium B) (Babizhayev & Bozzo Costa, 1994). Both media were adjusted to 290–300 mOsmol with NaCl and equilibrated with 95% air and 5% CO_2. When significant concentrations of oxygen scavengers or other agents were added, the change in osmolarity was compensated by decreasing the amount of NaCl used to adjust the media back to the required osmolarity following modification. The rabbit lenses were incubated in 3.0 ml of medium per lens at room temperature. The tests of lens integrity during incubations, to indicate that the lens is functioning normally, were performed as previously reported (Babizhayev, Deyev, & Chernikov, 1992). When necessary, the lens incubation media contained the liposome suspension (0.5 mg/ml). Fixed aliquots of media (50–500 μl) were taken out at different times from the organ cultures of lenses for measurements of LPO products. Generally, the total incubation time was 3 h.

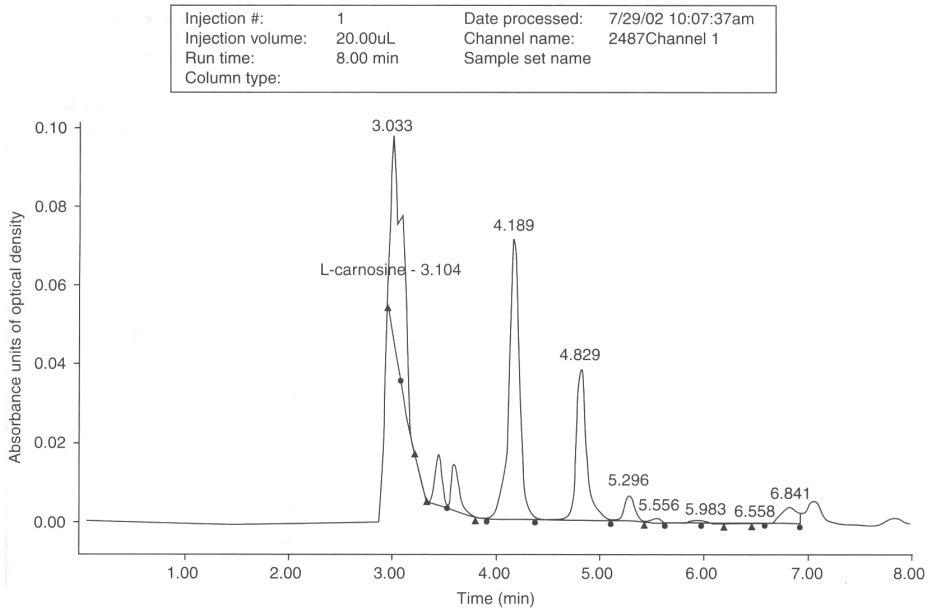
Apparent pK values of typical histidyl-imidazole in proteins and of imidazole groups of histidine-related compounds were measured according to Abe (2000).

RESULTS

NAC as a Time Release (carrier) Version of l-Carnosine in Ophthalmic Applications with Carboxymethylcellulose

An important chemical difference between carnosine and NAC is that carnosine is relatively insoluble in lipids (fats and fatty compounds), whereas NAC is relatively soluble in lipids (as well as in water). This means that NAC may pass through the lipid membranes of the corneal tissue and cellular structures more easily than carnosine, and may thereby gain the access more readily to the intraocular aqueous humor. NAC can gradually release carnosine, which then exerts its beneficial effects (Babizhayev et al., 1996). In this section of the study, we considered whether NAC acts in the ophthalmic formulation with lubricants (including carboxymethylcellulose) when topically administered to the eye as a time release carrier (prodrug) of L-carnosine. Because the eye is an important site of exposure to xenobiotics, it is not surprising that it possesses the important enzymes capable of metabolizing the foreign peptide species instilled to the eye. The cornea displays a regional distribution of the peptidase metabolic activities toward endogenous and exogenous peptide substrates topically administered to the eye (Sharma & Ortwerth, 1987). The findings suggest that, in order to deliver short-chain peptides intraocularly from topical solution instillation, it will be necessary to control with the specific excipient contents in a codrug ophthalmic formulation, manage the ocular tissue activity of aminopeptidases principally and, to a lesser extent, the activity of dipeptidyl peptidase and dipeptidyl carboxylpeptidase (Kashi & Lee, 1986). The aminopeptidase enzyme activity was reported to be inhibited by bestatin, amastatin, puromycin, bacitracin, sulfhydryl reagents, and metal chelators (such as EDTA) (Babizhayev & Meguro, 2004). The hydrolysis of L-carnosine released during its pass through the cornea to the aqueous humor has been reported to occur in rabbit cornea and conjunctival tissues (Babizhayev et al., 1996; Babizhayev, Yermakova, Semiletov, & Deyev, 2000).

The HPLC pattern of an extract of the aqueous humor obtained 30 min after instillation to the rabbit eye of ophthalmic formulation containing 1% NAC, lubricants carboxymethylcellulose, glycerine in the borate buffer confirms that the peak characteristic of L-carnosine has a concentration and a retention time (3.1 min) clearly distinct from NAC (6.0 min) and from the dead time of the column (Figure 5). This identified peak of L-carnosine was quantified and the data processor integrated that virtually all NAC after the overall extraction efficiency is converted into the L-carnosine compound with a retention time of 3.1 min (Figure 5). The data on the L-carnosine-related product were blanked with the control placebo data applied to the matched eyes of the control rabbits



	Peak name	RT (min)	Area (μV*sec)	Area (%)	Height (μV)	Height (%)	Amount	Units
1		3.033	251 219	17.42	52 493	21.24		
2	L-Carnosine	3.104	186 774	12.95	45 509	18.42	7.079	ug/mL
3		3.453	64 253	4.46	12 971	5.25		
4		3.610	67 747	4.70	11 790	4.77		
5		3.880	1 270	0.09	359	0.15		
6		4.189	493 858	34.24	71 709	29.02		
7		4.829	269 502	18.69	38 783	15.70		
8		5.296	43 914	3.04	6 341	2.57		
9		5.556	5 970	0.41	1 041	0.42		

FIGURE 5. High-performance liquid chromatography (HPLC) of extract of aqueous humor aspirated 30 min after the instillation of ophthalmic formulation with 1% *N*-acetylcarnosine, carboxymethylcellulose, and glycerine lubricants into the rabbit eye.

FIGURE 6. Typical rabbit eye with a normal cornea.

(Figure 6). The mean ratio of L-carnosine (C)/(NAC) relevant to the L-carnosine release in the aqueous humor 30 min after instillation of Formulation A (Can-C™) with 1% NAC into the rabbit eye corresponded to C/NAC = 6.64 ± 0.06 (n = 8, where n = number of the examined treated rabbit eyes; only right eyes were treated). In the control placebo formulation-treated eyes, the same indices could not be quantified at statistically significant rate. Concentrations of imidazole products in the aqueous humor corresponded to those of intact rabbit eyes and referred to baseline values of L-carnosine 0.19 ± 0.10 μg/ml related products variously detected in extracts from normal animals. When control formulations contained only 1% NAC dissolved in a buffered solution or contained 1% NAC and added preservative thimerosol, benzyl alcohol, or phenyl ethyl alcohol dissolved in a buffered solution (Formulations C–E, see Section: Formulations and Animals), the C/NAC ratio corresponded to 1.99 ± 0.05 (n = 7), 1.94 ± 0.12 (n = 5), 1.98 ± 0.05 (n = 5), 1.95 ± 0.05 (n = 5), relatively. Ophthalmic preservatives, especially Parabens included in the ophthalmic Formulation F (CE Marked eye drops, see Appendix 1), increased the concentration of L-carnosine in the aqueous humor after topical administration of IVP Officially registered brand of NAC eye drops to the rabbit eyes, demonstrating the effects of corneal absorption promoters. Previously, the purpose of several studies was the modification of ocular permeability of peptide drugs by absorption

promoters, including either EDTA, paraben, saponin, or benzalkonium chloride (Sasaki et al., 2000; Sasaki, Tei, Nishida, & Nakamura, 1995). These promoters enhanced the permeability of hydrophilic molecules through the cornea and conjunctiva. The promoting effects of the absorption promoters on the conjunctival peptide drug penetrations were not as strong as those on the corneal penetrations. The promotional effects of absorption promoters on the corneal drug penetration apparently increased with an increase in penetrant molecular weights, although those on the conjunctival drug penetrations did not depend on the molecular weights (Sasaki et al., 2000). The results of this study supportively demonstrate that in ophthalmic formulations containing lubricants carboxymethylcellulose, glycerine in the borate buffer, both Parabens used in the CE Marked eye drops Product or benzyl alcohol ophthalmic preservatives contained in the Can-C™ formula increase the intraocular absorption of ocularly applied L-carnosine prodrug. The NAC CE Marked eye drops Formulation F, which included Parabens preservatives showed little irritation on rabbit eyes and better tolerability than Formulation A including benzyl alcohol according to blinking measurements. However, it is not widely accepted that the benzyl alcohol preservative is not suitable for eye preparations because it may lead to irritation of the eye. Purified benzyl alcohol is preferred. When present, the preservative is contained in the composition in an amount of 0.3% and such composition may be used as either a common eye drop and a specific drug or both in the treatment of cataracts (Testa, Iuliano, Morton, & Longoni, 1987).

Prevention of the Peptide Drug Enzymatic Hydrolysis

The results indicate that ophthalmic preservatives used in the developed by IVP ophthalmic Formulations Can-C™ and officially registered eye drops brand are useful for the intraocular and systemic delivery to the eye of ocularly applied NAC acting as the ophthalmic prodrug of L-carnosine.

Our data demonstrate that topical administration of pure L-carnosine (1% solution) to the rabbit eye (instillation, subconjunctival injection) does not lead to accumulation of this natural compound in the aqueous humor over 30 min in concentration exceeding that in the placebo-treated matched eyes, and its effective concentration is exhausted more rapidly (Babizhayev et al., 1996; Babizhayev et al., 2000) .

The purpose of our experiments was to investigate the effect of absorption promoters on the ocular corneal permeability of NAC/carnosine peptides. EDTA and paraben preservatives were used as absorption promoters in the officially registered ophthalmic codrug Formulation F tested. These promoters enhanced the permeability of NAC and released L-carnosine molecules through the cornea and conjunctiva. The predicted responses of the corneal and conjunctival penetrations to these promoters are useful in controlling the extent and pathway of the ocular and systemic absorptions of instilled NAC prodrug in designed ophthalmic formulations thereof. Utility of enzyme inhibitors in the codrug formulation to modulate transport and metabolism of NAC prodrug appears to be a promising strategy for enhancing drug transport across cornea. This concept was employed by IVP in a codrug design of the officially registered NAC lubricant eye drops to provide improved physicochemical stabilization properties of L-carnosine principle released from NAC, and controlled drug release of the dipeptide molecules upon a peptidase hydrolysis in corneal and conjunctival tissues. In the preliminary study, the leucine aminopeptidase enzyme was characterized as a single polypeptide with a molecular mass of 42,977.2 kDa, as determined by matrix-assisted laser desorption ionization and time-of-flight mass spectrometry, and was found to be thermostable at 90°C for up to 1 h. Its optimal pH and temperature were observed to be 7.6–7.8 and 60°C (Deejing, Yoshimune, Lumyong, & Moriguchi, 2005). The aminopeptidase enzyme activity was strongly inhibited by EDTA, 1,10-phenanthroline, dithiothreitol, beta-mercaptoethanol, iodoacetate, and bestatin; and its apoenzyme was found to be reactivated by Co(2+) (Babizhayev & Meguro, 2004). Accordingly, the officially registered codrug design of NAC eye drops contains together in formulation two or more synergistic compounds (carboxymethylcellulose, paraben, and EDTA preservatives) in order to improve the NAC prodrug delivery properties bioconvertible into the L-carnosine peptide principle in the aqueous humor during its release from NAC upon hydrolysis in conjunctival and corneal tissues. Results from the studies described in this section provide valuable industrial information for optimization of the codrug design and ophthalmic formulation in order to achieve the sustained release of described peptide molecules NAC/L-carnosine) for the ocular diseases therapy.

Antioxidant Activity of NAC Versus L-carnosine in the Liposome Peroxidation System. Metabolic Bioactivating Antioxidant Activity of NAC

The comparative antioxidant activity of NAC and L-carnosine was assessed in the liposome peroxidation system (acting as oxidative lipid membrane substrate) catalyzed by Fe^{2+} + ascorbate (Figures 7A and B). The accumulation kinetics of molecular LPO products such as MDA and liposomal conjugated dienes and trienes are shown in Figure 7A–C. The results demonstrate that the LPO reactions in the model system of lipid membranes are markedly inhibited by L-carnosine. The effective concentrations of L-carnosine are 10 and 20 mM. Data on the biological effectiveness of L-carnosine as antioxidant preventing PC liposomal or linoleic acid peroxidation in physiological concentrations ranges of 5–25 mM have already been published (Babizhayev, 1996; Babizhayev & Bozzo Costa, 1994; Babizhayev et al., 1996). Figure 7A shows that the level of TBA-reactive substances (TBARS) reached at 5-min incubation decrease in the presence of L-carnosine (10 or 20 mM) at 10 min and at later time points (20 mM), which must be because of a loss of existing TBARS or peroxide precursors of

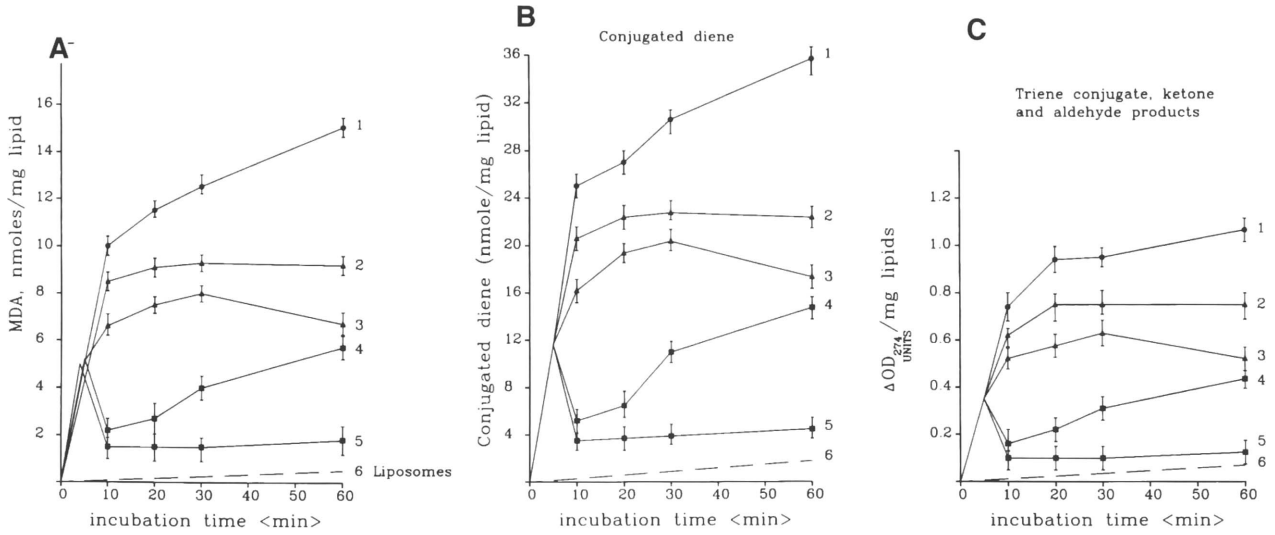


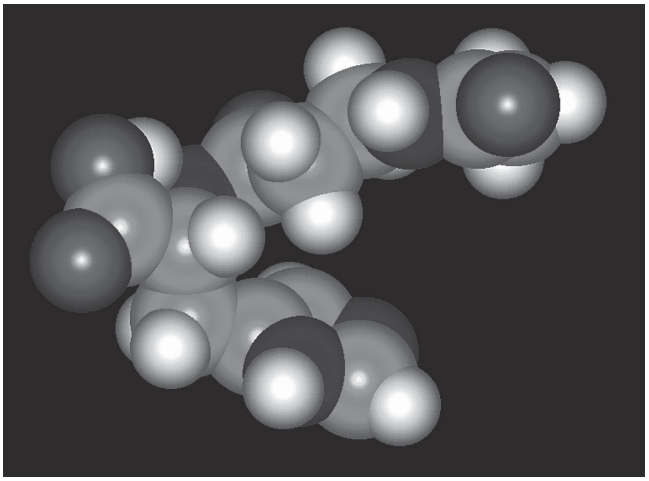
FIGURE 7. Accumulation of lipid peroxidation products [TBA-reactive substances (TBARS), measured as malonyl dialdehyde (MDA)] (A), diene conjugates (B), triene conjugates and ketone and aldehyde products (274 nm absorbing material) (C) in liposomes (1 mg/ml) incubated for 60 min alone (6, dotted line) and with addition of the peroxidation-inducing system of Fe^{2+} + ascorbate (1). Antioxidants *N*-acetylcarnosine (NAC) (10 or 20 mM) (2, 3) or L-carnosine (10 or 20 mM) (4, 5) were added at the fifth minute of the incubation period to the system containing the peroxidation inducers. Samples were taken at zero time and at time intervals indicated in the Figures and were used immediately for measurement of TBARS (see Section Peroxidation Reaction System). A similar amount of sample was partitioned through chloroform and used for detection of conjugated dienes and trienes dissolved in 2–3 ml of methanol–heptane mixture (5:1 vol/vol).

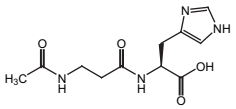
MDA and not because of a decreased formation of peroxide compounds. The ability of the histidine-containing compound NAC to inhibit the (Fe^{2+} + ascorbate)-induced oxidation of PC liposomes was compared with that of equimolar concentrations of L-carnosine. The antioxidant activity of 10 and 20 mM NAC corresponded to 38 and 55% inhibition of LPO for the two concentrations after 60-min incubation. NAC exhibited less antioxidant protection than L-carnosine, corresponding to 60 and 87% of the equimolar (10 or 20 mM) L-carnosine inhibition percentage. However, because NAC can act as a time release version metabolized into L-carnosine during its cross through the cornea to the aqueous humor (but not oral use), the bioactivating antioxidant activity of NAC converted to L-carnosine in vivo application is significantly increased. Once released from NAC, L-carnosine in the aqueous humor might act against peroxidation of the lens during its target pharmaceutical use.

The prodrug NAC approach has been utilized to enhance the ocular delivery of L-carnosine (Figure. 8A and B). The NAC prodrug approach is one of the most promising in ophthalmology and viable strategies currently being investigated in this study for ocular drug delivery. One aspect of the current research in ocular drug delivery is focusing on the design and contents of NAC (Figure 8A) ophthalmic prodrug, which explores the drug metabolism capability in ocular tissues and aqueous humor. Careful consideration and understanding of ocular tissue metabolic processes within the eye and site-specific cornea/conjunctiva tissues has important implications for controlling the activity of considered therapeutic peptide agents, and for providing the potential for intraocular antioxidant bio-activation of certain NAC prodrug formulations and designed codrugs, thus enabling significant improvements in efficacy and the minimization of local and systemic side effects.

The biological effectiveness of L-carnosine (Figure 8B) as specific scavenger for activated oxygen species was assessed in the crystalline lens-induced LPO system (Figure 9). Transparent rabbit lenses were incubated in the various media containing 0.5 mg/ml liposome suspension as the oxidation substrate, and the kinetics of the LPO reaction were estimated by measuring MDA, liposomal conjugated dienes and trienes making appropriate corrections for liposome autooxidation (Babizhayev & Bozzo Costa, 1994). In a background study in the absence of the lens, virtually no oxidation of liposomes took place during 180 min (Figure 9, curve 6). In the presence of the lens, however, a marked increase in the concentration of different molecular LPO products was demonstrated for the appropriate time of incubation both in glucose-free (A) or in glucose-containing (B) culture media ((Babizhayev & Bozzo Costa, 1994); Figure 9, curve 1). The level of MDA accumulation after incubation of rabbit lenses for 3 h was 3.5- and 5.3-fold higher than that of normal human or mouse lenses. The larger normal lenses (rabbit or human) have more epithelial cells rich in reduced glutathione (GSH), which are primarily metabolically active, and these lenses (or their specific organelles mitochondria) can generate the leaking active oxygen species and lipid peroxides more rapidly than cataractous lenses, with their exhausted pool of reductants, or tiny mouse lenses (Babizhayev & Bozzo Costa, 1994; Huang, Tang,

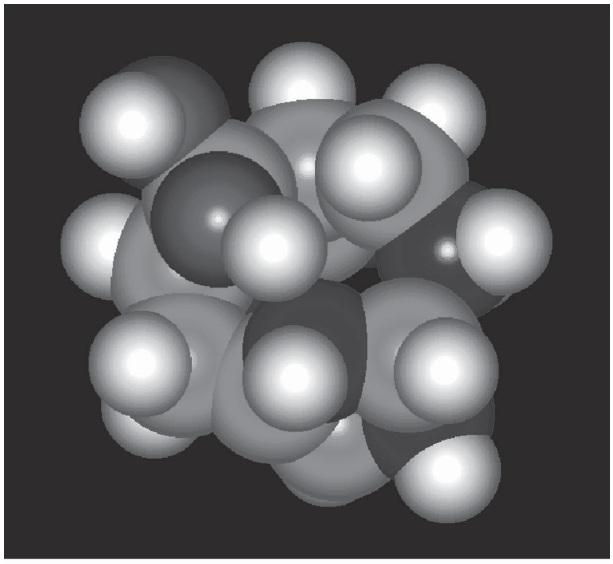
a





N-acetylcarnosine

b



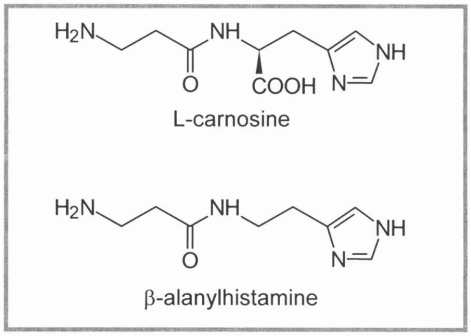


FIGURE 8. Structures of *N*-acetylcarnosine (A) and carnosine (B) shown as chemical structures and energy-minimized structures (space-filling model). (A) Chemical name: *N*-acetyl-L-carnosine $C_{11}H_{16}N_4O_4$; molecular weight: 268.27; (B) L-carnosine is $C_9H_{14}N_4O_3$; molecular weight: 226.20. Low-energy 3-D conformations of carnosine, carcinine, and *N*-acetylcarnosine were derived using the PM_3 method of the MOPAC 6.0 program (Stewart MOPAC Air Force Academy: Boulder, CO 80840). The precise energy minima conformations were determined by semi-empirical Quantum Mechanics. This technique structures a pool of energetically accessible shapes especially suitable for dipeptides comparative to large protein molecules. The program is supplemented with ZINDO/1 computer software for estimation of chelating properties of dipeptides and related compounds. The conformational geometry optimization was carried out using the revised computer program (Stewart, 1989a, b)

FIGURE 8. (Continued).

Yappert, & Borchman, 2006). In some cases, a small decrease in the liposomal MDA concentration after incubation for 2 h was observed. This may be connected with MDA utilization by the lens itself (interaction of MDA with amino group or its lowering by lenticular aldehyde dehydrogenase) (Babizhayev & Bozzo Costa, 1994). A considerable reduction in the accumulation rate of the liposomal LPO products was found after the addition of catalase (900 U/sample) (Figure 9, curve 3). This suggests a role of H_2O_2 in promotion of LPO by the lens. Addition of superoxide dismutase (SOD; 114 U/sample) to the incubation medium of the lens led to a marked reduction of the liposomal MDA level (Figure 9, curve 4), suggesting that the lens could generate $O_2^{-\bullet}$ in the surrounding medium. Addition of the ADP–Fe complex to the incubation medium of the lens decreased accumulation of TBA-reactive material in liposomes by 33–50%, indicating decomposition of the accumulated TBARS. Almost total inhibition of TBA-reactivity in the liposomes occurred after the addition of the chelating agent 1 mM EDTA, which eliminates free and accessible metal ions from the peroxidizing system (Figure 9, curve 5). L-Carnosine has been shown to act as a good scavenger of the lipid peroxyl ($LOO^{\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals (Aruoma, Laughton, & Halliwell, 1989; Babizhayev, 1989b Babizhayev et al., 1994). The presence of 5 mM L-carnosine in the rabbit lens/liposome-containing medium decreased the TBA-reactivity by approximately 25% at 2 h incubation (Figure 9, curve 7).

Study of L-Carnosine Uptake by the Lens

To study the kinetics of L-carnosine penetration into the lens, the isolated rabbit lens was placed and incubated in a medium containing L-carnosine (5–15 mM). Incubation of lens preparations with histidine dipeptide led to its accumulation in the lens tissue (Figure 10). It can be concluded that L-carnosine penetrates the barrier of the lens capsule when present in the aqueous humor at effective concentrations. The presence of L-carnosine in transparent crystalline lenses was detected, and

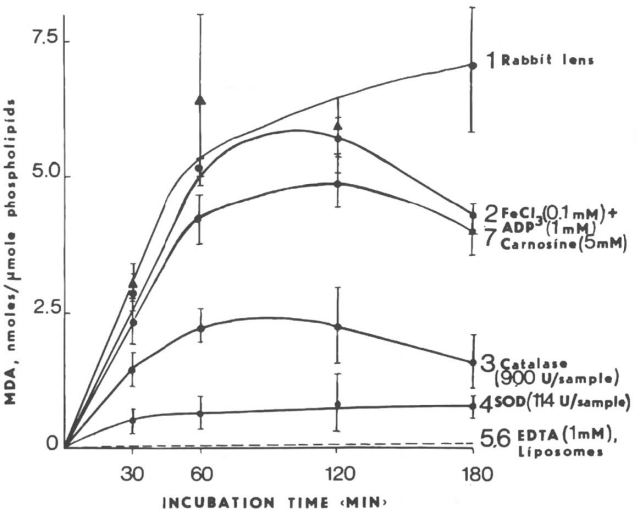


FIGURE 9. Effect of various oxygen radical scavengers on lipid peroxide formation in liposomes added to the incubation medium of the normal rabbit lens. In a total volume of 3.0 ml, the incubation mixture of the lens contained medium A or B, 0.5 mg/ml liposome suspension and concentrations of scavenger as indicated. Because the oxidation ratios in the glucose-containing or free standard culture media were similar, mean values of malonyl dialdehyde (MDA) concentrations are given for a representative experiment, with the error bars indicating the standard deviation obtained in both media for each group of 3–5 lenses.

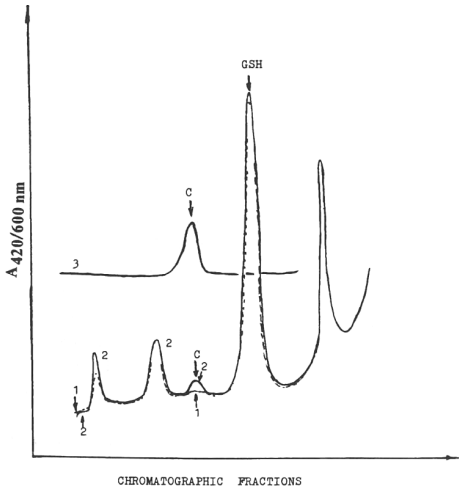


FIGURE 10. Absorbances of the extracted rabbit lens components after TLC separation. Curve 1 (broken line), extract of the control lens incubated for 60 min in Hank's medium. Curve 2, extract of the lens incubated for 60 min in a medium containing l-carnosine (5 mM). Curve 3, a standard preparation containing L-carnosine (1 mM) in Hanks' medium. C, L-carnosine maximum; GSH, reduced glutathione.

its concentration in this case was about 25 µM in normal human lenses and 0.89 ± 0.1 mM (n = 4) in rabbit lenses (Babizhayev, 1989b; Boldyrev, Dupin, Bunin, Babizhayev, & Severin, 1987; Jay, Miller, Morrison, & O'Dowd, 1990). At different stages of cataract development, the level of L-carnosine fell, reaching about 5 µM in ripe human cataracts (Babizhayev, 1989b Boldyrev, Dupin, Bunin, Babizhayev, & Severin, 1987; Jay, Miller, Morrison, & O'Dowd, 1990). Thus, L-carnosine that finds its way into the aqueous humor can accumulate in the lens tissue for a reasonable period of time.

Tissue and Intracellular Buffering of NAC (L-Carnosine) in the Cornea

NAC/L-carnosine provide intracellular pHi buffering to stimulate anaerobic energy formation. The structural integrity of the cornea is maintained by an active transport system, which depends on metabolism (Figure 11A). Corneal hypoxia and acidosis during contact lens wear is significant under a variety of wearing conditions and, thus, must be considered when evaluating the morphological or functional changes that occur following long-term contact lens wear (Figures 11A and B). The pH of corneal epithelium, stroma epithelium, and aqueous humor decreased significantly with lenses up to Dk/L = 100. Hypoxia had large effects on the corneal epithelium and stroma, but had no effect on the endothelium or aqueous. Conversely, carbon dioxide accumulation caused significant acidosis in all compartments measured (Bonanno, 1996). The intracellular non-bicarbonate buffering of corneal tissue is associated with the imidazole group that exists in histidine residues of constituent corneal tissues, tear and aqueous humor proteins, in free L-histidine, and in histidine-containing dipeptide(s) such as topically applied NAC lubricant eye drops (time release version of L-carnosine). Because the pK values of these imidazole groups are close to pHi, one of the two nitrogens of the imidazole ring can be protonated in the physiological range of pH. Thus, imidazole groups are utilized as potent proton-buffering constituents in corneal tissues. The regulatory process keeping pHi close to the pK values of imidazole groups is called "alphastat regulation." Its role is to maintain α-imidazole relatively constant [α-imidazole being defined as non-protonated imidazole/(non-protonated imidazole + protonated imidazole)]. Typical α_{imid} is conserved at a value of about 0.55 in intracellular fluid (Abe, 2000). Inorganic orthophosphate also serves as a typical inorganic buffer component in addition to imidazole compounds (Table 2). Acetylation of L-carnosine, L-histidine, or carnosine derivatives does not change the capacity of imidazole compound to bind the protons in the region of neutral and weak acid pH values (Boldyrev, 1998). From the data presented and discussed in this article, carnosine appears to be an efficient intracellular pH buffer, heavy metal chelator, potent anti-glycating agent, and regulator of many specific receptors and enzymes (Babizhayev, Deyev, Yermakova, Remenshchikov, & Bours, 2006).

Our clinical observations demonstrate that the feeling of "dry eye" is one of the most frequently reported symptoms by contact lens wearers. Epithelial lactate production increases during periods of corneal hypoxia, which causes an osmotic imbalance leading to increased stromal hydration. Further findings

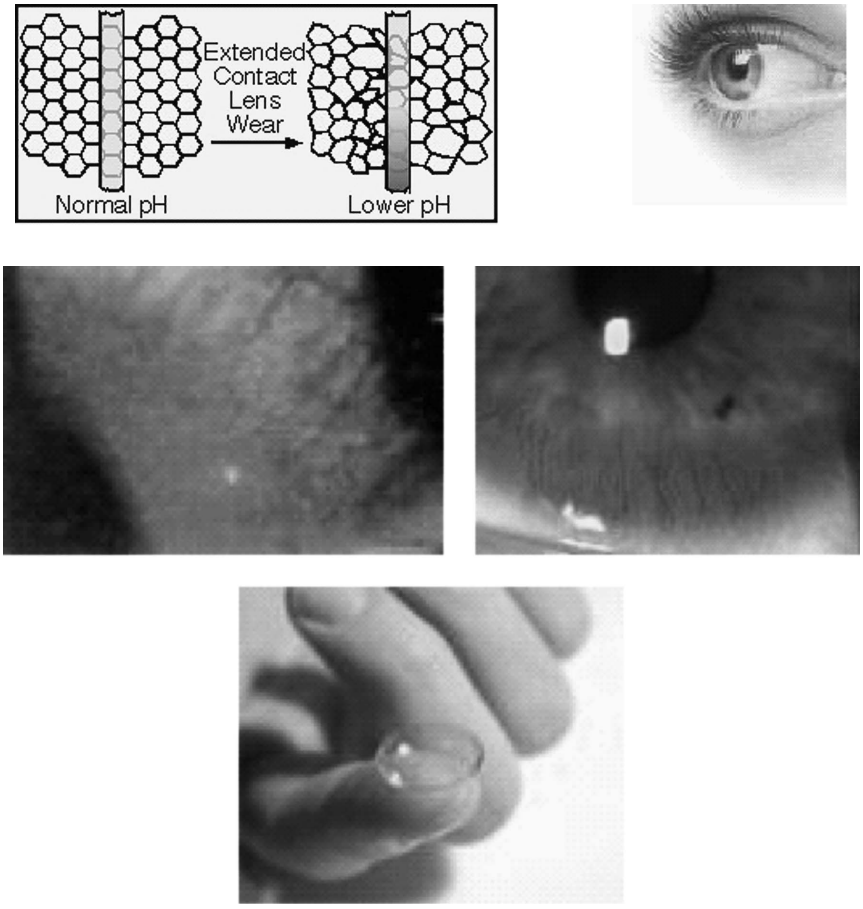


FIGURE 11. Corneal complications during contact lens wear (A) Contact lens wear can cause corneal complications such as ulcerative keratitis, neovascularization, epithelial microcysts, endothelial polymegethism, reduced mitotic activity, altered wound healing, and reduced corneal function. Some of these complications can lead to permanent visual impairment. The prevention of these complications requires an understanding of the fundamental mechanisms responsible for changes in corneal structure and function. Hypoxic or extended wear (sleeping with the lenses on, referred to as "closed-eye contact lens wear") can result in corneal acidosis, which alters corneal structure and function. (B) Histidine derivatives *N*-acetylcarnosine and mostly their bioactivating analog dipeptide L-carnosine exert universal antioxidant properties. The data support the clinical application of developed *N*-acetylcarnosine lubricant eye drops to compensate corneal acidosis during contact lens wear.

obtained at IVP Laboratories indicate that extended wear of hydrogel contact lenses reduces stromal keratocyte density. The mechanisms for this alteration may include hypoxic, cytokine-mediated, or mechanical effects. The role of released carnosine in corneal disorders indicates on relation to tissue buffering, osmoregulation and antioxidation. These features make the developed NAC ophthalmic prodrug lubricant eye drops useful to treat the contact-lens-induced stromal acidosis that cause corneal complications. Enhancement of the stability of the pre-lens tear film arising from the instillation of another re-wetting solutions was found to be transient. It was concluded that these lubricants are *unlikely* to provide a physical basis for prolonged symptomatic relief. The information obtained from our data can further an understanding of the etiology of corneal complications resulting from contact lens wear and will also improve present contact lens treatment strategies to help eliminate those lens-wearing conditions where stromal acidosis is likely to place the patient at risk.

DISCUSSION

The topical administration of NAC as bioactivating antioxidant for vision in the developed and patented design of lubricant eye drop formulations delivers pure L-carnosine and allows its increased intraocular absorption into the aqueous humor surrounding the lens, thus enabling significant improvements in drug efficacy and the minimization of side-effect from either local or systemic drug absorption/bioavailability to the eye, and also creates optimization effects in the cornea during the prolong time course of a contact lens wear. Knowledge

TABLE 2
Apparent pK Values of Imidazole Groups (See Also Abe, 2000 for Further Support)

Substance	pK
Typical histidyl-imidazole in proteins	6.5 (25°C)
Adjacent to acidic (–) group	7–8 (25°C)
Adjacent to basic (+) group	5–6 (25°C)
L-Histidine	6.21 (20°C)
Carnosine	7.01 (20°C)
Inorganic orthophosphate	6.88 (20°C)

pK measurements were performed at temperatures specified in parentheses.

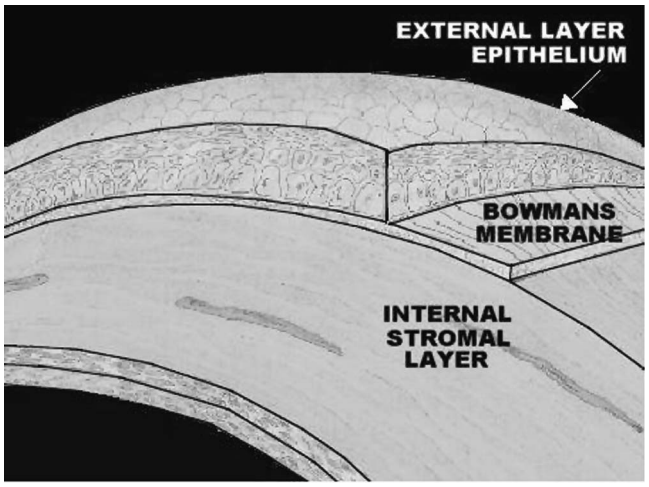


FIGURE 12. The structure of the normal eye cornea.

of these processes is important for ophthalmologists and optometrists to achieve eye drop indication clinical benefits for their patients and to professionals involved in the research of design, delivery, and metabolism of ophthalmic drugs. The obtained data demonstrate a specific method and design of ophthalmic formulations for prevention or treatment of cataract or another eye disease, comprising topically applying to a mammal in need of said treatment the developed prodrug/codrug aqueous ophthalmic compositions comprising NAC or a pharmacologically acceptable salt of NAC, in combination with a lubricant (cellulose compound) and corneal absorption promoter(s) in an amount effective to increase intraocular absorption of L-carnosine into the aqueous humor. The results support the hypothesis that the eye is susceptible to exposure to endogenous and exogenous substrates, and therefore must possess the capacity for biotransformation of topically applied peptide compounds. It is known that the cornea can tolerate high concentrations of topically applied compounds (Figure 12). The relative lack of metabolic activity present in the crystalline lens allowing the accumulation of the dipeptide L-carnosine in its tissue is not surprising, given the limited blood supply to this tissue. Glutathione is present in the lens, without which the presence of significant levels of potentially harmful xenobiotics can occur.

Ocular prodrug administration of NAC in the patented codrug formulations has proven in this study to be an effective method for drug delivery to the anterior segment of the eye of the bioactivating antioxidant L-carnosine. The NAC prodrug during its pass through the conjunctival and corneal tissues would then be metabolized by resident enzymes, *N*-acetylesterase and *N*-acetyltransferase, to the parent compound (Anderson, Davis, & Wei, 1980; Babizhayev et al. 1996). The results suggest that the developed codrug system ensures the best ocular bioavailability and an excellent tolerance of the active peptide principle and effectively inhibits the peroxidation reactions during cataractogenesis and cataract-associated disorders in veterinary practice and in human clinical medicine. Oxidation of membrane lipids could directly or indirectly alter the molecular structure of lens membranes. Development of peroxidative reaction in lipid membrane phase is accompanied by the accumulation of fluorescent products, which have fluorescence excitation and emission spectra parameters similar to those of cataractous fluorophores (Babizhayev, 1989c).

Which are the mechanisms of intermolecular protein cross-links formation in cataract? Dialdehyde-bifunctional reagents are characteristic of LPO products, and their interaction with free aminogroups entails Schiff's base forming and, subsequently, inter- and intramolecular cross-links. It should be pointed out that formation of phospholipid intermolecular cross-links has been registered in this study by characteristic fluorescence of lipid extract from cataractous lens. Covalent cross-linking of lenticular proteins in cataract may be possible also as a result of their interaction with the lipid-free radicals appearing in the course of LPO process. Cross-linking of biomolecules by the reaction of the carbonyl groups of MDA and amino groups of amino acids, proteins, nucleic acids and their bases, and phospholipids produces lipofuscins, fluorescent Schiff's base conjugates resulting in formation of high molecular weight aggregates chronically accumulated in cataracts and increased in ripe cataracts as well as inactivating enzymes in the lens (Babizhayev, 1989c; Bhuyan & Bhuyan, 1984).

The primary molecular LPO products, i.e., phospholipid hydroperoxides and their in vivo indicator, diene conjugate, represent the major change in the lipid composition of aqueous humor during cataract formation. At the stages of ripe cataract, end fluorescent LPO products are distinctly detected (Babizhayev, Deyev, & Linberg 1988). Crystalline lens is well equipped with antioxidant defenses showing some specificity in their action. LPO in the lens incubation system was decreased in the presence of free radical scavengers and enzymes that degrade H_2O_2 (EDTA, SOD, catalase, L-carnosine, and chelated iron). The most effective agent was

EDTA, which chelates the free metal cations required to generate $O_2^{-\bullet}$radicals that initiate the free radical process culminating in LPO. The mechanism of metal-catalyzed oxidation reactions of the lens reductants (GSH, ascorbate, lens crystallins containing SH groups) was proposed as a basic for release of free radicals or lipid hydroperoxides in the lens medium, and the rates and final levels of oxidant formation by lenses were found inversely dependent to their integral degrees of clouding (Babizhayev & Bozzo Costa, 1994). Because LPO is clinically important in many of the pathological effects and aging, new therapeutic modalities should treat the incessant infliction of damage to the lens cells and biomolecules by reactive lipid peroxides and oxygen species and ”refashion” the affected lens membranes in the lack of important metabolic ”detoxification” of phospholipid peroxides (Babizhayev, 2004b; Babizhayev, Deyev, Yermakova, Brikman, & Bours, 2004; Babizhayev, Yermakova, Deyev, & Seguin, 2000; Babizhayev et al., 2001; Babizhayev et al., 2002;). L-Carnosine and its ophthalmic bioactivating antioxidant prodrug form NAC are part of this group of products (Babizhayev, 2004b; Babizhayev et al., 2000; Babizhayev et al., 2001; Babizhayev et al., 2002; Babizhayev et al., 2004; Babizhayev et al., 2006). These compounds act as universal antioxidants with established ability to give efficient protection against LPO both in the lipid phase of cellular membranes and in the aqueous environment (protecting proteins, DNA, sugars from oxidative damage) (Babizhayev et al., 2006). Various protective antioxidant enzymes such as SOD or catalase can only react with their substrates in aqueous environment (Halliwell & Gutteridge, 1985).

The ophthalmic topical application of pure L-carnosine (1% solution) to the rabbit eyes (instillation, subconjunctival injection, ultrasound-induced phonophoresis) to produce the desired effect, whether this is therapeutic or prophylactic, did not lead to the intraocular absorption or accumulation in the aqueous humor of this dipeptide compound (Babizhayev et al., 1996; Babizhayev et al., 2000). Thus, the pharmaceutical compounding and formulating of NAC drug into suitable dosage prodrug/codrug forms for human and veterinary topical ocular administration is important according to the developed ophthalmic formulations (Babizhayev, 2004a) describing a combination of pharmacologically active NAC with a cellulose compound to enhance ocular metabolic activity, to reduce side effects of pure L-carnosine, and to modify the prodrug action in a way to make it more suitable for the treatment of cataracts.

After absorption in the aqueous humor from the ophthalmic time release NAC carrier, l-carnosine must pass into the lens along with results of provided studies gaining an access to prevent and manage the oxidative damage to the lens cells and tissues. In this study, we have in vitro assessed the uptake of l-carnosine in the crystalline lens as antioxidant at the viable contents produced during the chronic usage of the prodrug form required for a patient to self-medicate (Babizhayev, 2004a). The visualized effects of 1% NAC with lubricant carboxymethylcellulose on the course of age-related cataracts in the double-blind randomized clinical trials were demonstrated by the author’s group (Babizhayev, 2004b; Babizhayev et al., 2000; Babizhayev et al., 2001; Babizhayev et al., 2002; Babizhayev et al., 2004; Babizhayev et al., 2006). These observations strongly suggest that NAC may prevent and reverse cataracts. The mechanism for this observation may involve carnosine’s ability to disaggregate glycated α-crystallin protein (Seidler, Yeargans, & Morgan, 2004). In the cataractous lens, cross-linking of proteins by any means increases their effective molecular weight and produces light scattering and consequent lenticular opacity. The production of such high molecular weight protein complexes by disulfide bridges and covalent links with dialdehydes has been implicated in the formation of senile and other cataracts (Babizhayev & Deev, 1986). Both types of cross-linking may be caused by depletion of the lens’ reduced glutathione and accumulation of LPO products in the lens tissue. The results of our studies support that l-carnosine released from its ophthalmic prodrug NAC during its topical application to the eyes with cataracts is able to prevent the loss of reduced glutathione and to remove the secondary LPO products in biological systems (Babizhayev et al., 2004; Babizhayev, 2006). This, in turn, may lead to dissociation of the intermolecular protein cross-links because of glutathione-protein thiol-disulphide exchange mechanism and utilization of lipid peroxides and dialdehydes derived from LPO process, anchoring protein-lipid complexes in the lens (Babizhayev, 2006). A possibility exists from our studies that carnosine is reacting directly with MDA and other aldehydes/ketones. Indeed, carnosine has been shown to protect against MDA-induced cross-linking and toxicity, and a hydroxynonenal–carnosine adduct has recently been characterized, providing further evidence for carnosine’s potential as an aldehyde scavenger (Aldini, Carini, Beretta, Bradamante, & Facino, 2002). The presented results can be explained in part by the adduction of the various LPO products directly by carnosine following de-acetylation of NAC. The published results suggest that histidine is the representative structure of l-carnosine for an anti-cross-linking agent, containing the necessary functional groups for optimal protection against cross-linking agents (Hobart, Seibel, Yeargans, & Seidler, 2004).

The normal metabolic processes are essential for cell growth replacement and, in the case of the corneal epithelium and endothelium, for the maintenance of the ionic pump mechanism, which is responsible for maintaining the state of corneal hydration. In this study, we evaluated the hypothesis that contact-lens-induced stromal acidosis causes corneal complications. A goal of this study was to develop a practical clinical method or process to compensate stromal pH so that these research findings may have clinical applications. We are currently in the process of analyzing the completed clinical database. Intracellular pH [pH(i)] is an important modulator of the corneal function. Because it is readily influenced by metabolic processes, pH(i) is controlled physiologically. The data support the clinical application of developed NAC lubricant eye drops

to compensate corneal acidosis during contact lens wear, which is significant under a variety of wearing conditions and, thus, must be considered when evaluating the morphological or functional changes that occur following long-term contact lens wear. Intracellular H(+) corneal buffering is adjusted and occurs through a shuttling of the intrinsic mobile buffers such as acetylated carnosine and its bioactivating ophthalmic ingredient carnosine low molecular weight imidazole compounds.

LPO reactions are widely involved in the genesis of ophthalmic disorders, such as cataract, glaucoma, inflammatory, corneal, retinal, systemic disorders having a component of oxidative stress in their genesis (Babizhayev & Deyev, 1989). These are the most widely used application sites for drugs and pharmaceutical products besides ophthalmic surgery. But mainly products are swallowed in order that the active ingredient can be absorbed from the gut and can gain access to the blood stream and in this way, secondary to the eye. Although some parts of the eye are richly supplied with blood vessels, others including the crystalline lens are not. Moreover, the response of systemic drugs and oral supplements can be variable as other gut contents can influence the absorption of alimentary-administered remedies and particularly, being mostly peptidergic in nature products, are susceptible to preoteolytic breakdown by proteases, dipeptidases, encountered during internal digestion in the gastro-intestinal tract or transport in the blood stream, and consequently have too limited half-life upon systemic application.

CONCLUSION

This study presents a pharmacokinetic evidence suggesting that the developed ophthalmic prodrug and codrug NAC-sustained time-release forms of delivery are most effective to make L-carnosine bioavailable for the intraocular absorption in the aqueous humor. This is by far the most effective route of administration of L-carnosine for the eye and forwarding it to the lens tissues. Topically applied ophthalmic formulation produces effective levels of L-carnosine in the anterior segment of the eye. The response of the patient to the drug will to a great extent depend on the concentration of L-carnosine that is available at the site of action near the lens in the aqueous humor. The relationship between the dose or concentration administered and the final L-carnosine concentration at the focus of action near the lens in the anterior segment of the eye is the resultant of normal pharmacokinetic processes of NAC deacetylation, which are themselves subject to the effect of other agents and excipients (such as carboxymethylcellulose, vitamins, buffers, preservatives, pH adjusters) (Babizhayev, 2004a; Babizhayev & Meguro, 2004).

Our studies document the optimal bioavailability of an ophthalmic formulation including 1% NAC with a lubricant carboxymethylcellulose and predict further benefits of the application of this drug in prevention and reversal of age-related cataracts in human and in canine eyes (Babizhayev, 2004b; Babizhayev, 2006; Babizhayev et al., 2000; Babizhayev et al., 2001; Babizhayev et al., 2002; Babizhayev et al., 2004; Babizhayev et al., 2006). Other than senile cataract, NAC may have other clinical benefits. The unique and patented NAC lubricant eye drops formula could also provide beneficial results with the following eye disorders (Babizhayev, 2006):

- Presbyopia;
- Open-angle primary glaucoma (in combination with beta-blockers);
- Corneal disorders;
- Computer vision syndrome;
- Eyestrain;
- Ocular inflammation;
- Blurred vision;
- Dry eye syndrome;
- Retinal diseases;
- Vitreous opacities and lesions;
- Complications of diabetes mellitus and other systemic diseases;
- Contact lens difficulties, particularly with soft contact lenses. (not only do the lubricants in the Can-C NAC eye-drop help to make wearing contact lenses more comfortable, but NAC is also believed to reduce the build up of lactic acid in the eye, thus enabling the lens to be left safely in the eye for longer).

NAC prodrug and codrug ophthalmic formulations applied topically to the eye, and moreover, its controlled time released-ophthalmic ingredient L-carnosine exerts anti-glycation, bioactivating antioxidant properties in the lens and cornea as a scavenger of lipid peroxides, singlet oxygen and OH·radicals and spatial aspects of intracellular pH regulation for vision.

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APPENDIX 1. PACKAGING MATERIAL AND THERAPEUTIC INDICATIONS OF IVP OFFICIALLY REGISTERED BRAND OF NAC EYE DROPS

CE-Marked NAC eye drops officially registered in all countries of the EC for the free sales through the pharmacies.

Package:	Flac. collirio 5 ml

The granted certificate is called "APPROVAL OF QUALITY ASSURANCE SYSTEM FOR PRODUCTION AND/OR STERILIZATION", and it states that the quality assurance system for the production (patented NAC eye drops containing carboxymethylcellulose) is in accordance with the requirements of the EC Directive 93/42/CEE.

IVP Officially registered brand of **NAC** eye drops **is a medical device Sterile solution of carboxymethylcellulose. Warning: Discard the product 30 days after first opening.**

INDICATIONS AND DOSAGE: see internal insert

Keep Out of the Reach of Children

Store the product at a temperature not higher than 25°C (Figure A1)

Codice a barre

IVP brand of Eye Drops

Sterile solution of carboxymethylcellulose

FIGURE A1. Anti-cataract, anti-glare marketed brands of *n*-acetylcarnosine lubricant eye drops worldwide patented by Innovative Vision Products, Inc.

INDICATIONS

Protective, eutrophic, moisturizing, lubricating eyedrops.

Thanks to the antioxidative and anti-free radicals properties of its components, it protects the ocular tissues (crystalline lens, cornea, conjunctiva) from the oxidative stress because of environmental factors and from the assaults of cellular lipidic peroxidasis, and it makes easy the restoration of the natural epithelial trophism.

CE

IVP Officially registered brand of *N*-acetylcarnosine eye drops

Sterile solution of carboxymethylcellulose

Protective, eutrophic, moisturizing, lubricating eye drops

Bottle of 5 ml

Clarastill

STERILE A

Description

This IVP eye drops Product is a watery sterile solution at 0.3% of carboxymethylcellulose to be used on ocular tissues for its protective, eutrophic, moisturizing, lubricating action. Thanks to the anti-oxidative and anti-free radical properties of its components, this medical device protects the ocular tissues (crystalline lens, cornea, conjunctiva) from the consequences of oxidative stress because of unfavorable environmental factors (mechanical stress because of contact lenses, solar radiation, smoke, powder, air conditioning, computer radiations), and it makes easy the recover of the natural epithelial trophism.

The saline solution, base of the medical device, would remain in contact with the ocular surface only for few seconds with a transient benefit. By adding glycerine to the solution, an extraction of water from ocular tissues is obtained (moisturizing action) with the consequent formation of a thin watery film on the corneal surface which in presence of carboxymethylcellulose is englobed in the grid of the cellulose polymer. So a hydro-gel with film and viscous-elastic properties is formed.

In the medical device, the concentration (0.30%) of carboxymethylcellulose has been appropriately studied to grant to the obtained hydro-gel rheologic characteristics equal to those of the lachrymal liquid and a good adhesiveness on ocular

mucosa with a consequent protective, eutrophic, moisturizing, and lubricating effect without causing sight blurring.

IVP Officially registered brand of NAC eye drops is characterized by the following chemical-physical properties: it is perfectly transparent, mixable with water (and with lachrymal liquid) in all proportions; it does not form insoluble precipitates with the metal salts or with organic remnants eventually present in the lachrymal liquid; it has a pH and an osmolarity value perfectly compatible with the ocular tissue and with the lachrymal liquid and for this reason, it has a high tolerability.

Composition. Carboxymethylcellulose 0.3%, methyl *p*-hydroxybenzoate 0.030%, propyl *p*-hydroxybenzoate 0.012%, glycerin, disodium edetate, NAC 1%, sodium chloride, sodium tetraborate, potassium bicarbonate, purified water.

Instruction for use. Instill 1–2 drops of this Product solution in the lower conjunctival fornix of the eye pushing lightly the bottle.

It is possible to administer the product more times during the day, if necessary.

Precautions

- The product does not contain salts of quaternary ammonium (benzalkonium, cetrimide), for this reason it can be used also by wearers of rigid and soft contact lenses.
- Do not touch the eye with the bottle tip.
- Do not use in case of infection of the eyes.
- Do not use together with other pharmaceutical products for ophthalmic use.
- Do not use in case of known incompatibility toward whatever component of the product.
- Do not use the product after the expiry date printed on its box.
- Discard the product 30 days after first opening.

Storage conditions

Store the product at a temperature not higher than 25°C.

After first opening, store the product into the refrigerator at +2 + 8°C.

Warning: Keep out of the reach of children.

Packaging: 5 ml bottle

This product is a medical device